



Genomics-Assisted Crop Improvement

Volume 2

Genomics Applications in Crops

Rajeev K. Varshney
Roberto Tuberosa
Editors

 Springer

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Vol 2: Genomics Applications in Crops

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FOREWORD TO THE SERIES: GENOMICS-ASSISTED CROP IMPROVEMENT

Genetic markers and their application in plant breeding played a large part in my research career, so I am delighted to have the opportunity to write these notes to precede the two volumes on 'Genomics-assisted crop improvement'. Although I am not so old, I go right back to the beginning in 1923 when Karl Sax described how 'factors for qualitative traits' (today's genetic markers) could be used to select for 'size factors' (today's QTLs and genes for adaptation). But it was clear to me 40 years ago that even then plant breeders clearly understood how genetic markers could help them - if only they actually had the markers and understood the genetics underlying their key traits. It was not clear to me that it was going to take until the next century before marker-aided selection would become routine for crop improvement.

In the 1960s only 'morphological' markers were available to breeders. As a research student at Aberystwyth, I worked with Des Hayes at the Welsh Plant Breeding Station when he was trying to develop an F₁ hybrid barley crop based on a male sterility gene linked to a DDT resistance gene. The idea was to link the male fertile allele with susceptibility and then kill the fertile plants off in segregation populations by dousing the field with DDT. Rachel Carson's 'Silent Spring' ensured that idea never flew.

Then I moved to the Plant Breeding Institute in Cambridge where anyone working alongside the breeders in those early days could not help but be motivated by breeding. Protein electrophoresis raised the first possibility of multiple neutral markers and we were quick to become involved in the search for new isozyme markers in the late 1970s and early 1980s. Probably only the linkage between wheat endopeptidase and eyespot resistance was ever used by practical breeders, but we had an immense amount of fun uncovering the genetics of a series of expensive markers with hardly any polymorphism, all of which needed a different visualisation technology!

During this same period, of course, selection for wheat bread-making quality using glutenin subunits was being pioneered at the PBI, and is still in use around the world. These were the protein equivalent of today's 'perfect' or 'functional' markers for specific beneficial alleles. Such markers - although of course DNA-based, easy and economical to use, amenable to massively high throughput and available for all key genes in all crops - are exactly where we want to end up.

Proteins were superseded by RFLPs and in 1986 we set out to make a wheat map, only with the idea of providing breeders with the effectively infinite number of mapped neutral markers that they had always needed. We revelled in this massively expensive job, funded by a long-suffering European wheat breeding industry, of creating the first map with a marker technology so unwieldy that students today would not touch it with a bargepole, let alone plant breeders. This was, of course, before the advent of PCR, which changed everything.

The science has moved quickly and the past 20 years have seen staggering advances as genetics segued into genomics. We have seen a proliferation of maps, first in the major staples and later in other crops, including 'orphan' species grown only in developing countries. The early maps, populated with isozyme markers and RFLPs, were soon enhanced with more amenable PCR-based microsatellites, which are now beginning to give way to single nucleotide polymorphisms. These maps and markers have been used, in turn, to massively extend our knowledge of the genetic control underlying yield and quality traits. The relatively dense maps have allowed whole genome scans which have uncovered all regions of the genome involved in the control of key adaptive traits in almost all agricultural crops of any significance.

More amazing is the fact that we now have the whole genome DNA sequences of not one but four different plant genomes - *Arabidopsis*, rice and poplar and sorghum. Moreover cassava, cotton, and even maize could be added to the list before these volumes are published. Other model genomes where sequencing has been started include *Aquilegia* (evolutionary equidistant between rice and *Arabidopsis*), *Mimulus* (for its range of variation) and *Brachypodium* (a small-genome relative of wheat and barley).

Two other components deserve mention. The first is synteny, the tendency for gene content and gene order to be conserved over quite distantly related genomes. Ironically, synteny emerged from comparisons between early RFLP maps and probably would not have been observed until we had long genomic sequences to compare had we started with PCR-based markers that require perfect DNA primer sequence match. The possibility of being able to predict using genetic information and DNA sequence gained in quite distantly related species has had a remarkable unifying effect on the research community. Ten years ago you could work away at your own favourite crop without ever talking to researchers and breeders elsewhere. Not so today. Synteny dictates that genome researchers are part of one single global community.

The second component is the crop species and comparative databases that we all use on a daily basis. The selfless curators, that we have all taken for granted, deserve mention and ovation here because, while the rest of us have been having fun in the lab, they have been quietly collecting and collating all relevant information for us to access at the press of a button. This is a welcome opportunity to acknowledge these unsung heroes, and of course, their sponsors.

The practical application of markers and genomics to crop improvement has been much slower to emerge. While endopeptidase and the glutenin gels continue to see

use in wheat breeding, marker-aided selection (MAS) using DNA markers has, in both public breeding and the multinationals, emerged only in the last few years and examples of new varieties that have been bred using MAS are still few and far between. This will change, however, as the cost of marker data points continues to plummet and the application of high-throughput methods moves the technology from breeding laboratories to more competitive outsourced service providers.

The post-RFLP period and the new opportunities for deployment of economical high-throughput markers are the subjects of these volumes. The first volume deals with platforms and approaches while the second covers selected applications in a range of crop plants. The editors, Rajeev Varshney and Roberto Tuberosa, are to be congratulated on bringing together an authorship of today's international leaders in crop plant genomics.

The end game, where plant breeders can assemble whole genomes by manipulating recombination and selecting for specific alleles at all key genes for adaptation is still a very long way off. But these two volumes are a unique opportunity to take stock of exactly where we are in this exciting arena, which is poised to revolutionise plant breeding.

A handwritten signature in black ink, appearing to read 'Mike Gale', with a large, stylized initial 'M'.

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FOREWORD

According to the World Bank, approximately 1.2 billion people in absolute poverty live on less than US\$ 1 per day, while nearly twice this number live on less than US\$ 2 per day. About 90% of the world's poor comprise rural, resource-poor farmers and their families, and landless poor who depend on agriculture for their livelihoods. However, poor farmers live and work in regions that continue to be important sources of genetic diversity. While poor farmers, in general, cannot use "modern" (high-input) crop varieties, selected for optimal performance within a narrow range of highly managed environmental conditions, the more wealthy ones have replaced a wide range of traditional crops and varieties with a limited number of "modern" varieties of major crops.

However, it must be said to the credit of resource-poor farmers that they plant their own seeds (landraces) and manage their farms in a manner that allows the varieties to evolve. They select plant types rather than varieties based on their own observations and specific needs. They are in a way, responsible for maintaining the genetic diversity that is essential to the continued evolution and adaptation of plant genotypes.

They are therefore well placed to supply formal plant breeding systems with new genetic material that is urgently needed. Their empirical knowledge of the characteristics of specific plant types could help breeders identify the source of valuable traits for introgression into elite crop varieties. In this context, plant breeders need to screen germplasm from regions of low-resource agriculture but rich in genetic diversity and from seed banks for traits they consider useful, and then find ways to introgress the desirable traits into the varieties they are developing.

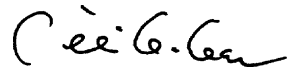
In recent years, genomics, the modern science of genetics, has been providing breeders with new tools and novel approaches to perform their tasks with high precision and efficiency. For example, applications of molecular markers in breeding through marker-assisted selection (MAS) have already been demonstrated in several crop species to develop improved varieties with better agronomic traits and enhanced resistance or tolerance to biotic or abiotic stresses. Indeed, MAS is being used widely both in developed and developing countries, and is enabling breeders to make use of unconventional plant materials.

Tremendous progress has been made in genome science in recent years. For example, the complete genomes for several plant species e.g., rice and sorghum, have already been sequenced, and similar efforts are underway for many other crop

species. Comparative and functional genomics approaches are helping scientists to better understand gene functions and to more effectively tailor the desirable genotype so that improved varieties can be released in a more timely fashion to farmers. If these new varieties prove to be appropriate for resource-poor farmers, then farmers could use them to enhance their livelihood security. This is probably the most obvious way to demonstrate how genomics empowers poor farmers.

This volume deals with applications of genomics towards crop improvement. I am glad to note the variety of opinions and experiences that the editors of this volume have gathered - eminent scientists representing international agriculture centers, advanced research institutes and national agricultural research systems from several countries - providing a treasure trove of information.

I am sure the book will be useful for the community involved in applying genomics research for crop improvement as well as for teachers and students to enhance their knowledge in the latest tools and approaches to genomics.



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PREFACE

Genomics, dealing with the collection and characterization of genes and analysis of the relationships between gene activity and cell function, is a rapidly evolving, interdisciplinary field of study aimed at understanding and exploiting the biological information encoded in DNA. The genomics toolbox includes automated genetic and physical mapping, DNA sequencing, bioinformatics software and databases, transcriptomics, proteomics, metabolomics, and high-throughput profiling approaches. Indeed, the past two decades have witnessed spectacular advances in genomics. For example, at the dawn of the genomics era, *Arabidopsis* was chosen as the first model genome for sequencing, which was then quickly followed by the sequencing of other model genomes (rice for monocots, *Medicago* and *Lotus* for legume crops and poplar for tree species) and crop species (soybean, cassava, sorghum, etc.). While new crops (e.g. maize, wheat, finger millet, etc.) are being added to the list for sequencing the genome or gene space, the generated sequence data are being analyzed in parallel for characterizing the genes and validating their functions through comparative and functional genomics approaches including bioinformatics, transcriptomics, and genetical genomics. Candidate genes are becoming increasingly useful for the development of markers for assaying and understanding functional diversity, association studies, allele mining, and most importantly, marker-assisted selection. Therefore, genomics research has great potential to revolutionize the discipline of plant breeding in order to face the challenges posed by feeding an ever-growing human population expected to top 10 billion by 2050, while decreasing the environmental footprint of agriculture and preserving the remaining biodiversity.

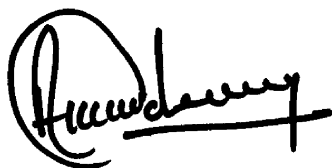
Several high-throughput approaches, genomics platforms, and strategies are currently available for applying genomics to crop breeding. However, the high costs invested in, and associated with, genomics research currently limit the implementation of genomics-assisted crop improvement, especially for autogamous and/or minor and orphan crops. This book presents a number of articles illustrating different contributions which genomics can offer to unravel the path from genes to phenotypes and vice versa, and how this knowledge can help to improve crops' performance and reduce the impact of agriculture on the environment. Each article shows how structural and/or functional genomics can improve our capacity to unveil and deploy natural and artificial allelic variation for the benefit of plant breeders. Volume 1, entitled "Genomics Approaches and Platforms", presents state-of-the-art genomic

resources and platforms and also describes the strategies and approaches for effectively exploiting genomics research for crop improvement. Volume 2, entitled “Genomics Applications in Crops”, presents a number of case studies of important crop and plant species that summarize both the achievements and limitations of genomics research for crop improvement.

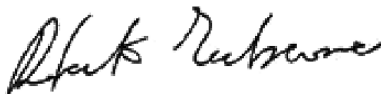
More than 90 authors, representing 16 countries from five continents have contributed 16 chapters for Volume I and 18 chapters for Volume II (see Appendix I). The editors are grateful to all the authors, who not only provided a timely review of the published research work in their area of expertise but also shared their unpublished results to offer an updated view. We also appreciate their cooperation in meeting the deadlines, revising the manuscripts, and in checking the galley proofs. While editing this book, we received strong support from many reviewers (see Appendix II) who provided useful suggestions for improving the manuscripts. We would like to thank our colleagues and research scholars, especially Yogendra, Rachit, Mahender, Priti, and Spurthi working at ICRISAT for their help in various ways. Nevertheless, we take responsibility for any errors that might have crept in inadvertently during the editorial work.

The cooperation and encouragement received from Jacco Flipsen and Noeline Gibson of Springer during various stages of the development and completion of this project, together with the help of Rajeshwari Pal of Integra Software Services for typesetting and correcting the galley proofs, have been instrumental for the completion of this book and are gratefully acknowledged. We also recognize that our editorial work took away precious time that we should have spent with our respective families. RKV acknowledges the help and support of his wife, Monika and son, Prakhar (Kutkut) who allowed their time to be taken away to fulfill RKV’s editorial responsibilities in addition to research and other administrative duties at ICRISAT. Similarly, RT is grateful to his wife Kay for her precious help in editing and proof-reading a number of manuscripts.

We hope that our efforts will help those working in crop genomics as well as conventional plant breeding to better focus their research plans for crop improvement programs. The book will also help graduate students and teachers to develop a better understanding of this fundamental aspect of modern plant science research. Finally, we would appreciate receiving readers’ feedback on the errors and omissions, if any, as well as their suggestions, so that a future revised and updated edition, if planned, may prove more useful.



Rajeev K. Varshney



Roberto Tuberosa

COLOR PLATES

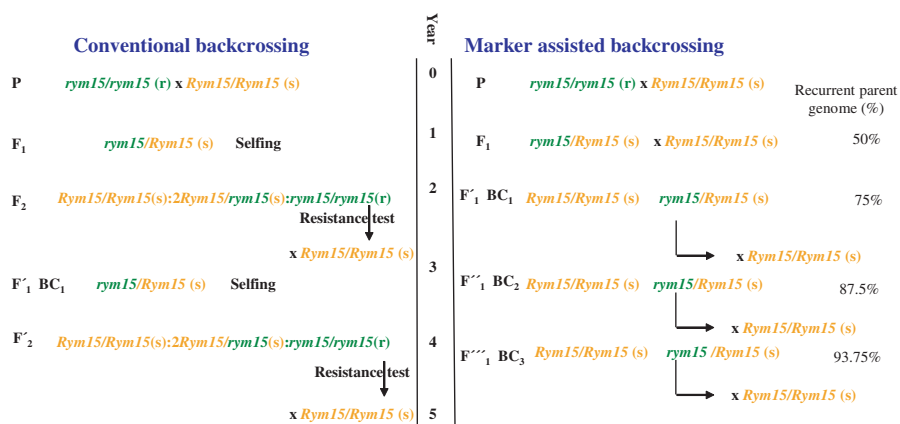


Plate 1. Comparison of conventional and marker-assisted backcrossing programmes for the incorporation of *rym15* (from Ordon et al. 1999, mod.) (See Fig. 1, on page 88)

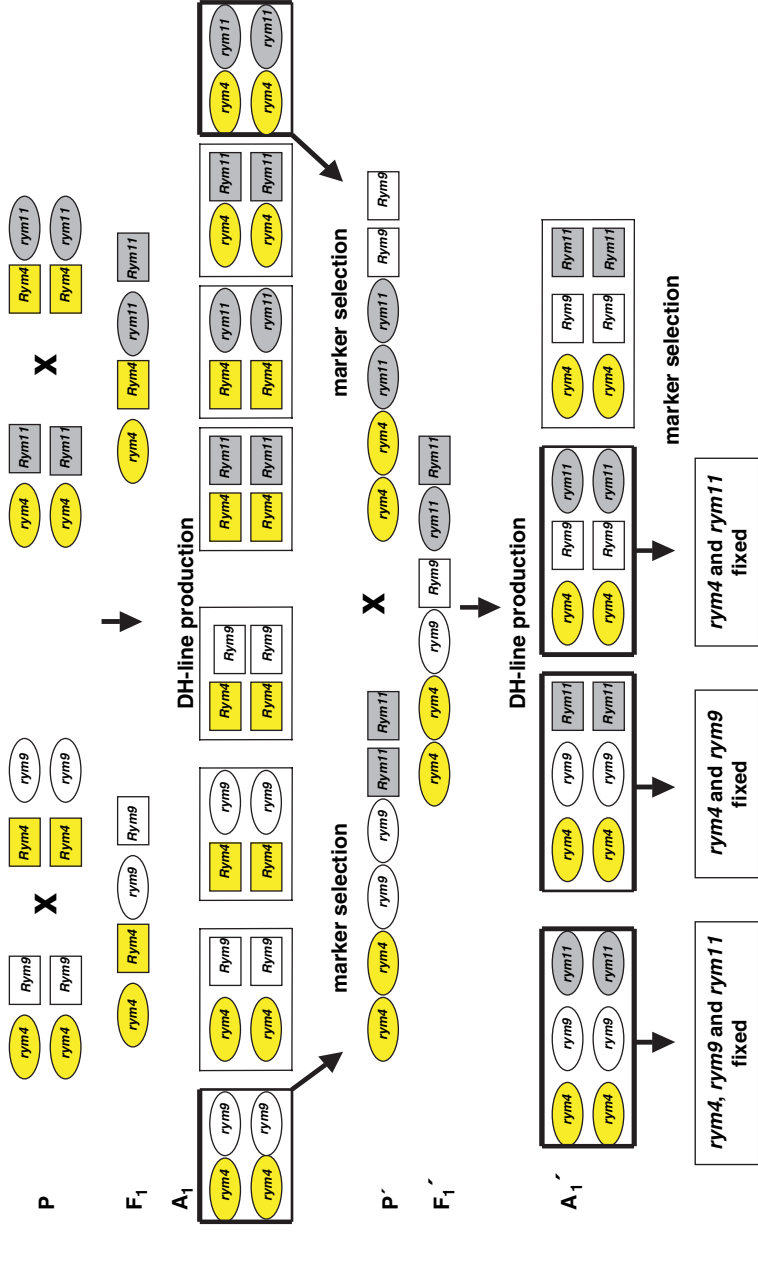
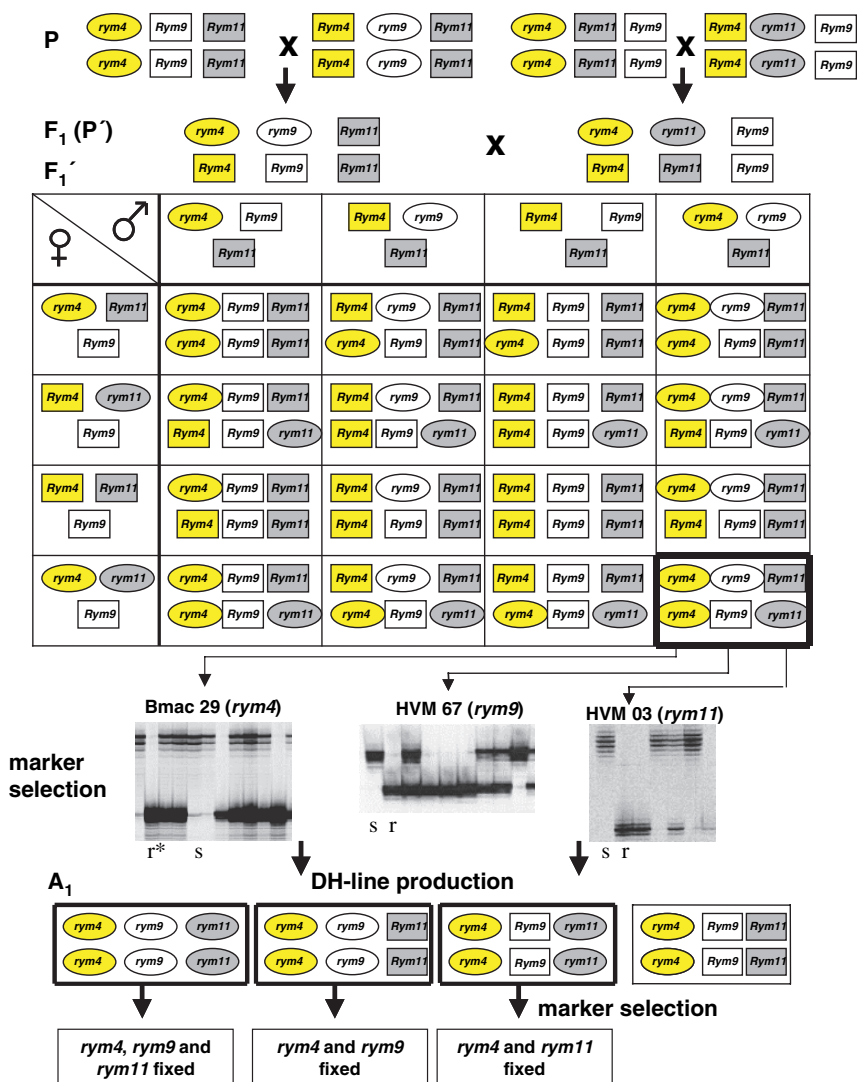


Plate 2. Scheme of pyramiding resistance genes *rym4*, *rym9* and *rym11* by two haploidy steps (Werner et al. 2005) (See Fig. 2, on page 91)



* r=allele of the resistant parent, s=allele of the susceptible parent

rym = resistance encoding allele; *Rym* = susceptibility encoding allele

Plate 3. Scheme of pyramiding resistance genes *rym4*, *rym9* and *rym11* by one haploidy step (Werner et al. 2005) (See Fig. 3, on page 92)

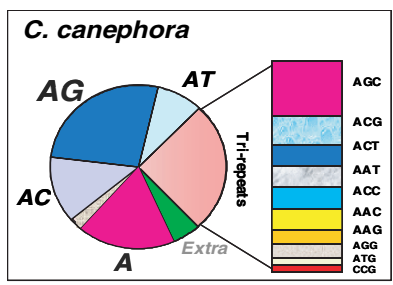
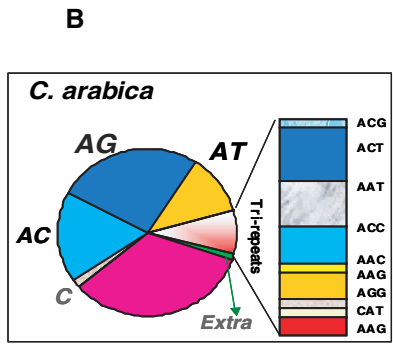
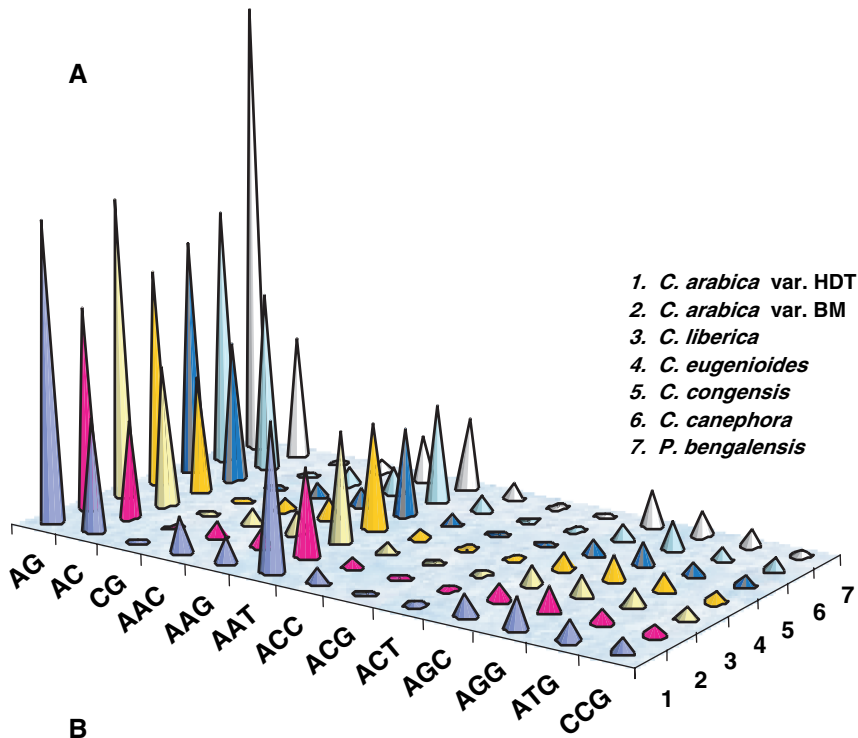


Plate 4. Relative abundance of different SSR motifs in genomes of different coffee species as revealed by: A) semi-quantitative Southern hybridization based slot-blot analysis, and B) *in-silico* sequence analysis of >1000 SSR positive clones from *C. arabica* and *C. canephora* (robusta) small-insert genomic libraries. Note almost similar comparable pattern/relative frequencies of different SSRs across coffee species and also between the two approaches of evaluation (our unpublished data) (See Fig. 1, on page 405)

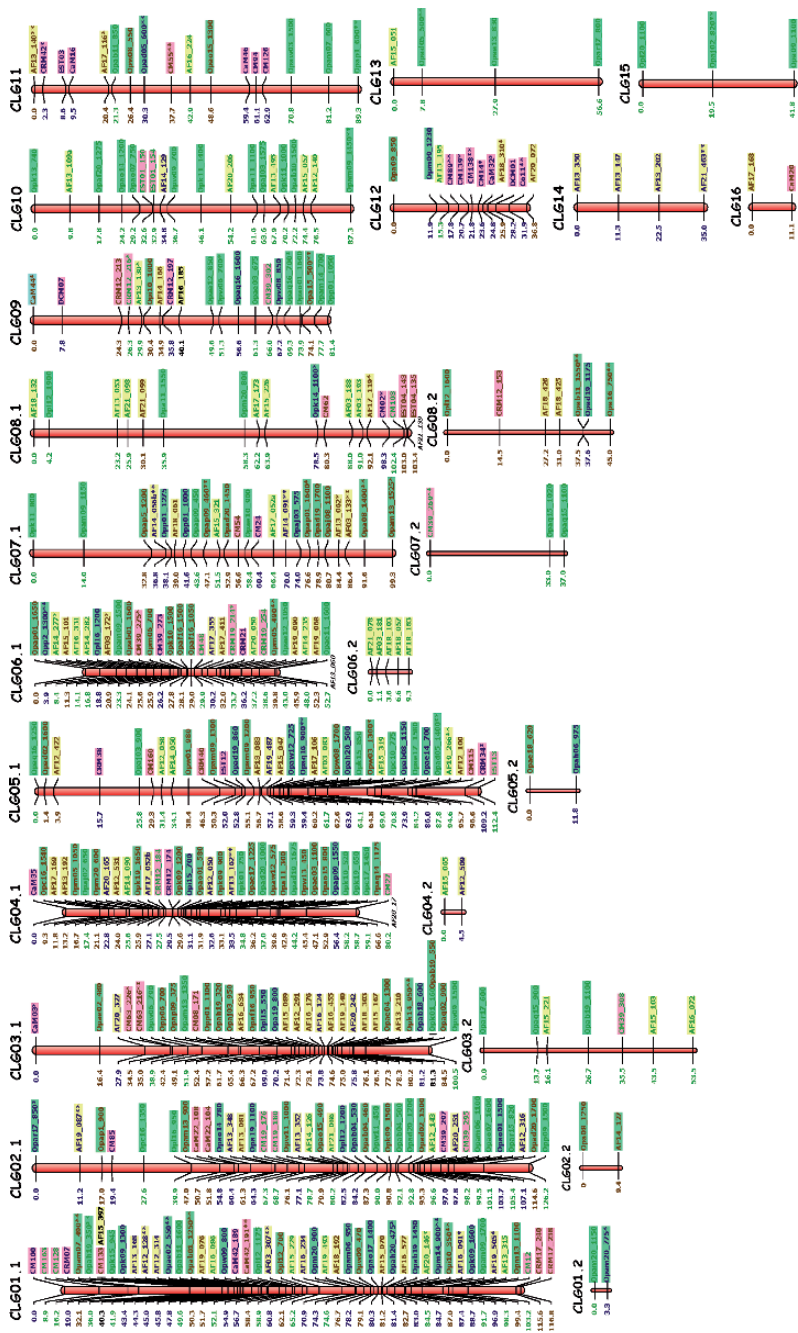


Plate 5. An integrated framework linkage map of robusta coffee (*C. canephora*) developed using a *pseudo-testcross* strategy and DNA markers (AFLP, RAPD, SSRs). The map comprises 11 major and 5 minor linkage groups, and has a total of 374 mapped markers. Prefixes Op-, AF- in the marker names indicates RAPD, AFLP respectively, while CM-/CR-/CaM- represents the mapped SSRs. The small groups shown below some of the major CLGs, represent the floating markers of the respective CLG placed in an 'Accessory group/map' (unpublished, Hendre 2006) (See Fig. 2, on page 413)

CHAPTER 1

MICROSATELLITE AND SNP MARKERS IN WHEAT BREEDING

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Abstract: Bread wheat (*Triticum aestivum* L.) is one of the most important crop plants. Due to its hexaploid nature consisting of three different genomes (A, B and D) and its large genome size of approximately 15 billion base pairs, it is also one of the most complex crop genomes. This has rendered the use of molecular markers in wheat genome analysis and breeding slow and difficult. Mainly, through the use of microsatellite or SSR (simple sequence repeat) markers, wheat molecular marker analysis has gained momentum during the last ten years. The advantage of microsatellite markers is that they detect an unsurpassed level of polymorphism in this recently polyploidised organism with a generally low level of sequence variation. Furthermore, a large proportion of the microsatellite markers is genome-specific, thus amplifying a defined single product from one of the three wheat genomes. Currently, 2,000 to 2,500 mapped microsatellite markers are available for the wheat genome. With microsatellite markers, the chromosomal position of many relevant breeding traits such as disease resistance genes and quality traits has been identified and they are increasingly used in marker-assisted selection during wheat breeding. For the future, high expectations are being put into another marker type that is called single nucleotide polymorphisms (SNPs) since their number in the wheat genome should be much higher and cost-efficient, highly multiplexed technologies are available for the analysis of SNP markers in plants. SNP marker development and use are, however, still in their infancy. Based on recent results, we discuss here the advantages and disadvantages of SNPs compared to microsatellite markers for future wheat breeding.

1. INTRODUCTION

Hexaploid bread wheat ($n = 21$) consists of three genomes with a basic chromosome number of $n = 7$ for each genome. The three genomes are derived from *Triticum urartu* (A-genome), an unknown species from the *Sitopsis* section (B-genome)

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and *Triticum tauschii* (D-genome) respectively (Feldman and Levy, 2005). The hexaploid bread wheat genome is one of the largest crop genomes with, all three genomes combined, approximately 15 billion base pairs (Arumuganathan and Earle, 1991) and each gene usually occurring in at least three copies. It is estimated that each of the three wheat genomes contains approximately 40.000 to 50.000 genes. More than 80% of the DNA of bread wheat consists of repeated DNA sequences with transposons and retrotransposons representing the highest proportion (Devos et al., 2005). Intensive genetic analyses showed that synteny perturbations between the three genomes are correlated with recombination rates along chromosomes (Akhunov et al., 2003a,b). Physical mapping through the use of deletion lines led to the conclusion that the genes are not evenly distributed along the chromosomes but are predominantly located in telomeric regions (Qi et al., 2004). However, first data on the DNA level obtained through the comparative sequencing of large DNA regions have shown that the three genomes differ considerably at their DNA sequence level predominantly caused by the presence/absence of retrotransposons and other repetitive sequences but also through the shuffling of genes within the individual genomes (Wicker et al., 2001; Appels et al., 2003; Keller et al., 2005).

Over more than fifteen years, molecular markers have been used in wheat genome analysis. Initially, with the RFLP technology, first genetic maps of wheat have been constructed for all 21 chromosomes. The advantage of the RFLP technology in wheat genome analysis includes usually the detection of one copy of the respective locus in each of the three wheat genomes simultaneously so that very large numbers of loci can be assigned to the wheat chromosomes or specific chromosomal regions through the use of deletion lines (Qi et al., 2004). Another advantage of the RFLP analysis is that through the hybridization of probes from closely related other Gramineae, it was possible to deduce that the wheat genomes and the genomes of other Gramineae, such as rice, maize, rye and barley share a very similar order of genes called synteny on the chromosomal level (Van Denze et al., 1995; Gale and Devos, 1998). RFLP analysis has however, only in a few cases been used for segregation analysis in wheat breeding except for the mapping of genes in wide crosses. This is mainly due to the fact, that the level of RFLP polymorphism is very low in wheat breeding material. Other PCR-based marker analyses such as multilocus systems, for example, AFLP have also not been widely used in wheat since this system also identifies only a limited level of genetic polymorphism in breeding material and routine analysis is being further complicated through the large genome size and the amount of repetitive sequences (Manifesto et al., 2001; Zhou et al., 2002).

2. MICROSATELLITE MARKERS

Only through the advent of microsatellite or SSR markers, the use of molecular markers has gained momentum in the last ten years since the first SSR markers have been described for hexaploid wheat (Devos et al., 1995; Röder et al., 1995; Bryan et al., 1997). Microsatellite markers are based on tandemly repeated DNA

sequences of short repetitive motives (e.g. poly CA, poly CT, poly AT and other repeated sequences of 3–5 bases). The variability of microsatellite sequences in a genome is not based on point mutations but on the variation in the number of the simple sequence repeats. Such variation occurs approximately ten times more frequently through processes such as slippage during replication or unequal crossing over (Hancock, 1999). This makes SSRs the most suitable and polymorphic marker system in species with a low level of polymorphism such as wheat.

2.1. Wheat Microsatellite Markers

The first large set of microsatellite markers for the wheat genome has been published in 1998 (Röder et al., 1998b). With these markers it could be shown that microsatellite markers have a number of characteristics that make them the currently best-suited marker system for the analysis of hexaploid wheat. Wheat microsatellite markers detect a much higher level of variability than RFLPs and other marker types (e.g. AFLPs), especially in closely related wheat germplasm and varieties such as they are used for breeding. Approximately 50% of the wheat SSR markers detect only a specific locus on one of the three genomes and thus are genome-specific. If they amplify from more than one of the three wheat genomes, the amplified fragments are often clearly distinguishable on high resolution gels. SSR markers are multiallelic and detect up to more than 30 different alleles in the wheat germplasm for a given locus which makes them in their information content significantly superior to biallelic marker systems (Plaschke et al., 1995; Röder et al., 2002). The distribution of microsatellite markers along the wheat chromosomes does not significantly differ from that genes, which show partial clustering at the physical end of the chromosomes, although most of the currently used SSR markers are not in genes (Röder et al., 1998a). Finally, wheat microsatellite markers are amenable to high-throughput so that large numbers of markers can be analyzed with large numbers of plants. This make them the preferred marker system for marker assisted selection and mapping in wheat and for breeding (Koebner et al., 2001; Koebner and Summers, 2003).

Over the last eight years, large sets of wheat microsatellite markers have been developed from various sources. At Gatersleben, most of the approximately 1.000 identified SSR markers are derived from genomic clones generated from single-copy sequences out of hypomethylated regions (Röder et al., 1998b; Pestsova et al., 2000; unpublished results). Other microsatellite markers, such as the wmc markers, are derived from libraries of wheat sequences that were generated through a variety of enrichment procedures (Song et al., 2002; Somers et al., 2004). Recently, a considerable number of wheat microsatellite markers have been identified from microsatellite motives identified within EST sequences through bioinformatic data mining (Eujayl et al., 2002; Holton et al., 2002; Gao et al., 2004; Yu et al., 2004; Peng and Lapitan, 2005). In general, the developed wheat SSR markers are of different amplification quality and have a varying number of detected loci. Nevertheless, it becomes now clear that microsatellite markers generated

from microsatellite motives within ESTs are usually more highly conserved and can be used through a wider range of germplasm (Zhang et al., 2006) but on the other side are also significantly less polymorphic than markers derived from genomic sequences since EST-SSR contain on average less repeating units of the microsatellite motif (Varshney et al., 2005).

Currently, the mapping of approximately 2.000 to 2.500 SSR markers on the 21 wheat chromosomes has been published in a variety of publications (Table 1). The precise number of mapped SSR markers in wheat is at present not easily determinable since the published maps show considerable overlap in the used SSR markers and frequently in different mapping populations different loci on the three homeologous chromosomes have been identified. Furthermore, in a number of cases only the number of detected loci is specifically described. Finally, no comprehensive sequence comparison between the sequences used for marker generation and no mapping data integration has been performed for all markers so that it is possible that a considerable number of microsatellites markers that detect the same sequence has been developed independently in different laboratories, especially in the case of EST-SSRs mined from public databases. Figure 1 shows a map generated with more than 1.000 unique SSR markers generating 1169 mapped microsatellite loci which was established in collaboration between the IPK and TraitGenetics representing currently the genetic map with the largest number of unique wheat SSR markers.

In wheat breeding, SSRs are increasingly being used as the marker backbone for a variety of purposes. These include the localization of individual genes onto the 21 wheat chromosomes such as, for example, disease resistance genes or genes affecting other agriculturally important traits. A large variety of papers has been summarized in a review (Röder et al., 2004) and a website (<http://maswheat.ucdavis.edu>). Furthermore, wheat microsatellite markers have been used for the localization of a large set of QTLs (quantitative trait loci) affecting morphological and agronomically important traits (e.g. Perretant et al., 2000; Börner et al., 2002; Huang et al., 2003; 2004). Other examples for important mapped QTLs are loci affecting resistance against the scab disease caused by *Fusarium* for which several populations have been used (Anderson et al., 2001; Buerstmayr et al., 2002; del Blanco et al., 2003; Bai and Shaner, 2004). For these QTLs, currently large SSR marker assisted selection projects are in progress. Further applications for which wheat microsatellite markers have been used include the characterization of large numbers of wheat varieties grown in Europe and North America, as well as the characterization of wheat lines from germplasm collections for the determination of genetic diversity over time (Donini et al., 2000; Christiansen et al., 2002; Huang et al., 2002; Pestsova and Röder, 2002; Röder et al., 2002). Many of these aspects have recently been published in a number of reviews (Koebner et al., 2001; Koebner and Summers, 2003; Röder et al., 2004). Finally, it is very likely, that wheat microsatellite markers will in the future also be used as backbone for the anchoring of a physical map of the wheat genome onto the genetic map and within the map-based isolation procedure for genes from the wheat genome, as it has been described already in a few cases (Stein et al., 2000; Keller et al., 2005).

Table 1. Published SSR maps in wheat with the number of mapped loci

Lab-designator of mapped SSRs (Number of mapped SSR loci)	Mapping population	Remarks	Reference
gwm (279)	Opatá 85 × W7984 (ITMI-population)	SSRs isolated from genomic libraries of A, B and D genomes	Röder et al. (1998)
psp (53)	CS × synthetic population	SSRs isolated from genomic libraries of A, B and D genomes	Stephenson et al. (1998)
gdm (46)	ITMI-population	SSRs isolated from genome D (<i>Ae. tauschii</i>)	Pestsova et al. (2000)
wmc (66)	ITMI-population	SSRs isolated from enriched genomic libraries of A, B and D genomes	Gupta et al. (2002)
DupW (22) cfd (84)	ITMI-population Courtot × CS population	SSRs isolated from ESTs SSRs isolated from genome D (<i>Ae. tauschii</i>)	Eujayl et al. (2002) Guyomarc'h et al. (2002)
cnl, ksum (149)	ITMI-population, physical mapping	SSRs isolated from ESTs	Yu et al. (2004)

(Continued)

Table 1. (Continued)

Lab-designator of mapped SSRs (Number of mapped SSR loci)	Mapping population	Remarks	Reference
wmc, gwm, gdm, cfa, cfd, barc (1235)	Consensus map	SSRs from various sources	Somers et al. (2004)
cfa, cfd, gpw, ksu, gwm, barc (725)	Physical mapping on multi-tetrasomic and deletion lines	SSRs from various sources	Sourdille et al. (2004)
xx-SSR (101)	ITMI-population and WSpop and LHpop	SSRs isolated from ESTs	Gao et al. (2004)
cwem (48 as PCR-based markers of total of 266 eSSR loci) barc (347)	Physical mapping on multi-tetrasomic and deletion lines	SSRs isolated from ESTs	Peng and Lapitan (2005)
hbg, hbe, hbd (250)	ITMI-population, physical mapping Kitamoe × Münstertaler, intraspecific DH-population	SSRs isolated from sheared genomic libraries of A, B and D genomes SSRs isolated from genomic libraries and ESTs	Song et al. (2005) Torada et al. (2006)

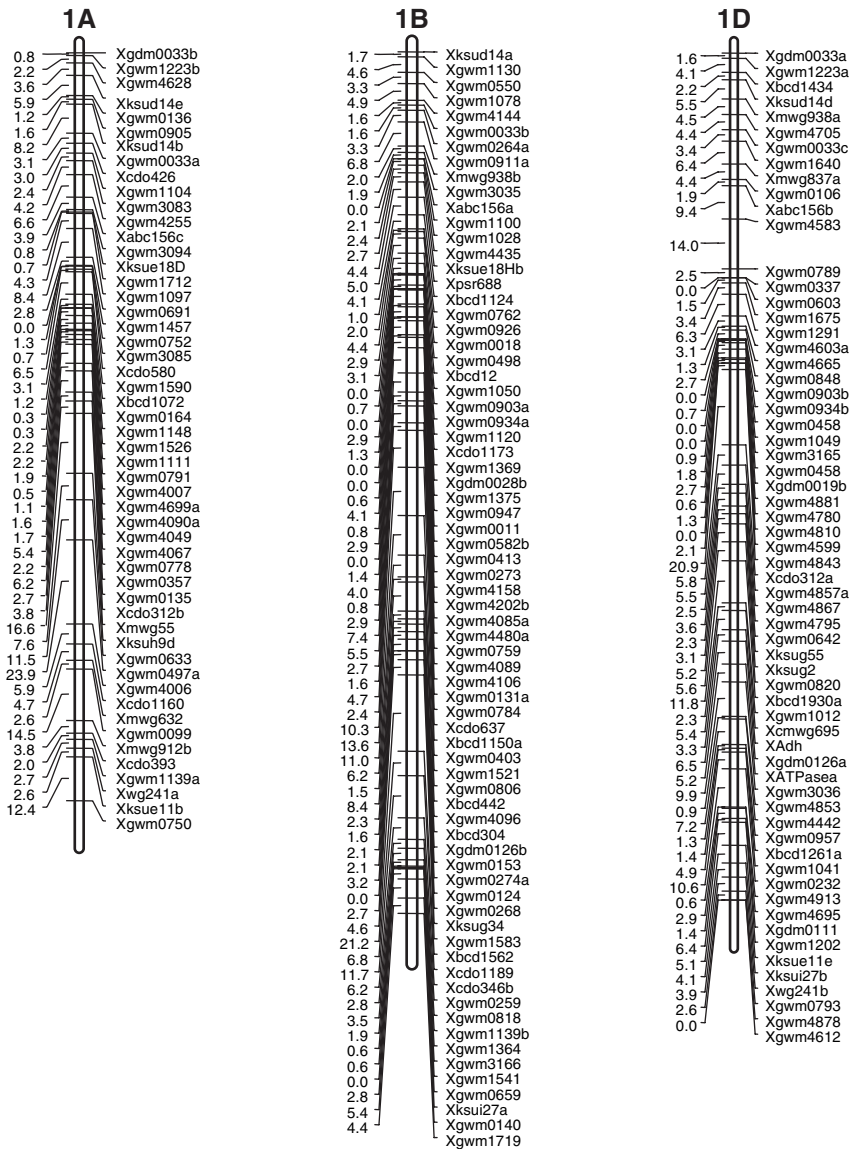


Figure 1. Molecular linkage map of wheat based on 70 recombinant inbred lines derived from the cross Oyata \times W-7984, the so-called ITMI-population (International Triticeae Mapping Initiative). The microsatellite loci carrying the lab designators “gwm” (Gatersleben wheat microsatellite) or “gdm” (Gatersleben D-genome microsatellite) are placed in a framework of previously mapped RFLP markers

In summary, wheat SSR will still be the tool of choice for wheat genome analysis and wheat breeding over the next several years, especially if the number of useful markers can be increased. Plant breeding companies are increasingly using the

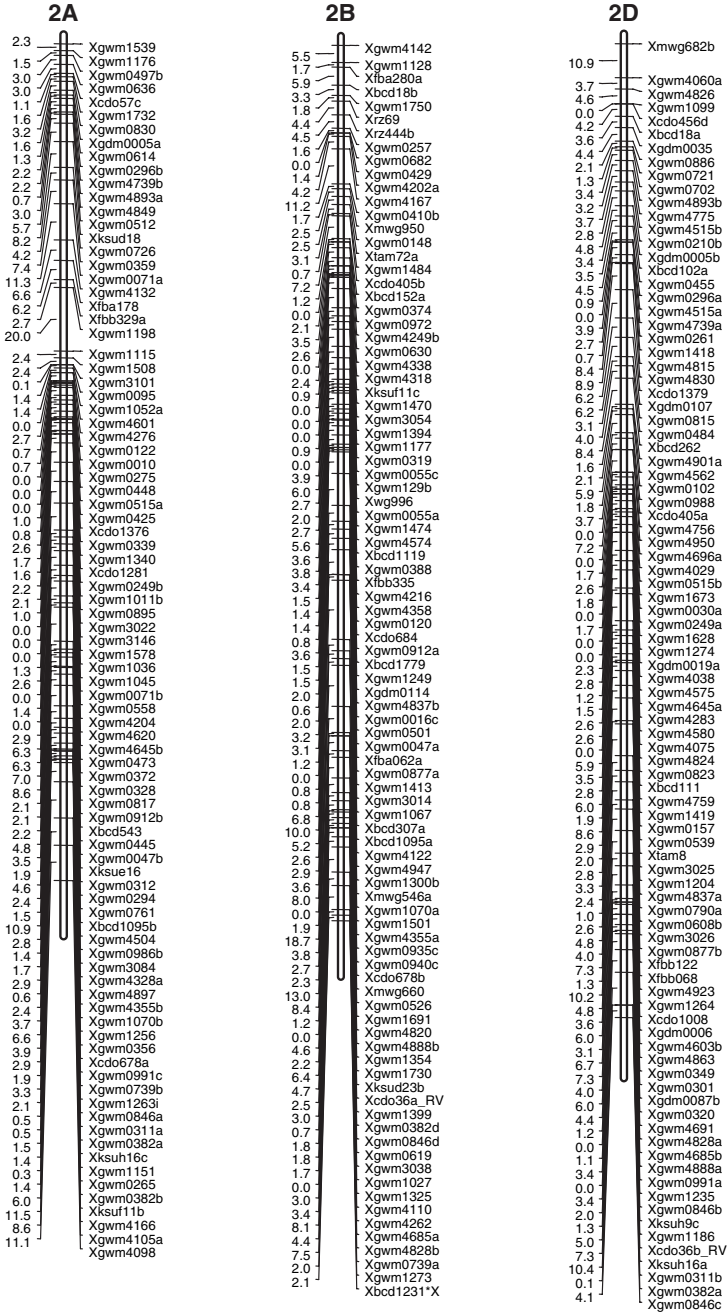


Figure 1. (Continued)

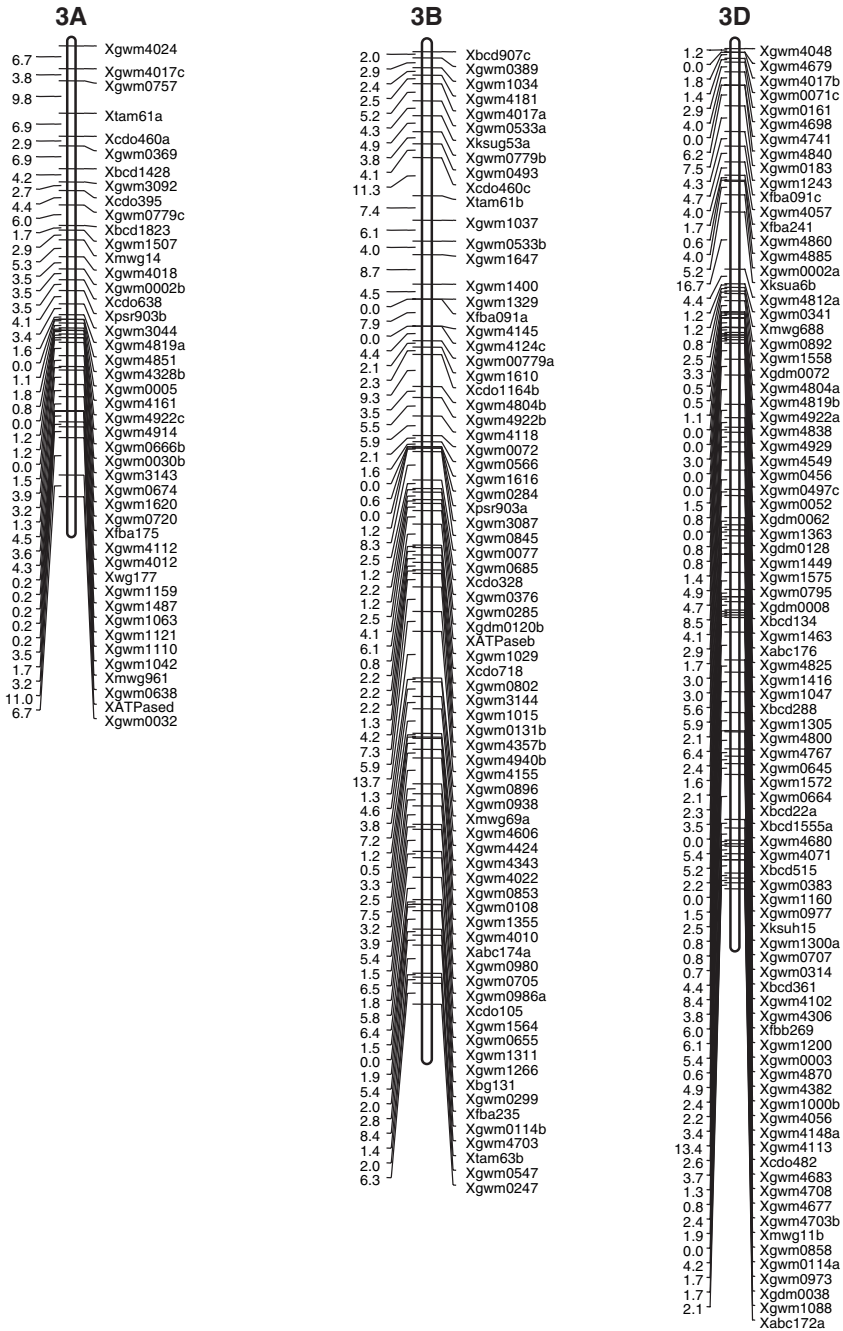


Figure 1. (Continued)

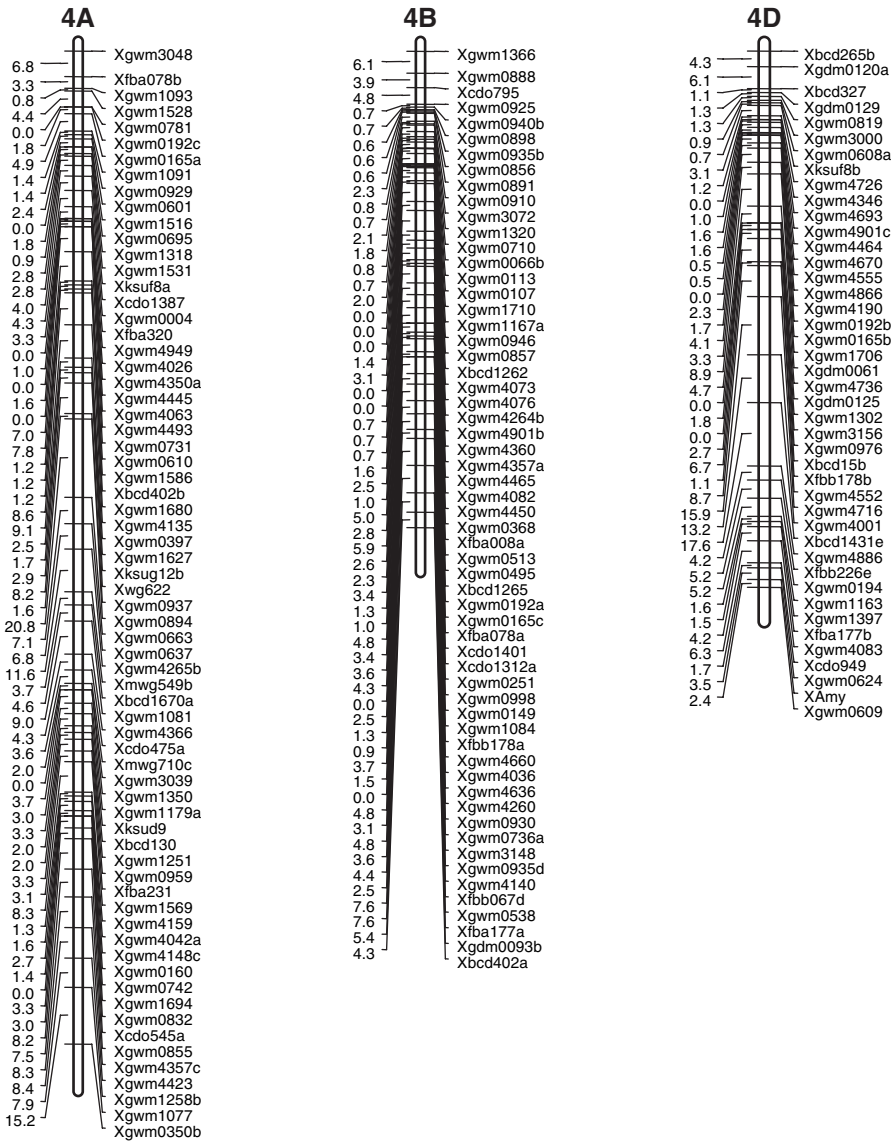


Figure 1. (Continued)

advantages of wheat microsatellite markers during marker-assisted selection and backcrossing of important traits into elite material and the development of new varieties (Koebner and Summers, 2003; Powell and Langridge, 2004). Specific examples for such applications are also described in other chapters of this book.

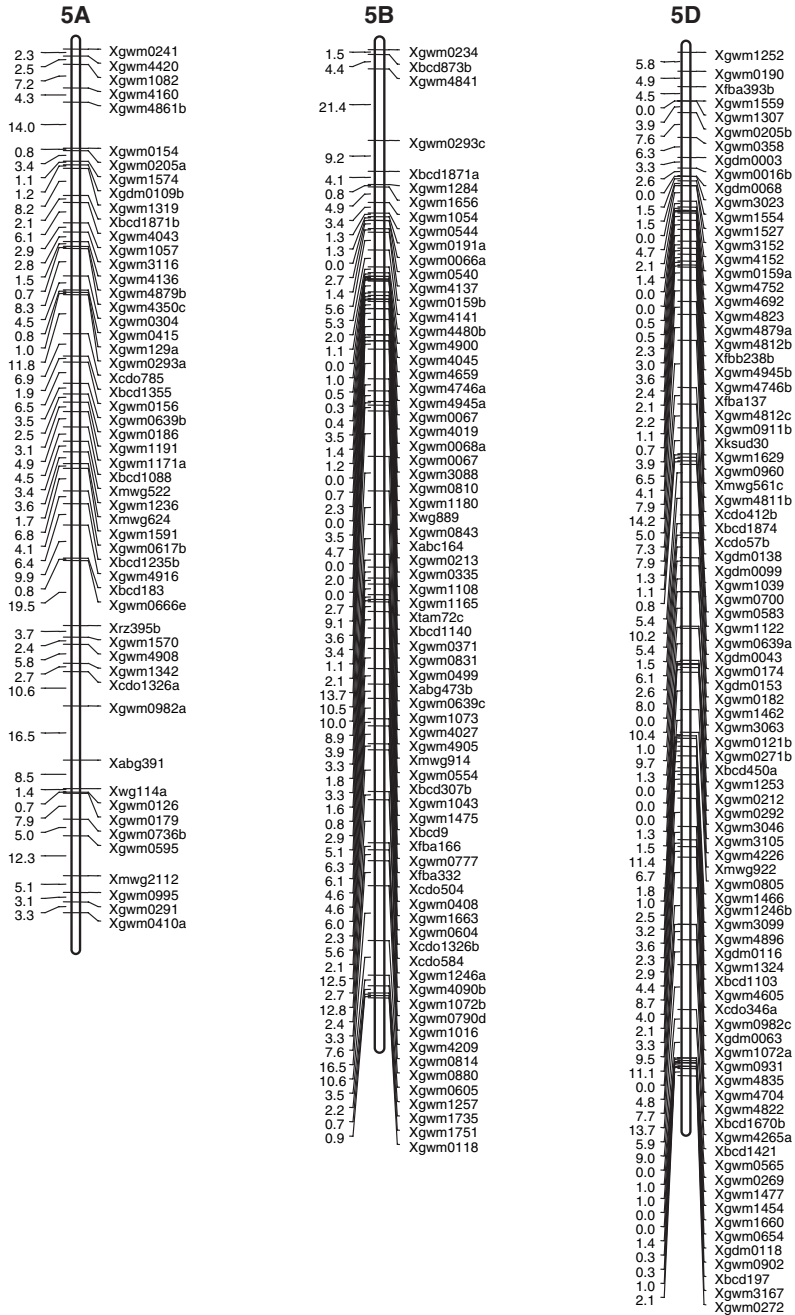


Figure 1. (Continued)

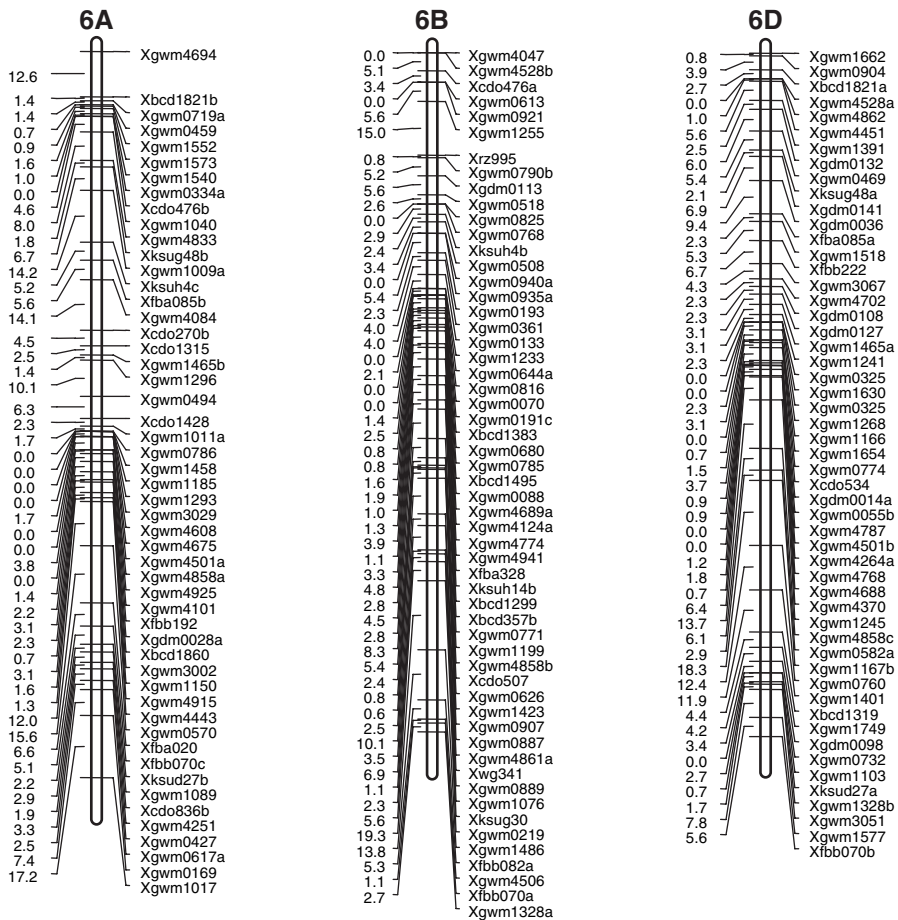


Figure 1. (Continued)

3. SINGLE NUCLEOTIDE POLYMORPHISMS OR SNP MARKERS

SNP markers are based on the variation of a specific nucleotide at a given sequence position between individuals. Predominantly, such variation occurs as biallelic alternative bases or as insertion/deletions of individual or small numbers of nucleotides. SNPs have in the last years gained considerable interest due to the fact that they are the smallest unit of genetic variation and being the basis of most genetic variation between individuals, they occur in virtually unlimited numbers. SNPs in coding sequences create furthermore the possibility of changes in the amino acid sequence within a protein (if they are not silent) and might have an effect on protein function and thus monogenic or polygenic traits associated with the expression of such genes (Johnson et al., 2001). SNP analysis has been spearheaded in human genome

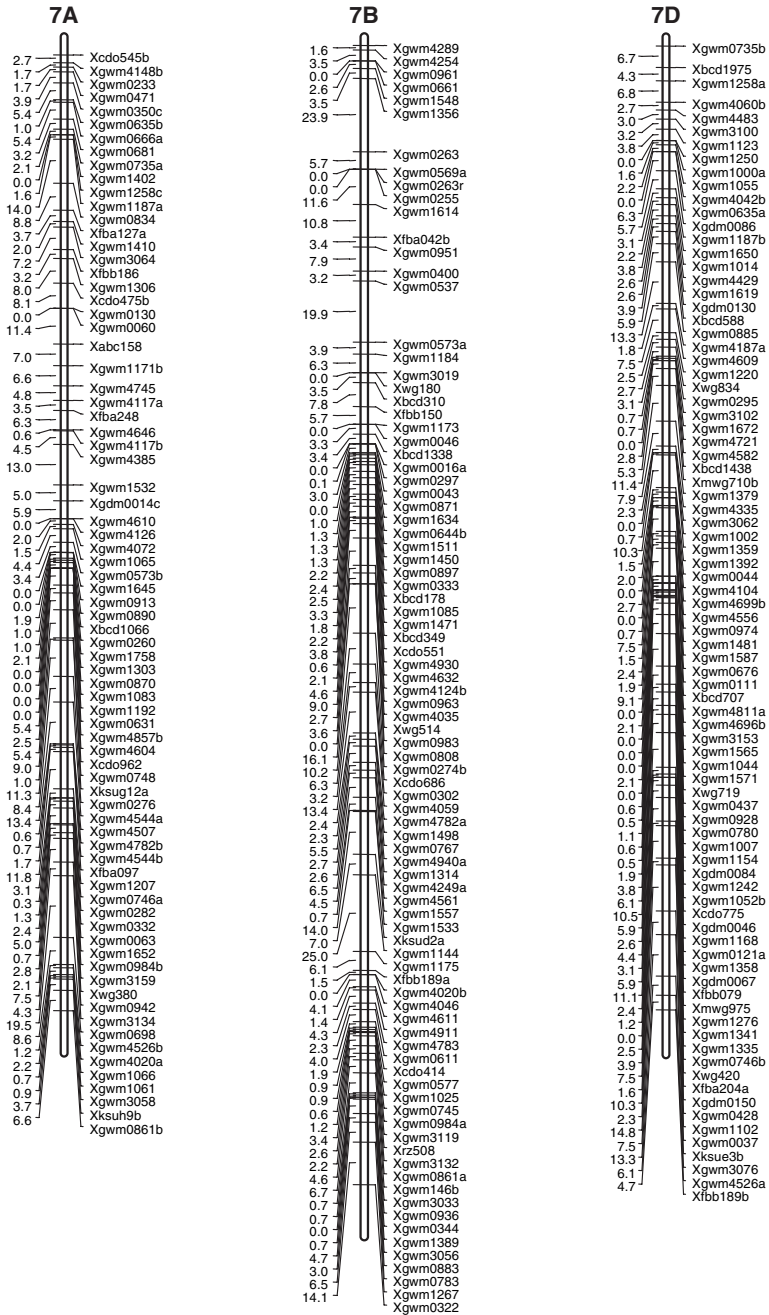


Figure 1. (Continued)

analysis, where meanwhile more than 2 million SNPs have been identified which represent in their entirety the majority of the genetic variation within the single copy and expressed part of the human genome (Sachidanandam et al., 2001).

The interest in SNP markers has further accelerated through the development of cost-efficient, high-throughput multiplexing (many markers being analyzed simultaneously) analysis techniques based on chips or other array techniques. Up to several million SNPs can now be analyzed simultaneously in the human genome with costs of a few cents per individual locus. Furthermore, individual SNPs can also be analyzed through a variety of technologies that permit the determination of individual genotypes in an unsurpassed speed and accuracy such as, for example through the use of fluorochrome-based analysis technologies that create data in real-time (e.g. Taqman, Ampliflour, Invader) and/or in a quantitative fashion (Gut 2001, 2004; Kwok, 2001).

Further hopes regarding the use of SNPs in routine analyses have been raised through the identification of haplotypes. Haplotypes are closely linked SNPs which occur along a chromosome in clearly defined structures or patterns (alleles) that extend over hundreds of base pairs or even several kilobases. The haplotype structure of SNPs alleviates the problem of scoring an extremely large number of SNPs by not requiring the analysis of each individual SNP in a genome but only of a limited number for genome coverage. Considerable efforts have been put into the identification of the haplotype pattern of the human genome (<http://hapmap.org>) but at present it is still not clear how efficient the analysis of haplotypes is for the identification of quantitative traits (Johnson et al., 2001; Foster and Sharp, 2004).

In plants, large scale SNP development and analysis project have been performed predominantly in diploid crop plants such as maize (Ching et al., 2002), barley and soybean (Zhu et al., 2003) where meanwhile more than 1.000 genes with SNPs were identified each. These data have demonstrated that SNPs are present in large numbers in crop plants and that they share similar features (e.g. presence as haplotypes) as in other eukaryotic species (Rafalski 2002a,b; Kahl et al., 2005).

3.1. SNP Identification in Wheat

SNP markers are usually identified through the comparative sequencing of individual lines or varieties or the bioinformatics analysis of EST data generated from a variety of lines (Rafalski, 2002a,b). With the advent of complete genome sequences for a number of plants such as in the model organisms *Arabidopsis thaliana* or rice (*Oryza sativa*), SNPs can be readily identified in basically unlimited numbers in single-copy DNA once such information is available.

Due to its large genome sequence, complete genome sequencing of the wheat genome is still at least five years and probably more years away, so that these resources will not be available in the near future. Furthermore, SNP identification through comparative sequencing is not easily possible in the hexaploid wheat genome. The main reason is the fact that with a normal primer pair usually the locus is being simultaneously amplified from the three different genomes so that even

when only a single fragment is identified after PCR amplification, the sequencing of that product is composed of three different sequences which make the analysis of the data at least extremely difficult. In many cases, the sequence is impossible to determine since one of the three genomes harbours insertions/deletions pushing the sequence out of frame for that genome. With these problems, direct sequencing is not feasible on a routine basis in wheat and other more complex approaches for SNP identification have to be used.

The most direct way of identifying SNPs in the wheat genome is the bioinformatic mining of wheat ESTs that are available in the respective EST databases. Approximately 500.000 ESTs from the wheat genome have been deposited in the EST databases making the wheat genome one of the best-sampled plant genomes (e.g. Lazo et al., 2004). Through concerted efforts of the international wheat community, these ESTs have been sampled from a variety of lines or cultivars so that SNPs between these lines could be identified. One of the pitfalls of EST sequence data is that the sequence quality is usually not better than 99% or a Phred-score of 20 meaning that on average 1 base out of 100 is incorrect. Thus, comparing individual sequences to each other for SNP identification is not easily possible and further complicated by the presence of highly related sequences from the other two genomes. This problem can be circumvented by the clustering of all sequence data generated from a wheat gene. EST clusters with a sufficiently deep sequence cover (several copies of ESTs from each of the three genomes) permit the identification of a consensus sequence from each of the three genomes and these sequences can be compared to similar clusters for the same gene derived from other wheat accessions. Since the sequences of the three genomes can be readily discriminated in such a comparison due to their higher sequence variation, SNPs in the individual copies derived from the three (A, B and D) genomes can be identified in a quite reliable fashion. Several years ago, an initiative has been started to analyze sequence clusters of more than 1.000 wheat genes in that way. However, until now beside the results of a pilot study, no detailed data regarding the outcome of that study and the level of polymorphism have been published probably due to the complexity of such an approach (Somers et al., 2003).

Another approach towards the identification of SNPs is the reduction of the hexaploid wheat genome into a diploid by the use of genome-specific primers. The average sequence difference between genes in the three individual wheat genomes is in the range of a few percent. Genome-specific primers permit the amplification of PCR products from only one specific wheat chromosome and thus make comparative sequencing of PCR products from individual lines feasible. Furthermore, through its inbreeding nature, such sequences from individual genomes are readily analyzable since only one allele should be amplified. Genome-specific primers can be generated from clustered EST data through the generation of primers with genome-specific 3'-ends. Since the coding sequences of wheat genes are highly conserved, frequently one of the two genome-specific primers is being derived from the less-conserved 3'-non-coding end of the respective gene. The pitfall of this approach is however, that not all potentially genome-specific primers do in

fact amplify a genome-specific product. Usually, several primer pairs have to be tested for obtaining one good genome-specific PCR product, thus making the approach quite laborious through the requirement of large-scale bioinformatic data processing and primer testing with a yield of usually less than 50% of functional genome-specific primers. One advantage of wheat in this approach is that through the use of nullitetrasomic lines, it is quite easy to confirm the genome-specific nature of an amplification product and simultaneously assign this product to a specific wheat chromosome. Currently, this approach is being used by the wheat HapMap-project (<http://wheat.pw.usda.gov/SNP>), where large numbers of potentially genome-specific primers have been generated and tested for SNP identification in hexaploid wheat and its diploid and tetraploid ancestors. At TraitGenetics, we have also used this approach in a pilot study towards the identification of SNPs in hexaploid bread wheat where we have generated more than 200 confirmed genome-specific primer pairs (unpublished results).

Further methods for the identification of SNPs in wheat are also used. For the analysis of specific candidate genes in a number of lines or varieties with respect to the occurrence of SNPs, it is possible to add a cloning step after the amplification of the respective PCR product from the three different genomes for each line and then determine the DNA sequence of a representative number of clones from each line. This approach creates probably the most complete data set for a given gene without the need of generating genome-specific primers but it is not easily amenable to high-throughput SNP detection in large numbers of genes. Due to its low level of SNPs in wheat varieties that will be described below, approaches to identify wheat genes that contain SNPs within breeding germplasm will probably gain more interest in the future. Such approaches could be, for example, the identification of single feature polymorphisms (SFPs) through the use of chip-technologies by means of comparative analysis of wheat cultivars or the use of heteroduplex analyses techniques (e.g. nuclease treatment or denaturing HPLC) for a first screening (Martins-Lopes et al., 2001). Although these techniques will require sophisticated bioinformatics to discriminate polymorphisms between the three wheat genomes and SNPs between lines, they might in the future provide an important tool for the pre-selection of genes with useful SNPs in wheat, however, without alleviating the problem of generating genome-specific PCR products at a later time. Another way of identifying SNPs in genome-specific sequences has been the sequencing of amplified microsatellite-flanking regions. This approach has recently also enabled the identification of a number of SNPs adjacent to microsatellite sequences (Ablett et al., 2006).

3.2. SNP Polymorphism in Wheat

The level of DNA polymorphism in hexaploid bread wheat is quite low when looking at fragment length polymorphism via RFLP analysis. Since point mutations are also the basis of SNPs, it can be expected that the frequency of SNPs is also low in hexaploid bread wheat. First data on SNPs have demonstrated that the SNP

frequency in the hexaploid wheat germplasm is in the order of 1 SNP per 540 base pairs (Somers et al., 2003). With that, the SNP frequency in bread wheat is at least five times lower than in maize (Ching et al., 2002; Rafalski, 2002a). Comparable levels of SNP polymorphism are being found, for example, in tomato and soybean (Zhu et al., 2003) both being crops that have passed through severe bottlenecks during domestication and breeding. For wheat, a reason for a low level of SNPs is also that hexaploid bread wheat is a recently generated polyploid with less than 10,000 years of divergence since its generation. An SNP frequency of one SNP per 540 base pairs would mean that on average in each sequenced gene fragment from an EST approximately one SNP would be located. This is however not the case since in many cases SNPs are clustered in 3'-untranslated regions and introns, so that a considerable amount of sequenced amplicons does not contain SNPs. Furthermore, the currently published data are based on germplasm that has been collected from all over the world and also contains highly polymorphic lines such as Chinese Spring or synthetic wheat lines which have added diversity to the wheat germplasm through the recreation of hexaploid wheat through the artificial hybridization of *Triticum turgidum* with *Aegilops tauschii* (Caldwell et al., 2004).

For applications in plant breeding, the used molecular markers have to be polymorphic in the respective breeding germplasm. European, North American or Australian wheat germplasm does only contain a fraction of the entire genetic variation of hexaploid bread wheat. Thus, for the use of SNP markers, it is important to determine the actual level of sequence polymorphism in such breeding germplasm. Since SNPs are usually biallelic markers it is necessary for practical purposes that the identified SNPs do show a high allele frequency. The allele frequency of the minor allele for an SNP should be at least 0.2 or 20% in order to be useful in actual wheat marker analysis since SNP occurring in only one or a few lines are not generally useful. We have performed a pilot study regarding the level of SNP polymorphism in a number of wheat lines predominantly representing well characterized European germplasm through the comparative sequencing of 202 genome-specific amplicons. These data have demonstrated that only 75 (37%) of the sequenced genome-specific amplicons showed at least one SNPs in a set of 12 wheat lines of which 4 were non-European wheat lines. An analysis of the eight European wheat varieties that were selected to represent the range of the European wheat germplasm based on microsatellite data, demonstrated that approximately two thirds of the identified SNPs were also present in the European wheat breeding material. Many of the identified SNPs were not identified in only one line but in at least 2 lines indicating a relatively high allele frequency of these SNPs in the wheat germplasm. This is also substantiated by the fact that SNPs that were found in the four exotic wheat lines were quite frequently also present in the wheat varieties suggesting that with respect to SNPs at least the European wheat breeding material and varieties do cover a considerable amount of the nucleotide polymorphism that is found in the entire hexaploid wheat gene pool. The comparative sequencing approach has also shown that in case of more than 1 SNP occurring in an amplicon, these are usually present in the form of well defined haplotypes.

In most cases only two haplotypes were observed but in some cases up to four haplotypes were observed.

In summary, in only one out of 4 sequenced amplicons, one or more SNPs were identified in European wheat germplasm indicating that a large number of genome-specific amplicons need to be investigated in order to cover the wheat genome at a reasonable density. For example, if a density of on average one informative SNP per 10 centiMorgan interval between two representative European wheat lines should be needed, one would need to have access to a large number of sequenced gene fragments. With an estimated polymorphism level of 20% between two average wheat European wheat lines and a genome size of approximately 3.500 to 4.000 centiMorgan for the three genomes together, this would require the availability of sequence data from nearly 10.000 genome-specific amplicons to have a sufficiently high number of genes with SNPs available.

3.3. Detection Methods for SNPs in Wheat

Another point that is important concerning SNP analysis in wheat is the mode of detecting individual SNPs or large numbers of SNPs through multiplexed systems. Currently, there are large numbers of individual systems available that can be used for SNP analysis in plants (e.g. Lee et al., 2004; Giancola et al., 2006). With respect to wheat, basically no publications regarding SNP detection on a large scale exist so that it is not entirely clear what system would be best-suited. Furthermore, the hexaploid wheat genome will also make SNP analysis quite difficult. Most SNP assay systems are based on the identification of individual SNPs either in individual or multiplexed systems with two major technological approaches for that purpose (Gut 2001, 2004; Kwok, 2001). One approach requires the amplification of the respective locus with a set of specific primers followed by the actual assay by means of various detection systems. For example, the frequently used TaqMan system is employing for this a fluorescent dye and a linked quencher, that are separated from each other during the detection process. In Pyrosequencing, a minisequencing procedure is performed with a detection primer that is a few bases upstream or downstream of the actual SNP. Primer extension requires an assay primer that is directly adjacent to the SNP which will be extended by incorporating a fluorescently labelled dideoxynucleotide. The other approach does not require the amplification of specific fragments via PCR. Such techniques are mostly used in a multiplex fashion in order to detect large numbers of SNPs at many different loci. Examples for these are chip-based systems that require the hybridization of genomic DNA onto oligonucleotides which are capable of identifying the two alleles of an SNP through specific hybridization to one or the other allele. The oligo-ligation assay (OLA) system catalyzes the allele-specific ligation of two oligonucleotides. Again another technique that does not require prior amplification is the nuclease-based Invader-technology.

Both technological approaches will have their problems with regard to SNP analysis due to the fact that the respective SNPs have to be detected in the wheat

genome that contains three highly related genomes. Specifically, SNP systems that do not require a prior PCR step have the problem that usually all three wheat genomes directly adjacent to a given SNPs are identical. This means that the analysis primer will bind to more than one genome. As a result of that a codominant scoring of SNPs will be impossible or require an extremely quantitative system that in the worst case needs to be able to discriminate a 6:0 from a 5:1 and from a 4:2 ratio (6:0 - hybridization to all three genomes with the investigated genome homozygous for allele 1; 5:1 – hybridization to all three genomes with one genome heterozygous; 4:2 – hybridization to all three genomes with one genome homozygous for allele 2). At present, it is not clear whether this can be achieved with sufficient accuracy so that in most cases SNPs detected with these non-PCR-based systems could only be scored as presence/absence and thus providing only 50% of the actual information compared to a codominant marker. SNP detection methods that require a PCR amplification step prior to the actual SNP assay do not face this problem because for the amplification of the actual locus, genome-specific primers could be used. In that way only one of the genomes will be assayed and only the three allelic states also observed in a diploid organisms will be present so that codominant scoring will be possible. In principle, genome-specific primers already exist in case of SNP identification via comparative sequencing but the amplified fragments for comparative sequencing are usually large in order to obtain as much sequencing information as possible and for SNP analysis fragments should be amplified for most technologies that are less than 100 base pairs since larger fragments result frequently in unequal amplification and require the optimization of the amplification conditions for each individual marker making high-throughput analysis difficult.

4. OUTLOOK TOWARDS THE USE OF SNPS IN WHEAT BREEDING

The future use of SNPs in wheat breeding has to be regarded from different angles. The general use of SNPs in wheat breeding as a replacement of other markers (especially microsatellite markers) is still far away. Based on the first data towards SNP identification and SNP frequencies in wheat breeding material, it will need quite some time until a sufficiently large number of SNPs with a good allele frequency will be identified. This will require the coordinated efforts of many different laboratories since, for a reasonable genome coverage a minimum of 10.000 genome-specific amplicons have to be investigated for the presence of SNPs. Although first efforts have been made to identify SNPs in wheat on a large scale through the wheat HapMap project (<http://wheat.pw.usda.gov/SNP>), it is very likely that such an effort will require several years until completion. Furthermore, it is at present not clear what technology could be used for assaying SNPs on a large and cost efficient scale in hexaploid wheat since all currently available technologies have problems with SNP analysis in hexaploid species. Thus it is very likely that over the next five years genome-wide SNP analysis will not be used widely in wheat breeding efforts.

In the short term, SNP markers will however gain significantly more importance for the analysis of individual genes. With established techniques for the generation of genome-specific primers and comparative sequencing, SNP analysis with a variety of techniques that involve a pre-amplification step with genome-specific primers will certainly be used for the analysis of specific genes that have a known influence on specific traits or have to be considered as interesting candidate genes. Examples for this have already been published such as the SNP analysis of the grain hardness locus (Giroux and Morris, 1998; Huang and Röder, 2005), the analysis of storage protein loci which control aspects of milling and baking quality (Zhang et al., 2003; Ravel et al., 2006) and other genes (Ellis et al., 2002; Yanagisawa et al., 2003; Blake et al., 2004). It is certain that we will see more progress being made in the near future towards the SNP analysis of such genes in wheat breeding since such individual analyses can be performed in a cost-efficient way on large numbers of individual plants once such assays have been developed and optimized. The same will be true for markers tightly linked traits of interest in wheat breeding which are converted to SNP markers for high-throughput analysis.

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CHAPTER 2

MOLECULAR MARKERS AND QTL ANALYSIS FOR GRAIN QUALITY IMPROVEMENT IN WHEAT

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Abstract: Molecular marker technology is playing an increasingly important role in the selection of wheat lines with improved quality attributes. This is due to the identification of molecular markers tightly linked to chromosome regions involved in the control of important quality characteristics such as dough properties, grain hardness, semolina and flour colour, grain protein content and starch composition, which strongly influence wheat end use, and its nutritional and market value. Marker assisted selection (MAS) will increase the efficiency of the breeding process, particularly when phenotyping requires laborious and time-consuming analyses, performed in advanced generations because of the relatively large amount of grain required. Moreover, the implementation of MAS allows the selection of individuals carrying the favourable alleles at the target loci, and also the pyramiding of favourable QTL alleles from different sources and for different traits. This notwithstanding, the progress obtained until now in applying MAS to quality characteristics has been slow compared to other traits.

1. INTRODUCTION

Durum and bread wheats are the major foods for much of the human population and are mainly consumed as processed products because of the unique functional properties they confer to the derived foods. Improving end-use quality has become of increasing importance to wheat breeders over the past few decades with an emphasis on developing cultivars (cvs.) of durum and bread wheat for specific applications such as bread (leavened, flat and steamed), other baked goods (cakes,

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cookies, crackers, etc.), pasta and noodles, and a wide range of other products of restricted regional uses. Processing and end-use quality of wheat-based products are influenced by several factors such as protein content and composition, starch, kernel hardness and lipids.

Some of these traits are complex (i.e. controlled by several genes and influenced by environmental factors and management procedures), while others are simply inherited. The selection of superior breeding lines with improved quality characteristics, as well as the dissection of their complexity, are being greatly favoured by the establishment and exploitation of biochemical and molecular markers (Gale 2005; Howitt et al. 2006). The ultimate aim is to tailor wheat cvs. with improved quality and to satisfy the specific end-use requirements of the market and consumers.

2. GRAIN PROTEIN CONTENT

Grain protein content (GPC) is one of the major quality traits in durum and bread wheat strongly associated to end-use performance of derived products. In bread wheat, the existence of a linear relationship between protein content and loaf volume have been reported (Finney and Barrimore 1948); similarly, in durum wheat it has been clearly established that high protein content and strong gluten are major factors for producing pasta with superior cooking properties, better cooking firmness and tolerance to overcooking (D'Egidio et al. 1990; Marchylo et al. 1998). The variation induced by environmental factors and the negative association with grain yield have hindered progress in increasing grain protein content by traditional breeding approaches, but the availability of genetic stocks such as aneuploids (see Snape et al. 1995 for advantages in locating QTL's using single chromosome recombinant lines) and large gene pools together with marker-mediated approaches are leading to the improvement of this trait.

Genes to improve protein content have been sought among wheat related species and promising materials have been detected in the tetraploid wild wheat progenitor *T. turgidum* ssp *dicoccoides* with values ranging from 14 to 29% protein (Avivi 1978; Grama et al. 1984). In particular, the accessions FA15-3 of *T. turgidum* ssp *dicoccoides* identified by Avivi (1978) has been used to introduce the high protein content trait into durum and bread wheats. Joppa and Cantrell (1990) developed a set of disomic substitution lines in the durum wheat cv. Langdon (LDN); in each line a pair of chromosomes of LDN were replaced by their homeologues derived from the *T. dicoccoides* accession FA15-3. The analysis of a population of recombinant substitution lines (RSLs) obtained from the cross between the high GPC line LDN(DIC-6B) and the cv. LDN led to the identification of a quantitative trait locus (QTL) for GPC on the short arm of chromosome 6B in the *Xmwg79-Xabg387* interval (Joppa et al. 1997). This QTL, designated *QGpc.ndsu.6Bb*, accounted for approximately 66% of the variation in protein content in this cross, suggesting that a major gene or a closely linked group of genes were segregating in this population.

Additionally, the significant increase in GPC was associated with an increase in the pasta quality (Joppa et al. 1991), while not significantly affecting grain yield or

kernel weight (Cantrell and Joppa 1991). Following these results, Khan et al. (2000) developed PCR-based markers to facilitate the introgression of the *QGpc.ndsu.6Bb* region into commercial tetraploid and hexaploid varieties: the microsatellite markers *Xgwm508* and *Xgwm193* proved particularly useful to monitor the introgression of the *T. dicoccoides* chromatin containing the high GPC gene(s). More recently, using field trials with ten replications and a large set of secondary RSLs obtained from the cross between LDN and RSL65, in a line carrying the complete *T. dicoccoides* segment flanked by the *Xgwm508* and *Xgwm193* markers, the source of GPC variation was mapped as a single Mendelian locus within a 2.7-cM region encompassed by the restriction fragment length polymorphism (RFLP) markers *Xcdo365* and *Xucw67*. This locus has been designated *Gpc-B1* (Olmos et al. 2003). To further delimit the location of *Gpc-B1*, the microcolinearity between rice and wheat present in this region was used by Distelfeld et al. (2004, 2006). This approach resulted in narrowing the position of the *Gpc-B1* locus to a 0.3-cM region flanked by PCR markers *Xucw79* and *Xucw71* and in identifying a codominant PCR marker (*Xuhw89*) tightly linked to the *Gpc-B1* locus. A candidate gene (a transcription factor regulating senescence) has now been cloned (Uauy et al. 2006), thus providing the possibility of using perfect markers in marker-assisted selection (MAS) programmes.

Mesfin et al. (1999) used three hard red spring wheat recombinant lines to identify genomic regions associated with high protein content inherited from *T. turgidum* ssp *dicoccoides*. A single region associated to high GPC was detected with five RFLP markers located near the centromere on chromosome 6B. It is possible that this high protein content QTL corresponds to the locus already described by Joppa et al. (1997); in fact, both research groups used the same *T. turgidum* ssp *dicoccoides* accession as donor of high GPC.

Khan et al. (2000) compared the *T. dicoccoides* accession FA15-3 and Glupro, a hexaploid high GPC cv. obtained from a three-way cross involving two bread wheat cvs. and FA15-3 (donor of the GPC gene/s), to determine the size of the *dicoccoides* chromosome 6B segment transferred to the bread cv. Using microsatellites, amplified fragment length polymorphism (AFLPs) and RFLPs, the authors showed that two separate segments from the *dicoccoides* accession were transferred to Glupro chromosome 6B, one in the distal region of the long arm and the other in the proximal region of the short arm, this latter encompassing the GPC QTL with the highest LOD identified by Joppa et al. (1997).

In a different study, Blanco et al. (1996) tested a set of 65 recombinant inbred lines developed by single seed descent from the cross between the durum wheat cv. 'Messapia' (low protein content) and the wild tetraploid *T. dicoccoides* accession MG 4343 (high protein content) to locate QTLs controlling GPC. One QTL was identified on each of the chromosome arms 4BS, 5AL, 6BS and 7BS, and two distinct QTLs were identified on chromosome 6A. According to the environment considered, the six QTLs explained from 49.2 to 56.4% of the phenotypic variation for GPC. Only some of the markers were found to be negatively associated with grain yield and/or seed weight in one or two environments. An additional locus

was detected on chromosome 7A (Blanco et al. 2002) when the same lines were cultivated in different environments and years.

Moreover, Blanco et al. (2006) used the backcross inbred lines approach to introgress *T. dicoccoides* high GPC alleles in durum wheat and to identify molecular markers linked to chromosome regions controlling high GPC. Using SSRs and AFLPs, three QTLs with major effects on GPC were detected on chromosome arms 2AS, 6AS and 7BL, identified by the markers *Xcfa2164*, *XP39M37(250)* and *Xgwm577*, respectively.

Prasad et al. (1999) tested a set of RILs derived from a cross between two bread wheat genotypes PH132 (high GPC) and WL711 (low GPC) and identified a sequence-tagged microsatellite site (STMS) *Xwmc41* associated to a QTL for protein content (designated *QGpc.ccsu-2D.1*), located on the chromosome arm 2DL. The same research group identified additional molecular markers linked to QTLs for high GPC: a significant association was found between *Xwmc415* and a QTL designated *QGpc.ccsu-5A.1* (Harjit-Singh et al. 2001).

Additional studies evidenced several major GPC QTLs on different chromosomes in the cultivated and the wild wheat gene pools and proved allelic relationships between some of the detected loci (Snape et al. 1995; Sourdille et al. 1999; Perretant et al. 2000; Turner et al. 2004).

3. GLUTEN PROTEINS

It is now accepted that the amount and composition of gluten proteins (the viscoelastic mass remaining after washing the dough with salt solutions) is mainly responsible for qualitative differences, such as pasta-making and bread-making properties, among durum and bread wheat cvs. (see Shewry et al. 2003, for a comprehensive review on gluten proteins). Gluten proteins are composed of two fractions, gliadins and glutenins, mainly differing for their capability to form polymers: gliadins are monomeric molecules, while glutenins are polymers containing different subunits connected by intermolecular disulphide bonds. When glutenin polymers are reduced and released components separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), two major groups are identified which have been designated as High Molecular Weight Glutenin Subunits (HMW-GS) and Low Molecular Weight Glutenin Subunits (LMW-GS). Technological properties of both durum and bread wheat doughs are positively correlated with the molecular size of glutenin polymers, which are highly variable and can reach millions of daltons.

The gliadin fraction includes proteins which are subdivided into α/β -, γ - and ω -types according to their N-terminal amino acid sequences, where disulphide bonds, if present, are intramolecular. Genes encoding gliadin components are located on the short arm of the homoeologous groups 1 and 6 chromosomes of the A, B and D genomes, at the *Gli-1* and *Gli-2* loci.

HMW-GS are encoded by genes present at the *Glu-1* loci on the long arms of the homoeologous group 1 chromosomes. Each *Glu-1* locus contains two tightly linked

genes encoding for two types of subunits (x- and y-type characterised by high and low molecular weight, respectively) differing in structural characteristics such as the number of cysteine residues, and the size and composition of the repetitive domain. The y-type gene present at the *Glu-A1* locus is always silent in tetraploid and hexaploid cultivated wheat, whereas the x-type gene at the same locus and the y-type at the *Glu-B1* locus are expressed only in some cvs.; this leads to variation in the number of subunits from three to five in bread wheat and from two to three in durum wheat. SDS-PAGE of seed proteins has been extensively used to assess HMW-GS composition of bread wheat cvs. Allelic variation has been detected at each *Glu-1* locus and the analysis of crosses between lines with different HMW-GS composition allowed the identification of subunits affecting breadmaking properties of different bread wheat cvs. In particular, the subunit pair 5+10 and 2+12 encoded by genes present at the *Glu-D1* locus have been correlated with good and poor breadmaking properties, respectively. The superiority of the pair 5+10 may be due to the additional cysteine residue present in subunit 1Dx5 at the beginning of the repetitive domain; this residue would promote the formation of glutenin polymers of larger size, thus endowing dough with increased strength (Greene et al. 1988; Lafiandra et al. 1993).

Similar differential effects of allelic subunits, encoded by the *Glu-A1* and *Glu-B1* loci have also been reported (Payne 1987).

LMW-GS are encoded by multigene families located on the homoeologous group 1 chromosomes at the *Glu-3* loci (*Glu-A3*, *Glu-B3* and *Glu-D3*) tightly linked to the *Gli-1* loci. As compared to the HMW-GS, the LMW-GS proved to be more difficult to study and characterise due to their heterogeneity and a range of different analytical tools have been used to elucidate their complexity and role in quality aspects. LMW-GS have been subdivided into the B, C and D types according to their biochemical characteristics (Jackson et al. 1983). Moreover, the B type subunits can be divided into three groups (LMW-m, LMW-s and LMW-i) based on the first amino acid residue (Met, Ser and Ile, respectively) of the mature protein. The LMW-i type has been detected more recently than the others and seems to be mainly encoded by genes present at the *Glu-A3* locus (Cloutier et al. 2001; Ikeda et al. 2002; Zhang et al. 2004). Additionally, this last group of subunits shows striking structural differences compared to the LMW-m and LMW-s groups as they lack an N-terminal region and all the cysteines are localized in the C-terminal region, though sharing the same number of cysteine residues (eight) with the other LMW-GS types. This difference in cysteine distribution might impact glutenin polymer formation and, more in general, gluten interactions and be responsible for quality differences. The C and D type subunits are composed mainly of proteins related to α/β -, γ - and ω -gliadins which have variable numbers of cysteine residues. It has been proposed that they are incorporated into the polymeric network by virtue of unpaired cysteines (D'Ovidio and Masci 2004).

Several studies have indicated that the allelic variation at the *Glu-3* loci is also associated to dough quality both in bread and durum wheat, and ranking of different alleles has been reported (see Juhász and Gianibelli 2006 for a review).

The availability of gene sequence data for both LMW- and HMW-GS made it possible to generate molecular markers, known as functional or perfect markers, closely linked or located within the gene of interest (Andersen and Lubberstedt 2003; Varshney et al. 2005), thus making available powerful tools to incorporate superior alleles in breeding materials.

In durum wheat, LMW-GS encoded by genes present at the complex *Glu-B3* locus have been associated to both pasta cooking properties and bread making properties (Boggini and Pogna 1989). The two major alleles (*LMW-1* and *LMW-2*) detected at this locus are related to poor and good gluten viscoelastic properties, respectively (Payne et al. 1984; Pogna et al. 1990).

In the attempt to develop molecular markers capable of discriminating durum wheat genotypes with the *LMW-1* or *LMW-2* allele and thus providing an alternative approach to electrophoretic techniques, D'Ovidio (1993) developed a pair of oligonucleotide primers that produced two main PCR amplification products in all the analysed genotypes. The PCR fragment of lower size, common to all the durum genotypes tested, was not assigned to any specific chromosome, whereas the other was polymorphic between the lines with different allelic LMW-GS. Subsequently, D'Ovidio and Porceddu (1996) developed a new set of primers yielding a single amplification product, ranging in size between 780 (*LMW-1*) and 830 bp (*LMW-2*), capable of differentiating durum wheat cvs. with poor or good pasta making properties.

More recently, efforts from different research groups resulted in the development of bread wheat specific primers that permitted to amplify, isolate and study several LMW-GS genes associated to specific *Glu-3* loci, thereby contributing to the detailed description of their structural features. Moreover, these primers are capable of differentiating homo- and homoeoalleles thus making available molecular markers suitable to follow the introgression of superior alleles in breeding programmes (Zhang et al. 2004; Ikeda et al. 2006; Long et al. 2006; Zhao et al. 2006). In particular, Zhang et al. (2004) isolated and characterised the complete coding sequence of one LMW-GS gene for each of the seven alleles (from *Glu-A3a* to *Glu-A3g*) present in different bread wheat cvs. All sequences were classified as i-type LMW-GS genes based on the presence of an N-terminal isoleucine residue and eight cysteine residues located within the C-terminal domain of the predicted, mature amino acid sequence. Comparison of gene sequences obtained from different alleles showed a wide range of sequence identity between the genes, with between 1 and 37 single nucleotide polymorphisms and between one and five insertion/deletion events. Allele-specific PCR markers were designed based on the DNA polymorphisms identified between the LMW-GS genes, and these markers were validated against a group of bread wheat cvs. containing different *Glu-A3* alleles. Using the same approach Zhao et al. (2006) developed PCR markers for the identification of a few *Glu-D3* alleles.

Although SDS-PAGE has been invaluable for studying HMW-GS, it has some limitations, such as co-migration of certain subunits or difficulty in detecting differences in expression levels, resulting in incorrect identification of alleles with

different functional effects. Additionally, glutenin composition analysis can be carried out only on the mature grains. To overcome these limitations, allele specific PCR markers, based on DNA polymorphism present among the glutenin subunit genes, have been obtained to assess allelic variation at the *Glu-1* loci and to provide high-throughput analysis of different alleles in breeding materials. Differently from SDS-PAGE, PCR can be performed on leaf samples collected during the vegetative growth stages; this in turn allows for the selection of the genotypes with the desired allele composition before flowering.

D'Ovidio and Anderson (1994) used the presence of some differences in the nucleotide sequences of the 1Dx2 and 1Dx5 glutenin genes to develop two oligonucleotides specific for the subunit 1Dx5. Similarly, in the attempt to identify bread wheat lines carrying the superior pair of subunits 5+10, a codominant PCR marker was developed by Smith et al. (1994). Subsequently, primer specific for homo- and homoeo-allelic HMW-GS genes have been developed by several research groups (D'Ovidio et al. 1994, 1995; Lafiandra et al. 1997; Ahmad 2000; De Bustos et al. 2000, 2001; Radovanovic and Cloutier 2003).

The usefulness of molecular markers appears particularly relevant to discriminating different alleles for HMW-GS associated to the *Glu-B1* locus. In a study carried out on several Canadian bread wheat cvs., Marchylo et al. (1992) reported that, though allelic subunits 1Bx7 and 1Bx7* could not be distinguished on the basis of their elution times, when analysed by reversed phase high performance liquid chromatography, the proportion of the first subunit was significantly higher than the latter. D'Ovidio et al. (1997) suggested that the results of Marchylo et al. (1992) could be due to a gene duplication involving the gene coding for the 1Bx7 subunit. Recent electrophoretic and chromatographic analyses of wheat cvs. and landraces from around the world revealed the large diffusion of the overexpressed 1Bx7 allele designated *Glu-B1a1* and its positive impact on dough technological properties (Butow et al. 2003, 2004; Vawser and Cornish 2004). Butow et al. (2004) utilised a co-dominant PCR marker, designed to amplify the 1Bx MAR region 750 bp upstream of the coding region of the *Glu-B1* gene, to discriminate bread wheat materials with the over-expressed subunit Bx7 from those with the subunit 1Bx7*. All the cvs. and landraces showing increased 1Bx7 expression (i.e. with the *Glu-B1a1* allele) produced a fragment longer than that of the other cvs. (~563 vs. 520 bp), indicating the presence of a 43 bp insertion in the MAR of the *Glu-B1a1* allele. A marker for the negative selection of the poor quality allele Bx6 was developed by Schwarz et al. (2004) using the DNA polymorphisms between the coding sequences of the x-type HMW-GS alleles 1Bx6, 1Bx7 and 1Bx17, whereas a set of dominant and codominant markers have been developed by Lei et al. (2006) for specific HMW-GS genes encoding 1By-type subunits. These markers resulted in an enhanced discrimination of alleles at the *Glu-B1* locus and particularly between alleles with contrasting effects on quality and difficult to screen by SDS-PAGE.

A further breakthrough in the use of PCR-based markers for the selection of HMW-GS alleles is represented by the introduction of the multiplex approach, in which more than one target sequence is amplified using more than one primer

pair, in order to score simultaneously for the presence of different homo- or homoeo-allelic variants (Ahmad 2000; Ma et al. 2003). Salmanowicz and Moczulski (2004) developed a multiplex PCR method to identify bread wheat genotypes with specific HMW-GS allele composition at the *Glu-1* complex loci (*Glu-A1*, *Glu-B1* and *Glu-D1*) by capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection. DNA fragments amplified using two triplex primer sets were well separated both by agarose slab-gel electrophoresis and CE-LIF; minor differences among the sequences of 1Ax2, 1Ax null, 1Bx6, 1Bx7, 1Bx17 and 1Dx5 genes have been revealed. CE-LIF can thus be an efficient alternative to standard procedures for early selection of useful wheat genotypes with good bread-making quality. In fact, compared to gel electrophoresis, CE-LIF requires samples of smaller volumes and results can be obtained in less than 20 minutes.

Novel alleles at the *Glu-3* and *Glu-1* loci have been identified in germplasm collections of tetraploid and hexaploid wheat and in related wild species, thus providing variation suitable for exploitation in wheat quality improvement (Shewry et al. 2006; D'Ovidio and Masci 2004).

4. STARCH

Starch is the major constituent of flour and semolina and accounts for 65–75% of wheat grain dry weight. Starch is composed of two types of polymers (amylose and amylopectin) whose relative amounts and structures are responsible for its unique physical and chemical properties with strong influences on functional properties of flour or semolina and on its specific uses in the food and manufacturing industries (Zeng et al. 1997; Yoo and Jane 2002). Amylose is an essentially linear α -1,4 glucan and contributes about 20–30% to the total starch, while amylopectin is a branched α -1,4 glucan containing about 5% α -1,6 branch points and constitutes the remaining 70–80% of the total starch. A number of enzymes are involved in starch synthesis with at least five known isoforms of starch synthases (SS, as reviewed by James et al. 2003). Four of these isoforms are involved only in amylopectin synthesis, with two forms of branching and debranching enzymes. The granule bound starch synthases (GBSSI or waxy proteins) are the sole starch synthases responsible for amylose synthesis in storage tissues (Nakamura et al. 1993). In bread wheat there are three waxy proteins with a molecular weight ranging from 59 to 60 kDa (Murai et al. 1999), designated Wx-A1, Wx-D1 and Wx-B1 encoded by three genes (*Wx-A1*, *Wx-D1* and *Wx-B1*) located on chromosome arms 7AS, 7DS and 4AL, respectively. The latter was originally located on chromosome 7BS before a translocation occurred between 7BS and 4AL chromosomes during wheat evolution (Miura and Tanii 1994; Yamamori et al. 1994). The different effects of the three waxy genes on the amylose content has also been assessed, with the *Wx-B1* gene showing the highest effect, followed by the *Wx-D1* and the *Wx-A1* gene (Miura et al. 1994, 1999).

Electrophoretic studies of bread and durum wheats led to the identification of partial waxy mutant lines, characterised by the lack of one or two waxy proteins.

Null *Wx-A1* and *Wx-B1* alleles have been found in Asian, European and North American wheat cvs. In particular, null alleles at the *Wx-A1* locus are fairly common in bread wheat from Japan, Korea and Turkey, while null alleles at the *Wx-B1* locus are very common in bread wheat from Australia and India (Yamamori et al. 1994, 1998). On the contrary, null alleles at the *Wx-D1* locus seem to occur more rarely (Yamamori et al. 1994; Boggini et al. 2001; Monari et al. 2005). Crossing of selected genotypes has permitted the combination of the different null alleles detected with the production of the entire set of partial and total waxy lines (Nakamura et al. 1995; Urbano et al. 2002). These genetic materials are of interest due to their impact on quality aspects and to their possible use in non-food applications. In fact, flour with reduced amylose content extends the shelf life of various baked products (Graybosch et al. 1998; Lee et al. 2001) and produces higher quality Asian noodles (Miura et al. 1994). In this respect, partial waxy wheat have been shown to be particularly suitable for the production of certain Asian noodles such as the Japanese Udon or white salted noodles, typically produced using medium-protein soft wheat. Wheat suitable for the production of Udon noodles typically has starch or flour with high swelling volumes and high peak pasting viscosity which have been associated with reduced amylose content and presence of GBSS null alleles, and in particular with the null *Wx-B1*. These results have been obtained by SDS-PAGE analysis of granule bound starch proteins, an approach which is particularly time consuming, as starch isolation is required before electrophoretic analysis, thus limiting the number of samples to be processed. In addition, electrophoretic results show poor resolution of different protein components and can only be carried out on mature grains. Thus, great efforts were devoted to the development of several PCR markers to study and assist the introgression of the waxy trait in advanced breeding lines.

A recessive PCR marker was developed by Briney et al. (1998) to identify lines null at the *Wx-B1* locus; it was thus possible an accurate starch quality identification using small amounts of leaf tissue or single seeds, enabling the screening of large segregating populations. Shariflou et al. (2001) developed a perfect codominant marker to discriminate the normal and mutant null alleles at the *Wx-D1* locus originated from the Chinese landrace 'Baihoumai'. Moreover, five primer pairs were designed by McLauchlan et al. (2001) using the available gene sequences to target each of the three waxy homoeoallele loci. Multiplex PCR has been used by Nakamura et al. (2002) to screen a large number of wheat samples from different countries in order to study waxy mutations and trace their origin.

Similarly to waxy proteins, mutant lines lacking one of the three possible Starch Synthase II (SSII) proteins have been identified by Yamamori and Quynh (2000) and bread lines lacking all these three proteins have been produced (Yamamori and Quynh 2000). In these lines, the apparent amylose content is significantly higher than that of the wild type (Yamamori et al. 2006). High amylose wheat flours show lower swelling and lower peak viscosity than waxy and normal wheat flours. Products made using SSII-deficient wheat, with a 35–40% amylose content,

have slightly increased levels of resistant starch. Resistant starch (RS) refers to the portion of starch and starch products that resist digestion as they pass through the gastrointestinal tract.

At present, there is a lot of interest in resistant starches because they have a low glycaemic index and reduce the risk of type 2 diabetes and obesity. They also act as a functional fiber, being fermented in the large bowel by the gut bacteria that produce short-chain fatty acids (especially butyric acid) which are responsible for the beneficial effects for the human gut by reducing the risk of colorectal cancer. Additionally, high-amylose starch is low in energy and in total carbohydrate content and seems to slightly improve cooked pasta firmness (Brouns et al. 2002).

Shimbata et al. (2005) characterised the mutations occurring at each SSII locus and identified allele-specific PCR-based markers for each locus. The markers developed were capable of distinguishing among heterozygous, homozygous null and homozygous wild type plants thus facilitating the introgression of mutated alleles in elite cvs.

5. KERNEL HARDNESS

Kernel hardness refers to the texture of the endosperm. Generally, the grain of bread wheat is identified as being either soft or hard. Grain hardness is a very important trait affecting milling, baking and end-use quality of wheat. Soft wheat kernels fracture more easily, release numerous intact starch granules and produce fine-textured flours with reduced starch damage (Giroux and Morris 1997). During milling of hard wheat, fractures yield clean, well-defined particles (Maghirang and Dowell 2003) with larger mean particle size, forming coarser-textured flours with a higher level of starch damage compared to soft wheat. The tetraploid durum wheat has the highest grain hardness and therefore the highest starch damage after milling. Since damaged starch granules absorb more water than undamaged granules, hard wheat is preferred for yeast-leavened products, whereas soft wheat is preferred for the production of cookies and cakes.

Among the different methods used to measure grain hardness, the two most popular with breeders are the Near-Infrared Reflectance Method, and the Single-Kernel Characterization System (AACC 2003a) because they are easy to use and produce reliable results.

Studying the inheritance of grain hardness, Symes (1965) showed that the difference between hard and soft wheat was due to a single major gene and to modifying minor genes. The *Hardness* locus (*Ha*) is located on the short arm of chromosome 5D (Mattern et al. 1973). Although the locus is named *Hardness*, the dominant trait is softness.

As far as the biochemical basis of hardness is concerned, Greenwell and Schofield (1986) found a 15-kDa protein named “friabilin” on the surface of water-washed wheat starch. The friabilin in water-washed starch is present in a higher amount in soft wheat than in hard wheat and is absent in durum wheat (very hard). According to recent studies, the 15-kDa protein complex consists of at least five components:

puroindoline-a, puroindoline-b, grain softness protein (respectively coded by the *Pina-D1*, *Pinb-D1* and *Gsp-1* genes) and two alpha-amylase inhibitors (Clarke and Rahman 2005). The *Pina-D1*, *Pinb-D1* and *Gsp-1* genes have been found to be tightly linked to the *Ha* locus at the distal end of chromosome 5DS (Giroux and Morris 1997; Tranquilli et al. 2002). The wild-type puroindoline genes are associated with soft kernel texture (Lillemo and Morris 2000). On the other hand, it has been demonstrated that in all the hard wheat examined there was a mutation either in *Pina-D1* or *Pinb-D1*, but not in *Gsp-1* (Giroux and Morris 1997; Lillemo and Morris 2000; Morris et al. 2001).

Although it is well documented that the *Ha* locus on the chromosome arm 5DS is the main determinant of kernel hardness (Sourdille et al. 1996; Campbell et al. 1999; Perretant et al. 2000; Igrejas et al. 2002), it was also shown that this locus alone and the tightly-linked genes *Pina-D1* and *Pinb-D1* cannot explain all the phenotypic variation of this trait in the different mapping populations. Many authors used QTL analysis to genetically dissect the complexity of the hardness trait. As a result, other QTLs with minor effect on grain hardness were detected on different chromosomes: 2A, 2D, 5B, 6D with single-factor effects and 5A, 6D, 7A with interacting effects (Sourdille et al. 1996), 3A (Campbell et al. 1999), 1A and 6D (Perretant et al. 2000), 2B and 6B (Galante et al. 2001), 5D in a position not coincident with the *Ha* locus (Igrejas et al. 2002) and 3BL (Narasimhamoorthy et al. 2006). The QTLs detected using bi-parental mapping populations do not provide the complete information as to the genetic control of a complex trait. Moreover, the QTL effect is estimated comparing two alleles in one genetic background only. From the breeders' standpoint it is much more interesting to characterise a range of different alleles in a germplasm collection including accessions derived from different crosses involving more than just two parents, as occurs in an ordinary breeding programme. Recently, Crepieux et al. (2005) presented results of a QTL mapping study carried out in a real wheat breeding population. Using the approach of the variance component (VC) based on the identity by descent (IBD), they detected two QTLs for grain hardness: one is likely to be associated to the *Ha* locus on the 5DS chromosome arm, the other is on the 1D chromosome close to the *Glu-D1* locus. However, in this latter case, they could not rule out the possibility of an artefact caused by the storage protein. Applying the "mixed-model analysis" to the same set of data, Arbelbide and Bernardo (2006) detected two markers strongly associated with kernel hardness in the multiple-marker analysis. One of the markers was very close to the *Pinb-D1* gene and therefore to the *Ha* locus. The second marker was close to the end of the 1AS chromosome arm, where the *Glu-A3* locus is located. A marker close to the QTL on 1D detected by Crepieux et al. (2005) was significant only in the single-marker analysis.

The QTL analysis is a first step towards the identification of the genes underlying each QTL. For a more efficient wheat breeding approach, the availability of reliable markers tightly linked to the QTL(s) or, even better, to the gene(s) of interest is very useful.

The puroindoline proteins a and b form the molecular basis of a large part of the wheat grain hardness (Morris et al. 2001). The availability of gene-specific primers allows the full-length amplification of the *Pina-D1* and *Pinb-D1* genes showing the sequence differences between alleles, which might have a different role in wheat breeding (Massa et al. 2004).

It has been demonstrated that the different *Pina-D1* and *Pinb-D1* alleles have a great influence on milling properties and also affect bread baking traits (Martin et al. 2001; Eagles et al. 2006). It is also known that, in synthetic hexaploid wheat, puroindoline alleles from *Aegilops tauschii* produce an endosperm softer than the one of soft common wheat (Gedye et al. 2004). A decrease in kernel hardness in substitution lines carrying additional copies of puroindoline genes from *Triticum monococcum* was found by Tranquilli et al. (2002), who also demonstrated that the simultaneous deletion of both puroindoline loci in common wheat increased its hardness to the level typical of durum wheat. They also showed that *Gsp-1* genes do not have a critical role on grain texture. There is also the possibility that new alleles of the puroindoline genes will be discovered, increasing the amount of tools available to modulate the hardness trait. On the other hand, it is well known that a part of the variation in kernel texture is not caused by the puroindoline genes.

As previously discussed, QTLs affecting grain textures were found on different chromosomes analysing recombinant inbred, double haploid lines (Sourdille et al. 1996; Campbell et al. 1999; Perretant et al. 2000; Galande et al. 2001; Igrejas et al. 2002) and backcross lines (Narasimhamoorthy et al. 2006). There is the possibility that even more QTLs and markers will be discovered using a novel approach that allows the analysis of lines deriving from different crosses, as happens in conventional breeding programmes (Crepieux et al. 2005; Arbelbide and Bernardo 2006).

The information already available on QTLs, genes and markers related to grain hardness is going to increase in the near future offering the breeders a wealth of tools enabling them to modulate the hardness trait taking also into account its influence on milling and baking quality. It is also possible to extend the range of variation of the trait in both bread and durum wheat resulting in the possibility to obtain supersoft or superhard varieties with novel end uses.

6. FLOUR/ SEMOLINA COLOUR

Another important quality parameter of bakery and pasta products is colour. In fact, natural colour is an important feature of bread, noodle and durum wheat pasta. In particular, the presence of yellow colour in the wheat flour is detrimental for bakery products made with common wheat, while it is desirable for the production of yellow alkaline noodles and for the majority of the durum wheat-based products. Thus, breeders have to apply different selection criteria depending on the wheat quality class being selected. In this respect, durum wheat genotypes with kernels containing a high level of yellow pigment and a low level of undesired components producing darker colours are today being selected.

Lepage and Sims (1968) pointed out that the yellow colour in durum wheat extracts is attributable to carotenoids, mainly to the xanthophyll lutein and its fatty acid esters (*ca.* 85% free lutein, 10% lutein monoesters and 5% lutein diesters). Wildfeuer and Acker (1968) reported that carotene accounts for 1% of the total carotenoid content in durum wheat semolina. Hentschel et al. (2002), analysing eight durum wheat cvs., showed that the fraction of carotenoids (only luteins and small amounts of zeaxanthin) accounts for 30–50% of the total yellow pigment content so they argued that other compounds of still unknown structure contribute considerably to the colour of the grains. Conversely, in a similar study Fratianni et al. (2005) evidenced the prevailing role of carotenoids, with lutein being the main compound, followed by zeaxanthin and β -carotene, with only a small fraction attributable to other compounds.

High levels of natural yellow colour in wheat flour or in semolina and thus in the end-use products can be primarily reached by selection for the natural carotenoid pigments accumulated throughout the inner and outer kernel layers. However, yellow pigments in final products such as noodles and pasta can be substantially broken down, during the manufacturing process, by the action of lipoxygenases (LOX activity), as well as peroxidases and polyphenoloxidases, the latter being mainly responsible for the formation of undesired brown colour components (Porceddu 1995; Dexter and Marchylo 2000; Peña and Pfeiffer 2005). Polyphenol compounds are also responsible for the red colour present in the kernel outer layers of some bread and durum wheat cvs. (Himi et al. 2005), but usually these compounds are not present in flour/semolina. It has been reported that in some durum wheat genotypes, a low LOX activity is more relevant to obtain yellow pasta as compared to the pigment content of the kernels (Borrelli et al. 1999).

Many analytical techniques (AACC 2003b), mainly based on chemical pigment extraction (e.g. HPLC) or light reflectance, have been proposed to measure the yellow pigment content in whole meals, semolina and pasta; it is thus possible to choose the methodology to be used according to the aim, the level of precision and the speed required. Results obtained on durum wheat samples by means of an automatic reflectance instrument (Minolta Chroma Meter CR-200) based on the color-space system (CIE 1986), the HPLC procedure and the standard water-saturated butanol (WSB) method have recently been compared by Fratianni et al. (2005). The authors found highly significant correlations among the measurements obtained from semolina samples using the three different techniques, while the relationships between the yellow index (b^* parameter) and the pigment content as determined by HPLC on the whole meals were strongly influenced by the sample characteristics. It was thus suggested that the reflectance determinations on whole meal samples, although fast and safe, provide only relative and not absolute values. This notwithstanding, due to its relatively low-cost and easy management, the Minolta instrument is widely used by breeders to measure the yellow index (b^*) of several hundreds of whole meal/semolina samples per day. Further, it is suitable to analyse small flour samples, thus allowing breeders to select for high yellow pigment in early segregating generations.

Notable sources of genetic variation suitable for increasing yellow colour in durum wheat kernels have been found, in addition to the primary gene pool, mainly in *Hordeum chilense* (Martin et al. 2000; Ballesteros et al. 2005) and especially *T. monococcum* which is characterised by high amount of luteins (Abdel-Aal et al. 2002). Recently released durum cvs. generally meet the yellow quality requirements and often can be used to produce semolina stocks suitable to enhance the semolina quality; this notwithstanding a wealth of important landmark durum cvs. that have been/are still largely used in breeding programmes, are characterised by low yellow pigment content, like cv. Creso in Italy and other cvs. derived from Altar 84. A high genetic variability for yellow pigment content in the whole meal has been found when analysing a wide collection including 325 durum wheat accessions representing the most important improved durum gene pools (Maccaferri et al., data not published).

Although several environmental factors have been shown to influence the yellow pigment content (Borrelli et al. 1999) and the presence of genotype by environment interaction, this quality trait can be considered highly heritable (Parker et al. 1998) with the heritability coefficient reaching values even equal to 0.90 in durum wheat; further, the genetic control of this traits is mainly due to additive effects (Nachit et al. 1995).

Studies on the genetic control of yellow pigment content in wheat, and especially in durum wheat, were scarce until a few years ago. In recent years, the development of DNA-based molecular markers has provided powerful tools to identify the genetic factors underpinning the variation of quantitatively inherited traits (Lee 1995; Tuberosa et al. 2002). Several QTL studies have now addressed the genetic dissection of yellow pigment content in wheat and some information are available on-line in the MAS-dedicated web site at <http://maswheat.ucdavis.edu>. A detailed summary of the results so far obtained as to the QTLs for yellow pigment content in semolina/wheat flour and for lipoxygenase (LOX) activity affecting yellow colour and brightness of the end-use products (noodles and pasta) is reported in Table 1. Overall, it can be underlined that the natural variation present in the wheat elite germplasm (both tetraploid and hexaploid) is mainly controlled by a relatively low number of genetic factors: in durum wheat, the regions located in the groups 5, 6 and 7L appear to account for a large portion of variation across homoeologous chromosomes and populations. Only chromosome regions located in group 3 seem to play an important role in bread wheat while not influencing the yellow pigment content in durum wheat. Moreover, it is worth mentioning that QTLs for LOX activity, which have a significant impact on the end-product quality colour (yellowness and brightness), were co-locating with the corresponding enzyme-coding genes.

In principle, once QTLs for the trait of interest have been identified, introgression of the favourable alleles and their pyramiding into elite germplasm (e.g. parental lines, populations, etc.) becomes possible through MAS (Ribaut and Hoisington 1998; Young 1999). However, to date only a few successful applications of MAS for the improvement of quantitative traits have been described (Hu et al. 1997; Ragot

Table 1. Summary of the major chromosome regions harbouring QTLs for (A) yellow pigment content in semolina/wheat flour and (B) for lipoxygenase (LOX) activity affecting yellow colour and brightness of the end-use products. QTLs most probably identified across homoecologous regions and/or different mapping populations have been listed within dashed horizontal lines

Chrom.arm	Mapping population ⁽¹⁾	Parent with plus allele	Markers/chromosome regions	R ² (%)
A				
1BS	K/UC	–	QTL interval: 10–30 cM from the chrom top	10
2BL	L/P	–	<i>Xgwm382-Xwmc167</i>	–
2D	CD87/K	–	<i>Xwmc25a-P44/M54-3</i> ⁽²⁾	12
3AS	CD87/K	–	<i>XksuB8-Xwmc50-Xpsr598-P44/M61-4-P40/M61-3</i>	17
3AS	S/Y	Schomburgk	<i>Xbcd828</i>	13
3BS	C/H	–	<i>Xgwm285-P40/M42-2-P40/M44-2-Xcdo583</i>	–
3BS	S/T	–	<i>Xgwm285-P40/M42-2-P40/M44-2-Xcdo583</i>	20
4AS	J/C	–	<i>XPaccMcga-3</i>	9
4BL	USA/S	–	<i>Xgwm1084</i>	9
5AS	L/P	–	<i>Xgwm293</i>	–
5BS	L/P	–	<i>Xwmc149-Xgwm234-Xgwm443</i>	–
5AL	J/C	–	<i>Xbcd926</i>	10
5BL	S/T	–	<i>Xstm286-Xgwm499-P36/M40-2-P36/M40-1Xgwm639B</i>	12
5D	C/H	–	–	–
6A	CD87/K	–	–	13
6AS	K/UC	–	QTL peak at 30 cM from the chrom. top	19
6AL	L/P	–	<i>XDUPw167</i>	–
6AL	USA/S	–	<i>Xgwm1150</i>	7
7BS	USA/S	–	<i>Xgwm573-Xgwm1184-Xgwm3019</i>	13
7AL	Om2*/ <i>T. dic.</i>	Omrabi5	<i>XmcaaEacg198</i>	6
7AL	S/Y	Schomburgk	<i>Xcdo347-Xwg232, plus AFLP marker: Xwua2,16, 18, 26, 35, 39, 56STS from AFLP Xwua26</i>	54
7AL	C/H	–	<i>Xfba349-Xcdo347-Xgwm344-128-Xpsr121-Xpsr680a-XksuH9c, plus 7 AFLP markers</i>	–
7AL	K/UC	Kofa	QTL peak interval at +170 cM from the chrom. top	40
7AL	L/P	–	<i>Xgwm332-Xgwm282-Xgwm344-Xbarc267</i>	–
7AL	Om2*/ <i>T. dic.</i>	Omrabi5	<i>Xgwm63e</i>	13
7BL	CD87/K	–	<i>Xpsr680</i>	10
7BL	K/UC	Kofa	QTL peak interval at +150 cM from the chrom. top	16
7BL	K/W	Kofa	<i>TdPsy1 + SSR and DARt markers</i>	–
7BL	L/P	–	<i>Xbarc267-Xbarc1073-XDUPw398</i>	–

(Continued)

Table 1. (Continued)

Chrom.arm	Mapping population ⁽¹⁾	Parent with plus allele	Markers/chromosome regions	R ² (%)
7BL	Om2*/ <i>T. dic.</i>	Omrabi5	<i>Xgwm344</i>	53
7EL	7A-7EL Ag1-23/ Aconchi// 3* UC1113	<i>Lophopyrum ponticum</i>	<i>Xwg420-Lr19-STSLr19-130- Xmwg2062-Xpsr148-Xpsr680- Xpsr121-Xpsr687-Y</i>	
7H ^{ch}	Tritordeum	<i>Hordeum chilense</i>		–
2H ^{ch}	Hordeum chilense			–
B				
4BS	J/C	Jannah Khetifa	Polymorphism at the <i>Lpx-B1</i> locus	–
4BS	K/UC	Kofa	QTL peak coincident with the <i>Lpx-B1</i> locus	–
2BS	J/C	–	<i>Xrz444</i>	–

– : data not reported.

⁽¹⁾Mapping population

Bread wheat:

S/Y = Schomburgk/Yarralinka (150 F4:F5 lines); Parker et al. 1998; Parker and Langridge 2000

C/H = Cranbrook/Halberd (163 Double haploid – DH – lines); Mares and Campbell 2001

CD87/K = CD87/Katepwa (180 DHs); Mares and Campbell 2001

S/T = Sunco/Tasman (163 DHs Mares and Campbell 2001)

Durum wheat:

J/C = Jannah Khetifa/Cham1 (110 Recombinant inbred lines – RILs –); Hessler et al. 2002; Nachit et al. 2001

L/P = Latino x Primadur (RILs); Somma et al. 2004

USA/S = USA x Svevo (249 RILs); Jurman et al. 2004

Om2*/*T. dic.* = Omrabi5/*T. dicoccoides* 600545//Omrabi5 (114 Backcross inbred lines – BDL –); Elouafi et al. 2001

K/W = Kofa/W9262-D063 (155 DHs); Pozniak et al. 2006

K/UC = Kofa/UC1113 (RILs); Zhang et al. 2006

Genetic stocks:

7A-7EL Ag1-23/Aconchi//3*UC1113 (7^a-7EL Recombinant backcross lines); Zhang et al. 2005

7H^{ch} (Addition lines); Alvarez et al. 1998

2H^{ch} (*Hordeum chilense* lines) Atienza et al. 2004

⁽²⁾Chromosome region associated also to polyphenol oxidase (PPO) activity influencing noodle brightness in the S/T population.

et al. 2000) due mainly to weak associations (in terms of genetic distance) between markers and target QTLs, unpredictable QTL effects across different background and/or the high costs of MAS (Salvi et al. 2001; Koebner 2003; Peleman and van der Voort 2003). Certainly, a more encouraging picture for MAS emerges considering single-gene traits such as disease resistance (Bus et al. 2000; Witcombe and Hash 2000) or even major QTLs, accounting for a sizeable portion of phenotypic variation and validated throughout different elite genetic backgrounds. In this respect, yellow pigment content, thanks also to the abundance of polymorphic locus-specific SSR

markers developed by the private and public wheat community in the latest years (Somers et al. 2004; Song et al. 2005), appears as a very interesting candidate for MAS applied to breeding programmes, especially in durum wheat.

As an alternative to traditional QTL studies involving one or more mapping populations, association mapping (AM) studies involving a large number (at least 100–200) of possibly unrelated accessions provides the opportunity to uncover the most important QTLs regulating the variation for the trait of interest within the germplasm of a particular crop (Rafalski and Morgante 2004; Breseghello and Sorrells 2006). In simple terms, the primary objective of AM is to detect correlations between genotypes and phenotypes, based on linkage disequilibrium (LD), i.e. the non-random combination of alleles at two genetic loci. This approach appears to be highly suited for the study of the yellow pigment content, thanks to a) the high level of variation present in the wheat germplasm, b) the usually high heritability values of the trait, and c) the good knowledge of the genetic bases of the carotenoids and xanthophyll biosynthesis pathways. In the elite durum wheat germplasm accessions, the large extent of LD (at the cM level, within 5–10 cM on average, Maccaferri et al. 2005, 2006) suggests that whole-genome search for chromosome regions harbouring QTLs can already be attained by using a high number (several hundreds) of neutral molecular probes, already available in the public domain (e.g. SSR markers; <http://wheat.pw.usda.gov/GG2/index.shtml>).

Alternatively, as already explored especially in maize, single genes known to play a crucial role in the carotenoid pathway can be searched for single feature polymorphisms/marker haplotypes associated to variation in yellow pigment content. In this respect, the genes and enzymes included in the carotenoid biosynthesis pathway are well known (Cunningham and Gantt 1998), while this is only partially true for the regulation mechanisms (von Linting et al. 1997; Gallagher et al. 2004; Cervantes-Cervantes et al. 2006).

In maize, Wong et al. (2003) found major QTLs controlling the accumulation level of different carotenoids and suggested that the genetic variation at key precursors in a common biosynthetic pathway/regulatory region may have quantitative effects on more than one compound. Moreover, they evidenced co-locations between QTLs for carotenoid accumulation in maize kernels and strong candidate genes, namely phytoene synthase and carotene desaturase. Subsequently, exploiting panels of genetically diverse accessions suitable for AM, Palaisa et al. (2004) elucidated the pattern of diversity and LD at *Y1* gene by exploring a large sequence window of several hundreds of kb up- and down-stream of the phytoene synthase gene and showed the presence of significant associations with the trait and extensive as well as selective sweeps caused by selection.

The isolation of candidate genes from the carotenoid biosynthesis pathway have also been attempted in wheat: Cenci et al. (2004) isolated BAC clones (from the tetraploid wheat Langdon) containing the three major genes (phytoene synthase, *PSY*, phytoene desaturase, *PSD*, and zeta-carotene desaturase, *ZDS*) and mapped some of the isolated clones using deletion stocks: *PSY* mapped on group 5, *PDS* on group 4 and *ZDS* on group 2. Only recently, Pozniak et al. (2006) identified a second

copy of *PSY* in the durum wheat genome mapping in the 7AL/7BL homoeologous regions harbouring the major yellow pigment content QTL in wheat. These wheat sequences are good candidates for more in depth analyses of genetic diversity and LD analysis in the wheat germplasm.

The alternative approach of searching for co-localizations between known QTLs and putative candidate genes/sequences by exploiting the conserved rice-wheat synteny is also feasible (Francki et al. 2004). However, because in a first attempt this approach failed to identify the presence of a sequence related to wheat *PSY* in the hortologous region of rice, careful in evaluating the patterns of co-linearity at small interval levels and in identifying the putative candidate genes/sequences should be used.

7. CONCLUSIONS AND OUTLOOK

The development of lines combining the desired expression of qualitative traits, such as those herein discussed, and acceptable yield levels still represents a major challenge for all the breeders involved in wheat selection programmes.

The availability of molecular markers closely linked to the QTL/genes of interest will allow for the implementation of MAS not only to select the individuals carrying the favourable alleles at the target loci, but also to pyramid favourable QTL alleles from different sources. MAS offers the opportunity for improving the efficiency of the breeding process, particularly when phenotyping alone does not guarantee the identification of the desired genotype or, alternatively, when labourious and time-consuming analyses are required to perform the phenotypic evaluation. Moreover, quick DNA extraction protocols coupled with high-throughput genotyping based on the scoring of markers which do not require the use of gels (Salvi et al. 2001) will streamline MAS at a lower cost.

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CHAPTER 3

MOLECULAR APPROACHES AND BREEDING STRATEGIES FOR DROUGHT TOLERANCE IN BARLEY

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Abstract: Barley genotypes, in particular landraces and wild species, represent an important source of variation for adaptive traits that may contribute to increase yield and yield stability under drought conditions, and that could be introgressed into improved varieties. Traits that have been investigated include physiological/biochemical and developmental/ morphological traits. Yield performance under drought is particularly a complex phenomenon, and plants exhibit a diverse range of genetically complex mechanisms for drought resistance. Quantitative trait loci (QTL) studies with and without *H. spontaneum* have shown that developmental genes, notably those involved in flowering time and plant stature show pleiotropic effects on abiotic stress tolerance and ultimately determine yield. Problems associated with the hybridization of *H. spontaneum* such as alleles with deleterious effects on field performance could be best addressed in the advanced backcross (AB-) QTL analysis. It was interesting to see that in AB-QTL populations like in balanced populations major QTL overshadowed minor QTL-alleles. Nevertheless, crosses with *H. spontaneum*, AB-QTL populations and association studies with *H. spontaneum* have also identified new alleles and genes that are related to abiotic stress tolerance. In order to identify genes that are related to drought tolerance microarrays analysis to monitor gene expression profiles for plants exposed to limited water environment is performed. Several studies with rapid dehydration treatment have shown that osmotic-stress-inducible genes could explain the response to drought stress in plants. Another development is the identification and use of nucleotide polymorphisms (SNP) in genes related to abiotic stress tolerance. An understanding of the combined function and expression of genes involved in various abiotic stresses, could help identify candidate genes underlying QTL of interest.

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1. INTRODUCTION

Drought continues to be a challenge to agricultural scientists in general and plant breeders in particular, despite many decades of research (Blum, 1996). Drought, or more generally, limited water availability is the main factor limiting crop production. Although it reaches the front pages of the media only when it causes famine and death, drought is a permanent constraint to agricultural production in many developing, and an occasional cause of crop losses in developed, countries. The development, through breeding, of cultivars with higher harvestable yield under drought conditions would be a major breakthrough (Ceccarelli and Grando, 1996). However, the ability of some plants to produce a higher economic yield under drought than others is a very elusive trait from a genetic point of view. This is because the occurrence, severity, timing, and duration of drought vary from location to location and in the same location from year to year. Cultivars successful in one dry year may fail in another, or cultivars resistant to terminal drought may not be resistant to intermittent drought, or to drought occurring early in the season (Turner, 2002). To make matters worse, drought seldom occurs in isolation; it often interacts with other abiotic (particularly temperature extremes) and biotic (root diseases and nematodes being the most obvious examples) stresses (Ceccarelli et al. 2004). In addition, areas with a high risk of drought (and/or other abiotic stresses) generally have low-input agriculture (Cooper et al., 1987). Thus, even though breeding for drought tolerance is complex, it is worsened by its interactions with the other stresses.

2. APPROACHES TO IMPROVEMENT IN DROUGHT TOLERANCE

2.1. Breeding Approaches

Two contrasting philosophies have been used to breed crops for drought tolerance. The first uses selection under optimum growing conditions, and is based on the assumption that an increased yield potential will have a carryover effect when the improved cultivars are grown under less favorable conditions. The second uses direct selection in the presence of drought within the target environment, and can take two forms: (i) selection for physiological or developmental traits (analytical breeding), and (ii) direct selection for grain yield (empirical or pragmatic breeding).

The first philosophy has failed to produce convincing results. In barley, scientists consistently found a negative relationship between yield potential and yield under stress conditions (Van Oosterom et al., 1993). The circumstantial evidence of the absence of a spillover effect is that drought continues to affect negatively agricultural production worldwide, despite the spectacular increases in crop yields obtained through breeding under optimum conditions.

Breeding based on traits associated with drought tolerance has been very popular. Traits that have been investigated include physiological/biochemical and developmental/morphological traits. The physiological/biochemical traits include

osmotic adjustment, osmotic potential, water soluble carbohydrates, proline content, stomatal conductance, epidermal conductance, canopy temperature, relative water content, leaf turgor, abscisic acid content, transpiration efficiency, water use efficiency, carbon isotope discrimination, and retranslocation. The developmental/morphological traits include leaf emergence, early growth vigor, leaf area index, leaf waxiness, stomatal density, tiller development, flowering time, maturity-rate, cell membrane stability, cell wall rheology, and root characteristics.

In barley, scientists at ICARDA have found that the traits more consistently associated with higher grain yield under drought are growth habit, early growth vigor, earliness, plant height under drought, peduncle length, and short grain-filling duration (Acevedo and Ceccarelli, 1989). The analytical approach has been very useful in understanding which traits are associated with drought tolerance and why. The approach has however been less useful in developing new cultivars that show improved drought tolerance under field conditions. This is because, as mentioned earlier, under field conditions, drought varies in timing, intensity, and duration. Therefore, it is the interaction among traits that determines the overall crop response to the variable nature of the drought stress, rather than the expression of any specific trait (Ceccarelli et al., 1991). Although breeding for drought tolerance based on direct selection for grain yield in the target environment (empirical or pragmatic breeding) appears intuitively as the most obvious solution, it has faced the criticism because the chances of progress appears slow and remote. Two of the major problems with selection in stress environments are precision of selection and existence of several target environments. Each environment is characterized by its own specific type of drought and generally a combination of stresses.

The issue of precision of selection under moisture stress conditions has been addressed by several papers, which have focused attention on the magnitude of heritability (Ceccarelli, 1994). A review of literature (Ceccarelli, 1996a) has shown the absence of a consistent relationship between grain yield (as a measure of the intensity of the stress) and the magnitude of heritability. Recently, vom Brocke et al. (2002) confirmed that in pearl millet, it is possible to detect genetic differences even under severe moisture stress conditions. Furthermore, considerable progress has been made in both experimental design and statistical analysis, which improve the estimate of experimental error (Singh et al., 2003). Therefore, it is possible to combine precision and relevance by conducting trials in the target environment even when it is a stress environment.

The second issue is how to deal with the multitude of target environments. This is intimately associated with broad and specific adaptation, which has been debated in plant breeding since the early 1920s and is still highly controversial. One of the causes of controversy is the definition of stress environment, which is different for different crops. For example, in a country like Syria, with a large spatial variability of rainfall, bread wheat, durum wheat, and barley are grown within short distances in progressively drier environments with some overlapping. Therefore, a stress environment for bread wheat is moderately favorable for durum wheat, and a stress

environment for durum wheat is moderately favorable for barley and lentil. At the drier end, barley is the only rainfed crop, while the other cereals are only grown under irrigation.

Another cause of controversy is the confusion between adaptation over time and adaptation over space, even though the distinction is of fundamental importance. Adaptation over time (also called stability or dependability) refers to the performance of a cultivar in a given location over several years. If the cultivar performs consistently better than a reference cultivar, it is said to be stable. Adaptation over space refers to the performance of a cultivar in several locations. If the cultivar performs consistently better than a reference cultivar, it is said to be widely adapted. It can be argued that wide adaptation over time (also defined as stability) is more important to farmers than wide adaptation over space. The latter is, for obvious reasons, the major concern of seed producers.

Breeding for specific adaptation is particularly important in crops predominantly grown in unfavorable conditions, because unfavorable environments tend to be more different from each other than favorable environments. Breeding for specific adaptation to unfavorable conditions is often considered an undesirable breeding objective, because it is usually associated with a reduction of potential yield under favorable conditions. This issue has to be considered in its social dimension and in relation to the difference between adaptation over space and adaptation over time.

2.2. Choice of Germplasm

Landraces are still the backbone of agricultural production in many developing countries, and for crops grown in stress environments. The reasons farmers prefer to grow only landraces or continue to grow landraces even after partial adoption of modern cultivars are not well documented. These include quality attributes, seed storability, and the ability of landraces to produce some yield even in difficult conditions where modern varieties are less reliable.

The value of landraces in barley (Grando et al., 2001) and several other crops (Brush, 1999) is well documented in Syria. The comparison between barley landraces and modern cultivars in different conditions, ranging from severe stress (low input and low rainfall) to moderately favorable conditions (use of inputs and high rainfall) has given several indications (Table 1).

- Landraces produce more yield than the modern cultivars under low-input and stress conditions.
- The superiority of landraces is not associated with mechanisms to escape drought stress, as shown by their heading date.
- Within landraces, there is considerable variation in grain yield under low-input and stress conditions, but all the landraces-derived lines produce some yield whereas some modern cultivars do not.
- Landraces are responsive to inputs and rainfall, and the yield potential of some lines is high, though not as high as modern cultivars.

Table 1. Grain yields (kg/ha) of pure barley lines derived from Syrian barley landraces and modern cultivars at three levels of drought stress^a in northern Syria (Ceccarelli 1996b)

Environment	Landraces (N = 44)	Modern (N = 206)	Difference	p ^b
Stress	1038	591	447	<0.01
Intermediate	3105	3291	-186	ns
Non-stressed	4506	6153	-1647	<0.01

^aAs defined by average precipitation and soil fertility

^bBased on t-tests for groups of unequal size; ns = not significant

- It is possible to find modern cultivars that produce yields almost as high as landraces under low-input and stress conditions, but the frequency is very low. Selection conducted only under high input conditions is likely to miss most of the lines that would have performed well under low-input conditions (Table 1).

2.3. Decentralized Selection

Selection for specific adaptation to drought stress in barley has been implemented at ICARDA initially by expanding the evaluation of early segregating populations in dry sites and by modifying the breeding method from a classical pedigree method to a modified bulk (Ceccarelli, 1996b). The most important results of this choice have been the re-evaluation of the role of landraces in breeding for drought tolerance (described earlier), and the discovery of the importance of the wild progenitor of cultivated barley, *Hordeum vulgare* ssp. *spontaneum*, as a source of tolerance to extreme levels of drought. Gas exchange observations made at anthesis in a wet site showed that *H. spontaneum* has widely open stomata, higher net photosynthesis, and lower pre-dawn leaf water potential at this stage of development than cultivated barley. The ability of some accessions of *H. spontaneum* to tolerate extreme levels of drought stress was evident during the severe drought of 1987. Two lines of *H. spontaneum* were the only survivors in the breeding nurseries at a site that received only 176 mm rainfall (Grando et al., 2001).

These lines had some photosynthetic activity early in the morning, even though six times less than in the absence of stress. The stomata were open and the pre-dawn leaf water potential was negative. At the same time, the stomata of the black-seeded local landrace Arabi Aswad, considered by farmers to be very resistant to drought, were closed even though the pre-dawn leaf water potential was slightly higher than in *H. spontaneum*. By midday, the stomatal conductance of *H. spontaneum* decreased, the net photosynthesis became negative, while the stomatal conductance of Arabi Aswad was zero.

Decentralized selection, i.e., selection in the target environment, is mostly based on grain yield. Grain yield is considered as the integrated response of a number of physiological, morphological, and developmental attributes. These

attributes allow the particular genotype or population to perform better in the particular type of drought encountered in a specific site and year, and in a combination of that particular type of drought with other stresses. The genotypes or populations selected under this specific set of conditions are then tested for a second season in a number of sites, and if again successful, for a third season.

The process makes use of the large annual variability, which characterizes stress locations. Thus, in a short period such as three years, and in seven to ten sites, there is a high probability that a genotype or population is exposed to a number of different types of drought in terms of occurrence, timing, duration, and severity. Therefore, the process progressively accumulates favorable alleles for performance under various conditions (Figure 1).

The process relies heavily on grain yield, but we also consider other attributes such as early vigor, habit of growth, flowering time, plant height, tillering, and, when extreme stress conditions occur, leaf rolling and senescence. The process combines the characteristics of the empirical and analytical approaches, and results in a slow but steady increase in yield under a combination of stresses. Figure 1 shows an example of this steady increase using a number of lines developed between 1985 and 2000. The yield increased from less than 0.96 t/ha in Harmal to 1.4 t/ha in Arta. Arta, however, has the major handicap of becoming very short under drought stress. A reduction of plant height is undesirable because it forces farmers to harvest by hand, which is much more expensive than mechanical harvesting. The use of *H. spontaneum* and one of the tallest selections from the black-seeded landrace, Zanbaka, has produced nearly 1.5 t/ha yield with a plant height that is only marginally lower than that of Zanbaka.

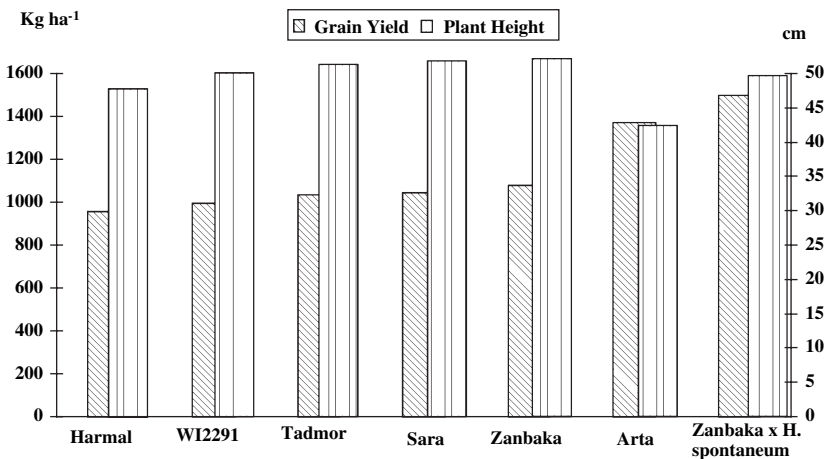


Figure 1. Grain yield and plant height of barely lines developed during the last 15 years (rainfall 197 mmm)

2.4. Conclusion

Barley breeding for stress environments is possible, provided it is conducted with strategies and methodologies that have little in common with those used in breeding for favorable environments. Adaptation over time can be improved by breeding for specific adaptation to a particular stress environment. This can be achieved by taking advantage of the temporal variability of stress environments, which permits exposure of the same breeding material to variable combinations of stresses over a (relatively) short period. We are aware that this is fundamentally different from the modern trend of plant breeding towards broad adaptation over space. The difference represents the contrasting interests of farmers and seed companies. Farmers are interested in cultivars that are consistently superior on their farm, regardless of how they perform at other locations or in other countries.

Recent advances in plant genomics have enabled the dissection of various molecular mechanisms involved in drought, cold and salt stress tolerance and in identifying various genes involved in these stress tolerance. Information generated in genomics should be integrated into practical plant breeding. Various genes identified in both model and crop plants could be used for developing stress tolerant plants through either marker-assisted selection or direct gene transfer.

3. MOLECULAR APPROACHES TO DROUGHT TOLERANCE

The advent of molecular markers has revolutionized the genetic analysis of crop plants and provided valuable new tools to identify chromosome regions influencing tolerance to abiotic stresses. Marker technologies and saturated marker maps allow the location of genomic regions or quantitative trait loci (QTL) with significant effects on drought tolerance or yield stability under adverse environmental conditions. The identification of these QTL with linked markers allows the breeders to use marker-assisted selection as a complementary tool to traditional selection. In addition, QTL mapping is a first step towards unraveling the molecular basis of drought resistance, i.e., by map-based cloning (Frary et al., 2000), with the ultimate goal of assigning functions to genes.

Yield performance under drought is particularly a complex phenomenon, because yield itself is a quantitative trait, and plants exhibit a diverse range of genetically complex mechanisms for drought resistance, including mechanisms of drought escape, drought avoidance, and drought tolerance (e.g., osmotic adjustment). This complexity may explain why most QTL studies of field-grown barley have so far been performed in favorable conditions, while only few studies have performed QTL analyses for agronomic performance under drought stress (Teulat et al., 2001b; Baum et al., 2003; Talamé et al., 2004; Forster et al., 2004; Long et al., 2001). These studies have shown that developmental genes, notably those involved in flowering time and plant stature have pleiotropic effects on abiotic stress resistance and ultimately determine yield potential.

In order to reduce complexity, physiological traits that have an influence on yield performance under drought were investigated in several studies on osmotic

adjustment, relative water content and carbon isotope discrimination conducted on field-grown barley (Teulat et al., 2002) and under controlled conditions (Teulat et al., 1998, 2001a).

In addition, the application of expression analyses and cloning techniques to the analysis of stress response has allowed the detection of a number of candidate genes whose expression is affected under stress (Cattivelli et al., 2002; Diab et al., 2004; Choi et al., 2000, 2002).

The following paragraphs focus on QTL studies for plant height, flowering time, and yield in relation to drought stress. Special emphasis is on studies using exotic barley germplasm, as wild barley being adapted to drought environments represents a promising resource for the improvement of drought tolerance. Therefore, recent association tests with wild barley grown in the fertile crescent (Ivandic et al., 2002, 2003) and QTL studies on advanced backcross populations tested in stressed and favorable conditions are discussed (Pillen et al., 2003, 2004; Matus et al., 2003; Li et al., 2005; Li et al., 2006; Talamé et al., 2004; von Korff et al., 2004, 2006; Hori et al., 2005). For balanced populations, only QTL analyses conducted under drought stress are presented (Teulat et al., 2001b; Baum et al., 2003; Talamé et al., 2004; Forster et al., 2004; Long et al., 2003). In addition, studies using physiological traits as well as recent advances in the discovery of genes involved in stress tolerance are introduced.

3.1. Chromosome Regions for Drought Tolerance and Developmental Traits in Balanced Populations

Agronomic performance in balanced populations was tested under drought prone conditions of West Asia, North Africa, and Australia. Baum et al. (2003) and Grando et al. (2005) used 194 RILs, randomly selected from a population of 494 RILs, and tested them in the 1996–97 and 1997–98 cropping seasons at ICARDA's research stations near Tel Hadya and Breda in Syria. Total rainfall in Tel Hadya in 1996–97 and 1997–98 was 433.7 and 410.5 mm, respectively, whereas in Breda it was 230.8 and 227.4 mm, respectively. The map extended over 890 cM and contained 189 marker loci, including one morphological marker locus (*btr* = brittle rachis), 158 AFLP loci and 30 SSR loci. Teulat et al. (2001a,b, 2003), Forster et al. (2004), and Diab et al. (2004) used a progeny of 167 two-row barley recombinant inbred lines (RILs), and the two parents Tadmor (selected by ICARDA from Arabi Aswad) and ER/Apm (an ICARDA breeding line released in Tunisia, Morocco and Libya), which were grown in three Mediterranean sites (Montpellier, France; Meknes, Morocco and Le Kef, Tunisia). The trial in Montpellier was conducted under two water treatments in 1999; 360.5 and 458 mm. For the two other sites, the plants were grown under rainfed conditions, in Meknes in 2000 (254.6 mm) and 2001 (267.4 mm) and in Le Kef in 2001 (282.8 mm). One hundred and thirty-three markers covered 1500 cM of the genome. Diab et al. (2004) added more candidate genes and ESTs to the available map. Ellis et al. (2002) and Forster et al. (2004) tested the Derkado/B83-12/21/5 doubled haploid population consisting of

156 doubled haploid two row barley lines in Meknes and Kef additionally at two sites (irrigated and non-irrigated) in Egypt. The cross was originally designed to investigate quality, yield and disease resistance traits, but has now been used to investigate over 60 traits including abiotic stress tolerance (Ellis et al., 2002). Derkado/B83-12/21/5 carry the semi-dwarf gene *sdw1* and *ari-e.GP*. One hundred and fifty-two markers covered 1426 cM of the eight linkage groups (3Ha, 3Hb, Ellis et al. 2002). Long et al. (2003) tested the 110 recombinant inbred lines of the cross Mundah/Keel at different sites in South Australia in 1999–2001. The cross was between a parent with superior adaptation on sandy soils low in fertility (Mundah) and parent with poor adaptation (Keel). Fifty-four markers (14 AFLPs, 12 RFLPs, 28 SSRs) were mapped in this population.

The developmental trait, heading date, in barley is controlled by three genetically independent mechanisms of the photothermal response, i.e., day length (photoperiod), plant response to a sum of temperature over a period (earliness *per se*), and response to a period of low temperature at the initial stages of plant development (vernalization) (Ellis et al., 1990). The major genes for photoperiodic response are the *Eam1* or *Ppd-H1* location on chromosome 2H (bin 4, Laurie et al., 1995) and *Ppd-H2* on chromosome 1H (bin 14) (Laurie et al., 1995). Earliness *per se* (*eps*) genes are minor genes, which influence onset of flowering independent of temperature or light. They have been identified as *eps2S* on 2H (bin 6), *eps3L* on 3H (bin 13), *eps4L* on 4HL, *eps5L* on 5H (bin 6), *eps6L.1* and *eps6L.2* on 6H (bin 7, 13), *eps7S* on 7H (bin 3) and *eps7L* on 7H (bin 12) (Laurie et al., 1995). *Vrn-H1* on chromosome 5H (bin 11), *Vrn-H2* on 4H (bin 13), and *Vrn-H3* on 1H (bin 13) determine the vernalization requirement in barley (Laurie et al., 1995; Dubcovsky et al., 1998, Figure 2).

For days to heading a few major chromosomal locations have been repeatedly identified in the above mentioned populations (Figure 2). QTL have been identified on 7H (bin 2-3) (Baum et al. 2003; Long et al., 2003), 3H (bin 12) (Baum et al., 2003; Teulat et al., 2001), 2H (bin 3-7) (*Ppd-H1*, Baum et al., 2003) 2H (bin 11-13) (Baum et al., 2003; Teulat et al., 2001), and 5H (bin 6) (Baum et al., 2003, Figure 2). Under better rainfall conditions the locations on 3H (bin 12), 2H (bin 3-7) (*Ppd-H1*) and 2H (bin 13) were more important in the Arta/*H. spontaneum* 41-1 population whereas under drier conditions the 7H (bin 3) location was more important (Baum et al., 2003). At the 7H location, *H. spontaneum* 41-1 contributed the drought escape allele. In the Tadmor//ER/Apm population 2H (bin 13), 3H (bin 12), 5H (bin 11) and 7H (bin 9) are the important loci. Forster et al. (2004) pointed out that for the Tadmor//ER/Apm populations flowering time variation in the RILs under non-vernalization conditions may be due to the *Vrn-H2* locus on 4H, and this may account for a cluster of QTL in this region on chromosome 4H, even though no QTL for heading date was among the QTL. Tadmor, however, has vernalization requirements which might not always be met in North African sites.

One of the most important characters for the extreme drought conditions with annual rainfall less than 200 mm is the plant height (under drought stress). Here, alleles from *H. spontaneum* 41-1 become very useful to increase plant height. QTL

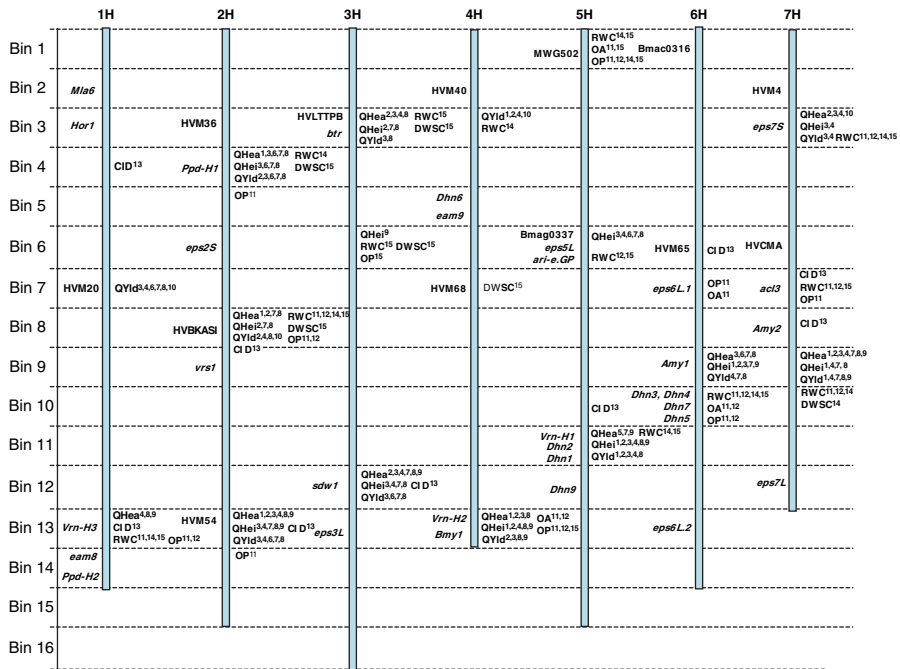


Figure 2. Genetic map of barley with locations for selected QTLs for agronomic and physiological traits. The genetic map is based on the Steptoe x Morex bin map by Kleinhofs and Graner (2001). Markers and loci have been assigned to the bin with the help of reference markers. Marker names are given on the left side of the chromosome bar. QTL for agronomic traits and physiological traits are presented on the right side of the bar. Selected QTL are shown for yield (QYld), heading date (QHea), plant height (QHei), physiological traits: relative water content (RWC), osmotic adjustment (OA), osmotic potential (OP), water-soluble carbohydrate concentration (WSC) and carbon isotope discrimination (CID). References: 1 Pillen et al., (2003), 2 Pillen et al., (2004), 3 Baum et al., (2003), 4 Talame et al., (2004), 5 Hori et al., (2005), 6 Li et al., (2005), 7 Li et al., (2006), 8 von Korff et al., (2006), 9 Teulat et al., (2001b), 10 Long et al., (2003), 11 Teulat et al., (1998), 12 Teulat et al., (2001a), 13 Teulat et al., (2002), 14 Teulat et al., (2003), 15 Diab et al., (2004)

with major effects have been identified by Baum et al. (2003) on chromosomes 2H (bin 13), 3H (bin 12, *sdw1*), and 7H (bin 3). These might be more specific to *H. spontaneum* 41-1 background. In the Tadmor//ER/Apm population, major QTL for plant height were located on 2H (bin 13), 3H (bin 6), 4H (bin 13), 6H (bin 10) (Teulat et al., 2001). Plant height was not reported in the Mundah/Keel population. As expected, *sdw1* (3H) and *ari-e.GP* (5H) had major effects on plant height in the Drekado/B83-12/21/5 population. Both of them were also associated with most of the traits assessed, such as seedling establishment, plant vigour, peduncle length, thousand kernel weight and developmental rates between growth stages. Additionally, *ari-e.GP* (5H) was associated with shoot weight, root weight, shoot carbon isotope discrimination, and yield. *Sdw1* was associated with shoot carbon

isotope discrimination, root nitrogen isotope discrimination and grain nitrogen (Ellis et al., 2002; Forster et al., 2004).

In the Arta/*H. spontaneum* 41-1 population, QTL for grain yield and biological yield have been identified and located at the *btr* location on 3H (bin 3), and the *sdw1* (bin 12) locus in the part of the population with non-brittle lines. In both locations, pleiotropic effects for several other characters were identified. Other grain yield QTL were identified on 4H (bin 13), 5H (bin 11), and 7H (bin 3) (Baum et al., 2003). Teulat et al. (2001b) identified QTL for grain yield on chromosome 4H (bin 13); further on chromosome 7H (bin 9). In the Mundah/Keel population (Long et al., 2003) grain yield QTL were identified on chromosome 1H (bin 7), 2H (bin 8) and 4H (bin 3). In the Drekado/B83-12/21/5 population, *sdw1* was associated with grain yield in the three North African sites (Forster et al., 2004).

Cold and frost tolerance is a necessary requirement for barley production in the Fertile Crescent. In the Arta/*H. spontaneum* 41-1 population eight QTL for cold damage were identified. For the QTL with minor effects (2H, 4H and 6H), the allele from the *H. spontaneum* line showed the better protection. For all other QTL, the allele of Arta showed better ability to protect the plant from cold damage. Three of the four alleles with a high effect on cold damage were localized on chromosome 5H at the known locations for frost tolerance QTL (Pan et al., 1994; Reinheimer et al., 2004; Francia et al., 2004). Additionally, a QTL on 7H was identified. This is in agreement with the observation of Karsai et al. (2004), whose survey of *H. spontaneum* accessions of the Middle East origin showed some degree of vernalization response.

3.2. Genes Controlling Stress Tolerance

Cellular dehydration is common among the abiotic stresses and plants exhibit common molecular responses to them. Accumulation of a family of proteins known as dehydrins (*Dhns*) is one of the prominent components of this adaptive process (Close 1997). *Dhns* typically accumulate in different tissues in response to low temperature, drought, salinity, or abscisic acid (ABA) application and during the late phase of embryogenesis. Dehydrin genes have been mapped in the barley genome and some of them have been shown to co-segregate with stress tolerance QTL (Cattivelli et al., 2002). Three *Dhn* loci (*Dhn1/Dhn2* and *Dhn9*) are located in the same region of chromosome 5H where *Vrn-H1*, cold and salt tolerance QTL and ABA accumulation QTL have been mapped. Similarly, the *Dhn* cluster comprising *Dhn3*, *Dhn4*, *Dhn5* and *Dhn7* and the locus of *Dhn8/pAF93* on chromosome 6H are associated with drought tolerance QTL (Teulat et al., 2003). The recently identified *Dhn10* gene is located on 3H between saflp106 and ABG4 and *Dhn6* on chromosome 4H (Choi et al., 2000). Thirteen barley *Dhn* loci (Choi et al., 1999, 2000; Choi and Close 2000) have been identified and located on the different barley chromosomes.

Single-gene expression studies and transcriptome profiling in response to drought and cold revealed the induction of 'effector' genes responsive to a single stress

such as the barley *Cor14b* or *Arabidopsis* *Rd22* and genes whose expression changed in response to both drought and cold (e.g. barley *Dhn* genes or *Arabidopsis* *Rd29a*). This suggests the existence of crosstalk between distinct stress-signaling pathways (Shinozaki et al., 2003) as quoted by Tondelli et al. (2006). Several transcription factors regulating stress inducible expression of the above mentioned effector genes via interaction with cis-acting elements in promoter regions have been isolated and characterized. The barley *HvCbf3* gene, ortholog of the *Arabidopsis* *CBF/DREB1* gene has been mapped to a position more proximal to the *Dhn1/Dhn2* – *Dhn9* interval. Skinner et al. (2006) investigated and mapped homologs of the *Arabidopsis* low temperature regulatory genes CBFs to determine if there were candidates for winterhardiness-related quantitative trait loci (QTL). Twelve of the CBF genes were located on 5HL and 11 of them formed two tandem clusters. *AtFRY1* and *AtICE1* are regulators of CBF genes. The barley orthologs were mapped to chromosome 7H. TC147474 might be a positional candidate gene for the drought tolerance QTL located on chromosome 7H (Teulat et al., 2001). The *TC147474* (*FRY1*) might co-locate with the QTL for kernel weight and earliness in the same population.

3.3. Putative Traits for Drought Tolerance: Physiological Traits

Breeding for drought tolerance based on traits associated with drought resistance, but easier to select for than grain yield, has been and still is very popular. Some physiological responses have been observed in plants induced by drought stress (Ludlow and Muchow, 1990).

Osmotic adjustment (OA) has been found to be one of the physiological mechanisms associated with plant tolerance to water deficit (Blum, 1988). Relative water content (RWC) is a measure of plant water status resulting from a cellular water deficit. RWC is an appropriate estimate of plant water status as affected by leaf water potential and OA. Carbon isotope discrimination is another method used to screen for drought tolerance. The correlation between water use efficiency and carbon isotope discrimination has been extensively studied in several crops including wheat (Farquhar and Richards 1984; Condon et al., 1987) and barley (Araus et al., 1997).

The chromosomal location of some traits related to drought tolerance is known. Differentially expressed sequence tags related to drought tolerance have been mapped in the Tadmor//ER/Apm RIL population by Diab et al. (2004). Osmotic potential, OP100 (is the osmotic potential under full turgor) and RWC are components of OA and they are highly correlated (Teulat et al., 1997). For that reason, OA is considered a drought-related trait (Teulat et al., 2001a). Several chromosomal regions related to variation in OA and water status were detected. Two QTL were identified for OA, one on chromosome 3H and one on 5H (Teulat et al., 2001; Diab et al., 2004) (Figure 1).

The genomic regions with QTL clusters for physiological traits were found on 1H (bin 13), 2H (bin 8), 5H (bin 1), 6H (bin 10) and 7H (bin 7) (see Figure 1). For example, QTL for RWC, OA and OP were all mapped to approximately the

same chromosomal location on 6H (Teulat et al., 1998, 2001a, 2003; Diab et al. 2004). This locus on 6H matches the location of the candidate genes *Dhn3*, *Dhn4*, *Dhn7*, *Dhn5*, which are expressed during drought and/or cold stress (Cattivelli et al.; 2002). Genomic regions with effects on OA, OP and RWC coincided with QTL for flowering time, height and yield as for example on 2H (bin 8) and 4H (bin 13) (Figure 1). The co-location of the QTL for DWSC100 (difference in water-soluble carbohydrate concentration between well-watered and stressed plants) and RWC in more than one genomic region, 2H (bin 4, bin 8) and 3H (bin 3), under water stress suggests that the accumulation of WSC (water-soluble carbohydrate concentration) may be important for plants to maintain their RWC. QTL for carbon isotope discrimination (CID) did also overlap with loci identified for RWC, OA and OP (1H, 2H, 7H). In addition, CID was located close to genomic regions with relevance for agronomic performance such as on 2H (bin 8) and 3H (bin 12).

3.4. Dormancy and Desiccation Tolerance

Seed dormancy in wild barley is an abiotic stress tolerance characteristic because in nature seed dormancy allows plants to escape drought and high temperatures in the warmer summer months (Snape et al., 2001). Drought, salinity, and freezing are stresses that lead to cellular dehydration through different mechanisms. The common component of water stress is evident in shared molecular responses to these stresses. Examples of such responses are common genes induced by all three types of stress and the importance played by the phytohormone abscisic acid (ABA). The coincidence of dormancy QTL with the abiotic stress tolerance QTL indicates that there might be a common physiological basis for dormancy (Zhang et al., 2005). One may infer that dormancy QTL are associated with those QTL that control abiotic stress tolerance. QTL have been identified on chromosomes 4, 5, and 7H in the Steptoe/Morex mapping population, chromosomes 1 and 5H in the Harrington/TR306 population, chromosomes 2H, 3H, and 5H in the Triumph/Morex population, and chromosomes 2H, 5H, and 7H in the Harrington/Morex population (Zhang et al., 2005). Zhang et al. (2005) identified QTL that are coincident with abiotic stress tolerance QTL in a cross with *H. spontaneum*: CD1H2 with a salt and drought tolerance QTL on chromosome 1H, CD2H with a drought tolerance QTL on chromosome 2H, CD4H with a salt tolerance QTL on chromosome 4H, CD5H1 and CD5H2 with a salt and a cold tolerance QTL, and CD7H3 with a drought tolerance QTL (Cattivelli et al., 2002).

3.5. Association Mapping for Drought Tolerance in Natural Populations

Wild barley from the Fertile Crescent shows a high phenotypic and genetic diversity and distinct changes in allele frequencies along climatic and geographic gradients. Ivandic et al. (2000) demonstrated that wild barley accessions sampled from a 100 m transect in Israel exhibited marked differences in response to water deficit. The same barley population with 52 different accessions was genotyped with 33

evenly distributed SSRs and phenotyped for developmental, morphological and yield-related traits under well watered and water stress conditions (Ivandic et al., 2003). The authors found significant associations for all studied traits, in particular at Bmac181 (4H) close to the dehydrin locus *Dhn6* and Bmac18 (6H) adjacent to the gene cluster *Dhn3*, *Dhn4*, *Dhn5*, *Dhn7*. In a previous association study, Ivandic et al. (2002) tested association of flowering time and adaptation to site of origin ecology and geography with 33 SSRs in 39 wild barley accessions from Israel, Turkey, and Iran. Flowering time was tested with and without vernalization and in long and short day. Significant associations for flowering were primarily detected close to known locations of earliness-per-se genes (*eps*) while none of the major photoperiod or vernalization response genes were detected in the wild barley germplasm. In addition, nine of the 14 significant associations with ecology and geography were also significant for flowering time, which underlines the importance of plant development in adaptation. The authors concluded that wild barley is characterised by different adaptation mechanisms and novel alleles as compared to cultivated barley. Association mapping in genetically diverse germplasm may thus form an effective strategy for detecting genomic regions and novel alleles from wild barley involved in adaptation to stressed environments.

4. ADVANCED BACKCROSS POPULATIONS

Wild barley has often been considered a promising resource for the improvement of agronomic and quality traits as well as stress tolerance. Ellis et al. (2000), for example, postulated that exotic barley being adapted to a wide range of environments offers the prospect of a goldmine of untapped genetic reserves. Nevo et al. (1992) demonstrated that wild barley harbours considerably more genetic variation than the cultivated species, and that many exotic alleles are associated with adaptation to specific environments with different abiotic stress conditions. As such, the challenge remains to identify allelic variation for these genes in exotic germplasm, with the most beneficial pleiotropic effects on abiotic stress resistance, and to introduce them into breeding programs. However, the improvement of agronomic performance with exotic germplasm is not straightforward, because wild barley harbours many alleles with deleterious effects on field performance.

With the advanced backcross quantitative trait locus (AB-QTL) mapping approach, Tanksley and Nelson (1996) developed a strategy, which allows a targeted transfer of favorable exotic alleles into elite breeding material. Through this approach, specific exotic alleles derived from the exotic donor are tagged with molecular markers and tested for association with agronomic traits. Since QTL detection is carried out in advanced backcross populations, problems associated with considerable phenotypic variation, linkage drag and epistasis in interspecific crosses are reduced. In addition, identified favourable QTL alleles can be rapidly transferred into QTL-NILs (near-isogenic lines) and used for breeding as well as for the analysis of gene function. In the following paragraphs, the performance of exotic alleles in advanced backcross populations is discussed for flowering time,

plant height and yield as relevant indicators for field performance under extreme and more favourable climatic conditions.

Several studies have employed the AB-QTL strategy to introgress exotic barley alleles into barley cultivars and examine agronomic performance, quality and disease tolerance (Pillen et al., 2003, 2004; Matus et al., 2003; Talamé et al., 2004; Forster et al., 2004; Li et al., 2005, 2006; Hori et al., 2005; von Korff et al., 2004, 2005, 2006; Yun et al., 2006, Figure 2). Whereas Pillen et al., (2003, 2004), Talamé et al., (2004), Forster et al., (2004), Li et al., (2005, 2006), von Korff et al., (2005, 2006) and Yun et al., (2006) concentrated on the analysis of phenotypic data from extensive field or greenhouse trials, Matus et al. (2003), Hori et al. (2004) and von Korff et al. (2004) focused more on the development of advanced backcross populations and detailed characterization of the genetic structures of these new genetic resources. Matus et al. (2003) developed recombinant chromosome substitution lines (140 BC₂F₆) derived from 'Harrington x Caesarea 24-26', analyzed them for agronomic, domestication-related traits and malting quality in California and conducted association test with 47 SSR markers. Hori et al. (2005) generated recombinant chromosome substitution lines, a BC₃F₁ (134 lines) and a derived DH (93 lines) population, from the cross Haruna Nijo x H602. The populations were phenotyped for seed dormancy and several agronomic traits in one environment and genotyped with 25 SSRs and 60 ESTs. Von Korff et al. (2004) developed the two BC₂DH populations 'S42' from 'Scarlett x ISR42-8' (301 lines) and 'T42' from 'Thuringia x ISR42-8' (84 lines). The authors described the genomic architecture of two derived sets of candidate introgression lines as revealed with 98 and 65 SSRs.

Pillen et al. (2003, 2004) conducted the first thorough analysis of the agronomic performance of exotic barley germplasm. They genotyped two BC₂F₂ populations Apex x ISR101-23 (136 lines) and 'Harry x ISR101-23' (164 lines) with 45 and 50 SSRs, respectively. They field-tested them for agronomic traits and malting quality parameters in two consecutive years and at three different locations in Germany. A agronomic performance of the exotic germplasm under drought conditions was analyzed by Talamé et al. (2004). They examined a selected set of 123 DH lines derived from a BC₁F₂ Barke x HOR11508 in Italy, Morocco, and Tunisia (Forster et al., 2004). They studied the DH lines for agronomic traits and conducted a QTL analysis with 54 polymorphic AFLP markers and 59 SSRs.

Li et al. (2005) performed an AB-QTL analysis in 181 selected BC₃DH lines derived from the spring barley cultivar, Brenda, and the exotic accession, HS213. The population was tested in field trials in two consecutive seasons and at two different locations in Germany, and genotyped with 60 SSRs, revealing that only 61% of the lines carried a detectable introgression. Von Korff et al. (2005, 2006) phenotyped 301 BC₂DH lines of the population 'S42' for agronomic performance and disease resistance in two consecutive years and at four different locations in Germany. Li et al. (2006) tested 200 BC₃F₂ derived lines from the cross Brenda x HS584 at two locations over four years and conducted a QTL analysis with 108 SSRs. Yun et al. (2006) developed an AB-population (BC₂F_{6,8}) from the cross

Harrington x OUH602, phenotyped the population for seedling disease resistance, and conducted a QTL analysis with a set of 111 SSRs.

The exotic donors used in these studies were derived from Israel (Pillen et al., 2003, 2004; Li et al., 2005, 2006; Matus et al., 2003; von Korff et al., 2004), Greece (Talamé et al., 2004), and the Caspian Sea region (Hori et al., 2005). Their selection was primarily based on per se performance, origin, and passport data. QTL analyses, however, have shown that the phenotype of a plant is only a modest predictor of its genetic potential, especially with respect to quantitative traits (Tanksley et al., 1996). Accordingly, von Korff (2005) selected the donors based on agronomic performance of backcross progeny derived from crosses between ten barley cultivars and ten *H. vulgare* ssp. *spontaneum* accessions, rather than on per se performance of the wild barley accessions. Despite the rich collections of exotic barley, the challenge remains to develop strategies for effectively sampling novel and favourable exotic alleles and introducing them into elite germplasm.

The potential use of wild germplasm for the improvement of agronomic traits is still a matter of controversy. Matus et al. (2003) and Li et al. (2004) did not detect favourable exotic alleles for yield. They characterised the use of wild barley as disappointing from a plant breeding perspective, but suggested that exotic introgression libraries may be a valuable tool for gene discovery. In contrast, Pillen et al. (2003) found that at 34% of all QTL, the exotic allele improved agronomic performance. Similarly, von Korff et al. (2006) detected favorable exotic alleles at 36% of all QTL in the BC2DH population 'S42'.

Pillen et al. (2003) and von Korff et al. (2006) reported that the maximum average yield increase associated with an exotic QTL allele resulted in an average yield improvement of 7.7% and 7.1 %. Pillen et al. (2003) explained the lower favorable effect of exotic alleles on yield compared to the strong effect of exotic alleles in tomato (34%) (Fulton et al., 1997) and rice (17%, 18%) (Xiao et al. 1996, Xiao et al. 1998) with different breeding systems. In barley, homozygous backcross lines were analyzed (Pillen et al., 2003, 2004, Matus et al., 2003, Li et al., 2004). Fulton et al. (1997) and Xiao et al. (1998), however, compared hybrids in tomato and rice, where heterosis effects between elite and exotic germplasm may have contributed to the yield increase. Talamé et al. (2004), however, identified exotic QTL alleles in barley, causing an average yield increase of 17%, which is consistent with results in tomato and rice. This higher yield increase can possibly be explained by a selection against negative agronomic characters during the establishment of the AB-population. The exotic introgressions in AB-populations, however, are still relatively large, an average introgression size of 38.6 cM (Matus et al. 2003) and 35 cM (von Korff et al. 2004) was reported, and they contain thousands of genes. Therefore, selection against deleterious characters risks the elimination of linked alleles with a potentially beneficial effect. On the other hand, if major alleles with deleterious effects are removed, effects of minor alleles may be more easily detectable. Notorious examples here are the two complementary genes on chromosome 3H, *Br1* and *Br2* (Azhacuvél et al. 2006) with the dominant exotic

allele causing the formation of a brittle rachis with a strong negative effect on yield performance.

In addition, all these AB-QTL analyses reported a strong segregation of flowering time and plant stature in their respective populations. Wild barley is characterized by early and heterogeneous flowering and prostrate growth behavior, which is presumably an adaptation strategy to drought-prone environments. Correspondingly, the majority of exotic alleles caused early flowering with the strongest effect consistently found at a QTL on 2HS (Pillen et al., 2003; Li et al., 2005, 2006; von Korff et al., 2006). This region harbors the photoperiod response gene *Ppd-H1*, which promotes early flowering under long day conditions (Laurie et al., 1995). At other loci, however, the exotic allele clearly delayed flowering, for example, at QTL close to the vernalization response genes *Vrn-H1* (Hori et al., 2005; Li et al., 2006), *Vrn-H3* (Talamé et al., 2004; von Korff et al., 2006) and *Vrn-H2* (Pillen et al., 2003, 2004; Korff et al., 2006). An effect of the exotic allele on heading date was also repeatedly found on 2H, bin 13 (Pillen et al., 2003, 2004; Talamé et al., 2004; von Korff et al., 2006), 3H, bin 3 close to the *btr* loci (Pillen et al., 2004; Talamé et al., 2004; von Korff et al., 2006), 3H, bin 12 and adjacent to the *sdw1* locus (Pillen et al., 2004; Talamé et al., 2004; von Korff et al., 2006; Li et al., 2006) and 7H, bin 9 (Pillen et al., 2003, 2004; Talamé et al., 2004; von Korff et al., 2006).

AB-populations also show a large variation for plant height. Talamé et al. (2004) found a maximum variation in plant height of between 88 and 144 cm in Morocco. Von Korff et al. (2006) reported an average plant height in the population 'S42' across eight environments of between 63 cm and 110 cm. Major plant height QTL were located on 2H, bin 8 (Pillen et al., 2004; von Korff et al., 2006; Li et al., 2006), 3H, bin 12 (Talamé et al., 2004; von Korff et al., 2006), 4H, bin 13 (Pillen et al., 2003, 2004; Talamé et al., 2004; von Korff et al., 2006) and 5H, bin 6 (Talamé et al., 2004; Li et al., 2005; von Korff et al., 2006; Li et al., 2006). Corresponding candidate genes are the semi-dwarf genes *sdw3*_[2H] (Gottwald et al., 2004), *sdw1*_[3H], and *ari-e.GP*_[5H], and the flowering loci *Ppd-H1*_[2H] and *Vrn-H2*_[4H]. At the majority of QTL-loci, the exotic allele increased plant height, in particular at QTL close to the candidate genes *sdw1* and *ari-e.GP*, but at the QTL on 2HS and 4HL the exotic allele consistently reduced plant height.

Under drought conditions, heading is negatively and plant height positively correlated with yield, indicating that tall early heading genotypes present a good yielding capacity under water limiting conditions. Indeed, the strongest favorable effects of the exotic germplasm on yield were found under drought conditions in Tunisia and Morocco (Talamé et al., 2004). The same authors, however, observed that the exotic alleles with a delay in flowering time showed a favorable effect on yield, indicating that the favorable effect on yield under conditions of limited water was not due to drought escape but to an increase in yield potential. Similarly, in the AB-population 'S42' the exotic introgression on 4HL showed a favorable effect on yield under drought conditions, although this QTL-allele postponed flowering (Hamam et al., in prep).

In AB-QTL studies, major QTL loci often showed pleiotropic effects on a number of different traits and resulted in a strong clustering of QTL in particular on 2HS, 3HL, 4HL, and 5HL. The exotic introgression at the semi-dwarf locus *sdw1*, for example, affected, next to flowering time, plant height, yield and thousand grain weight (Pillen et al., 2003; Li et al., 2005; von Korff et al., 2006; Li et al., 2006, Figure 2). Similarly, QTL close to the *Vrn-H2* locus influenced heading date, plant height, and yield (Pillen et al., 2003; Talamé et al., 2004; von Korff et al., 2006). Although the donor and recipient germplasm differed between the cited AB-QTL studies, the exotic alleles exhibited predominantly the same qualitative effect at these major QTL for heading date, plant height, and yield. The exotic alleles are thus often similar in their effects and clearly different from the elite alleles. Wild barley thus harbors novel genetic variability for these key loci. The AB-QTL strategy allows the selection of major genes/alleles from the exotic gene pool with the most beneficial pleiotropic effects, especially in stress environments, and introduces these into breeding programs while eliminating negative alleles such as brittleness.

Eshed and Zamir (1994) demonstrated that introgression lines in tomato are a powerful tool for map-based cloning (Frary et al., 2000) and the discovery of gene function by transcriptome and metabolome analysis (Schauer et al., 2006). In barley, advanced backcross populations enable the fast generation of such introgression lines as demonstrated by von Korff et al. (2004) and Hori et al. (2005). Von Korff et al. (2004) selected from each of the BC₂DH populations 'S42' and 'T42' and Hori et al. (2005) from the BC₃F₁ population Haruna Nijo x H602, a minimal set with 49, 43, and 19 introgression lines, respectively, which cover a large percentage of the exotic genome in overlapping exotic segments. Further backcrossing and establishment of nearly isogenic lines generate a valuable resource for validating the effects of exotic QTL-alleles, introducing them into elite cultivars, map-based cloning of verified QTL, and ultimately for the study of gene function.

5. IDENTIFICATION OF FUNCTIONAL GENES RESISTANT TO DROUGHT BY MICROARRAY

A number of genes have been described that respond to drought at the transcriptional level (Bray 1993; Seki et al., 2002; Cheong et al., 2003; Liu and Baird, 2004). The functions of some genes have been predicted from sequence homology with known proteins and are thought to have a role in protecting the plant from water deficit. Some important stress-inducible genes have been used to improve the stress tolerance of plants by gene transfer (Xu et al., 1996; Pellegrineschi et al., 2002, 2004; Abebe et al., 2003; Umezawa et al., 2006). Although a large number of drought-induced genes have been identified in a wide range of plant species, a molecular basis for plant tolerance to water deficit remains far from being completely understood (Ingram and Bartels, 1996; Bruce et al., 2002).

There is widespread interest in using microarrays to monitor gene expression profiles for plants exposed to limited water environment. Several studies with rapid dehydration treatment have shown that osmotic-stress-inducible genes could explain

the response to drought stress in plants (Seki et al., 2001; Ozturk et al., 2002; Seki et al., 2002; Shinozaki et al., 2003; Talamé et al., 2003; Ueda et al., 2004; Zhang et al., 2004). It is also thought that these gene products may play important roles in the adaptation of plants.

It has become evident that multiple stress responses are necessary for plants to endure severe stress conditions (Nakashima and Yamaguchi-Shinozaki, 2006). It is possible for a single transcription factor to control the expression of many target genes through the specific binding of the transcription factor to *cis*-acting element in the promoters of their respective target genes. Nakashima and Yamaguchi-Shinozaki (2006) described the expression mechanisms of osmotic stress-responsive genes for the presence of multiple regulons in Arabidopsis. The dehydration-responsive element-binding protein (DREB1)/C-repeat (CRT)-binding factor (CBF) regulon is involved in osmotic stress-responsive gene expression, and the other regulons including the DREB2 regulon are involved in osmotic stress-responsive gene expression. It is expected that biotechnological efforts, which strive to control gene expression within these regulons, will improve tolerance against multiple stresses in plants.

5.1. Responsive Genes to Osmotic-/Drought-Stress in Barley Under Seedling Stage

Compared with the model plant, barley, a major crop plant, is one of few rainfed crops in the dry areas. Thus, it is a good target for the study of the mechanisms of drought tolerance in crop plants. However, until now, there are only few reports on the use of barley cDNA micro-array for transcriptional analysis under dehydration (Ozturk et al., 2002) and osmotic stress (Ueda et al., 2004) conditions. Response to dehydration in barley (*Hordeum vulgare* L. cv. Tokak) was monitored by micro-array of 1463 DNA elements (Ozturk et al., 2002). Transcripts that showed significant up-regulation under dehydration stress were exemplified by jasmonate-responsive, metallothionein-like, late-embryogenesis-abundant (LEA) and ABA-responsive proteins. The most drastic down-regulation in a category was observed for photosynthesis-related functions. Under osmotic stress, Ueda et al. (2004) found that 52 genes were differentially expressed in a total of 460 non-redundant cDNA microarray, 22 of which were up-regulated, such as sucrose synthase, GAPDH, alanine aminotransferase, lipoxygenase, and PMP3. Thirty genes, including sugar transporter, HSP70, serine/threonine kinase, PEPC, actin, water channel 2, etc., were down-regulated. Although information regarding expression profiles in response to the development of abrupt and severe water stress is useful, studies that impose slower, more realistic rates of dehydration are needed.

In an attempt to meet this need, Talamé et al. (2003) compared gene expression patterns between several water stress treatments in young barley plants. From the tissues of treated and control plants, mRNA was extracted, labeled, and hybridized to micro-arrays containing 1,463 DNA elements derived from barley cDNA libraries (Ozturk et al., 2002). Micro-array analysis with mRNA extracted from water

shock and control tissues largely confirmed the changes in the expression level of transcripts as reported earlier by Ozturk et al. (2002). Nearly 15% of all transcripts were either up- or down-regulated under conditions of rapid drought stress. Transcripts that showed significant up-regulation included jasmonate-responsive, metallothionein-like, late-embryogenesis-abundant (LEA), and ABA-responsive proteins. In the second experiment, water-stressed plants showed significant changes in transcript abundance after the seventh day of water deficit when the relative water content was ca. 90–93%. The greatest change in expression was observed after eleven days of treatment. As in the water shock experiment, expression profiles were analyzed by cluster analysis. It was shown that the majority of treatment effects were due to transcript changes in genes whose putative function involved protein synthesis, and turnover early in the experiment followed by later induction of known stress-responsive transcripts.

5.2. Transcriptional Analysis Between Drought-Tolerance and Sensitive Genotypes at the Reproductive Stage

The grain yield of barley is likely to be reduced a little if drought stress occurs during the vegetative period. However, it is vulnerable to drought stress during the reproductive growth stage, which is usually characterized by terminal drought stress (Ceccarelli et al., 2004). Understanding the molecular mechanism of drought tolerance during the reproductive growth stage may facilitate identification of novel drought tolerance genes for genetic improvement of barley cultivars. Meanwhile, the different genotypes contrasting in drought tolerance should have different mechanisms in response to drought stress. Therefore, it is meaningful to investigate and identify the genes that are differentially expressed between drought-tolerant and -sensitive genotypes under water deficit conditions at the reproductive stage, and to gain insight into the processes involved in stress responses by analyzing their expression profiles.

Exploration for differentially expressed genes responsive to drought has been carried out at the International Center for Agricultural Research in the Dry Areas (ICARDA) using two barley genotypes of Tadmor and WI2291 as experimental material (Guo et al., 2005). A greenhouse experiment was conducted in an ICARDA temperature-controlled greenhouse with 16 hours daylight at 30°C and 8 hours dark period at 20°C with pots to estimate drought tolerance of two barley genotypes. Drought stress was imposed at the heading stage by reducing available water in the soil from 70% (non-stress condition) to 10% (severe drought stress). Two physiological traits chlorophyll content and Fv/Fm (maximal fluorescence (F_m)/variable fluorescence (F_v)) and grain yield were measured. Tadmor showed significantly less yield loss than WI2291 under drought stresses, and there was almost no difference in the reduction of grain yield between moderate and severe drought stress in Tadmor, but there were pronounced differences in WI2291 (Figure 3). The ratio of F_v/F_m indicates the potential photochemical yield of PS II and quantum efficiency, and it is an important index for evaluating the photosynthesis efficiency (Figure 3).

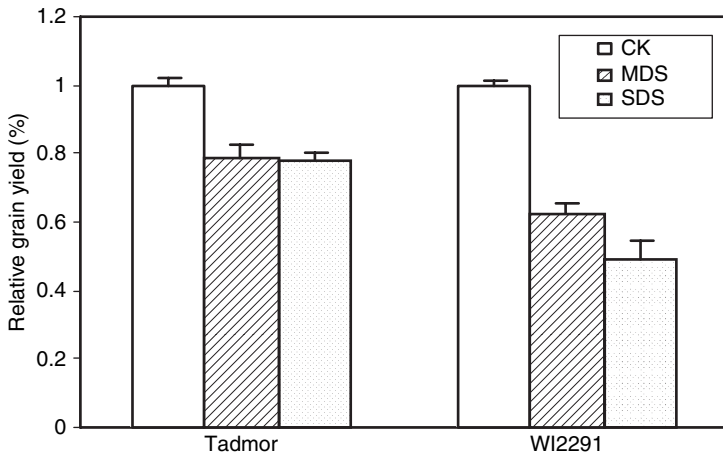


Figure 3. Grain yield of Tadmor and WI2291 under the three moisture regimes in the soil (means \pm SE)

- CK = well-watered conditions, 70% available water in the soil
- MDS = moderate drought stress, 35% available water in the soil
- SDS = severe drought stress, 10% available water in the soil

In this study, the chlorophyll content and the ratio of F_v/F_m slightly decreased with time and almost remained constant in control leaves in both varieties through the 13-day period after treatment (Figure 4). However, the chlorophyll content and the ratio of F_v/F_m obviously decreased after 7 and 9 days in severe drought stress (SDS), 9 and 11 days in moderate drought stress (MDS) for Tadmor and after 5 and 7 days in SDS, 7 and 7 days in MDS for WI2291 after imposing water deficit. The chlorophyll content and the ratio of F_v/F_m decreased more in WI2291 than in Tadmor. These results confirm the field observation that Tadmor is more tolerant to water deficit stress than WI2291.

For gene expression analysis through 22K of Affymetrix GeneChip Barley 1 arrays, total RNAs were isolated from the flag leaves of Tadmor and WI2291 at the 3rd day of severe drought stress and non-stress treatment at post-anthesis stage. Sample processing, hybridization, and scanning of Affymetrix Barley Genome Arrays were performed through the standard Affymetrix protocol (Affymetrix GeneChip® Expression Analysis Technical Manual). Gene ontology analysis was conducted by HarvEST Barley 1.34 and GO functional categorization at TAIR (The Arabidopsis Information Resource). The results showed that some functional genes in both genotypes have similar response to water deficit stress in gene expression profiles, 77 of those genes were found to be significantly differentially expressed in Tadmor and WI2291 after drought stress. These genes may only be common genes responsive to drought stress since they appeared in both the tolerant and sensitive genotypes. The gene expression profiles were further analyzed for exploration of specific genes in response to drought stress through the comparison of gene expression profiles to drought stress between Tadmor and WI2291, 372 genes

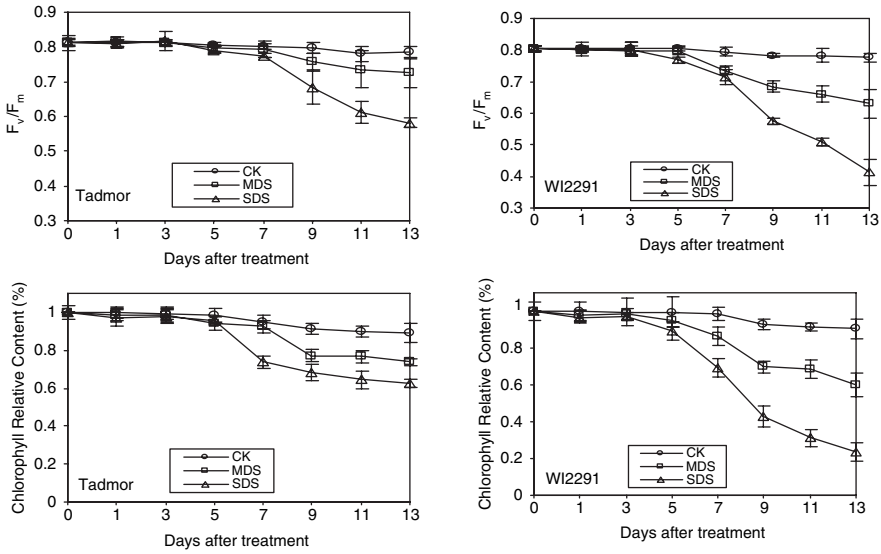


Figure 4. Chlorophyll fluorescence parameters (F_v/F_m) and chlorophyll contents of Tadmor and WI2291 under well-watered conditions (mean \pm SE of five individual measurements)

were found to be significantly differentially expressed between two genotypes at post-anthesis drought.

Under drought stress different varieties of barley should respond to this stress with changing expression of sets of genes regardless of whether they are tolerant or sensitive to drought. Some of these differentially expressed genes may only be responsive/induced genes without contributing to the tolerance, while some of them may be specific genes that allow the plant to adapt to the stress. A number of genes showed in both lines, Tadmor and WI2291 a differential expression under drought. The results indicated that the genes like dehydration-responsive protein RD22, early drought-induced protein, heat shock protein-like, dehydrin 3, protein kinase HvPKABA1, protein kinase SPK-3, pyrophosphate-dependent phosphofructokinase, etc., increased significantly with severe drought stress of post-anthesis stage in both varieties. These genes could putatively only be responsive/induced genes to drought stress. The comparative analysis of gene expression profiles between Tadmor and WI2291 indicated that some genes were regulated by drought stress and could putatively be the specific/responsible genes for drought tolerance in Tadmor. These include serine/threonine kinase-like protein, hepatoma-derived growth factor, inositol-3-phosphate synthase, microsomal signal peptidase, chlorophyll *a/b*-binding protein 1A, ribulose-bisphosphate carboxylase activase, pathogenesis related protein-1 and 4, PRB1-2, PRB1-4, ABC transporter family protein, organic anion transporter, glutathione S-transferase, OsGSTT1, etc.. All these results may provide new insights for the response of plants to water deficit stress.

6. SNPS FOR THE SELECTION OF ABIOTIC STRESSES

Single nucleotide polymorphisms (SNP) result from a single base mutation, which substitutes one base for another, are biallelic in nature and less polymorphic than the multiallelic microsatellites. However, the extraordinary abundance of SNPs in the genome largely offsets the disadvantage of their being biallelic and makes them the most attractive molecular marker system developed so far (Gupta et al., 2001). The allelic frequencies of a given SNP may vary in different populations. On average, an SNP is believed to be present in every 100 to 300 bases in any genome (Gupta et al., 2001). Their wide distribution in the genome, coupled with the availability of several high throughput assay systems, makes SNPs good candidates as genetic markers for both mapping and association genetic studies in plants (Wilson et al., 2003).

The development of new varieties tolerant to abiotic and biotic stresses is an essential part of the continued barley improvement program. Rostoks et al. (2005) developed more than 2000 genome-wide barley SNPs from eight diverse barley accessions. The genes used for SNP discovery were selected based on their transcriptional response to a variety of abiotic stresses such as high salt, drought, low temperature, low nitrate, and water logging. More than 300 SNPs were found in three mapping populations (Oregon Wolfe Dominant x Oregon Wolfe Recessive, Steptoe x Morex and Lina x *Hordeum spontaneum* 92) in an integrated linkage map incorporating a large number of RFLP, AFLP, and SSR markers. The integrated map showed good agreement with the published maps in terms of marker order, and known abiotic stress QTL mapped in relevant crosses. A number of stress responsive genes mapped to chromosome 5H and could potentially be involved in salt and low temperature tolerance. These include cyclic nucleotide-gated ion channels (scsnp46137, scsnp14060, scsnp14759, scsnp22879), putative heat shock proteins (scsnp00892, scsnp01204, scsnp02771, scsnp6096), M-type thioredoxin (scsnp03171), putative protein kinase (scsnp14350), putative multidrug, and toxic compound extrusion efflux transporter (scsnp15296). An understanding of the combined function and expression of genes involved in various abiotic stresses, which could help identify candidate genes underlying the QTL of interest is important.

7. CONCLUSIONS

Numerous QTL studies for agronomic performance have been conducted in favorable environments using balanced populations. Although drought tolerance has been recognized as one of the prime breeding goals to increase food production in arid areas, only very few QTL studies are available which analyzed agronomic performance in drought stressed environments. For agriculture in the Mediterranean region *H. spontaneum* plays a major role as the main source of drought tolerance, and first results of QTL studies with exotic germplasm look promising. A number of favorable exotic alleles for agronomic performance were detected, in particular,

in the studies conducted under drought (Baum et al., 2003, Talame et al., 2004) (Figure 2). However, further QTL studies on drought stress using land races and exotic barley as donors are clearly needed to make efficient use of the vast collection of germplasm resources. QTL studies with and without *H. spontaneum* have shown that developmental genes, notably those involved in flowering time and plant stature show pleiotropic effects on abiotic stress tolerance and ultimately determine yield. Important loci in this regard are the semi-dwarf gene *sdw3* (bin 7 on 2H), the flowering locus *Ppd-H1* (2H, bin 4) and the vernalization requirement *Vrn-H2* (4H, bin 13) (Figure 2). It was shown that *H. spontaneum* offers novel allelic variance at these loci. These alleles from wild barley can be exploited for the improvement of drought tolerance in elite germplasm by selecting those exotic alleles with the most beneficial pleiotropic effects on drought resistance.

Problems associated with the hybridisation of *H. spontaneum* such as alleles with deleterious effects on field performance, i.e. the brittle rachis loci *btr*, could be best addressed in the advanced backcross QTL analysis. It was interesting to see that in AB-QTL populations like in balanced populations major QTL overshadowed minor QTL-alleles. Nevertheless, crosses with *H. spontaneum*, AB-QTL populations and association studies with *H. spontaneum* have also identified new alleles and genes that are related to abiotic stress tolerance. Their description and exploitation in breeding lines might lead to the availability of a wider gene pool for adaptation to drought tolerance.

In order to better understand which genes are involved in drought and other abiotic stresses in the dry areas two approaches are important. Appropriate genetic resources and populations using new donors such as exotic barley and landraces have to be generated. Here, first the establishment of introgression lines (mainly used in relation to introgressions of *H. spontaneum* into *H. vulgare*) and their testing in dry areas would give more insight into the effect of certain favorable alleles. Second, much larger populations need to be tested in order to be able to identify confounding effects through major QTL loci. Furthermore, as arid areas may be characterized next to drought by very different environmental conditions, a thorough selection of test environments and comprehensive phenotyping are indispensable.

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CHAPTER 4

MOLECULAR MARKERS FOR GENE PYRAMIDING AND DISEASE RESISTANCE BREEDING IN BARLEY

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Abstract: Barley (*Hordeum vulgare* L.) is one of the oldest crop plants and among the most important cereals worldwide which is cultivated from the polar circle to the tropics. Barley crops can be infected and severely damaged by many fungal, viral and bacterial pathogens as well as insect pests. Therefore, breeding for disease and pest resistance is of special importance in barley in order to prevent or reduce yield losses. Furthermore, genetic resistance allows the reduction of agrochemical applications and thereby greatly contributes to environment and consumer protection. Resistance breeding has already been very successful in the past and provided many resistant or tolerant barley varieties highly adapted to adverse growing conditions. The genetic basis of such resistance depends on the respective pathogen or pest: Many cases of monogenic (major gene) resistance have been described but oligo- or polygenic types of resistance are also widespread and appreciated by breeders and growers due to their superior durability. Today, molecular markers are available for many major resistance genes and resistance QTL (quantitative trait loci) against a wide range of pathogens in barley. Such markers are the basis for an efficient marker assisted selection (MAS) in scientific breeding research and commercial barley breeding. Furthermore, marker assisted backcrossing procedures allow an enhanced incorporation of resistance genes derived from non-adapted germplasm (e.g. *H. bulbosum*). Beyond this, pyramiding of resistance genes by marker-assisted combination breeding may lead to longer lasting resistance and enable the further use of resistance genes already overcome by single strains or isolates of the respective pathogen. This chapter reviews the present state of the art concerning markers available for major resistance genes and resistance QTL in barley and exemplifies marker assisted backcrossing and pyramiding strategies in the pathosystem barley – barley yellow mosaic virus disease caused by BaYMV and BaMMV.

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1. INTRODUCTION

Next to wheat, maize, and rice, barley (*Hordeum vulgare* ssp. *vulgare*) which is grown world-wide on an area of about 57 million hectares (FAO 2005) is number four of the world's cereal crops being very important for livestock feeding and human alimentation. Like in other crops, severe yield losses occur in barley every year due to fungal and viral diseases and as a result of insect pests. Therefore, breeding for disease and pest resistance has to be regarded as a major goal of barley breeding reducing yield losses and contributing to a sustainable environment and consumer friendly barley production due to reduced pesticide applications.

As a result of intensive breeding, modern barley cultivars combine pathogen resistances with high grain yield and superior seed quality. In general, combining of resistances or the introgression of new resistance genes from unadapted germplasm or related species, respectively, is achieved by sexual recombination, i.e. crossing of parental lines followed by phenotypic selection in the segregating offspring. In this case the success of breeding entirely depends on extensive field or glasshouse tests for resistance to the respective pathogens. But, as barley is damaged by many pathogens which often show a rapid adaptation to its hosts resistance genes, breeding for resistance is a very complex task and the identification of desired recombinants expressing resistance to most diseases by phenotypic selection only has nearly reached the limits of manageability. But, today plant biotechnology methods allow to improve the efficiency of selection and to accelerate the whole breeding process: for example, anther or microspore culture starting from F_1 donor plants immediately leads to homozygous doubled haploid plants facilitating easier and more accurate selection than in conventional segregating F_2 populations (Friedt & Ordon 2004, Werner et al. 2006). In addition, especially the development of molecular marker systems and the isolation of resistance genes transferring selection to some extent from the phenotypic to the genotypic level offer new opportunities for a more efficient breeding for resistance.

Since barley is grown from the polar circle to the tropics it is hit by a wide range of different fungal and viral diseases. Consequently, many corresponding resistance genes and quantitative trait loci (QTL) have been tagged already. In the following chapter an overview is given on such major resistance genes and QTL for resistance and possibilities to use respective markers in different marker based selection schemes are elucidated.

2. MOLECULAR MARKER TAGGED RESISTANCE GENES AND RESISTANCE QTL IN BARLEY

The development of molecular markers starting from restriction fragment length polymorphisms (RFLPs, Botstein et al. 1980), via the application of PCR-based procedures (Saiki et al. 1985, 1988), has dramatically increased the possibilities of an efficient application of marker based selection procedures in plant breeding. Meanwhile, closely linked PCR-based markers have been developed for many major resistance genes against important pathogens of barley (Table 1).

Table 1. List of mapped major resistance genes in barley based on Graner (1996) and Graner et al. (2000a), modified and extended

Resistance gene	Chromosomal location	Reference(s)
<i>Powdery mildew (Blumeria graminis)</i>		
<i>mlo</i>	4HL	Hinze et al. 1991, Büschges et al. 1997
<i>Mlg</i>	4HL	Görg et al. 1993, Kurth et al. 2001
<i>Mla</i>	1HS	Schüller et al. 1992, Schwarz et al. 1999, 2002, Wei et al. 1999, Zhou et al. 2001, Haltermann et al. 2001, 2004, 2006,
<i>mlt</i>	7HS	Schoenfeld et al. 1996
<i>Mlj</i>	5HL	Schoenfeld et al. 1996
<i>Mlf</i>	7HL	Schoenfeld et al. 1996
<i>MlLa</i>	2HL	Hilbers et al. 1992, Giese et al. 1993, Mohler & Jahoor 1996
<i>Mlh</i>	6H	Hilbers, cit Graner 1996
<i>MlHb</i>	2HS	Pickering et al. 1995, Graner et al. 1996b
<i>MI(TR)</i>	5H	Falak et al. 1999
<i>Mli</i>	1HL	Jahoor, cit Graner 1996
<i>Puccinia graminis</i>		
<i>Rpg1</i>	7HS	Horvath et al. 1995, Penner et al. 1995, Han et al. 1999, Brueggemann et al. 2002
<i>rpg4</i>	5HL	Borovkova et al. 1995, Kilian et al. 1997
<i>Puccinia hordei</i>		
<i>Rph2</i>	5HS	Borovkova et al. 1997
<i>Rph3</i>	7HS	Park et al. 2003
<i>Rph4</i>	1H	Collins et al. 2001, Park et al. 2003
<i>Rph5</i>	3H	Mammadov et al. 2003, 2005
<i>Rph7</i>	3HS	Graner et al. 2000b, Brunner et al. 2000, 2003, Scherer et al. 2005
<i>Rph9</i>	5HL	Borovkova et al. 1998
<i>Rph12</i>	5HL	Borovkova et al. 1998, Park et al. 2003
<i>Rph15</i>	2HS	Weerasena et al. 2004
<i>Rph16</i>	2HS	Ivandic et al. 1998, Perovic et al. 2004
<i>Rph19</i>	7HL	Park & Karakousis 2002
<i>Puccinia striiformis</i>		
<i>rpsGZ</i>	4H	Yan & Chen 2006
<i>Rhynchosporium secalis</i>		
<i>Rh (Rrs1)</i>	3HL	Graner & Tekauz 1996, Penner et al. 1996, Reitan et al. 2002, Genger et al. 2003
<i>Rhy</i>	3HL	Barua et al. 1993

(Continued)

Table 1. (Continued)

Resistance gene	Chromosomal location	Reference(s)
<i>Rh2</i>	7HS	Schweizer et al. 1995, Schmidt et al. 2001
<i>Rrs4</i>	4HL	Patil et al. 2003
<i>Rrs13</i>	6HS	Abbott et al. 1995
<i>Rrs14</i>	1H	Garvin et al. 2000
<i>Rrs15</i>	7HL	Genger et al. 2005
<i>Pyrenophora teres</i>		
<i>Pt.a</i>	3HL	Graner et al. 1996
<i>Pt.d</i>	2HS	Graner & Tekauz, unpubl., cit. Graner et al. 2000
<i>Rpt4</i>	7H	Williams et al. 2004
<i>Cochliobolus sativus</i>		
<i>Rcs5</i>	7HS	Steffenson et al. 1996
<i>Typhula incarnata</i>		
<i>Ti</i>	1HS	Graner et al. 1996
<i>Pyrenophora graminea</i>		
<i>Rdg1</i>	2HL	Thomsen et al. 1997
<i>Rdg2</i>	7HS	Tacconi et al. 2001, Bulgarelli et al. 2004
<i>Heterodera avenae</i>		
<i>Ha2</i>	2HS	Kretschmer et al. 1997
<i>Ha4</i>	5HL	Barr et al. 1998
<i>Barley stripe mosaic virus (BSMV)</i>		
<i>Rsm</i>	7HS	Edwards & Stephenson 1996
<i>Barley yellow dwarf virus (BYDV)</i>		
<i>Ryd2</i>	3HL	Collins et al. 1996, Paltridge et al. 1998, Ford et al. 1998
<i>Ryd3</i>	6H	Niks et al. 2004
<i>Barley yellow mosaic virus (BaYMV), Barley mild mosaic virus (BaMMV)</i>		
<i>rym1</i>	4HL	Okada et al. 2004
<i>rym3</i>	5HS	Saeki et al. 1999, Werner et al. 2003a
<i>rym4</i>	3HL	Graner & Bauer 1993, Ordon et al. 1995, Weyen et al. 1996, Stein et al. 2005, Kanyuka et al. 2005
<i>rym5</i>	3HL	Graner et al. 1999a, Pellio et al. 2005, Stein et al. 2005, Kanyuka et al. 2005
<i>rym7</i>	1HS	Graner et al. 1999b
<i>rym8</i>	4HL	Bauer et al. 1997
<i>rym9</i>	4HL	Bauer et al. 1997, Werner et al. 2000
<i>rym10</i>	3HL	Graner et al. 1995,
<i>rym11</i>	4HL	Bauer et al. 1997, Nissan-Azzous et al. 2005
<i>rym12</i>	4HL	Graner et al. 1996a
<i>rym13</i>	4HL	Werner et al. 2003b
<i>Rym14^{Hb}</i>	6HS	Ruge et al. 2003
<i>rym15</i>	6H	Le Gouis et al. 2004
<i>Rym16^{Hb}</i>	2HL	Ruge-Wehling et al. 2006

Besides major resistance genes many QTL contributing to resistance against important pathogens have been detected. In contrast to resistance encoded by major genes it is consensus and to some extent supported by evidence that quantitative resistance is more durable *per se*. In the past, studies on quantitative, oligo- or polygenically inherited resistance have mainly focused on epidemiological studies and biometrics, but today efficient tools such as molecular marker techniques and sophisticated software packages are available, facilitating the dissection of quantitative resistance into individual Mendelian loci and the determination of locus-specific PCR-based markers, especially SSRs (Ramsay et al. 2000, Maccaulay et al. 2001) and the AFLP-technique (Voss et al. 1995) facilitating an accelerated construction of genetic maps in comparison to RFLPs (Graner et al. 1991), has enhanced QTL detection. Consequently, the number of disease resistance QTL has risen considerably and may rise in the future due to the development of more efficient finger print techniques in barley e.g. DArTs (Wenzl et al. 2004).

With respect to *Blumeria graminis* the causal agent of powdery mildew, first QTL have been mapped in barley in the beginning of the 1990s on chromosome 7H and 5H (Heun 1992). Since that time many additional QTL have been detected all over the barley genome (Saghai Maroof et al. 1994, Backes et al. 1995, Thomas et al. 1995, Backes et al. 2003), some of them mapping in the vicinity of major resistance genes e.g. on chromosome 1H (Backes et al. 1996, Emebri et al. 2005). Using Advanced Backcross QTL analysis (AB-QTL), QTL for resistance to powdery mildew, leaf rust (*Puccinia hordei*) and scald (*Rhynchosporium secalis*) have been detected in *H. spontaneum* (v. Korff et al. 2005).

For *P. hordei* three QTL conferring leaf rust resistance at the seedling stage (explaining 55% of the phenotypic variance), and 5 QTL for resistance in the adult plant stage (explaining 60% of the phenotypic variance) with two QTL in common have been identified (Qi et al. 1998, 2000, van Berloo et al. 2002). In additional studies it turned out that only one QTL had a substantial effect in both developmental stages, and it was shown that some of the QTL exhibit isolate-specific reactions. In addition, one QTL for resistance to leaf rust has been identified in the position of the *Rph16* gene (Kicherer et al. 2000). In a very recent approach, a QTL for resistance to *P. hordei* has also been detected using association mapping (Kraakman et al. 2006).

With regard to yellow or stripe rust (*P. striiformis* f. sp. *hordei*) first QTL had been detected on chromosomes 5HL and 4HL (Chen et al. 1994). In later studies these QTL were confirmed and additional QTL have been detected (Toojinda et al. 1998, 2000, Castro et al. 2002, Vales et al. 2005) and combined in order to improve resistance ("pyramided", see Castro et al. 2002, 2003a, 2003b, 2003c).

Regarding resistance to *Pyrenophora teres*, the causal agent of barley net blotch, 7 QTL responsible for resistance in the adult stage accounting for 67% of the phenotypic variation, and 3 QTL for resistance in the seedling stage explaining 47% of the phenotypic variance were detected by Steffenson et al. (1996). In more recent studies, QTL for resistance against this pathogen have been located on

chromosome 6H (Manninen et al. 2000, Cakir et al. 2003, Emebri et al. 2005). Ma et al. (2004) detected two QTL on chromosomes 6HS and 2HS explaining about 70% of the phenotypic variance. Analysing adult plant resistance to the spot form of net blotch (*Pyrenophora teres* f. *maculata*), QTL on chromosomes 7H, 4H and 5H were identified (Williams et al. 2003). Furthermore, it has been shown that resistance to spot blotch *Cochliobolus sativus* (anamorph *Bipolaris sorokiniana*) at the adult plant stage is governed by two QTL explaining 70% of the phenotypic variance (Steffenson et al. 1996). Differences in QTL localisation for resistance to spot blotch were detected using different DH populations (Bilgic et al. 2005).

For resistance to scald (*Rhynchosporium secalis*) QTL were mapped on chromosomes 2HL (Backes et al. 1995), 3H, and 7H (Bjørnstad et al. 2004) as well as on 3H, 4H and 6H (Jensen et al. 2002). With respect to *Pyrenophora graminea*, the causal agent of barley leaf stripe, QTL were located on chromosomes 7HL, 2HS and 4HS (Pecchioni et al. 1996, 1999, 2000). Meanwhile, additional isolate and non isolate-specific loci were identified (Arru et al. 2002, 2003).

Among the fungal diseases of barley, Fusarium head blight (FHB, caused by *Fusarium* sp.) has recently gained evident importance. In this respect, three distinct regions contributing to resistance have been detected on chromosome 2H (Mesfin et al. 2003). QTL influencing mycotoxin (DON) content have also been detected on chromosome 2HL (Dahleen et al. 2004) and an additional major FHB QTL on chromosome 6H. Some of these QTL have been validated in different crosses and environments (Canci et al. 2004). In recent studies QTL for resistance to FHB have again been located on chromosome 2H, but additional QTL have been detected on the short arm of chromosome 5H (Hori et al. 2005) and on 4H (Horsley et al. 2006).

Besides these pathogens of major importance, QTL for resistance to bacterial leaf streak caused by *Xanthomonas campestris* pv. *hordei* (Attari et al. 1998) and to the blast disease caused by *Pyricularia oryzae* (Sato et al. 2001) have been detected. In addition to genetic loci for fungal and bacterial diseases, QTL for tolerance against *Barley yellow dwarf virus* (BYDV) have been identified (Toojinda et al. 2000, Scheurer et al. 2001). For BYDV-PAV and BYDV-MAV seven QTL were mapped and substantial QTL x environment interaction was observed (Toojinda et al. 2000). Scheurer et al. (2001) used a German isolate of BYDV-PAV and detected two QTL on chromosomes 2HL and 3HL in the region of the *Ryd2* gene together explaining 46% of the phenotypic variance. An additional QTL accounting for more than 70% of the phenotypic variance for tolerance to the German BYDV-PAV isolate has recently been mapped on chromosome 6H (Niks et al. 2004) and has been named *Ryd3* due to the large portion of phenotypic variance explained. QTL for resistance to the vectors of BYDV, i.e. several aphid species, have been detected on barley chromosomes 1H and 7H (Moharramipour et al. 1997).

It is interesting to note that some QTL map in chromosomal regions of barley where previously major resistance genes had been identified. For example, this has been shown for powdery mildew (Backes et al. 1996, 2003), yellow rust, leaf rust (Thomas et al. 1995, Kicherer 2000), and net blotch (Richter et al. 1998) resistances.

3. MARKER BASED STRATEGIES IN BREEDING FOR RESISTANCE

As described above, closely linked markers are available for many major resistance genes of barley as well as for numerous QTL. Besides this, some major resistance genes like *mlo* (Büschges et al. 1997), *Mla* (Haltermann et al 2001, Zhou et al. 2001), *Rpg1* (Brueggemann et al. 2002, Nirmala et al. 2006), or *rym4* (Stein et al. 2005, Kanyuka et al. 2005) have been isolated already, facilitating the development of allele specific functional genetic markers. These markers facilitating selection on the allele level as well as closely linked markers can be used in marker based selection procedures on the single plant level, i.e. in early generations, independently from the natural occurrence of respective pathogens in the field and from labor and time consuming greenhouse tests. Based on the known position of respective genes and QTL it can be calculated whether these are combinable at all and which population size is needed to combine these genes. In applied barley breeding e.g. markers for BaMMV/BaYMV resistance genes have been widely applied (Schiemann & Backes 2000) or for BYDV-resistance (Ovesna et al. 2000). Besides the application in mere marker based selection procedures, respective markers can be used to enhance backcrossing procedures and to pyramid resistance genes.

3.1. Marker Assisted Backcrossing

An incorporation of recessive resistance genes derived from exotic or un-adapted germplasm normally requires long-lasting backcrossing procedures to combine these resistances with other agronomic traits, in particular superior yield. Incorporating recessive resistance genes by phenotypic selection takes twice as long as incorporating dominant genes, since a selfing generation is required after each backcross (BC) step for the phenotypic identification of homozygous recessive resistant plants to be used in the next BC cycle. However, by using co-dominant markers, like SSRs, heterozygous carriers of the resistance encoding allele to be used for the next BC step can be detected directly in F_1 , thereby saving one year for each BC cycle. The same holds true for dominant markers generating an additional fragment linked to the resistance encoding allele (cf. Ordon et al. 1999). Differences between conventional selection and a MAS procedure are exemplified in Figure 1 for the BaMMV resistance gene *rym15*. The time saving of this procedure becomes even more evident in the case that barley mild mosaic virus (BaMMV) and barley yellow mosaic virus (BaYMV) resistances are not encoded by the same but by different genes, like in the case of the Japanese variety 'Chikurin Ibaraki 1', carrying the BaMMV resistance *rym15* on chromosome 6H (Le Gouis et al. 2004) and a separate BaYMV resistance gene on chromosome 5H (Werner et al. 2003a). Since BaYMV is not efficiently transmissible by mechanical means, an additional selfing generation is needed to obtain a sufficient quantity of seeds for field testing, while in the MAS approach the track of both genes can be followed simultaneously. The use of markers leads

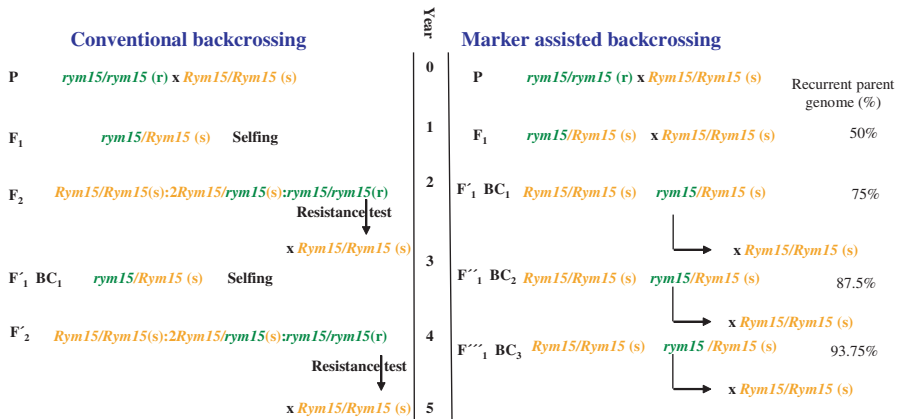


Figure 1. Comparison of conventional and marker-assisted backcrossing programmes for the incorporation of *rym15* (from Ordon et al. 1999, mod.) (See Plate 1)

to a drastic enhancement of BC procedures which may be further improved if the genomic portion of the recurrent parent is determined additionally. Results in different species have shown that there may be a significant deviation from the 75% genomic portion of the recurrent parent expected in the BC₁ generation (Powell et al. 1996, Uptmoor et al. 2006), thus demonstrating the usefulness of genotype-based selection.

3.2. Pyramiding Resistance Genes

Monogenic (qualitative) resistance, i.e. resistance conferred by a single major gene (Leach et al. 2001), is assumed to be non-durable in most cases, with exceptions like *mlo* resistance, due to the generally high mutation rate in plant pathogens (Takken & Joosten 2000, Kiyosawa 1982, Kloppers & Pretorius 1997). This can lead to the selection of new pathogen strains, able to overcome the effect(s) of an individual resistance gene. Additionally, the probability of disease resistance breakdown is increased by the large-scale and long-term cultivation of varieties carrying single genes enabling the pathogen to overcome the resistance (Singh et al. 2001). Thus, on the one hand screening for new sources of resistance is needed (Huang et al. 1997), followed by the detection, tagging and incorporation in adapted cultivars. On the other hand, strategies have been developed for extending the useful life-time of single resistance genes in cultivars in combination with creating cultivars with broader resistance-spectra, i.e. the accumulation of host resistance genes in a single line or cultivar, a procedure called pyramiding (Nelson 1978). Besides the extension of resistance durability the strategy of gene pyramiding is also applied for increasing the level and spectrum of disease resistance (Huang et al. 1997, Kinane & Jones 2000), leading to broad-spectrum resistance. This is of special interest, because resistance genes often differ in their specificity against the

various pathogen strains so that their combination can result in a broader spectrum of resistance than conferred by each gene alone.

For pyramiding, markers facilitating the simultaneous selection of several resistance genes and avoiding the need of repeated inoculation with different isolates followed by phenotyping are especially useful. By phenotypic selection alone it appears very demanding, difficult or even impossible to reliably combine and detect multiple resistance genes in one genotype, due to dominance and epistasis effects and/or simply masking of one gene's effect by another or others.

Attempts for pyramiding resistance genes have meanwhile been carried out in barley for several pathosystems. For example, pyramiding of different scald resistance genes resulted in improved resistances to the fungal pathogen *R. secalis* in comparison to lines carrying a single resistance gene: While Brown et al. (1996) performed the pair-wise combination of genes for resistance to scald by the use of linked isozyme markers, Raman et al. (1999) developed AFLP markers for this purpose. Pyramiding of genes *rym3* and *rym5* was achieved in Japan by using isozyme markers and phenotypic selection procedures (Saeki et al. 1999). Besides pyramiding major genes, trials were also carried out to combine positive QTL-alleles, e.g. Castro et al. (2000, 2003a, 2003b) created pyramids of resistance QTL for stripe rust of barley (*Puccinia striiformis* f.sp. *hordei*) on chromosomes 4H, 1H, and 5H with the objective of determining the relationship between QTL number and level of resistance. Pyramiding was carried out by using microsatellite markers, and a reduction of disease severity has been observed in genotypes with positive alleles at two or more loci. Similar results have recently been obtained by Richardson et al. (2006).

Pyramiding of major resistance genes may be of special importance in the future especially aiming at an extension of the utility of major resistance genes. For example, many resistance genes against the barley yellow mosaic virus complex have been described (Table 1). However, out of the recessive genes derived from the primary *H. vulgare* gene-pool only *rym11* is still effective against all strains known in Europe (Kanyuka et al. 2004, Habekuß et al. 2005). *Rym4* is not effective against BaYMV-2, *rym9* is ineffective against BaYMV and BaYMV-2, and *rym5* is not effective against BaMMV-SIL (Kanyuka et al. 2004) and the new strain of BaMMV (BaMMV-TASL) recently identified in Germany (Habekuß et al. 2005).

Studies on combining resistance genes against the barley yellow mosaic virus complex have focused on genes for which molecular markers were identified since the 1990s, i.e. *rym4*, *rym5*, *rym9* and *rym11* (Bauer et al. 1997; Graner et al. 1999a). While *rym4* and *rym5* map to the same marker interval in the telomeric region of chromosome 3HL (Graner et al. 1999a, Pellio et al. 2005) and represent alleles of the same gene, i.e. the eukaryotic translation initiation factor 4E (*Hv-eIF4E*, Stein et al. 2005), *rym9* is located in the telomeric and *rym11* in the centromeric region of chromosome 4HL (Bauer et al. 1997; Nissan-Azzouz et al. 2005). For pyramiding such recessive genes doubled haploid (DH) populations are better suited than segregating F₂ populations, since homozygous recessive

genotypes are much more frequent among DHs. In addition, dominant markers are as informative as co-dominant ones in such populations. Therefore, DH lines from crosses comprising the following gene combinations were generated: *rym4 x rym9*, *rym4 x rym11*, *rym5 x rym11*, *rym5 x rym9*, *rym9 x rym11*. Starting from these crosses two different strategies have been followed involving one or two cycles of DH production (Werner et al. 2005). For the first, F_1 derived DH populations were screened with appropriate closely linked PCR-based markers in order to identify plants carrying both genes in a homozygous recessive state, theoretically present at a frequency of 25%. These genotypes were inter-crossed followed by a DH line production step and re-analyses by the molecular markers to identify genotypes homozygous recessive for two or three resistance genes, respectively (Figure 2).

For the second strategy, F_1 plants having one resistance gene in common, e.g. [*rym4 x rym9*], [*rym4 x rym11*], were inter-crossed and at least 100 F_1 kernels were produced for each cross (Figure 3, Werner et al. 2005). Genotypes homozygous recessive at the common resistance locus were identified using respective SSR markers. The selected genotypes were then screened for heterozygosity at the other resistance loci and DHs were produced from the resultant selections (Figure 4, Werner et al. 2006). In theory 6.25 plants of the 100 F_1 plants should carry the expected genotype. Since less than the expected number of plants were identified in four of the crosses, genotypes were also selected that were heterozygous at all three resistance loci to enrich the pool. Among the DH offspring of these plants 20 out of 107 carried *rym4*, *rym9* and *rym11* and 27 out of 187 tested carried *rym5*, *rym9* and *rym11* in homozygous recessive states. In addition, DH-lines carrying all possible two-gene-combinations were achieved simultaneously (for details cf. Werner et al. 2005).

Since DH line production itself is time-consuming and costly the second strategy, including only one DH step, is considered more efficient than the first strategy with two DH steps. Although there are no cost differences concerning molecular analyses it should be noted, that in the one step strategy, co-dominant markers are a prerequisite because the first selection step takes place in heterozygous genotypes. For all other steps of both strategies dominant markers are as informative as co-dominant ones since heterozygous genotypes are absent in DH populations. Due to the appearance of the resistance breaking BaMMV-strain (Kanyuka et al. 2004, Habekuß et al. 2005) this strategy has gained actual importance. For example, combining the gene *rym5*, which at this time represents the sole basis of European barley cultivars resistant to BaMMV, BaYMV and BaYMV-2, with *rym9* effective against BaMMV and BaMMV-SIL (Kanyuka et al. 2004) should result in resistance against all yellow mosaic inducing viruses currently known in Europe.

In practical barley breeding pyramiding using molecular markers has to be repeated in each crossing cycle since the combined resistance genes will segregate in the progeny. However, this holds true only until respective major genes will be fixed within the actual breeding material, e.g. in Europe.

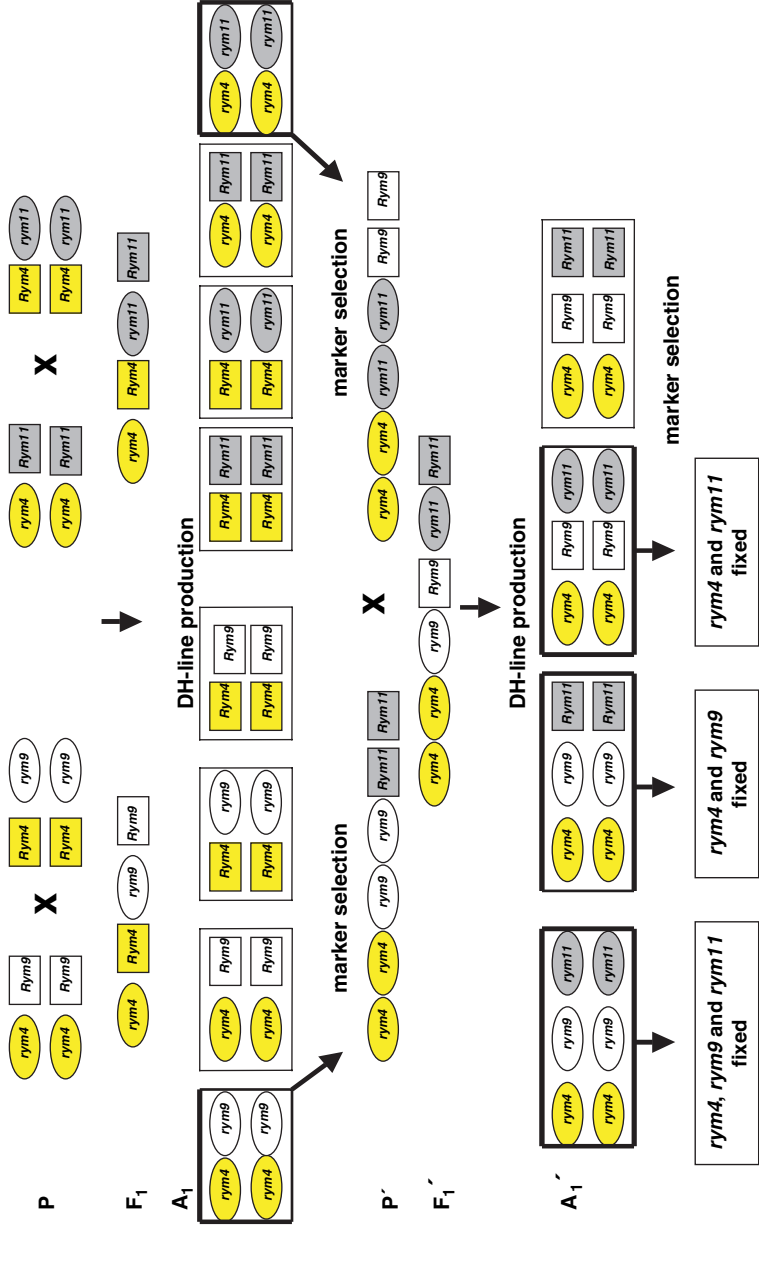
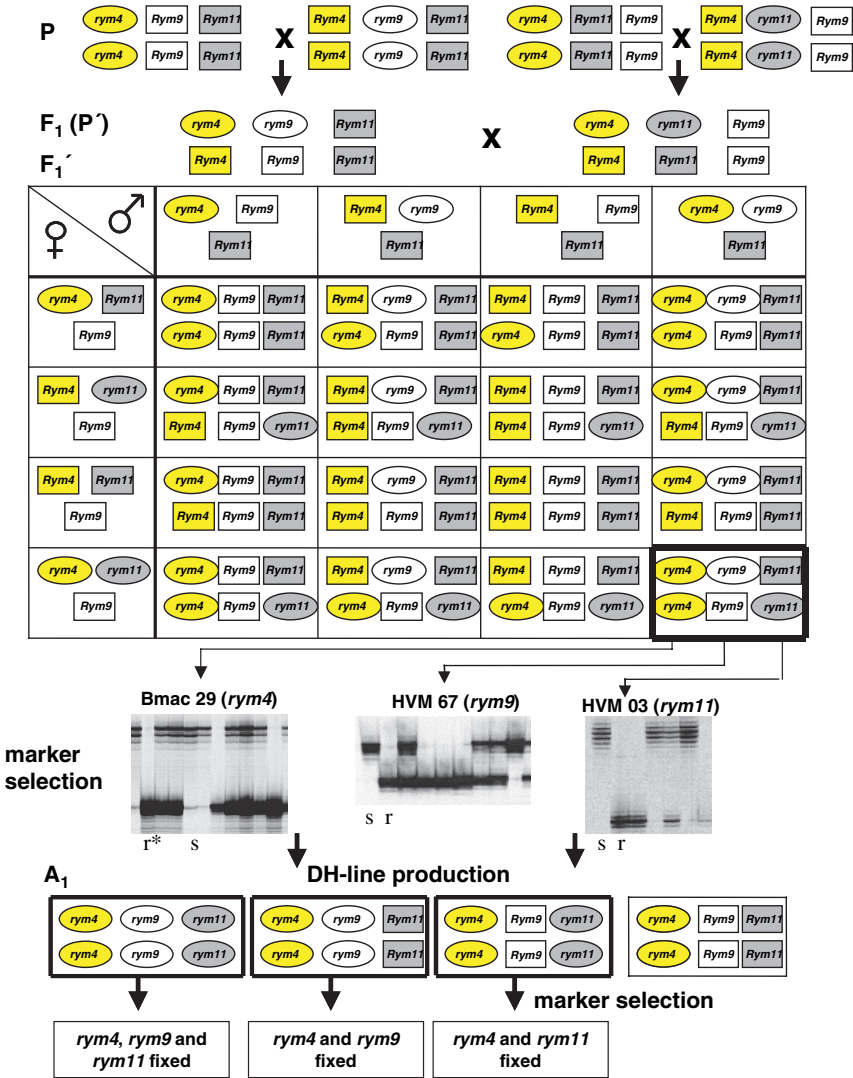


Figure 2. Scheme of pyramiding resistance genes *rym4*, *rym9* and *rym11* by two haploidy steps (Werner et al. 2005) (See Plate 2)



* r=allele of the resistant parent, s=allele of the susceptible parent

rym = resistance encoding allele; *Rym* = susceptibility encoding allele

Figure 3. Scheme of pyramiding resistance genes *rym4*, *rym9* and *rym11* by one haploidy step (Werner et al. 2005) (See Plate 3)

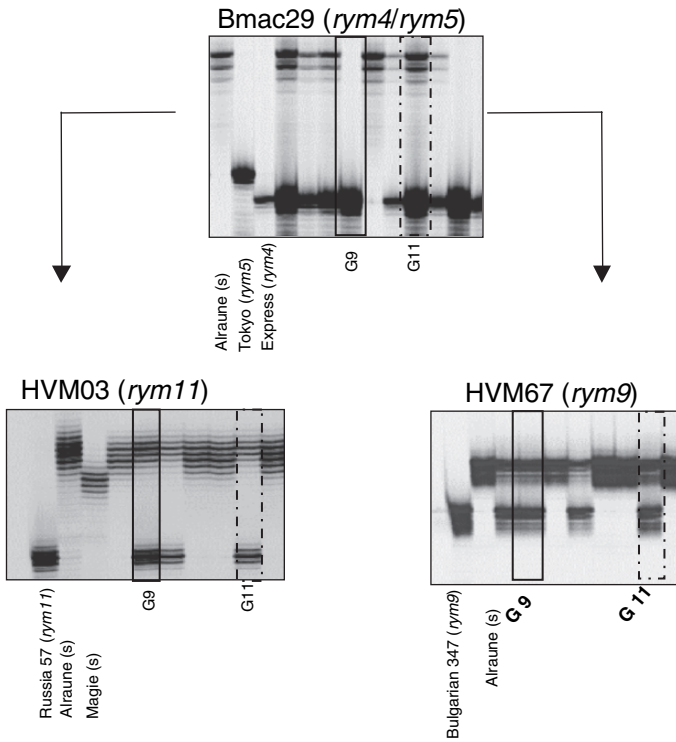


Figure 4. Scheme for selecting F_1 genotypes homozygous at the resistance locus in common (*rym4*) and heterozygous at the others (*rym11/rym9*, G9) and of genotypes heterozygous at all three loci (G11) as the starting point for pyramiding three and two resistance genes, respectively, by one DH step (Werner et al. 2006)

4. CONCLUSIONS AND FUTURE PERSPECTIVES

The results presented elucidate that molecular markers are available for many major resistance genes and QTL in barley facilitating – besides mere marker based selection procedures which are already applied in practical barley breeding for resistance to many pathogens e.g. BaMMV/BaYMV (Schiemann & Backes 2000) or BYDV (Ovesna et al. 2000) – the implementation of new breeding strategies like marker assisted backcrossing procedures or pyramiding of resistance genes. In the last few years the number of isolated resistance genes has risen considerably. However, the availability of expression profiling techniques and knowledge on synteny between the sequenced rice genome and barley will lead to an enhanced isolation of resistance genes in this species and a deeper understanding of respective pathosystems in the future. The isolation of genes involved in resistance and/or tolerance will transfer breeding for resistance in barley to the next level facilitating the identification of novel alleles and their directed use in molecular breeding strategies in order to enhance resistance.

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CHAPTER 5

CLONING GENES AND QTLs FOR DISEASE RESISTANCE IN CEREALS

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Abstract: A number of resistance genes against biotrophic pathogens recently have been cloned from wheat and barley. These include the barley stem rust resistance gene *Rpg1*, the leaf rust resistance genes *Lr10* and *Lr21* in wheat and several alleles from the highly diverse powdery mildew resistance loci *Mla* in barley and *Pm3* in wheat. In addition, the durable and recessive *mlo* gene also conferring powdery mildew resistance as well as the viral resistance genes *rym4 / rym5* were isolated from barley. There are many advanced projects in a number of research groups aimed at the isolation of additional resistance genes, including some quantitative trait loci with major effects on resistance against biotrophic and necrotrophic pathogens. The availability of these genes for transgenic approaches as well as the development of highly diagnostic markers to test for the presence of the gene in plants will allow new breeding strategies. Resistance breeding, possibly more than breeding for any other major trait, will benefit enormously and rapidly from this new molecular information: a rapid diagnosis of resistance genes as well as a rational combination of qualitative and quantitative resistance factors based on molecular knowledge will become feasible in the next decade.

1. INTRODUCTION

Plant pathogens are causing losses both by reducing the quantity of the harvested crop as well the quality of the product. It is estimated that in wheat, barley and rice around 10% of the total crop is destroyed by plant diseases. Yield losses reach 30% considering also pests and weeds (Oerke and Dehne 1997). Quality problems of the harvested product can be due to shrivelled seed which are frequently found as a consequence of the infection by leaf pathogens such as mildews, rusts,

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septoria, stagonospora blotch as well as many others. There also are direct quality problems caused by pathogens. In cereals, *Fusarium* species are the major culprit for mycotoxin contamination from the harvested grain, causing economic losses and in the worst case human and animal health problems. Consequently, the improvement of resistance against the *Fusarium* pathogens has been one of the main goals in classical and molecular pathology during the last decade.

Disease resistant plants are essential for sustainable agriculture. Although fungicides are available against many of the important cereal diseases, they are not affordable for farmers in many parts of the world, are sometimes difficult to apply because of weather conditions and can cause environmental problems. Historically, the applied goal of the genetic and later the molecular analysis of naturally occurring disease resistance has been to support and to improve resistance breeding in crop plants. However, the study of the classical and molecular genetics of disease resistance in plants also has been a scientifically rewarding area of basic research, revealing elementary mechanisms in the interactions of plants with pathogens at different levels. These studies have also resulted in the discovery of surprising similarities between pathogen defense in plants and defense mechanisms involved in innate immunity of animals and humans (Nuernberger et al. 2004).

There are recent reviews on the topic of cereal resistance genes which describe in some more detail several aspects which can only briefly be discussed in this chapter (Ayliffe and Lagudah 2004; Keller et al. 2005). Here, we will focus on the cloning of resistance genes and QTL in cereal species in the narrower sense (wheat, barley, rye and oat), but occasionally data and discussion on cereals in the wider sense (including the two crops maize and rice) will be included.

2. SINGLE GENE RESISTANCE VS. QUANTITATIVE RESISTANCE: TWO GENETICALLY DIFFERENT TYPES OF RESISTANCE AND THEIR CONSEQUENCES FOR BREEDING STRATEGIES

The studies on the inheritance of resistance revealed that there are two clearly distinct genetic mechanisms for disease resistance: monogenic resistance is based on single genes or at least single genetic loci whereas quantitative resistance depends on two or more genes. Genes involved in quantitative resistance have additive genetic effects, with none of them individually conferring sufficient resistance to be of agronomic interest. In most cases (for some exceptions see below), it was found that single resistance genes conferred complete resistance but were only active against certain races of the pathogen, i.e. they showed a genetic interaction with genes from the pathogen. If such a gene conferred a complete resistance against all known pathogen races, large-scale cultivation frequently resulted in the appearance of new, virulent pathogen races possibly created by mechanisms of recombination

or mutation. Alternatively, these virulent races might have been present at very low frequencies in the pathogen populations already before introduction of a particular resistance gene. Thus, single gene based, race-specific resistance is often rapidly overcome by new pathogen races, thereby limiting its use as a single defense line in a crop species. A possible use of resistance genes in a more sustainable way in breeding is based on combination of genes (McDonald and Linde 2002). This is achieved by combining (“pyramiding”) several resistance genes in the same cultivar, or by using line or cultivar mixtures. There, different *R* genes are present in different plant lines and the combined planting of the lines results in an overall improvement of resistance without putting an enormous selection pressure on the pathogen.

Single gene, race-specific resistance is based on a direct or indirect recognition event between the product of the plant resistance gene and the effector protein encoded by the avirulence gene of the pathogen. This interaction is studied intensively both in model plants as well as in crop species and in some cases is now quite well understood (see e.g. Schulze-Lefert and Bieri 2005 and references therein). At the genetic level, resistance genes are often clustered at some loci in the genome or exist as different alleles, each allele conferring resistance towards a specific set of pathogen races carrying the corresponding avirulence allele. In contrast to this resistance based on major single genes, quantitative resistance rarely shows a genetic interaction with the pathogen and thus is effective against all races of a particular pathogen species. It slows down the disease development by increasing latency period, lowering sporulation frequency or other parameters related to the development of the epidemic. Quantitative resistance shows a relevant environmental dependence which makes breeding for it cost- and labor intensive in large-scale breeding programmes, particularly if a number of quantitative traits have to be incorporated into the same elite genetic material.

Although plant pathogen interactions are sometimes simplified by stating that quantitative resistance is durable, whereas this is not the case for single-gene based resistance, one should keep in mind that this is not always the case. There are several examples of single genes which have been effective for many years: these include the *Sr2* stem rust resistance gene in wheat, the *mlo* powdery mildew resistance gene in barley and the *N* gene against tobacco mosaic virus. In addition, there are indications that certain quantitative resistances can also erode and become less effective, albeit not in the rapid form as observed for race-specific resistance genes in the boom-and-bust cycles (McDonald and Linde 2002).

Resistance breeding in crop plants depends on both major *R* gene as well as quantitative resistance. Monogenic resistance genes are easy to work with but are frequently not durable as described above due to rapid selection of pathogen races which overcome the specific resistance. Consequently, quantitative resistance is an attractive alternative although it is difficult to handle in a breeding program if there are no diagnostic markers available for selection of the relevant chromosomal regions. The application of molecular markers has allowed the

genetics of quantitative resistance to be determined and quantitative trait loci involved in resistance have been identified and mapped. The focus of current research is on cloning a large number of quantitative as well as major resistance genes from different cereal species and the understanding of their function and evolution.

3. MAP-BASED CLONING OF CEREAL DISEASE RESISTANCE GENES

Currently, the only grass genome which is completely sequenced is the rice genome. It was proposed early on as the model genome for all grass species based on large-scale synteny between rice and other species. The possible use and the pitfalls of using rice as a genomic model for gene cloning in other grasses have been described in two recent reviews and shall not be repeated here (Bennetzen and Ma 2003; Xu et al. 2005). In summary, rice provides an excellent model to saturate genetic regions of interest with molecular markers. In addition, it can provide candidate genes for further study, but it has to be kept in mind that resistance genes are poorly conserved at orthologous positions in grass genomes (Leister et al. 1998; Guyot et al. 2004). The rice genome can not replace barley and wheat genomic tools as, at least for resistance genes, a physical contig spanning the locus has to be derived from the species, or even better, from the cultivar of interest. An alternative “bridging” genome could possibly be the genome of *Brachypodium distachyon*, a species which is more closely related to wheat than rice and will be sequenced in the near future.

Once the first resistance genes had been cloned from model organisms, it became clear that many of them encoded proteins with conserved domains such as nucleotide binding site (NBS) and leucine rich repeat (LRR) domains. Many studies subsequently described the isolation of resistance gene analogs (RGAs, genes encoding proteins with domain structures very similar to those encoded by functional resistance genes) using PCR amplification based on primers developed from conserved regions. A large number of cereal RGAs were subsequently isolated and characterized from several species (see e.g. Leister et al. 1998). It is disappointing that until now none of these RGAs was proven to be an active resistance gene by functional analysis in wheat and barley. Amazingly enough and in a sort of contradiction to the unsuccessful RGA approaches, the recently cloned cereal resistance genes have mostly turned out to encode NBS-LRR type of proteins. The failure of the RGA strategy for cloning of resistance genes is possibly due to the large number of RGAs present in cereal genomes. In rice, around 600 RGAs were found, and this class of genes accounts for approximately 1% or more of the rice genes (Bai et al. 2002; Monosi et al. 2004). A similar number can be expected for the barley genome and each of the three genomes of hexaploid wheat. Thus, the sheer number of RGAs makes the identification of a specific gene difficult. Consequently, all the known wheat and barley resistance genes have been isolated by map-based cloning and not by homology with the rice genome or the RGA approach.

Map-based cloning in cereal genomes is challenging because of the large genome sizes (5×10^9 bp in barley, 1.6×10^{10} bp in bread wheat) and the high content of repetitive DNA making chromosome walking tedious. The steps towards cloning of a gene include (1) the establishment of a large population segregating for the trait of interest, (2) high resolution mapping of the gene using molecular markers and (3) establishment of a physical contig of BAC clones containing flanking markers of the gene. Both for marker development as well as physical mapping, the availability of genomic resources is crucial. In wheat and barley, high density maps and a large number of markers, mainly highly polymorphic microsatellite markers as well as EST sequences are available (reviewed in Keller et al. 2005). In an analysis for wheat published by McIntosh et al. (2003), there were 5537 RFLP loci, 1620 microsatellites and 2049 protein loci and genes controlling phenotypic traits. This makes the wheat genome one of the most densely mapped plant genomes, except for those with a completely known genome sequence. More information is available on the Graingene web site <http://wheat.pw.usda.gov/GG2/index.shtml>.

There is a growing list of wheat and barley BAC libraries available for physical mapping. For barley, there are BAC libraries from cultivars Morex and Cepada Capa, with several others currently being made (Yu et al. 2000; Isidore et al. 2005a). Two libraries from hexaploid wheat cultivar Chinese Spring are available as spotted filter sets for hybridization screening (Allouis et al. 2003, Shen et al. 2005). In addition, pooled PCR screenable BAC libraries have been constructed from hexaploid wheat cv. Glenlea (Nilmalgoda et al. 2003), as well as from single chromosomes (Safar et al. 2004) and from pooled chromosomes (Janda et al. 2004) of cv. Chinese Spring. The BAC library of hexaploid Chinese Spring as well as the chromosome 3B specific BAC library have recently become available for general use (see <http://cnrgv.toulouse.inra.fr/ENG/collection.html>). Genomic studies in the complex hexaploid wheat genome can be simplified by specific analysis of one of the three individual wheat genomes. These can be accessed through the diploid A, B, and D-genome donors. Therefore, a number of BAC libraries have been constructed from diploid ancestors of wheat, e.g. for the A genomes of *T. monococcum* (Lijavetzky et al. 1999) and *T. urartu* (Akhunov et al. 2005), for the wild grass which is most similar to the B genome (*Aegilops speltoides*, Akhunov et al. 2005) and for the D genome (*Aegilops tauschii*, Moullet et al. 1999, Luo et al. 2003, Akhunov et al. 2005), as well as from the tetraploid AB genomes (Cenci et al. 2003). Given the observed large differences in haplotypes of different varieties in barley (Scherrer et al. 2005) and wheat (Isidore et al. 2005b), it will be very helpful for the cloning of a specific gene if several or many BAC libraries are available. Then, the BAC library from the cultivar which has the most closely related haplotype can be used. Map-based cloning has resulted in the isolation of a number of fungal disease resistance genes in wheat and barley. The isolated genes are listed in Table 1. In addition, the table also lists the cloned maize and rice disease resistance genes against maize rust and rice blast/bacterial blight. Below, we discuss in more detail the cloning of the wheat and barley genes.

Table 1. Cloned disease resistance genes in wheat, barley, rice and maize

Species	Gene	Disease	Type of gene	Reference
Wheat	<i>Lr10</i>	Leaf rust	NBS-LRR	Feuillet et al. 2003
Wheat	<i>Lr21</i>	Leaf rust	NBS-LRR	Huang et al. 2003
Wheat	<i>Pm3a-g</i>	Alleles against specific races of powdery mildew	NBS-LRR	Yahiaoui et al. 2004 Srichumpa et al. 2005 Yahiaoui et al. 2006
Barley	<i>mlo</i>	Powdery mildew	7 transmembrane domain protein	Büschges et al. 1997
Barley	<i>Mla</i> alleles 1,6,7,10,12,13	Alleles against specific races of powdery mildew	NBS-LRR	Zhou et al. 2001 Halterman et al. 2001 Halterman et al. 2003 Shen et al. 2003 Halterman and Wise 2004
Barley	<i>Rpg1</i>	Stem rust	Tandem kinase	Brueggemann et al. 2002
Maize	<i>Rp1-D</i>	Leaf rust	NBS-LRR	Collins et al. 1999
Rice	<i>Xa1</i>	Leaf blight	NBS-LRR	Yoshimura et al. 1998
Rice	<i>xa5</i>	Leaf blight	TFIIA γ	Iyer and McCouch 2004 Jiang et al. 2006
Rice	<i>Xa-21</i>	Leaf blight	RLK	Song et al. 1995
Rice	<i>Xa-26</i>	Leaf blight	RLK	Sun et al. 2004
Rice	<i>Pib</i>	Rice blast	NBS-LRR	Wang et al. 1999
Rice	<i>Pi-ta</i>	Rice blast	LRD	Bryan et al. 2000
Rice	<i>Pi-9</i>	Rice blast	NBS-LRR	Qu et al. 2006

3.1. Powdery Mildew Resistance Genes in Cereals: The Well Studied Cases of the Barley *Mla* and Wheat *Pm3* Allelic Series of Resistance Genes

Barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) is a biotrophic fungal pathogen of economical importance in most barley growing areas. Breeding for resistance against this disease already has a long history, such that a high number of resistance genes (denominated *Ml* genes) have been identified in the cultivated and wild gene pools of barley (Jorgensen 1993; Weibull et al. 2003). The *Mla* locus (first described in the cultivar “Algerian” = *Mla1*) was shown to encode a large number of alleles specifying race specific resistances that are distinguishable based on the differential response of barley lines towards a set of different mildew isolates. A total of 32 *Mla* alleles have been defined genetically (*Mla1* to *Mla34*; *Mla4* was shown to be an independent locus and renamed *Mlk1*; *Mla15* is identical with *Mla7*) (Jorgensen 1993; Weibull et al. 2003), and many more can be expected in as yet uncharacterized germplasm. The *Mla* locus is thus among the most variable disease resistance loci found in plants.

Initially, a physical map spanning the *Mla* locus was established using the cv. Morex BAC library, which was the only barley BAC library available at that time (Wei et al. 1999). The BAC contig encompassing 261 kb represented 0.25 cM in an F₂ population of 1800 individuals used for high resolution mapping. The contig was fully sequenced and annotated (Wei et al. 2002). The cv. Morex sequence revealed the presence of 8 NBS-LRR resistance gene homologs (RGHs) that were grouped in three families based on sequence similarity. A function in disease resistance could not be assigned to any of these candidate genes as Morex does not express a known powdery mildew resistance gene at the *Mla* locus. Thus, a functional *Mla* gene had to be isolated from another barley accession. In a second step, this was done concomitantly, yet independently, for both *Mla1* and *Mla6* using different approaches.

To isolate *Mla1*, a cv. Algerian derived *Mla1* near-isogenic line was γ -ray mutagenized to recover *Mla1* mutants. Two mutants showed an altered RFLP banding pattern when hybridized with selected probes derived from the Morex contig. One of these probes was a RGH and was subsequently used to isolate the *Mla1* sequence from a cosmid library of a *Mla1* expressing barley line. The *Mla1* gene identity was confirmed through genetic complementation in a single cell transient expression assay (see below). *Mla1* encodes a coiled-coil nucleotide-binding-site leucine-rich-repeat (CC-NBS-LRR) type of R-protein of 958 amino acids with 11 imperfect LRRs (Zhou et al. 2001). To isolate *Mla6*, the corresponding LRR regions of different Morex RGH family members were amplified from a *Mla6* expressing cv. Franger derived near-isogenic line. These LRR fragments were then hybridized to a cDNA library developed from the same accession. Only probes from one RGH family (the same as for *Mla1*) identified clones in the library. These cDNAs corresponded to one potentially full-length CC-NBS-LRR protein of 956 amino acids. A genomic clone from the same gene was isolated through a PCR based screen of a cosmid library constructed from the same accession. The identity of *Mla6* was confirmed similarly as *Mla1* (Halterman et al. 2001). The *Mla1* and *Mla6* gene function was later corroborated in transgenic barley. An 8kb genomic fragment of either one of the genes was able to confer complete, race specific resistance that was indistinguishable from the respective donor lines (Bieri et al. 2004).

Subsequently, further *Mla* alleles were isolated more easily: *Mla12* was isolated from a cosmid library (Shen et al. 2003) and *Mla13* from a cDNA library (Halterman et al. 2003) using the previously isolated *Mla* genes as probes. *Mla7* and *Mla10* were amplified by a RT-PCR strategy with primers designed from sequences conserved in the previously isolated genes (Halterman and Wise 2004). Sequence comparison of *Mla* coding regions revealed that they are closely related (>93% overall identity) and represent gene variants (probably alleles) of a common progenitor. The N-terminal part of the proteins is much more conserved (>98% identical) than the C-terminal part (>82%). Accordingly, the region determining the difference in recognition specificity between *Mla1* and *Mla6* could be mapped in a domain swap experiment to the C-terminal part of the protein encompassing the non-LRR C-terminus plus

three to nine LRRs (Mla6 and Mla1, respectively; Shen et al. 2003). The same region also has a number of hypervariable residues that likely are under diversifying selection.

In bread wheat, the *Pm3* locus encodes one of the earliest identified allelic series for wheat disease resistance. Ten *Pm3* alleles (*Pm3i* to *j*) have been genetically identified in wheat lines or cultivars originating from four continents (Zeller and Hsam 1998). Each of them confers race-specific resistance to wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*). The first of these alleles identified at the molecular level was the *Pm3b* allele which was cloned using a positional cloning strategy (Yahiaoui et al. 2004). Two wheat species with lower ploidy levels, the diploid wheat *T. monococcum* and the tetraploid *T. turgidum* cv. *durum*, provided models for the genome of hexaploid wheat and allowed the establishment of a physical contig spanning the *Pm3* locus. Although the haplotypes at the *Pm3* locus differed dramatically between the three levels of ploidy, a large resistance gene-like family was consistently found at the *Pm3* locus and a candidate gene cosegregating with *Pm3b* was identified. A single mutant showing no obvious large deletion at the *Pm3* locus showed a single base-pair deletion in the coding region of the candidate gene, and no mRNA could be detected from the candidate gene by RT-PCR in this mutant. In the transient assay described below for powdery mildew resistance genes, the identity of the candidate gene as *Pm3b* was demonstrated by its race-specific interaction with the powdery mildew pathogen (Yahiaoui et al. 2004).

For the isolation of the remaining *Pm3* genes, haplotype analysis of ten lines carrying different *Pm3* alleles was performed. In all these lines a conserved genomic region delimited by markers co-segregating with *Pm3b* was identified, including a structurally very well conserved, *Pm3b*-like gene (Srichumpa et al. 2005). Based on this haplotype conservation, six additional *Pm3* resistance alleles (*Pm3a*, *c*, *d*, *e f*, and *g*) were isolated using a PCR-based strategy (Srichumpa et al. 2005; Yahiaoui et al. 2006). The *Pm3* resistance alleles encode a coiled-coil nucleotide binding site leucine rich repeat type of protein. Haplotype conservation in *Pm3* lines and high sequence conservation (more than 97% sequence identity) observed between the *Pm3* resistance genes indicated that all *Pm3* specificities form a true allelic series. Highly specific functional markers derived from the allelic sequences now allow for the detection of individual *Pm3* alleles with complete accuracy in wheat breeding programs (Tommasini et al., 2006). In addition to the functional alleles, a susceptible variant of the *Pm3* genes was identified. The comparison of the sequence of the *Pm3* resistance alleles with the susceptible allele *Pm3CS* provided important information on the evolutionary processes that generated the resistance alleles. One group of four *Pm3* resistance alleles showed few, clearly delimited, polymorphic sequence blocks of ancient origin, possibly derived from gene conversion. A second group of three alleles differed from *Pm3CS* by only two to five mutations, all non-synonymous and all in the LRR-encoding region. Transient transformation assays confirmed that these few differences in the LRR domain of *Pm3* proteins are responsible for the specificity of resistance against different powdery mildew isolates. Moreover, the very high sequence conservation

between these alleles and the susceptible *Pm3CS* allele together with the absence of synonymous mutations between all these genes indicated a recent evolution of the *Pm3* resistance genes probably after wheat domestication 10,000 years ago (Yahiaoui et al. 2006).

Interestingly, both the *Mla* and *Pm3* genes are located in the distal region of chromosome group 1 (1HS and 1AS, respectively) and both loci are characterized by a large number of functionally different alleles. However, the two loci are not orthologous as the *Mla* probes derived from barley sequences map about 5 cM from the wheat *Pm3b* gene in a wheat genetic map (Zhou et al. 2001 and our unpublished results). In addition, the two genes are not similar at the sequence level (except for the overall domain structure of CC, NBS, LRR domains). We conclude that *Mla* and *Pm3* represent a case of convergent evolution where quite different genes were recruited to confer resistance against very similar pathogens. It is relevant to study at the molecular level the direct or indirect mechanisms of MLA and PM3 protein interaction with fungal avirulence gene products and the subsequent signal transduction. It is also interesting to note that two different genetic loci have evolved into major resistance genes in the two closely related crop species wheat and barley. This strongly suggests that for most resistance loci it will be necessary to isolate them from the crop species where they are active as there is very low functional similarity between orthologous genes even in closely related species. Thus, the isolation and comparison of the *Mla* and *Pm3* genes has resulted in exciting new findings and demonstrates that, in addition to putative practical applications, it is well worth isolating resistance genes from crop plants instead of limiting this type of work to model species.

3.2. The *mlo* Powdery Mildew Resistance in Barley

Classical monogenic resistance is race-specific and genetically dominant or co-dominant. The *Mla* and *Pm3* genes described above are typical examples of such resistance genes. In barley, there is a second and highly unusual recessive resistance against powdery mildew based on the absence of the *mlo* gene. Absence of *Mlo* confers a broad spectrum resistance which is active against most powdery mildew races in barley and has been durable for a long time. The *mlo* haplotype introgressed in many European spring barley lines is derived from a natural gene silencing event found in Ethiopian landraces. The complex molecular basis of this mutation has recently been identified and is described in Piffanelli et al. (2004). Resistance based on the *mlo* gene was obtained in all powdery mildew susceptible barley lines which were mutagenized and screened for resistance. Therefore, it seems that loss of the *Mlo* gene is sufficient to confer this broad spectrum resistance which is based on the formation of cell wall appositions. The *mlo* gene was actually the first gene cloned from a large cereal genome by map-based cloning (Büschges et al. 1997). This was based on a segregating F₂ population with more than 2000 individuals and the identification of closely linked AFLP markers. In this early time of gene cloning in barley there was no large insert library available yet and the authors made a YAC

library from cultivar Ingrid which has an *Mlo* genotype. The *Mlo* gene could be mapped to a short physical sequence of approximately 30 kb, revealing only one good candidate gene for *Mlo*. PCR-based sequencing of the *Mlo* gene candidate in several independently generated *mlo* mutants from different genetic background revealed mutations in the coding region in all of them. This mutant analysis as well as experiments detecting intragenic recombination events demonstrated the identity of the cloned gene as *Mlo*. The MLO protein is encoded by 12 exons and contains 533 amino acids, with a number of putative transmembrane domains. The analysis of MLO function in barley as well as in *Arabidopsis* is currently a very active area in research and ideally this type of resistance could also be developed in other cereal species, particularly wheat.

3.3. Rust Resistance Genes from Wheat and Barley

There are three rust diseases in wheat and barley: leaf rust, stem rust and stripe rust. Although there are more than 200 resistance genes described against these diseases in the two crops (see e.g. McIntosh et al. 1995), until now only the three leaf rust resistance genes *Lr1*, *Lr10* and *Lr21* have been cloned from wheat. In addition, one stem rust resistance gene, *Rpg1*, has been isolated from barley.

Similar to wheat genes *Pm3* and *Lr10* as well as the barley *Mla* gene, the wheat *Lr21* gene is located in the distal region of the short arm of a group 1 chromosome, in this case 1DS. It was isolated by map-based cloning, using a shuttle mapping strategy between the diploid species *Aegilops tauschii*, the D-genome donor of bread wheat, and the hexaploid bread wheat genome (Huang et al. 2003). *Lr21* had originally been introgressed into bread wheat from an *Ae. tauschii* line collected in Iran. High-resolution mapping was done in the hexaploid recipient wheat, whereas the large insert clones (in this case cosmid clones) were generated from the diploid *Ae. tauschii* donor line. Although one recombinant, which later turned out to be intragenic, complicated the identification of a candidate gene considerably, Huang et al. (2003) could identify a promising candidate gene which was then confirmed to be *Lr21* based on stably transformed transgenic wheat lines. The *Lr21* gene encodes a 1080 amino acid protein of the NBS-LRR type. In the amino terminal domain it has a highly unusual 151 amino acid domain which is not found in other NBS-LRR proteins. The LR21 protein has only 13 imperfect leucine-rich repeats, which is relatively short compared to other NBS-LRR type of resistance genes, but very similar in size compared to other LRR domains in plant proteins (De Lorenzo et al. 2001; Baumberger et al. 2001).

The *Lr10* leaf rust resistance gene is located on chromosome 1AS and was described in the gene pool of hexaploid wheat but not in any diploid relative. A high resolution genetic map was established based on 3120 F2 plants. Closely linked markers were used to identify BAC clones from the region of interest. At the time of this work, there were only BAC libraries available from the A^m genome from *T. monococcum* and the D-genome from *Ae. tauschii*. As the *Lr10* gene is located on chromosome 1AS, the library from *T. monococcum* DV92 was used to establish

a physical contig around the gene (Stein et al. 2000). Two chromosomal walking steps were necessary to establish a contig including flanking markers. These steps were difficult because of the highly repetitive nature of the wheat genome, and the development of specific probes for the isolation of overlapping clones was time-consuming. Sequencing of the relevant part of the contig revealed the presence of two NBS-LRR candidate genes between the flanking markers (Wicker et al. 2001). It later turned out that the choice of the diploid wheat for establishing a contig had been a lucky one, as the line *T. monococcum* DV92 has a haplotype at the *Lr10* locus which is very similar to the one in *Lr10* containing hexaploid wheat line (Feuillet et al. 2003). It was also found later that most hexaploid wheat lines have a haplotype with a large deletion at the *Lr10* locus and the discovery of the gene would have been impossible in a BAC contig from such a line (Isidore et al. 2005b).

Using EMS mutagenesis, three independent mutations were identified in one of the candidate genes, demonstrating that the affected gene is *Lr10* (Feuillet et al. 2003). Transformation with the two candidate genes only resulted in resistant and fertile plants when both candidate genes were used, a fact that is still not completely understood (Feuillet et al. 2003). The *Lr10* gene encodes a CC-NBS-LRR type of protein with 919 amino acids and 14 imperfect leucine-rich repeats. Amazingly, diversifying selection was shown to act on the N-terminus of the gene, whereas the LRR domain was more conserved. This contrasts with the finding in other resistance genes (McDowell et al. 1998; Meyers et al. 1998). *Lr10* is clearly different in sequence compared to other cloned cereal resistance genes. In particular, it is more closely related to the Arabidopsis *Rpm1* bacterial resistance gene than to the second wheat leaf rust resistance gene cloned, *Lr21* (Feuillet et al. 2003). *Lr10* is similar to *Rpm1* in two more aspects: first, both genes are present only as one copy in the genome which contrasts with the observed gene clusters at many other resistance loci in cereals (e.g. *Mla*, *Pm3*, *Lr21*). Second, for both genes there is a characteristic presence or absence polymorphism in the gene pool (Isidore et al. 2005b): a wheat cultivar either has an *Lr10* gene or a very closely related gene or it has a large deletion, very similar to the *Rpm1* locus. The molecular relevance of these similarities for resistance gene function of these two genes remains to be determined. Very recently, a third leaf rust resistance, *Lr1*, has been cloned but this work has not yet been published (S. Cloutier, B. McCallum, M. Jordan, C. Feuillet, B. Keller, unpublished data).

A single rust resistance gene has been isolated from barley: the *Rpg1* stem rust resistance gene located on chromosome 7HS which confers a highly valuable and durable (since 1942) resistance in many North America cultivars. The gene originates from an old Swiss landrace from the Canton Lucerne and was cloned by Brueggeman et al. (2002). The story of the *Rpg1* cloning is one of several good case studies revealing the limits of the use of the rice genome for cloning of disease resistance genes (summarized by Ayliffe and Lagudah 2004). For *Rpg1* cloning, a high resolution map of 8,518 gametes was used as well as a BAC contig of 330 kb established by chromosome walking from the cultivar Morex which

contains *Rpg1*. High resolution mapping delimited the interval containing *Rpg1* to 110 kb. Two candidate genes with sequence similarity to receptor-kinase like genes were identified and one was putatively assigned as being *Rpg1* by comparing allelic sequences from susceptible and resistance cultivars. The RPG1 protein is encoded by 14 exons and contains 837 amino acids (Brueggeman et al. 2002). For a resistance protein it has a highly unusual protein structure with two tandem kinase domains but lacking any LRRs. It remains to be seen if it is a membrane-bound protein or localized intracellularly. The proposed *Rpg1* gene was transformed into the susceptible barley cultivar “Golden Promise”, making it resistant to stem rust and confirming that the candidate gene was *Rpg1* (Horvath et al. 2003). Interestingly, and similarly to *Lr10* (Feuillet et al. 2003), transgenic plants showed an increased resistance compared to the line carrying the endogenous gene. While this can be attributed to overexpression by a heterologous promoter in the case of *Lr10*, *Rpg1* was under control of its own promoter and the reason for increased resistance in transgenic plants remains unclear.

3.4. The *Rp1* Common Rust Resistance Locus in Maize

The *rp1* locus conferring resistance to maize common rust (*Puccinia sorghi*) is a complex locus on the distal end of the short arm of maize chromosome 10. Genetic studies showed that different *Rp1* specificities could recombine, demonstrating the complexity of this locus (Saxena and Hooker 1968). Recombination events in the *rp1* complex have been associated with the generation of variants with novel phenotypes. Some of these variants have non-specific reactions to maize rust and display lesion mimic phenotypes (Hu et al. 1996), some confer a reduced level of resistance, others show novel race specificities (Richter et al. 1995). The *Rp1-D* specificity was isolated using a transposon tagging strategy combined with a candidate gene approach (Collins et al. 1999). In a first step, resistance gene analogs were amplified from the maize genome and one of them, PIC20 hybridized with a small gene family at the *rp1* locus. For the final identification of *Rp1-D*, independent mutants using *Mutator* and *Ds* transposons were obtained for the *Rp1-D* gene. In one family of *Rp1-D* mutants generated using the *Mutator* element, a *Hind*III restriction fragment of 5 kb hybridized to the *Mutator* probe and cosegregated with the mutant allele. In the *Ds* mutants of *Rp1-D*, the PIC20 probe was used to detect presence or absence of insertions at the *Rp1-D* locus. PIC20 detected insertions of the *Ds* transposon in a *Rp1-D*-mutant and excision in three *Ds* revertants. The mutated sequences were then isolated from λ genomic DNA libraries and were found to correspond to the same gene. The *Rp1-D* gene encodes a NBS-LRR type of protein of 1292 amino acid (Collins et al. 1999). In contrast to a majority of NBS LRR proteins, RP1-D does not show a TIR or a leucine zipper motif at the N terminus. The *Rp1-D* haplotype was found to contain 9 paralogous genes. Only one is functional and is located at the most distal end of the cluster (Smith et al. 2004). Genes at the *rp1* locus often mispair at meiosis which should result in the creation of new haplotypes or new recombinant genes. Recently, recombination events have been identified at the *rp1*

locus which resulted in the generation of a novel specificity (Smith and Hulbert 2005). Based on haplotype analysis of the recombinants, the novel resistance is believed to be the result of a combination of existing *Rp1* genes by intergenic recombination rather than the result of the creation of a new gene.

3.5. Viral Resistance Genes *rym4* / *rym5* in Barley

In winter barley, viral infection by the two bymoviruses *Barley Yellow Mosaic* (BaYMV) and *Barley Mild Mosaic Virus* (BaMMV) can cause considerable yield loss. This group of viruses is transmitted by a soil-borne fungus, *Polymyxa graminis*, which is almost impossible to fight with fungicides. In the barley gene pool, there are recessive resistance genes against BaYMV and BaMMV such as the *rym4* / *rym5* complex on chromosome 3HL. Recently, these genes were shown to be allelic and they were isolated by a map-based cloning strategy (Stein et al. 2005). First, a contig of 650 kb was established at the *Rym4/Rym5* locus. It did not span the gene despite considerable efforts to extend the contig to include flanking markers on both sides of the resistance gene. The region turned out to have (i) a suppressed recombination and (ii) to be extremely gene poor. It was completely sequenced and represents the largest contiguous sequence in barley and wheat that has been analysed to date (Wicker et al. 2005). A gene encoding a typical eukaryotic translation initiation factor 4E was located on the contig and represented a very promising candidate for the resistance gene. When the gene for *Hv-eIF4E* from a susceptible barley line was transformed into a virus resistant cultivar, the transformants turned out to be susceptible, confirming that the cloned gene was the susceptibility factor. Comparative sequencing of *Hv-eIF4E* in 56 barley accessions revealed a low degree of polymorphism in the gene pool. Both for the *rym4* and *rym5* alleles two characteristic and diagnostic polymorphisms were detected, suggesting an independent origin of the two resistance alleles. The amino acid changes caused by the DNA polymorphisms were all located on the surface of the protein model for eIF4E in close proximity of the cap-binding domain (Stein et al. 2005). The viral RNAs encode a “virus genome-linked protein” (VPg) with binding affinity for eIF4E but the exact molecular mechanism of this interaction and its consequences resulting in susceptibility or resistance remain elusive. Interference with viral translation or viral movement are both possible explanations. The characterization of amino acids putatively involved in resistance or susceptibility should greatly facilitate the determination of the molecular basis leading to the particular outcome of the virus-host interaction. The results described by Stein et al. (2005) clearly suggest that each of the *eIF4E* genes in cereal genomes represents a good candidate for having allelic variants conferring viral resistance.

3.6. Ongoing Single Gene Isolation Projects in Wheat, Barley and Rye

In addition to the genes which have already been cloned from the complex wheat and barley genomes, there is an increasing number of projects targeting other

relevant disease resistance loci in wheat, barley and rye. This work is profiting from the improved genomic resources described earlier, as well as from an increased interest in genetic resistance of cereals. An exhaustive description of all the ongoing projects would be beyond the scope of this chapter, but a few of the cloning projects which are well advanced will be described in a bit more detail and should give a representation of the type of genes that will possibly be cloned in the near future.

The short arm of rye chromosome 1RS is homoeologous to the group 1S chromosome arms in barley and wheat and carries a number of economically important resistance genes against stem, leaf and stripe rust as well as powdery mildew. The 1RS chromosome translocation is present in many bread wheat cultivars and, therefore, these resistance genes are present in a broad set of elite wheat varieties. Mago et al. (2005) have recently described their progress in the isolation of the *Sr31*, *Lr26* and *Yr9* genes on chromosome 1RS. They used two wheat lines each with a different rye translocation (one of which did not carry the resistance genes) for high resolution mapping. This elegant approach circumvented the problem of the lack of recombination within the rye segment in a normal cross between two wheat lines, of which only one carries the translocation. A first result from recombination and deletion mapping experiments was the observation that the three resistance activities are caused by three physically distinct, but closely linked genes. Although a homolog of the barley *Mla* gene mapped very closely to the resistance gene cluster, a deletion mutant lacking all three resistance activities retained all *Mla* markers. This suggests that *Sr31*, *Lr26* and *Yr9* are not related to *Mla*. Nevertheless, the high density map around the genes as well as the physical knowledge of the barley *Mla* region should simplify the cloning of these genes in the near future.

The isolation of an additional stem rust resistance gene in wheat, *Sr2*, is well advanced. *Sr2* confers partial and recessive resistance against stem rust, being analyzed genetically as a QTL (see below). The gene has conferred durable resistance over many decades and thus represents a very important target for cloning. Because *Sr2* can be scored with certainty under suitable environmental conditions, it can be mapped at high resolution similar to single genes. *Sr2* is completely linked, or pleiotropic, with pseudo-black chaff, a phenotypic marker which causes dark pigmentation on the stem internodes and glumes. *Sr2* maps to chromosome 3BS – a region where QTL for *Fusarium* resistance and Stagonospora resistance also are found as well as the leaf rust resistance gene *Rph7* in barley (see below). This region is the focus of an international effort to assemble and map large physical contigs for sequencing. The cloning of *Sr2* will be greatly supported by this effort. Using mapping information from the rice genome, Kota et al. (2006) have recently mapped *Sr2* at high resolution with the closest flanking markers spanning an interval of 0.4 cM, and the same authors started to build a physical contig from this locus (Spielmeyer and Lagudah, personal communication).

In barley, there are also many advanced map-based cloning projects. Only three of them will be mentioned. In contrast to wheat, no leaf rust resistance gene has yet been isolated from barley. However, the two genes *Rph5* and *Rph7* have been

located to very small genetic intervals in different regions of chromosome 3 HS (Mammadov et al. 2005; Scherrer et al. 2005). The *Rph5* gene has been located in a small 0.3 cM genetic interval in the barley map and the syntenic rice genomic region was established (Mammadov et al. 2005). The *Rph7* gene was located in a genetic interval of 0.13 cM and a physical BAC contig containing the flanking markers was established from a barley cultivar containing the *Rph7* gene (Scherrer et al. 2005). The complete BAC sequence was determined but no obvious candidate gene could be identified. Thus, *Rph7* possibly represents a new type of resistance gene. Finally, the advance in the isolation of the barley *Rdg2a* gene against the hemi-biotrophic seed-borne pathogen *Pyrenophora graminea* (leaf stripe) should be mentioned. *Rdg2a* was located in a 0.14 cM interval between two resistance gene analogs and might therefore be a member of a larger RGA cluster at this locus (Bulgarelli et al. 2004).

There is also progress in the characterization of genes involved in resistance against necrotrophic cereal pathogens. These pathogens are often characterized by production of toxins which result in necrosis of the host tissue. Genetically, sensitivity is conferred by a dominant gene, suggesting that a specific receptor/transporter is responsible for the observed sensitivity. Thus, the recessive allele is desired in breeding material. The wheat *Tsn1* gene belongs to this class of genes and confers sensitivity to the proteinaceous, host-selective toxin Ptr ToxA produced by the tan spot fungal pathogen (*Pyrenophora tritici repentis*). The group of J. Faris has recently mapped this gene at high resolution in an interval of 0.8 cM and started to establish BAC contigs from this region (Lu et al. 2006; Lu and Faris 2006). The same group has also made significant progress in the isolation of the *Snn1* sensitivity gene for a toxin produced by the *Stagonospora nodorum* glume blotch pathogen. This gene has been mapped in a population of 2,050 gametes to a genetic interval of 0.9 cM. Using the current genetic resolution, a BAC contig of 250 kb cosegregates with the *Snn1* gene (J. Faris, personal communication).

4. CLONING OF RESISTANCE QTL FROM CEREALS

Breeding for quantitative, horizontal resistance is a promising approach to achieve durable resistance. Quantitatively acting defence genes do not confer a high degree of resistance to pathogens, but they slow down disease development. The molecular isolation of QTLs from cereal genomes is not a prerequisite for breeding, but an effective breeding program would profit enormously from diagnostic and very closely linked molecular markers for QTLs. Positional cloning requires intermediate steps, like the generation of molecular markers which are genetically tightly linked to the target locus. These markers can then be used in traditional breeding programmes (non-transgenic) to introgress the QTL into different genetic backgrounds. In addition, QTL cloning and functional analysis will be essential to learn more about the molecular mechanisms that result in partial resistance. As described above, gene isolation in cereal genomes is difficult, and QTL isolation is further complicated by the small phenotypic effect of a specific locus. Dissection of the phenotypic

variance, validation of single loci in recombinant, near isogenic backgrounds and testing in replicated trials are essential steps to achieve a precise map location of a specific QTL. Near isogenic lines for a specific QTL represent ideal biological material to also characterize the molecular or physiological mechanisms that lead to enhanced resistance. Recently, an excellent discussion on QTL cloning in plants was published and more details on the technology for QTL isolation are found there (Salvi and Tuberosa 2005).

The development of collections of near isogenic genotypes sharing the same QTLs for horizontal resistance and differing for a number of major resistance genes could result in highly valuable breeding material and be an efficient combination of the two different genetic resources. Thus, the high resolution mapping and cloning of resistance QTLs will form the basis of new strategies for resistance breeding which could not systematically be followed in the past because of a lack of tools to detect the different loci involved in resistance. Currently, despite the many studies focused on mapping resistance QTL, only a few of them are at a stage where cloning of the underlying gene is within reach.

A short summary on some advanced cloning projects for disease resistance QTL is given in Table 2. The table only lists examples of QTLs for which one or several labs are intensively working on high-resolution mapping and cloning. Typically, these large projects are made in some form of national or international collaborations. In wheat, a QTL for *Fusarium* head blight (FHB) resistance, *Qfhs.ndsu-3BS* from the resistant cv. Sumai 3 has been located in a very small region on chromosome 3B (Liu and Anderson 2003a,b) and the isolation of this gene can be expected in the near future. FHB (scab) is a very serious problem for wheat production. The disease is caused by several species of the genus *Fusarium*, and the International Maize and Wheat Improvement Center (CIMMYT) has described FHB to be a major limiting factor to wheat production in many parts of the world (Dubin et al. 1997). The fungus attacks the spike mostly at the flowering stage, and with favourable weather conditions, infection can result in serious yield losses. Crop quality is often compromised by harmful mycotoxins that are produced by the fungus in diseased grain. Fine mapping of *Qfhs.ndsu-3BS* gene is ongoing using the rice genome as a model to derive new markers for the wheat 3BS region. QTL cloning and

Table 2. Advanced projects for cloning disease resistance QTL and durable resistance genes in wheat

Target gene	Chromosomal location	Disease	QTL/single gene	Reference
<i>Qfhs.ndsu-3BS</i>	3BS	<i>Fusarium</i> Head blight	QTL	Liu and Anderson 2003b
<i>Sr2</i>	3BS	Stem rust	QTL/single gene	Kota et al. 2006
<i>Lr34</i>	7DS	Leaf rust	QTL	Schnurbusch et al. 2004b; Spielmeyer et al. 2005
<i>Qsnb.sfr-3BS</i>	3BS	<i>Stagonospora</i> <i>nodorum</i>	QTL	Schnurbusch et al. 2004a

characterization will shed light on the molecular mechanism underlying resistance to this major disease (Liu and Anderson, 2003a,b). A similar approach has been undertaken to map and isolate the horizontal slow rusting resistance loci *Lr34/Yr18* (Schnurbusch et al. 2004b, Spielmeier et al. 2005), *Lr46/Yr29* (Rosewarne et al. 2006), and *Sr2* (Kota et al. 2006) as well as a major QTL for *Stagonospora nodorum* glume blotch resistance which is also located on chromosome 3BS (Schnurbusch et al. 2004a). The first two loci located on chromosomes 7DS and 1BL, respectively, are conferring quantitative and durable resistance to both leaf and stripe rust. *Lr34/Yr18* was also described to be linked to resistance against powdery mildew and *Barley Yellow Dwarf Virus*.

5. PROBLEMS FOR GENE IDENTIFICATION AND VALIDATION: GENOMIC RESOURCES ARE MOSTLY NOT FROM THE SPECIFIC CULTIVAR CARRYING THE RESISTANCE GENE OR QTL OF INTEREST

Once an *R* gene or a resistance QTL has been located in a physical interval, there is still the problem of the identification of the resistance gene from a number of candidate genes. As described above, there is only a very limited number of cultivars or lines for which genomic BAC libraries are available in cereals, mostly because of the genome size of these crops which make the development of such resources costly. Thus, there are hundreds of specific resistance genes identified in wheat and barley, as well as dozens of resistance QTL, all identified in different cultivars of the species. In contrast, there are only one or very few BAC libraries available for each cereal species, making it unlikely that there is a specific library of the line carrying the resistance gene of interest. The major question then is the following: does the genomic region of the cultivar from which the BAC library was made accurately corresponds to the region in the cultivar in which genetic mapping was performed? Or in other words, does the BAC cultivar contain an allelic form of the gene giving resistance? Are the two haplotypes similar enough to each other? This is not a trivial point as there are a number of cases where the haplotypes in susceptible cultivars simply do not contain the region of interest because of deletion events. This was found e.g. for the *Rpm1* Arabidopsis gene against *P. syringae* (Grant et al. 1998) and the *Lr10* leaf rust resistance gene in wheat (Feuillet et al., 2003). In the latter case, most modern hexaploid varieties carry the deletion haplotype, including the two cultivars from which BAC libraries are available: Renan and Chinese Spring. Thus, *Lr10* was only cloned by the fortunate situation that there was a BAC library available from diploid wheat *T. monococcum* cv. DV92 which turned out to have the same haplotype at the *Lr10* locus as the hexaploid wheat lines carrying the gene (Wicker et al. 2001, Feuillet et al. 2003, Isidore et al. 2005b). In other situations, particularly in the case of resistance gene clusters, the haplotypes at these loci differ dramatically between different cultivars. One of the best studied large and complex resistance loci, the *Dm3* locus is not from cereals but from lettuce. Nevertheless, it is probably a good example for the

enormous within-species or within-genus variability of resistance loci. The *Dm3* downy mildew resistance locus shows a great variability in the gene cluster of NBS-LRR genes, with cultivars having from 9 to 39 genes (Kuang et al. 2004). The actual line carrying the active *Dm3* gene has a cluster of 32 copies of the gene. A similar situation is found at the *Rp1* resistance locus which also shows a dramatic variability of the number of copies in the gene cluster (Collins et al. 1999; Smith et al. 2004). In such cases, in the absence of a BAC library from a cultivar with the gene, it is difficult to identify the actual resistance gene. Several strategies have been developed to solve this problem: cosmid and cDNA libraries from the lines carrying functional *Mla* resistance alleles were used for the isolation these genes (Halterman et al. 2001; Zhou et al. 2001). In a second strategy, a non-gridded BAC library of the cultivar of interest can be made. This was successfully done in the case of the *Rph7* leaf rust resistance gene in barley present in the cultivar Cepada Capa, although the nature of the resistance gene is still not clear (Isidore et al. 2005b, Scherrer et al. 2005). Non-gridded libraries can be screened using pooling strategies. Although this approach is not quite as efficient as the use of a fully arrayed or pooled BAC library, such libraries are very valuable and cost effective if only a single gene needs to be isolated from a specific line.

It is not yet clear if resistance gene loci are particularly variable among cultivars of a specific species or if variability, and thus the problems of cultivar specific haplotypes described above, is a general problem of gene and QTL isolation in crop species. Given recent evidence in maize (Fu and Dooner 2002, Brunner et al. 2005, Morgante et al. 2005) and barley (Scherrer et al. 2005), there is considerable variability at many genetic loci among cultivars of the same species. This might be mostly due to gene fragments moved by helitron transposons (Morgante et al. 2005) and therefore have little phenotypic consequences. However, the detailed aspects of intra-species variability has to be determined in much more detail to learn if haplotype variability will represent a common problem for cereal gene and QTL isolation. It might also be worth to consider the construction of BAC libraries for several wild accessions from a particular species. This might result in a good representation of the haplotypes originally present in the gene pool and provide the necessary basis for molecular work on most haplotypes present in modern cultivars.

6. VALIDATION OF CANDIDATE RESISTANCE GENES

Map-based cloning can delineate a resistance gene or resistance QTL to a small region of the genome. However, in most cases it will not result in only one candidate gene but in a number of genes which might be the gene of interest. Validation remains a thorny and often underestimated part of map-based cloning projects which today, with more and better tools available than just a few years ago, can result in relatively rapid progress towards the identification of the candidate genomic region.

In the case of race-specific, major resistance genes which are frequently encoded by NBS-LRR type of genes, the identification of a single NBS-LRR gene in the genetic interval carrying the resistance gene makes it likely that this is actually

the gene of interest. Nevertheless, such a simple situation is rather the exception than the rule as NBS-LRR genes frequently occur in gene clusters, making the identification of the actual *R* gene problematic (see e.g. Yahiaoui et al. 2004) and in many cases, more sophisticated approaches are needed. Once one or more candidate genes have been identified for a specific resistance gene or QTL, there are a number of possible ways to confirm or exclude its identity as a resistance factor. Ideally, functional null mutants are available. Sequence analysis of candidate genes in these mutants can rapidly reveal whether the candidate gene is the resistance gene. The barley *mlo* powdery mildew resistance gene was quickly confirmed this way, as well as the barley *Mla1* and *Mla12* alleles (Shen et al. 2003; Zhou et al. 2001), the *Lr10* leaf rust resistance gene in wheat (Feuillet et al. 2003) and the wheat powdery mildew resistance gene *Pm3b* (Yahiaoui et al. 2004). Knock out mutants are much more easily identified for major resistance genes than for resistance QTL. Nevertheless, for major resistance QTL such as the leaf rust resistance locus *Lr34*, mutants have been identified and will be highly useful once candidate genes are identified (R. Singh, CIMMYT, personal communication).

Powdery mildew is a highly relevant pathogen in several cereal crops. Its interaction with wheat or barley occurs exclusively in epidermal cells which can be transiently transformed by particle bombardment (Douchkov et al. 2005; Schweizer et al. 1999; Shirasu et al. 1999). Using marker genes such as genes encoding beta-glucuronidase or green fluorescent protein, the transformed cells can be identified. By cobombardment with a candidate resistance gene and subsequent infection with a specific race of the powdery mildew pathogen, transformed cells can be examined for compatible or incompatible interaction with the pathogen. This allows for a test of whether a candidate gene confers resistance and has been successfully used for the *mlo* gene (Shirasu et al. 1999) and the *Mla* alleles (Halterman et al. 2001; Shen et al. 2003; Zhou et al. 2001) in barley as well as for the *Pm3* allelic series in wheat (Srichumpa et al. 2005; Yahiaoui et al. 2006; Yahiaoui et al. 2004). Such transient assays are of course extremely valuable but are currently restricted to young seedlings and the epidermal tissue. Many pathogens, including the rusts, interact mainly with mesophyll cells and transient expression is not feasible for functional analysis of candidate resistance genes against these pathogens.

Stable transformation in cereals such as wheat and barley as well as maize is now routine but still tedious and time consuming before results can be obtained in the T_1 or T_2 generation. In barley, stable *Agrobacterium*-mediated transformation is mostly restricted to the cultivar Golden Promise, whereas in wheat, particle bombardment is still the most widely used method. In wheat, several sublines of cultivar Bobwhite, the Canadian cultivar Fielder as well as a number of other cultivars are transformed in different projects. The restricted availability of lines which can be transformed efficiently is a potential problem if a specific gene should be tested. For example, both cultivar Fielder as well as cultivar Bobwhite S26, which can be easily transformed, already contain several leaf rust resistance genes, among them the *Lr10* gene (Feuillet et al. 2003). Thus, there is still a need to have a broader set of genotypes that can be transformed. This will increase the chance

of finding a line which is highly susceptible against the specific disease of interest, allowing reliable phenotyping of transgenic plants containing candidate genes.

Recently, virus-induced gene silencing (VIGS) based on the *Barley Stripe Mosaic Virus* (BSMV) has been developed as a new tool for functional gene analysis in barley and wheat (Holzberg et al. 2002, Scofield et al. 2005). In this system, short fragments (up to 1200 nucleotides) are cloned into the γ -genome of the tripartite viral genome. After infection of the plants at the young seedling stage (barley or wheat), the target gene is effectively silenced. In the case of a candidate gene actually being the resistance gene, after infection of seedling genotypes carrying the resistance gene of interest with the relevant pathogen race, we expect silencing of the resistance gene and susceptibility. The power of VIGS in cereals was recently shown for the wheat *Lr21* leaf rust resistance gene which was efficiently silenced by VIGS and seedlings became susceptible (Scofield et al. 2005). This confirmed the results previously obtained by stable transformation (Huang et al. 2003). Thus, candidate genes whose function can be determined at the seedling stage can be efficiently silenced and resistance genes can be identified. There are two possible problems with this system which have to be considered in its application: first, in the barley gene pool there is widespread resistance against BSMV, preventing its use in these lines. Therefore, for each barley line to be used for VIGS, it has to be established first that it is susceptible to the virus. In wheat cultivars, resistance to BSMV is rare or absent, making VIGS more straightforward (S. Bieri and B. Keller, unpublished data). The second problem is related to the fact that *R* genes are frequently organized as gene clusters of closely related genes at a specific locus. Several tandemly oriented and highly similar copies of NBS-LRR genes represent a considerable problem for VIGS identification of the correct gene. As VIGS is silencing all genes which are >80% homologous (Holzberg et al. 2002), all members of an *R* gene family at a specific locus will be silenced and VIGS will only be useful for confirming that the gene of interest is a member of the gene family, but will not help to identify the particular family member which is the resistance gene. Possibly, the use of more specific 3' untranslated regions of individual gene members of the family can solve this problem.

7. OUTLOOK

The improvement of genomic resources for application in cereals promises to make map-based cloning of resistance genes and resistance QTL an increasingly important topic of research. After many decades of classical resistance breeding there is an enormous pool of lines carrying well defined resistance traits against most of the important diseases in cereals. This is probably one of the broadest and best characterized genetic resources in higher plants, but it still waits to be explored for a better molecular understanding of plant disease resistance. Given the many ongoing map-based cloning projects for resistance genes and QTLs, we will see a rapid progress in the isolation of genetic factors contributing to different types of resistances in the next years. Map-based cloning and the identification

of candidate genes will possibly also profit from association mapping which has a high potential for genetic resolution in suitable populations. It remains to be determined how easy it will be to assemble good, diverse populations derived from the wheat breeding pool for association mapping. However, the first successful association mapping study in wheat has recently been published (Bressegello and Sorrells 2006). Cloning of resistance genes in cereals will not only contribute to better molecular understanding of disease resistance but also, as the work is done in crop plants, to improved resistance breeding. Two main approaches will support breeding: first, it will become easier to combine resistance factors by marker-assisted selection and second, transgenic approaches which are based on a better molecular understanding of the fundamental processes underlying disease resistance will become feasible.

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CHAPTER 6

MAIZE BREEDING AND GENOMICS: AN HISTORICAL OVERVIEW AND PERSPECTIVES

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Abstract: The 1909 publication of G. H. Shull was the dawn of the modern era of maize breeding. Since that time maize has been unique as a major crop of the world and a very important species for basic research. Most of the knowledge from that research has been either worthless or inaccessible to maize breeding programs and actual improvement of maize. Recently, this relationship has changed and the connections between basic research and breeding have improved. The previous 10-15 years have been especially eventful with the advent of transgenic maize and DNA sequence information for maize germplasm. There is much to learn about the maize genome. Only a small fraction of the 30,000 to 60,000 genes and other sequences have functions assigned to them on the basis of direct experimentation. The advent of the genomics era of maize breeding, even at such an early stage, has clearly underlined our abilities to empirically assess all of the promising genetic options and questions that are raised by new sources of information. Field-based phenotyping and the prioritization of phenotyping have become much more important in the genomics era of maize breeding. The gains and pains of the initial decades of maize breeding have been well documented for some traits and production environments. But, with the exception of improved resistance to biotic and abiotic stress as a basis for genetic gains in grain yield, very little fundamental information and few validated mechanisms have been identified from that era. The next era of maize breeding should be fundamentally different in the sense that the strategies will rely more on a scientific method; consequently, some of the reasons for success or failure will be known and considered for devising more efficient methods of maize improvement.

1. INTRODUCTION

This review presents a summary and critical assessment of the achievements, prospects and limitations of genomics research for maize improvement, i.e., genomics-assisted breeding (GAB) and genomics-assisted selection (GAS). This has

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become a reality for maize improvement in some areas of the world and it has introduced new scientific and social considerations. Presently, science-based breeding of maize still occurs within the context of a mostly unknown genome and dynamic environments and furthermore, it is expensive at some stages of implementation and maintenance.

The origin of the modern era of maize breeding began roughly 100 years ago with Shull's experiments (Shull 1909). For much of the past century, maize has played a unique role among plant species because of its pivotal role as a major crop worldwide and a very important species for basic research. In particular, the previous 15 years have been especially eventful with the advent of transgenic maize and "low cost" DNA sequence information for maize germplasm. This is only the beginning as we are just starting to understand the content and organization of the maize genome, the interconnecting circuitries among genotype, environment and phenotype, and how to translate such intellectual wealth into beneficial forms.

Breeding and genomics are rather broad and encompassing topics. Therefore, the only certainty of this review is that it will miss or omit some important information or perspectives. Under this respect, some definitions may be helpful in order to reveal my perspective and bias. Plant breeding has always been the genetic adaptation of plants to the desires and needs of human societies in the context of agricultural systems. Genomics, with all of the subcategories, is the attempt to conduct comprehensive assessments and functional annotation of an organism's genome and gene products. Together, plant breeding and genomics form a complementary approach to aspects of systems biology in agriculture as the former has relied heavily on phenotypic analyses while the latter has emphasized genotypic and molecular analyses. Both approaches are necessary and increasingly synergistic.

The foundation of GAB for maize was prepared through a series of social, technical and scientific events spanning several decades. Detailed accounts of some of those events have been presented in recent publications (Charles 2001; Troyer 2004; Crosby et al. 2006). In the U.S., agricultural subsidy programs, the Plant Variety Protection Act of 1970 and the *Chakrabarty v. Diamond* ruling of 1980 are examples of important social events that either encouraged or enabled investments in plant breeding and genomics by corporations and venture capitalists. For maize, those investments and the security provided by the rule of law were vital to the development of GAB. In science and technology, examples of significant developments or discoveries include methods for plant regeneration from cell cultures of maize in the '70s, cloning and sequencing the first maize genes in the '80s, the introduction of the first molecular markers in the '80s and early '90s, and the advancements in computational biology and field plot equipment. Enabled by those events and developments, the first transgenic maize hybrids were first sold in the U.S. in the mid '90s (Troyer 2004). Consequently, some aspects of maize breeding have changed significantly. With respect to maize, those events are presented in their approximate chronological order in this chapter.

2. GENOMICS-ASSISTED BREEDING FOR MAIZE: TRANSFORMATION

In this section, aspects of some of the more influential developments in biotechnology and bioinformatics will be summarized and evaluated with respect to aspects of maize breeding and improvement. Generic schemes and considerations for genomic-assisted breeding have been presented (Varshney et al. 2005). When possible, reviews of methods and technology have been provided.

New and superior germplasm has always been a disruptive and driving force in a competitive seed market. For example, when inbred line B73 was developed, the research directors of the two leading seed companies in the U.S. informed Wilbert A. Russell, who with Lowell Penny developed that inbred, that their companies could not use B73 in their programs (W.A. Russell, personnel communication). However, soon thereafter, other companies used B73 to produce superior hybrids and all other companies were forced to do the same. Decades later, the value and impact of a few transgenes were similarly underestimated (Charles 2001).

Methods for genetic transformation and a few transgenes for resistance to insects and herbicides have been major assets and disruptive forces for maize breeding programs for various reasons which will be discussed in the section on maize breeding (Lee 1999; Crosbie et al. 2006; Table 1). Here, the methods and their consequences will be discussed. Technical reviews of maize transformation methods for the nuclear genome have been published (Armstrong 1999; Kaepler 2004; Torney et al. 2006). Transformation of the mitochondrial and chloroplast genomes, while desirable for some circumstances is less advanced and will not be considered herein (Maliga 2004).

In the public sector, *Agrobacterium*-based transformation of immature zygotic embryos has become a method of choice; although biolistic methods of transformation are still used (Torney et al. 2006). An important advantage of *Agrobacterium*-based is a higher frequency of apparently unrearranged, single-copy insertions of the transgene(s) into the maize nuclear genome. That advantage is important for GAB because it facilitates assessments and predictability of the relationship between genotype and phenotype (e.g. fewer problems with silencing) with respect to the transgene and it should simplify evaluations required by regulatory agencies.

One unique problem with that method is unintentional transfer and integration of portions of the bacterial plasmid into the maize genome: up to 70% of the events may result in stable integration of the intended transgene and portions of the plasmid (Shou et al. 2004). The effects of such unwanted DNA sequences on gene expression and phenotype were not determined but they would be a consideration in subsequent research, development and regulatory review.

Although *Agrobacterium*-mediated transformation of maize represents a significant advance, several serious limitations remain with respect to the target tissue, length of the DNA sequence delivered, the sites of integration and genotypic specificity (Lee 1999; Kaepler 2004; Cheng et al. 2004; Torney et al. 2006). The tissue culture-based methods of transformation are labor intensive, may result in

Table 1. Assessment of genomics platforms and related infrastructure for their utility in maize breeding¹

Infrastructure	Not Important	Important	Very Important	Comments ²
DNA sequence analysis	0	0	15	
Transcript analysis	12	3	0	Need more information on actual gene function.
Proteomics	12	3	0	
Analytical services ³	0	1	14	
TILLING	13	2	0	Maize has a naturally high level of DNA sequence polymorphism.
Insertional Mutagenesis	9	3	3	Concerns about intellectual property and patent claims.
Transformation	0	1	14	Not always able to transform the desired genotype but vital for validation and product development.
Genetic Map	4	3	7	
Complete Genomic sequence	2	5	8	
Physical Map	8	4	3	
Informatics	0	0	15	
Comparative Genomics	3	6	6	
Modeling and Simulation of phenotype	7	4	4	Need more information on actual gene functions and interactions between internal and external factors for effective modeling.
Off-season breeding facilities	0	0	15	
Wide-area field testing network	0	0	15	
Twin-plot field combines	0	0	15	

¹ Survey conducted May through October 2006 of 15 research managers from six multinational maize breeding programs.

² Text in the "comments" column indicates that more than half of the responses included a similar statement; thus, there is some evidence of consensus for that comment.

³ Analytical services are high-throughput analyses of chemical composition of plant parts.

somaclonal variation and require considerable time to proceed from transformed cell to seed; essentially, the methods are very similar to those established in the 1970s. Pollen-based methods would greatly improve this aspect of GAB but they have not been developed despite numerous efforts in this direction.

There has been, however, more progress in other components of transformation technology. The length of the delivered DNA sequence, while routinely in the range of a few kilobase pairs, has the potential to be extended to several tens of kilobases (Hamilton et al. 1996). The ability to introduce longer sequences of DNA

would facilitate GAB by accelerating the introduction and assessment of novel DNA sequences, combinations of genes and regulatory sequences and the establishment of transgene linkage blocks or artificial chromosomes (Lee 1995).

Most analyses of transgene integration in maize indicate that the desired sequences insert into and are transmitted by the nuclear genome (as opposed to the mitochondrial or chloroplast genomes). However, the ability to direct the transgenes to specific sites, or to eliminate certain sequences via site-specific recombination has not become routine for maize; but, incipient and potentially transferable technology has been reported for some plant species. Working models of site-specific recombination based on Cre-lox were demonstrated several years ago (Ow 2002). Recently, zinc-finger nucleases have been used to achieve a higher frequency of site-specific recombination in tobacco but tests in maize have not been reported (Wright et al. 2005). Given that so much of the maize nuclear genome consists of frequently methylated sequences derived from transposable elements which may vary greatly among maize inbred lines (Fu and Dooner 2001), it seems reasonable to be concerned about the integration sites of the potentially valuable transgenes and the costs of evaluating such seemingly random events. But at this time, it appears as if each event integrates into a unique site in the maize nuclear genome and hence, potentially introduces a unique set of variables for that given chromosomal context in a given genotype. Methods that may minimize one aspect of such variation, position effect variegation, have been developed by including putative matrix attachment regions in the constructs (Brouwer et al. 2002). The lack of control on the site of integration is certainly a source of inefficiency for GAB of maize since it necessitates evaluating additional constructs and events.

A narrow spectrum of maize genotypes are directly amenable to current methods of transformation (Torney et al. 2006). Such genotypic specificity hinders complementation tests needed for functional genomics. For maize GAB, while it is always desirable to conduct genetic investigations directly with a choice of elite and commercially valuable germplasm, meaningful assessments can be made with less elite but transformable germplasm and promising candidate events are subsequently and quickly introduced into the appropriate gene pool (Armstrong et al. 1995). Attempts to broaden the range of transformable genotypes have achieved some success through QTL mapping and breeding (Armstrong et al. 1992) and bioinformatic-assisted transformation with genes known to influence developmental processes (Lowe and Gordon-Kamm 2004).

Maize transformation is far from ideal for GAB. The time and cost required to proceed from idea to commercial varieties may exceed 10 years and US\$ 100 million; with about half of that time involved in transformation work. Currently, depending on the level and specificity of gene product needed for the desired phenotypic effect, it is necessary to produce and evaluate several constructs and hundreds of events to identify candidates for breeding and commercialization. The demands of product reliability are greater than that required for most aspects of basic research and the number of options to assess are increasing with the knowledge of genomes and their interactions with the environment. Perhaps with better understanding, a

higher form of knowledge, some of the assessments may be conducted through computational biology and simulation. Although maize transformation alone may never be completely adequate to efficiently evaluate all of the leads emerging from genomics, it will be the best option for assessing the most important ones.

3. GENOMICS-ASSISTED BREEDING FOR MAIZE: MOLECULAR MARKERS AND DIAGNOSTICS

In contrast with maize transformation methods, the detection and utilization of nuclear DNA polymorphism is hardly a limiting step in some maize breeding programs (Ragot and Lee 2006; Table 1). Single nucleotide polymorphism has become the method of choice for GAB of maize. The maize nuclear genomes of elite gene pools seem to contain a sufficiently high frequency of variation for SNPs and the improving annotation of maize nuclear genomes provide enough sites to survey for nearly all applications of marker-assisted selection (MAS) and GAB of maize (Ching et al. 2002). In most breeding programs, DNA markers are used to some degree and in the largest maize breeding programs, the information is used at all stages of research and development for fingerprinting, selection for simple and complex traits and predictions of parents and their progeny (Crosbie et al. 2006; Ragot and Lee 2006).

Relative to indels and simple sequence repeats, SNPs may require assessments at more sites to provide the same degree of discrimination but that may be easily accommodated by the next generation of PCR-free methods of detection. Of course, such platforms for detecting DNA polymorphism are costly and technically dynamic (Syvanen 2001). This has required some rather expensive experimentation with platform-optimization that may be reasonable and possible only for the largest and more competitive programs and markets.

The current SNPs should only become better as their information content improves through functional genomics and genome annotation. Such 'functional' markers, derived from characterized sequence motifs and related validation will be an advance since many of the current SNPs have not been assigned a physiologically significant purpose through direct experimentation (Andersen and Lubberstedt 2003). For maize, the prospects for functional markers have been recently reviewed (Shi et al. 2005).

A potential, perhaps temporary, limitation of functional markers is that they focus on genic DNA sequences, and only the primary DNA sequence. Therefore, variation due to changes in methylation, local and long-distance regulatory sequences and relatively new categories of sequence (e.g. miRNA) will be missed (Lee 1999; Lee 2006). Components of the maize nuclear genome are frequently (de)methylated and the sequence space between genes is commonly tens of thousands of base pairs. Consequently, much functional information could reside in a large sequence space that is recalcitrant to superficial analyses, as indicated by the results recently reported in the cloning of *Vgt1*, a QTL that influences the transition from the

vegetative to the reproductive stage of maize (Salvi et al. 2007; see also Salvi and Tuberosa in Volume 1).

Advances in the development of functional markers will be achieved through the various platforms established for functional genomics and annotation. In maize, systems for transposon mutagenesis, EST resources, microarrays and TILLING have been established to complement the Maize Genetic Stock Center and other germplasm repositories. These resources, primarily playgrounds for basic research removed from GAB, have been described and listed (Shi et al. 2005; Lawrence et al. 2005). The current utility of those platforms for maize breeding suggests that further development is needed (Table 1). Also, association mapping has emerged as a potential complement of other methods although its utility in reference populations of elite germplasm will be diminished by the limited generations for recombination and potentially, by population structure (Yu and Buckler 2006). Clearly, association mapping must be allied with populations of simpler family structure (Laird and Lange 2006). The value of such resources and methods for GAB of maize, while yet to be realized, has been demonstrated in principle. For example, positional cloning of sequences underlying QTL has become a reality for well-integrated maize programs (see Salvi and Tuberosa, Volume 1) 2002). Also, a very promising technique for directed allele-silencing, RNAi has been used to produce a potentially simpler version of Quality Protein Maize (Segal et al. 2003). The development of DNA sequence databases, deeper and with improving annotation, should make RNAi and related approaches a more important option for functional genomics and GAB of maize, both of which are huge challenges when one considers that a complete DNA sequence of bacteriophage lambda was available in 1982 and functional analysis of such a simple system is still in progress (Lee 1999).

But, all journeys begin with a single step. With the genomic sequence of gene-enriched regions and ultimately most of a single maize genotype (B73) due in 2008, the informational infrastructure should be a relatively rich resource. The value of such a resource was demonstrated long ago in one of the first significant events of primordial GAB for maize, modification of the codons of *cryIA(b)* and *cryIA(c)* (Perlak et al. 1991). This is one of the first examples of bioinformatics and computational biology for GAB and it contributed to a significant achievement in maize improvement (Armstrong et al. 1995). Also, simple bioinformatic tools demonstrated that many maize ESTs contained simple sequence repeats and could be readily converted to functional markers (Senior et al. 1996). The value of bioinformatics and comparative genomics has been demonstrated in recent advances in maize transformation based on research with *Arabidopsis thaliana* (Lowe and Gordon-Kamm 2004). The *LEC1* gene and its relationship to ectopic development of somatic embryos was initially described in *Arabidopsis*. That observation led to the hypothesis that a similar gene with a similar function could exist in maize. The *Arabidopsis LEC1* DNA sequence helped identify a maize homologue which was demonstrated to elicit a positive tissue-culture response from previously recalcitrant maize inbreds. In the private sector, there is a steady stream of candidate genes from model systems, other plants and their maize homologues that are under

evaluation for efficacy in GAB and critical stages of those investigations are enabled and expedited by contemporary genomic platforms, bioinformatic tools and well-annotated databases such as those listed at MaizeGDB and similar sites (Lawrence et al. 2005; Table 1).

4. THE EMERGING MAIZE NUCLEAR GENOME AND GAB

Since 1997, I have reviewed features of the maize genome that present challenges for GAB and each time, as more information emerges, the list of challenges increases (Lee 1999a; 1999b; 2006). As we know, the maize genome is large, an ancient and fractionated tetraploid, volatile and contains somewhere between 30,000 to 60,000 genes embedded in various categories of DNA sequences, most of which were derived from transposable elements (San Miguel et al. 1996; Gaut and Doebley 1997; Haberer et al. 2005). The duplicate sequences and their products present a degree of structural and functional redundancy that hinders many attempts to link genotype with phenotype. In addition, noncollinearity of gene-like sequences and transposons are commonly observed in the elite maize gene pool (Fu and Dooner 2002; Brunner et al. 2005). If such noncollinearity proves to be of much functional significance then it may suggest a more informative basis for establishing maize gene pools and it may be related to phenomenon such as hybrid vigor (M. Lee, in preparation).

There is much to learn about the maize genome. Only a small fraction of the 30,000 to 60,000 genes has been assigned functions on the basis of direct experimentation. Many of the clues to function will come from comparative genomics and computational biology; but in the end, they must be validated in the right genotypes of the right species in the right environments. Also, there is a huge difference in the estimated number of maize genes and the actual number of maize ESTs in public and private repositories: most EST collections contain a few hundred thousand 'unique' sequences. What could explain the difference between the estimates and the collections? One explanation may be that a portion of maize genes, like those of humans, produce more than one transcript through alternate splicing and start/stop sites and antisense transcription (Lee 2006). Recent preliminary investigations in maize suggest that antisense transcripts are frequently detected (Morrow et al. 2006; Jia et al. 2006). Of course, assessing the function(s) of such transcripts will be the next step and it will more difficult than their initial identification. Other categories of transcripts (e.g. miRNA and siRNA) may also be contributing to the difference between gene number estimates and the number of ESTs of maize (Brodersen and Voinnet 2006). For maize, this is a largely unexplored and poorly-annotated aspect of the genome but there is at least one case in which miRNA has a clearly established function in development (Lauter et al. 2005).

The nonexpressed (to our knowledge) components of the maize genome are also mostly unexplored as well. The large distances between genes and gene islands of maize are mostly composed of highly variable sequences derived from transposons; all of which have the potential to contain regulatory sequences that could influence

expression of native genes and transgenes. Embedded in that mixture of intergenic DNA sequence are the known (i.e. consensus) and some unknown regulatory sequences. Identifying such sequences and their functions has been difficult for reasons that have been described (Lee 2006). A good example of the strategy and effort required to discover such sequences has been described for the *B* locus (Stam et al. 2002) and the *Vgt1* QTL (Salvi et al. 2007). In both cases, sequences involved in the regulation of transcription of the coding region were located more than 50 kilobase pairs upstream from the start of transcription. Given the structure of the maize genome, such long-distance regulation may be common but difficult to discern. Recently, a preliminary investigation at the *tb1* locus has also identified some evidence of long-distance regulation of expression (Clark et al. 2006). No doubt, if the lessons from careful investigation of regulatory networks in other species are of any predictive value for plants, these types of sequences and their regulatory effects will be important for GAB of maize (Davidson 2006).

Other nonexpressed DNA sequences may also influence genetic variation and effects in GAB era. For example, matrix or scaffold attachment regions (MARs or SARs) of various types have been shown to be important variables in mammalian gene expression. In maize, putative MARs have been identified and their effects on transgene expression have been assessed to a limited degree (Brouwer et al. 2002). The extent to which such sites vary among genotypes and how they influence gene expression and contribute to phenotype remains to be determined. Perhaps, the foundation for addressing some of those mysteries in a systematic manner will become apparent with the acquisition of an increasing amount of gene-related genomic sequence information once the sequencing of the maize genome is completed.

The phenotypic and genotypic volatility of the maize genome has been documented repeatedly; yet, parsimonious underlying mechanisms and explanations have not been validated. The phenotypic instability of some inbred lines has been described and suggests that, even when inbreds originate from doubled haploids (i.e. monoploids) significant changes in the genome may and do occur each sexual generation (Bogenschutz and Russell 1986). Facets of the potential genomic changes have been documented for a sample of inbred lines (Gethi et al. 2002). Such volatility could be significant for GAB of maize with respect to germplasm registration, aspects of intellectual property and maintenance of some germplasm collections.

Among the many gaps in knowledge and areas of limited exchange between maize breeding and maize genomics, the most important topic that has received the least attention is the analysis of interactions between the genotype and the environment (i.e. G x E). Perhaps, the platforms for such analyses have been only recently established or need to be developed. Admittedly, such analyses are difficult, risky and time-consuming relative to more focused investigations on molecules and their possible influences on phenotype. By their nature, basic geneticists and genomicists usually try to conduct experiments with materials and methods that maximize the genetic and minimize the environmental effects on the phenotype.

Plant breeders will seek similar information but in a much wider set of environments, the target environment of the cultivars and hybrids. For maize GAB, an improved understanding of the plant's receptors and 'senses' and subsequent responses will be important for modeling, prediction methods, gene design, testing strategies and data interpretation.

Two simple examples of the effects of temperature illustrate the potential significance of considerations of GxE for GAB. In maize, the activity of the transposon Ac2 seems to increase in ears exposed to higher temperature (Osterman 1991). In *Antirrhinum*, the activity of *Tam* transposons decrease 10-1000 times at higher temperatures (Coen et al. 1989). No doubt, other fundamental processes that influence genome content and stability are influenced by signals from the environment. In maize and other plants, there are many temperature-sensitive alleles and phenotypes but the basis of the sensitivity has been determined in only a few instances. Research on alleles of the *APETALA3* (*APT3*) gene of *Arabidopsis* has shown that temperature sensitivity of the phenotype of the *ap3-1* allele, which ranges from mutant to near wild type, is determined by RNA processing and the splice variants produced under different conditions (Sablowski and Meyerowitz 1998). Unfortunately for maize GAB, the generation time, size and genetic nature of the plant hinder such carefully-controlled studies. Those circumstances will be a significant problem for the next phase of research in maize genomics and explorations of the maize genome; genotype by environment interactions.

5. SOME CHANGES IN MAIZE BREEDING WITH GENOMICS-ASSISTED BREEDING

The recent changes in maize breeding procedures and structure have been discussed (Lee 1995; Lee 1999; Betran et al. 2004; Kaeppler 2004; Troyer 2004; Crosbie et al. 2006; Ragot and Lee 2006). For maize breeding programs in competitive markets and cash-based economies supported by clear 'rule of law' the changes have been extensive. Some of the changes in personnel and infrastructure have been discussed in a previous section herein. Gradually, some of the agents of change have been adapted to and adopted by other markets, economies and regions in accordance with their conditions.

Perhaps the most obvious cause of change has been transgenic maize hybrids (aka GMO maize). Transgenic maize not only elevated societies' awareness of maize breeding, it also required maize breeding programs to develop parallel breeding programs, transgenic and nontransgenic (Lee 1999; Crosbie et al. 2006; Ragot and Lee 2007). Each transgene, and perhaps combinations of transgenes, must be reviewed by various national trade and regulatory agencies and the acceptance of transgenic maize has varied greatly among regions and countries. Some of the potential and actual risks of unintentional or poorly coordinated release of transgenic maize were clearly demonstrated in the late 1990s when StarLink maize hybrids were sold and grown prior to the completion of some regulatory procedures and approval (Troyer 2004). Consequently, maize breeding programs have adapted by

backcrossing transgenes into commercial and pre-commercial inbred lines in order to produce near-isogenic versions, transgenic and nontransgenic, of a given hybrid. The rapid and relatively precise backcrossing has been enabled by the development of suitable platforms for collecting DNA marker data and the use of either winter nurseries or greenhouses for producing several generations of maize per year. When functioning as an integrated system, such backcrossing has been invaluable by reducing the time required for product assessment and development.

Exotic germplasm has acquired an additional meaning in maize breeding. The potential gene pool of maize has truly expanded to include virtually any organism or design imaginable. In the private sector, assessing and sorting the many options presented by comparative genomics and model systems have become significant components of maize breeding. Considerations such as position effects, genetic background (Fu and Dooner 2002) and possible interactions of new sequences in old genomes have become routine for some maize breeding programs.

The advent of transgenic maize and some transgenes have influenced maize breeding programs in various ways. Their impact on sales and market share has been mentioned previously. The transgenes for resistance to *Ostrinia* and *Diabrotica* are good examples of how a few genes may influence an entire program. When the transgenes for insect resistance were initially backcrossed into a relatively wide array of elite germplasm, breeders observed that plant growth and development in some families of inbreds or genetic backgrounds declined when the transgene was introgressed. So, the genomes of those inbreds, or components of their genomes may be excluded from some aspects of breeding and product development. The initial backcrossing of those few transgenes may have revealed features of the genetic structure within maize gene pools that were unpredictable because very new genes and gene products were being introduced into relatively old genomes. Once the transcriptomics and metabolomics platforms mature then it should be worthwhile to establish large-scale profiles of maize gene pools based on transcript and metabolites profiles (Table 1). Such information may minimize the surprises related to introduction of truly new transgenes and it should enable modeling and simulation methods that have better predictive ability.

The primary effect of the transgenes for resistance to *Ostrinia* and *Diabrotica* provided indirect benefits such as a lower incidence of disease in the stalk and grain and a root system more capable of maximum use of soil water. Such indirect benefits have partially enabled an increase in the planting density used by farmers. As planting densities continue to increase, breeders have discovered that certain families of inbreds have root systems that fail under some conditions. Actually, breeders have been making such discoveries for several decades prior to the advent of transgenic maize but at least some of the recent observations and changes in germplasm have been accelerated by the introduction of a few transgenes that have minimized some sources of variation so that maize breeders could identify previously obscured sources of variation.

Transgenes have also renewed the debate regarding the type of inbred line to use as a tester or common parent when evaluating populations and candidate inbreds

for hybrid performance. The direct and indirect effects of some transgenes could obscure some weaknesses in the stalk, as well as resistance to biotic stress and root systems of the candidate inbreds and populations. That would reduce the selection pressure and the opportunities to observe the native features of the germplasm.

Another important change has been the utilization of DNA sequence polymorphism and associated information. Several programs in the private sector have established centralized service laboratories capable of rapid, large-scale and cost-effective (per data point) collection of DNA marker data so that such information may be collected for all inbred lines and populations at various stages of breeding (Ragot and Lee 2006). Such information has obvious utility for quality control, assessments of genetic relationships, establishment of intellectual property and some stages of selection (Smith and Smith 1992; Niebur et al. 2004).

The capacity to screen tens of thousands of accessions at a similar number of sites in the genome provides a new and targeted approach to germplasm evaluation and use of exotic germplasm within the maize gene pool. On the basis of knowledge about relationships between DNA sequence and properties of gene products, it should be possible to conduct sequence searches or design assays to search for specific sequences within certain genes to determine if such a motif exists in the native gene pool. Then, the few candidate alleles could be quickly and precisely introgressed into the elite, reference population(s) (Lee 1995). For some phenotypes, this approach could be a viable alternative to transgenic approaches and it would reduce some of the concerns and costs of that option.

Improvements in the acquisition of DNA marker data have revolutionized two old and reliable methods of maize breeding, backcrossing and recurrent selection. Marker-assisted backcrossing, already described and reviewed elsewhere (Ragot and Lee 2006), has become a standard component of breeding programs that need to introgress transgenes, native genes and QTL. That development was quite predictable (Lee 1995). In contrast, the ability to conduct marker-assisted recurrent selection (MARS) for QTL and complex traits such as grain yield has been a surprise of GAB era. There are several good reasons why MAS should not succeed in such situations (Holland 2004). The initial reports of MAS for complex traits and QTL in maize were mostly negative with the exception of the special case of sweet corn (Moreau et al. 2004; Edwards and Johnson 1994). Subsequent studies on a larger scale in several elite reference populations indicate that MARS for QTL and complex traits was an effective and competitive strategy relative to phenotypic selection (Johnson 2004; Crosbie et al. 2006). The changing fortunes of MAS should encourage reviews and reconsideration of other failed attempts to use DNA markers at other stages of selection such as choice of parents and methods for predicting and testing hybrids (Bernardo 1994). The advent of functional markers and new insights into patterns of gene expression of inbreds and hybrids could be important components of improved methods to those significant challenges of maize breeding (Lee 1999; Swanson-Wagner et al. 2006).

Two final components for GAB of maize should be briefly described, namely the places where maize breeding occurs and the people who conduct the breeding.

The personnel involved and their roles in some of today's maize breeding programs would not be recognizable to an experienced maize breeder of 30 years ago. GAB of maize truly depends on the contributions of lawyers, computational biologists, bioinformaticians, information technologists, biochemists, cell biologists, developmental biologists, molecular biologists as well as the previously recognizable collection of statisticians, pathologists, entomologists, geneticists, physiologists and plant breeders. The intellectual skill sets of a contemporary maize breeder have expanded to include association mapping and Bayesian-based statistics while the resources for educating the next generation of plant breeders have decreased. The stages of maize breeding have been organized into a series of activities resembling an assembly line of a factory in which a breeder may be responsible for the development of new populations and the early generations of selection while other colleagues would focus on later stages of hybrid testing and commercialization. The extent, to which the various specialists collaborate, especially in the private sector, has been a key to some of the early success of GAB of maize.

The places in which maize breeding occurs have also changed with GAB (Ragot and Lee 2006). Structures and climates that facilitate the production of 3-4 generations per year have become much more important components of infrastructure. In several programs, methods that produce doubled-haploids have been implemented to various degrees as a means of reaching homozygosity as quickly as possible (Melchinger et al. 2005). With DNA markers and associated information, it has become possible to conduct effective selection in environments other than the target environment; a huge change for maize breeding. Also, components of valuable germplasm may be created in any laboratory. The next great commercially valuable transgene will probably originate from a thermal cyclor and a method based on DNA shuffling (Stemmer 1994). Maize breeding has changed and the roles of the maize breeder have also changed.

With this collection of new tools, information and personnel, maize breeding programs have initiated projects to address some of the most challenging limitations encountered by crop-based agriculture. Indications from the private sector suggest that genes for improved tolerance to water deficits have been isolated from *Arabidopsis* and have shown promising results in maize. In parallel, the CGIAR's Generation Challenge program has been supporting genomics-based investigations towards improved drought tolerance of maize (<http://www.generationcp.org/vw/index.php>). Also, the HarvestPlus program has initiated genomics-based and MAS programs for biofortification of maize grain (Pixley et al. 2006). In the near future, GAB and GAS of maize should deliver germplasm with improved protein composition, resistance to parasites such as *Striga* and the capacity to eliminate toxins (e.g. mycotoxins) produced by pathogens. Decades of well-intentioned conventional selection have achieved limited success for those phenotypes.

The arrival of the genomics era of maize breeding was immediately preceded by a significant increase in the rate and volume of field-based assessments of candidate hybrids. The increase was driven by advances in computational power,

plot equipment, modifications to off-season nurseries and an overall expansion in the infrastructure that supports maize breeding (Smith et al. 1999 and Table 1). Maize breeding had become a year-round activity by the late 1980s. The advent of the genomics era of maize breeding, even at such an early stage, has clearly stressed programs' abilities to empirically assess all of the promising genetic options and questions that are raised by new sources of information. So, field-based phenotyping and the prioritization of phenotyping has become much more important in the genomics era of maize breeding (Table 1). In some regards, this is not new for some programs because testing combinations of inbred lines in hybrids has been a challenge for decades (Smith et al. 1999). As with the challenge of predicting performance of untested hybrids, some of the many questions and opportunities raised by the early stages of genomics will be addressed through modeling and simulation to predict aspects of plant growth and development in response to changes in genetic and environmental variables (Hammer et al. 2006). It is certainly premature to assess the impact of such efforts on maize breeding because the current models are based on limited information (Table 1). However, it is clear that such activity will become more important to maize breeding because it is simply too expensive and probably impossible to directly assess a fraction of the many options that will be enabled by the maturation of the genomics era.

In some ways, maize breeding has come a long way since the initial reports of Shull and colleagues nearly 100 years ago. The gains and pains of artificial selection in maize have been well documented for some traits and production environments (Duvick et al. 2004; Duvick 2006). But, with the exception of improved resistance to biotic and abiotic stress as a basis for genetic gains in grain yield, very little fundamental information and few (if any) validated mechanisms have been reported to account for those gains. The next era of maize breeding should be fundamentally different in the sense that the strategies will be based more on a scientific method and some of the reasons for success or failure should be known and incorporated into more efficient methods of maize improvement.

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CHAPTER 7

MOLECULAR MARKERS AND MARKER-ASSISTED SELECTION IN RICE

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Abstract: The status of rice as a model crop and the sequencing of the *indica* and *japonica* genomes have provided breeders with the necessary tools for marker assisted breeding. Simple Sequence Repeat (SSR) markers are easily available for any region of the genome, and candidate gene markers are being developed rapidly. The likely targets of MAS include yield and agronomic traits, cooking and nutritional quality, and resistances to abiotic and biotic stresses. MAS for gene pyramiding for disease and insect resistances is being widely used. For major genes and QTLs of larger effect, marker assisted backcrossing (MAB) is an effective method for developing improved versions of widely-grown “mega” varieties. Developing submergence tolerant mega varieties is a good example of how the MAB approach can result in significantly improved mega varieties within two to three years. The use of markers in more conventional breeding nurseries has been limited by cost, but is beginning to be applied for some traits like grain quality. Lower-cost marker methods combined with large-scale gene discovery will increase the use of MAS over the next decade.

1. INTRODUCTION

As a model cereal crop, rice has benefited greatly from the advances in plant genomics. The completed genome sequence of *japonica* cultivar Nipponbare and the complete draft DNA sequence of *indica* cultivar 93-11 have provided the basis for advanced gene discovery, complementing the detailed investigations made for the dicot *Arabidopsis*. The existence of multiple mutant collections, cDNA and EST databases, and whole-genome chips has enabled large-scale functional genomics applications (Leung and An 2004). Some cultivars, particularly of the *japonica* type, can be readily transformed through the *Agrobacterium* approach, permitting gene validation through complementation and overexpression studies.

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The mapping of genes controlling agronomic traits coupled with the widespread availability of easy to use simple sequence repeat (SSR) markers (McCouch et al. 2002) and quick DNA extraction methods (Xu et al. 2005b) has provided breeders with an excellent opportunity to apply marker assisted selection (MAS) methods in rice. However, most rice breeders have not taken advantage of this new approach, due to insufficient laboratory facilities, lack of trained staff, high costs, or lack of clear objectives amenable to MAS. With continued cost reductions, and a focus on introgressing useful genes into elite backgrounds, the wider adoption of MAS, and marker assisted backcrossing (MAB) in particular, is expected for rice. The following review will focus on general strategies for MAS in rice, and will emphasize traits where there is a clear advantage for using the technique.

2. MOLECULAR MARKERS FOR RICE BREEDING

Molecular markers can have a number of applications in agriculture, and their application in rice improvement has been recently reviewed (Mackill and McNally 2004; Collard and Mackill 2007). Rice scientists have greatly benefited by the high quality genome sequence of *japonica* cultivar Nipponbare (IRGSP 2005), and the less-refined sequence of the *indica* cultivar 93-11 (Yu et al. 2002). In addition to the large number of validated SSR markers (McCouch et al. 2002 and at <http://www.gramene.org/>), the IRGSP identified 18,828 class I SSRs throughout the genome (IRGSP 2005). These markers are also effective with most wild species used as sources of new genes or alleles (Gao et al. 2005). The costs and ease of high-throughput DNA extraction are declining (Xu et al. 2005b), making it more practical to apply MAS methods in breeding populations.

The major requirement for application of molecular markers in rice improvement is that a gene or QTL of significant effect be mapped with a high degree of accuracy, and that the gene is effective in the desired genetic background. Furthermore, the gene should not have negative effects on other traits. In initial mapping studies, a gene is generally mapped at low resolution using small populations (<200). For MAS, more tightly linked markers are required. Hence, the gene should be fine-mapped at a higher resolution. With populations typically higher than 400, genes can be mapped at less than 1 cM resolution. This will usually yield markers that are sufficient for MAS. However, further analysis resulting in positional cloning of the gene will be beneficial for MAS. If the candidate gene is identified, markers within or closely linked to the gene can be developed. These markers will not undergo recombination in breeding populations, and they are usually diagnostic of the favorable allele in a wide range of germplasm. If the functional nucleotide polymorphism is identified this will lead to a more powerful, gene-based or functional marker (FM) (Andersen and Lubberstedt 2003), sometimes referred to as a "perfect marker". However, with SSR markers still in wide use, the SSR markers within or adjacent to the gene will remain valuable for MAS.

A marker that is non-recombinant with the target gene can be used to reliably predict the presence of the gene in segregating populations. A codominant marker is preferred so that heterozygotes can be detected in backcross generations. The marker can be used in combination with flanking markers to reduce the effect of linkage drag when transferring only the desired QTLs or genes into an elite genetic background (Tanksley et al. 1989; Frisch et al. 1999b; Hospital 2001). In combination with selection against unlinked donor markers, this method can be used to quickly and cleanly transfer QTLs or genes into elite cultivars, providing the best assurance that the traits of the recurrent parent are accurately recovered. This is the most practical application of markers in rice improvement. Markers can also be used in normal breeding nurseries, although the number of plants or progeny grown in such nurseries is usually much higher, making it more costly.

New types of markers promise to bring down the cost of MAS and make it more attractive for breeders to use. Single Nucleotide Polymorphisms (SNPs) are the most common type of polymorphism in plants. For the two rice subspecies, 80,127 polymorphic sites were identified (IRGSP 2005). High throughput assays for SNPs are being developed to allow more widespread use of these markers. Another promising marker is the Single Feature Polymorphism (SFP), short deletions that are detected using oligo arrays hybridized with genomic DNA to perform whole-genome scans (Hazen and Kay 2003).

3. TARGET TRAITS FOR MARKER ASSISTED SELECTION

The following discussion will cover only those genes or traits where MAS is a realistic option in current breeding programs. This requires that the trait is generally an important objective in rice breeding programs. It also requires that valuable genes or QTLs have been identified, and that MAS offers a significant advance over what could be achieved without the use of markers (i.e. through strictly phenotypic selection). This survey is not considered exhaustive, and emphasis is on the more important breeding objectives and on recent literature. More detailed information on mapped genes is available at the Gramene web site (<http://www.gramene.org/>).

The decision on which traits are of relevance for MAS is not trivial and has been made in this article based on which traits seem likely to benefit most from using markers. This means that a number of traits are left out, but might be of particular relevance for specific breeding objectives. An example would be flowering duration, which is considered an important agronomic trait. The elegant work by Yano and colleagues has identified the most important genes underlying QTLs for flowering duration and photoperiod sensitivity (Izawa et al. 2003). Most breeders can easily and accurately measure flowering duration in their breeding materials and would not consider this trait a high priority for MAS. However, where whole-genome monitoring is used to combine the essential requirements for a suitable variety, QTLs for flowering duration could be selected

for. MAB methods might also be appropriate if a breeder would like to change the duration of a very desirable cultivar by introducing a different heading date allele.

3.1. Grain Yield and Agronomic Traits

3.1.1. Grain yield

Grain yield is one of the most important breeding objectives, but in practice it is often the most difficult to improve on. Historically, the major advances in increasing yield of rice came from the increase in harvest index brought about largely because of the development of semidwarf cultivars in the 1960s and 1970s (Peng et al. 2000). Some increases in biomass have been observed in more recent cultivars (Peng et al. 2000), but increasing the yields further for tropical rices has been very difficult. In general, hybrid rice is thought to be a more promising approach to increasing the yield potential in rice over the existing inbred cultivars. There have been some recent advances in raising the yield potential through the ‘super hybrid’ rice developed in China (Peng et al. 2004).

Grain yield does not appear to be a very promising target for marker assisted selection due to the complicated genetic inheritance. The QTLs almost always have small effects and are specific to genetic background (Guo et al. 2005). It is not uncommon to find larger effects of QTLs where one of the parents is a low-yielding type, but it is simplistic to think that these alleles would confer a significant advantage in the genetic background of modern elite varieties. Some researchers have noted QTLs of larger effect for specific yield components. For example Mei et al. (2006) found some useful QTLs for number of spikelets per panicle, which was related to number of secondary panicle branches. Takai et al. (2005) detected QTLs of moderate effect for grain filling percentage. A QTL for yield potential on chromosome 5, *rg5*, which represented an allele from the *indica* cultivar Kasalath studied in the background of *japonica* Nipponbare, was thought to be of use in different genetic backgrounds (Ishimaru et al. 2005).

Many researchers have applied the Advance Backcross QTL (ABQTL) approach (Tanksley and Nelson 1996) to rice for discovering QTLs with positive effects on agronomic traits derived from wild species (Xiao et al. 1998; Moncada et al. 2001; Septiningsih et al. 2003; Thomson et al. 2003; Lee et al. 2005b). Tian et al. (2006) used an accession of ‘Dongxiang’ wild rice (*O. rufipogon*) to develop introgression lines with small segments transferred to an elite cultivar. In general, the lines with more introgressed segments from wild rice performed poorly; however, some favorable alleles were identified for grain number on chromosomes 5, 8 and 9. They found that QTL analysis in the BC2F2 generation was only a moderate predictor of the effects in the introgression lines.

By map-based cloning Ashikari et al. (2005) isolated a cytokinin oxidase conferring higher grain number and yield. The Null allele for this gene (OsCKX2) conferred higher grain number, and they developed NILs through a MAB approach.

This gene was combined with the major semidwarf gene *Sd1*, corresponding to a mutant allele at a GA_{20} oxidase gene (Sasaki et al. 2002), in a process they termed “tailor-made crop improvement”.

3.1.2. Heterosis and hybrid rice

Attempts to identify QTLs affecting heterosis have had some success, although some authors have noted that it is difficult to find a correlation between F1 performance and parental genotypes (Li et al. 2001; Luo et al. 2001). You et al. (2006) observed that main effect QTLs for agronomic traits could contribute cumulatively to genetic variation, and larger effects could be observed in the hybrids, with relatively lower contributions of epistatic effects. A region near the *Sd1* locus on chromosome 1 seemed to have the largest effect. Mei et al. (2005), in two BC and a RIL population using Lemont (*japonica*) and Teqing (*indica*) indicated that the primary genetic basis for heterosis was overdominance from epistatic loci, while Abdelkhalik et al. (2005) suggested it was from the cumulative effects of partially dominant loci. Hua et al. (2003) found little overlap between heterotic effects and trait performance. They found that heterosis could be explained by single-locus effects in combination with dominance by dominance interaction at the two-locus level.

The relatively complicated genetic control of yield and heterosis has not allowed the identification of promising markers for MAS; however, the production of hybrid rice involves several concomitant traits that are readily amenable to MAS. Most currently grown hybrids rely on the cytoplasmic male sterility (cms) system, which requires that the male parent possess genes for restoration ability. These restorer genes are very tedious to manipulate in breeding because they can only be assayed in mature plants from testcross progeny. For the widely used WA type cms, the *Rf3* gene has been mapped on chromosome 1 (Zhang et al. 1997) and an additional locus has been mapped on chromosome 10 (Yao et al. 1997). For the Chinsurah Boro II type cms, the *Rf1* gene has been cloned (Yao et al. 1997), and Wang et al. (2006) found that the locus involved two related PPR (pentatricopeptide repeat) genes (*Rf1a* and *Rf1b*).

The 2-line system of hybrid rice production involves genetic male sterility genes that are sensitive to temperature and/or photoperiod. This includes the genes designated *tms2* (Lopez et al. 2003), *tms3* (Lang et al. 1999), *tms4* (Dong et al. 2000), *tms5* (Wang et al. 2003b), and *tms6* (Wang et al. 2004; Lee et al. 2005a). With one or a combination of these genes, a breeding line is developed that will be fertile under low-temperatures and/or short daylength for seed maintenance, but will be sterile under higher temperatures or longer daylengths for hybrid seed production.

Wide compatibility (WC) is a very useful trait for conferring spikelet fertility to the hybrids from wide crosses (usually *indica* × *japonica* crosses). Several WC genes have been identified, but the *S5* gene on chromosome 6 has been most widely used (Ikehashi and Araki 1986). Candidate genes for this locus have been reported (Ji et al. 2005; Qiu et al. 2005). Several other QTLs in the WC cultivar Dular have been mapped and targeted for MAS (Wang et al. 2005). Another useful gene for hybrid rice is elongated uppermost internote (*eui*), a recessive tall allele that can be

used in the female parent to increase panicle exertion for hybrid rice production. Several groups have reported the *eui1* gene to be a putative cytochrome P450 gene (Luo et al. 2006; Ma et al. 2006; Zhu et al. 2006).

3.1.3. Other agronomic traits

A number of agronomic traits of value in breeding programs have been identified. These include seedling vigor (Kanbar et al. 2006), seed longevity or dormancy (tolerance of pre-harvest sprouting) (Wan et al. 2006; Yamaguchi et al. 2006), and grain shattering (Li et al. 2006). While these are all useful traits, it is not clear if they would be high-priority targets for MAS in rice breeding programs.

3.2. Grain Quality and Nutrition

3.2.1. Cooking quality and grain appearance

Both cooking quality (taste and texture, largely) as well as grain appearance are high-priority traits in nearly all rice breeding programs. Rice starch is composed of amylose and amylopectin, and the percentage of amylose is an important characteristic of cooking quality. Amylose content is largely determined by the *wx* locus on chromosome 6, and in particular on the amount of Wx protein (granule-bound starch synthase, GBSS) present (Wang et al. 1995). Varieties with near zero amylose and no Wx protein are called glutinous or waxy, and are used for special foods such as desserts and snacks. High amylose cultivars (>25%), with high levels of Wx protein, are common in *indica* rices, and cook dry and fluffy, often becoming hard after cooling. Low amylose cultivars (15–20%) is soft and sticky; nearly all temperate *japonica* cultivars have low amylose. Intermediate amylose (20–25%) is widely preferred in many parts of the tropics. These rices are soft but not sticky. The level of Wx protein is related to the level of mature Wx mRNA, which is related to the stage of intron 1 excision from pre-mRNA (Cai et al. 1998; Isshiki et al. 1998). A single nucleotide polymorphism in the splice site of the leader intron of *Wx* in the amplified fragment explained 80% of the variation in apparent amylose content in a diverse sample of rice varieties (Ayres et al. 1997). A microsatellite marker closely linked to *wx*, containing a variable number of CT repeats, is closely associated with amylose content and grain quality. Larkin and Park (2003) found that two SNPs in exons 6 and 10 were associated with differences in apparent amylose content and viscosity properties of rice cultivars.

Gelatinization temperature (GT) is another important quality predictor, with intermediate GT being a preferred objective for many consumers. Waters et al. (2006) found two polymorphisms in the starch synthase II (SSIIa) gene (*Alk* on chromosome 6), that explain differences in GT. Umemoto and Aoki (2005) found SNPs in this gene that affected activity and starch granule association of SSIIa. In QTL studies of quality parameters, the *Wx* locus appears to have a larger effect, but a QTL at *Alk* is also observed (Fan et al. 2005). In a QTL analysis by Ge et al. (2005) QTLs associated with the *Wx* locus affected water absorption of cooked rice.

Fragrance is an important trait for many breeding programs, and a major gene on chromosome 8 (betaine aldehyde dehydrogenase 2, *BAD2*) has been positionally cloned (Bradbury et al. 2005a), with fragrance representing a loss of function caused by an 8-bp deletion. A perfect marker has been developed from this polymorphism for MAS (Bradbury et al. 2005b). Cooked kernel elongation is a desired trait of some consumers, especially for Basmati rices, and a gene has been mapped to chromosome 8 (Ahn et al. 1993).

Grain size, shape and appearance are very important quality factors, with most consumers preferring specific size and shape characteristics. These factors are generally under the control of QTLs of small effect, although a few with higher effect have been observed. For example, a QTL on chromosome 3 for grain length and weight has been noted in several studies (Li et al. 2004; Ge et al. 2005) and a gene underlying a QTL in the same region was recently cloned (Fan et al. 2006). Grain chalkiness is nearly always considered an undesirable trait; again its genetic control is complicated. But a QTL on chromosome 8 was found to be important in chalkiness and some other quality traits (Wan et al. 2005). While the genes for starch properties seem to be excellent candidates for MAS, the more complicated genetic control of appearance traits, and the fact that they are more easily assessed visually, has meant that MAS is not as useful.

3.2.2. *Nutritional properties*

As of this writing there are few nutritional traits that have been mapped in rice. QTLs for cadmium concentration in brown rice when grown in Cd-affected soil, have been identified (Ishikawa et al. 2005). This has been proposed as a means of developing cultivars with tolerance to soils high in Cd. Low phytic acid mutants have been studied because of their potential for improved quality animal feed, as well as the potential for low phytic acid cultivars to improve uptake of Fe in micronutrient deficient populations. One low phytic acid mutant *Lpa1* was mapped to a 47-kb region on chromosome 5 (Andaya and Tai 2005).

Iron deficiency anemia is a major health problem of rice consumers in Asia, and cultivar differences have been observed (Haas et al. 2005). One of the complications of this trait is that differences are much more pronounced in brown rice than in milled rice, which is the major form consumed. Identification of QTLs controlling Fe and Zn content is a high priority and would greatly aid in developing cultivars with higher micronutrient density.

3.3. **Resistance to Abiotic Stresses**

3.3.1. *Drought tolerance*

Tolerance of abiotic stresses are a primary target for MAS, because of the difficulty in screening for them, and also because a number of QTLs with fairly large effect have been found (Mackill 2006). Among the abiotic stresses, drought is by far the most widely prevalent in rice, and it is also one of the most complicated genetically and physiologically. There has been considerable effort to map QTLs

for drought tolerance and associated traits, including root growth. In general, these have failed to identify QTLs with major effects. Some of the early work on mapping for drought tolerance component traits has been reviewed previously (Nguyen et al. 1997; Mackill et al. 1999; Price 2002). While a number of QTLs have been identified for component traits, especially root depth and thickness, these traits have not been easy to associate with drought tolerance as measured by grain yield under stress.

Zou et al. (2005) identified QTLs in a RIL population from the cross of intolerant Zhenshan 97B with IRAT109, an upland drought tolerant rice. Two QTLs for grain yield under drought were detected on chromosome 2, with LOD scores around 4.2. Yue et al. (2005) studied the same population under two soil types: sandy soil and paddy (flooded) soil. They used Drought Response Index (DRI, Pantuwan et al. 2002), that can remove the effects of differences in genetic yield potential and flowering time, as well as relative yield (RY) to estimate drought tolerance. QTLs for DRI and RY mostly had small effect, and were not strongly related to root traits. One QTL on chromosome 10 contributed 32% of the phenotypic variance for RY. Root traits played a more important role under the sandy soil conditions. The same population was studied for stress at the reproductive stage in tubes (Yue et al. 2006). QTLs on chromosomes 2 and 9 from IRAT109 consistently conferred an advantage for RY. The QTL on chromosome 9 was also associated with higher spikelet fertility, which was a good indicator of drought tolerance.

At IRRI, a backcrossing scheme using multiple donor lines of diverse origin was employed to generate populations with multiple introgressions in diverse backgrounds (Li et al. 2005b; Lafitte et al. 2006). Segregating generations were selected under severe drought stress. In progeny selected for tolerance, a number of chromosomal segments were associated with improved tolerance, as well as other traits. A number of chromosomal regions selected appeared to be similar to QTLs detected in previous studies (Li et al. 2005a). In a study using Teqing as the *indica* recurrent parent and Lemont as the tropical *japonica* donor, a number of alleles from Lemont imparted improved tolerance in random introgression lines (Xu et al. 2005a).

Most drought tolerance studies indicate that there are multiple QTLs with small effect on the trait. There has thus been an attempt to identify genes that are differentially expressed between tolerant and susceptible types, and correlate these with consistently mapped QTLs. For example, Gorantla et al. (2005) observed 13 drought response genes close to known QTLs. If the candidate genes underlying QTLs can be identified this would make selection for multiple QTLs more efficient in breeding populations. Nevertheless, a search for QTLs of larger effect has continued in order to find those that would impart a higher level of tolerance when transferred into improved lines. Some recent results indicate that selecting parents with a higher level of drought tolerance shows promise in identifying more effective QTLs (G. Atlin, personal communication). Even with QTLs of moderate effect, the benefits would be substantial if they could be transferred into high-yielding and widely accepted cultivars.

3.3.2. *Salinity tolerance and other soil nutrient stresses*

Tolerance to salinity is desirable at both the vegetative and reproductive stage, and tolerance at the two stages does not seem to be correlated. At the vegetative stage, large differences have been observed in tolerance levels. A major QTL named *Saltol* controlling 64–80% of the phenotypic variability from the tolerant cultivar Pokkali was identified on chromosome 1 (Bonilla et al. 2002), and the QTL seems to be present in other varieties (Takehisa et al. 2004). This QTL has been fine-mapped and is being used in MAB strategies to improve the salinity tolerance of important cultivars (G. Gregorio, personal communication). A gene from the tolerant cultivar Nona Bokra at a similar location was recently identified through positional cloning (Ren et al. 2005). This selective transporter was named *SKCI* and was involved in maintaining K⁺ homeostasis. Mapping of tolerance at the reproductive stage is still underway.

A number of fairly strong QTLs have been mapped for other soil-related stresses, and these show great promise for MAS (Wissuwa 2005; Mackill 2006). QTLs for tolerance to Fe toxicity (Wan et al. 2003; Shimizu et al. 2005), P deficiency (Ni et al. 1998; Wissuwa et al. 1998), and Al toxicity (Nguyen et al. 2003) are among the more promising. Among these, the P deficiency QTL has been fine-mapped (Wissuwa et al. 2002) and is being transferred in a MAS program. QTLs for ability for rice to tolerate low N levels have been identified, and the trait appears to be quite complex (Lian et al. 2005). However there has been considerable interest in this trait because of environmental concerns from over-fertilization and high cost of N fertilizer for many rice farmers.

3.3.3. *Submergence tolerance*

Tolerance to excess water includes tolerance of complete submergence and tolerance to gradually rising water levels that stagnate for one or more months. In the latter situation, deepwater rices are suitable where water levels go beyond 50 cm. Under these situations, rapid internode elongation is usually needed. A major QTL was mapped on chromosome 12 for early elongation ability in deepwater rice (Tang et al. 2005). However, submergence tolerance is much more useful, being appropriate for the large areas of rainfed lowland rice where short-term flooding is common.

Submergence tolerant cultivars can survive 2 weeks or more of complete submergence, whereas most cultivars die within a week. The most tolerant cultivars (e.g. FR13A, Kurkaruppan, Goda Heenati) are from Orissa, India, or Sri Lanka, and their tolerance is controlled at the *Sub1* locus on chromosome 9 (Xu et al. 2006). Three ethylene-response-factor (ERF)-like genes at this locus have been identified, two of which are induced by submergence. The *Sub1A* gene conferred tolerance when overexpressed in a variety that lacked the gene. This gene seems to be only present in *indica* cultivars. SNP markers are available from the sequence that can be used for MAS. Some other QTLs have been identified in FR13A that confer additional levels of submergence tolerance (Nandi et al. 1997), but the *Sub1* locus accounts for about 70% of the phenotypic variation for survival under submergence.

Tolerance to submergence at the germination stage (anaerobic germination) seems to be independent of tolerance during vegetative stage growth. Tolerance at germination would be desirable both for protecting germinating seeds from unexpected flooding caused by heavy rainfall and for strategies of deliberate submergence to control weeds in direct-seeded rice. A QTL study measured shoot length under water and found five QTLs, mostly of relatively small effect (Jiang et al. 2004). Studies with varieties with a higher level of tolerance are underway and may yield QTLs of larger effects.

3.3.4. *Low-temperature tolerance*

Cold tolerance at the booting stage (about 12 days before heading) is an important breeding objective in temperate areas or in the high-elevation tropics. *Japonica* rice cultivars are more tolerant than *indicas*. Takeuchi et al. (2001) identified three QTLs in a cross between two *japonicas*; one of the QTLs on chromosome 7 contributed 22% to the phenotypic variation. Dai et al. (2004) also detected a strong QTL on chromosome 7 from a Yunnan land race. Another QTL on chromosome 4 was detected by introgressing the chromosomal fragment from the tolerant tropical *japonica* Silewah into the *japonica* variety Hokkai241. The gene *Ctb1* has been finely mapped and candidates identified (Saito et al. 2004). Andaya and Mackill (2003b) identified eight QTLs in a RIL population between a *japonica* cold tolerant variety M-202 and an *indica* susceptible variety IR50. The two strongest QTLs were on chromosomes 2 and 3.

At the vegetative stage low temperature causes chlorosis and even death of the plants. This is a problem in some temperate areas and in the subtropics where rice is grown in the winter season as a double crop. A strong QTL for cold-induced necrosis and wilting tolerance has been identified on chromosome 12 (Andaya and Mackill 2003a). In another *indica* × *japonica* cross (Jileng 1/Milyang 23), Han et al. (2004) detected different QTLs for leaf discoloration under 12°C water temperature, and they did not detect the same strong QTL on chromosome 12. They found a larger QTL on chromosome 1.

3.4. Resistance to Biotic Stresses

3.4.1. *Disease resistance*

Blast (*Magnaporthe grisea*) is the most widespread fungal disease of rice and has attracted major efforts to map both major gene and quantitative (partial) resistance. For most breeding objectives in the tropics, quantitative resistance seems to be the best option, because of the risk of breakdown of major gene resistance. Partial resistance is controlled by multiple QTLs of relatively small effect (Wisser et al. 2005; Wu et al. 2005). In practical breeding programs for tropical lowland rice, it has not been too difficult to develop varieties with sufficient partial resistance, but the small effects of most QTLs has made it difficult to apply MAS. Association of known PR genes with QTLs is seen as a means of finding more useful markers for selecting for partial resistance to blast (Liu et al. 2004).

At least 40 major genes have been mapped, and candidates have been found for some (Chen et al. 2005). Characterizing the individual R genes for their race specificity has provided information for pyramiding combinations of genes to obtain more broad spectrum resistance, which is thought to be more durable. Fine-mapping and identification of good PCR-based markers for the important resistance genes has provided useful tools for MAS (Fjellstrom et al. 2006).

Bacterial blight (*Xanthomonas oryzae*) has also been well studied, and at least 30 genes are known. Compared to blast, major gene resistance has been more durable for bacterial blight. A number of resistance genes have been identified by map-based cloning, beginning with the well-known *Xa21* gene (Song et al. 1995). These genes have been the focus of efforts for fine-scale mapping and MAS. Some recent examples include *Xa2* (He et al. 2006), *xa13* (Chu et al. 2006) and *Xa27* (Gu et al. 2005).

Tungro is the most serious virus disease, being caused by two viruses, spherical and bacilliform. A resistance gene for the spherical virus was mapped on chromosome 4 (Sebastian et al. 1996). Four resistance genes are reported in the Gramene database, but they have not all been documented in refereed journal articles. Rice Yellow Mottle Virus is an important disease in Africa. Two QTLs on chromosomes 7 and 12 showing complementary epistasis have been mapped and used in MAS (Ahmadi et al. 2001).

3.4.2. *Insect resistance*

Major attention has been given to the brown planthopper (BPH), which can cause devastating crop losses under conducive conditions. At least 18 genes have been mapped and/or fine-mapped (Jena et al. 2006). Quantitative trait loci have also been mapped (Sun et al. 2005) with the objective of attaining resistance that would not break down with evolution of new biotypes. Resistance to green leaf hopper (GLH) has also received some attention, mostly as a vector of tungro virus (Sebastian et al. 1996; Wang et al. 2003a). Green rice leaf hopper is a pest in temperate areas, and at least six resistance genes are known. CAPS markers for MAS for resistance gene *Grh3* were developed (Saka et al. 2005).

Stem borers are one of the most important insect pests of rice, and no strong sources of resistance have been identified. Efforts for developing resistant varieties is focused more on transgenics with Bt toxin genes.

4. MAS EXAMPLES IN RICE

Examples of the use of MAS in rice breeding programs have been limited as reported in the published literature. Reasons for this scarcity are numerous and include

- Cost of applying genotyping methods, especially in large breeding populations
- Limited number of major genes or QTLs that will be beneficial in local germplasm

- Lack of traits that have been fine-mapped with good selectable markers
- Novelty of methods and long-term nature of MAS projects: most of them are still ongoing

MAS activities are often not published in journals because they are perceived as applied breeding activities and in many cases documentation may be insufficient for full data analysis. This is unfortunate, because there is a need to properly document these activities to provide rice scientists with up-to-date information on how MAS can be applied in practice. One can get a better idea of the current extent of activities in MAS by browsing the abstracts presented at the 2005 International Rice Genetics Symposium, available at <http://www.irri.org/rg5/>.

The prerequisites to apply effective MAS can be considered generally as: mapping the gene or major QTLs for the trait, fine-scale mapping, validation of the effect of the gene/QTL in appropriate genetic background, and development of diagnostic markers for the target gene (Collard and Mackill 2007). The current applications of MAS in rice breeding can be considered under three general categories: gene pyramiding, marker assisted backcrossing, and use of markers in standard breeding populations. Molecular markers are also useful for evaluation of breeding material and cultivars (Collard and Mackill 2007), but these are not covered in the present article.

4.1. Gene Pyramiding

Pyramiding genes for resistance to diseases has been the most common application of MAS in rice. The major benefit obtained from the use of markers in gene pyramiding is the ability to detect plants with multiple genes whose effects are difficult to separate phenotypically. Collard and Mackill (2007) documented five reports in the literature, mostly for blast and bacterial blight, and many more are in progress (for examples see: <http://www.irri.org/rg5/Abstracts.pdf>). The chief advantage of these pyramids is that they combine the resistances for many races of the pathogen achieved from the different spectra of the component resistance genes. In blast, a number of gene combinations are thought to give a very strong level of resistance, especially combinations of *Pi1* and *Pi2*. Markers specific for the *Pi2/Piz/Pi9* gene complex on chromosome 6 are likely to be useful for breeding programs seeking higher levels of blast resistance (Fjellstrom et al. 2006).

Pyramiding major genes for bacterial blight should be valuable, because the durability of resistance genes is relatively longer, and some R genes have been very effective. *Xa4*, *xa5*, *xa13*, and *Xa21* have been commonly used (Huang et al. 1997; Joseph et al. 2004). For blast, the concept also seems to apply; however, the durability of blast resistance genes has been very low. This has led to concern that even gene pyramids could be overcome by more complex *Magnaporthe* races. At present there is not sufficient information to draw firm conclusions. In the irrigated lowland tropics, partial resistance is usually sufficient. In areas of higher blast pressure (temperate rice or the tropical uplands), major gene pyramids seem

an appropriate, although not fail-safe strategy. Narayanan et al. (2004) combined two blast resistance genes *Pi1* and *Piz⁵* (*Pi2*) with the bacterial blight resistance transgene *Xa21*.

In many applications of gene pyramiding, the objective is actually to transfer the target genes into an existing variety, thus representing a combination of pyramiding with MAB. As described in the next section, one potential drawback to this is the compounding of linkage drag over several genes. With two to three gene pyramids this may not be a major drawback, but certainly it will be for more complex pyramids.

Gene pyramids have been proposed for other traits aside from resistance. For example, Nas et al. (2005) combined three genes controlling temperature sensitive male sterility in an attempt to derive lines with different behavior for sterility induction.

4.1.1. *Marker assisted backcrossing*

The MAB approach is a highly effective, yet underused strategy for applying markers in rice breeding programs. The advantages of MAB for rice breeding include:

- Lower population sizes than conventional breeding that require less genotyping
- The existence of widely-grown rice cultivars (“mega varieties”) that possess the essential features of high grain quality, yield and local adaptation, but lack specific traits such as stress tolerance
- Existence of major QTLs and major genes that could add value to many elite rice cultivars

Tanksley et al (1989) pointed out the potential of using markers to precisely introduce a target gene into a desired cultivar. Theoretical considerations involved in reducing linkage drag and the contribution of background markers have been described (Frisch et al. 1999b; 1999a). Collard and Mackill (2006) divided the process of MAB into three components: use of a tightly linked marker or gene marker (perfect marker) for foreground selection, use of flanking markers for “recombinant selection”, and use of background markers for selection against the donor genome at unlinked loci.

Most rice workers have employed MAS without rigorous use of background markers or flanking markers. There are several reports of combining pyramiding and MAB for bacterial blight resistance: three resistance genes in BC3F2 (Sanchez et al. 2000), three resistance genes in BC2F3 (Singh et al. 2001), and two resistance genes in BC1F3 (Joseph et al. 2004). Liu et al. (2003) transferred the blast resistance gene *Pi1* into the widely used restorer line Zhenshan 97 using background markers to remove the donor genome. Ahmadi et al. (2001) applied foreground selection for two QTLs for resistance to RYMV, in combination with background selection. After the BC3 generation, the proportion of the recipient genome was about 95% and the segment around the two QTLs was about 20 cM. Steele et al. (2006) performed a complicated assembly of multiple QTLs related to drought and fragrance. While the objective was to combine five QTLs over three backcross generations while

removing unnecessary or undesirable donor segments, only one of the four drought-related QTLs (for root length) functioned in the background of the recipient cultivar Kalinga. Toojinda et al. (2005) combined genes for submergence tolerance (*Sub1*), and resistance to BB, BPH, and blast, as well as some quality traits, into two widely grown varieties, Khao Dawk Mali 105 and RD6 over four backcrosses. The extent of donor genome still present was not mentioned.

The most systematic studies reported are those of Q. Zhang and colleagues for transferring bacterial blight resistance into widely grown cultivars. A fragment of 3.8 cM surrounding the *Xa21* gene was introduced into Minghui 63 restorer line (Chen et al. 2000) and a similar fragment was introduced into the restorer '6078' (Chen et al. 2001) within three backcross generations. The process involved selection of BC progeny possessing the target locus, selection of progeny with recombination on one side of the locus (recombinant selection), and selecting among the remaining progeny for fewer number of unlinked donor segments. Work at IRRI has shown that the same approach can be applied to QTLs that have been fine-mapped. The *Sub1* QTL for submergence tolerance was introgressed into the widely grown cultivar Swarna (Xu et al. 2006), and in the BC3 progeny the introgressed fragment was limited to 2.3 Mb of DNA (Neeraja et al., submitted).

One of the applications of MAB is the introduction of transgenes into a desirable cultivar. The transformation system is efficient for only a few cultivars and is not very easy in *indica* rice cultivars. So generation of transgenics and selection of the desirable event will usually occur in an easily transformable cultivar, and needs to be backcrossed into other cultivars. This is currently the approach being used to introduce the vitamin A trait (Golden Rice) into the mega varieties in Asia (Al-Babili and Beyer 2005; Paine et al. 2005).

One drawback to the above approach is the intensive amount of genotyping that must be done. Large backcross populations, usually on the order of 500 plants or more, must be produced to make sure that there are sufficient plants to carry out background selection after the foreground and recombinant selection have been performed. With flanking markers of 3–5 cM, the foreground/recombinant selection will limit the population to less than 5% of the total backcross progeny. If very large populations are used, the process could be completed by BC2, but the number of molecular data points required would be lower for BC3 (Frisch et al. 1999a). Despite the intensive genotyping required, the approach has much to commend it, especially considering the difficulty of developing improved varieties that can replace the existing mega varieties. These varieties have many desirable features and changes to their genetic makeup more often result in deficiencies, even when they occur on a small scale. The approach is certainly not proposed as a replacement of conventional breeding efforts for developing new varieties. On the other hand, it gives greater assurance that the intense efforts undertaken for MAB would result in improved cultivars that have the characteristics that farmers want. This is particularly important considering that most varieties released for cultivation are not ultimately adopted by a large number of farmers (Mackill 2006).

4.1.2. *Use of markers in breeding nurseries*

The incorporation of MAS into breeding nurseries has been problematic. In early generations, where MAS could have the greatest impact, most breeders handle hundreds of thousands, and even millions of plants. This presents a practical hurdle for DNA extraction and marker analysis. For most traits, visual or simple chemical screens are generally sufficient. More widespread use of MAS in early generations will require less expensive procedures.

In later generations, MAS can be applied for traits that are difficult to assess phenotypically. An example that appears practical at the moment is for grain quality, where a limited number of genes appear to be crucial to determining the basic properties of starch that affect texture, probably the most important quality trait. Knowing the alleles at the *waxy* and *Alk* loci for example, the breeder can obtain basic information on the desirability of breeding lines, which could normally not be obtained until advanced evaluation of fixed lines. These markers are currently being employed at IRRI in F5 to F6 lines before they are evaluated intensively in replicated yield trials.

Another example of the value of markers is in hybrid rice breeding. With the existing 3-line method of hybrid rice seed production, the male parent is required to possess restoring ability, usually controlled by two dominant genes for the widely used WA cms system. Testing for restoring ability is a laborious process that involves making test crosses to a cms line and assessing the fertility of the progeny. Having a marker system that predicted at least one of the *Rf* genes greatly reduced the amount of testing needed for this trait (M. Sattari et al., unpublished data).

For rice blast, it has been mentioned that partial resistance is probably the most effective strategy for the tropical lowlands. Many varieties have the necessary level of resistance and so it is not difficult to develop new lines that have this resistance. However, accurate assessment of partial blast resistance is not easy to obtain without repeated and careful evaluation. The genetic control of this partial resistance is complex, and the QTLs do not have large effects. For this reason, the standard approaches of MAS seem inadequate to assist breeders in their selection work. This would perhaps be different if markers that were diagnostic of partial resistance alleles were available. There is evidence that the level of partial resistance can be attributed to the number of favorable alleles of PR-related genes that are associated with the QTLs identified for partial resistance (Liu et al. 2004; Wu et al. 2004).

5. PROSPECTS

At the moment, the use of MAS in rice is occurring mostly for pyramiding resistance genes and MAB. Increasing use of these methods is expected, particularly for MAB. More widespread use of MAS is also expected with the improvement of methods for marker analysis and identification of candidate genes for economic traits. Rice gene discovery will progress rapidly. As can be seen from examples presented here, development of perfect markers is very beneficial for practical application of MAS.

Costs of DNA extraction have been reduced and it is not difficult to obtain samples for tens of thousands of plants, which should facilitate the entry of MAS into the conventional breeding process. Of particular note is the potential for lower-cost whole genome scans, which would allow more targeted assembly of genes and chromosomal regions into the predicted favorable genotypes. Low-cost array-based methods of assessing hundreds or thousands of markers will be available in the near future, making the greater use of MAS in rice inevitable.

The current focus on gene discovery should result in identification of an increasing number of genes and genetic pathways that are responsible for complex economic traits such as grain yield. One of the reasons that large population sizes are emphasized in traditional plant breeding is that the genetic control of target traits is not known, and the assembly of favorable alleles is undertaken on a trial and error basis. A more targeted allele assembly implies that population development is aimed at assembling specific alleles and fewer populations should be needed to achieve the desired result.

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CHAPTER 8

APPLICATION OF GENOMICS FOR MOLECULAR BREEDING IN RICE

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Abstract: Rice is arguably the most important food crop of the world and due to its small genome size compared to other major cereals, rice was selected as model crop species for decoding of its full genome. The international rice genome sequencing project (IRGSP), a consortium of laboratories from ten different countries, has generated a very high quality map based sequence of the 12 chromosomes of *japonica* rice cultivar 'Nipponbare' and made it available in the public domain. A whole genome draft sequence of *indica* type rice variety '93-11' has also been reported by the Beijing Genomics Institute. Annotation and comparative analysis of these and other partial genomic sequences has provided a wealth of information to the rice geneticists and breeders. Simple Sequence Repeat (SSR) markers are now easily available for any region of the rice genome. SSR markers have also been derived from the expressed sequence tags (ESTs) and unigene sequences, which correspond to the expressed component of the genome and thus have greater potential in comparative genome analysis. Furthermore, millions of single nucleotide polymorphism (SNP) and insertion-deletion (InDel) markers have already been identified in rice. Saturation of the genome with such sequence based SSR and SNP markers is accelerating fine mapping and map-based cloning of genes, and thus, development of gene-based allele-specific markers. Rice improvement programs are expected to benefit greatly from the use of these markers in near future.

1. INTRODUCTION

The genetic information in plants is stored in three sub-cellular compartments namely nucleus, chloroplast and mitochondria. The nuclear genome is much larger and far more complex in both structure and function than the two organelle genomes.

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Beginning from the second decade of the last century, conscious efforts have been made to understand structure and function of the plant nuclear genome employing tools and techniques of Mendelian genetics and cytology. As a result, genes with visible phenotypic effects could be arranged in linear order in different linkage groups based on genetic recombination. The chromosome numbers of most of the important plant species were determined. Chromosome pairing behavior in interspecies hybrids was used to identify possible diploid progenitors of many of the present day allopolyploid crop species. Discovery of DNA as genetic material and elucidation of its double helical structure during 1950s gave a new dimension to the study of genomes. Development of molecular techniques such as DNA cloning and sequencing that followed these discoveries, paved the ways for a detailed analysis of genome structure and function. Automation in laboratory instrumentation facilitating high throughput sample analysis coupled with an array of robust computational methods enabled complete sequencing of several large eukaryotic genomes including that of human, *Drosophila*, *Arabidopsis* and rice. The availability of high quality whole genome sequence provided a thorough understanding of the genome structure and evolution patterns. Novel means of understanding the genome functions could be designed by utilizing the DNA sequence information. These developments led to the birth of a new discipline in biology aptly named as "Genomics".

The widely grown cultivated rice (*Oryza sativa* L.) behaves like a true diploid with gametic chromosome number 12. Rice genome was initially characterized by cytological analysis at mitotic metaphase and chromosome pairing at pachytene stage of meiosis. Kuwada (1910) first reported the chromosome number of cultivated rice as $2n=24$, using both mitotic and meiotic cells. Shastri et al. (1960) thoroughly examined pachytene chromosomes of rice and numbered the chromosomes in decreasing order of their length. Morinaga (1937, 1939) first analysed the genomes of different rice species and proposed five different genomes from A to E. Based on several earlier linkage mapping studies using morphological markers, Nagao and Takahashi (1963) proposed 12 possible linkage groups corresponding to the haploid chromosome complement of rice. Cytogenetic stocks such as trisomics and reciprocal translocations were used to establish relationship between these linkage groups and the 12 chromosomes of rice (Iwata 1986). With the construction of molecular marker based high-density linkage maps, use of mapped markers to integrate genetic and physical maps and whole genome sequencing, a wealth of information is now available for the rice researchers, which can be very effectively utilized for various applications in rice genetics and breeding. The main objective of this short review is to highlight the application of genomics for molecular breeding in rice.

2. EARLY PROGRESS IN RICE MOLECULAR GENETICS

The first attempt to characterize the rice genome using molecular markers was made in the year 1988. McCouch et al. (1988) cloned single/low copy genomic sequences and used those as probes in southern hybridization to generate the first

set of restriction fragment length polymorphism (RFLP) markers. Based on segregation of these markers in a small F_2 population of 50 individuals derived from *javanica* \times *indica* cross, the molecular map of the rice genome was constructed that contained 135 RFLP markers and covered a total length of 1389 cM. Rice genome had approximately 50% single copy sequences and was less C-methylated than maize and tomato genomes. This work greatly accelerated rice genetics research worldwide. Many genes of agronomic importance for both qualitative and quantitative traits could be mapped and localized into marker intervals using this map. Following this landmark development, several RFLP maps of rice genome were reported. High-density linkage maps were subsequently made using larger sets of RFLP markers by two groups independently; one at the Cornell University, USA (Causse et al. 1994) and the other at the Rice Genome Research Program, Japan (Kurata et al. 1994; Harushima et al. 1998). The Cornell group extended the first RFLP map by McCouch et al. (1988), while the RGP group used a different set of random genomic clones and cDNA clones prepared from callus and roots as probes in RFLP to develop the map. Kurata et al. (1994) mapped 1383 DNA markers using 186 F_2 plants derived from a cross between a *japonica* variety, Nipponbare, and an *indica* variety, Kasalath. Additional RFLP markers were developed by using cDNA clones from green and etiolated shoots and 934 new markers were added to the map using the same F_2 population after omitting 42 markers for various reasons. This gave a total of 2275 markers mapped to 1174 discrete positions covering 1521.6 cM of the rice genome (Harushima et al. 1998). Subsequently, the Japanese group further enhanced the map density (<http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html>) using 3267 DNA markers and almost all the RFLP markers were converted to Cleaved Amplified Polymorphic Sequence (CAPS) markers. These maps have been of great value in both genetic and physical mapping of genes and map-based sequencing of the rice genome.

RFLP markers are not very convenient to use in high throughput genotyping applications. Plant breeding requires a faster, simpler and more easy-to-use method of analysis. Therefore, while the RFLP maps were being saturated with additional markers, simple sequence repeats (SSRs) markers were being designed for the rice genome (Wu and Tanksley 1993; Zhao and Kochert 1992, 1993). SSRs, also called as microsatellites, are tandemly arranged repeats of 1–6 nucleotide long DNA motifs that frequently exhibit variation in the number of repeats at a locus. As the flanking sequences may be unique, once SSR loci are cloned and sequenced, primers can be designed from the flanking sequences. The resultant sequence tagged microsatellite usually identifies a single locus. Because of high mutation rate of SSRs, these markers are often multi-allelic that often allows resolution on agarose gels. SSRs are also visualized on sequencing gels where single repeat differences can be resolved and, thus, all possible alleles detected. SSRs provide highly informative markers because they are co-dominant, generally highly polymorphic and highly reproducible between laboratories. These are amenable to high-throughput genotyping and thus useful for construction of high-density linkage maps, gene mapping and marker-assisted selection. Screening of a rice genomic library with

poly(dG-dA).(dC-dT) and poly(dG-dT).(dC-dA) probes by Wu and Tanksley (1993) indicated that (GA)_n repeats occurred, on average, once every 225 kb and (GT)_n repeats once every 480 kb. DNA sequencing of a set of ten randomly selected microsatellites indicated that the numbers of repeats ranged from 12 to 34 and that the patterns of microsatellites in rice were similar to those of humans and other mammals. Multiple alleles, ranging from 5 to 11, were observed at the microsatellite loci in a set of 20 rice accessions. This study was extended further by Panaud et al. (1996) who added 20 new microsatellite. Chen et al. (1997) estimated 5700–10,000 microsatellites in rice genome, with the relative frequency of different repeats decreasing with increasing size of the motif. A map with 120 microsatellite markers that were well distributed throughout the 12 chromosomes of rice was constructed. Cross-transferability of these markers to the wild relatives of the cultivated rice *O. sativa* revealed their utility in monitoring of gene introgression in breeding programmes (McCouch et al. 1997). The SSR map of the rice genome was further extended by adding 188 new markers derived partly from random genomic library (97 SSRs), and partly from known genes (26 SSRs) or EST (65 SSRs) sequences (Temnykh et al. 2000). The GA and CCG motifs were found most frequent among di- and tri-nucleotide SSRs in the genic regions of rice genome. There was uniform distribution of these markers irrespective of their sequence origin. These markers were successfully used to analyze genetic diversity (Yang et al. 1994; Olufowote et al. 1997; Cho et al. 2000), identify elite rice varieties (Singh et al. 2004) and to locate genes and QTLs on rice chromosomes using both intra- and interspecific crosses (Xiao et al. 1998; Bao et al. 2000; Zou et al. 2000; Bres-Patry et al. 2001; Moncada et al. 2001). However, a high-density rice genome map, which is required for fine scale mapping and map based cloning of genes, could not be constructed using SSR markers. This was primarily because of the difficulties in the marker development, as laborious iterations of genomic DNA library screening with SSR probes and sequencing of a large number of SSR positive clones were required.

3. DECODING OF THE RICE GENOME AND ITS IMPACT ON MARKER AND GENE DISCOVERY

Multiple international efforts were aimed at complete decoding of the rice genome. The International Rice Genome Sequencing Project (IRGSP; <http://rgp.dna.affrc.go.jp/Seqcollab.html>), with partner institutions from ten different countries namely, Japan, United States, China, France, Taiwan, India, Thailand, Korea, Brazil and the United Kingdom was officially launched in February 1998. The objective of this international collaborative effort led by Japan was to accelerate the sequencing of the genome of *japonica* rice cultivar Nipponbare by pooling resources and manpower, and to ensure public access to the high quality sequence data. IRGSP followed a map-based clone-by-clone approach for sequencing and contemplated to finish the genome sequencing by 2008. The multinational company Monsanto also carried out sequencing on contract at the University of Washington, Seattle, USA, following the same approach as IRGSP and announced that it had

sequenced the Nipponbare genome in April 2000 (<http://www.rice-research.org>). Although incomplete, the sequence was very informative, and Monsanto shared the sequence information with academic researchers and with the IRGSP. In January 2001, another multinational company Syngenta reported phase-I draft sequence of the same japonica cultivar Nipponbare, which was published in 2002 (Goff et al. 2002). It had contracted Myriad Genetics in Salt Lake City, Utah, USA, for sequencing using whole-genome shotgun approach. The fourth effort that surprised the world was that by the Beijing Genomics Institute (BGI), China, where sequencing of *indica* line 93–11 was carried out independently following a similar approach to that of Syngenta. BGI announced its plan to sequence in May 2000, and had the rough phase-I draft by October 2001, which was published in 2002 (Yu et al. 2002) simultaneously as that by Syngenta. While the sequence by Syngenta was not available for public, the *indica* sequence was placed in the public domain by the BGI. The sequence assembly by Syngenta has about 6X genome coverage and that by BGI now has a 6.28X coverage. Based on these assemblies, the rice genome size was predicted to be 433 Mb for *japonica* and 466 Mb for *indica*.

The IRGSP for its sequencing strategy required a physical map of the rice genome based on artificial chromosomes. A yeast artificial chromosome (YAC) physical map covering 63% of the rice genome was first constructed (Saji et al. 2001). However, instability, high chimera frequency, and difficulties in manipulation and purification make YAC clones difficult substrates for genome sequencing. Subsequently, low-copy number bacterial clones, namely bacterial artificial chromosomes (BACs) and P1-derived artificial chromosomes (PACs), were preferred for sequencing of the rice genome. Construction of a BAC library in *indica* rice cv Teqing and fingerprinting of 21,087 BAC clones from this library was reported (Tao et al. 2001). However, very limited genetic anchoring of the BAC contigs was carried out by this group. Subsequently, two deep-coverage BAC libraries from *japonica* rice cv Nipponbare were constructed, fingerprinted with HindIII and assembled into contigs, which were physically anchored onto the rice chromosomes by overgo hybridization, DNA gel blot hybridization, and *in silico* analysis (Chen et al. 2002). This physical map consisted of 65,287 fingerprinted BAC clones (including 2778 singletons) thought to represent 20-fold redundant coverage of the genome. The Rice Genome Research Programme, Japan constructed a transcript map with 6,591 EST markers (Wu et al. 2002), which were then used to screen the PAC and BAC libraries. Employing both Finger Printed Contigs (FPC) and Sequence-Tagged-Connector (STC) approaches, RGP constructed physical maps of rice chromosomes 1, 2, 6, 7, 8 and 9 (Wu et al. 2003). These two BAC and PAC based physical maps, were used to identify clones in the minimum tiling path. The physical gaps in the BAC/PAC tiling path were bridged using a variety of substrates, including PCR fragments, 10-kb plasmid and 40-kb fosmid clones. Individual PAC/BAC clones in the minimum tiling path were shotgun-sequenced and locally assembled. A completed phase-II and high-quality draft of the Nipponbare genome was announced by IRGSP on 18th December 2002. The phase II sequences of the BAC/PAC clones, however contained certain gaps, poor quality

regions, mis-assemblies, and single strand coverage regions. Several 'finishing' protocols were employed to fill the gaps and improve the sequence quality to an accuracy of 99.99%. Mis-assembly was resolved by manual detection, selection of a representative sub-clone, and full sequencing of the sub-clone. Manual editing and re-sequencing of some regions were carried out to improve coverage and the sequence quality. After two years of finishing work, the completion of rice genome sequencing was officially declared in December 2004 and published in August 2005 (IRGSP 2005). A total of 3,401 BAC/PAC clones were sequenced by the IRGSP to average tenfold sequence coverage, assembled, ordered and finished to a sequence quality of less than one error per 10,000 bases. The total assembled nucleotide sequence is 370.7 Mbp (Build 3.0 Pseudomolecules). Taking into account the estimated size of gaps, the entire rice genome is estimated to be 388.82 Mbp. This contrasts the genome size estimated earlier based on draft Phase I sequences by Syngenta and BGI. The high quality sequences obtained by IRGSP covered about 95% of the whole rice genome and 98.9% of the euchromatin region, while the remaining 5% corresponded to mostly centromere and heterochromatin regions.

The sequence of the rice genome has revealed many interesting features. A total of 37,544 non-transposable-element-related protein-coding genes were detected, with gene density of one per every 9.9 kb. This is the largest number of genes identified in plant and animal genomes that have been completely sequenced so far. About 71% of these genes are homologous to *Arabidopsis* genes and 29% exist in clusters. The number of genes that appeared to be unique to rice and the other cereals was 2,859, which might differentiate monocot and dicot lineages. Of the 37,544 predicted protein-coding genes, 17,016 could be supported by full-length cDNAs available in rice (Kikuchi et al. 2003). The density of expressed genes was greater on the distal portions of the chromosome arms except for the highly heterochromatic short arms of chromosomes 4, 9 and 10. About 0.2% of rice genome corresponded to sequences, which are highly homologous to the chloroplast and mitochondrial DNA that suggested repeated transfer of the organellar DNA to the nuclear genome. The transposon content of rice was about 35% with representatives from all known transposon superfamilies. Thus, the high quality sequence generated by the IRGSP provides finer details about the structure and evolution of the rice genome. Besides, this sequence resource is of immense value in many aspects of rice genetics and breeding: i) the predicted gene models would serve as the base for a functional analysis of the rice genome. Each of these genes can be characterised individually through RNAi technology. Gene chips based on these sequences have already been designed for use in whole genome expression profiling to identify and understand the role of genes in complex biological processes such as yield and abiotic stress tolerance. About 50,000 Tos17 retrotransposon insertion lines carrying 5,000,000 insertions have been produced in rice (Miyao et al. 2003). By comparing the sequences flanking these insertion sites with the rice genome sequences 11,487 target loci have been mapped on the 12 chromosomes. It has been observed that 3,243 of the predicted genes carry one Tos17 insertion each. Phenotypic alteration in these mutants can easily be associated with the disrupted

gene, thereby defining its biological function. ii) A large number of sequence based molecular markers such as SSRs and SNPs have been designed that practically saturate the genome. These markers can be used to map and tag genes for both qualitative and quantitative traits at very fine scale into short physical intervals, which in turn is helping isolation of genes by positional candidate approach. iii) Once genes are identified and functionally validated, it would be possible to identify new alleles in the germplasm by association mapping and thus enable designing allele specific markers. Use of such functional markers in crop breeding will make selection most efficient. iv) Rice is a small genome member of the grass family that includes many large genome members such as wheat, barley and maize. Synteny among the genomes of the family members is known. It is therefore possible to extend the rice gene information to other important cereal crops. In the following sections, we further elaborate on the development of sequence-based markers and their use in rice molecular breeding.

4. DEVELOPMENT AND USE OF SSR MARKERS

4.1. SSR Markers from the Genome Sequences of Rice

The first attempt to design new SSR markers *in silico* based on the rice genome sequence was made by Temnykh et al. (2001) using 74,127 BAC-end sequences (<http://www.genome.clemson.edu/projects/rice/>) with an average length of 500 bp, five complete BAC sequences generated by the US Rice Genome Sequencing Program (<http://www.tigr.org/tdb/rice/>) and 22 complete PAC sequences developed by the Rice Genome Research Program in Japan (<http://rgp.dna.affrc.go.jp/GenomeSeq.html>). SSRs were identified in a total of 57.8 Mb DNA sequence and classified as class I (>20bp repeat length) or class II (>12 bp <20 bp). The frequency of the class I SSRs in the BAC end-sequences was one per every 40 Kb, whereas in BAC and PAC sequences, the frequency was much higher (one SSR in every 16 kb). Class II SSRs were estimated to occur every 3.7 kb in BAC ends and every 1.9 kb in fully sequenced BAC and PAC clones. Microsatellites with poly(AT)_n repeats represented the most abundant and polymorphic class of SSRs but were frequently associated with the Micropon family of miniature inverted-repeat transposable elements (MITEs) and were difficult to amplify. Temnykh et al. (2001) designed a new set of 200 Class I SSR markers and integrated into the microsatellite map of rice, providing immediate links between the genetic, physical, and sequence-based maps. With this, a total of 500 mapped SSR markers rigorously evaluated for amplification, map position, and allelic diversity became available for the rice researchers. Availability of genomic DNA sequences in the public domain eliminated the efforts that would have been otherwise required for genomic library construction, repeated screening of library, and sequencing of clones and thus accelerated SSR marker development.

Monsanto also analysed its draft sequence for SSR motifs and identified 6,650 di-, tri-, and tetranucleotide repeats of ≥ 24 bp flanked by 100 bp of unique sequences on

each side (<http://www.rice-research.org>). Temnykh et al. (2002) designed primers for 2,041 of these SSRs identified by Mansanto and another 199 SSRs (≥ 20 bp) identified in the IRGSP Phase II BAC/PAC sequences. Of these 2,240 new Class I SSR markers developed *in silico*, 1,825 could be mapped on the 12 rice chromosomes by e-PCR. Genetic mapping using 295 markers revealed that 95% of the e-PCR results were correct. Fifty-six SSR markers (2.8%) hit BAC/PAC clones on two or more different chromosomes and appeared to be multiple copy. Similar to earlier observations based on library screening, the highest proportion of these new markers corresponded to poly(GA) motifs (36%), followed by poly(AT) (15%) and poly(CCG) (8%) motifs. All di-, tri- and tetra-nucleotide motifs were represented among these markers, with the exception of three GC-rich classes, poly(GC), poly(GGCC) or poly(GGGC) motifs. AT-rich SSRs had the longest average repeat tracts, while GC-rich motifs were the shortest. In combination with the pool of 500 previously mapped SSR markers, a total of 2,740 SSR markers were made available for rice, which accounted for approximately one SSR every 157 kb of the rice genome.

Based on the draft sequence of Syngenta, Goff et al. (2002) identified a total of 48,351 SSRs with minimum length of 15 nucleotides at an frequency of one SSR every 8kb. More than 7,000 SSRs were found in the predicted genes, most of these (92%) being trinucleotides, so that length changes should not disturb the open reading frame. Rice genome could be however, saturated with long potentially hypervariable (≥ 20 bp) SSRs, by using the high quality genome sequences (IRGSP 2005). A total of 18,828 Class I di, tri and tetra-nucleotide SSRs, representing 47 distinctive motif families, were identified and annotated in the rice genome. Their physical positions could be defined in relation to widely used RFLP markers (Causse et al. 1998; Harushima et al. 1998) and previously published SSRs (McCouch et al. 2002). There was an average of one hypervariable SSRs in every 19.6 Kb sequence, with the highest density of markers occurring on chromosome 3 (one SSR/17.9 Kb) and the lowest occurring on chromosome 4 (one SSR/24.4 Kb). It is difficult to compare this observation with that of Goff et al. (2002) due to the difference in the quality of the genome sequence, its coverage and minimum length of the Class I markers in these two studies. Large number of SSR markers developed from the finished genome sequence of rice provides a unique opportunity to the rice researchers world over for fine mapping of genes, location of QTLs into short marker intervals and marker assisted selection.

4.2. SSR Markers from the EST/Unigene Sequences of Rice

Another source of SSR markers is the expressed sequence tag (EST) databases, which are continuously growing in size in most crop species (<http://www.ncbi.nlm.nih.gov/EST>). These EST databases can be mined for microsatellite motifs that would serve as locus-specific markers. Development of EST-based microsatellite markers thus would involve considerably lower cost and effort. Besides, such markers being derived from the conserved expressed

component of the genome are expected to show greater cross transferability between species and genera (Varshney et al. 2005). The unavailability of genomic and cDNA sequences, thus would not limit the development of robust microsatellite markers and various marker-based applications in related species and genera.

A major disadvantage of the EST-derived microsatellites is the sequence redundancy that yields multiple sets of markers at the same locus. However, more recently the random EST sequences are being assembled into unique gene sequences called unigenes (<http://www.ncbi.nlm.nih.gov/unigenes>) that circumvents the problem of redundancy in EST databases. For example, 41,600 EST sequences in barley have been assembled into just 1,240 unigenes. The unigene-based microsatellite markers (UGMS markers) would therefore, have the advantages of unique identity and positions in the transcribed regions of the genome. With the availability of large unigene databases, it is now possible to systematically search for microsatellites in the unigenes. The UGMS markers can be used for accurately assaying functional diversity in the natural populations and the available germplasm collections as well as for comparative mapping and evolutionary studies as anchor markers. Recently, Parida et al. (2006) mined SSRs in 48.8 Mb unigene sequences of rice and carried out a detailed analysis of their nature, frequency and genomic distribution. About 40% of the unigenes contained SSRs (excluding monomeric repeats) at a frequency of one SSR in every 3.6Kb of sequence. The proportion of the trinucleotide repeats was highest (80%) among the repeat motifs present in the unigenes, of which 85% were found within the ORFs, 12% in 5' UTRs and 3% in 3'UTRs. Among the dinucleotide repeats, GA was most common (21.2%) and CG was least (3%) frequent. Interestingly, (GA)_n polynucleotides usually occurred in regions with balanced (40–50%) GC content, which favours robust PCR amplification (Temnykh et al. 2001) and therefore could be used for efficient genotyping applications. A total of 13,230 SSRs longer than 12 nucleotides, which included 2,780 Class I types, were identified and primers were designed for their amplification. The Class I SSR markers were physically mapped on the rice genome. The overall map density of these markers was one in every 141 kb and thus considered as a high-density physical map. The size of physical interval however varied from 174 bp to 3.26 Mb with the chromosome 3 having maximum UGMS markers (364) and thus the highest average map density (99 kb) followed by chromosome 1 (355 markers, 121 kb). The least number of markers (128) was placed on chromosome 11 giving the lowest map density (221 kb). Cross transferability of a sizable number of rice class I markers to other cereals namely wheat, barley, maize and sorghum suggested utility of these markers in comparative mapping of cereal genomes and genes.

5. DEVELOPMENT AND USE OF SNP MARKERS

Single nucleotide polymorphisms (SNPs) are the most abundant form of molecular markers present in any genome and rice is no exception. They arise due to spontaneous mutations in the DNA leading to base transitions (purine to purine

and pyrimidine to pyrimidine substitutions) or transversions (purine to pyrimidine and pyrimidine to purine substitutions). Small insertions and deletions (InDels) of one or two bases are also quite common and often are clubbed together with SNPs. While class I SSR loci are present on an average at every 19.6 kbp in the rice genome (IRGSP 2005), the SNPs are present at every 100 bp or less (<http://www.ncbi.nlm.nih.gov/projects/SNP/>), and therefore are most useful for fine mapping of genes and QTLs for the agronomic traits in order to develop tightly linked DNA markers for molecular breeding applications. Due to very low frequency of the spontaneous mutations and much greater possibility of base transitions as compared to transversions, SNPs are often bi-allelic in nature. Though, theoretically it is possible to have up to four alleles at any base position and we do see tri- and tetra-allelic SNPs but rarely. In contrast, multiple alleles are common at the individual SSR loci due to differences in the number of repeat units generated by replication slippage and unequal crossing over. A group of SNPs present in a single gene or small stretch of DNA, called haplotype, may have 2^n number of possible combinations, giving them a high discriminating power and multiple alleles at the gene level. Similar to the SSRs, large number of SNPs occur in the intergenic spaces, but these are not likely to have any direct effect on the traits of agronomic importance. The genic SNPs on the other hand may cause single amino acid substitutions, premature termination of the amino acid reading frame or a frame shift in case of InDels, thus directly affecting the function of the genes, some times leading to drastic phenotypic consequences at the trait level. Many genic SNPs are present at the redundant base positions in the amino acid codons, and therefore have no functional significance, but these can still be used as gene based perfect markers for molecular breeding.

Decoding of the genomes of *japonica* rice variety 'Nipponbare' and *indica* rice variety '93-11' and availability of BAC-end sequence data from *indica* rice variety 'Kasalath' has led to the identification of more than 3.9 million rice SNPs and this information is now available in the public domain at the NCBI website (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). Furthermore, the rice genomics revolution has led to accumulation in the public databases of more than 1.1 million expressed sequence tags (ESTs) from different varieties of rice, a number that is second only to human ESTs, and these provide another important resource for discovering SNPs in the expressed genic sequences of rice. Feltus et al. (2004) aligned draft sequences of rice varieties 'Nipponbare' and '93-11', and after filtering multiple copy and low quality sequences, they identified 408,898 SNPs/InDels. These include only a subset of the available SNPs, in particular excluding large number of SNPs that might occur in repetitive DNA, but with higher likelihood that this subset is useful. Direct sequencing of a random sample of these SNP sites suggested that $79.8\% \pm 7.5\%$ of the *in silico* discovered SNPs were real. These SNPs were present in all the twelve rice chromosomes but were not randomly distributed across the genome as 566 rice genomic regions had unusually high (48.6 Mb, 13.6% of the genome) or low (64.7 Mb, 18.1% of genome) polymorphism rates (<http://www.plantgenome.uga.edu/snp>). The international rice genome sequencing

consortium identified 80,127 polymorphic SNP/InDel sites that distinguish between the *japonica* rice variety 'Nipponbare' and *indica* variety 'Kasalath' for which 15.6 Mb of high quality BAC-end sequence data was generated (IRGSP 2005).

The most challenging task ahead is validation of these large numbers of SNPs through wet lab experiments, and establishing their association with the traits of agronomic importance so that they can be used in practical rice breeding. Most of the SNPs in the public domain databases were identified by *in silico* analysis of the available sequences and have rarely been validated in wet lab conditions. Now we have many novel high throughput techniques for SNP genotyping, and these are being employed increasingly for SNP validation. Restriction fragment length polymorphisms (RFLPs), the earliest type of molecular markers, in fact are due to SNPs leading to loss or gain of restriction sites. The RFLPs represent only a fraction of the total SNP content in a genome and the technique is quite tedious for regular use in rice breeding. Now it has been replaced by analysis using high throughput capillary electrophoresis of PCR amplified DNA fragments, resolution of small size differences between the allelic DNA bases using MALDI-TOF or hybridization of labeled DNA samples to large number of oligonucleotide probes immobilized on glass slides (micro arrays) and nano-size beads (bead arrays). The discovery and development of SNP markers requires techniques like DHPLC, TILLING and most importantly re-sequencing of the allelic DNA fragments, but after validation the subsequent analysis of the SNP loci in different rice varieties and segregating populations can be done using a number of techniques. The most common among these are CAPS, SSCP, SNUPe, DHPLC and MALDI-TOF mass array. Most recently hybridization of sample DNAs to synthetic oligonucleotide probes immobilized on micro or nano size beads is offering unprecedented throughput. These techniques will allow screening of large number of segregating genotypes simultaneously for the presence of favorable alleles of a number of genes in early generation of the breeding program, hence compressing the breeding cycle as well as breeding population for actual replicated field trails.

The present limitations in the molecular breeding of rice are: (i) non-availability of tightly linked or actual gene-based markers for the traits and (ii) high cost of analysis per sample. The first limitation is getting removed rapidly as several genes of agronomic importance are being discovered with the help of rice genome sequence information. The cost of analysis per sample is also coming down with the introduction of marker multiplexing and wider use of the high throughput genotyping techniques. Although the initial cost of the equipments are quite high, establishment of genotyping service facilities will easily take care of this. High throughput SNP mapping projects have already been initiated at IRRI through international collaboration in partnership with private service provider companies like Illumina (http://www.illumina.com/products/prod_snp.ilmn) and Perlegen Sciences Inc. (www.perlegen.com). In due course of time one can see large number of validated SNPs and haplotypes of rice in a way similar to the human SNP database developed by the International HapMap consortium (2006).

6. DISCOVERY OF CANDIDATE GENES AND GENE BASED MARKERS

The most significant impact of the availability of high quality rice genome sequence information has been on rapid discovery of genes of agronomic importance and elucidation of the underlying allelic differences at the level of gene sequence that are directly responsible for the differences in the traits at the morphological level. This is helping development of gene-based perfect markers for molecular breeding applications. These markers will not recombine with the trait because marker itself is responsible for the differences in the trait. A partial list of such genes identified in rice using map-based cloning approach is given in Table 1. This list is likely to grow very fast because intensive research is going on around the world, both in public and private sectors, to identify many more such genes for yield components, quality traits and stress tolerance (both biotic and abiotic) in rice. One difficulty is that, apart from disease resistance genes, most of the important agronomic traits e.g. yield, quality and abiotic stress tolerance, show quantitative inheritance due to involvement of many genes with small individual effects and role of gene into gene (epistasis) and gene into environment interactions. We do not yet have many validated markers for these traits that can explain major part of the phenotypic variation in the segregating populations.

Two different approaches have been used for the identification of candidate genes for these complex quantitative traits. First approach uses forward genetics where two parents with contrasting phenotypes, e.g. salt tolerant and salt susceptible rice varieties, are first crossed to develop a mapping population of about 200 F₂, RILs (recombinant inbred lines) or BILs (backcross inbred lines). After identification of the QTL intervals for the salt tolerance using this population, a much larger F₂ population of typically more than 1000 individuals developed from intercrossing of

Table 1. Genes for important agronomic traits cloned recently using the rice genome sequence information

S. no.	Trait	Gene	Reference
1.	Bacterial leaf blight resistance	<i>Xa 21</i> (NBS-LRR type receptor kinase)	Song <i>et al.</i> (1995)
2.	Plant height	<i>Sd 1</i> (gibberellin-20-oxidase)	Sasaki <i>et al.</i> (2002)
3.	Amylose content	<i>Sbe 3</i> (starch branching enzymes)	Liu <i>et al.</i> (2004)
4.	Grain number	<i>OsCKX2</i> (cytokinin oxydase)	Ashikari <i>et al.</i> (2005)
5.	Salt tolerance	<i>SKC1</i> (a HKT type transporter)	Ren <i>et al.</i> (2005)
6.	Grain aroma	<i>BAD2</i> (betaine aldehyde dehydrogenase 2)	Bradburry <i>et al.</i> (2005)
7.	Blast resistance	<i>Pik^h</i> (NBS-LRR type protein)	Sharma <i>et al.</i> (2005)
8.	Submergence tolerance	<i>Sub1</i> (Ethylene response factor-like protein)	Xu <i>et al.</i> (2006)
9.	Lodging tolerance	<i>Lsi 1</i> (Silicon transporter)	Ma <i>et al.</i> (2006)
10.	Seed shattering	<i>qSH 1</i> (BEL1-type homeobox)	Konishi <i>et al.</i> (2006)

QTL-NILs (differing for a single QTL only), is then used for fine mapping of the individual QTL interval to identify and validate the function of the candidate gene (Ren et al. 2005). Availability of rice genome sequence with complete information on the genes present in specific physical/genetic interval is of great help in this process and many key genes have been cloned during the last two years as compared to only limited examples of map-based cloning before the genomic era (Table 1). The second genomic approach is based on the gene expression profiling of the rice varieties and land races that are known to possess the genes of agronomic importance e.g. 'Pokkali' for salt tolerance (Kawasaki et al. 2001; Sahi et al. 2006) and 'N22' for drought tolerance (Gorantla et al. 2005). Analysing differential expression of genes with and without stress has led to identification of several hundred probable candidate genes that could be responsible for the phenotypic difference at the trait level. Unfortunately, it has not been possible to narrow down the number of candidate genes to a small number due to common stress response pathways and cross talks between alternate response pathways. A combination of the genetic fine mapping and differential expression profiling is likely to speed up gene discovery for complex agronomic traits in rice by drastically bringing down the number of candidate genes from several hundreds to tens, as described for the identification of candidate genes for ovariole number in case of fruit fly (Wayne and McIntyre 2002).

7. PROSPECTS OF HIGH THROUGHPUT MARKER-ASSISTED SELECTION (MAS) IN RICE

Molecular markers are used as gene tags to select for desirable genotypes. It offers several advantages over conventional phenotype based selection: i) Selection can be practiced at any stage of plant growth, ii) Creation of selection environment is not required, iii) Several useful genes can be combined in one genotype irrespective of nature of interaction among them, iv) Heterozygous carriers of useful recessive alleles can be identified easily, and v) by judicious combination of background and foreground selection, it is possible to save time and effort. Marker assisted selection in rice is being practiced for a few traits including resistance against bacterial leaf blight and blast diseases (Sanchez et al. 2000; Singh et al. 2001; Joseph et al. 2004; Liu et al. 2003; Toojinda et al. 2005). Molecular mapping of many other useful genes although has been reported, the identified markers are yet to be used in MAS. Many of the linked markers were RFLPs, which are not amenable to automation and thus a large number of samples cannot be handled in a short time. Moreover, absence of tightly linked markers that are polymorphic enough to be used in several different segregating populations has limited the use of markers in rice breeding. The availability of a large number of sequence based SSR and SNP markers that saturate the rice genome would be of considerable value in fine mapping of target genes. Use of these markers is expected to eliminate the problems due to recombination and lack of polymorphism. Besides, genotyping of a large population using a set of markers can be achieved in a short time since both the

marker classes allow complete automation. For instance, one of the primers in case of SSR markers can be labeled using fluorescent dye and the amplified products can be sized quickly using an automated fragment analyzer. Multiplexing of a set of primer pairs in PCR would significantly increase the speed of analysis (Coburn et al. 2002). Similarly, SNP analysis is completely automated and thus would allow very high throughput genotyping. Since there is no involvement of cost in marker development, routine use of these markers in rice breeding is feasible provided some initial investment is made on laboratory infrastructure. However, MAS is not a complete replacement for evaluation of phenotype in the field. It is recommended that MAS for difficult-to-phenotype traits needs to be combined with phenotypic selection for easy-to-phenotype traits for deriving maximum benefit from the vast genomic resources including molecular markers available in rice. Integration of molecular markers in rice breeding is a necessity for imparting far greater efficiency to the development of superior genotypes.

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CHAPTER 9

MARKER-ASSISTED SELECTION IN SORGHUM

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Abstract: Sorghum [*Sorghum bicolor* (L.) Moench] is an important food and feed crop in many parts of the world, and has potential uses in the biofuels industry. Compared to most other cereals, sorghum is more tolerant to many abiotic stresses, including heat, drought, and flooding, making it an ideal crop for growing on marginal lands as demands for food, feed, and energy increase. Though it is generally stress-tolerant, the true potential of sorghum can only be realized through concerted genetic improvement programs. The use of DNA-based markers for the genetic analysis and manipulation of important agronomic and stress-tolerance traits is becoming an increasingly useful tool in sorghum improvement. The known germplasm of sorghum is incredibly diverse, and molecular markers are being used to assess this diversity to help manage large germplasm collections, and to make these collections more useful to breeders. Molecular markers have been used in sorghum to identify quantitative trait loci (QTL) for many complex traits, including pre-flowering and post-flowering drought tolerance, early-season cold tolerance, and resistance to the parasitic weed *Striga*. However, progress in utilizing these QTL had been limited by the lack of a standard genetic map and a common nomenclature for the various linkage groups of sorghum. Fortunately, the genetic map of sorghum has recently become standardized, and has also been linked to physical chromosomes. The use of a common map will facilitate the exchange of marker and QTL information between sorghum research groups. This will allow independent validation of QTL and should expedite efforts to use these QTL for the development of improved sorghum cultivars through marker-assisted selection and trait introgression. Newer, faster marker technologies based on single nucleotide polymorphisms (SNPs), and mapping methods based on linkage disequilibrium (association mapping), will soon become useful tools for future efforts to improve this important crop.

1. INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is an important crop in many parts of the world and is grown for food, feed, and industrial purposes. Sorghum ranks fourth among grain crops in the United States, behind maize (*Zea mays* L.), soybean

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[*Glycine max* (L.) Merr.], and wheat (*Triticum aestivum* L.) in total production (FAO 2004). It is also a major crop in many parts of Africa and some Asian countries. Compared to most other cereals, sorghum is more tolerant to many abiotic stresses, including heat, drought, and flooding. Combined with its potential use in the emerging biofuels industry, sorghum is an ideal candidate for a more concerted crop improvement program as agriculture is pushed to more marginal lands, and food and energy demands increase. The use of DNA-based markers for the genetic analysis and manipulation of important agronomic traits is becoming an increasingly useful tool in sorghum improvement.

DNA markers have the potential to enhance the operation of a plant breeding program through a number of ways. Genetic fingerprints can be used for the identification of genetic stocks and cultivars, and for the verification of pedigree information (Soller and Beckmann 1983). Comparison of the unique genetic signatures of germplasm accessions has allowed breeders to assess the amount of diversity in their breeding material and to determine relatedness between individuals (Deu et al. 1994; de Oliveira et al. 1996; Yang et al. 1996; Menkir et al. 1997; Abu Assar et al. 2005). As genotyping technologies have improved, very dense genetic maps of sorghum have been created (Menz et al. 2002; Bowers et al. 2003). The ability to associate quantitative phenotypic data with genetic maps has helped to increase our understanding of the inheritance of complex agronomic traits in sorghum (Pereira and Lee 1995; Tuinstra et al. 1996; 1997), which is beginning to lead to marker-assisted plant breeding. However, the application of this technology is still relatively new, and it may take some time before marker-assisted selection (MAS) becomes a routine operation in most sorghum breeding programs. The sorghum genome is expected to be completely sequenced in the very near future and opportunities for integrating genetic maps with the physical map of the crop should increase the effectiveness of knowledge-based breeding of some of the more difficult traits in sorghum. This review summarizes the knowledge base generated and experience gained in the identification and analysis of quantitative trait loci (QTL) and MAS in sorghum both in our own research program as well as in the greater sorghum research community.

2. GENETIC DIVERSITY ASSESSMENT IN SORGHUM GERMPLASM

Analyses of the extent and distribution of genetic variation in a crop are essential for understanding the evolutionary relationships between germplasm accessions and for sampling genetic resources more systematically for both breeding and conservation purposes. Traditionally, genetic resources in sorghum are classified by taxonomists based on a set of morphological characteristics. Cultivated sorghum (*S. bicolor* subsp. *bicolor*) is divided into five major races, namely *bicolor*, *guinea*, *caudatum*, *kafir*, and *durra*, and the ten possible hybrid groups among these races (Harlan and de Wet 1972; Harlan and Stemler 1976; de Wet 1978). There are several 'wild' subspecies within *S. bicolor*, and races within these subspecies as well, many of

which harbor potentially valuable traits. However, the morphological characteristics used in the classification of sorghum into different races are conditioned by relatively few genes. On the other hand, important traits which are related to habitat adaptation, and which exhibit enormous variability among sorghum germplasm, are complex and quantitatively inherited. Also, closely related accessions could potentially look very different in the field, and some accessions with similar appearance may actually be of very different origins. Hence, classifying germplasm accessions based solely on a few discrete morphological characters may not provide an accurate indication of the genetic diversity present within the sorghum germplasm.

Several studies have been conducted to characterize the genetic diversity among the various races of sorghum (Deu et al. 1994; de Oliveira et al. 1996), and within smaller collections from specific regions (Yang et al. 1996). Because both natural and human selection efforts should have contributed to the current genetic differences in sorghum, we hypothesized that geographic distribution would have a greater effect on genetic diversity than racial classification. To test this hypothesis, we used molecular markers to analyze genetic diversity among the five major races of cultivated sorghum (Menkir et al. 1997). One-hundred ninety accessions representing each race and each of the major geographical centers of distribution were randomly selected from the world collection of sorghum maintained by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). The 190 accessions were divided into 38 sets, in each of which five randomly-selected accessions from all five races were included to ensure parallel comparison of races and to minimize biases while scoring marker products. A total of 82 random amplified polymorphic DNA (RAPD) primers were used for DNA amplification. Only 53 primers produced clearly scorable markers, generating a total of 220 loci. A high level of genetic variation was detected among the sorghum accessions. The results of our study indicated that within-race genetic diversity was high for race *bicolor*, and *guinea*, and low for race *kafir*. Partitioning the genetic variation further revealed that 86% of the total genetic variation occurred among individual accessions and only 14% among races. Examination of the degree of association of accessions with their geographic areas of origin indicated that only 13% of the total genetic variation was attributable to divergence among regions. In spite of the limited differentiation among regions, the extent of genetic diversity within and among regions showed some trends. Though represented by a large number of accessions, Southern African germplasm exhibited the least amount of genetic diversity suggesting a narrow genetic base of accessions from this region. By contrast, sorghums from West Africa exhibited a high level of genetic diversity with a least number of accessions. Genetic diversity in Central and Eastern Africa, as well as the Middle East, was as high as that observed in accessions from West Africa.

Simple sequence repeat (SSR) markers are becoming more popular for diversity analysis studies in sorghum because of their repeatability, simplicity, and ability to be semi-automated. Also, their multiallelic nature makes these markers more informative than most other marker types. Abu Assar et al. (2005) used only 16 SSR

markers to assess the diversity present within a collection of 96 sorghum accessions representing very different geographies and races. Each accession showed a unique genetic signature, demonstrating the utility of SSRs for differentiating accessions. By applying the unweighted pair-grouped method with arithmetic average (UPGMA) clustering to the genotypic data, the accessions tended to cluster based on geographic origin, and within these clusters accessions of similar morphological classification tended to also congregate together.

Germplasm collections at most programs often become very large through time, containing too many accessions for a single breeder to assess in a practical manner. Thus, a system for assembling a smaller number of specific accessions more methodically to establish a so-called 'core collection' has been proposed. The ideal core collection should encompass all of the genetic diversity contained within the larger collection, represented by fewer individual accessions. Such a core collection would serve as a starting point for breeders interested in finding new sources for specific traits. Core collections can be easily evaluated by a breeder for specific characteristics, and if desirable individuals are found they can be utilized in a breeding program, and related accessions from the larger collection can be evaluated if the breeder so desires. However, developing such an informative panel for a core collection can be a challenge. In general, core collections need to be small enough to be of practical use, while containing a significant portion of the genetic diversity of the species represented by the whole collection.

Grenier et al. (2000a) were among the first to describe phenotype-based methods for assembling a core collection from landrace sorghum collections. They described three different methods of subsampling. The first sampling scheme involved stratification of the sorghum collection held at ICRISAT based on photoperiod sensitivity, followed by random sampling. The second scheme was based on quantitative characteristics, and the third was based on geographical origin and other specific traits. All accessions in each subset were genotyped for 15 SSR markers, using semi-automated allele sizing (Grenier et al. 2000b). Similar measures of genetic diversity were obtained for all three sampling strategies. The mean number of alleles per locus was similar for all three sampling methods, as were both the mean and range of diversity indices for all loci. One locus in particular had relatively low diversity, but the level was comparable in all three samples. A similar number of rare alleles were also retained by each sampling strategy, though a few rare alleles were detected in only one sample. Nonetheless, it was concluded that all three sampling methods retained a similar level of genetic diversity, representative of the global set (all three samples combined).

In addition to maximizing genetic diversity in a breeding program or core collection, elimination of redundant accessions is also important for reducing the time and costs required to maintain large collections. Dean et al. (1999) used the same 15 SSR markers to examine the genetic diversity within 19 accessions listed as 'Orange' in the US national sorghum collection. Their analysis revealed two different groups of identical entries, which could potentially be pooled to eliminate redundancy. They also found many nearly identical entries, and concluded that

some of these could also be pooled. The total number of 'Orange' accessions could be reduced to 10, with only a 10% loss of genetic diversity among the entries. In addition, because they genotyped five individual plants from each entry, they found that one accession was not homogeneous, and may have been contaminated with seeds from another source. Thus, the determination of genetic purity of accessions or breeding lines is another important use for molecular markers in sorghum. A large scale genetic diversity project involving molecular characterization of 3000 sorghum accessions at 50 SSR loci is underway at ICRISAT in collaboration with CIRAD and CAAS (R.K. Varshney, personal communication).

3. GENETIC MAPPING IN SORGHUM

The first group of genetic linkage maps of sorghum consisted primarily of RFLP markers derived from maize probes (Hulbert et al. 1990; Whitkus et al. 1992; Binelli et al. 1992; Melake-Berhan et al. 1993). Comparison of these maps with those of maize revealed a high degree of synteny between the two genomes. Whitkus et al. (1992) and Melake-Berhan et al. (1993) also noted that many of the probes which mapped to a single locus in sorghum were duplicated in maize, suggesting possible duplication events in the evolution of maize after its divergence from sorghum. These early maps, however, did not contain enough markers to resolve ten linkage groups, which is the haploid chromosome number for sorghum. Chittenden et al. (1994) published the first 'complete' linkage map of sorghum with ten linkage groups using mostly sorghum-derived RFLP probes, and some from maize. This map was based on an inter-specific cross (*S. bicolor* BTx623 × *S. propinquum*), mapped in the F₂ generation. That same year, Pereira et al. (1994) also published a linkage map of sorghum with ten groups. A 'composite' map using the genotypic data from two recombinant inbred (RI) populations was published by Dufour et al. (1997) with linkage group designations following those of Pereira et al. (1994). This map contained 199 markers on 13 linkage groups and was later supplemented in subsequent publications with the addition of more RFLP and AFLP markers (Vos et al. 1995), as well as with morphological markers, reducing the number of linkage groups to 11, with two very small unlinked clusters (Boivin et al. 1999). Xu et al. (1994) also published a map of sorghum using RFLP probes primarily derived from sorghum, and some from maize. This map contained 190 markers on 10 major linkage groups, and four smaller ones. This map was based on the genotypes of 50 F₂ plants from a cross between IS3620C and BTx623. Several later studies improved upon this map by addition of more loci. Using 137 RI lines from this same cross, Peng et al. (1999) generated a linkage map containing 323 mapped loci on 10 linkage groups. The total length of this map was 1,347 cM. Bhatramakki et al. (2000) reported the addition of 147 SSR loci to this map using the same RI population, changing the total map length to 1,406 cM. Though these maps were useful tools for mapping of quantitative trait loci (QTL), the lack of agreement between maps from various research groups, as well as relatively poor map quality, made comparison of results with other studies or research groups very difficult. Clearly, there arose a need among the sorghum research community for a consensus map.

More recently, two very dense genetic linkage maps of sorghum have emerged. Menz et al. (2002) added AFLP markers to the IS3620C \times BTx623 map of Bhattarakki et al. (2000) to create a very dense linkage map containing 2,926 loci on 10 linkage groups with a total genetic distance of 1,713 cM. Shortly thereafter, using the interspecific cross (*S. bicolor* BTx623 \times *S. propinquum*) of Chittenden et al. (1994), another dense linkage map was generated. This map contained 2,512 loci on 10 linkage groups, and is based entirely on RFLP probes (Bowers et al. 2003). Interestingly, the total genetic distance of this map was much shorter than the map by Menz et al. (2002), at only 1,059.2 cM. This discrepancy may be due to the fact that the interspecific population was mapped in the F_2 generation and the intraspecific population was mapped as RI. However, in a recent paper these two maps were aligned by identifying and mapping markers common to both populations (Feltus et al. 2006). A common nomenclature for the 10 chromosomes of sorghum has also been established, and the 10 linkage groups have been assigned to these chromosomes by the use of fluorescent in-situ hybridization (FISH) of sorghum genomic BAC clones containing mapped marker loci (Kim et al. 2002; 2005). The relationships among the various linkage groups reported in previous studies were also reported by Kim et al. (2005), so that these groups can also be assigned to chromosomes and compared to each other. A common nomenclature for sorghum chromosomes and linkage groups should facilitate the cross-validation of QTL studies by different research groups by making direct comparisons of mapped QTL possible.

4. QTL IDENTIFICATION IN SORGHUM

Molecular markers have been used to identify and characterize QTL associated with many different traits in sorghum, including plant height and maturity (Pereira and Lee 1995), traits associated with domestication (Paterson et al. 1995), disease resistance (Gowda et al. 1995), insect resistance (Nagaraj et al. 2005; Deu et al. 2005), and drought tolerance (Tuinstra et al. 1996; 1997). Identification of QTL often leads to further investigations to identify the underlying gene or genes through fine mapping and map-based cloning. When successfully implemented, such studies provide valuable insight into the genetic mechanisms controlling complex, and often economically important, traits. However, from a practical plant breeding standpoint, QTL are usually identified for the purpose of finding linked molecular markers that can be utilized in trait introgression for crop improvement, and often the specific underlying genes are not identified. For the purposes of this review, examples of QTL identification for tolerance to biotic and abiotic stresses important in sorghum are highlighted.

4.1. Identification of QTL for Striga Resistance

Several parasitic plant species of the genus *Striga* are major pests of sorghum in parts of Africa, often causing complete loss of the crop in severe infestations.

Because efforts to control the pest through chemical or cultural means have been met with limited success and are often not practical in poor areas, developing crops with genetic resistance is currently the best strategy for dealing with *Striga* infestation. However, field resistance to *Striga* is a complex quantitative trait that has been difficult to address via conventional plant breeding approaches. We have had a *Striga* resistance breeding program at Purdue University for several years and have employed a mix of biotechnological approaches to address this problem. We have broken *Striga* resistance down into its component parts using laboratory assays, and have identified several mechanisms by which sorghum can avoid or resist parasitism by *Striga*, each of which could be manipulated genetically in the development of resistant cultivars.

The most thoroughly studied mechanism is decreased production of germination stimulants by host roots. *Striga* seeds require a chemical signal from the roots of a potential host in order to germinate. Several compounds have been identified from the exudates of sorghum roots, and from roots of other host and non-host plant species, that have been shown to stimulate germination of *Striga* seeds in the laboratory (Cook et al. 1966; 1972; Hauck et al. 1992; Siame et al. 1993). Some sorghum varieties produce abnormally low amounts of these compounds, resulting in decreased *Striga* germination compared to normal varieties (Vogler et al. 1996). Once germinated, *Striga* also requires the presence of an additional factor to initiate formation of a haustorium, a rootlike structure that penetrates the host root. A sorghum mutant that exhibits decreased levels of haustorial initiation in *Striga* seedlings has also been reported (Mohamed 2002), but the essential chemical compound has not been identified. A third mechanism, described as an 'incompatible response' has also been reported, in which *Striga* seedlings attach to host roots, but fail to develop (Mohamed 2002). This mechanism is also not yet clearly understood. Recently Mohamed et al. (2003) described another *Striga* resistance mechanism, a hypersensitive response (HR) to haustorium invasion, in the roots of certain sorghum varieties, most notably the cultivars 'Framida' and 'Dobbs', and a wild accession (*Sorghum verticilliflorum* (Steud.) Stapf), designated P47121. Hypersensitive response is a common mechanism of resistance to microbial pathogens among diverse plant species, and is characterized by rapid necrosis of the cells surrounding the site of infection (Agrios 1988). In general, the hypersensitive response is also associated with the production of many different compounds, including reactive oxygen species, phytoalexins and other phenolic compounds, and pathogenesis-related proteins. Following the hypersensitive response in sorghum roots, most attached *Striga* seedlings fail to develop. It has been shown through classical Mendelian genetics that the hypersensitive response in sorghum roots is conferred by two dominant genes, designated *Hrs1* and *Hrs2* (Hypersensitive response to *Striga*) (Mohamed 2002). However, the functions of these genes are not known, and the underlying physiology of the hypersensitive response to *Striga* is currently under investigation.

Field-based selection for *Striga* resistance must be conducted in areas where *Striga* is endemic, because the pest must be kept under containment elsewhere. Also,

the inherent variability in field-based selection for Striga resistance may further hinder selection efforts. Though highly informative for characterizing specific Striga resistance mechanisms currently available, laboratory assays particularly for post-attachment mechanisms are not amenable to phenotyping a large number of RI lines or breeding material. Thus, the identification of molecular markers for specific Striga resistance mechanisms will facilitate faster introgression and pyramiding of these traits into sorghum breeding lines.

Using a recombinant inbred population, a genetic linkage map was constructed with over 230 DNA markers, an estimated map size of 1,628 cM, and an average interval of 9.5 cM between adjacent loci. The parents of this population were a Striga-resistant caudatum SRN39 and a highly susceptible Chinese kaoliang 'Shan Qui Red' (SQR). Based on laboratory assay data, the locus for the low germination stimulant (*lgs*) production (Vogler et al. 1996) was mapped at 11.8 cM from an RFLP marker *pio200725*-BamH1, and 13.5 cM from *ssr617g*, an inter-simple sequence repeat (ISSR) marker (Ibrahim 1999). We also used the linkage map to place putative QTL for Striga resistance using phenotypic data from field evaluation of this mapping population against *Striga hermonthica* and *Striga asiatica*. Single marker analyses detected six QTL for resistance to *S. hermonthica* and five QTL for resistance to *S. asiatica*. The QTL detected for resistance to *S. hermonthica* accounted for 37% of the variation in resistance, and QTL detected for resistance to *S. asiatica* accounted for 49% of the variation in resistance. Two of these QTL were on the same linkage group as the *lgs* locus. Interval mapping confirmed most of the QTL detected by single marker analysis (Ibrahim 1999). Based on a series of field evaluations of two independent RI populations, Haussmann et al. (2001) produced evidence that other minor loci play a quantitative role in the production of Striga germination stimulants in sorghum, and that different populations of Striga react differently to germination stimulant levels. Another study by this group placed the *lgs* locus on linkage group I (chromosome 6), and a significant QTL for field resistance to Striga also mapped to this region, helping to confirm the location of this QTL (Haussmann et al. 2004). However, the nearest mapped DNA marker in this population is approximately 30 cM away. Saturation of this region of the genome with more molecular markers is needed, so that closer markers can be identified for use in marker-assisted breeding. Several other QTL for field resistance to Striga were also identified in this and another population (Haussmann et al. 2004).

Dissecting Striga resistance into simpler components based on laboratory assays has enhanced efficiency of breeding crop cultivars with improved resistance to the parasite as well as in gene identification. We have mapped the low haustorium initiation factor (*lhf*) locus in an F_{2:3} population derived from a cross between SQR and a wild sorghum accession P78, in which the low haustorium initiation trait was identified. The *lhf* locus maps to linkage group F (chromosome 9), approximately 19.3 cM from SSR marker *txp359* (Mohamed 2002). This locus would be a good candidate for marker-assisted introgression into breeding lines or locally adapted cultivars if a more tightly linked marker were identified. Although Mendelian segregation ratios indicated two loci conferring HR to Striga, only one locus has

been mapped to date. A gene for HR, derived from the wild accession P47121, resides on linkage group B (chromosome 2), 7.5 cM from *txp96* (Mohamed 2002). Though a closer marker would be desirable for marker-assisted introgression, only 7.5 % recombination should be an acceptable margin for error in a Striga resistance breeding program given the high heritability of this trait.

4.2. Identification of QTL for Drought Tolerance

Tolerance to abiotic stress is generally complex and highly influenced by environmental factors. Because of this, selection for improved abiotic stress tolerance is often difficult, and gains from selection are usually quite low. Drought tolerance is one such trait, and is especially important in sorghum, given its exceptional drought tolerance relative to other crops, and its importance in semi-arid parts of the world. The genetic and physiological mechanisms that condition the expression of drought tolerance in sorghum and other crops are still poorly understood. Controlled by many genes and dependent on the timing and severity of moisture stress, drought tolerance has been difficult to study and characterize. Identification of QTL and associated molecular markers, based on carefully managed replicated tests, should facilitate improvement of drought tolerance in sorghum, and also help to increase our understanding of the underlying physiological and genetic mechanisms controlling this complex trait in sorghum, with possible application in other related crops.

A number of physiological and biochemical traits have been hypothesized to enhance drought tolerance. Yet, only a few of these mechanisms have been demonstrated to be causally related to the expression of tolerance to drought under field conditions. As with Striga resistance, we have made a slow but significant progress via empirical breeding of sorghum for drought tolerance by breaking this complex trait into specific phenological stages of plant development, namely early-season (seedling), midseason (pre-flowering), and late-season (post-flowering) drought responses. Using this approach, we have identified sorghum germplasm that are uniquely pre-flowering or post-flowering drought tolerant and a few that combine tolerance at both stages. We have developed new improved drought tolerant sorghum lines in diverse and elite germplasm backgrounds. Our breeding and selection efforts were based on reliable phenotypic markers associated with morphological and yield-related symptoms that occur at pre-flowering and post-flowering stages of plant development as long as moisture stress treatments are induced in a timely manner. Some of these marker traits are simply inherited, while others appear quantitative, rendering them amenable to QTL marker analysis and marker-assisted introgression. Molecular markers linked to QTL for drought tolerance could be used to increase the efficiency of breeding efforts to select sorghum germplasm with enhanced drought tolerance.

We conducted a number of studies using a RI population derived from a cross between Tx7078, which shows pre-flowering drought tolerance, and B35, which shows post-flowering tolerance. A genetic map was constructed using RAPD and RFLP markers, and the RI lines were carefully evaluated for response to drought

in a series of pre-flowering or post-flowering stress environments, both of which were in turn compared to fully irrigated controls. Pre-flowering drought tolerance was assessed by measuring grain yield under drought stress, yield stability (drought versus irrigated), seed set stability, and plant height stability. Single marker analysis identified six QTL associated specifically with pre-flowering drought tolerance. Despite the fact that drought stress was much more severe in the first growing season, QTL by environment effects were relatively small, suggesting that these QTL are stable across varying levels of drought stress or other confounding climatic factors. In addition, some markers showing significant association with yield or seed set under irrigated conditions also showed significance for drought tolerance (Tuinstra et al. 1996). Assessment of post-flowering drought tolerance in sorghum by measuring grain yield under drought stress, yield stability, seed weight stability, and stay-green score was established in a study conducted under carefully managed drought stress conditions (Wanous et al. 1991). The term 'stay-green' refers to delayed senescence which is associated with post-flowering drought tolerance irrespective of the maturity of the genotype. Twelve QTL for post-flowering drought tolerance were identified by single marker analysis (Tuinstra et al. 1997b). Six QTL for stay-green were identified on five linkage groups. QTL on three of these linkage groups were also positively associated with grain yield under irrigated conditions. This indicates that there may be a physiological link between the expression of stay-green under post-flowering drought and grain yield under non-drought conditions.

Because of the importance given to the stay-green trait as a readily visible and heritable drought response trait, several additional studies have been conducted to characterize and map this trait in various populations. Three of these studies (Crasta et al. 1999; Xu et al. 2000; Subudhi et al. 2000) also used B35 as the source of the stay-green trait. In a recent review comparing these studies, Sanchez et al. (2002) named four major stay-green QTL derived from B35 that were consistently identified: *Stg1* and *Stg2* (chromosome 3), *Stg3* (chromosome 2) and *Stg4* (chromosome 5). Two of these, *Stg1* and *Stg3*, correspond to two QTL identified by Tuinstra et al. (1997b). A QTL for stay-green was also identified by Tao et al. (2000) at the same location as *Stg3* in a RI population derived from a cross between QL41 and QL39. QL41 is a stay-green line derived from B35 so this discovery is not surprising, however the other three QTL were not identified in this study, but several additional stay-green loci were found, some of which were associated with QL39. Using the stay-green line SC56, which is unrelated to B35, Kebede et al. (2001) found stay-green QTL at the same locations as *Stg1* and *Stg4*. They also detected a stay-green QTL on chromosome 1, which appears to correspond to a QTL found by Tuinstra et al. (1997b) and by Crasta et al. (1999). These results indicate possible commonalities between stay-green mechanisms from diverse sources. More recently Haussmann et al. (2002) analyzed the stay-green phenotype at various phenological stages by measuring the percentage of leaf area remaining green at weekly intervals (extrapolated to 15 d, 30 d, and 45 d) after flowering during drought conditions in two RI populations. The stay-green source line E36-1, which is unrelated to B35

and SC56, was common to both populations. Five to nine QTL were identified for each of the three time points in each population, and several of these overlapped, suggesting that some aspects of stay-green may be regulated in a stage-specific manner, while others are not. Three QTL derived from E36-1 were common to both populations. One QTL, which was previously identified in other studies with B35 (Tuinstra et al. 1997b; Crasta et al. 1999; Subudhi et al. 2000) was detected on chromosome 2, though it is different from *Stg3*. Most other stay-green QTL found by Haussmann et al. (2002) appear to be unique to E36-1. From the results of multiple studies it is clear that there are several sources of this trait, and likely several mechanisms by which sorghum can remain green and photosynthetically active during post-flowering drought stress. Some of these mechanisms may be common among the germplasm while others seem to be unique to a particular source. Thus, there appears to be great potential for the use of marker-assisted selection to pyramid multiple stay-green genes from these various sources into drought-tolerant breeding lines and cultivars.

4.3. Identification of QTL for Early-Season Cold Tolerance

Cool temperatures during the early growing season are a major limitation to growing sorghum in certain regions of the United States and other temperate areas. Tolerance to early-season cold temperatures is a major trait that is needed in many of the sorghum production areas of the United States. The trait is essential for early-season stand establishment to take advantage of the extended growing season that would ensue if the crop is planted and established early. Increased grain yields have in fact been correlated with improved seedling vigor in sorghum (Chhina and Phul 1987; Cisse 1995). Improved tolerance to early-season cold temperatures would also help to protect the crop from stand reductions caused by unexpected cold periods in late spring. We have found that several sorghum landraces from China exhibit higher emergence and greater seedling vigor under cool conditions than typical American varieties (Cisse and Ejeta 2003; Singh 1985). Unfortunately, Chinese landrace sorghums also possess other undesirable traits, such as susceptibility to most leaf diseases. Directed introgression of seedling cold-tolerance genes from these landraces into elite, high-yielding lines, without the undesirable traits from the donor parents, could be expedited by marker-assisted selection.

To identify markers for seedling cold tolerance, we conducted QTL analyses using a population of RI lines derived from a cross between 'Shan Qui Red' (SQR), a cold-tolerant Chinese line, and SRN39, a cold-sensitive caudatum. The RI lines were evaluated for percent germination under cold and optimal conditions in growth chambers, and for percent emergence and seedling vigor scores (Maiti et al. 1981) in the field under early and later plantings. Analysis of the phenotypic data showed a high degree of heritability for all traits measured (Gunaratna 2002), suggesting that gains from selection for seedling cold tolerance should be significant. However, the correlation between percent germination under controlled conditions and percent field emergence was weak, suggesting that seed germination and emergence are

genetically distinct processes. Composite interval mapping (Zeng 1993; Zeng 1994) identified one QTL associated with germination under optimal conditions on linkage group B (chromosome 2) near marker *txp348*. Several QTL associated with percent emergence and seedling vigor in the field after both early and more optimal plantings were also identified. A region of linkage group A (chromosome 1) near *txp43* was significantly associated with percent emergence and seedling vigor after both early and later plantings, suggesting that the same genetic mechanism may play a critical role in both emergence and subsequent early seedling growth under various conditions. Another QTL for percent seedling emergence after early and later plantings was located on group B near *txp211*, indicating that seedling emergence at different temperatures may be controlled by the same genetic mechanism. However, one QTL for seedling vigor was identified on group D (chromosome 4) near *txp51*, which was specific for early planting only, suggesting a temperature-specific gene for vigor. Interestingly, the QTL for emergence and vigor in the field did not overlap with the QTL for germination, supporting our conclusion that germination and emergence are under the control of different genes (Knoll et al. 2007).

5. MARKER-ASSISTED SELECTION AND INTROGRESSION

When genetic markers are found to be associated with a trait of interest, those markers can be exploited to expedite the development of new cultivars through marker-assisted selection (MAS), or to efficiently improve existing cultivars through marker-assisted introgression of traits. The effectiveness of MAS was first demonstrated by Tanksley and Hewitt (1988) in selection for soluble solids and pH in tomato fruit. More recently, Zhou et al. (2003) used SSR markers to select for a major QTL for scab resistance in two different populations of wheat, and achieved a significant reduction in the percentage of infected spikelets in families carrying the QTL. Yousef and Juvik (2002) backcrossed three QTL markers for seedling emergence into three elite sweet corn inbreds, and then tested the effects of the markers in the resulting BC₂F₁ progeny. They found favorable increases in emergence for two of the markers in all three genetic backgrounds, and the third marker showed favorable increases in two of the populations, thus validating the QTL and the utility of MAS for emergence.

Though marker-assisted selection has been shown to be an effective tool, it is not meant to be a substitute for conventional phenotype-based selection, but rather a means to enhance the efficiency of breeding. Molecular markers can help to increase the efficiency of selection for traits that are otherwise difficult to assess, or in traits such as disease resistance when a uniform and optimal level of inoculation is required, or when the trait of interest is recessive, or is expressed late in the life cycle of the plant (Arús and Moreno-González 1993). Molecular markers also allow for environmentally neutral selection, which means that selection can proceed at off-season sites, and in years with unusual weather. However, the greatest potential of marker-assisted selection appears to be in accelerating the rate of gain from selection for desirable genotypes and in the manipulation of QTL that condition

complex economic traits. Effective use of marker-based selection or marker-assisted introgression should significantly decrease the amount of time required by plant breeders to develop new cultivars. For MAS to be effective, the marker and trait should be as tightly linked as possible to minimize recombinations between the marker and the gene of interest. If a tightly linked marker cannot be found, an alternative approach is to select two flanking markers instead. Selection based on molecular markers is particularly useful in the introgression of specific traits into existing cultivars through repeated backcrossing. In addition to selecting for the markers of interest from the donor parent, a breeder can also select for recovery of recurrent parent alleles elsewhere in the genome to hasten recovery of the recurrent genome (Arús and Moreno-González 1993), especially if there are known markers for specific traits in the recurrent parent. However, phenotypic selection is also essential to recovery of the desired characteristics of the recurrent parent, and should not be overlooked.

5.1. Validation of Putative QTL for Marker-Assisted Selection

Identification of QTL associated with important agronomic traits is essential. However, it is only valuable if confirmation is made of the robustness of the markers through repeated evaluation across populations and environments. We recently completed such a validation experiment for an important agronomic trait in sorghum. To test the utility of linked markers for identifying important QTL for early-season cold tolerance in sorghum in varying genetic backgrounds and environments, we chose three closely linked SSR markers (*txp43*, *txp51*, and *txp211*), representing the three most significant QTL for seedling emergence and/or vigor using two segregating populations. Because 'Shan Qui Red' (SQR) was the original source of the cold-tolerance genes, it was used as a parent in both of these populations: Tx2794 × SQR F₂ and Wheatland *ms*₃ × SQR BC₁F₂. Individual F₃ or BC₁F₃ families were genotyped for the three markers, and five representatives of each genotypic class (27 classes total) were randomly chosen for field testing. The field trials were planted about one month earlier than normal planting time for sorghum at West Lafayette, IN in 2004 and 2005. Percent emergence, seedling vigor scores, and stand biomass were measured. Statistical analyses indicated that the SQR allele of *txp43* had favorable effects on seedling vigor in both populations, and on emergence in the Tx2794 population. A large positive effect of the SQR allele of *txp51* was observed in the Tx2794 population for both vigor and emergence. Slight genotype by environment interaction was observed for *txp51* in the Wheatland population. Marker *txp211* had small but significant effects on seedling vigor and emergence in both populations. Various other interactions between loci were also significant, most notably the interaction of *txp43* and *txp51*, which strongly favored the SQR genotypes at both loci for multiple traits. This study (Knoll and Ejeta 2007) validated QTL markers in different genetic backgrounds, and demonstrated the utility of MAS for a quantitative trait, cold tolerance, evaluated in the field. The results also indicate that these markers should be useful tools for future selection

efforts. In fact, *txp43* and *txp51* from SQR are currently being introgressed into various lines in our breeding program in an effort to develop sorghum hybrids with improved early-season performance.

5.2. Development and Analysis of Near-Isogenic Lines Differing for QTL Markers

Although QTL analysis in our drought tolerance studies identified regions of the sorghum genome that condition the expression of drought tolerance, it provided limited information concerning the expression of individual QTL. Analysis of near-isogenic lines (NILs) that differ at QTL can be an effective approach for the detailed mapping and characterization of individual loci. The usual procedure for developing NILs requires multiple generations of tedious backcrossing and phenotypic or marker-assisted selection at each generation to select individuals carrying the locus or loci of interest. Therefore, the use of NILs in analysis of important agronomic traits has been limited because of the time and effort required to develop them. We developed a simpler procedure for developing NILs for any region of the genome that can be analyzed with molecular markers (Tuinstra et al. 1997a). Using the single-seed descent method of plant breeding, a collection of 98 F_2 individuals were advanced to the F_5 generation. At this point, the expected heterozygosity is 6.25%, meaning most loci in the genome are fixed. At this point, eight to ten individual plants were selfed from each F_5 line, and these were in turn maintained as individual lines. The result, at the $F_{5:8}$ generation was a collection of 98 heterogeneous inbred families (HIFs) each consisting of 8 to 10 NILs. Each individual NIL was then genotyped for markers of interest. An advantage of this method over development of NILs by repeated backcrossing is that this is the only generation that must be genotyped. Using families derived from F_5 lines, approximately 6.25% of the families should be segregating for any given marker. The effects of the segregating marker can then be determined by planting NILs in replicated field trials and comparing the phenotypes of the individual NILs within each family.

Using this procedure we developed sets of NILs for four RAPD markers linked to QTL associated with yield under drought environments and other morphological traits associated with drought tolerance. Three HIFs were identified as segregating for each of the four RAPD markers. Within each family, two NILs were chosen for each marker allele. These NILs were planted in replicated field trials at different locations under pre-flowering drought, post-flowering drought, and fully irrigated conditions. Grain yield was measured in all treatments. Seed set, xylem water potential, and osmotic potential were measured in pre-flowering drought treatments. Seed weight and stay-green score were measured in the post-flowering drought treatments. Seed set and seed weight were measured in the irrigated treatments for estimation of the stability of these traits in their respective drought treatments. In most cases, NILs contrasting for a specific locus differed in phenotype as predicted by QTL analysis. Associations between several markers and grain yield in both drought and non-drought conditions were confirmed (Tuinstra et al. 1998). Some

QTL, however, were only confirmed in one environment, for example the stay-green effect of marker *tM5/75* was confirmed in one environment, but was not statistically significant in another. In some instances, new associations were revealed by analysis of NILs. For example, marker *tH19/50* was found to be associated with stay-green in two environments, though this association was not identified in the original QTL analysis. Further analyses indicated that differences in agronomic performance may be associated with effects of heat tolerance, water status, and expression of stay-green, suggesting that these loci mediate the expression of drought tolerance via different biological mechanisms.

6. FUTURE PROSPECTS FOR MAS IN SORGHUM

The recent publication of dense genetic maps for sorghum, the alignment of those maps, the adoption of a common nomenclature for the linkage groups of sorghum, and assignment of linkage groups to physical chromosomes should facilitate the exchange of genetic marker and QTL information among members of the sorghum community. New molecular marker technologies, especially high-throughput methods, will continue to enhance our ability to generate DNA marker data, though accurate measurement of phenotypic traits will continue to be even more important. A new chip-based technology, named Diversity Arrays Technology (DArT), has been developed which will allow rapid detection of large numbers of single nucleotide polymorphisms (SNPs) in many different plant species without the need for extensive prior sequence information (Jaccoud et al. 2001; Kilian et al. 2003). A DArT array is currently available for sorghum (DArT P/L 2006). Although DArT analysis does not require prior genomic sequence information, the sequence of any specific marker locus can be readily determined by sequencing the corresponding DNA clone from the array. This sequence could then be used to create a marker that is more amenable to routine genotyping. This ability to generate and process large amounts of genotypic data may permit large scale association mapping studies. Association mapping is based on the linkage disequilibrium (LD) within natural or assembled populations, and has been used by human geneticists to associate regions of the human genome with various diseases (Collins et al. 2004). The greatest potential use of this technique for plant geneticists and breeders will be the ability to screen populations or collections of germplasm to identify potential QTL and genetic markers for MAS, without using traditional linkage mapping populations. However, there are some disadvantages of this method compared to mapping in experimental populations. First, there is a higher probability of both Type I and Type II errors (Brescghello and Sorrells 2006), especially if the extent of linkage disequilibrium is less than the density of markers used. Thus, a large number of markers should be analyzed to saturate the map to a sufficient density, often to a resolution of less than one centiMorgan (cM). SNP-genotyping microarrays containing thousands of known loci are currently available to human geneticists (Affymetrix, Inc. 2006), but are not yet available for crop plants, though DArT

(Jaccoud et al. 2001; Kilian et al. 2003) may offer an acceptable alternative. Stratification within the assembled collection of germplasm may also affect estimates of association between traits and genotypes, and should be determined prior to association mapping. Mapping traits based on linkage disequilibrium in germplasm collections will be complimentary to traditional QTL mapping based on recombination within experimental populations. The results of one type of study could be used to confirm the results of the other. Though the future possibilities for MAS in sorghum and other crops appear nearly limitless, it has been suggested that the greatest limitation will not be generating genotypic data, but accurately measuring phenotypes. QTL determination and their potential utility in breeding programs are only as good as the quality of the phenotypic data employed in their generation.

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CHAPTER 10

MOLECULAR GENETICS AND BREEDING OF GRAIN LEGUME CROPS FOR THE SEMI-ARID TROPICS

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Abstract: Grain legumes are important crops for providing key components in the diets of resource-poor people of the semi-arid tropic (SAT) regions of the world. Although there are several grain legume crops grown in SAT, the present chapter deals with three important legumes i.e. groundnut or peanut (*Arachis hypogaea*), chickpea (*Cicer arietinum*) and pigeonpea (*Cajanus cajan*). Production of these legume crops are challenged by serious abiotic stresses e.g. drought, salinity as well as several fungal, viral and nematode diseases. To tackle these constraints through molecular breeding, some efforts have been initiated to develop genomic resources e.g. molecular markers, molecular genetic maps, expressed sequence tags (ESTs), macro-/micro- arrays, bacterial artificial chromosomes (BACs), etc. These genomic resources together with recently developed genetic and genomics strategies e.g. functional molecular markers, linkage-disequilibrium (LD) based association mapping, functional and comparative genomics offer the possibility of accelerating molecular breeding for abiotic and biotic stress tolerances in the legume crops. However, low level of polymorphism present in the cultivated gene pools of these legume crops, imprecise phenotyping of the germplasm and the higher costs of development and application of genomic tools are critical factors in utilizing genomics in breeding of these legume crops.

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1. INTRODUCTION

1.1. Importance of Legume Crops

Grain and forage legumes are grown on some 180 million hectares, or 12% to 15% of the Earth's arable surface (source: FAO Database [<http://apps.fao.org/page/collections>]). They account for 27% of the world's primary crop production, with grain legumes alone contributing 33% of the dietary protein nitrogen needs of humans (Vance et al., 2000). Grain legumes are key components in the diets of resource-poor people in the developing world; especially those who are vegetarian because of choice or cannot afford to supplement their diets with meat. Grain legumes are a rich source of essential vitamins, minerals, and important amino acids like lysine (Duranti and Gius, 1997; Grusak, 2002). Last but not least, grain legumes can also contribute to the Nitrogen balance of soils where they are grown, making them an indispensable component of the sustainability of the system. Another attractive feature is their ability to fix atmospheric nitrogen in the soil by virtue of their symbiotic association with *Rhizobium* bacteria (Schulze and Kondorsi, 1998; Serraj, 2004), thus reducing the need for N-fertilizers in the cropping systems. Legumes often attract higher market prices than other staple crops, making them an important source of income for farmers.

Legumes belong to the taxonomic family *Fabaceae*, containing over 18,000 species divided into the three sub-families Mimosoideae, Caesalpinoideae and Papilionoideae. Legume species have been cultivated for millennia all over the world because of the nutritional value of their seeds as mentioned above. Among different legumes, soybean (*Glycine max* L.) is the major single contributing species, which is used for multiple applications in the food and feed industries. Others, such as chickpea (*Cicer arietinum* L.), common bean (*Phaseolus vulgaris* L.), groundnut or peanut (*Arachis hypogaea* L.), cowpea (*Vigna unguiculata* L.) and pigeonpea (*Cajanus cajan* L.) contribute significantly to the diets of large numbers of people in Asia, Africa, and South America. The high nutritional value of legumes is achieved by the presence of a wealth of secondary metabolites and in the capacity of legumes to establish a symbiosis with the soil bacteria *Rhizobium*, which supplies nitrogen to the plant in exchange of carbohydrate supply to the microsymbiont (Dixon and Sumner, 2003, Desbrosses et al., 2005). The symbiosis results in the formation of root outgrowth called nodules, which can have different types of shape depending on plants. That symbiosis gets preferentially established under low N conditions, and gets inhibited under excess nitrogen, although certain species are able to obtain most of their nitrogen from the symbiosis in environments that do contain nitrogen. Nodules host the *Rhizobium* bacteria, which differentiate in the nodules into symbiotic bacteroids, and are the site of catalysis of dinitrogen into ammonia by the microbial enzyme nitrogenase. As an energy source to achieve N fixation, the bacteria obtain dicarboxylic acids from the host plant. By a complex amino-acid cycle the reduced nitrogen is provided to the plant (Lodwig et al., 2003) where it is accumulated into proteins. Thus legumes can also help replenish nutrient-depleted soil.

1.2. Legume Crops in the Semi-Arid Tropics

The semi-arid tropics (SAT) covers parts of 55 developing countries where the 75–180 day growing period has a mean daily temperature of more than 20°C. The dry semi-arid tropics have very short growing seasons, separated by very hot and dry periods in which growth without irrigation or stored soil moisture is impossible. Natural soil fertility is often low, in part because soils are highly weathered by the dry-hot and humid-hot cycles, and pest and disease pressure can be intense. Farmers face further substantive risks, even within the growing season, as there are irregular periods of drought and high evaporative demand which can seriously compromise crop productivity. Based on 1996 statistics, the SAT is home to about 1.4 billion people, of which 560 million (40%) are classified as poor, and 70% of the poor reside in rural areas (Ryan and Spencer 2001).

Although a number of crops are grown in SAT areas, among legume crops, chickpea, groundnut common bean, cowpea and pigeonpea provide key components in the diets of resource-poor people in the developing world. We, at ICRISAT, together with our National Agricultural Research System (NARS) partners are engaged on crop improvement in chickpea, groundnut and pigeonpea, therefore in this article we discuss the advances in the area of genetics and genomics applied to breeding in only these three legume crops. In the first instance, a brief introduction of these crops is given in following sections.

1.2.1. Chickpea (*Cicer arietinum* L.)

Chickpea is the third most important grain legume globally, and second in importance in Asia. It is also an important legume crop in Eastern and Southern Africa. About 90% of the global area and 88% of production is concentrated in Asia. Chickpea has one of the best nutritional compositions of any dry edible legume, and is mainly used for human consumption. The *desi* type (colored seed coat) is usually de-hulled and split to make *dhal* or flour (*besan*), while *kabuli* types (white or cream-colored seed coat) is often cooked as whole grain. The haulms are used for animal feed. Chickpea improves soil fertility through nitrogen fixation (up to 140 kg N/ha). Chickpea area has slightly decreased globally, but has been stable at 9 M ha in Asia for the past 25 years. However, production in Asia has increased by 39% due to a 32% increase in productivity. Even then, the current average yield in Asia (0.8 t/ha) is low, and far below the potential yield (5 t/ha), or research station yields (3.5 t/ha). The global demand for chickpea in 2010 is estimated at 11.1 Mt (up from the current 8.6 Mt). A combination of productivity enhancement through crop improvement enhanced with biotechnological tools, integrated crop management and expansion of area to new niches and production systems are needed to achieve this target.

According to van der Maesen (1987), the cultivated chickpea has been taxonomically placed in the genus *Cicer*, which belongs to the family *Fabaceae* and its monogeneric tribe *Cicereae* Alef. Presently, the genus consists of 43 species divided into 4 sections, namely *Monocicer*, *Chamaecicer*, *Polycicer* and *Acanthocicer*.

This classification is based on their morphological characteristics, lifestyle and geographical distribution (van der Maesen, 1987). Eight of these *Cicer* species share the annual growth habit with chickpea are of particular interest to breeders.

1.2.2. Groundnut (*Arachis hypogaea* L.)

Groundnut is an important food and cash crop for the resource-poor farmers in Asia and Africa. It is primarily grown for edible oil (48–50%) as well as for direct consumption as food by people. Groundnut haulms are excellent fodder for cattle, and groundnut cake (after oil extraction) is used as animal feed. It contributes significantly to household food security and cash income through the sale of groundnut products. Groundnut productivity in Western and Central Africa (WCA) and Eastern and Southern Africa (ESA) is below the world average yield of 1.4 t/ha. Although groundnut productivity in Asia (1.8 t/ha) exceeds the world average, it is still lower than the yields in developed countries (3 t/ha). The area under groundnut in ESA has increased dramatically from 2.3 to 3.3 M ha during 2000 to 2004. In Asia, the area under groundnut is increasing in China and Vietnam, but is declining in India during 1991–2004. There has been a slight decline in area in WCA. Although global productivity has shown a positive trend, much more needs to be achieved in future.

The genus *Arachis* belongs to the family *Fabaceae*, subfamily *Papilionaceae*, tribe *Aeschynomeneae*, subtribe *Stylosantheae*. Cultivated groundnut (*Arachis hypogaea* L.) can be botanically classified into two subspecies, *hypogaea* and *fastigiata* that are distinguished based on branching pattern and distribution of vegetative and reproductive nodes along lateral branches. Each subspecies is again divided into two botanical varieties; subsp. *hypogaea* into var. *hypogaea* (virginia) and var. *hirsuta* and subsp. *fastigiata* into var. *fastigiata* (valencia), var. *vulgaris* (spanish), var. *peruviana* and var. *aequatoriana* (Karpovickas and Gregory, 1994).

1.2.3. Pigeonpea (*Cajanus cajan* L.)

Pigeonpea is a versatile and multipurpose crop. It is one of the major food legumes in the tropical and sub-tropical regions in Africa, Asia and the Caribbean countries. Its green pods and seeds are consumed as a vegetable, and the dry grains are cooked whole or after dehulling (as *dhal*). The foliage is used as fodder, and the dry sticks are used for fencing, thatching, and as firewood. It fixes atmospheric nitrogen, and the extensive leaf fall adds organic matter to the soil. Dry grain is also used for animal feed. About 90% of the global pigeonpea area (4.4 M ha) is in Asia (about 86% in India). Other major countries where pigeonpea is grown are Myanmar, Nepal, Bangladesh, Pakistan and China. In Sub-Saharan Africa (SSA), pigeonpea is grown in Uganda, Kenya, Malawi, Mozambique, Zimbabwe, Zambia, South Africa, Sudan and Ethiopia; but reliable statistics are not available. Pigeonpea production has shown only a marginal increase during the past two decades (2.2 to 2.9 million t during 1980–98). However, productivity has remained stagnant at 0.7 t/ha, mostly because it is intercropped with cereals or cotton and receives no or little inputs; or

Table 1. Characteristics and genomics data available for some SAT legumes

	Chickpea	Groundnut	Pigeonpea
Species name	<i>Cicer arietinum</i> L.	<i>Arachis hypogaea</i> L.	<i>Cajanus cajan</i> L.
Ploidy level and chromosome number	2n = 2x = 16	2n = 4x = 40	2n = 2x = 22
Genome size ¹	931 Mbp	2891 Mbp	858 Mbp
SSR markers	~700 (Winter et al., 1999; Huettel et al., 1999; Sethy et al., 2003,2006b; Lichtenzweig et al., 2005; Choudhary et al., 2006; Varshney et al., unpublished; Bhatia et al., unpublished results)	~700 (Hopkins et al., 1999; He et al., 2003; Ferguson et al., 2004; Moretzsohn et al., 2004; Palmieri et al., 2005; Mace et al., unpublished; D. Bertoli, Brazil, pers. commun.; S. Knapp, pers. commun.)	~100 (Burns et al., 2001; Odoney et al. 2007)
BAC libraries	3.8 X (Rajesh et al. 2004), 7 X (Lichtenzweig et al. 2005)	6.5–9.0 X (Yuksel and Paterson, 2005)	–
ESTs	~2000 (NCBI, Buhariwalla et al., 2005)	~7538 (NCBI, Luo et al., 2005; S. Knapp, pers. commun.)	More than 884 (NCBI) (Gaikwad et al. unpublished)
Gene arrays	768- features microarray (Coram and Pang, 2005a), SAGE Gene Chip (P. Winter, Germany, pers. commun.)	400 unigene array (Luo et al., 2005)	

¹ As per estimate of Royal Botanic Gardens, Kew, UK (<http://www.rbgekew.org.uk/cval/>)

² NCBI = <http://www.ncbi.nlm.nih.gov/>

gets relegated to marginal and poor soils, often where no other crop can be grown. Additionally, pigeonpea has also generally a poor harvest index.

Pigeonpea belongs to the *Cajaninae* sub-tribe of the economically important leguminous tribe *Phaseoleae* that contains soybean (*Glycine max* L.), common bean (*Phaseolus vulgaris* L.) and mungbean (*Vigna radiata* L.) (Young et al., 2003). The genus *Cajanus* comprises 32 species most of which are found in India and Australia although one is native to West Africa. Pigeonpea is the only cultivated food crop of the *Cajaninae* sub-tribe and has a diploid genome.

A brief overview on genome size, ploidy level, existing genomics resources in chickpea, groundnut and pigeonpea is given in Table 1.

2. CHALLENGES IN SUSTAINABLE CROP PRODUCTION OF SAT LEGUMES

2.1. Abiotic Stresses

Abiotic stresses severely limit agricultural production. There is a clear consensus that drought is among the most severe stress for legume production in SAT regions

of Asia and Africa while salinity is the second ranked constraint in the production of these legumes in some Asian countries.

2.1.1. *Drought*

The SAT regions are characterized by short and erratic rainfall (and then long periods with virtually no rain), where crops grown under rainfed conditions suffer from both intermittent and terminal drought stress, and crop grown in residual moisture after the rain suffer terminal drought, thus incurring major yield losses. Water deficit is one of the most severe stresses for sustainable crop production. Worldwide, yield losses each year due to drought are estimated to be around US\$500 million (Sharma and Lavanya, 2002).

Water capture by roots and water-use efficiency are probably two important components of the yield architecture, as defined by Passioura (1977) that are important for crops growing under terminal drought conditions. These two traits are the classical component of what is called 'drought avoidance', and which means getting more water or using it more efficiently). Drought avoidance is considered to be the major trait of interest to expand production to presently uncropped areas and post-rainy fallows in SAT regions. Although roots have already proved to be beneficial for yield under terminal drought (chickpea, Kashiwagi et al., 2004), there is a need to understand better how root traits contribute to drought avoidance, and a need to explore them in those crops where little information on roots has been acquired (e.g., groundnut). Specifically, there is a need to understand the dynamics of roots, how roots contribute to the overall water budget, and more interestingly how they contribute at the time of grain filling, and how they contribute at the time of flowering. Recent studies at ICRISAT indicate that deeper rooting correlates with a higher harvest index (HI) in chickpea in conditions of more severe drought (Kashiwagi et al., 2004, 2006). This might be related to the root being able to supply water during flowering and allowing less flower drop because of water deficit. Water use efficiency (WUE) or more specifically transpiration efficiency (TE) is another trait that is being addressed in groundnut at ICRISAT by using different biotechnological, physiological and breeding approaches. For TE, there is also a need to understand better the mechanisms that lead to better TE, if we ever want to reach the genes involved.

2.1.2. *Salinity*

Soil salinity is an important limiting factor for crop yield improvement, which affects 5–7% of arable lands, i.e. approximately 77 million ha worldwide. Most crops are sensitive to salt stress at all stages of plant development, including seed germination, vegetative growth and reproductive growth, although the latter stage is certainly the most sensitive across many crops. Legumes, in general, are sensitive to salinity, and within legumes, chickpea, fababean and pigeonpea are more sensitive than other food legumes. The salinity problem is increasing, in particular in areas where irrigation is a common practice (Ghassemi et al., 1995). Though management options exist to alleviate salt effects, these are often in contradiction with the

immediate economic choices of the concerned farmers; thus crop improvement for salt tolerance appears to be the best and economic alternative.

The problem of salinity is basically two-fold. In one case, soil is saturated with sodium (Na) and soil pH remains within an optimal range for crop growth. This type of salinity refers to coastal or dryland salinity. These are soils that get saturated with sodium because an existing saline ground water table rising (proximity to the sea or salt that has accumulated in the soil profile), bringing the salt to the surface. In a second case, soil is both saturated with Na (exchangeable sodium percentage, ESP, > 6) and pH has reached levels above 8.5–9.0. This type of salinity is also called transient salinity, and is thereafter referred to as *sodicity* or *sodic soils*. In this case, the sodium saturation brings about the same effect as salinity, but the high pH dramatically affects the availability of micronutrients (low availability/solubility of micronutrient salts at these pH levels), the soil structure and porosity (poor drainage, tendency for water logging, little oxygenation because of saturation of the exchange complexes in the soil by sodium). In the past, most studies have focused on *salinity*, and only a few on *sodicity*.

Despite the importance of salinity in crop production worldwide and the abundant knowledge on the effect of salinity on plant growth and development, there has surprisingly been little effort to breed for improved salinity tolerance, with the exceptions of wheat, rice, barley, alfalfa and claims of soybean. Breeding tolerant crop varieties is therefore urgently needed.

2.2. Biotic Stresses

The major biotic factors of SAT legumes are diseases and insect pests. The chickpea diseases of major importance are ascochyta blight (caused by the necrotrophic fungus *Ascochyta rabiei* (Pass.) Lab.), fusarium wilt (caused by *Fusarium oxysporum* f. sp. *ciceris*), Botrytis gray mold and root rots caused by *Sclerotium* and *Pythium*. Majority of these diseases affect all aerial parts of the plant. Among the pigeonpea diseases, sterility mosaic (viral disease), fusarium wilt (caused by the fungus *Fusarium udum* Butler), and phytophthora blight (*Phytophthora drechsleri*) are major diseases causing significant losses of pigeonpea yield. In groundnut, rust, late leaf spot, and early leaf spot are serious diseases worldwide, which cause 50–60% pod yield loss. Rust and late-leaf spot often occur together and the pod yield loss can exceed 70% in the crop. Besides adversely affecting pod yield and its quality, these foliar diseases also affect haulm (fodder) yield and quality. Whereas the level of resistance available in cultivated groundnut to rust is very high, for early- and late-leaf spot, it is low. Wild *Arachis* species harbour many useful resistance genes against various diseases and insect pests. Of the important biotic constraints specific to sub-Saharan Africa (SSA), the groundnut rosette disease (GRD), vectored by aphids, is endemic to the continent and its adjoining islands and epidemics occur often throughout SSA, reducing groundnut production and crippling rural food security.

More than 200 species of insects feed on pigeonpea and chickpea, of which pod borer (*Helicoverpa armigera*), spotted pod borer (*Maruca vitrata*), pod fly (*Melanagromyza obtuse*), pod sucking bugs (*Clavigralla* spp., *Nezara viridula*) and the bruchid (*Callosobruchus* spp.) are most important economically (Singh et al., 1990). *Helicoverpa* causes an estimated loss of US\$ 317 million in chickpea and pigeonpea (ICRISAT, 1992), and possibly over US\$ 2 billion on other crops worldwide. A conservative estimate is that over US\$ 1 billion is spent on insecticides to control this pest. Therefore, in addition to the huge economic losses caused directly by the pest, there are several indirect costs from the deleterious effects of pesticides on the environment and human health (Sharma, 2001). These insect pests feed on various plant parts such as leaves, tender shoots, flower buds, and immature seeds. It has been difficult to breed for *Helicoverpa* resistance in chickpea and pigeonpea because sources with a high level of resistance are not available in the cultivated species of these legumes. Recent studies show potential of utilizing the wild species in insect pest resistance breeding programme as these have shown higher levels of resistance.

3. UTILIZATION OF PLANT GENETIC RESOURCES (PGRS)

Availability and characterization of suitable germplasm is a critical factor for utilizing genetic variation in crop breeding. Fortunately for all the three legume species mentioned in the article, a large number of accessions are present in different genebanks throughout the world (Dwivedi et al., 2006). For instance, ICRISAT, under an agreement with FAO, holds 16,853 cultivated and 117 wild accessions of *Cicer* species, whereas the International Centre for Agricultural Research in Dryland Areas (ICARDA), Syria, under the same FAO agreement, maintains 8,342 cultivated and 255 wild accessions. Other institutions holding chickpea germplasm are the National Bureau of Plant Genetics Resource (NBPGR), India (14,566 accessions); Centre for Legume Improvement in Mediterranean Area (CLIMA) (4,351 accessions) and AusPGRIS (7922 accessions) in Australia; United States Department of Agriculture (USDA), USA (4,662 accessions); and the Seed and Plant Improvement Institute, Iran (4,925 accessions). The European *Cicer* database contains 3,700 cultivated accessions from 11 countries (Pereira et al. 2001). For groundnut, ICRISAT holds, under the same agreement with FAO, 14,126 accessions of cultivated peanut and 293 accessions of wild *Arachis* species from 93 countries. Other institutions holding large numbers of peanut accessions are the National Research Centre for Groundnut (NRCG), India (7,935 accessions) and the USDA Southern Regional Plant Introduction Station, USA (6,233 accessions). In the United States, wild *Arachis* species are maintained at North Carolina State University, Raleigh (250 accessions) and at the Texas Agricultural Experiment Station (TAMU), Texas (300 accessions). For pigeonpea, ICRISAT holds under the agreement with FAO 12,398 pigeonpea accessions of cultivated and 314 accessions of wild species from 74 countries. Other institutions holding substantial amounts of pigeonpea germplasm

include the NBPGR (5,454 accessions) in India and the USDA, Southern Regional Plant Introduction Station (4,116) in USA.

3.1. Core and Mini-Core Collections

Despite the availability of a large number of germplasm, only limited numbers of accessions have been used in breeding programme not only in SAT legumes but other crop species as well (Dwivedi et al., 2006). One of the main reasons for this fact may be the large sizes as well as non-availability of information on germplasm collections. Core collections present a manageable and cost-effective entry point into germplasm collections for identifying parental genotypes with new sources of disease and pest resistance or abiotic stress tolerance. Evaluation of core collections is usually the most efficient and reliable means of carrying out an initial search of the germplasm collections. For instance, early evaluation of limited number of germplasm accessions led to premature conclusion that no variability for salinity tolerance existed in chickpea (Saxena, 1984). However, recent screening of large number of germplasm accessions, including the chickpea mini-core collection, revealed very large variation, readily usable for breeding purposes (Vadez et al., 2006). Evaluation of larger amounts of germplasm through multi-location trials is both very expensive and time consuming; large-scale generation of accurate and precise evaluation data from such trials is generally not possible, thus dramatically reducing the probability of identifying desirable material. Core collections usually consist 10% of the entire germplasm collection that represents the collections variability (Brown, 1989). These representative subsample collections are developed from the entire collection, using all available information on accessions including the origin and geographical distribution plus characterization and evaluation data. Ten percent of most crop germplasm collections are a much more feasible amount of material for intensive and precise evaluation.

Most core collections have been designed from global or regional collections held within international agricultural research centers or national program gene banks, while a few have also been developed for wild accessions (Tohme et al., 1996). After evaluating a total of 16,991 chickpea accessions for 13 traits and 14,310 groundnut and 12,153 accessions of pigeonpea for 14 traits each, the core collections of chickpea, groundnut and pigeonpea with 1,956 (Upadhyaya et al., 2001a), 1,704 (Upadhyaya et al., 2003) and 1,290 accessions (Reddy et al., 2005), respectively have been developed at ICRISAT. In addition, the core collection of 505 genotypes of chickpea was developed after analysis of 3,315 genotypes (Hannan et al., 1994). Similarly for groundnut, an USDA core collection with 831 genotypes after evaluating 7,432 accessions for 24 traits (Holbrook et al., 1993) and an Asian core collection based on evaluating 4,738 genotypes for 15 traits (Upadhyaya et al., 2002) are available. Although these core collections have been useful for identifying diverse sources for traits of interests and broadening the genetic base of cultivars for a crop (Upadhyaya et al., 2001b, 2006a; Krishnamurthy et al. 2003;

Serraj et al., 2004), even a core collection can be too large so a further reduction is also valuable providing it is not associated with losing too much of the spectrum of diversity. Upadhyaya and Ortiz (2001) developed a strategy for sub-sampling a core collection to develop a mini-core collection, based on selecting 10% of the core accessions representing the variability of larger collection of species. In this process, the core collection is evaluated for various morphological, agronomic, and quality traits to select a 10% subset from this core subset (i.e., 1% of the entire collection) that captures a large proportion (i.e. more than 80% of the entire collection) of the useful variation. Selection of core and mini-core collections is based on standard clustering procedures used to separate groups of similar accessions combined with various statistical tests to identify the best representatives. The mini-core collection developed at ICRISAT for chickpea consisted of 211 accessions (Upadhyaya and Ortiz, 2001), while the groundnut (Upadhyaya et al., 2002) and pigeonpea (Upadhyaya et al., 2006b) mini-core consists of 184 accessions and 146 accessions, respectively. Both core or mini-core germplasm collections have been used for identifying a range of germplasm with beneficial traits for use in breeding programs (see Dwivedi et al., 2006 for references). Increasing concern of trade and food processors for consistent and better quality and physical specifications, however, suggest further characterization of core or mini-core collections for quality and market traits.

3.2. Molecular Characterization of PGRs

The core or mini core collections have been developed based on morphological or agronomic traits; little information is available on molecular genetic diversity present in the germplasm collection. Molecular characterization of germplasm is a particularly useful tool for assisting genebank curators to better manage genetic resources, helping them to identify redundant germplasm and to provide scientists with the most diverse germplasm for applications in research and breeding (Bretting and Widrechner, 1995; Virk et al., 1995; Brown and Kresovich, 1996; van Treuren et al., 2001; Upadhyaya et al., 2006b). Accessions with the most distinct DNA profiles are likely to contain the greatest number of novel alleles (Tanksley and McCouch, 1997). As a part of the Generation Challenge Programme (GCP) of the CGIAR, molecular characterization of global composite collections of the SAT legumes is in progress at ICRISAT. For example, genotyping of about 3000 chickpea accessions (Upadhyaya et al., 2006a) with 50 SSR markers and 1000 groundnut accessions with 20 SSR markers, in collaboration with ICARDA (Syria) and EMBRAPA (Brazil) respectively has already been completed. Molecular characterization of 1000 pigeonpea accessions at 20 SSR loci is in progress. These studies provide estimates on genetic diversity and the population structure of the germplasm that can be used to define the most diverse collection, called 'reference collection' for using in association mapping studies (see later).

4. MOLECULAR BREEDING FOR SAT LEGUMES

Legume breeders have made major contributions to combat the problem of both abiotic and biotic stresses in the past but the pace and extent of improvements must be dramatically increased to attend to parallel demands. Recent advances in the area of biotechnology have offered the tools in the form of molecular markers to assist the breeding practices (Jain et al., 2002). Molecular markers are powerful diagnostic tools that detect DNA polymorphism both at the level of specific loci and at the whole genome level (reviewed by Azhaguvel et al., 2006). As compared to morphological traits/markers, molecular markers have several advantages as they are phenotypically neutral and are not influenced by pleiotropic and epistatic interactions, and their expression is not dependent on plant age/part (Jones et al., 1997). In fact the use of molecular markers in improving the breeding efficiency in plant breeding was suggested as early as in 1989 (Tanskley et al., 1989; Melchinger, 1990). In this regard, once linkage between a gene for the agronomic trait of interest and marker locus is established, then DNA diagnostic tests can be used to guide plant breeding (Morgante and Salamini, 2003; Gupta and Varshney, 2004). The selection of useful lines for breeding with the help of linked molecular markers is called marker-assisted selection (MAS). Use of MAS is especially advantageous for traits with low heritability where traditional selection is difficult, expensive or lack accuracy or precision.

A variety of molecular markers exist, such as RFLPs (*Restriction Fragment Length Polymorphisms*, Botstein et al., 1980), RAPDs (*Random Amplification of Polymorphic DNAs*, Williams et al., 1990), AFLPs (*Amplified Fragment Length Polymorphisms*, Vos et al., 1995) and microsatellites or SSRs (*Simple Sequence Repeats*, Tautz, 1989). Among the different classes of molecular markers, SSR markers are often chosen as the preferred markers for a variety of applications in breeding because of their multiallelic nature, codominant inheritance, relative abundance and extensive genome coverage (Gupta and Varshney, 2000). More recently, markers such as SNPs (*Single Nucleotide Polymorphisms*, Rafalski, 2002) and DArT (*Diversity Array Technology*, Killian et al., 2005) have been added to list of preferred marker systems for breeding.

MAS in breeding has revolutionized the improvement of temperate field crops (Koebner, 2004; Varshney et al., 2006) and will have similar impacts on breeding of tropical legume crops, particularly for traits where phenotyping is only possible late in the season, and where screening of traits is difficult or prohibitively expensive. Breeding for enhanced drought and salinity tolerance is notoriously difficult due to the genetic complexity of these traits, the high genotype-by-environment interaction and the difficulties of carrying out precise phenotypic evaluation under field conditions. Part of the problem comes from the difficulty to assess the relative contribution of different traits on the yield under terminal drought. Thus, these are traits where MAS could greatly enhance the effectiveness and impact of plant breeding programs.

4.1. Molecular Tools for SAT Legume Genomics

Molecular markers and molecular genetic linkage maps are the prerequisites for undertaking molecular breeding activities. However, the progress towards development of a reasonable number of molecular markers and molecular genetic maps for cultivated species has been very slow in almost all the three legume crops discussed in this chapter. One of the main reasons for this fact may have been the low level of genetic diversity present in the cultivated gene pools of these species, at least with the detection tools that are currently available. Nevertheless, because of the development of more sophisticated molecular tools, some progress has been made in the area of molecular mapping in these legume species.

4.1.1. Chickpea

The beginnings of the linkage map development in chickpea were based on morphological and isozyme loci. However, their small numbers and the fact that expression of these markers is often influenced by the environment, makes them unsuitable for routine use. Further, there is an extremely low level of polymorphism among genotypes of cultivated chickpea (*C. arietinum* L.). Therefore, interspecific crosses (*C. arietinum* × *C. reticulatum*, *C. arietinum* × *C. echinospermum*) were exploited for developing genetic linkage maps (Gaur and Slinkard, 1990a, 1990b). The earlier maps were sparse and represented less than 30 loci mapped in a very small portion (about 250 cM) of the chickpea genome (Gaur and Slinkard 1990a, 1990b; Kazan et al. 1993). Integration of molecular markers into genetic linkage maps in chickpea was started with the work of Simon and Muehlbauer (1997). Due to the lack of more recently available molecular markers, Simon and Muehlbauer (1997) employed RFLP and RAPD markers that showed limited polymorphism in the cultivated species (Udupa et al., 1993; Banerjee et al., 1999).

Subsequent development of SSR or microsatellite markers revolutionized genetic analysis and opened new possibilities for the study of complex traits in plant species especially crops like chickpea having a narrow genetic background. As a result, several hundred SSR markers have been developed in chickpea (Huettel et al., 1999; Winter et al., 1999; Sethy et al., 2003, 2006a, 2006b; Lichtenzweig et al., 2005; Choudhary et al., 2006). The majority of these markers have been mapped using interspecific mapping populations (Winter et al., 1999, 2000; Tekeoglu et al., 2002; Pfaff and Kahl, 2003). A genetic map constructed from an interspecific cross, however, may not represent the true recombination distance map order of the cultivated genome due to uneven recombination of homeologous chromosomes and distorted genetic segregation ratios (Flandez-Galvez et al., 2003a). Therefore, in the framework of targeting traits of breeding importance, molecular genetic linkage maps, with SSR markers, have been developed using intraspecific mapping populations from the cultivated gene pool (Cho et al., 2002, Flandez-Galvez et al., 2003a). The genetic linkage maps developed to date with DNA based molecular markers in chickpea are summarized in Table 2.

Table 2. Important genetics maps available for some SAT legume crops

Mapping population	Features of genetic map	Genome coverage	Reference
Chickpea			
F_2 , interspecific (<i>C. arietinum</i> × <i>C. reticulatum</i>) and F_2 , interspecific (<i>C. arietinum</i> × <i>C. echinospermum</i>)	7 linkage groups with 3 morphological and 26 isozymes	200 cM	Gaur and Slinkard, 1990a, 1990b
F_2 , interspecific (<i>C. arietinum</i> × <i>C. reticulatum</i>)	8 linkage groups with 5 morphological and 23 isozymes	257 cM	Kazan et al., 1993
F_2 , interspecific (<i>C. arietinum</i> × <i>C. reticulatum</i>) and F_2 , interspecific (<i>C. arietinum</i> × <i>C. echinospermum</i>)	10 linkage groups with 9 morphological, 27 isozyme, 10 RFLP and 45 RAPD loci	527 cM	Simon and Muehlbauer, 1997
RIL, interspecific (<i>C. arietinum</i> 'ICC 4958' × <i>C. reticulatum</i> 'P148977')	11 linkage groups with 120 STMS loci	613 cM	Winter et al., 1999
RIL, interspecific (<i>C. arietinum</i> 'ICC 4958' × <i>C. reticulatum</i> 'P148977')	16 linkage groups with 118 SSR, 96 DAF, 70 AFLP, 37 ISSR, 17 RAPD, 8 isozyme, 3 cDNA, 2 SCAR and 3 morphological marker	2,078 cM	Winter et al., 2000
RIL, interspecific (<i>C. arietinum</i> 'FLIP 84-92C' × <i>C. reticulatum</i> 'P148977')	9 linkage groups with 89 RAPD, 17 ISSR, 9 isozyme, and 1 morphological marker	982 cM	Santra et al., 2000
F_2 , interspecific (<i>C. arietinum</i> 'ICC 4958' × <i>C. reticulatum</i> 'P148977')	8 linkage groups, integration of 55 SSR and 1 RGA	1,175 cM	Tekeoglu et al., 2002
RIL, intraspecific (<i>C. arietinum</i> 'ICCV 2' × <i>C. arietinum</i> 'JG62')	14 linkages groups with 68 SSR, 34 RAPD, 4 ISSR, and 5 morphological markers	297 cM	Cho et al., 2002
RIL, intraspecific (<i>C. arietinum</i> 'ILC 1272' × <i>C. arietinum</i> 'ILC3279')	8 linkage groups with 52 SSR, 3 <i>Ascochyta blight</i> resistance loci	–	Udupa and Baum, 2003
RIL, interspecific (<i>C. arietinum</i> 'ICC 4958' × <i>C. reticulatum</i> 'P148977')	incorporated 47 DR gene specific markers to Winter et al. (2000) 2500 cM, total 296 markers, 12 linkage groups	2,500 cM	Pfaff and Kahl, 2003
F_2 , intraspecific (<i>C. arietinum</i> 'ICC 12004' × <i>C. arietinum</i> 'Lasser')	8 linkage groups with 54 SSR, 3 ISSR, 12 RGA loci	535 cM	Flandez-Galvez et al., 2003a

(Continued)

Table 2. (Continued)

Mapping population	Features of genetic map	Genome coverage	Reference
F_2 interspecific (<i>C. arietinum</i> 'Lasseter' × <i>C. echinospermum</i> 'PI527930')	8 linkage groups with 14 SSR, 54 RAPD, 9 ISSR, 6 RGA loci	570 cM	Collard et al., 2003
RIL, intraspecific (<i>C. reticulatum</i> 'PI 359075' × <i>C. arietinum</i> 'FLIP 84-92C')	11 linkage groups with 53 SSRs	–	Cho et al., 2004
RIL, intraspecific (<i>C. arietinum</i> - two populations, CA2139 × JG62, CA2156 × JG62)	10 linkage groups with 118 RAPD, 13 SSR, 3 ISSR, and 4 morphological markers	–	Cobos et al., 2005
RIL, interspecific (<i>C. arietinum</i> 'Hadas' × <i>C. reticulatum</i> 'Cr205')	9 linkage groups with 91 SSR, 2 CytP450 markers	345 cM	Abbo et al., 2005
Groundnut			
F_2 , interspecific (2x) (<i>A. stenosperma</i> × <i>A. cardenassi</i>)	11 linkage groups with 117 RFLP loci	1,063 cM	Halward et al., 1993
BC interspecific (2x) (<i>A. stenosperma</i> × (<i>A. stenosperma</i> × <i>A. cardenassi</i>))	11 linkage groups with 167 RAPD loci	800 cM	Garcia et al., 1995
BC interspecific (4x) (<i>A. batizocoi</i> × (<i>A. cardenassi</i> × <i>A. dtogoi</i>))	23 linkage groups with 370 RFLP loci.	2,210 cM	Burrow et al., 2001
F(2:3), intraspecific (<i>A. hypogaea</i>) (4x) ICG 12991 (Spanish) × ICG SM 93541	5 linkage groups with 12 AFLP loci	139.4 cM of the genome	Herselman et al., 2004
F_2 interspecific (A Genome, 2x) (<i>A. duranensis</i> × <i>A. stenosperma</i>)	11 linkage group with 204 SSR loci	1,231 cM	Moretzsohn et al., 2005
F_2 interspecific (B Genome, 2x) (<i>A. ipaensis</i> × <i>A. magna</i>)	11 linkage group with 94 SSR loci	754.8 cM	Gobbi et al., 2006; D. Bertoli, Brazil (pers. communication)
Pigeonpea			
F_2 , interspecific (<i>C. cajan</i> × <i>C. scarabaeoides</i>)	~200 DArT loci	–	A. Killian, Australia (pers. communication)

Two independent interspecific-derived populations have been extensively employed for genetic linkage map development in chickpea: (i) *C. arietinum* 'ICC 4958' × *C. reticulatum* 'PI489777' at the University of Frankfurt, Germany, (ii) *C. arietinum* 'FLIP 84-92C' × *C. reticulatum* 'PI599072' at Washington State University, Pullman, USA. Among the different types of molecular markers developed for chickpea, SSR markers have proved very useful in linkage mapping and formed the basis for the map initially developed by Winter et al. (1999) that spanned a distance of 613 cM and consisted of 120 SSR markers. This map was greatly extended by Winter et al. (2000) and subsequently by Pfaff and Kahl (2003) with his addition of 47 defense response (DR) genes. The extended map covers a distance of 2500 cM arranged in 12 linkage groups and represents the most extensive linkage map in chickpea. Relatively smaller maps derived from intraspecific (within *C. arietinum*) crosses, have been developed and are being extended (Cho et al., 2002,2004; Flandez-Galvez et al. 2003a; Cobos et al., 2005).

In summary, a reasonable number of SSR markers representing the entire chickpea genome are available at present. The repository of SSR markers for chickpea is being extended by serious efforts by developing new microsatellite markers at NIPGR (Sethy et al., 2003; Chaudhary et al., 2006) and ICRISAT, Patancheru. For instance, a set of about 200 SSRs has been developed at NIPGR (Bhatia et al. unpublished). Similarly sequencing of a microsatellite enriched library of a chickpea (*C. arietinum*) genotype ICC 4958 at ICRISAT, in collaboration with University of Frankfurt, provides another set of about 200 SSRs that can be used to develop markers (Varshney et al., unpublished data). Therefore immediate priority should be accorded to saturation of the existing 'reference' intraspecific as well as interspecific genetic maps with the presently available >500 new (unmapped) SSR markers (Lichtenzveig et al., 2005; Sethy et al., 2006a,b; Choudhary et al. 2006; Bhatia et al., unpublished results; Varshney et al., unpublished results).

4.1.2. Groundnut

The paucity of DNA polymorphism in cultivated groundnut posed a considerable obstacle to genetic mapping in groundnut. For instance, earlier studies using RAPD and RFLP approaches detected limited DNA variation in *Arachis* species (Kochert et al., 1991; Halward et al., 1992; Paik-Ro et al., 1992). The use of a synthetic amphidiploid TxAG-6 (Simpson et al., 1993) made possible the generation of the first molecular map representing the entire tetraploid genome of groundnut. The discovery of a high level of polymorphism between the cultivar Flourrunner and the parents of TxAG-6 by RAPD analysis (Burrow et al., 1996) was followed by RFLP analysis showing 83% polymorphism on a per band basis (Burrow et al., 2001). By using 78 BC₁F₁ lines generated from the cross (TxAG-6 × Flourrunner), mapping of 220 cDNA probes integrated 370 RFLP loci into 23 linkage groups. The total length of the first tetraploid map was 2210 cM, which was slightly greater than twice the length (1063 cM) of the map previously reported from a cross between two A-genome diploid species (Halward et al., 1993). The common markers mapped

in both crosses showed a high degree of collinearity between the diploid and tetraploid chromosomes (Burrow et al., 2001). These studies have been summarized in the database PeanutMap (<http://peanutgenetics.tamu.edu/cmap>; Jesubatham and Burrow, 2006).

In terms of mapping the diploid genomes of *Arachis*, the first genetic map was constructed by Halward et al. (1993) based on the 87 F₂ lines derived from a cross of *A. stenosperma* × *A. cardenasii* and contained 117 RFLP loci on 11 linkage groups with a genome coverage of 1400 cM. RFLP analysis is time consuming and labor intensive. RAPD and AFLP were used to detect DNA polymorphism in several studies in different germplasm collections (He and Prakash, 1997; Subramanian et al., 2000; Dwivedi et al., 2001; Raina et al., 2001; Milla et al., 2005), but represent dominant markers with low information content. As a result of extensive efforts of several laboratories, a large number of microsatellite markers have been generated in groundnut (Hopkins et al., 1999; He et al., 2003; Ferguson et al., 2004; Moretzsohn et al., 2004; Mace et al., unpublished; D. Bertoli, Brazil, pers. commun.; S. Knapp, USA, pers. commun.). The availability of more than 500 SSR markers in groundnut provides the opportunity to integrate these markers into a genetic linkage map. However, these markers have been integrate only in the AA- genome map (Moretzsohn et al., 2005) by using an F₂ population obtained from a cross between two diploid species with AA genome (*A. duranensis* and *A. stenosperma*). The genetic map had 80 SSR loci on 11 linkage groups covering 1231 cM. Similar efforts to prepare a genetic map for BB genome are underway in Brazil. As of now, the genotyping of a F₂ population derived from cross between *A. ipaensis* (KG30076) and *A. magna* (KG30097) has resulted in development of 11 linkage groups with 94 markers (Gobbi et al. 2006). As a part of Generation Challenge Programme (GCP) of CGIAR, preparation of the first genetic map for tetraploid cultivated groundnut species is in progress at ICRISAT. However, the lower level of polymorphism between the parental genotypes of existing mapping populations (e.g. TAG24 × ICGV 86031 developed at ICRISAT, GPBD4 × TAG24 developed at UAS Dharwad) poses a serious problem. Nevertheless, we expect to prepare the partial/genome wide map with about 100 SSR loci (Varshney et al., unpublished results). The progress in the area of genome mapping of *Arachis* species is summarized in Table 2.

4.1.3. Pigeonpea

In case of pigeonpea, molecular markers (RFLPs) were used as early as 1994 to study genetic diversity in wild species using nuclear DNA probes (Nadimpalli et al., 1994). Subsequently, Ratnaparkhe et al. (1995) attempted to study DNA polymorphism in cultivars and wild species. The level of polymorphism among the wild species was extremely high, while little polymorphism was detected within *C. cajan* accessions. In order to characterize a few putative cytoplasmic male sterility lines, maize mitochondrial DNA (mt DNA) specific probes were used in RFLP analysis (Sivaramkrishnan et al., 1997). Recently, AFLP analysis was carried out with a few cultivars and two wild species (*Cajanus volubilis*, *Rhynchosia bracteata*)

using two *EcoRI* and 14 *MseI* primers (Punguluri et al., 2006). The two wild species shared only 7% bands with the pigeonpea cultivars, whereas 87% common bands were seen among cultivars. The cluster analysis revealed low polymorphism among pigeonpea cultivars and very high polymorphism between cultivated pigeonpea and its wild relatives. Similar results were obtained in a very recent analysis using DArT markers (Yang et al., 2006).

In terms of development of SSR markers, about 10 SSR markers are available in public domain (Burns et al., 2001). To develop a resource of microsatellite markers for pigeonpea, primer pairs were generated for 39 microsatellite loci at ICRISAT. These markers (19 polymorphic loci) yielded an average of 4.9 alleles per locus while the observed heterozygosity ranged from 0.17–0.80 with a mean of 0.42 per locus (Odeney et al., 2007). However, to the best of our knowledge, there is no report on any genetic mapping in pigeonpea. In collaboration with ICRISAT, some efforts are underway to develop the first generation map for pigeonpea based on DArT markers at DArT Pty. Ltd. (A. Killian, pers. commun.).

4.2. Trait Mapping and Marker-Assisted Selection

Marker-assisted selection (MAS) offers great promise for improving the efficiency of conventional plant breeding. Molecular markers are especially advantageous for traits where conventional phenotypic selection is difficult, expensive, or lacks accuracy or precision. Molecular mapping and identification of molecular markers associated with genes and QTLs for traits are prerequisites for the MAS. As mentioned above, though not excellent, some progress has been made in the area of development of molecular markers or construction of genetic maps in chickpea and groundnut. As a result, molecular markers linked to a few abiotic or biotic stress tolerance/resistance as well as agronomic traits have been identified recently.

4.2.1. Chickpea

Genetic mapping in chickpea has focussed on tagging agronomically relevant genes such as ascochyta blight resistance (Tekeoglu et al., 2002; Udupa and Baum, 2003; Collard et al., 2003; Flandez-Galvez et al., 2003b; Millan et al., 2003; Cho et al., 2004; Iruela et al., 2006), fusarium wilt resistance (Benko-Iseppon et al., 2003; Sharma et al., 2004) and yield-influencing characters such as double podding and other morphological characters (Cho et al., 2002; Rajesh et al., 2002; Abbo et al., 2005; Cobos et al., 2005). Progress in the area of mapping of ascochyta blight resistance has been summarized recently by Millan et al. (2006). Since apparently all major blight resistance QTLs are tagged with SSR markers, pyramiding of resistance genes via MAS should now be feasible and awaits its proof-of-principle. The genetic control of this disease bred into cold tolerant germplasm would be a major breakthrough for yield increases in Mediterranean-type environments in many parts of the world.

In order to address the issue of drought tolerance through molecular markers, more than 1500 chickpea germplasm and released varieties were screened for

drought tolerance at ICRISAT. The most promising drought tolerant variety was ICC 4958 that had 30% more root volume than the popular variety Annigeri (Saxena et al., 1993); therefore, root traits were considered important parameters to improve the drought tolerance (Kashiwagi et al., 2006). Selection for root traits is very difficult, since it involves laborious methods such as digging and measuring root length and density. Molecular tagging of major genes for root traits may enable MAS for these traits and could greatly improve the precision and efficiency of breeding. In this direction, a set of 257 recombinant inbred lines (RILs) was developed from the cross Annigeri \times ICC 4958 at ICRISAT and glasshouse-evaluated to identify molecular markers for root traits. After screening the parental genotypes with over 250 STMS and 100 EST markers and the mapping population with 57 polymorphic markers, a QTL flanked by STMS markers TAA170 and TR55 on LG 4A was identified that accounted for maximal phenotypic variation in root length (33%), root weight (33%) and shoot weight (54%) (Chandra et al., 2004). Genotyping of two other mapping populations (ICC 4958 \times ICC 1882 and ICC 8261 \times ICC 283), which have larger genetic variation than Annigeri \times ICC 4958 with SSR markers is in progress at ICRISAT.

For improving cold tolerance, AFLP markers have been linked to the trait using bulked segregant analysis of F_2 progeny of a cross between the chilling sensitive cultivar Amethyst and the chilling tolerant ICCV 88516 (Clarke and Siddique, 2003). Candidate AFLP markers were converted into SCAR markers (Paran and Michelmore, 1993) to overcome the limitations of the dominant AFLP marker system. The most promising primers were based on a 560 bp fragment containing a simple sequence repeat (3 bp repeat microsatellite) with nine repeats in the susceptible parent and ten repeats in the tolerant parent. The three-base difference was visualised on a vertical acrylamide gel, and was very useful in the selection of chilling tolerant progeny derived from crosses between ICCV 88516 and Amethyst. Unfortunately, there has been no success in applying these SCAR markers to other breeding materials.

In the case of flowering, a major gene (*efl-1*) for time of flowering was reported by Kumar & van Rheenen (2000), and another one (*ppd*) by Or et al. (1999). The latter gene controls time to flowering through photoperiod response (Hovav et al., 2003). Cho et al. (2002) mapped a QTL for days to 50% flowering to LG 3. Another QTL was also located on this linkage group in an interspecific RIL population and explained 28% of the total phenotypic variation (Cobos et al., 2005).

In addition to the above mentioned traits, molecular mapping for other traits is in progress in many laboratories. For instance, SSR-based genotyping and phenotyping of one mapping population (ICCV 2 \times JG 62) is in progress at NIPGR and ICRISAT to identify the molecular markers associated with salinity tolerance.

4.2.2. Groundnut

There are very few genetic maps available based on cultivated groundnut genotypes. The available maps, based on interspecific crosses, will be useful in locating specific

genes of interest in the interspecific crosses and also providing valuable information about genome organization and evolution. However, these markers will be of less value in elite cultivated germplasm, in which very little polymorphism exist.

Although marker-trait association has been little used within *A. hypogaea*, even with the limitations afforded by present technologies, it has much potential for introgressing genes from closely related *Arachis* species into the cultivated genome. For instance, Garcia et al. (1995) showed introgression of genes from *A. cardenasii* into *A. hypogaea* in 10 of 11 linkage groups on the diploid RFLP map developed by Halward et al. (1993). Subsequently, Garcia et al. (1996) used RAPD and SCAR technologies to map two dominant genes conferring resistance to the nematode by using the mapping population derived from the cross *A. hypogaea* x *A. cardenasii*. Burrow et al. (1996) identified RAPD markers linked to nematode resistance in another interspecific cross involving the species *A. hypogaea*, *A. batizocoi*, *A. cardenasii* and *A. diogeni*. Such linkage of RAPD markers with components of early leaf spot and corn rootworm resistance was shown in another interspecific cross (Stalker and Mozingo, 2001). By using the BSA approach with an F₂ population derived from the cross (ICG 12991 × ICGVSM 93541) and phenotyping the F₃ population, twenty putative AFLP markers were identified of which 12 mapped to five linkage groups. Interestingly, mapping of a single recessive gene on linkage group 1 (3.9 cM from a marker originating from the susceptible parent) explained 76% of the phenotype variation for aphid resistance. AFLP markers were used to establish marker-trait association for tomato spotted wilt virus resistance in groundnut (Milla 2003). Marker-trait association studies for several other traits, e.g. water use efficiency (WUE), rust and late leaf spot (LLS) are underway at ICRISAT and UAS Dharwad.

4.2.3. *Pigeonpea*

Higher level of heterogeneity and very low level of genetic variation in cultivated pigeonpea has hampered development of genetic maps and marker-trait association analysis. Recently, the use of RAPD markers through BSA approach showed association of two RAPD loci with fusarium wilt resistance (Kotresh et al., 2006). It is anticipated that development of higher number of polymorphic SSR markers and DArT arrays (A. Killian, pers. commun.) in pigeonpea will facilitate trait mapping in the near future.

5. NOVEL GENETIC AND GENOMICS APPROACHES

New technologies promise to resolve constraints that have been limiting the impact of linkage based molecular mapping. Such modern genomics approaches have been used in some cereal and other plant species, and legume improvement can be benefited by exploring such approaches.

5.1. Association Mapping and Advanced Backcross QTL (AB-QTL) Analysis

In general, a low level of polymorphism has been a major constraint in developing genetic maps in the legume crops mentioned in this chapter. Further, species like pigeonpea, which is of regional importance in Asia and Africa, has not been explored at the international level. Non-availability of resistance sources in cultivated gene pools of these species for several fungal and viral diseases, e.g., pod borer in chickpea and pigeonpea, sterility mosaic in pigeonpea, aflatoxin in groundnut, and the difficulties of crossing cultivated species with wild species are other barriers that hampered the development of appropriate mapping populations in these legume species. Novel approaches, based on classical genetics, like linkage disequilibrium (LD) based association mapping (Hirschhorn and Daly, 2005), advanced back-cross QTL (AB-QTL) analysis (Tanksley and Nelson, 1996) offers the possibility to overcome at least a few barriers. For instance, an appropriate natural population, genebank or breeding material may be used in LD-based association analysis. In this regard, emergence of novel marker systems such as SNPs and DArTs and developments in this direction for the mentioned legume species would make it possible to undertake candidate gene sequencing (using SNP assays) as well as whole genome scanning (using DArTs) based approaches for association analyses. In contrast to the numerous linkage disequilibrium (LD) studies in human and other mammals, there are very few publications on this topic in agriculturally important crops including legumes (Virk et al., 1996; Beer et al., 1997; Pakniyat et al., 1997; Forster et al., 1997; Igartua et al., 1999; Remington et al., 2001; Thornsberry et al. 2001; Turpeinen et al. 2001; Hansen et al. 2001; Sun et al. 2001, 2003; Skot et al., 2002; Ivandic et al., 2002, 2003; Amirul Islam et al., 2004; Zhu et al., 2003; Simko et al., 2004). Traditionally the plant community has been reticent to use LD mapping believing that it can lead to spurious and non-functional associations due to mutation, genetic drift, population structure, breeding systems and selection pressure (Hill and Weir, 1994; Pritchard et al., 2000). However, most of these limitations are being overcome in recent mammalian studies by following precautions that minimize circumstantial correlations and maximize the accuracy of association statistics (Yu et al., 2006; Yu and Buckler, 2006; Ersoz et al., 2007). Unfortunately the real value of LD mapping in legume species remains to be demonstrated as most of the reports to date are based on small population sizes or a limited number of markers and generally lack validation.

Advanced-backcross QTL analysis (AB-QTL), proposed by Tanksley and Nelson (1996), involves transferring the QTLs of agronomically important traits from a wild species to a crop variety. In this approach, a wild species is backcrossed to a superior cultivar with selection for domestication traits. Selection is imposed to retain individuals that exhibit domestication traits such as non-shattering. The segregating BC_2F_2 or BC_2F_3 population is then evaluated for traits of interest and genotyped with polymorphic molecular markers. These data are then used for QTL analysis, potentially resulting in identification of QTLs, while transferring these QTLs into adapted genetic backgrounds. The AB-QTL approach has been

evaluated in many crop species to determine if genomic regions (QTLs) derived from wild or unadapted germplasm have the potential to improve yield (for a review, see Varshney et al., 2005). However, the wild species chromosome segments masked the magnitude of some of favourable effects that were identified for certain introgressed alleles (Septiningsih et al., 2003). Thus, yield promoting QTL did not have a substantial contribution to the phenotype and the best lines were inferior to commercial cultivars in some studies. In tomato, however, the pyramiding of independent yield promoting chromosome segments resulted in new varieties with increased productivity under normal and stress conditions (Wang D. et al., 2004). One disadvantage is that the value of the wild accession for contributing useful QTL alleles is unknown prior to a major investment in mapping. Nevertheless, the approach holds a great potential to harness the potential of wild species for crop improvement in case of legume species where only low level of genetic variation and source of resistance/tolerance to biotic/abiotic stresses exist in the cultivated gene pool.

5.2. Transcriptomics and Functional Genomics

Functional genomics has revolutionized biological research and is predicted to have a similar impact on plant breeding through the evolution of marker-assisted to genomics-assisted breeding (Varshney et al., 2005). The salient challenge of applied genetics and functional genomics is the identification of the genes underlying a trait of interest so that they can be exploited in crop improvement programmes. Among legume species, much work in terms of development of functional genomics resources such as ESTs, genome sequencing, array development has been done either in model species like lotus (*Lotus japonicus* L.) and medicago (*Medicago truncatula* L.) or major species like soybean. In contrast, only a limited number of ESTs have been generated so far in legume species of SAT (Table 1). These ESTs can be used to develop the molecular markers as shown in chickpea (Buhariwalla et al., 2005) and groundnut (Luo et al., 2005) as well as to develop cDNA arrays. At NIPGR, the chickpea ESTs are being developed from seeds (both developing and maturing) and symbiotic root nodules in association with *Mesorhizobium ciceri*. So far about 1000 seed specific unigenes have been identified (unpublished results). The most striking feature of these ESTs is that, majority of them are putative or unknown proteins. The use of suppression subtractive hybridization (SSH) to prepare the subtracted cDNA library of 7-day old symbiotic root nodules lead to the identification of three putative genes regulated during symbiotic relationship with *M. ciceri*. Further validation with Northern analysis has lead to the identification three putative genes up-regulated during symbiotic association in a temporal manner.

The macro- and micro-arrays based on EST/gene sequence information have been successfully utilized in many plant species for understanding the basic physiology, developmental processes, environmental stress responses, and for identification and genotyping of mutations. Recently in chickpea, a small array with 768 features has been developed (Coram and Pang, 2005a) that has been used to identify genes

responsible for ascochyta blight resistance (Coram and Pang, 2005b, 2006), drought and salinity tolerance (E. Pang, pers. commun.). The candidate genes identified by EST sequencing (and gene prediction) and functional genomics approaches can be further verified through real time PCR analysis (Luo et al., 2005) and genetical genomics/ expression genetics approaches (Jansen and Nap, 2001; Varshney et al., 2005) after conducting gene expression analysis in quantitative fashion using segregating mapping populations. By analyzing the expression levels of genes or clusters of genes within a segregating population, it is possible to map the inheritance of that expression pattern. The QTLs identified using expression data in a mapping population are called e(xpression)QTLs. The eQTLs can be classified as *cis* or *trans* acting based on location of transcript compared to that of the eQTL influencing expression of that transcript (de Konig and Haley, 2005). Because of this feature, eQTL analysis makes it possible to identify factors influencing the level of mRNA expression. The regulatory factor (second order effect) is of specific interest because more than one QTL can be putatively connected to a *trans*-acting factor (Schadt et al., 2003). Thus, the mapping of eQTLs allows multifactorial dissection of the expression profile of a given mRNA or cDNA, protein or metabolite into its underlying genetic components as well as localization of these components on the genetic map (Jansen and Nap, 2001). In recent years, in many plant species, the genetical genomics approach has demonstrated its power (see Kirst and Yu, 2007).

Another powerful approach of gene discovery is 'Serial Analysis of Gene Expression (SAGE)' (Velculescu et al., 1995) that utilizes the advantage of high-throughput sequencing technology to obtain a quantitative profile of gene expression which measures not the expression level of a gene, but quantifies a 'tag' which represents the transcriptome product of a gene. A tag for the purpose of SAGE, is a nucleotide sequence of a defined length, directly adjacent to the 3'-most restriction site for a particular restriction enzyme. The data product of the SAGE technique is a list of tags, with their corresponding count values, and thus is a digital representation of cellular gene expression. Based on the length of tags, several modified forms of SAGE, e.g., MicroSAGE, MiniSAGE, LongSAGE and SuperSAGE, have been developed (Sharma et al., 2007). In fact, by using SuperSAGE methodology, over 220,000 SuperTags describing the differential transcription profiles of chickpea roots and nodules have already been sequenced at University of Frankfurt (G. Kahl, pers. commun.). Targeted gene-expression chips are being developed by adding SuperTag oligonucleotides derived from the most informative genes expressed differentially under stress- and non-stress conditions and from large-versus small root systems to a gene expression chip (P. Winter, pers. commun.).

In groundnut, recent activities in the area of functional genomics have produced a gene chip with 400 unigenes after cluster analysis of 1825 ESTs and used for identifying the genes associated with disease resistance and drought tolerance (Luo et al., 2003, 2005). Further to validate the microarray and EST data by EST-discovery, real-time PCR analysis was conducted for 10 specific genes (Luo et al.,

2005). The use of suppression subtractive hybridization (SSH) to prepare the subtracted cDNA libraries and identify the genes regulated during interaction with the fungus *Cercosporidium personatum* (causing the disease late leaf spot) is in progress in Brazil (Nobile et al., 2006). To understand the molecular mechanisms of drought tolerance, the use of differential expression of mRNA transcripts and proteins are underway at Florida A & M University (Katam et al., 2006). With the development of more functional genomics resources in SAT legumes, it is anticipated that the use of functional genomics and expression genetics approaches may help the community to dissect the complex traits and devise strategies for crop improvement.

5.3. Comparative Genomics

In recent years, the availability of ESTs and genome sequence data for model legumes i.e. medicago (*M. truncatula*), and lotus (*L. japonicus*) and major crop legumes like soybean has opened the possibilities of transfer of information from model to crop legumes and vice-versa (Gepts et al., 2005, Young et al., 2005). Identification of putative orthologs from related genomes will facilitate comparative genomics and comparative genetic mapping. Using 274 unique low copy gene specific markers from *M. truncatula* and *G. max*, Choi et al. (2004, 2006) have demonstrated that gene-specific markers are transferable across *Papilionoid* legume species may find utility in phylogenetic relationship assessment at different, but overlapping, taxonomic levels. Moreover, majority of these markers (85.3%) are also linked to the legume genetic maps. Similarly, Gutierrez et al. (2005) have studied the conservation of 209 EST-SSR markers from the model legume *M. truncatula* in three major European crop legumes i.e. faba bean (*Vicia faba*), pea (*Pisum sativum*) and chickpea and have reported 36%–40% transferability range for this class of markers. Recently, extensive efforts have been made to develop bioinformatics tools and pipelines after exploiting the genomics resources of model species as well as other legume species and as a result about 450 cross species markers have been developed (Fredslund et al., 2005, 2006a, 2006b). For many markers, the map position in lotus and/or medicago is known and in other legume species such as groundnut, soybean, chickpea, these markers are being mapped. These studies will provide more anchor points to relate different legume genomes. Moreover, the identification of the cross-genera transferable legume SSR markers will cut down the cost and labor associated with development of SSR markers in the orphan legumes and will help in comparative mapping and map-based cloning of orthologous genes. Since the EST-SSR markers reveal very less polymorphism in legumes (Gutierrez et al. 2005), the alternative source is the genome specific genomic SSRs. By virtue of their long polymorphic microsatellite repeat stretches and the variable microsatellite flanking region, the genomic microsatellites are a promising source of cross-transferable markers in self-pollinating legume species (Sethy et al., manuscript in preparation). The levels and patterns of conservation of

Cicer genomic SSR markers across model, crop and fodder legumes have demonstrated that the genomic SSRs find a mean average transferability of nearly 25% across *M. truncatula*, *L. japonicus*, soybean, pea, lentil, pigeonpea, blackgram, mungbean and *Trifolium alexandrinum* (Figure 1) and often conserved in the model plant *A. thaliana*. Moreover, the *Cicer* markers have been demonstrated to be

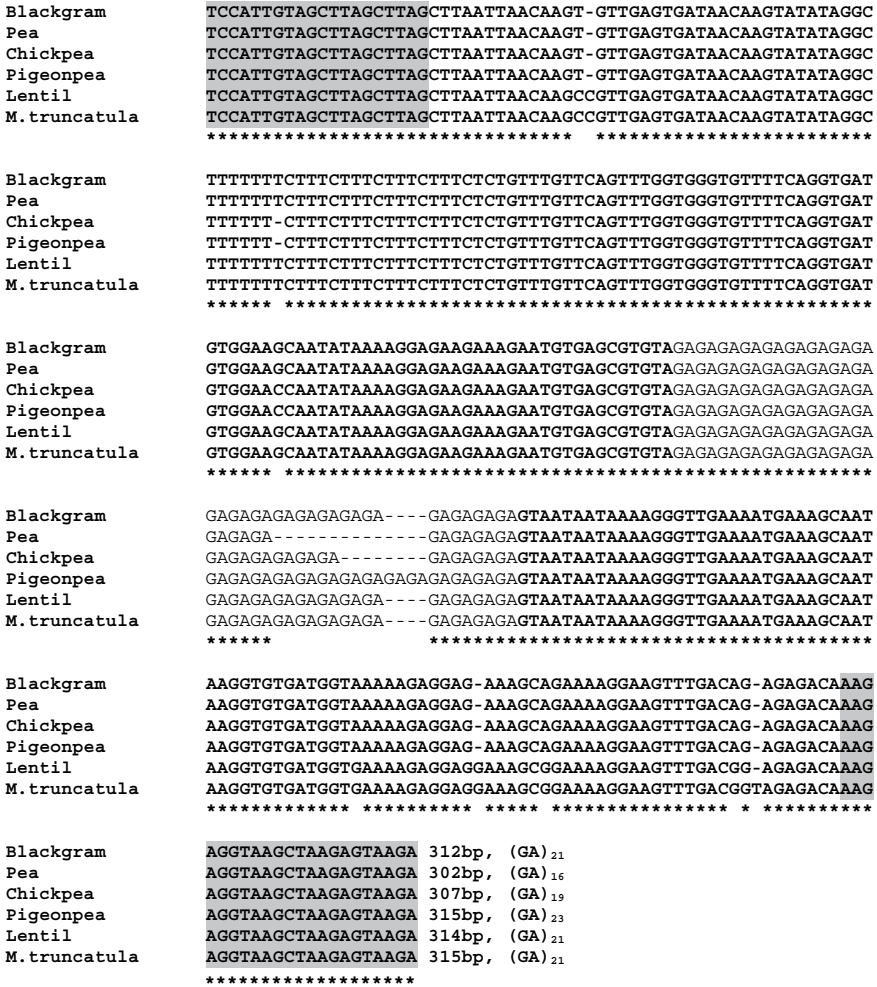


Figure 1. Multiple sequence alignment of the size variant alleles of the legume accessions at the chickpea STMS marker NIPGR19 locus. Accessions of *M. truncatula* (SA27783), blackgram (IC342955), lentil (IC383669), pea (RFP16) and pigeonpea (IC347150) along with chickpea (Pusa362) are analyzed. The asterisks indicate similar sequences and ‘-’ indicate alignment gaps. The repeat region is indicated in boldface and shadowed boxes indicate conserved primer binding sites. Allele sizes and repeat motifs are mentioned at the end of the sequence

polymorphic even within *M. truncatula*, soybean and blackgram opening the possibility of comparative mapping and generation of a consensus legume genetic map (Sethy et al., unpublished results).

6. TOWARDS A BRIGHT FUTURE OF MOLECULAR BREEDING IN SAT LEGUMES

Traditional cropping systems across the world have depended on the rotation of cereal and legume crops. However, with increasing intensification of agriculture during the twentieth century, there has been a substantial emphasis on cereals as the pre-eminent food commodity in national production and international trade. In turn, this has been reflected by a continuous and cumulative increase in funding for research and breeding of cereal crops (Goff and Salmeron, 2004) that has resulted in the state-of-the-art in legumes falling further and further behind. Nevertheless, progress in the genomics of two legume species, medicago and lotus, as model genomes offers the potential for real technological leap-frogging amongst legume crops.

Although during the past few years, significant progress has been made in the area of genomics of SAT legume crops as a large number of molecular (SSR) markers and ESTs have been developed, there is still a need to develop more SSR, SNP or DArT markers and dense genetic maps for the species mentioned in this chapter. Further the generation of some BAC and BIBAC libraries in case of chickpea and groundnut offers the possibility to develop genome wide or local physical maps to isolate genes for resistance/tolerance to biotic/abiotic stresses as well as agronomic traits (Yuksel et al., 2005). Thus molecular breeding through existing tools in combination with continuous incremental changes such as improvements in genetics and biometrics, plus revolutionary changes including automation of breeding trials and computerization of phenotyping will be very useful for legume improvement (Dwivedi et al., 2006). In addition to linkage based trait mapping, several other approaches such as LD-based association mapping, AB-QTL analysis, transcriptomics and functional genomics can be used to identify the molecular markers or candidate genes for traits of interest in breeding. Beyond its increased power of selection, marker or genomics-assisted breeding offers additional advantages in the economics of scale both in terms of cost and time as very different traits can be manipulated using the same technology. The proof-of-function of candidate genes can be obtained by using TILLING (Targeting Induced Local Lesions In Genomes, see Till et al., 2007) population, while the EcoTILLING approach may be used for allele mining to improve the traits. Allele mining for candidate genes should provide superior alleles and haplotypes for the traits (Varshney et al., 2005).

Recent studies show strong correlation between the degree of synteny and phylogenetic distance in legumes (Young et al., 2003; Wang M.L. et al., 2004; Choi et al., 2004). Therefore, advances in the area of genomics of medicago and lotus may be used to transfer information on genes involved in nitrogen fixation and other physiological processes of agronomic importance in SAT legume crops by utilizing

the comparative genomics approach combined with bioinformatics. However, the extent to which genetic knowledge from model systems will readily translate into economic impact in related crops remains to be empirically demonstrated (Thro et al., 2004; Koebner and Varshney, 2006). Genomics research in the legume crops together with model systems will soon routinely define the location of genomic regions controlling a target trait as well as identify underlying candidate genes and their sequences through mapping, mutation analysis and transcriptomics. Based on this new knowledge it will be possible to develop highly precise DNA markers for selection or introgression of desired traits. While the newly developed genetic and genomics tools will certainly enhance the prediction of phenotype, they will not entirely replace the conventional breeding process.

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CHAPTER 11

GENOMICS APPROACHES TO SOYBEAN IMPROVEMENT

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Abstract: Soybean, *Glycine max* (L.) Merr., has become the major source of edible vegetable oils and high protein feeds for livestock in the world. A native of Eastern Asia, soybean was introduced into the USA and South America where it has become the most economically important agricultural crop and export commodity. In recent years, as demand for soybean increased due to the values of seed oil and protein, as well as industrial and nutraceutical uses, it has received more attention by scientists aiming to the development and employment of genomic technology for soybean improvement. Several DNA marker systems, such as restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR), and single nucleotide polymorphism (SNP), were integrated into the soybean genetic linkage map, which has been successfully utilized for mapping quantitative trait loci (QTL) linked to desirable traits and marker-assisted breeding of disease resistance and seed composition. The availability of a large number of expressed sequence tags (EST) and BAC sequences facilitated the discovery of new SNP and SSR markers in soybean toward the construction of high resolution genetic maps. Integrated genetic and physical maps will provide an invaluable resource for gene identification and positional cloning of important quantitative trait loci in soybean. Functional genomics has emerged as a new and rapidly evolved discipline to identify and understand gene functions via an integrated approach which includes transcriptomics, proteomics, metabolomics, translational genomics, and bioinformatics. The completion of whole soybean genome sequencing is anticipated in a few years. The availability of the soybean genome sequences in combination with the integrated genetic and physical maps will be invaluable resources providing soybean researchers powerful and efficient genomic tools to identify and characterize genes or QTLs for agronomic traits of soybean. As a result, it facilitates marker-assisted breeding and soybean improvement.

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1. INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is an important leguminous crop producing the most valuable source of protein and vegetable oil closest to the optimum dietary essential amino acids profiles for human and animal nutrition (Lusas 2004). Domestication of the crop likely occurred between the 11th and 15th century BC, or perhaps earlier, from northeast China, and subsequently reached China's southern region and neighboring countries such as Korea, Japan, Thailand, the Philippines, etc. (Hymowitz 2004). It was not until the 18th century when soybean was introduced into Europe. In the western hemisphere, soybean was first brought into North America in 1765 and then into Central and South America during the mid 1900s (Hymowitz 2004). Since then, soybean has become a major economic crop for soybean producing areas.

Worldwide, the expansion in soybean production is likely due to the increased value of protein and oil in soybean seeds. Among over 19,000 soybean accessions of USDA-ARS Soybean Germplasm Collection (<http://www.ars-grin.gov/cgi-bin/npgs/html/stats/genus.pl?Glycine>), it was reported that soybean has a wide range of genetic variation in protein and oil contents. The range of protein content is from 34.1 to 56.8% of dry seed mass, with a mean of 42.1%, while the range of oil content is 8.3 to 27.9%, with a mean of 19.5%. Between these two seed composition traits, there exists a widely known, strong negative correlation (Hurburgh et al. 1990), indicating that efforts to improve protein content often results in decreased oil content, and vice versa. It is extremely rare to find germplasm in which the content for both protein and oil is relatively high (Wilson 2004). Thus, soybean workers who have extended great efforts to improve these important seed traits normally accept a sacrifice of one trait while attempting to improve the other.

The soybean production in the world has been steadily increasing for the last ten years, rising from 127 million metric tons in 1995 to over 209 million metric tons in 2005 (FAO, 2006, <http://faostat.fao.org>). Among the countries growing soybean, the United States led production at 82.8 millions tons in 2005, equaling approximately 40% of the world's total soybean production. The other two major soybean producing countries exist in South America. Brazil and Argentina produced 50 and 38 million tons, respectively, and accounted for approximately 42% of the world's total production in 2005 (www.soystats.com). It is noted that although the soybean is a native of China and has been widely utilized as valuable protein source in people's diets in many other countries of Asia, soybean production in this area was only 11% of the world total. A 2005 report indicated that China produced 17 million tons (8% of the world total) with an average seed yield of 1.78 t/ha and India produced 6.6 million tons (3% of the world total) with an average yield of 0.96 t/ha (FAO, 2006, <http://faostat.fao.org>). In the European Union and Eastern Europe, the two largest soybean producers are Italy and Russian Federation. Each produced less than a half million tons, or 0.5% of the world total.

Since domestication, soybean seeds have been an essential and dominant protein source for human nutrition in Asian countries. Several nutritious foods were

developed from soybean. Among these, miso, soybean sauce, soybean milk, tempeh, and tofu were the most traditional soybean-based foods (Hymowitz 2004). In recent years, efforts have been made to validate nutritional and pharmaceutical values of soybean protein. In nutrition studies, Birt et al. (2004) reported that consumption of soybean-based foods reduces cancer, blood serum cholesterol, osteoporosis and heart disease. Thus, soybean products have been recommended as a source of supplemental protein for the human diet in the United States (<http://www.soyfoodsillinois.uiuc.edu>). Besides human consumption, soybean is also an important protein source for animal feed. In 2004, soybean provided the source for 67% of the world's protein meal (Lucas 2004). In the USA, protein meal production has steadily increased over the last ten years (www.soystats.com) due to increasing demand of soybean meals for the livestock and poultry industries.

Among oilseed crop plants such as peanut, rapeseed, and sunflower, soybean provided 57% of the world's total oilseed production (www.soystats.com). Preference for soybean oil may largely be due to the fact that soybean seed has a different composition than other legumes having relatively high levels of protein and oil and lower overall carbohydrate content. The oil portion of soybean is composed primarily of five fatty acids. Palmitic and stearic acids are saturated fatty acids and comprise 15% of the oil. In addition, soybean oil is rich in unsaturated fatty acids such as oleic, linoleic, and linolenic, which make up 85% of the oil in seed. Soybean is also a good source of the minerals, vitamin B, folic acid, and isoflavones which are attributed to slowing cancer development, heart disease, and osteoporosis (Wilson 2004).

In addition to valuable protein and oil source for human foods and animal feed, soybean also has numerous industrial uses and applications. Since the 1920s many US laboratories and universities have investigated potential industrial applications of soybean meals and oil. A large spectrum of soybean-based industrial uses have been adopted, for instance, building and construction materials, cleaners, concrete, plastics, paper, engine oils, printing inks, paints, lubricants, hydraulic fluids, pesticides, cosmetics, pharmaceuticals (Lusas 2004). In particular, since the late 1970s fatty acids in soybean oil were employed for biodiesel development, in which over 113 millions liters were consumed in the US in 2004 (www.soystats.com).

2. MOLECULAR MARKER SYSTEMS AND GENOMIC MAP

2.1. Taxonomy and Cytogenetics

Soybean was first described by Linnaeus (1737) as *Phaseolus max* based on his specimen and *Dolichos soja* based upon descriptions of other writers. Several years later, he realized that these two designations were actually the same plant. Since then, the correct nomenclature of soybean has been a subject of long debates among plant taxonomists (Hymowitz and Singh 1987). After thoroughly re-examining specimens of the soybean, Merrill (1917) proposed *Glycine max* as a new designation for soybean and this nomenclature has to date been widely

accepted. The genus *Glycine* is divided into two subgenera *Glycine* (wild perennials) and *Soja* (annuals).

Perennial subgenus *Glycine* has 22 species with chromosome number ($2n$) varying from 40 to 80 and geographical distribution mainly in Australia and neighboring islands, such as Indonesia, Papua New Guinea (Hymowitz 2004). Of these, species *G. tomentella* with four cytotypes ($2n = 38, 40, 78, 80$) and widely distributed in Asia and Australia has been intensively employed in soybean breeding programs due to valuable gene resources and cross-compatibility (Singh and Hymowitz 1999).

Annual subgenus *Soja* has two species, wild annual soybean *Glycine soja* and cultivated soybean *Glycine max*. Both species have the same chromosome number ($2n = 40$) and genome designation (GG) (Hymowitz 2004). Ohashi et al. (1984) proposed the scientific name of the wild annual soybean to be changed to *G. max* (L.) Merr. subsp. *soja* (Sieb. and Zucc.) Ohashi. Yet, the proposal in new nomenclature has not been popularly adapted. The US National Plant Germplasm System (USDA-GRIN, <http://www.ars.grin.gov>) and Hymowitz lab still remain the original nomenclature, *G. soja*, for this wild annual soybean (Hymowitz 2004).

The cultivated soybean, *G. max*, is a true domesticate and morphologically extremely variable in leaf shape, flower color, seed coat color, seed size and weight. This diversity of the cultivated soybean is perhaps due to the development of land races taking place for thousand years in East Asia. These land races containing specific traits have been grown by individual farmer families for generations for various uses including food, animal feed, and medicinal herbs. Today, these land races provide invaluable sources of generic diversity within soybean germplasm collections (Hymowitz 2004).

In addition to these two annual soybean species, a third form known as *G. gracilis* has been proposed (Skvortzow 1927) and a subject of long debates. Many studies showed that this new proposed species was a variant of *G. max* (Hermann 1962; Shoemaker et al. 1986) based upon the fact that there was no cross-ability barrier among *G. soja*, *G. max* and *G. gracilis*; hybrid seeds germinated normally; and F1 plants were totally pollen- and seed-fertile (Singh and Hymowitz 1989). Subsequently, Wu et al. (2001) used SSR markers to evaluated genetic diversity among *Glycine* species and suggested that *G. gracilis* should be a separate species from *G. max*. However, neither the International Legume Database (ILDIS, <http://www.ildis.org>) nor USDA-GRIN recognizes *G. garcillis* as a separate species.

Both cultivated soybean and its annual relative, *G. soja*, have a diploid chromosome number of $2n = 40$. However, Hymowitz et al. (1998) reported most genera in the *Phaseolae* tribe have a genome complement of $2n = 22$, suggesting that *Glycine* was probably derived from a diploid ancestor with $n = 11$. It was believed that through thousands of years of evolution aneuploid loss occurred, yielding $n = 10$, followed by polyploidization events caused by additions, deletions, mutations, and rearrangements to generate a current diploid number of $2n = 40$ (Lackey 1980). In spite of polyploidization most regions of soybean genome act like diploid. By using primary trisomics approach, in which one additional chromosome of genome, $2x+1=41$, was generated for each of 20 chromosomes, soybean

cytogeneticists were able to locate genes onto a specific chromosome and associate molecular linkage groups (MLGs) with specific chromosomes. With a subset of 20 trisomics (Xu et al. 2000) and SSR markers, Zou et al. (2003) assigned 11 LGs to respective soybean chromosomes. However, this approach has encountered difficulty in making crosses, generating a large numbers of hybrids, counting and identifying chromosomes. It is anticipated that with bacterial artificial chromosome (BAC) clones and fluorescent in situ hybridization (FISH) approach (Hans and Jackson 2006), existing MLGs can soon be assigned to 20 corresponding chromosomes of soybean.

The soybean genome consists of ~1100 Mbp and is relatively larger than the genome of *Arabidopsis* and rice, but much smaller than the genome of maize and barley (Arumuganathan and Earle 1991). It is believed that due to the polyploidization event(s) occurring in an ancient ancestor, the soybean genome has a high percentage of internal duplication regions distributed among the chromosomes (Pagel et al. 2004). When using RFLP probes in a mapping study, Shoemaker et al. (1996) reported more than half of the RFLP probes detected multiple hybridizing fragments, indicating the ancient polyploid nature and duplication segments in the soybean genome. With the construction of BAC clones and the FISH technique, Hans and Jackson (2006) were able to anchor BAC clones to soybean linkage groups and identify duplicated regions of the soybean genome. However, the polyploid nature and extent of segmental duplications in the soybean genome result in a number of drawbacks for efforts to conduct whole genome sequencing and assembly due to repetitive DNA sequences.

2.2. Molecular Marker Systems

Development of new soybean varieties through conventional breeding is time and labor intensive and also takes large amounts of space in the greenhouse and field for evaluation. These constraints limit the number of plants which can be evaluated in the field each season and it takes about five to eight years to develop a variety. Breeders select traits of interest based on phenotypic performances and most of the traits are influenced by the environment. Dekkers and Hospital (2002) mentioned that selection based on genotype will greatly increase breeding efficiency. Plant breeders are interested in new technologies that are cost effective and improve efficiency of crop breeding. With the advancement of molecular marker technology, selection is based on DNA markers which are tightly linked with a trait(s)/gene(s) of interest rather than phenotype alone. Molecular markers have complemented the traditional breeding and have increased efficiency and reliability in breeding programs.

During the last decade, development of several molecular marker systems facilitated identification of genetic variation present in genomic DNA sequences of various crop plants, including soybean. Among these systems, restriction fragment length polymorphism (RFLP) (Botstein et al. 1980), random amplified polymorphic

DNA (RAPD) (Williams et al. 1990; Welsh and McClelland 1990), DNA amplification fingerprinting (DAF) (Caetano-Anolles et al. 1991), amplified fragment length polymorphism (AFLP) (Vos et al. 1995); and simple sequence repeat (SSR) or microsatellites (Litt and Luty 1989) are most commonly employed in soybean molecular genetic analysis. Recently, significant efforts are directed forward SNP marker development.

2.2.1. *Restriction fragment length polymorphism*

As the first generation of molecular markers to be used in animal and plant molecular genetic mapping, RFLPs are caused by changes in DNA sequence of genome, such as mutations, insertions, or deletions of DNA fragments. These changes result in the gain, loss, or movement of some restriction sites, which is the basis for generating RFLPs. These DNA markers are bi-allelic codominant; thus, a unique locus and chromosomal position can be precisely identified with a specific probe that could be either genomic DNA fragment, cDNA, or expressed sequence tags (ESTs). RFLP markers are robust and accurate markers for genomic genotyping; this genotyping technique, however, is a labor-intensive and expensive procedure. Therefore, many improvements have been made for the RFLP analysis to be a cost-efficient, high-throughput approach (Nguyen and Wu 2005).

In soybean, RFLPs were first utilized as DNA probes to differentiate soybean cultivars (Apuya et al. 1988, Keim et al. 1989). Later, Keim et al. (1990) published the first RFLP-based map of the soybean genome using an F₂ mapping population derived from a cross of *G. max* (A81-356022) and *G. soja* (PI468916). The mapping work was subsequently expanded by Shoemaker and Olson (1993) with the addition of 355 RFLP loci. In a separate investigation, Rafalski and Tingey (1993) mapped more than 600 RFLP loci in a different cross of cultivated soybean and wild soybean. It was reported the level of RFLP polymorphism was relatively low, 33%, despite the wide cross of cultivated and wild soybean employed (Shoemaker et al. 2004). Moreover, the duplicated nature of the soybean genome is an additional factor confounding genotype analyses in soybean. Despite complications, a number of studies were conducted using RFLP markers to characterize genetic variation in soybean germplasm and map quantitative trait loci (QTL) for agronomic traits (Keim et al. 1990; Concibido et al. 1996; Lee et al. 1996; Mian et al. 1998). To date, 709 RFLP loci were mapped to 20 MLGs of soybean genome (Song et al. 2004).

2.2.2. *Random amplified polymorphic DNA*

The advent of the polymerase chain reaction (PCR) technology facilitated the subsequent development of many PCR-based DNA marker systems for genetic mapping and DNA fingerprinting. Among these, RAPD marker was the pioneer of the second generation of PCR-based molecular markers. The assay requires no prior knowledge of DNA sequence for detection of genetic variation and amplified fragments can be simply separated in agarose gel electrophoresis. For PCR amplification, a single arbitrary primer is added to a reaction. The primer randomly anneals to homologous sequences in the genome. Amplicons throughout the genome are

targeted and amplified if the primer also anneals to sequences on complementary strands not far from the 3' end of the other primers (Welsh and McCelland 1990). Because the primers are relatively short in sequence (10 nucleotides), they possibly hybridize to genomic sites at which they fortuitously match or almost match; as a result, multiple products with relatively complex pattern are produced under conditions of low stringency with a single random primer. However, the RAPD assay has disadvantages in the procedure. For instance, there is a problem of reproducibility within and between laboratories over time, making the marker information difficult to share and repeat (Nguyen and Wu 2005). With these drawbacks, RAPD has not been widely employed for genetic mapping and genomic fingerprinting. In soybean, there have been attempts to use RAPD assay for construction of genetic map. Ferreira et al. (2000) incorporated 106 RAPD markers into a framework of 250 existing RFLP loci using recombinant inbred lines from a cross of cultivated soybeans.

2.2.3. *Amplified fragment length polymorphism*

Combining the advantages of RFLP analysis and PCR technology, the AFLP assay was developed (Vos et al. 1995) and widely used for genetic map and genomic fingerprinting. Like RAPD markers, the AFLP assay requires no prior knowledge of genomic DNA sequence. Restriction sites of genomic DNA are digested with two restriction endonucleases to generate restriction fragments. The DNA adaptors are ligated to the ends of the restriction fragments and PCR amplifications are performed to amplify selected subpopulations of the pool of fragments. Selectivity results from the addition of two or three arbitrary nucleotides to the 3' ends of the PCR primers. Between these primers, the forward primer is labeled with radioactive ^{33}P . The amplified restriction fragments can be separated on denaturing polyacrylamide gels and visualized by means of autoradiography (Vos et al. 1995) or silver staining (Chalhoub et al. 1997). When PCR products are labeled with different fluorescent dyes, the amplified restriction fragments can be visualized with automated DNA sequencer (Myburg et al. 2001).

In soybean, AFLP markers were used to construct a genetic map using a subset of 42 RILs from a cross of cultivated soybean (Keim et al. 1997). This map has a total of 650 AFLP loci mapped to several linkage groups. One of the drawbacks of AFLP markers is the clustering of high density markers resulted from the use of *EcoRI/MseI* restriction enzymes (Young et al. 1999). However, marker clustering appeared to be eliminated when AFLP markers were generated with *PstI/MseI* restriction enzymes (Keim et al. 1997). Because the large amount of marker data can be generated with a single AFLP assay without the need for prior knowledge of DNA sequence, AFLPs have proven useful for saturating specific genomic regions using bulked segregant analysis (Michelmore et al. 1991) or for comparison of near-isogenic lines for a trait of interest (Muehlbauer et al. 1991).

2.2.4. *Simple sequence repeat*

Simple sequence repeat or microsatellite markers (Litt and Luty 1989), the most popular PCR-based DNA markers, were developed based upon genetic variation

in the number of repeat units (motifs), which are composed of 1~6-bp short DNA sequences, such as dinucleotide repeats (AT) n or (CT) n , and trinucleotide repeats (ATT) n (Li et al. 2002). It has been shown that the SSR markers were abundant, highly polymorphic, codominant and distinguish multiple alleles within a plant species (Cregan et al. 1999a; Cho et al. 2000; Eujayl et al. 2002; Thiel et al. 2003). These characteristics make SSR technology an ideal marker system for DNA fingerprinting, genetic mapping, studies of genetic diversity, population genetics, and marker-assisted selection (MAS). However, the SSR technology has major drawbacks of high cost of development, prior knowledge of the flanking sequence for designing locus-specific PCR markers. Moreover, the difficulty, which frequently challenges researchers, is to distinguish alleles that differs only one or a few repeat units in size. In order to overcome these technical difficulties, alternative approaches of allelic variation detection are recommended, for instance, radioisotope is used to label one of primers and PCR products are separated in denaturing polyacrylamide gels (Shan et al. 1999). Currently, with the development of fluorescence-based genotyping system, the PCR products that are labeled with different fluorescent dyes can be multiplexed based upon allele size range and dyes for semi-automated detection using capillary electrophoresis DNA sequencer systems.

In soybean, Akkaya et al. (1992) and Morgante and Olivieri (1993) reported the discovery of several SSR markers in a set of 38 *G. max* and five *G. soja*. These authors also demonstrated attractive features of the molecular marker system, such as high levels of polymorphisms, codominant inheritance, and locus specificity, which have been also found in many other plant genomes (Broun and Tanksley 1996; Sanchez de le Hoz et al. 1996; Lu et al. 2005). Subsequently, several reports (Cregan et al. 1994; Maughan et al. 1995; Morgante et al. 1994; Rongwen et al. 1995) detected very high levels of allelic variation, for instance one locus with 26 alleles among groups of 91 cultivated soybean and five wild soybean genotypes. Since then, the SSR markers have provided an excellent complement to RFLP markers for use in soybean molecular biology, genetics, and plant breeding research (Shoemaker et al. 2004). A large set of more than 600 SSR markers were first developed and mapped in an integrated molecular linkage map of soybean (Cregan et al. 1999a). The development of additional SSR markers was subsequently continued and up to date there are more than 1000 SSR loci (Song et al. 2004) were mapped to 20 homologous linkage groups (LGs) presumed to be associated with the 20 pairs of soybean chromosomes (USDA-ARS SoyBase website: <http://soybase.agron.iastate.edu>). With the ongoing efforts in soybean BAC end and whole genome shotgun sequencing, additional SSR markers could be easily identified and mapped.

2.2.5. *Single nucleotide polymorphism*

Recently, a novel molecular marker system was developed and successfully employed in human and mice genomic projects (Collins et al. 1998; Kwok et al. 1996; Lindblad-Toh et al. 2000). This marker system based upon changes in single

Transitional SNP

Wild type: 5' -TGTTATCCAT**C**ACCTTTTCCCTCA-3'
Mutant line: 5' -TGTTATCCAT**T**ACCTTTTCCCTCA-3'

Transversional SNP

Wild type: 5' -AGATTTAATT**A**TGGTAAAACCTAGGA-3'
Mutant line: 5' -AGATTTAATT**C**TGGTAAAACCTAGGA-3'

Indel

Wild type: 5' -GGGCATTCT**TATA**TTCATTCAAATT-3'
Mutant line: 5' -GGGCATTCT . . . TTCATTCAAATT-3'

Figure 1. SNPs and Insertion/Deletion (Indels) are prevalent sequence variations between individuals of all animal and plant species. These variations are consequences of either transitional or transversional mutation events

DNA base between homologous DNA fragments or small insertions and deletions (indels), which was collectively called single nucleotide polymorphisms (SNPs) (Figure 1). The single nucleotide changes were due to point mutations, one of the many types of mutations that naturally occur in genomes with relatively low mutation rates (Kahl et al. 2005). With high level of polymorphism and even distribution across genomes, SNPs are valuable gene-based molecular markers suitable for high-throughput, automated genotyping. The great potential use of SNPs for genetic mapping of complex traits, pharmacogenetics, and medical diagnostics has received much attention in medical sciences (Gu et al. 1998; McCarthy and Hilfiker 2000).

Following remarkable progress made in the human and animal genome projects, SNPs markers have been widely investigated and developed in many important plant genomes, such as *Arabidopsis* (Kawabe et al. 2000; Kuittinen and Aguade 2000), barley (Kanazin et al. 2002; Rostoks et al. 2005), rice (Zhao et al. 2002; Monna et al. 2006), maize (Tenailon et al. 2001; Vroh Bi et al. 2006), sunflower (Lai et al. 2005), and soybean (Zhu et al. 2003). In soybean, SNPs marker development was first reported by Scallan et al. (1987) when comparing 3543 bp of the *Gy4* glycinin locus plus flanking DNA in two genotypes. Since then, several additional SNPs were identified by independent research groups (Xue et al. 1992; Zhu et al. 1995). Recently, when comparing sequences of 143 DNA fragments derived from coding and noncoding regions, which was based upon sequence analysis of 25 diverse cultivated soybean genotypes, Zhu et al. (2003) showed that nucleotide diversity, indicated by θ value, was 0.00053 and 0.00111 in coding and noncoding DNA sequence, respectively. It indicated that SNP frequency in soybean genome was much lower than the similar estimates of *Arabidopsis* ($\theta = 0.0017$) (Olsen et al. 2002) and maize ($\theta = 0.0096$) (Tenailon et al. 2001). It was speculated that low level of linkage disequilibrium coupled with limited haplotype diversity in this investigation suggested that cultivated soybean genome is a mosaic of a

limited number of haplotypes (Zhu et al. 2003). The authors also suggested that SNP discovery focused on noncoding perigenic regions, where greater sequence polymorphism is present, will permit successful SNP diversity in soybean. The results of a subsequent study of SNP discovery in soybean supported the above opinions (Van et al. 2005).

For decades, random sequencing of gene transcripts has been recognized as simple and efficient method of identification of many the expresses genes in an organism (Putnay et al. 1983). Hence, these sequences, known as Expressed Sequence Tags (ESTs), have become valuable and useful source of DNA sequences in which SNPs can be efficiently discovered. In soybean, the Soybean EST Project database contained more than 391,034 publicly available ESTs from 84 libraries (www.ncbi.nlm.nih.gov/) as of August 2006. Employing this DNA sequence resource, Van et al. (2005) identified 97 SNPs present in coding sequence and noncoding regions in 15 soybean genotypes from Korea and the United States and concluded that a diverse set of soybean genotypes can be successfully utilized for SNP discovery.

Many efforts have been made to identify and map SNPs associated with traits of interest in soybean. Using a recombinant inbred line (RIL) population of two Korean soybean genotypes, Kim et al. (2004) identified three SNPs closely linked to lipoxygenase-2 gene (*Lx2*) in soybean seed, which is responsible for generation of grassy-beany and bitter flavors. Jeong and Saghai Maroof (2004) used allelic-specific PCR approach and detected two SNP markers tightly linked to two mosaic virus resistance genes, *Rsv1* and *Rsv3*, in soybean. Using a sequence of the *GmNARK* gene (<http://www.ncbi.nlm.nih.gov/>), which is responsible for supernodulation mutation in soybean, Kim et al. (2005) identified two SNPs for this gene and successfully developed PCR-based markers specific for these SNPs. Lately, Wu et al. (2005) and Hyten and Cregan (2006) developed several SNPs for *rhg1* and *Rhg4* loci responsible for SCN resistance in soybean. In addition to the existing SSR/RFLP-based soybean linkage map, 1,183 gene-based SNPs were mapped to 20 soybean MLGs (Cregan et al. 2006). The availability of the new integrated SSR/RFLP/SNP genetic map would provide soybean researchers valuable sources of markers for a diversity of applications, such as QTL mapping, MAS, positional cloning and association analysis.

2.3. Soybean Genetic and Physical Map

2.3.1. Genetic map

As mentioned above, soybean is thought to be the product of a diploid ancestor ($n = 11$), which underwent aneuploid loss ($n = 10$), polyploidization ($n = 20$) and diploidization. Thus, it is expected that any given gene will be present four times in the genome, which is a big challenge for the genomic and biological research in dissecting genes or genomic regions conferring to important agronomic traits. A good soybean genetic map, therefore, would be an important tool for molecular genetics analysis. Through over fifteen years' efforts, the soybean composite genetic

map is well developed. The map encompasses 20 linkage groups and contained 1,849 markers, including 1,015 SSRs, 709 RFLPs, 73 RAPDs, 24 classical traits, six AFLPs, ten isozymes, and 12 others (Song et al. 2004). With the development of thousands of SNP markers, a high density of genetic map will be coming soon.

2.3.2. *Physical map*

A physical map for the soybean cultivar 'Forrest' was constructed from 78,001 BAC and BIBAC representing 9.6 haploid genomes (Wu et al. 2004a). This map is under further development in attempts to reduce the number of contigs, create the minimum tile path, and add additional DNA markers (e.g., through BAC-end sequencing) (Shultz et al, 2006a; 2006b). Soybean cultivar 'Williams 82' was selected by the soybean genomics community as the representative cultivar for soybean genomics research and over 80% of EST sequences have been generated from this cultivar. This genotype is also being used for whole genomic sequencing. Therefore, construction of a physical map for the soybean cultivar 'Williams 82' is underway. Genome-wide physical mapping strategy based on BAC restriction fingerprinting has revolutionized genomics of large, complex genomes. The global fingerprinting approach employs a restriction fragment profile of a clone as its fingerprint and assumes that clones sharing a large number of, but not all, fragments are overlapping clones. Comparison of the fingerprints in a large population of clones is used to identify overlapping clones and is the basis for the construction of contigs. To generate fingerprints of DNA fragments cloned in BACs, two methods have been applied in soybean physical mapping, one is polyacrylamide (or DNA sequencing) gel-based fingerprinting (PAGFP) method for the 'Forrest' physical map (Wu et al. 2004a), and another one is a capillary-sequencer-based fingerprinting method, known as high information content fingerprinting (HICF) (Luo et al. 2003), for the 'Williams 82' physical map (unpublished data). The HICF method generates a more accurate and detailed clone fingerprint, and therefore produces better assemblies with less redundancy needed in the clone coverage (Nelson et al. 2005). The fingerprint data can be directly uploaded into an adapted version of Fingerprint Contig (Soderlund et al. 1997; 2001) software to assemble contigs based on restriction digest fingerprints, and the contigs are ordered and assigned to chromosomes based on anchored markers.

Integrated genetic and physical maps are extremely valuable for map-based gene isolation, comparative genome analysis, as sources of sequence-ready clones for genome sequencing projects and as important references for assembling of whole genome shot-gun sequences for the complex genomes. Accordingly, the public efforts are to develop resources to access the complete soybean genome through producing a high-resolution genetic map densely populated with SSR and SNP markers, constructing physical map segments with deep-coverage libraries of BACs, and through molecular markers to integrate the genetic and physical maps. In order to reach the goals, two important tools have being developed in the National Center for Soybean Biotechnology (NCSB) at University of Missouri-Columbia. The first tool is the six-dimensional BAC DNA pools, which is used for anchoring genetic

markers onto the physical map. The 49,152 clones of a Williams 82 BAC library that covers about 6 genome equivalents were pooled in six different dimensions creating 208 pools of BAC DNA. A PCR approach is used to screen these pools to identify BAC clone(s) containing genetically-mapped SSR markers and genes of interest. This resource will be fundamental to integrate the genetic and physical maps in an efficient and cost-effective manner, provide an invaluable resource for mapping genes or EST unigenes, and afford targeting genomic regions underlying various soybean traits including resistance to soybean cyst nematode, tolerance to abiotic stresses, and seed compositions for positional cloning. The second tool is a high density genetic map using a population of more than 700 RILs derived from the cross of Forrest and Williams 82 for integration of genetic and physical map in a fine scale.

The integrated physical, genetic, and cytogenetic map of the soybean genome will provide a 'highway' for anchoring gene sequences on physical map, isolation of a large number of genes and for many genetic and biological studies. The physical map can be readily accessed and will provide virtually unlimited numbers of DNA markers from any chromosome region or closely linked gene of interest. Synteny between soybean and legume species could also be used for isolation of agronomically important genes in other species such as *Medicago* or *Lotus* (Cannon et al. 2003; Yan et al. 2004; Mudge et al. 2005), for which whole genome sequences are available in GenBank.

3. FUNCTIONAL GENOMIC TOOLS AND RESOURCES

Plant functional genomics has emerged as a new and rapidly evolving scientific discipline to study the functions of genes. With the rapid advancement in the complete sequencing of genomes of an increasing number of plants (*Arabidopsis*, rice, *Medicago*, etc.), research priority has shifted from the identification of genes to the elucidation of their functions. The ultimate goal of functional genomics is the improved understanding of cell organization at different levels, from individual genes to groups of bio-molecules and complete genomes. Breakthroughs in understanding the relationship between genotype and phenotype will happen only through the integrated functional genomics approach. Recently, legume genomics has been focused primarily on the development of resources and information of two species considered to be model legumes, *Medicago truncatula* and *Lotus japonicus*, and soybean, the legume of principal economic importance in the United States (VandenBosch and Stacey 2003). A Joint Genome Institute (JGI) and soybean research community collaborative effort to sequence the soybean genome is underway and this will facilitate progressive completion of gene function analysis in soybean.

Very promising genomic tools including extensive EST resources and the availability of different kinds of microarray platforms are instrumental to build momentum toward gene function studies in soybean, and recently included reference species among legumes. In soybean, over 390,000 EST sequences are publicly available through various projects (<http://www.plantgdb.org>) and these

are expressed in different tissue and organ systems including developing seeds, seed coats, leaves, pods, roots, and various stages of plants regenerating from tissue culture. This resource includes the recently added drought stress specific and root tissue type specific ~15,000 EST sequences generated from the normalized, subtracted soybean root libraries from authors research group. Analysis of these ESTs contributed nearly 36,000 Unigene for soybean (www.tigr.org). Even though ESTs are useful for making a catalogue of expressed genes and the information, genome-scale collections of the full-length cDNAs of expressed genes are important for the analysis of structure and function of genes (Seki et al. 2004). So, in order to understand the gene regulatory network and also for various marker development projects it is necessary to build a strong soybean full length cDNA resource.

DNA microarray assays (Schena et al., 1995; Shalon et al. 1996) have provided an unprecedented opportunity for the generation of gene expression data on a whole-genome scale. The production of microarrays and the global transcript profiling of crop plants has revolutionized the study of gene expression and provides a unique snapshot of how these plants are responding to a particular stress/event. Both cDNA and oligoarrays are available for soybean gene expression profiling studies. The cDNA arrays available for soybean were constructed from low redundancy 'unigene' set of 27,513 clones that represent a variety of soybean cDNA libraries made from a wide array of source tissue and organ systems, developmental stages, and stress or pathogen-challenged plants (Vodkin et al. 2004). The construction of new sets of soybean long oligo arrays (70mer oligo sequence) is in pipe line from the above research group and these arrays contain a total of ~37,000 transcripts. At the same time GeneChip® soybean genome arrays are available to study gene expression of over 37,500 soybean transcripts. This short oligo array also contains transcripts for studying two pathogens important for soybean research. Specifically, the array includes probe sets to detect approximately 15,800 transcripts for the fungal pathogen of Phytophthora root rot (*Phytophthora sojae*) as well as 7,500 soybean cyst nematode (*Heterodera glycines*) transcripts (<http://www.affymetrix.com/products/arrays/specific/soybean.affx>).

The first generation of soybean cDNA microarrays containing approximately 4,100 Unigene ESTs derived from axenic roots, to evaluate their application and utility for functional genomics of organ differentiation in legumes (Maguire et al. 2002). Using a 9,280-cDNA clone array, Thibaud-Nissen et al. (2003) have identified 495 cDNA clones showing modulation of expression in response to 2,4-D treatment during the development of somatic embryos. Clustering the clones by similarity of expression profile over the course of this study allowed determining the timing of molecular events taking place during embryogenesis. In the same studies, they also found that oxidative stresses influence arrangement of new cells into organized structures which may depend on a genetically controlled balance between cell proliferation and cell death. Transcript profiling experiments conducted during *Pseudomonas syringae* pv. *glycinea* susceptible (S) and hypersensitive response-associated resistance (R) interactions in soybean identified differential expression pattern of plant-pathogen responsive genes and reported that the

R response generally showed a significantly greater degree of differential expression (Zou et al. 2005). Recently, Ainsworth et al. (2006) investigated the transcriptome response of soybean to elevated carbon dioxide [CO₂] in growing and fully expanded leaves using the cDNA microarrays. This study suggested that at the transcript level, elevated [CO₂] also stimulates the respiratory breakdown of carbohydrates, which likely provides increased fuel for leaf expansion and growth. Recently, the author's research group has identified several drought stress responsive and root and leaf tissue specific transcripts in soybean (Valliyodan et al. unpublished data) using GeneChip® soybean genome arrays from Affymetrix (www.affymetrix.com).

The use of microarray tool to analyze gene expression changes within the target plant is important for revealing the transcriptional regulatory networks which in turn contribute to understanding the responses of crop plants to various stresses and developmental changes, and crop improvement. Although the uses of gene expression profiling using microarrays are increasingly common, the integration of transcriptomics, proteomics and metabolomics represents the frontier of integrated functional genomics.

4. DISSECTING THE GENETICS OF THE TRAITS AND APPLICATIONS

4.1. Disease Resistance

Similar to other crop plants, several agronomically important traits in soybean, such as grain yield, disease resistance, and seed composition, are controlled by quantitative trait loci (QTL). These complex traits are environmentally sensitive; thus, it is often difficult to make progress in conventional breeding programs (Lee 1995). This is especially true for traits with low heritability. The development of DNA molecular marker technologies has facilitated the construction of genetic maps of many crop genomes, including soybean. A genetic analysis of the association of phenotypic trait and genotypic marker data from a population of lines segregating for genes conditioning quantitative traits provided the unique ability to dissect the complex trait into a set of discrete QTL (Tanksley et al. 1989), in which the contribution of an allele from each parent at a particular locus can be determined separately from the other loci affecting the trait. These important attributes provide an effective approach to pyramid desirable alleles for a complex trait into an adapted genetic background (Orf et al. 2004). Therefore, marker-based plant breeding coupled with traditional breeding techniques will be more effective and more efficient to introduce important agronomic traits into soybean.

4.1.1. Soybean cyst nematode

Soybean cyst nematode (SCN, *Heterodera glycines* Ichinohe) is among the most economically destructive pests of soybean in the United States and the world. The disease was first reported in Japan (1915), in Korea (1936), and in the US (1954) (Winstead et al. 1955). In the US, the most recent estimate shows that SCN causes

a 2 to 6% annual yield loss worth \$1 billion each year (Wrather et al. 2003). Early inheritance studies indicated that SCN resistance is genetically controlled by three recessive genes designated *rhg1*, *rhg2*, and *rhg3* (Caldwell et al. 1960) and one dominant gene designated *Rhg4* (Matson and Williams 1965). An additional dominant gene was later identified in PI88788 and designated as *Rhg5* (Rao-Arelli 1994). But genetic analysis of different sources of resistance showed that inheritance of SCN resistance was oligogenic and complex (Anand and Rao-Arelli 1989). Multiple alleles at a single locus could be involved in SCN resistance (Hancock et al. 1987; Hartwig 1985). Since then, many efforts have been made to screen soybean germplasm for resistance to SCN. Among the resistant sources identified, soybean line 'Peking' and plant introduction 'PI88788' were quickly deployed to incorporate resistant genes into new varieties in soybean breeding programs (Arelli et al. 2000). However, it was reported that certain field populations of SCN could overcome resistant cultivars, revealing the complex nature of host-pathogen interaction in soybean and SCN, which required the establishment of race-specific interrelationship for each resistant genotype and SCN race.

Advances in molecular genetics and biotechnology make it possible to identify and map genomic regions conditioning SCN resistance. As one of early molecular genetics analysis of SCN resistance, Concibido et al. (1994) reported two RFLP markers, pA85 and pB32, were significantly associated with SCN disease response and tentatively mapped to LGs A and K. In addition, a previously identified RFLP marker, pK69 (Boutin et al. 1992), was also found to be associated with SCN resistance and mapped to LG G (Concibido et al. 1994). Since then, several additional putative SCN resistant QTL were identified from a number of resistance sources, such as plant introductions, exotic germplasm, wild annual species (*Glycine soja*). Recently, when reviewing early QTL analysis of SCN resistance, Concibido et al. (2004) summarized a total of 16 putative QTL responsible for SCN resistance mapped on 17 different LGs of the soybean integrated molecular linkage groups (Cregan et al. 1999a). Of these, the QTL on LG G has been well characterized and proven to be the most important QTL because it contains the gene *rhg1*, which is capable of resistance to most of existing SCN races, 1, 2, 3, 5, 6, and 14 (Concibido et al. 1997; Yue et al. 2001a; 2001b). In addition to RFLP, AFLP, and RAPD markers reported to be associated with different genomic loci on LG G (Chang et al. 1997; Concibido et al. 1994; Kabelka et al. 2005; Meksem et al. 2001), Cregan et al. (1999b) mapped two SSR markers, BARC-Satt309 and BARC-Sat_168, at a distance of 0.4 cM to the gene *rhg1* and concluded that these two markers were highly effective in differentiating resistance and susceptible genotypes in SCN soybean breeding process. When mapping QTL for SCN resistance from PI90763 and PI404198A, Guo et al. (2006) also found the same genetic linkage of these SSR markers on LG G. It indicated that marker-assisted selection for SCN resistance can be highly efficient by deploying a small set of the SSR markers flanking within interval of the *rhg1* locus.

The second important QTL on LG A2 was located at the dominant gene *Rhg4*, which was reported to play a distinct role in resistance to SCN race 3

(Concibido et al. 1994; Heer et al. 1998; Webb et al. 1995). Similar to genetic linkage of the gene *rhg1*, various DNA molecular markers were shown to be associated with this LGA2-QTL containing the *Rhg4* gene (Concibido et al. 2004). Especially, Cregan et al. (1999b), Guo et al. (2006) mapped the SSR marker Satt632 close to this genomic region. In addition, the *i* locus, a morphological marker conditioning seed coat color, was also proven to be linked to *Rhg4* at a genetic distance of less than 1 cM (Webb et al. 1995). Using AFLP technology and bulk segregant analysis (BSA) to analyze resistant cultivar 'Forrest', Meksem et al. (2001) constructed a high-density genetic map of AFLP markers for the intervals carrying the genes *rhg1* and *Rhg4* and concluded that these two genes together explained over 98% of the resistance to SCN race 3. The findings were similar to the conclusion of a previously study that both these two genes are required for expression of complete resistance to SCN race 3 (Webb et al. 1995).

In attempts to characterize functional genomics of these two genes, Hauge et al. (2001) have reportedly cloned candidate genes for *rhg1* and *Rhg4* using positional cloning techniques. Both candidate genes are *Xa21*-like receptor kinases, which belong to R-genes conferring resistance to bacterial blight in rice (Zaitsev et al. 2001). Structures of these genes have an extracellular leucine-rich repeat (LRR) domain, showing similarity at the amino acid level to the *Cf-2* disease resistance gene, which confers resistance to leaf mold in tomato (Dixon et al. 1996). The results were confirmed in another patent application (Lightfoot and Meksem 2002). Even so, neither research group has reported their complementation studies to confirm that they have correct candidate genes. It is obvious that cloning and characterization of the candidate genes of *rhg1* and *Rhg4* will provide invaluable insight into the mechanisms of genetic resistance to SCN and might provide the knowledge needed to engineer novel resistance genes (Concibido et al. 2004).

With the availability of molecular markers closely linked to SCN resistance genes, such as Satt309, Satt163, Sat_168 (LG G), or Satt632, Sat_157 (LG A2), soybean breeders are capable of selecting resistant lines by genotyping breeding populations in a few days instead of growing progenies for greenhouse bioassay taking a month (Concibido et al. 2004). Each year around 20,000 soybean lines were genotyped for MAS in the SCN resistance breeding program of University of Missouri-Columbia (UMC) (Sleper, per. com.). Obviously, MAS enables breeders increasing the efficiency and speed of development of SCN resistant cultivars compared to conventional SCN breeding. However, in some occasions MAS for the *rhg1* gene has some difficulty due to lack of polymorphisms between parental lines for markers linked to this locus. Lately, the development of SNP genetic markers in soybean (Zhu et al. 2003) has provided soybean breeders an innovative approach to overcome the difficulty encountered. Wu et al. (2005) reported that several SNPs were identified for the *rhg1* and *Rhg4* loci in 30 parental genotypes commonly employed in the SCN resistance breeding program of UMC. With a sequenced region containing the *rhg1* gene, Hyten and Cregan (2006) demonstrated association analysis and discovered 67 SNPs throughout the region being significantly associated with SCN resistance. These findings indicate that SNP for SCN resistance would be molecular markers of choice in soybean

breeding programs and become more efficient as high-throughput SNP genotyping platforms such as Luminex flow cytometry (<http://www.luminexcorp.com>) and Illumina Beadstation (<http://www.illumina.com>) are employed.

4.1.2. *Phytophthora*

Phytophthora root and stem rot caused by fungal pathogen *Phytophthora sojae* was reported to occur in the US and other soybean production regions in the world, such as Brazil, Argentina, Australia, China, and South Korean (Grau et al. 2004). Studying the host resistance to this pathogen indicated that there are three types of resistance based upon inoculation experiments performed in host plant positions. For instance, the hypocotyl inoculation test detects single dominant genes, known as *Rps*, while the root inoculation test detects partial resistance, which is expressed as fewer rotted roots and slower disease progress (Tooley and Grau 1984). To date, eight dominant genes, designated as *Rps1* to *Rps8*, have been identified to convey resistance to 55 physiologic races of *P. sojae* (Burnham et al. 2003). Among these, *Rps1* and *Rps3* were reported to have multiple alleles (Grau et al. 2004). This indicated race-specificity of host resistance and genetic complexity of *P. sojae* population in soybean.

The availability of genomic genotyping technologies has facilitated the identification and mapping of DNA molecular markers associated with resistance to Phytophthora root and stem rot on the soybean genetic linkage map (Cregan et al. 1999a). Screening mapped RFLP markers in ‘Williams’ near-isogenic lines, Diers et al. (1992b) mapped the loci *Rps1*, *Rps2*, and *Rps3* on linkage groups (LG) K, L, and E, respectively. However, Demirbas et al. (2001) utilized SSR markers for genetic linkage analysis and mapped the loci *Rps1*, *Rps2*, *Rps3*, and *Rps4* on LGs N, J, F and G, respectively. Both research groups reported genomic locations of *Rps5* and *Rps6* were unclear. Lohnes and Schmitthenner (1997) used another set of RFLP markers to map locus *Rps7* in a ‘Clark x Harosoy’ isoline mapping population and reported this locus was closely linked to the RFLP marker R022-1 on LG N. The genomic location of the loci *Rps1* and *Rps7* on LG N was subsequently confirmed by Demirbas et al. (2001) and Weng et al. (2001), who utilized SSR markers and compared their linkage group to a consensus linkage group of the soybean (Cregan et al. 1999a). Burnham et al. (2003) reported a novel Phytophthora resistant locus, *Rps8*, in PI399073 from South Korea and mapped the locus on LG A2. Subsequently, however, the locus was re-located on LG F based on linkage analysis with SSR and RFLP markers (Gordon et al. 2006).

Among single-dominant *Rps* genes, *Rps1* locus was reported to carry five alleles (*Rps1-a*, *Rps1-b*, *Rps1-c*, *Rps1-d*, and *Rps1-k*) and confer resistance against a large number of *P. sojae* races (Schmitthenner et al. 1994). Thus, many efforts have been made to investigate molecular basis of this locus. By the means of primary trisomic analysis of cytogenetics, Gardner et al. (2001) was able to locate the locus *Rps1-k* on chromosome 3, corresponding to LG N of the integrated genetic linkage map of soybean. Bhattacharyya et al. (1997) and Kasuga et al. (1997) reported the high-resolution genetic and physical maps of the *Rps1-k* region and mapped

three AFLP markers tightly linked to the locus *Rps1-k* at a genetic distance less than 0.5 cM. The authors concluded that the *Rps1-k* was located at the end of an introgressed region. From a disease resistance gene-like sequence, Bhattacharyya et al. (2005) successfully cloned a family of CC-NBS-LRR-type sequences from the genomic region containing the locus *Rps1-k*. Among 12 candidate signaling proteins interacting with the resistance gene *Rps1-k*, Bhattacharyya and Gao (2006) reported that *Rps1-k-2* interactor regulates GmMcII, a gene responsible for hypersensitive cell death, which is associated with the expression of Phytophthora resistance.

Unlike the extensive studies conducted for single-dominant *Rps* genes, genetic information of partial resistance to *P. sojae* is limited. Recently, Burnham et al. (2003) developed three RIL mapping populations derived from the crosses of three susceptible parents and a partial resistant cultivar Conrad. The authors reported two QTL associated with *P. sojae* resistance were mapped to LG F and D1b+W of the soybean linkage groups. However, the authors also indicated that genomic locations of the QTL were different from the regions where the known *Rps* genes were mapped. This will pose a challenge to soybean breeders.

Employing single-dominant *Rps* genes for development of resistant cultivars has proven to be an economical and efficient measure to control this pathogen. However, as in many other host-pathogen systems, the fungal pathogen *P. sojae* has been reported to quickly adapt to specific *Rps* genes and as the result the host plant becomes vulnerable to a new physiologic race of the pathogen population. This is a great challenge to soybean breeders who have to continue accessing soybean germplasm to mine novel *Rps* genes for breeding programs. Besides single dominant gene-based breeding to control the disease, partial resistance has been shown to be effective against all races of *P. sojae* (Tooley and Grau 1986; Schmitthenner 1985). Anderson and Buzzell (1992) and Walker and Schmitthenner (1984) investigated resistance to *P. sojae* in several soybean lines and concluded that partial resistance is highly heritable and a quantitative trait. The authors suggested that selection for partial resistance combined with single-dominant *Rps* genes would provide long-term disease management of *P. sojae* in soybean.

Linkage analysis of DNA molecular markers and various *Rps* genes conditioning resistance to *P. sojae* in soybean can be useful to soybean breeders and pathologists to implement cost-efficient and successful MAS for Phytophthora resistance. Moreover, in addition to the identifications of molecular “tags” closely linked to the genes, markers flanking the loci of interest would provide an efficient tool for map-based cloning of the resistance genes, which in turn facilitates the development of SNP markers, a new generation of DNA molecular marker for soybean.

4.2. Abiotic Stress

Among the different abiotic stresses, drought, salinity, waterlogging/flooding and temperature stress (high and low) are the main factors reducing crop production worldwide and accounts for more than 50% yield losses (Boyer 1982; Bray et al.

2000). When plants are subjected to abiotic stresses, they activate different mechanisms to survive and sustain growth and yield. Thus, it is important to know what mechanisms exist to help plants surviving under severe stress. Plant traits linked to stress tolerance are multigenic, i.e. these traits are controlled by QTL. Here, progress on molecular mapping and QTLs analysis only for drought, salinity and flooding or waterlogging tolerance in soybean and their application in MAS for soybean improvement will only be presented.

Drought is a major limitation for soybean production. Crops often experience a water-limiting environment, thus the genetic improvement of a crop for drought resistance is a sustainable and economically feasible solution to reduce the problem of drought (Blum 1988). Drought tolerance is a complex phenomenon; dissection into individual component makes it easier to understand its genetic bases and also to apply this knowledge in MAS programs. Different authors described a number of drought related physiological traits in soybean from different perspectives (Raper and Kramer 1987; Nilsen and Orcutt 1996; Orcutt and Nilsen 2000; Purcell and Specht 2004). Purcell and Specht (2004) viewed drought from agro-physiological (i.e., plant biomass and grain yield), rather than eco-physiological (i.e., plant survival or competitiveness). Their consideration was based on soybean yield, thus a stress-related trait to be manipulated to lessen the reduction in soybean yield caused by the stress. They summarized a list of traits or physiological mechanisms impacting soybean yield via transpiration (T), water use efficiency (WUE) or harvest index (HI).

Molecular markers have been used to map the genomic location of genes/QTL for different traits related to drought, salinity and submergence tolerance in soybean (Table 1). As of August 2006, a total of 480 soybean genes and 1174 QTLs have been reported in the USDA-ARS soybean database (<http://soybase.org>). So far, very little progress has been made in the areas of abiotic stress tolerance but significant progress has been made for mapping many agronomic, pest resistant and seed composition related traits in soybean. Mian et al. (1996; 1998), using two mapping populations, cv. Young x PI416937 and S100 x Tokyo, identified a total of 5 QTLs for WUE, but only two QTLs linked to RFLP markers cr392-1n (LG J) and A489H (LG L) explained 13 and 14% of phenotypic variation, respectively. These two QTLs were found independently in two populations. Marker loci A489H (WUE2-1) also harbor QTL for leaf traits such as length, weight, width, shape, and area, plant yield and height, as well as seed oil content (<http://soybase.org>). Although yet to be confirmed in any other populations, WUE is no doubt an important locus to be used in MAS. An RFLP marker, A063E, was found common in both populations but phenotypic effect was less than 10%. Bhatnagar et al. (2005) identified a major QTL for slow wilting trait related to increased drought tolerance in soybean. The major QTL linked to SSR marker Sat_044 in linkage group K explained 17% of phenotypic variation. Specht et al. (2001) used 236 RILs developed from a cross between Minsoy x Noir 1 to determine the genetic basis of beta and carbon isotope discrimination (CID). They reported a QTL for CID on LG C2 with phenotypic contribution of <10% and with no effect on beta.

Table 1. Quantitative trait loci (QTL) identified for soybean drought, salinity and submergence/waterlogging tolerance

Reference	Mapping population, Number of lines and Population type	Trait studied number of QTL (in parenthesis)	Linked marker (s), linkage group (LG) and phenotypic variation (R ² %)
Drought			
Bhatnagar et al. (2005)	Jackson x KS4895 81 RILs	leaf wilting (1)	SSR marker; Sat_044, LG K, R ² =17
Specht et al. (2001)	Minsoy x Noir 1 256 RILs	yield (1)	SSR marker Satt205-Satt489, LG C2, R ² ~7
Mian et al. (1998)	S100 x Tokyo 116 F ₂	water use efficiency (1) (WUE)	RFLP marker A489H, LG L, R ² =14
Mian et al. (1996)	Young x PI416937 120 F ₄	water use efficiency (4) (WUE)	4 RFLP markers, B031-1n, A089-1, cr497-1, K375-1n; R ² ~ 8-13
Salt tolerance			
Lee et al. (2004)	S100 x Tokyo, 100 F ₂	salt tolerance (1)	SSR marker, Sat_091 LG N, R ² ~ 41-79
Flooding/waterlogging			
Reyna et al. (2003)	A5403 x Archer, RILs 9461 x Archer, NILs	flooding/waterlogging QTL effect evaluation	No effect of marker Sat_064
VanToai et al. (2001)	Archer x Minsoy 122 RILs Archer x Noir 1 86 RILs	waterlogging (1) waterlogging (1)	In both the populations, marker Sat_064 linked for improved plant growth R ² ~ 11-18, and grain yield R ² ~ 47-80

Cultivated soybean (*Glycine max*) is considered a salt sensitive crop whereas wild soybean (*Glycine soja*) is found to be tolerant (Singh and Hymowitz 1999; Luo et al. 2005; Kao et al. 2006); thus wild soybean (*G. soja*) can be used as resistance source for varietal improvement in soybean (*G. max*). So far, a major QTL for salt tolerance has been mapped in soybean (Lee et al. 2004). More than 100 F₂ populations were developed from a cross between S100 (salt tolerant) x Tokyo (salt sensitive). This QTL was linked to the SSR marker Sat_091 on LG N accounting for 41, 60, and 79% of the genetic variation for salt tolerance in the field, greenhouse, and combined environments, respectively. This data needs to be confirmed in another population before using in MAS program.

Waterlogging is also a major environmental stress reducing soybean growth and yield. Different authors have reported that yield can be reduced 17-43% if soybean is subjected to waterlogging at the vegetative stage, but greater yield reduction

(50–55%) occurs under waterlogging stress at the reproductive stage (Oosterhuis et al. 1990; Scott et al. 1989). Tolerance to waterlogging could increase yield and tolerance in soybean has been identified (VanToai et al. 1994). One major QTL for waterlogging has been reported and confirmed in two mapping population of soybean (VanToai et al. 2001). The QTL linked to SSR marker, Sat_064 on LG G explained phenotypic variance for plant growth 11–18% and yield 47–180%. Reyna et al. (2003) used Sat_064 genotypic data to create several NILs and these NILs were evaluated in southern background and found no effect for Sat_064. This variation may be due in part to the environment differences and the fact Sat_064 was originally identified in a northern genetic background. Several mapping populations have been developed at UMC for QTL mapping with the objectives to discover new QTLs and/or genes that confer tolerance to waterlogging under different genetic backgrounds and apply them for marker-assisted crop breeding.

Marker-assisted selection (MAS), has been successfully applied into soybean breeding program for SCN and other pest resistance traits, oil content and other traits. Monsanto and other private industries have incorporated MAS in their breeding programs and are making millions of selections annually, largely for insect and disease resistance genes using MAS. At the same time, public sectors are also using MAS for breeding programs on a limited scale. High density genetic map (saturated with molecular markers), tightly linked QTLs/genes with markers and confirmed QTLs/genes across the environments and in different genetic backgrounds are the most important criteria for effective MAS. MAS for abiotic stress related traits like drought, salinity and submergence tolerance have been initiated but not to extent of biotic stresses. Both private and public sectors are putting efforts on molecular breeding for abiotic stress tolerance.

4.3. Seed Composition

As earlier mentioned, soybean are grown as a source of protein and oil for agricultural, consumer, and industrial uses. In recent years, the value of soybean seed composition has extensively received attention for industrial applications and medical effects. These include increasing oil content for fitting the demand of biodiesel production, altering fatty acid or amino acid profiles, and developing healthy soybean seeds with high oleic acid, high isoflavone, low linolenic acid, or low phytate. However, the value of soybean to the producer is traditionally based on yield, and this trait has been the primary focus of most soybean breeding programs (Nichols et al. 2006). Recently traits that improve the value and functionality of soybeans such as modification of protein and oil to give greater utility in food, health, and industrial uses have been emphasized by soybean breeders and industries. Soybean breeding research has been directed toward modifying the fatty acid profile in the oil to expand uses in food and industrial applications. Modifying soybean oil to lower saturates to less than 7%, increasing oleic acid content to 50 to 60%, and lowering linolenic acid content to <3% would greatly improve soybean quality for greater functionality in food and industrial applications. Other areas of

emphasis include improving protein by modifying the amino acid profile, improving digestible phosphorus, and reducing anti-nutritional factors such as trypsin inhibitors and allergenic factors (Wilson 2004).

There are many traits whose genetic potential is masked by epistatic interactions between genes or by genes in the repulsion linkage phase. Molecular markers can assist selection for favorable alleles, dissect the QTL regions conferring favorable seed traits and breakdown the unfavorable linkage relationship at the genetic level (Lee 1995). DNA marker analysis can measure the effect of an individual locus at the allelic level. For example, the contribution of alleles from each parent at a particular locus can be determined separately from other loci affecting the trait. This provides an effective method to pyramid desired genes for complex traits or traits with low heritability into adapted varieties (Orf et al. 2004). Therefore, high-throughput molecular breeding techniques, coupled with traditional breeding approaches, will be more effective and efficient for introducing important quantitative traits into soybean.

So far, a large number of QTL related to soybean seed composition have been detected by QTL analysis with molecular markers. All major QTL with more than 10% of phenotypic contribution were summarized in Table 2. The QTL identified could be useful for developing soybean varieties with desirable component in the seed through MAS. QTLs conditioning protein content in soybean were widely investigated on 17 soybean populations and found to be located on the soybean genome except for LG B1, D1b, D2, J, and O (Table 2). A total of 61 QTLs for protein content were reported and 16 putative QTLs (~25%) accounting for more than 10% phenotypic variation were identified. Among them, ten QTLs were detected in more than two soybean populations. The trait for protein content is highly heritable, as shown in the locus on LG I (Satt127) with a maximum phenotypic contribution of 65% (Table 2). Fine-mapping studies have been used to clone the gene close to the Satt127 marker on LG I and resulted in QTL localization within 1.1 cM using BAC libraries (Joseph et al. 2004; Nichols et al. 2006). MAS of lines with or without positive alleles at the QTL on LG I was applied to compare the allelic effect and confirm the QTL resulting in a 56 g kg⁻¹ increase in protein content in lines carrying homozygous alleles from the high protein parent (Yates et al. 2004).

In addition to high protein content, soybean is also an important oil source for food and biodiesel. QTL for oil content are distributed throughout the soybean genome except for LG D1a, D1b, F, J, N, and O (Table 2). Thus, three LGs (D1b, J, O) do not carry genes for both protein and oil content. Of 19 QTLs identified, 11 (69%) and 19 (58%) QTLs were found to be associated with protein and oil content, respectively, commonly control both traits. Because strong negative correlations exist between protein and oil content, it needs to be determined genetically whether the negative relationship is due to pleiotropism or repulsion linkages between protein and oil alleles. Of 53 oil QTLs reported in SoyBase (<http://soybase.agron.iastate.edu>), 19 putative QTLs explain more than 10% of the phenotypic variation (Table 2).

Table 2. Major QTL ($R^2 \geq 10\%$) related to soybean seed composition

Trait	Population	LG	Flanking loci (cM) (a)	R^2 (%)	LOD	Reference
Protein content	A81-356022 x PI 468916 60 F _{2:3}	I	K011	42	-	Diers et al. 1992a
		E	SAC7_1	24	-	
		B2	A242_1	19	-	
		L	A023_1	16	-	
PI 437088A x Asgrow A3733 76 RILs F ₂ eight populations (b)		G	A245_2	12	-	Chung et al. 2003 Brummer et al. 1997
		I	OPAJ13a-OPAW13a	36	20	
		A2	A505_1	11	-	
		C1	A063_1	17	-	
		D1a	A398_1	28	-	
		E	B174_1	11	-	
Young x PI 416937 120 F ₄ (b)		G	A890_1	16	-	Lee et al. 1996
		I	A144_1	28	-	
		C1	Gc97_1	13	-	
		N	A071_2	11	-	
		B2	B142_1	10	-	
		H	A566_2	14	-	
PI 97100 x Coker 237 111 F ₂ (b)		K	A065_3	11	-	Lee et al. 1996
		H	B072	32	-	
Peking x Essex 200 F _{2:3}		F	B148	17	-	Qui et al. 1999
		C1	Satt578	12	-	
Noir 1 x Archer 240 RILs		L	Satt166	11	-	Orf et al. 1999
		A1	T155_1	15	-	
Minsoy x Archer 233 RILs		C1	SoyGPATR	12	-	Orf et al. 1999
		I	Satt127	65	-	
A81-356022 x soja PI 468916 BC ₃ Minsoy x Noir1236 RILs		M	Satt567	27	12.8	Sebolt et al. 2000 Specht et al. 2001

(continued)

Table 2. (continued)

Trait	Population	LG	Flanking loci (cM) (a)	R ² (%)	LOD	Reference
Cysteine	Essex x Williams 131 RILs	C2	Satt277-Satt202	28	9.8	Hyten et al. 2004
		F	Satt335-Satt144	18	4.4	
		K	Satt539-Satt102	24	4.3	
		M	Satt540-Satt463	13	3	
Methionine	N87-984-16 x TN93-99101 RILs	G	Satt570	20	3.5	Panthee et al. 2005
		B2	A953_IH-Satt560	12	3.5	
		F	Satt252	11	2.2	
		G	Satt235	13	2.8	
		F	Satt252	15	2.8	
Oil content	N87-984-16 x TN93-99101 RILs	G	Satt564	19	2.6	Panthee et al. 2006
		M	Satt590	23	2.4	
		I	A407_1	28	-	
		E	SAC7_1	43	-	
		B2	A242_1	39	-	
F ₂ eight populations (b)	PI 27890 x PI 290136 69 F _{2,5}	L	A023_1	32	-	Mansur et al. 1993
		A2	T153_I-A111_I (18)	36	5.5	
		K	BC1-A315_1 (26)	24	2.9	
		A1	A104_1	19	-	
		B1	A109_1	31	-	
		G	A584_1	19	-	
		H	A069_1	18	-	
		K	K387_1	16	-	
		D2	Cr142_1	13	-	
		C1	A063_1	13	-	
Archer x Minsoy 233 RILs	Minsoy x Noir 1 240 RILs	G	L154_1	17	-	Lee et al. 1996
		A1	T155_1	13	-	
		A1	Satt174	10	4	
		C1	SOYGPATR	11	3.3	
PI 97100 x Coker 237 111 F ₂ (b)	Young x PI 416937 120 F ₄ (b)	C1	A063_1	13	-	Lee et al. 1996
		D2	Cr142_1	13	-	

	Noir 1 x Archer 240 RILs	C2	Satt432	11	3.3	
		L	A489_1	19	6.1	
	Peking x Essex F _{2:3}	H	B072	21	-	Qui et al. 1999
	PI 437088A x Asgrow A3733 76 RILs	I	OPAJ13a-OPAW13a	26	14	Chung et al. 2003
	Essex x Williams 131 RILs	C2	Satt277-Satt460	32	12	Hyten et al. 2004
		L	Satt166-D11	10	3.3	
	N87-984-16 x TN93-99 101 RILs	M	Satt540-Satt463	12	3.6	Panthee et al. 2005
		D1b	Satt274	12	3	
		O	Satt420	15	3.5	
		O	Satt479	12	3.1	
Linolenic acid	A81-356022 x PI 468916 60 F _{2:3}	E	SAC7_1	31	-	Diers and Shoemaker 1992
		L	A023	26	-	
		K	A065_3	20	-	
Linoleic acid	A81-356022 x PI 468916 60 F _{2:3}	A1	A082_1	38	-	Diers and Shoemaker 1992
		E	A242_2	21	-	
		B1	A118_1	20	-	
		B2	A343_1	24	-	
Palmitic acid	A81-356022 x PI 468916 60 F _{2:3}	J	K375_1	18	-	Diers and Shoemaker 1992
	Cook x N87-2122-4	A1	Satt684	33	5-7	Li et al. 2002
Oleic acid	A81-356022 x PI 468916 60 F _{2:3}	A1	A170_1	23	-	Diers and Shoemaker 1992
		E	Pb	21	-	
		B2	A619_2	19	-	
Oligosaccharide	Keunolkong x Iksan 10 115RILs	L	Satt278	14	-	Kim et al. 2005
Cell wall polysaccharides	Minsoy x Archer 108 RILs	A1	Satt174-Satt211	17	4	Stombaugh et al. 2004

(continued)

Table 2. (continued)

Trait	Population	LG	Flanking loci (cM) (a)	R ² (%)	LOD	Reference
Fucose	Minsoy x Archer 108 RILs	A2	Satt119–Satt233	38	11	Stombaugh et al. 2004
Galactose	Minsoy x Archer 108 RILs	A1	Satt211–T155_1	27	7	Stombaugh et al. 2004
Glucose	Minsoy x Archer 108 RILs	O	Satt153–Scaa001	13	3	Stombaugh et al. 2004
Pectin	Minsoy x Archer 108 RILs	A1	Satt174–Satt211	22	6	Stombaugh et al. 2004
Sucrose content	V71-370 x PI 407162149 F _{2,3}	I	A144	12	–	Maughan et al. 2000
Phytate	Boggs-RR x CX1834-1-2 166 F ₂	L	Satt561	11	–	Walker et al. 2006
		N	Satt237	41	–	
Total isoflavone	AC756 x RCAT Angora 207 RILs	A1	Satt200	12	2	Primomo et al. 2005
		M	Satt201	26	5.5	
Daidzein	AC756 x RCAT Angora 207 RILs	A1	Satt200	15	2.1	Primomo et al. 2005
		M	Satt201	18	3.4	
	Essex x Forrest 100 RILs	A1	Satt276	10	2.7	Kassem et al. 2004
		N	Satt080	10	3.2	
Genistein	AC756 x RCAT Angora 207 RILs	M	Satt201	31	7.6	Primomo et al. 2005
	Essex x Forrest 100 RILs	B2	Satt063	38	2.9	Kassem et al. 2004
Glycitein	AC756 x RCAT Angora 207 RILs	F	Satt516	39	2.9	Primomo et al. 2005
	Essex x Forrest 100 RILs	B1	Satt251	50	11	Kassem et al. 2004
		N	Satt237	11	2.3	

(a) : Only independent QTL contributing >10% to the particular trait variation.

(b) : single factor analysis was employed.

There were reports on seven, six, five, and six QTLs for linolenic, linoleic, palmitic, and oleic acid, respectively, in soybean fatty acid (FA) composition in SoyBase (<http://soybase.agron.iastate.edu>), including three QTLs for linolenic acid on LG E, K, and L; three QTLs for linoleic acid on LG A1, B1, and E; three QTLs for palmitic acid on LG A1, B2, and J; and three QTLs for oleic acid on LG A1, B2, and E. Only one QTL on LG E conditions the content of three FAs such as linolenic, linoleic, and oleic acids. Recently, six QTLs associated with increased oleic acid were mapped on LG A1, D2, G, and L with SSR markers in a population of G99-G725 (low) x N00-3350 (high), and four of them were confirmed in a different population of G99-G3438 x N00-3350 (Monteros et al. 2004).

Soybean also has desirable carbohydrates (i.e., glucose, fructose, and sucrose) and undesirable carbohydrates (i.e., raffinose, stachyose). Of 17 markers significantly associated with seed sucrose content on seven LGs, only QTL on LG I was found to have a phenotypic contribution greater than 10% (Table 2). Based on a comparison of the reported QTLs for seed composition traits, it is likely to be a gene cluster or a major QTL with pleiotropic effect (Maughan et al. 2000). Among the seven genomic regions underlying QTLs for sucrose content, four regions (LG E, F, I, L) also affect protein content, and three regions (LG A2, I, L) are associated with oil content as well.

There are many bioactive soybean components that may contribute to the hypothesized health benefits of soybean but most attention has recently focused on the isoflavones because isoflavones in soybean seeds are believed to have preventive effects for several major hormone-dependent diseases in humans. Numerous publications and research studies on isoflavones have prompted a nationwide increase in the consumption of soy-based foods and supplements in the United States. QTL analysis identified several genomic regions (LGs A1, B1, B2, F, M and N) associated with isoflavone contents (Primomo et al. 2005; Kassem et al. 2004).

5. CONCLUSIONS AND PERSPECTIVES

As a valuable source of seed protein and vegetable oil for human and animal nutrient, soybean is the most economically important legume crop in many countries in the world. In addition, the values of soybean seed composition have received a sincere attention by soybean researchers to investigate for industrial applications and medical uses. Steadily increased world production and meal consumption of soybean for last ten years indicated that soybean crop continues to be an important leguminous crop plants significantly contributing to various aspects of agricultural production, animal industry, and in particular human health.

For decades, many efforts have been made to develop new soybean variety with improved nutritional and agronomic characteristics through conventional breeding approaches; however, the recent advances of DNA molecular marker technologies, such as SSR and RFLP, led to successful construction of the integrated genetic linkage map of soybean, in which genetic linkage of molecular markers were established in soybean genome. Newly gene-based SNP markers were integrated to

the pre-existing DNA molecular linkage map of the soybean would provide soybean researchers efficient genomic tools for identification and characterization valuable genes/QTL conditioning important agronomic traits and facilitating MAS programs.

Genome sequencing, in combination with various forward and reverse genetic approaches and “omics technologies”, has made possible the identification of more than 30,000 genes in soybean, the understanding of the molecular basis of soybean growth and seed development, which is beginning to revolutionize the way in which plant breeders have utilized for cultivar improvement. Functional genomics has become an important discipline to identify gene, gene function, and to understand the relationship between phenotype and genotype via integrated functional genomic approach. Important functional genomic tools are ESTs, microarray, proteomics, metabolomics, translational genomics and bioinformatics. The availability of EST sequences generated from cultivar ‘Williams 82’, a representative soybean genotype, facilitated entire genome sequencing and construction of a physical map, which is based upon a novel strategy of 6-D BAC DNA pools. In combination of a genetic map with high density of SSR and SNP markers, a high resolution physical map will be invaluable resources for map-based gene isolation, targeting genomic regions underlying various traits of interest such as disease resistance, abiotic stress, and seed composition.

Recently, a collaborative effort led by Department of Energy and Joint Genome Institute (DOE-JGI) has been initiated aiming to sequence the entire soybean genome in hopes that the sequence information can be available by the end of 2008. With the availability of the soybean sequence and integrated genetic and high quality physical maps, soybean researchers will be able to efficiently implement linkage disequilibrium and association analysis. As a result, QTL mapping, positional cloning, as well as MAS will be accordingly accelerated.

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CHAPTER 12

APPLICATION OF GENOMICS TO FORAGE CROP BREEDING FOR QUALITY TRAITS

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Abstract: Forage quality depends on the digestibility of fodder, and can be directly measured by the intake and metabolic conversion in animal trials. However, animal trials are time-consuming, laborious, and thus expensive. It is not possible to study thousands of plant genotypes, as required in breeding programs. Therefore, several indirect methods including near-infrared reflectance spectroscopy (NIRS) have been established to overcome this limitation. However, the ideal indirect system for the prediction of forage performance would be based on gene-derived “functional” DNA markers, allowing early selection ultimately without need of field trials, and being environment independent. In addition, once identified relevant genes controlling forage quality are targets for transgenic approaches. Substantial progress has recently been achieved in the development and application of genomic tools both in model species and major forage crops such as ryegrass and alfalfa. Key genes involved in developmental and biochemical pathways affecting forage quality such as cell-wall, lignin, fructan, and tannin biosynthesis have been isolated and characterized. For some of these genes, allelic variation has been studied in detail and sequence motifs with likely effect on forage quality have been identified by association studies. Moreover, transgenic approaches substantiated the effect of several of these genes on forage quality. Perspectives and limitations of these findings for forage crop breeding are discussed given expected further progress in forage crop genomics, but also the complexity of the trait complex forage quality, since typically species mixtures of heterogeneous and heterozygous genotypes are grown in the field.

1. INTRODUCTION

1.1. Direct Measurement of Forage Quality

In most forage grasses and legumes, above-ground parts are harvested before or during flowering. An exception is forage maize, harvested after seed-set. Animal feeding trials allow direct evaluation of forage crop quality via the target product

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such as milk or beef. Different parameters were developed such as Mega Joule “Net-Energy-Lactation” ((MJ) NEL) (Groß 1979, Weißbach 1993) for milk production, “Kilo Starch-Units” (kStE) (Zimmer et al. 1980) for beef production and “Metabolizable Energy” (ME) (Menke and Huss 1987), all reflecting the energy density (J/kg) of forage dry matter (Boberfeld 1986). However, animal trials are time-consuming, laborious, and thus expensive. It is not possible to study thousands of plant genotypes, as required in breeding programs.

1.2. Indirect Measurement of Forage Quality

Several indirect biological, chemical, and physical methods for quality evaluation have been developed. Biological methods for quality evaluation can be subdivided into field, *in vitro*, and enzymatic methods. Field methods score the expected nutritive value of plant communities (Boberfeld 1986) based on the species composition. In case of forage maize, the proportion of ears in total dry matter has been used for quality evaluation (Zscheischler 1990). A widely used *in vitro* rumen digestion analysis was developed by Tilley and Terry (1963) using a two-step procedure - first rumen liquor and subsequently peptic hydrochloric acid to estimate the *in vitro* digestibility of organic matter. Another *in vitro* test employing rumen liquor determines gas production, protein and fat content to estimate NEL or StE (Menke and Steingäß 1987). Enzymatic methods use cellulase together with peptic hydrochloric acid to estimate NEL and StE (Kirchessner and Kellner 1981).

Since digestibility is mainly limited by poorly digestible cell wall components, chemical methods for forage quality evaluation focus on the breakdown and characterization of cell wall fractions within the organic matter. Using detergents, Van Soest (1974) separated cell complexes into soluble cell content and insoluble “neutral detergent fibre” (NDF) representing mainly the cell wall fraction. By acidic detergents further fractionation into a lignin (“acid detergent lignin”: ADL) and a non-lignin fraction (ADF-ADL; ADF: “acid detergent fibre”) is possible. ADF values can be converted into NEL and StE estimates (Kirchessner and Kellner 1981).

All above mentioned approaches are too laborious for routine quality evaluation of large numbers as required in plant breeding. In contrast, by near-infrared reflectance spectroscopy (NIRS) (Norris et al. 1976) large sample numbers can be investigated with low effort. Infrared spectra of ground materials (1400 to 2600 nm) can be employed to estimate a number of quality parameters, once calibrations to animal trials, biological, or chemical methods have been established. For most of the above mentioned quality characters, NIRS calibrations have been established for the major forage crops (e.g., Ralph et al. 2004, Barriere et al. 2003). Moreover, in view of developing DNA-markers for forage quality traits, NIRS allows quality evaluation at large scale as required for QTL mapping or association studies.

1.3. Trait Complexity

Forage crop breeding is characterized by different layers of complexity. Several factors need to be considered when determining forage quality, such as the fed

animal species, the forage plant species, the cropping system, and the method(s) used for forage quality evaluation. In addition, the optimum diet of animals depends on the product, such as beef or milk for cattle. Ruminants have a much better capability to digest fibrous carbohydrates compared to monogastrics and to convert poor quality protein and nonprotein nitrogen sources (Van Soest 1974). Furthermore, intake and digestion by animals depends on forage properties such as its dry matter content, particle size, and the ensiling process.

At the feed or plant level, a given variety is often only part of the animal diet fed together with minerals, additives etc. If used for grazing, a single variety typically is only part of a mixture of different grasses and legumes. Further aspects adding to the complexity are different ploidy levels within crops such as in ryegrass, and symbiosis with endophytes or root nodule bacteria. In contrast, many genetic experiments are performed under simplified conditions to establish sound phenotype – genotype associations, preferably (i) at the diploid level, (ii) in monoculture, (iii) at *per se* level, and (iv) for single spaced plants. Therefore, a crucial question is, whether results obtained in “artificial” experimental situations are transferable and, thus, valuable to operational breeding programs.

2. FACTORS INFLUENCING FORAGE QUALITY

2.1. Organ, Tissue- and Cell Level

Forage maize breeding in Europe has originally been based on the concept that the best hybrids for grain production are also optimal for forage use. However, coefficients of determination between grain and forage yield ($r^2 < 0.25$) were not sufficiently high to justify selection on the basis of grain yield (Vattikonda and Hunter 1983). In addition, substantial genotypic variation for digestibility and yield of stover was found among hybrids suggesting that the non-grain portion of the maize plant offers considerable potential for the improvement in yield and forage of silage maize (Dhillon et al. 1990; Geiger et al. 1986).

It has been shown for different forage species that especially the extent and mode of lignification is limiting both digestibility and intake (Barriere et al. 2004). Thus, major breeding criteria for forage grasses such as *Lolium perenne* are besides a maximum animal intake per time unit and a high digestibility, (1) a high biomass yield and (2) a minimum need for cuttings. This can be achieved by late- or not-flowering and long-leaved grass types (high leaf elongation ratio) with high quality also in older leaf parts showing lack of senescence (stay green types) (Wilman et al. 2004).

2.2. Cell Wall and Cell Content

Digestibility of the ear fraction in maize is close to 90% in contrast to about 50% of stover (Zimmer et al. 1990). Within stover, the lignin fraction shows by far the lowest digestibility (about 7%) compared to cellulose and hemicellulose. Therefore, a promising target for the improvement of feed quality in silage maize and

forage grasses is the lignin biosynthetic pathway, including monolignol biosynthesis and polymerization of monolignols, the latter being performed by peroxidases and laccases (Ralph et al. 2004). The digestibility of grasses as a general trait becomes markedly reduced during the course of the growing season. This reduction is largely caused by an increase in the content of poorly digestible cell wall structural components. In parallel, there is a decrease in the content of soluble carbohydrates – “sugars”. Varieties of ryegrass with a high stable level of carbohydrates in the form of fructans have been shown to retain a high degree of digestibility throughout the growing season. Poorly digestible structural components create an imbalance between carbohydrate and protein levels during ruminant fermentation, leading to a loss of nitrogen (ammonia) to environment.

Forage legumes are highly digestible as compared to forage grasses. However, proteolysis and microbial deamination might lead to protein loss in the rumen, not fully compensated by post-ruminal absorption (Robbins et al. 2002). High digestion rates may result in protein foaming and rumen pasture bloat as a digestive disorder (Gruber et al. 2001). Moreover, stem lignification is also affecting forage legume quality (Reddy et al. 2005).

2.3. Biochemical Pathways

The lignin biosynthetic pathway is well characterized (Boudet et al. 1995), especially the common phenylpropanoid pathway starting with the deamination of phenylalanine and providing hydroxycinnamoyl CoAs. The enzymes involved in the common phenylpropanoid pathway are phenylalanine ammonia-lyase (PAL), cinnamate hydroxylase (C4H), coumarate hydroxylase (C3H), caffeic O-methyltransferases (COMT), ferulate hydroxylase (F5H), and hydroxycinnamate CoA ligases (4CL). In total 34 genes have been identified in the *A. thaliana* genome coding for enzymes in the monolignol biosynthesis (Raes et al. 2003). The end products of this common pathway, the hydroxycinnamoyl CoAs, are precursors of the major classes of phenolic compounds which accumulate in plant tissues, e.g. flavonoids, stilbenes, phenolamides as well as lignins. Subsequently, cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) are specifically involved in biosynthesis of the lignin monomers p-coumaryl, coniferyl, and sinapyl alcohol. In maize, genes for COMT (Collazo et al. 1992) and CAD (Halpin et al. 1998) have been isolated. Defect alleles of both genes have been shown to correspond to *brown midrib* mutations (COMT: *bm3* ; CAD: *bm1*) known for long time (Barriere and Argillier 1993). Several independent studies on *bm1* and *bm3* have proved already the concept of increasing silage quality by altering the lignin biosynthetic pathway (Barriere and Argillier 1993) and reducing lignin content. However, application of *bm* mutants in plant breeding has been hampered by their pleiotropic effects on yield characters and lodging.

Lignins exhibit a high degree of structural variability depending on the relative proportion of three monolignols, different types of interunit linkages, and the occurrence of non conventional lignin units within the polymer (Boudet and Grima-

Pettenati 1996). Polymerization of monolignols involves peroxidases and laccases in an oxidation step but is generally not well understood (Boudet et al. 1995). Laccase was the first enzyme shown to be able to polymerize lignin monomers *in vitro* (Freudenberg et al. 1958). Several studies indicated that laccase and laccase-like activities are closely correlated with lignin deposition in developing xylem (Davin et al. 1992). Nersissian and Shipp (2002) identified 19 laccases in the genome of *A. thaliana*. In case of peroxidases, association between allelic variation at one Prx locus with forage digestibility in maize has been demonstrated (Guillet-Claude et al. 2004b). Thus, a minimum of 70–80 genes code for enzymes that are directly involved in lignin formation, based on the small *A. thaliana* genome, which can be considered as candidate genes for cell-wall digestibility. Furthermore, gene families involved in cellulose or hemicellulose formation might as well affect cell wall digestibility (Barriere et al. 2003, Ralph et al. 2004).

Fructans are polymers of fructose, and have a general structure of a glucose linked to multiple fructose units (polyfructosylsucroses). In contrast to the uniform structure of bacterial fructans, plant fructans represent five major classes of structural distinct fructans according to the linkages between the fructose units; inulins, levans, inulin neoseries, levan neoseries, and mixed type levans. It is now known that four out of five different fructosyltransferases (FT), each with their own specificity, are needed to synthesize the wide variety of fructans found in plants; 1-SST (1-sucrose:sucrose fructosyltransferase), 1-FFT (1-fructan:fructan fructosyltransferase), 6-FFT (6-fructan:fructan fructosyltransferase), 6-SFT (Sucrose fructosyl 6-transferase) and 6G-FFT (fructan:fructan 6G-fructosyltransferase). Other enzymes that are involved in fructan degradation are fructanhydrolases (FH), and invertases. Fructanhydrolase is a beta-fructofuranosidase and can uncouple fructose units from fructans with sucrose as an end product. Invertase, which is active in the vacuole, cleaves one sucrose molecule into glucose and fructose. It's also capable of cleaving fructose molecules from smaller fructans. This fructan hydrolysing activity decreases with a higher degree of polymerization of the fructan. The type of fructans and FTs varies among different monocot species. Levan type fructans are abundant in *Triticum*, *Hordeum* and *Bromus*, whereas inulin types are characteristic to e.g. *Lolium* species. Interestingly, bifurcose (a product of 6-SFT and precursor to the levan type and one of the routes to the levan neoseries) has not been found in *Lolium* species. According to this, four enzymes would be necessary to account for the synthesis of the fructans identified in *L. perenne*, namely 1-SST, 1-FFT, 6G-FFT and 6FFT (Parvis et al. 2001). Genes coding respective enzymes in forage crops have been isolated recently (Gallagher et al. 2004, Chalmers et al. 2003).

For wool production under grazing conditions, the sulfur-containing amino acids methionine and cysteine are among the most limiting essential amino acids (Rogers 1990). Sunflower seed albumin 8 (SFA8) contains high levels of methionine and cysteine and is resistant to rumen degradation (McNabb et al. 1994). Chimeric constructs directing the SFA8 protein into endoplasmatic reticulum in tall fescue leaves resulted in an up to 0.2% accumulation of SFA8 in the soluble leaf protein fraction (Wang 2001).

A major role for reducing the high digestion rates in forage legumes has been assigned to condensed tannins (Gruber et al. 2001). Condensed tannins are polymeric flavonoids with protein-precipitating properties. Whereas high amounts of condensed tannins are detrimental to ruminant digestion, moderate levels (2–3% of dry matter) improve forage legume quality by reducing ruminal digestion rates and avoidance of protein foaming, and thus lead to higher rates of protein conversion into animal products (Robbins et al. 2002). Highly nutritious species such as white clover and alfalfa have a low level of endogenous tannins as compared to tanniferous forages like Lotus. The initial steps in condensed tannin biosynthesis belong to the general flavonoid pathway and include enzymes like chalcone synthase. Whereas genes coding for enzymes of the general flavonoid have been isolated for long time, the first genes coding for enzymes of the condensed tannin specific pathway such as leucoanthocyanidin reductase have been isolated more recently (Tanner et al. 2003). As for the lignin biosynthesis pathway, regulatory genes coding for transactors have been envisaged as targets to manipulate tannin content, such as myb- or myc-like genes (Gruber et al. 2001).

3. QTL FOR FORAGE QUALITY

Quantitative trait loci (QTL) mapping is a forward genetic approach, associating trait variation with genome regions, systematically covered with molecular markers. Several QTL mapping studies for forage traits have been conducted, e.g., in maize (Lübberstedt et al. 1997a, b, 1998; Ralph et al. 2004), and ryegrass (Cogan et al. 2005a).

3.1. Maize

The first published QTL in relation to forage quality were based on whole plant measurements (Lübberstedt et al. 1997a, b, 1998), in order to reflect agronomic performance. However, the ear fraction might mask some of the variation present for cell-wall digestibility in the stover. Nevertheless, analysis of multiple traits including dry matter yield (DMY), dry matter content (DMC), starch content (STC) and yield (STY), metabolizable energy content (MEC) and yield (MEY), and *in vitro* digestibility of organic matter (IVDOM) revealed the “architecture” of forage quality in whole plant samples. In total, 16 QTL were detected for IVDOM and 14 for MEY (Lübberstedt et al. 1997b, 1998) as the two major forage quality characters. MEY was closely correlated with DMY and plant height (PHT), i.e., biomass production, which was also reflected by several common QTL affecting these three characters. In contrast, IVDOM was negatively correlated with PHT, but positively with STC and MEC as also expressed by shared QTL regions. It was thus possible to identify those genome regions controlling IVDOM, independent of the starch (=ear) fraction.

Subsequently QTL for cell-wall digestibility and lignification traits were studied in different recombinant inbred line (RIL) populations (Mechin et al. 2001,

Chromo- some	BIN								
	01	02	03	04	05	06	07	08	09
1							4CL2 FSH	CCR1	
2								Px1	
3					myb2				
4					COMT PAL3				
5			4CL1 CAD 3	Px13					
6	COMT								
7									
8					MIRP1				
9		COMT 2							
10					CHH				

Figure 1. Assignment of mapped forage QTL (gray boxes; according to Ralph et al. 2004) to chromosomal bin regions (Gardiner et al. 1993) in maize. Dark gray boxes indicate “QTL hotspots”, i.e., genome regions detected more than once. Colocalized candidate genes (e.g. “lignin genes”) where included (abbreviations: see Ralph et al. 2004)

Roussel et al. 2002, Cardinal et al. 2003, Ralph et al. 2004). In total, 22 different QTL were identified for these characters (Figure 1), with some of the genome regions detected repeatedly (chromosomal bins 1.02, 1.03, 2.08, 3.05/06, 4.08, 4.09/10, 6.06, 9.02; Gardiner et al. 1993, Ralph et al. 2004). In many cases, QTL for cell-wall digestibility and lignin content co-localized. Moreover, several genes involved in monolignol biosynthesis mapped to the respective QTL regions including the isolated brown midrib genes coding for COMT (*bm3*) and CAD (*bm1*) (Ralph et al. 2004). However, each bin region of about 20 cM might still contain more than 1000 genes. Thus, additional evidence is required to proof which genes underlie those QTL controlling forage quality or lignification.

3.2. Forage Grasses and Forage Legumes

In forage grasses such as *Lolium perenne* both cell-wall digestibility especially of flower stems and high concentration of water soluble carbohydrates like fructans in leaves are considered as the main forage quality parameters (Yamada and Forster 2005). QTL for herbage traits in *L. perenne* have been determined for progeny of a paircross between a heterozygous and a doubled haploid genotype (Cogan et al. 2005b), forming the p150/112 reference population of the International *Lolium* Genome Initiative (ILGI). As major traits relating to ryegrass forage quality, *in vitro* dry-matter digestibility (IVDMD), neutral detergent fiber (NDF), estimated metabolisable energy (EstME), protein content (CP), and water soluble carbohydrates (WSC) have been determined on spaced plants employing NIRS calibrations. In total 42 QTL have been identified for these quality traits in six different experiments. Major QTL regions were located on linkage groups 3, 5, and 7. The QTL at linkage group 7 were located in the vicinity of three genes coding for enzymes involved in lignin biosynthesis, COMT, CCR1, and CAD.

QTL for WSC and WSC components have been identified in a *L. perenne* F₂ population (Turner et al. 2006), derived from contrasting grandparent plants with regard to WSC content. Plant samples were taken from tiller and leaf bases of spaced plants. While WSC was determined by a colorimetric assay, WSC components including concentrations of sucrose, glucose, fructose, and fructans at different degrees of polymerization were determined by HPLC. In total, 14 QTL for WSC and WSC components were detected, clustering on linkage groups 1, 2, 5, and 6 (Turner et al. 2006). Major QTL explaining more than 30% of the phenotypic variation were identified for WSC and fructan concentration on linkage group 6. Two loci coding for alkaline invertase were mapped to linkage group 6 in the vicinity of QTL for glucose and fructose concentration, which is in agreement with the biochemical role of alkaline invertase to break down sucrose into fructose and glucose.

Grasses can contribute to avoid malnutrition of ruminants, as shown for grass tetany (hypomagnesaemia) caused by grass varieties with low levels of magnesium. Yamada and Forster (2005) reported on the mapping of QTL for concentrations of multiple micro- and macronutrients determined by inductively-coupled plasma mass spectrometry in the p150/112 mapping population. In total, 45 QTL were identified, with major clusters on linkage groups 1, 2, 4, and 5, and QTL for magnesium content located on linkage groups 2 and 5.

In other forage grass species such as *Festuca* sp. and timothy, molecular markers and mapping populations have been developed more recently (e.g., Cai et al. 2003, Alm et al. 2003). Thus, results on forage quality in additional forage grass species can be expected in the near future.

Forage legumes are considered to be beneficial for the quality of forage mixtures including grasses. High digestion rates especially of the protein fraction might result in protein foaming or reduced conversion into ruminant biomass, which can be controlled by moderate levels of condensed tannins (see 2.3). Although marker-aided studies including QTL mapping have been performed in alfalfa (Brummer

et al. 2005), white and red clover (Sawbridge et al. 2003, Herrmann et al. 2006), investigations targeting forage legume quality are lacking so far.

3.3. Transferability of QTL

For some forage crops such as alfalfa, commercial varieties are mainly tetraploid although diploids also exist. In this case, QTL mapping in diploids is much more straightforward. However, QTL detected at diploid level might not be functional at the autopolyploid level. A well known example for differences in gene action at diploid and tetraploid level is the presence of gametophytic self-incompatibility in diploid potatoes, whereas autotetraploid potatoes generally are self fertile (Becker 1993). Furthermore, autopolyploids generally have enlarged cells and vegetative organs as compared to diploid forms (Becker 1993). This implies that tetraploid performance can only partly be predicted based on “diploid information”. Similarly, prediction of genotype or family performance to be grown in swards based on information obtained at the single-spaced plant level might be poor. Posselt (1984) reported a generally lower heterosis for agronomic traits for ryegrass in swards as compared to spaced plants. Furthermore, low correlations were found in ryegrass for seed yield components evaluated in plots versus single plants (Elgersma 1990). Thus, depending on the trait of interest, the mode of testing genotype or family performance is essential with regard to the transferability of information for breeding of superior varieties under practical conditions. Another topic relates to trait evaluation in monoculture or mixture with other species. The latter might be closer to agronomic practice but complicate QTL mapping. For all above mentioned questions it is important to know the correlations between experimental and agronomic performance.

Genome regions increasing GCA within a given synthetic are of highest priority for synthetic breeding. Hence, evaluation of testcross rather than per se performance (after cloning of mapped genotypes) will be preferable. In hybrid breeding, per se performance of inbreds is of minor interest compared to that of hybrid performance. In an experiment on mapping of QTL for forage traits in maize, four segregating populations were established within the flint heterotic pool and evaluated for forage traits after testcrossing to elite dent tester inbreds at the hybrid level (Lübberstedt et al. 1997a, b, 1998). The predictive value of QTL was evaluated by comparing QTL results across testers within one population or across populations using the same tester. The three small validation populations had zero, one, or both parent lines in common with the large calibration population. Generally, the number of common QTL across populations increased with the genetic similarity of mapping populations. Almost all QTL detected in the small independent sample were also detected in the calibration population, both derived from the same cross, or could be explained by linked QTL with reversed gene effects. Hence, from a breeders perspective small populations might be sufficient for detecting the most relevant QTL.

For unrelated mapping populations, about 70% of the detected QTL were specific to each population. However, consistency of QTL across populations as well as testers was highly trait-dependent. In conclusion, QTL or genes identified in

different populations or test systems (like plots versus single spaced plants) need re-evaluation in breeding populations under practical relevant conditions. No information is available so far about the transferability of forage grass or legume QTL from spaced plant to plot performance, or the transferability between populations.

4. FUNCTIONAL MARKERS

A concept for definition, development, application, and prospects of functional markers (FM) in plants has recently been published (Andersen and Lübberstedt, 2003). In contrast to anonymous genetic markers such as morphological, isozyme, or random DNA markers (microsatellites, AFLPs, RFLPs, etc), FM are derived from polymorphic sites within genes causally involved in phenotypic trait variation. The major drawback of anonymous genetic markers is that their predictive value depends on the known linkage phase between marker and target locus alleles (Lübberstedt et al. 1998). Thus, (quantitative) trait locus mapping is necessary for each cross de novo, as different subsets of QTL are polymorphic in individual populations, and linkage phases between marker and QTL alleles can disagree even in closely related genotypes. In contrast, once genetic effects have been assigned to functional sequence motifs, FM derived from such motifs can be used for fixation of gene alleles (defined by one or several FM alleles) in a number of genetic backgrounds without additional calibration. FM development requires (1) functionally characterized genes, (2) allele sequences from such genes, (3) identification of polymorphic, functional motifs affecting plant phenotype within these genes, and (4) validation of associations between DNA polymorphisms and trait variation. Finally, application of FM depends on the availability of robust marker assay technologies.

Accumulation of plant nucleotide sequences proceeds rapidly with almost 2.74 million entries deposited for maize at GenBank in April 2006 (<ftp://ftp.ncbi.nih.gov/genbank/gbrel.txt>). However, only a limited number of genes have so far been assigned to an “agronomic function” (Lübberstedt et al. 2005). Furthermore, while few projects are focused on comprehensive allele sequencing (<http://www.maizegenetics.net/>), the major effort has been spent on characterizing one or few genotypes per species. The purpose of the following chapter is to describe methods for isolation of “forage quality genes” while including the so far functionally characterized genes.

4.1. Gene Isolation

In case of forage quality, biochemical pathways and genes coding for enzymes of these pathways have been shown to contribute to forage quality (see 2.3). Therefore, these genes qualify for candidate genes to be validated for their role in respect to forage quality by hypothesis-driven “reverse genetics” approaches. Additional genes controlling forage quality can be identified by phenotype-based

“forward genetic” approaches. For both reverse and forward genetics, efficient high-throughput approaches have been developed in the past decade in the area of plant genomics, allowing for parallel evaluation of multiple genes.

4.1.1. *Mutants*

In maize, *bm* mutants have originally been identified based on their phenotypic effect on the coloration of midribs. Later it has been shown, that *bm* mutants have an increased digestibility, but inferior agronomic performance (Barriere and Argillier 1993). Two of the four *bm* genes have been isolated and shown to code for enzymes in the monolignol biosynthesis (Boerjan et al. 2003). More recently, *brittle stalk-2 (bk2)* coding for a COBRA-like protein has been shown to mediate cellulose-deposition in secondary cell-walls of maize (Ching et al. 2005). *Bk2* affects both the strength and lignin content of maize stems and by this likely forage quality. Consequently, genes involved in cell-wall biosynthesis and especially those in the monolignol biosynthesis as well as in the polymerization of lignins can be considered as candidate genes with respect to forage quality (Barriere et al. 2003).

Comprehensive knock-out mutant collections mainly based on the Mutator transposon have been and are being established (<http://www.mutransposon.org/project/>) in maize both for forward and reverse genetic screening of traits and genes. For example, the NSF-funded Maize Gene Discovery Project uses *RescueMu* plasmid rescue to create immortalized collections of insertion sites in *E. coli* (Lunde et al. 2003). Over 70.000 *RescueMu* flanking sequences have been sequenced, while cataloging mutant seed and cob phenotypes of 23.000 maize ears, 6.200 families of maize seedlings, and 4.000 families of adult maize plants carrying *MuDR/Mu* and *RescueMu* insertion allele. To obtain seed, users can search the web site database for insertions into genes of interest and then perform PCR or hybridization on column libraries to ascertain which plant has the mutation (Lunde et al. 2003). Respective knock-out mutant collections have not been reported so far for forage grasses or forage legumes.

4.1.2. *Transformation, RNAi, and virus induced gene silencing*

Transformation for in vivo validation of gene function is established for the major forage crops (Spangenberg et al. 2001). Transgenic manipulation of lignin biosynthesis in tall fescue confirmed the central role of genes coding for CAD and COMT in controlling forage quality in grasses (Chen et al. 2003, 2004). Down-regulation of both genes led to improved forage quality. Since additional genes involved in monolignol formation and in polymerization of monolignols have already been isolated from forage grasses (Lidgett et al. 2005, Gavnholt and Larsen 2002), validation of these genes with respect to forage quality can be expected in the near future. Likewise, genes in fructan metabolism in ryegrass such as fructosyl-transferases or invertases have been isolated (Chalmers et al. 2005). Transgenic modification of fructan metabolism in grasses has so far been restricted to introduction of alien (such as bacterial) genes (e.g., Ye et al. 2001).

In forage legumes, one focus is on generation of high levels of condensed tannins by use of genes involved in anthocyanidine biosynthesis (Robbins et al. 2005, O'Donoghue et al. 2005), using so far genes from the model legumes *Lotus ssp.* and *Medicago trunculata*. With respect to lignification, down-regulation of C4H and C3H showed even stronger effects on digestibility in alfalfa as compared to COMT and CAD (Reddy et al. 2005). The closest correlation to digestibility was found for lignin content, but not lignin composition.

Recently, RNAi (RNA interference) has emerged as method of choice to validate gene function in the context of plant development. The essence of RNAi is the delivery of double-stranded RNA (dsRNA) into an organism, or cell, to induce a sequence-specific RNA degradation mechanism that effectively silences a targeted gene (Waterhouse and Helliwell 2003). An important aspect of using RNAi in plant genomics is the delivery of the silencing-inducing dsRNA. This RNA can be delivered by stably transforming plants with transgenes that encode dsRNA. It can also be transiently delivered by bombarding plants with nucleic-acid-coated beads, by infiltrating plant cells with transgene-carrying *Agrobacterium tumefaciens* or by infecting plants with a virus, either on its own or together with a satellite virus (Waterhouse and Helliwell 2003). At least for genes involved in lignin biosynthesis, application of RNAi for generation of knock-out genotypes for gene function validation appears to be a very promising approach (Capell and Christou 2004). Transformation of maize and forage crops is not possible at high throughput. Alternatively, virus-induced gene silencing (VIGS) has been proposed for rapid *in vivo* gene function tests in maize based on maize streak virus, wheat streak mosaic virus, or barley stripe mosaic virus (Robertson 2004). In addition, VIGS has been established for legume species (Constantin et al. 2004). However, the usefulness of VIGS in relation to forage quality remains to be demonstrated.

4.1.3. *Model species*

Complete plant genomes have been sequenced for the model species *A. thaliana* and for rice (The *Arabidopsis* Genome Initiative 2000, Goff et al. 2002, Yu et al. 2002). Maize is turning into another plant model species, given the current activities towards completion of whole-genome sequencing and availability of multiple plant genomics related resources (<http://www.maizegdb.org/>). Within legumes, comprehensive functional genomics projects are underway for *Lotus corniculatus*, *L. japonica*, and *Medicago truncatula* (VandenBosch and Stacey 2003) as well as grain legumes such as soybean (Gepts et al. 2005).

Brachypodium distachyon has long been discussed as the ideal model species for forage grasses. Brachypodium is a temperate grass with many growth features in common and close phenotypic similarity to forage and turf grasses. Moreover, orthologous ryegrass ESTs show a substantially higher sequence homology to Brachypodium sequences as compared to rice, supporting a close evolutionary relationship between these two species (unpublished data). Brachypodium has many properties in common with the dicot model species *A. thaliana*, including a similar small genome size and 5 chromosomes per haploid genome,

a short life-cycle, and self-fertility (<http://www.brachypodium.org/>). Important resources (ESTs, BAC libraries, ecotype accessions) and methods (transformation) have already been established for *Brachypodium* (<http://www.brachypodium.org/>). Whereas *A. thaliana* has been accepted as model species (as indicated by its broad use in research) and proven to be useful during the last decade, *Brachypodium* has not found broad acceptance until recently. However, this will likely change after whole genome sequencing of *Brachypodium* has been announced (<http://www.jgi.doe.gov/sequencing/cspseqplans2007.html>). The genome sequencing of *Brachypodium* is expected to be completed and released to the public domain in 2008 (<http://www.jic.bbsrc.ac.uk/corporate/media-and-public/current-releases/060712.htm>).

The term “function” in functional genomics relates to some basic characteristics of genes (e.g. mutant phenotype, biochemical properties, expression pattern of selected genotypes). The ambition for *A. thaliana* is to characterize the function of all genes of this species until 2010 (http://www.arabidopsis.org/info/2010_projects/). Genes of interest can, in principle, be identified for any forage crop by exploiting information based on sequence homology or conserved map position provided from model species. While the usefulness of model species in reverse genetics approaches due to synteny relationships among genomes of related species (see 5.4) is obvious, their implementation in forward genetics strategies to identify novel genes (outside the pathways described under 2.3) controlling forage quality (“agronomic function”) especially of perennial crops might be limited.

Since genomic resources for major forage species have substantially increased over the past five years, they might serve as “model crops” for a broad range of related forage grasses or legumes. Meanwhile (5/2006) about 40.000 ESTs each have been released for *Festuca arundinacea* and *Trifolium pratense* (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). More than 10.000 ESTs have been published for the two *Lolium* species *L. multiflorum* and *L. temulentum*. Substantial numbers of ESTs have been generated for *L. perenne* (>70.000; Sawbridge et al. 2003, Asp et al. 2006), and *T. repens* (>30.000; Spangenberg et al. 2003), but not released so far. Furthermore, a comprehensive collection of “gene thresher” genomic sequences has been produced for *L. perenne* (<http://www.vialactia.co.nz/news/newsitem.asp?id=61>), and partly built into GRAMENE (<http://www.gramene.org/>). International consortia have been formed addressing the development of genomic tools including physical maps and large scale sequencing in *Lolium* and *Festuca* species (e.g., European *Lolium* and *Festuca* Initiative (ELFIN); <http://www.iger.bbsrc.ac.uk/elfin/>) as well as *Trifolium* species (International *Trifolium* Network (ITN), inaugurated 7/2005 at IGER, Aberystwyth, UK).

4.1.4. Sequence homology and synteny

An important question in forage grasses and legumes is, whether the model species investigated are phylogenetically close enough to extract meaningful information by comparative genomics. This seems to be the case within the grass

family (Devos and Gale 2000, <http://ukcrop.net/comparative.html>), but also legumes (Choi et al. 2004). Genes identified in, e.g., a single grass species might be directly used in different forage grasses by gene transfer or for identification of orthologous genes to develop allele-specific markers. An example is the conserved organization of vernalization genes in cereals and forage grasses (Yan et al. 2003, 2004, Jensen et al. 2005a, Andersen et al. 2006). However, identification of orthologues is not always straightforward, as illustrated for the *bm3* gene coding for COMT, mapping on chromosome 4 in maize (Figure 1) and linkage group 7 in ryegrass (Cogan et al. 2005b). According to Alm et al. (2003) describing the synteny between several grass species including maize and ryegrass, no orthologous genome regions between maize chromosome 4 and ryegrass linkage group 7 were discovered at the macrosynteny level. This missing relationship might be detected at the microsytenty level, once more sequence information is available. Moreover, exploitation of synteny to identify relevant loci across species might be complicated in case of gene families, such as in the case of laccases and peroxidases involved in the polymerisation of monolignols (Ralph et al. 2004). However, several relevant forage grasses or legumes are closely related, increasing the chance for transferability of synteny-based information within these two groups of species. A promising system for information transfer between different forage grasses as well as rice are *L. perenne* / *Festuca pratensis* introgression families exploiting high levels of polymorphism between both crossable species with markers or sequences assigned to the rice genome sequence for “introgression landing”, as demonstrated for the fine mapping and candidate gene identification for the *F. pratensis* stay green locus (King et al. 2005).

The original synteny studies have been based on RFLP mapping across species using cDNA probes (Devos and Gale 2000). Grass genomes were shown to be organized in a limited number of chromosome blocks. More recent availability of complete genome sequences in *Arabidopsis* and rice and sequenced BAC contigs in other species as well as comprehensive (mapped) EST collections led to re-evaluation of the synteny concept (Delseny 2004). These sequencing projects revealed numerous polyploidization events after speciation even in the small genome species *A. thaliana* (Blanc et al. 2003), but also in rice and maize (Salse et al. 2004). Furthermore, large genomes like the maize genome have been invaded by retrotransposons resulting in reshuffling of original ancestral genomes due to duplications, deletions, illegitimate recombination etc. (Delseny 2004). Since these events occurred after speciation of maize, substantial differences have been reported between larger allelic sequence stretches even within maize among different inbred lines (Fu and Dooner 2002, Brunner et al. 2005). An *in silico* alignment of genomic rice sequence with mapped maize EST sequences identified larger collinear chromosome regions between rice and maize in agreement with previous studies (Salse et al. 2004). However, fine-scale analysis revealed besides several duplicated regions numerous internal rearrangements within syntenic chromosome blocks. In conclusion, rice can be used to identify candidate genes in a target region identified in maize, but the order and number of genes might be altered at the microsytenty

level between rice and maize. The high flexibility regarding chromosome mutations (Brunner et al. 2005) might be specific for the maize genome.

4.1.5. *Map-based gene isolation*

In maize, map-based gene isolation of QTL controlling forage quality will become much more efficient with the availability of the whole genome sequence in a few years. All relevant additional resources needed for map-based gene isolation such as mapping populations, BAC libraries, markers at high density are already publicly available (<http://www.maizegdb.org/>).

In forage grasses and legumes, BAC libraries have been reported for *L. perenne*, *F. pratensis*, and *T. repens* (Farrar et al. 2005, Spangenberg et al. 2003, Donnison et al. 2005). Combined with the increasing availability of molecular markers in these species (see 3.2; Jensen et al. 2005b, Lübberstedt 2005), map-based gene isolation has generally become possible. In addition, large numbers of SSRs have recently been published for timothy (>300; Cai et al. 2003) and red clover (>1200; Sato et al. 2005).

4.1.6. *Expression profiling*

Microarray-based expression profiling enables the simultaneous study of several and ultimately all genes of an organism in one experiment. In maize, publicly available microarrays contain currently PCR fragments from more than 10,000 different ESTs (<http://www.maizegdb.org/microarray.php>), about 18,000 probe sets covering 15,000 different genes (<http://www.affymetrix.com/products/arrays/specific/maize.affx>), whereas long oligo microarrays include 58,000 different oligonucleotides (<http://www.maizearray.org/>). Since current maize microarrays do not cover all maize genes, complementary approaches can be employed to identify, e.g., rare transcripts, such as suppression subtractive hybridization (SSH) (Diatchenko et al. 1996). The SSH procedure enriches cDNA libraries for low-abundant and differentially expressed mRNAs by normalization (Diatchenko et al., 1996). Shi et al. (2005) used SSH combined with macroarray hybridization to identify differentially expressed genes in near isogenic lines for virus resistance genes in maize.

Expression profiling was employed to identify novel candidate genes involved in forage quality using maize unigene (cDNA) microarrays as well as macroarrays containing SSH derived EST fragments differentially expressed in a *Bm3/bm3* isogenic line contrast (Shi et al. 2006, Lübberstedt et al. 2006). About 10% of the sequences on the microarray were mapped *in silico* allowing an assignment of respective differentially expressed genes to maize chromosomes. "Stems" (more precisely: leaf sheaths) of 5–7 week old maize plants were compared in isogenic comparisons in three genetic backgrounds (KWa, KWb: inbreds from KWS Saat AG; public line F2) with respect to *bm1*, *bm2*, and *bm3*. Moreover, extremes of three QTL mapping populations differing in cell-wall digestibility were bulked and compared with respect to differential expression (Lübberstedt et al. 2006; Shi et al., 2007). Summarized over the different comparisons, several 1000 genes showed

significant differential expression in individual experiments. Moreover, most genes involved in monolignol biosynthesis showed differential expression in at least one of the *Bm/bm* isogenic comparisons. When comparing *Bm3/bm3* isogenic line contrasts in KWa, KWb, and F2, 53 genes were consistently differentially expressed. Although the *bm3* gene is located at chromosome 4, mapped differentially expressed genes in isogenic *bm3* contrasts were distributed across all maize chromosomes. Thus, isogenic contrasts for a well characterized candidate gene or genome region might reveal additional genes of interest based on differential expression involved in the same metabolic pathways or with regulatory function. For further investigations, differentially expressed genes cosegregating with forage quality QTL have high priority. In the study of *Bm/bm* isogenic contrasts (Shi et al. 2006), at least one differentially expressed gene was identified per chromosomal bin region (Gardiner et al. 1993) for each of the forage quality QTL described by Ralph et al. (2004).

For ryegrass and white clover, the International Transcriptome Initiative for Forage and Turf (ITIFT) for forage plant gene discovery based on expression profiling has been announced recently (Webster et al. 2005). Currently 15k cDNA unigene microarrays are provided for each ryegrass and white clover. Most likely, such microarrays will be useful for other closely related forage species such as *Festuca* ssp. Moreover, Affymetrix microarrays are meanwhile available for rice, wheat, and *M. trunculata* (<http://www.affymetrix.com/community/research/consortia.affx>), which should be useful in heterologous hybridization studies, as demonstrated, e.g., for alfalfa (Aziz et al. 2005, May 2005).

4.2. Allelic Diversity

Once genes affecting forage quality have been identified, the next step towards development of functional markers is the identification of polymorphisms within these genes causally affecting the target trait.

4.2.1. Association studies in maize

One major limitation in “classical” QTL studies is the low number of (typically 2–4) segregating alleles, adding to the QTL transferability problem. More recently, more complex population structures allowing simultaneous evaluation of multiple alleles at different levels of genetic resolution have been proposed (Thornsberry et al. 2001, Churchill et al. 2004). Assignment of an “agronomic function” to short sequence motifs can be achieved by candidate gene based association studies (Risch 2000). This approach is limited by linkage disequilibrium (LD), i.e. haplotype structures in the gene(s) of interest. However, for several genes a generally low LD was detected in maize (Remington et al. 2001; Flint-Garcia et al. 2003), including examples in elite materials (Zein et al. 2007). Thus, candidate gene-based association studies are promising in maize. In heterogeneous genotype collections associations identified for specific sites might be confounded with effects from other genome regions especially in case of population stratification (Pritchard et al. 2000), which needs to be taken into account for interpretation of results from association studies.

In a pioneering study, Thornsberry et al. (2001) demonstrated the feasibility of association studies in maize to identify sequence polymorphisms within genes affecting characters of agronomic significance. While taking population structure into account, nine SNP or INDEL polymorphisms were shown to significantly affect flowering time in a set of 92 diverse maize lines. In part, these results were confirmed in a collection of European elite inbred lines (Andersen et al. 2005). The major reason for non-significance of some of the nine polymorphisms identified very likely the much narrower genetic material investigated by Andersen et al. (2005) as compared to Thornsberry et al. (2001).

First reports on association studies for genes involved in cell wall biosynthesis confirm that these pathways are promising targets for identification of polymorphic sites associated with forage quality, and thus FM development (Barriere et al. 2003). Zein et al. (2006) investigated the sequence variation at the *Bm3* locus in a collection of 42 European maize inbred lines, contrasting with respect to stover DNDF and relevant for hybrid maize breeding in Central Europe. For association with forage quality, stover digestibility was determined in six environments between 2001 and 2003 in Germany (heritability >0.9). One INDEL polymorphism within the intron revealed significant association with stover digestibility (Lübberstedt et al. 2005). In a study of Guillet-Claude et al. (2004a), polymorphisms both in the AldOMT (= *Bm3*) and the CCoAOMT2 but not CCoAOMT1 coding genes showed significant association with maize digestibility. Moreover, polymorphisms in the maize peroxidase gene *ZmPox3* were also significantly associated with maize digestibility (Guillet-Claude et al. 2004b). In conclusion, availability of qualified candidate genes can be effectively converted into informative molecular markers by means of association studies. In maize, comprehensive association studies are ongoing in the group of E. Buckler (<http://www.maizegenetics.net/>), where 18 genes are studied in a panel of 102 maize genotypes, and within Genoplante (e.g. Guillet-Claude et al. (2004a)).

4.2.2. *Association studies in forage grasses and legumes*

Association studies based on candidate genes are especially promising in species with a generally low linkage disequilibrium (Flint-Garcia et al. 2003), as can be expected for outcrossing forage crops. Studies on systematic allele-sequencing and association studies in ryegrass are currently ongoing in the EU project GRASP (<http://www.grasp-euv.dk>, Xing et al. 2006), including genes involved in lignin biosynthesis (Lübberstedt et al. 2003). First results from a similar project aiming at development of gene-derived SNP markers in ryegrass, including genes from lignin and fructan biosynthesis, have been presented by Pointing et al. (2005) and Forster et al. (2005). SNP development and association studies have been initiated in white clover as well (Forster et al. 2006).

4.2.3. *TILLING*

TILLING (McCallum et al. 2000) can be employed to relate sequence polymorphisms with phenotypic variation. Variants for virtually all genes of interest in a

fixed genetic background can be produced by TILLING (McCallum et al. 2000). The advantage of TILLING as compared to association studies is that isogenic lines are compared avoiding statistical artefacts due to population structure effects. The disadvantage of TILLING is that establishing a comprehensive TILLING population covering most genes is quite laborious. Therefore, TILLING populations are usually restricted to one or few genetic backgrounds and the alleles fixed within the respective “background genotypes”. Thus, if a knock-out allele is fixed at a locus of interest, it might be not possible to identify revertants. Two TILLING populations for maize have been produced at Purdue (<http://genome.purdue.edu/maizetilling/>) in B73 and W22 background, available for the maize research community. Within the next few years, 150 maize genes will undergo systematic studies using this resource (<http://genome.purdue.edu/maizetilling/>). The development of TILLING populations in forage grasses and legumes is complicated by the lack of homozygous diploid inbred lines in most species, since both phenotypic and heteroduplex analysis in segregating offspring of heterozygous genotypes is impaired. Kardailsky et al. (2005) reported the first TILLING population in ryegrass. In the longer run, establishment of homologous recombination as established in moss (*Physcomitrella patens*) (Schaefer and Zryd 1997) would be desirable to generate isogenic genotypes with defined polymorphic differences and, if possible, in any genetic background.

5. IMPLEMENTATION OF MARKERS IN FORAGE CROP BREEDING

Independent of the breeding category, cultivar breeding includes three phases: I) generation of genetic variation, II) development of genetic components for producing new varieties (such as inbred lines in hybrid breeding), and III) testing of experimental varieties (Becker 1993). Molecular breeding benefits all three phases, but is also useful in the context of variety registration and protection as well as for the characterization and management of genetic resources. The predictive value of markers depends on whether they are random DNA markers, gene-derived or functional markers (Andersen and Lübberstedt 2003).

Markers are useful to establish heterotic groups (Riday and Brummer 2003), and to assign genotypes or populations to heterotic groups. This topic might become increasingly relevant, if forage crop breeding moves from population or synthetic breeding to hybrid breeding (Riday and Brummer 2003). Furthermore, markers might assist identification of genetically divergent parent genotypes or populations with a maximum usefulness (Lamkey et al. 1995, Schnell 1983) to generate better varieties. Finally, recurrent selection programs might benefit from the application of markers ensuring, e.g., a sufficient level of genetic variation over several selection cycles.

Marker-assisted selection (MAS) and backcrossing (MAB) are major applications of molecular markers. MAS is especially promising for traits with low heritability (Lande and Thompson 1990), whereas MAB allows tracing of favourable alleles, which is especially useful in case of recessive gene action. In case of MAB,

including transfer of transgenes across genotypes and populations, markers are useful for background selection (Frisch et al. 2001) to recover the elite parent background efficiently in a short time. For MAS, an increasing number of candidate gene-derived or even functional markers (Andersen and Lübberstedt 2003) will become available in the near future, as demonstrated for ryegrass recently (Faville et al. 2004). Functional markers will reduce the risk of a Type 3 error in declaring QTL-marker associations (Dudley 1993), i.e., declaring in case of a significant QTL the wrong marker allele as being linked to a favourable QTL allele. In addition, markers can be employed to predict the performance of components of hybrid or synthetic varieties using BLUP (Bernardo 2002).

During the final steps of variety production markers can be used to (i) reduce the amount of experimental testing, (ii) confirm hybridity, and (iii) fulfill DUS criteria in variety registration (Tommasini et al. 2003). Finally, gene bank collections might benefit from molecular markers to describe and maintain genetic resources, as well as to establish core collections.

FM would be superior as compared to anonymous markers for selection of, e.g. parent materials to build segregating populations, as well as subsequent development of inbred lines. FM would also be useful for variety registration based on presence/absence of specific alleles at morphological trait loci currently used to discriminate varieties. Rapid progress in sequencing projects, especially for maize, and genomics will shift the current bottleneck for FM development from the availability of candidate genes and the availability of allele sequence information to the assignment of “agronomic function” with sequence polymorphisms, which is currently not systematically considered in functional genomics projects. First reports on association studies in maize are promising. The number of sequence polymorphisms useful for FM development can be expected to increase substantially within the next 5–10 years with the availability of further association studies and TILLING experiments, initially in maize but subsequently for other forage grasses and legumes.

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CHAPTER 13

MOLECULAR MAPPING, MARKER-ASSISTED SELECTION AND MAP-BASED CLONING IN TOMATO

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Abstract: Significant progress has been made in molecular marker research in tomato, *Lycopersicon esculentum* Mill., including generation of markers, development of maps, mapping of genes and QTLs, and fine-mapping, characterization and map-based cloning of genes and QTLs. Numerous types of molecular markers have been developed in tomato, including RFLPs, RAPDs, AFLPs, SSRs, CAPS, ESTs, COSs and SNPs. Several molecular maps of tomato have been developed based on different interspecific populations, including the saturated linkage map based on a *L. esculentum* × *L. pennellii* cross. Markers and maps have been utilized extensively to map genes and QTLs controlling agriculturally and biologically important traits and for marker-assisted improvement of many simple-inherited traits such as disease resistance. Marker information also has been used for fine mapping and map-based cloning of several major genes and QTLs. Comparatively, little progress has been made in improving complex traits via marker-assisted selection. However, rapid advances in developing more efficient and resolving markers and in refining QTL positions are expected to lead to a greater use of marker technology for crop improvement in tomato.

1. INTRODUCTION

1.1. Botanical Description

The cultivated tomato, *Lycopersicon esculentum* Mill., is a worldwide-grown vegetable crop and the focus of a large agricultural industry. Botanically, tomato is a fruit as it develops from an ovary, however, in most places it is consumed as a vegetable. It belongs to the nightshade family Solanaceae, which is a diverse family consisting of ~96 genera and over 2800 species (Knapp et al. 2004). Solanaceae is the most variable of all crop families in terms of agricultural utility, the third most economically-important after grasses and legumes, and the most valuable in

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terms of vegetable crops. The family contains many of the commonly cultivated plants, including the tuber-bearing potato (*Solanum tuberosum* L.), fruit-bearing vegetables such as tomato, tomatillo (*Physalis philadelphica* L.), pepper (*Capsicum annum* L.) and eggplant (*Solanum melongena* L.), ornamental plants such as petunia (*Petunia hybrida* hort.) and Nicotiana (*Nicotiana tobacum* L.), medicinal plants such as Jimsonweed (*Datura stramonium* L.), *Capsicum* and *Nicotiana*, plants with edible leaves (e.g. *Solanum aethiopicum* L., *Solanum macrocarpon* L.), and various night-shades weeds. The tomato genus *Lycopersicon* is one of the smallest genera in Solanaceae, though the centerpiece for genetic and molecular research in the family.

The cultivated tomato was originally named *Solanum lycopersicum* by Linnaeus (Linnaeus 1753). In 1754, Miller separated tomatoes and designated the genus *Lycopersicon* and the species *esculentum* (roughly means “edible”) for the cultivated tomato (Miller 1754). Genus *Lycopersicon* was initially distinguished from *Solanum* by its distinct characteristics of anthers and leaves. However, phylogenetic relationships between *Solanum* and *Lycopersicon* have been the subject of a great debate for a long time, with many Solanaceae researchers recognizing *Lycopersicon* as a distinct genus while others suggesting its merge with *Solanum* (for a review see Spooner et al., 1993). More recently, a new taxonomic classification of tomato and re-adoption of *S. lycopersicum* for the cultivated tomato has been suggested based on molecular and morphological information (Knapp et al. 2004; Spooner et al. 1993). The other species of *Lycopersicon* also have been assigned or re-assigned to *Solanum*. In this chapter, however, due to the use and citation of numerous historical references and in order to be consistent with much of the literature, I follow the Miller’s classification and use original *Lycopersicon* names.

There are 9 known species within *Lycopersicon*, including the cultivated type *L. esculentum* and its wild form *L. esculentum* var. *cerasiforme* (Dun.) Gray, and the 8 wild species *L. pimpinellifolium* (Jusl.) Mill., *L. cheesmanii* Riley, *L. chmielewskii* Rick, Kes., Fob. & Holle, *L. chilense* Dun., *L. parviflorum* Rick, Kes., Fob. & Holle, *L. peruvianum* (L.) Mill., *L. hirsutum* Humb. and Bonpl. and *L. pennellii* (Corr.) D’Arcy (Rick 1976a; Rick 1979b). Although all species produce hermaphrodite flowers, a complete range of mating systems is found, from the almost completely autogamous *L. cheesmanii* and *L. parviflorum* to obligately outcrossed, self-incompatible biotypes of *L. chilense*, *L. hirsutum*, *L. peruvianum*, and *L. pennellii*. Self-fertility with various degrees of facultative outcrossing is found in *L. chmielewskii*, *L. esculentum*, *L. pimpinellifolium* and the self-compatible biotypes of *L. hirsutum* and *L. pennellii*. The 9 species have been grouped into two intra-crossable, inter-incrossable groups (or complexes): the “*esculentum* complex”, including *L. esculentum*, *L. esculentum* var. *cerasiforme*, *L. pimpinellifolium*, *L.cheesmanii* (including *L. cheesmanii* f. *minor*), *L. chmielewskii*, *L. parviflorum*, *L. hirsutum* (including f. *typicum* and f. *glabratum*) and *L. pennellii*, and the “*peruvianum* complex” including *L. peruvianum* and *L. chilense* (Rick 1979a). All species within the *esculentum* complex can be hybridized with the cultivated tomato relatively easily and most (except *L. hirsutum* f. *typicum* and some *L. pennellii*) are self-compatible. The two species within the *peruvianum* complex are extremely

diverse and represent a wealth of characteristics potentially valuable to tomato improvement. However, these species have been rather partial in their usefulness to cultivated forms due to various barriers present in sexual hybridization and gene transfer (Rick 1973). For example, they can be hybridized with members of the *esculentum* complex only by the application of techniques such as embryo rescue.

All tomato species are native to a small area in western S. America, between Ecuador and Chile (Rick 1976b). However, their natural habitat is highly variable, from very dry to very wet and from coastal to mountainous areas of more than 3300 m elevations (Warnock 1988). Fruit color varies depending on the species, from red and orange to yellow and green. Among the 9 species, only *L. esculentum* has become a domesticated crop (Rick 1978), which includes the common fresh-market and processing tomatoes, land races and primitive cultivars, as well as the wild cherry. Fruits of the cultivated species come in a wide range of shapes, sizes and colors. Some may be globe, round, flattened, oval, heart, or elongated and even square-shaped, and which may be red, pink, golden, yellow, striped, purple, green or white (Stevens and Rick 1986). The average weight for the garden tomato is 4 to 6 oz., but some varieties weigh up to 2 pounds. Both the wild cherry and currant tomatoes (*L. pimpinellifolium*) produce shiny, bright red berries whereas the Galapagos tomatoes (*L. cheesmanii*) produce dull purplish or yellow-orange berries (diameter \approx 1 cm). The rest of the wild species produce small, inedible green fruit.

All tomato species are diploid ($2N = 2X = 24$) and are similar in chromosome number and structure. Tomato's 12 chromosomes are highly differentiated and can readily be distinguished at pachytene. Tomato is very sensitive to any chromosome imbalance, and no aneuploid with euchromatic deficiencies can be maintained (Rick and Gill 1973). It is one of the most genetically characterized higher plant species and an excellent model system for basic and applied research. This is due to many reasons, including ease of culture, short life cycle, high self-fertility and homozygosity, great reproductive potential, ease of use for controlled pollination and hybridization, availability of a wide array of mutants and genetic stocks (<http://tgrc.ucdavis.edu/>; <http://www.sgn.cornell.edu/>), diploid with a rather small genome (0.86 pg, 950 Mbp) (Arumuganathan and Earle 1991), and amenability to asexual propagation and protoplast, cell and tissue cultures and whole plant regeneration thereof (McCormick et al. 1986). Members of *Lycopersicon* are easily transformed, and transgenic tomatoes are routinely produced using *Agrobacterium tumefaciens* (McCormick et al. 1986). Recent availability of high molecular weight insert genomic libraries (YACs and BACs) has facilitated map-based gene cloning in tomato. Moreover, advances in tomato EST database and genome sequencing have added additional tools for further expansion of basic and applied research in tomato.

1.2. Domestication and Genetic Variation

Peru was earlier widely accepted as the center of tomato domestication, however, "the bulk of the historical, linguistic, archaeological and ethno-botanical evidence favors Mexico as the source of the cultivated tomatoes" (Rick 1976b) and the

most likely center of domestication. It is not known exactly when domestication of tomatoes occurred, however, by the time the Spanish conquered Mexico in 1523, they were already domesticated (Rick 1978). Accordingly, the Spanish explorers found tomatoes in Central America and took seed to Europe, from where it was disseminated to many other parts of the world (Rick 1976b). A comparison of hereditary enzyme variants revealed much greater similarity between the older European cultivars and the primitive cultivars and cherry tomatoes of Mexico and Central America than between the European cultivars and the primitive plants of the Andean regions (Rick 1976b, 1991). The first record of tomatoes in Europe is credited to descriptions published in 1554 by Italian herbalist Pier Andrea Mattioli, while the first recorded mention of it in N. America was in 1710 (Rick 1978). However, in the U.S., commercial production of tomato in a small scale began in 1847 at Easton, Pennsylvania, and it grew to become a major vegetable crop in the mid 20th century.

The cultivated tomato has a narrow germplasm base, largely because of several population bottlenecks that occurred during domestication and evolution of modern cultivars (Rick 1976b). Although higher levels of variability are found in primitive cultivars in the native regions of tomato, only about 5% of the total genetic variation within *Lycopersicon* can be found within *L. esculentum* (Miller and Tanksley 1990; Rick and Fobes 1975). The related wild species of tomato, however, are a rich source of desirable genes and have been utilized extensively to improve agricultural characteristics of tomato, in particular disease resistance. In addition, there are thousands of monogenic mutants (<http://tgrc.ucdavis.edu/>; <http://www.sgn.cornell.edu/>), which are useful for genetics and breeding studies in tomato. Recent advancements in molecular markers and marker-assisted selection (MAS) have made tomato breeding via introgression from related wild species and mutants more efficient, as discussed in this chapter.

1.3. Economic Importance

The tomato has become an important crop worldwide in less than one century. Worldwide, it is the 2nd most consumed vegetable per person (after potato) (FAOSTAT 2005) and unquestionably the most popular garden vegetable. In the U.S., it is the 3rd most economically important vegetable crop (with a total farm value of \$2.062 B) after potato (\$2.564 B) and lettuce (\$2.064 B) (<http://www.usda.gov/nass/pubs/agr05/agstats2005.pdf>). In addition to tomatoes that are eaten directly as raw vegetable or added to other food items, a variety of processed products have gained significant acceptance. Although a tropical plant, tomato is grown in almost every corner of the planet. It is grown in greenhouses where summers are too cool for pollination or fruit set in outdoors. Major production countries in descending orders include China, U.S.A., Russia, Turkey, India and Italy (FAOSTAT 2005). Worldwide, in 2005 a total of 4,528,519 ha of tomato were harvested with a total production of 124,748,292 Mt (FAOSTAT 2005). In N. America, production occurs in the U.S., Canada and Mexico, comprising a total of

310,000 ha. In the U.S., total harvested area in 2004 was estimated to be 170,808 ha (505,60 ha fresh-market tomatoes valued \$1.34 B and 120,248 ha processing tomatoes valued \$0.72 B) (<http://www.nass.usda.gov:8080/QuickStats/index2.jsp>). California is by far the leading producer of processing tomatoes followed by Florida, which is also the leading state in producing fresh-market tomatoes (USDA 2005). Per capita consumption in the U.S. includes 31.7 kg of processing and 8.7 kg of fresh tomatoes (<http://www.ars.usda.gov/>). Although tomatoes do not rank high in nutritional value, by virtue of volume consumed, they contribute significantly to the dietary intake of vitamins A and C and essential mineral and nutrients. In the U.S. diet, for example, tomato ranks first among all fruits and vegetables as a source of vitamins and minerals (Rick 1980). Furthermore, tomatoes are the richest source of lycopene, a phytochemical that protects cells from oxidants that have been linked to cancer (Giovannucci 1999).

1.4. Past Breeding Achievements

Research to develop new cultivars of tomato with improved characteristics started more than 200 years ago in Europe. Effective breeding programs in the U.S., however, started a little over a century ago (Stevens and Rick 1986). Livingston is generally recognized as the first tomato breeder in the N. America, conducting breeding in 1870s (Tigchelaar 1986). Almost until the middle of the 20th century, tomato-breeding objectives included development of multipurpose cultivars to meet several needs, including fresh-market and processing industries. Since then, however, tomato breeding objectives have depended upon method of culture, that is, field or greenhouse-grown tomatoes and whether the product has to be used fresh or processed (Stevens and Rick 1986; Tigchelaar 1986). However, the universal goal of tomato breeding has been to increase fruit yield per unit area. Other essential characteristics common to both fresh-market and processing industries include disease resistance, broad adaptability, earliness in maturity, ability to set fruit at adverse temperatures, resistance to rain-induced cracking, tolerance to major ripe-fruit rots, adequate vine cover, fruit firmness and several other fruit quality characteristics. Specific traits needed in processing cultivars include compact, determinate plant habit suitable for machine harvest, ease of fruit separation from the vine (jointless character), and specific fruit quality characteristics such color, pH, total acidity, soluble solids, total solids, viscosity (consistency). In comparison, specific traits of interest in fresh-market cultivars include large, round fruit with adequate firmness and shelf-life, uniform fruit size, shape and color, appearance, freedom from external blemishes or abnormalities, texture, taste and flavor (Stevens and Rick 1986; Tigchelaar 1986). During the past several decades remarkable progress has been made in improving tomatoes for various important traits. For example, between 1920s and 1990s, fruit yield of processing cultivars in the U.S. increased from 10.1 to 72.4 tons/ha, a 7.2-fold increase (Warren 1998). A recent statistic by the USDA indicated processing tomato yield of ~102 tons/ha in the U.S. in 2004 (<http://www.nass.usda.gov:8080/QuickStats/index2.jsp>). Furthermore, most

recently developed cultivars possess up to 6 (in true-breeding lines) or 10 (in hybrids) disease-resistance attributes. These mainly include diseases for which major resistance genes have been identified, including fusarium wilt, verticillium wilt, root-knot nematode, alternaria stem canker, gray leaf spot, and some bacterial and viral diseases. All original characterizations and gene introgressions were through phenotypic selection (PS) and traditional breeding protocols. Even today much of the disease resistance breeding in tomato is through the use of the same protocols. However, there are major limitations when using traditional protocols for gene transfer, in particular transfer from wild to the cultivated species. Thus, tomato breeders have consistently sought more effective breeding approaches. During the past two decades, the use of molecular markers and MAS techniques has facilitated identification, mapping and transferring of many genes in tomato. The use of MAS is becoming a routine procedure in tomato improvement, in particular when breeding for disease resistance, as described in below.

1.5. New Breeding Approaches

Traditional protocols of plant genetics and breeding, which are based on PS and progeny testing, have been very effective in improving crop productivity and quality during the past several decades (Duvick 1996). These methods, however, are often time consuming and not without inherent difficulties. For example, the average length of a breeding project for a seed or vegetable crop, from hybridization and selecting the new genetic combinations to testing them in the field and introducing them in the market, is ~10–15 years. This lengthy process may not allow the time-sensitive needed increase in crop productivity in the future. Furthermore, for many desirable agronomic and horticultural characteristics, controlling genes may be found only in exotic genetic backgrounds, utilization of which often encounters difficulties. After interspecific hybridization, a major task becomes eliminating the great bulk of undesirable genes introduced from the wild donor. A series of backcrosses to the cultivated type alternated with concurrent inbreeding are required to select the desired combination of parental characteristics. During this process, however, some genes of interest from the wild donor may be lost or eliminated, limiting the breeding success. These and other problems associated with traditional breeding methods warrant the use of techniques that have higher resolutions.

An alternative approach to improving effectiveness of selection is to discover and use genetic markers that are associated with genes or quantitative trait loci (QTLs) that control the trait(s) of interest. Marker technology can facilitate determination of the number, chromosomal location, and individual and interactive effects of genes or QTLs affecting a trait. Following their identification and localization, useful genes or QTLs can be introgressed into desirable genetic backgrounds via MAS, or isolated via map-based cloning. MAS may not only speed up the process of gene transfer, but it also allow pyramiding of desirable genes and QTLs from different genetic backgrounds. This can be an effective approach to substantial crop improvement, more so than potentially feasible through PS. Furthermore, in *Lycopersicon*, where

most genetic variability is within the wild species, identification and transfer of genes from the wild into the cultivated type may be significantly facilitated by the use of marker technology. In below, the current status of markers and maps, gene and QTL mapping, marker-assisted breeding and map-based cloning in tomato is reviewed and discussed.

2. GENETIC MARKERS AND MAPS

2.1. Classical Markers and Maps

The potential value of genetic markers for use as indirect selection criteria has been known to breeders for more than 80 years, since morphological and physiological markers were used to tag and select for superior phenotypes. Although morphological markers are easy to monitor, their use in genetics and breeding studies is restricted due to factors such as limitation in number, expression of dominance or epistatic interactions, pleiotropic effects, and incomplete penetrance or expressivity. In tomato, there are over 1300 known morphological, physiological and disease resistance genes (Chetelat 2002). During 1970s and 1980s, the second-generation markers, namely isozymes, became popular and were used for genetic mapping in plant species. In tomato, 41 isozymic loci corresponding to 15 unique enzymatic reactions have been characterized (Tanksley 1993). While isozyme markers have several advantages, including the low cost and ease of use, environmental insensitivity, lack of pleiotropic effects and co-dominant inheritance, they are very limited in number and often not polymorphic among closely-related genotypes (Foolad et al. 1993). Although currently isozyme markers are not used widely, before the era of DNA markers they were considered highly useful in genetics and breeding studies.

The first classical linkage map of tomato, showing markers on all 12 linkage groups, was reported in 1968 and included a total of 153 morphological and physiological markers (Butler 1968). For the next several years, the map was expanded and by 1975 more than 258 morphological and physiological markers were assigned to tomato chromosomes (Rick 1975). At that time, tomato had one of the best linkage maps of any plant species. The classical map information in 1970s greatly facilitated mapping of isozyme loci. The first complete isozyme linkage map of tomato was published in 1980, which included 26 isozyme markers (Tanksley and Rick 1980). Currently, there are 36 known isozyme markers in tomato that have been mapped (Tanksley 1993). The latest published classical linkage map of tomato consisted of 285 morphological, physiological, isozyme and disease resistance genes mapped onto the 12 tomato chromosomes (Tanksley 1993; Chetelat 2002).

2.2. Molecular Markers and Maps

With the advent of DNA markers in 1980s and 1990s, many limitations associated with morphological and isozyme markers were overcome and genetic mapping

entered a new exciting and progressive era with the promise to significantly enhance the efficiency of plant genetics and breeding studies. During the past two decades, different types of DNA markers have been developed and evolved, including restriction fragment length polymorphisms (RFLPs) (Botstein et al. 1980), randomly amplified polymorphic DNAs (RAPDs) (Williams et al. 1990), amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995), variable number of tandem repeats (VNTRs or minisatellites) (Jeffreys et al. 1985), simple sequence repeats (SSRs or microsatellites) (He et al. 2003), cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel 1993), sequence characterized amplified regions (SCARs) (Paran and Michelmore 1993), expressed sequence tags (ESTs) (Adams et al. 1991), conserved ortholog set (COS) markers (Fulton et al. 2002), and single-nucleotide polymorphisms (SNPs) and insertion-deletion (InDels) markers (Landegren et al. 1998). Among crop species, tomato is very rich in the number and type of molecular markers. Currently there are over 1000 RFLP markers, most of which mapped to the 12 tomato chromosomes, and more than 162,000 ESTs (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato), of which only a small portion mapped to tomato chromosomes (http://www.sgn.cornell.edu/cgi-bin/search/markers/cos_list.pl). ESTs are derived from different cDNA libraries and their sequences are available on the web site of Solanaceae Genome Network (SGN; <http://www.sgn.cornell.edu>). In addition, several other markers, including SSRs (Villalta et al. 2005), CAPS (Ganal et al. 1998), RAPDs (Foolad et al. 1993) and AFLPs (Suliman-pollatschek et al. 2002) have been developed in tomato. For example, currently at least 148 SSR markers and 77 CAPS have been mapped onto the high-density tomato genetic map (Frary et al. 2005) (http://www.sgn.cornell.edu/cgi-bin/mapviewer/mapTop.pl?map_id=9).

The development and use of PCR-based markers in tomato has recently increased as they are generally cheaper, faster and less labor intensive than RFLPs. However, a significant issue in marker development in tomato is the limited polymorphisms among closely related genotypes. Such limitation restricts the use of DNA markers in many plant-breeding programs which focus on exploitation of intraspecific genetic variations. To overcome this problem, recently significant efforts have been devoted to the discovery and development of high-resolution markers such as SNPs and InDels, which can detect polymorphisms among genotypes within the cultivated species of tomato (Labate and Baldo 2005; Suliman-pollatschek et al. 2002). Such markers can have great utilities in tomato genetic and breeding studies that use populations derived from intraspecific crosses or between the cultivated and closely-related species such as *L. pimpinellifolium*. The growing tomato databases of DNA sequence, in particular the tomato ESTs, is providing information that are being used to develop more resolving genetic markers. It is expected that the availability of such markers will be on the rise for the next few years.

The first molecular map of tomato was published in 1986 containing 18 isozyme and 94 DNA markers (Bernatzky and Tanksley 1986). The first high-density map of tomato, comprising of 1030 markers, was published in 1992 (Tanksley et al. 1992). This map, constructed based on 67 F₂ plants of a *L. esculentum* × *L. pennellii*

Table 1. Genetic linkage maps of tomato (*Lycopersicon spp.*) developed based on different intra- and interspecific crosses

Linkage map	Population type ^a	Population size	Number of markers	Type of markers ^b	References
<i>L. esculentum</i> × <i>L. esculentum</i> var. <i>cerasif.</i> 1. Cervil × Levovil	F ₇ -RIL	153	377	RFLP, RAPD, AFLP	(Saiba-Colombani et al. 2000)
<i>L. esculentum</i> × <i>L. pimpinellifolium</i>					
• M82-1-7 × LA1589	BC ₁	257	120	RFLP, RAPD, morphological	(Grandillo and Tanksley 1996a)
• NC84173 × LA722	BC ₁	119	151	RFLP	(Chen and Foolad 1999)
• Giant Heirloom × LA1589	F ₂	200	90	RFLP, CAPS	(Lippman and Tanksley 2001)
• E6203 × LA1589	BC ₂ F ₆ -BIL	196	127	RFLP	(Doganlar et al. 2002)
• NC84173 × LA722	F ₁₀ RIL	119	191	RFLP, RGA	Foolad et al. (unpubl.)
• NCEBR1 × PSLP125	F ₂	172	256	RFLP, EST, RGA	Foolad et al. (unpubl.)
• NCEBR1 × PSLP125	F ₈ -RIL	172	255	RFLP, EST	Foolad et al. (unpubl.)
<i>L. esculentum</i> × <i>L. cheesmanii</i>					
1. UC204B × LA483	F ₂	350	71	RFLP	(Paterson et al. 1991)
2. UC204B × LA483	F ₇ -RIL	97	132	RFLP	(Paran et al. 1995)
<i>L. esculentum</i> × <i>L. parviflorum</i>					
1. E6203 × LA2133	BC ₂	170	133	RFLP, SCAR, morphological	(Fulton et al. 2000)
<i>L. esculentum</i> × <i>L. chmielewskii</i>					
1. UC82B × LA1028	BC ₁	237	70	RFLP, Isozyme	(Paterson et al. 1988)
<i>L. esculentum</i> × <i>L. hirsutum</i>					
1. E6203 × LA1777	BC ₁	149	135	RFLP	(Bernacchi and Tanksley 1997)

(continued)

Table 1. (continued)

Linkage map	Population type ^a	Population size	Number of markers	Type of markers ^b	Reference
2. E6203 × LA1777	NIL, BIL	111	95	RFLP	(Monforte and Tanksley 2000b) (Zhang et al. 2002)
3. NC84173 × PI126445	BC ₁	145	171	RFLP, RGA	
<i>L. esculentum</i> × <i>L. pennellii</i>					
1. VF36 <i>Tm</i> 2 ^a × LA716 (high-density map of tomato)	F ₂	67	1050	Isozyme, RFLP, morphological	(Pillen et al. 1996; Tanksley et al. 1992)
2. Vendor <i>Tm</i> 2 ^a × LA716	F ₂	432	98		(de Vicente and Tanksley 1993)
3. M82 × LA716	IL	50	375	RFLP	(Eshed and Zamir 1995)
4. VF36 <i>Tm</i> 2 ^a × LA716	F ₂	67	1242	AFLP, RFLP	(Haanstra et al. 1999)
5. E6203 (LA925) × LA716	F ₂	83	1,500	COS	(Fulton et al. 2002)
6. E6203 × LA1657	BC ₂	175	110	RFLP	(Frary et al. 2004b)
7. E6203 × LA716	F ₂	83	152	SSRs, CAPs	(Frary et al. 2005)
<i>L. esculentum</i> × <i>L. peruvianum</i>					
1. E6203 × LA1706	BC ₃	241	177	RFLP, SCAR	(Fulton et al. 1997)
<i>L. esculentum</i> var. <i>cerasif</i> × <i>L. pimpinellifolium</i>					
1. E9 × L5	F ₆ -RIL	142	132	SSR, SCAR	(Villalta et al. 2005)
<i>L. esculentum</i> var. <i>cerasif</i> × <i>L. cheesmanii</i>					
1. E9 × L3	F ₆ -RIL	115	114	SSR, SCAR	(Villalta et al. 2005)
<i>L. peruvianum</i> × <i>L. peruvianum</i>					
1. LA2157 × LA2172	BC ₁	152	73	RFLP	(van Ooijen et al. 1994)

^a BIL: backcross inbred line; NIL: near isogenic line; RIL: recombinant inbred line

^b AFLP: amplified fragment length polymorphism; CAPs: cleaved amplified polymorphic sequence; EST: expressed sequence tag; RAPD: randomly amplified polymorphic DNA; RFLP: restriction fragment length polymorphism; RGA: resistance gene analog; SCAR: sequence characterized amplified region; SSR: simple sequence repeat

cross, also displayed the chromosomal locations of 100 genes of known function or phenotype, including morphological, isozyme and DNA markers. A more saturated version of this map was published in 1996, reducing the inter-marker space to ≤ 1 cM (Pillen et al. 1996). Furthermore, the density of markers in this map has since increased, currently including about 2000 markers (http://www.sgn.cornell.edu/cview/map.pl?map_id=9). The haploid DNA content of the tomato genome is ~ 950 Mbp (Arumuganathan and Earle 1991). This means that on average 1cM genetic map distance in tomato amounts to ~ 750 kb. With the high-density molecular map of tomato, it is likely that any gene of interest, if segregating in this population, would be within one to few map units of at least one marker. However, many agriculturally important traits are not segregating in this population or many of the markers in this map are not polymorphic in other populations of tomato. Thus, during the past two decades several other molecular maps of tomato have been constructed, mostly based on interspecific crosses between the cultivated and various wild species of tomato (Table 1). Most of these maps are of low to moderate density and developed based on RFLP markers from the high-density map, though some other markers, such as RAPDs, ESTs, AFLPs, SSRs and resistance gene analogs (RGAs), also have been utilized. As alluded to before, for interspecific crosses between the cultivated tomato and closely-related wild species *L. pimpinellifolium* and *L. cheesmannii* identification of polymorphic markers is very challenging. For example, only $\sim 30\%$ of the RFLP markers in the high-density map detect polymorphism in *L. esculentum* \times *L. pimpinellifolium* crosses (Chen and Foolad 1999; Grandillo and Tanksley 1996a). In fact in a more recent study, $< 15\%$ of RFLP markers detected polymorphism between a Mexican accession of *L. pimpinellifolium* and *L. esculentum* (MR Foolad et al., unpubl.). Despite these and other challenges, however, to date linkage maps have been developed based on interspecific crosses between the cultivated and all wild species of tomato, except may be *L. chilense*.

To accelerate the use of markers and maps in tomato genetics and breeding studies, recently efforts have been made to develop maps based on mainly PCR-based markers. One such effort (Frery et al. 2005) has produced a map based on an F_2 population of a *L. esculentum* \times *L. pennellii* cross using a set of 76 SSRs and 76 CAPS (Table 1). Another significant effort currently underway in a few laboratories around the world is conversion of tomato RFLP markers to friendly PCR-based markers such as CAPS. Furthermore, a simultaneous effort is development of genetic maps based on intraspecific populations within the cultivated tomato.

3. GENE TAGGING AND QTL MAPPING

Tomato has been at the forefront of many crop species in terms of gene tagging and QTL mapping for agriculturally and biologically important traits. For simply-inherited traits, gene mapping in tomato started in 1930s (MacArthur 1934), however, tagging of single-gene agricultural traits with genetic markers and their use in tomato breeding started in 1970s. Rick and Fobes (1974) reported an association of root-knot nematode (*Meloidogyne incognita*) resistance with a rare form

of isozyme acid phosphatase, *Aps-1*¹. This association was later determined to be due to a tight linkage between nematode resistance gene *Mi* and the *Aps-1* locus on chromosome 6 (Medina-Filho 1980). Subsequently, linkages were reported between isozyme loci and genes controlling a few important traits in tomato, including male-sterility (Tanksley et al. 1984) and self-incompatibility (Tanksley and Loaiza-Figueroa 1985). Since then, marker tagging of many simple traits in tomato has been reported, as discussed in below.

The use of markers to identify and map genes or QTLs controlling complex traits in tomato started in 1980s. Earlier studies mainly used morphological and isozyme markers and early filial or backcross populations to identify QTLs for different quantitative traits. However, the first comprehensive and systematic use of markers to dissect genetic controls of complex traits in tomato was that of Paterson et al. (1988), in which a rather complete RFLP map was employed to map QTLs for various fruit quality characters. This study demonstrated, for the first time, that genetics of quantitative traits could be resolved into discrete Mendelian factors. Subsequently, QTL mapping became common in tomato genetics and breeding studies, where QTLs have been identified for numerous traits. It is difficult to provide a complete account of all genes and QTLs that have been identified and/or mapped in tomato. Rather a tabulated summary of major genes and QTLs that have been identified and mapped during the past two decades is presented (Tables 2–4) with a summary discussion of some major mapping studies.

3.1. Mapped Disease Resistance Genes and QTLs

Mapping disease resistance genes and QTLs has been the centerpiece of all mapping studies in tomato. Identification of markers associated with disease resistance in tomato started with the pioneering work of C.M. Rick and co-workers who identified a linkage between root-knot nematode resistance gene *Mi* and isozyme locus *Aps-1* (Rick and Fobes 1974). Since then genetic markers, in particular DNA markers, have been used extensively to tag or map major genes or QTLs for resistance to many fungal, bacterial, viral, and nematode diseases in tomato. In Table 2, all known mapped disease resistance genes and QTLs in tomato together with gene/QTL symbol, the causal agent of the disease, genetic source of the resistance and chromosomal location of the resistance gene or QTL are presented. The space limitation does not allow discussion of the procedures/methodologies employed to identify and map genes or QTLs for the different diseases. However, some general comments are as follow:

- With a few exceptions, most disease resistance genes and QTLs in tomato have been identified in the related wild species and mapped using interspecific populations.
- For some diseases, often multiple gene resources have been used to identify and map resistance genes and QTLs. Multiple resistance genes/QTLs from different sources may allow pyramiding of resistance using a MAS approach.
- For most horizontal resistance traits, often multiple QTLs have been identified in each study. In many cases, it has been difficult to determine the precise location

or actual effect or importance of each QTL in the original studies. Many studies have suggested development of near-isogenic lines (NILs) to determine such information.

- In most cases, early filial and backcross populations have been employed for gene/QTL mapping. More recently, however, advanced populations such as RILs, BILs and ILs have been used to provide better mapping resolutions (Table 2).
- Recently MAS has been extensively employed for disease resistance breeding in tomato, in particular in many seed companies. The utility of MAS for resistance breeding appears to have superseded its utility for any other trait in tomato, as described in below.

3.2. Mapped Insect Resistance/Tolerance Genes and QTLs

Comparatively less research has been done to identify or map genes and QTLs for insect resistance than disease resistance in tomato. This is due in part to many challenges involved in conducting controlled experiments on insect resistance. However, some mapping experiments have been conducted, much of which using *L. pennellii* accession LA716 as the resistance source. The multiple pest resistance of this accession is mediated by acylsugars exuded by type-IV glandular trichomes on the surface of the plants (Mutschler et al. 1996), which act as feeding deterrents for insects such as potato aphid, green peach aphid, tomato fruitworm and beet armyworm, and as feeding or oviposition deterrents for leafminer and silverleaf whitefly. Using an F₂ population of a *L. esculentum* × *L. pennellii* (LA716) cross, a total of five QTLs were detected on chromosomes 2, 3, 4 and 11 with association with one or more aspects of acylsugar production (Mutschler et al. 1996). Transferring subsets of 3–5 QTLs to a *L. esculentum* background resulted BC₃ progeny which exhibited accumulation of only low levels of acylsugars, prompting the researchers to speculate presence of other yet unidentified genetic factors controlling acylsugars accumulation (Lawson et al. 1997). In a different study, using an F₂ population of a cross between two *L. pennellii* accessions, a total of 6 QTLs were detected (Blauth et al. 1999), which were different from those identified in the previous study, further suggesting the complexity of genetic controls of acylsugar production. There is no report on transferring of these QTLs to the cultivated tomato.

Specific insect resistance genes often confer resistance to only one insect species or to a closely related species. However, the *Mi* gene, which confers resistance to a root-knot nematode, is an interesting exception. After *Mi* was cloned (Kaloshian et al. 1998) it was determined that it was the same as *Meu1*, a locus conferring resistance to the potato aphid, *Macrosiphum euphorbiae* (Rossi et al. 1998). Currently, *Mi* (*Meu1*) is the only insect resistance gene that has been cloned from a plant species. This gene is a member of nucleotide binding, leucine-rich repeat family of plant resistance genes, many members of which have been found to confer isolate-specific resistance to viruses, bacteria, fungi, and nematodes. However, *Mi* is the first example of a plant resistance gene active against two such distantly related

Table 2. Summary of disease resistance genes and QTLs (Q) mapped in tomato chromosomes

Disease	Gene/QTL	Pathogen	Resistance source	Mapping population ^a	Chromosomal location	References
Alternaria stem canker	Asc	<i>Alternaria alternata</i>	<i>L. pennellii</i>	F ₂	3	(Mesbah et al. 1999; van der Biezen et al. 1995)
	Anthraxnose	f. sp. <i>lycopersici</i>	<i>L. esculentum</i>	F ₂	Various Chromosomes	(Stommel and Zhang 1998)
	ripe rot	<i>Colletotrichum coccodes</i>	<i>L. peruvianum</i>	BC ₁	1,6,7,8,9,10	(Sandbrink et al. 1995)
Bacterial canker	<i>Cm 1.1-10.1</i> (Q)	<i>Calvibacter michiganensis</i> ssp. <i>michiganensis</i>	<i>L. peruvianum</i>	F ₂	5,7,9	(van Heusden et al. 1999)
	Three (Q)	<i>Calvibacter michiganensis</i> ssp. <i>michiganensis</i>	<i>L. hirsutum</i>	BC ₂ S ₅	2,5	(Coaker and Francis 2004; Kabelka et al. 2002)
Bacterial speck	<i>Rcm2.0</i> , <i>Rcm5.1</i> (Q)	<i>Calvibacter michiganensis</i> ssp. <i>michiganensis</i>	<i>L. pimpinellifolium</i>	NIL F ₂	5	(Martin et al. 1993b)
	<i>Pto</i>	<i>Pseudomonas syringae</i> pv. <i>Tomato</i> (Pst)	<i>L. pimpinellifolium</i>	NIL F ₂	5	(Salmeron et al. 1996)
Bacterial spot	<i>Prf</i>	Required for res. to Pst	<i>L. pimpinellifolium</i>	NIL F ₂	5	(Yu et al. 1995)
	<i>Rx-1</i> , <i>Rx-2</i> , <i>Rx-3</i> (Q)	<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	<i>L. esculentum</i>	BC ₁	1,5	(Ballvora et al. 2001)
Bacterial wilt	<i>Bs4</i>	<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	<i>L. pennellii</i>	F ₂	5	(Yang et al. 2005)
	<i>Rx-3</i> (Q)	<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	<i>L. esculentum</i>	F ₂ and BC ₃	5	(Danesheh et al. 1994; Thoquet et al. 1996)
Bacterial wilt	Several (Q)	<i>Ralstonia solanacearum</i>	<i>L. pimpinellifolium</i>	F ₂	3,4,6,7,10,11	(Danesheh et al. 1994; Thoquet et al. 1996)

	Two (Q)	<i>Ralstonia solanacearum</i>	<i>L. peruvianum</i>	F ₃	6	(Mangin et al. 1999)
Blackmold	Blackmold (Q)	<i>Alternaria alternata</i>	<i>L. cheesmanii</i>	NIL F ₂	2,3,9,12	(Robert et al. 2001)
Corky root rot	Py-1	<i>Pyrenochaeta lycopersici</i>	<i>L. peruvianum</i>		3	(Doganlar et al. 1998)
Cucumber mosaic v.	Cmr	CMV	<i>L. chilense</i>	BC ₁ -inbred	12	(Stamova and Chetelat 2000)
Early blight	I1 (Q)	<i>Alternaria solani</i>	<i>L. hirsutum</i>	BC ₁	1,2,5,8,9,10,11,12	(Foolad et al. 2002)
	I3 (Q)	<i>Alternaria solani</i>	<i>L. hirsutum</i>	BC ₁ S ₁	1,2,3,5,8,9,10,11,12	(Foolad et al. 2002)
	7 (Q)	<i>Alternaria solani</i>	<i>L. hirsutum</i>	BC ₁	1,2,3,5,8,9,10,11,12	(Zhang et al. 2003)
	7 (Q)	<i>Alternaria solani</i>	<i>L. pimpinellifolium</i>	RILs	1,3,4,5,6,9,11	MR Foolad et al, unpubl
Fusarium crown and root rot	Frl	<i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i>	<i>L. peruvianum</i>	F ₂	9	(Vakalounakis et al. 1997)
Fusarium wilt	I, I1, I2, I2C, I3	<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i>	<i>L. pimpinellifolium</i>	Different populations	7,8,11	(Sarfatii et al. 1991; Scott et al. 2004; Simons et al. 1998)
Gray leaf spot	Sm	<i>Stemphylium</i> spp.	<i>L. pimpinellifolium</i>	F ₂	11	(Behare et al. 1991)
Late blight	Ph-1	<i>Phytophthora infestans</i>	<i>L. pimpinellifolium</i>	BC ₁	7	(Pierce 1971)
Late blight	Ph-2	<i>P. infestans</i>	<i>L. pimpinellifolium</i>	F ₂	10	(Moreau et al. 1998)
Late blight	Ph-3	<i>P. infestans</i>	<i>L. pimpinellifolium</i>	F ₂	9	(Chunwongse et al. 1998)
Late blight	Ph-4	<i>P. infestans</i>	<i>L. pimpinellifolium</i>	RILs	2	Foolad et al. (unpubl.)
Late blight	Ph-5	<i>P. infestans</i>	<i>L. pimpinellifolium</i>	F ₂	1	Foolad et al. (unpubl.)
Leaf mould	lb1-lb12 (Q)	<i>P. infestans</i>	<i>L. hirsutum</i>	BC ₁	All 12 chrs.	(Brouwer et al. 2004)
	Cf-1, Cf-2,	<i>Cladosporium fulvum</i>	<i>L. hirsutum</i>	F ₂ , NIL F ₂ ,	1,6	(Balint-Kurti et al. 1994; Jones et al. 1993; Lauge et al. 1998)
	Cf-4, Cf-5,		<i>L. pimpinellifolium</i>	BC ₁		
	Cf-9					

(continued)

Table 2. (continued)

Disease	Gene/QTL	Pathogen	Resistance source	Mapping population ^a	Chromosomal location	References
Nematode (potato cyst)	<i>Hero</i>	<i>Globodera rostochiensis</i>	<i>L. pimpinellifolium</i>	NIL F ₂	4	(Ganal et al. 1995)
Nematode (root-knot)	<i>Mi-1</i> , <i>Mi-2</i> , <i>Mi-3</i> , <i>Mi-9</i>	<i>Meloidogyne</i> spp.	<i>L. peruvianum</i>	F ₂ , F ₃ , NIL F ₂ , BC _{1,2}	6,12	(Aarts et al. 1991; Ammiraju et al. 2003; Veremis et al. 1999; Yaghoobi et al. 1995)
Potyvirus	<i>pot-1</i>	Potyruses	<i>L. hirsutum</i>	ILs	3	(Parrilla et al. 2002)
Powdery Mildew	Lv <i>Ol-1</i> , <i>Ol-2</i>	<i>Leveillula taurica</i> <i>Oidium</i> <i>lycopersicum</i>	<i>L. chilense</i> <i>L. esculentum</i> <i>L. hirsutum</i>	F ₂ F ₂	12 4,6	(Chunwongse et al. 1994) (de Giovanni et al. 2004; Huang et al. 2000)
	3 (Q)	<i>Oidium</i> <i>lycopersicum</i>	<i>L. parviflorum</i>	F ₂	6,12	(Bai et al. 2003)
Tobacco mosaic virus	<i>Tm-1</i> , <i>Tm-2</i> ^a	<i>TMV/ToMV</i>	<i>L. hirsutum</i> <i>L. peruvianum</i>	F ₂	2,9	(Levesque et al. 1990; Ohmori et al. 1996; Vakalounakis et al. 1997)
Tomato mottle virus	One Gene	<i>ToMoV</i>	<i>L. chilense</i>	F ₂	6	(Griffiths and Scott 2001)
Tomato spotted wilt v.	<i>Sw-5</i>	TSWV	<i>L. peruvianum</i>	NILs, F ₂	9	(Stevens et al. 1995; Langella et al. 2004)
Tomato yellow leaf curl virus	<i>Ty-1 (Q)</i> , two other Q	<i>TYLCV</i>	<i>L. chilense</i> <i>L. pimpinellifolium</i> <i>L. hirsutum</i>	BILs, F ₂ , F ₃ , F ₄	6,11	(Chagué et al. 1997; Hanson et al. 2000; Zamir et al. 1994)
Verticillium wilt	<i>Ve</i>	<i>Verticillium dahliae</i>	<i>L. cheesmanii</i> <i>L. pennellii</i>	F ₂ , RILs, ILs	9	(Diwan et al. 1999; Kawchuk et al. 1998)

^a BIL: backcross inbred line; NIL: near isogenic line; RIL: recombinant inbred line

organisms belonging to two different phyla. Another study revealed that several isolates of potato aphid and green peach aphid (*Myzus persicae*) can overcome the resistance mediated by *Mi* (*Meu1*), limiting the use of this gene for aphid control in tomato (Goggin et al. 2001). In a more recent study, however, seven BILs, derived from crosses between two aphid susceptible *L. esculentum* lines and two aphid resistant accessions of *L. pennellii* and *L. hirsutum*, were identified exhibiting resistance to both types of aphid (Kohler and St. Clair 2005). These lines can be useful for developing tomato cultivars with genetic resistance to different types of aphid. In summary, although there has been limited research and progress in identification and mapping of genes/QTLs for insect resistance in tomato, the increasing restrictions on the use of pesticides and the advancements in marker technology are expected to change this in the direction of marker-assisted breeding of insect-resistant tomato cultivars.

3.3. Mapped Abiotic Stress Tolerance/Resistance Genes and QTLs

Most abiotic stress tolerance traits are complex, rendering crop improvement for such traits via traditional breeding unrewarding. Identification and use of genetic markers that are associated with stress related traits, however, is a promising approach to improve plant stress tolerance. During the past two decades, considerable efforts have been made to identify and map genes or QTLs conferring tolerance to different abiotic stresses in tomato, including salinity, drought and extreme temperatures (for a review see Foolad, 2005). Table 3 displays the major mapping activities in tomato, and below is a brief discussion of some of the studies.

More mapping studies have been done on salt tolerance than tolerance to any other environmental stress in tomato (Foolad 2005). For example, QTLs have been identified for salt tolerance during seed germination, vegetative growth and later stages (Table 3) and in different interspecific populations, in particular derived from *L. esculentum* × *L. pennellii* and *L. esculentum* × *L. pimpinellifolium* crosses. Comparisons of QTLs across populations and studies indicate that, for each trait, often similar QTLs were identified in populations derived from the same cross whereas across species some QTLs were conserved and others species-specific (Foolad and Chen 1998; Foolad et al. 1998b). Also, it seems that at each stage similar QTLs contribute to tolerance under different stress levels (Foolad and Jones 1991). Compared to germination and vegetative stages, limited research has been conducted to identify QTLs for salt tolerance during reproduction. However, various studies suggest that different QTLs control tolerance at different stages (Foolad 1999a, 2005). Nevertheless, QTLs for tolerance at different stages can be pyramided using a MAS approach to develop tomatoes with improved salt tolerance throughout the plant ontogeny.

Limited research has been conducted to identify genes or QTLs for cold tolerance in tomato. The only published research for cold tolerance during seed germination is that of Foolad et al. (1998a) in which a few QTLs were identified in an interspecific backcross population of a cross between *L. esculentum* and *L. pimpinellifolium*. More recently, two studies were undertaken to identify QTLs for cold tolerance

Table 3. Summary of abiotic stress tolerance/resistance QTLs mapped in tomato chromosomes

Stress	Specific trait ^a	Number of QTLs	Tolerance source	Mapping population ^b	Chromosomal location	References
Cold (low temp.)	SG	3	<i>L. pimpinellifolium</i>	BC ₁ S ₁	1,4	(Foolad et al. 1998a)
		5	<i>L. pimpinellifolium</i>	RILs	1,2,3,8,12	MR Foolad et al. unpubl.
	VG	3	<i>L. hirsutum</i>	BC ₁	6,7,12	(Vallejos and Tanksley 1983)
Drought	Sht. wlt., RAU	10	<i>L. hirsutum</i>	BC ₁	1,3,5,6,7,9,11,12	(Truco et al. 2000)
	SG	4	<i>L. pimpinellifolium</i>	BC ₁ S ₁	1,8,9,12	(Foolad et al. 2003a)
Salt	WUE	8	<i>L. pimpinellifolium</i>	RILs	1,2,3,4,8,9,12	MR Foolad et al. unpubl.
		3	<i>L. pennellii</i>	BC ₁ S ₁ , F ₃	Undetermined	(Martin et al. 1989)
	SG	5	<i>L. pennellii</i>	F ₂	1,3,7,8,12	(Foolad and Jones 1993)
		8	<i>L. pennellii</i>	F ₂	1,2,3,7,8,9,12	(Foolad et al. 1997)
		8	<i>L. pennellii</i>	F ₂	1,3,5,6,8,9	(Foolad and Chen 1998)
		7	<i>L. pimpinellifolium</i>	BC ₁ S ₁	1,2,5,7,9,12	(Foolad et al. 1998b)
		8	<i>L. pimpinellifolium</i>	RILs	1,2,3,4,8,9,12	MR Foolad et al. unpubl.
	VG	4	<i>L. pimpinellifolium</i>	BC ₁ S ₁	1,5,9	(Foolad and Chen 1999)
		5	<i>L. pimpinellifolium</i>	BC ₁	1,3,5,6,9	(Foolad et al. 2001)
		7	<i>L. pimpinellifolium</i>	RILs	1,3,4,5,7,8,9	MR Foolad et al. unpubl.
	Ion accumulation	6	<i>L. pennellii</i>	F ₂	1,2,4,5,6,12	(Zamir and Tal 1987).
	FN, FW, FY	Several	<i>L. pimpinellifolium</i>	F ₂	Undetermined	(Breto et al. 1994)

^a FN: fruit number; FW: fruit weight; FY: fruit yield; RAU: root ammonium uptake; SG: seed germination; Sht. wlt.: shoot wilting; VG: vegetative growth; WUE: water use efficiency

^b RIL: recombinant inbred line

during seed germination, one using a selective genotyping in a large ($N = 1000$) *L. esculentum* \times *L. pimpinellifolium* BC₁ population and the second using an F₉ RIL population ($N = 145$) of the same cross (MR Foolad et al., unpubl.). These studies verified all of the QTLs identified in the original study (Foolad et al. 1998a) and detected a few additional QTLs. A comparison of QTLs indicated that most QTLs were stable across populations whereas a few were population-specific. QTL mapping studies for cold tolerance during vegetative growth and reproduction are scarce. In one study, using BC₁ population of a cross between a cold-sensitive *L. esculentum* line and a cold-tolerant *L. hirsutum* accession, Vallejos and Tanksley (1983) identified 3 QTLs for growth at low temperatures. In another study, several QTLs were identified associated with shoot wilting and root ammonium uptake under chilling temperatures in a *L. esculentum* \times *L. hirsutum* BC₁ population (Truco et al. 2000).

Research to identify genes or QTLs for drought tolerance in tomato has also been limited. The only published report on drought tolerance during seed germination is that of Foolad et al. (2003a) in which 4 QTLs were detected in backcross progeny of a *L. esculentum* \times *L. pimpinellifolium* cross. As a follow-up, more recently an F₉ RIL population of the same cross was evaluated for drought tolerance during germination, where several QTLs were identified consistent with the results of the first study (MR Foolad et al., unpubl.). Limited research has been done to discern genetic controls of drought tolerance during vegetative and reproductive stages. To facilitate selection for low Δ (¹³C/¹²C discrimination) as a means to improve tomato drought tolerance, 3 QTLs associated with this trait were identified using filial and backcross progeny of a *L. esculentum* \times *L. pennellii* cross (Martin et al. 1989). However, it has not been determined whether selection for these QTLs would increase water use efficiency in tomato. There is no published research on QTLs for drought tolerance during reproductive stage.

Although several studies have investigated physiological and genetic relationships among tolerances to different environmental stresses in tomato, only a few studies have used QTL mapping techniques, which mainly focused on seed germination stage (Foolad 1999b, 2000; Foolad et al. 1999; Foolad et al. 2003b; Foolad et al. unpubl.). These studies, however, have indicated the presence of genetic relationships among cold, salt, and drought tolerance during seed germination in tomato. For example, a few QTLs were identified with effects on germination under two or three different stresses, which were referred to as stress-nonspecific QTLs. Also, a few QTLs were identified with effects on germination only under specific stress conditions, which were referred to as stress-specific QTLs. In summary, the various studies have indicated that some genes affect tomato seed germination under different stress conditions while others are more stress-specific.

3.4. Mapped Genes and QTLs for Flower- and Fruit-Related Characteristics

During the past few decades, markers and maps have been used to identify genes or QTLs for many flower- and fruit-related characteristics in tomato, as listed in

Table 4. It is obvious from the table that often several groups conducted research on the same or similar traits, or the same traits were studied using different interspecific populations. For example, QTLs for fruit weight (size), shape and soluble solids contents were identified in at least 20, 13 and 23 studies, respectively (Table 4). In below, the status of marker development for a few major fruit traits in tomato is reviewed and discussed.

3.4.1. *Fruit size*

Although there are variations in fruit size in the cultivated tomato, differences are much greater between the cultivated (with fruit size as big as 1000 g with 10 or more locules) and wild species (with fruit size as small as 1 g with only 2 locules). Therefore, most QTL mapping studies have employed interspecific populations. Although traditional breeding studies had suggested simple genetic control for tomato fruit size, molecular marker research has revealed the presence of approximately 30 QTLs for this trait, mapping to different chromosomes (Table 4) (Chen et al. 1999). Many studies, however, have identified same QTLs for this trait, and the most recent studies have not detected any novel QTL (Lippman and Tanksley 2001). Also, many studies have indicated co-localization of QTLs for fruit size and total (or soluble) solids contents (e.g. see Chen et al., 1999), confirming the presence of a negative correlation between these two traits (Georgelis et al. 2004). One of the major QTLs for fruit size in tomato, *fw2.2*, which was detected in many studies (Table 4), has been cloned (Frery et al. 2000), as discussed in below.

3.4.2. *Fruit shape*

There are great variations in fruit shape in tomato, ranging from oblate to round to ovate (blocky or elongated) to pear shape. Earlier studies had identified several genes controlling fruit shape in tomato, including *pr* (pyriform), *o* (ovate), *bk* (beaked tomato), *n* (nipple-tip tomato), *f* (fasciated) and *lc* (for locule number). During the past two decades, however, a few of these genes and several other genes and QTLs affecting fruit shape have been located on tomato chromosomes and/or cloned (Tables 4 and 6). For example, a major fruit-shape QTL (*fs8.1*) differentiating fresh-market (round) and processing (blocky) tomatoes was mapped on chromosome 8 (Grandillo et al. 1996) and later cloned and characterized (Ku et al. 2000). *fs8.1* exert its effect on fruit shape by changing the length of carpels during pre-anthesis, resulting in longer and larger fruit. Similarly, a major fruit-shape QTL (*ovate*), controlling the transition from round to pear-shaped fruit was mapped (Ku et al. 1999) and cloned (Liu et al. 2002). The overall results from different studies indicate that much of the variation in tomato fruit shape is controlled by a few major loci and the great variation observed in the cultivated species is most likely the result of allelic variation at these loci (van der Knaap and Tanksley 2003).

3.4.3. *Fruit color/nutritional-value*

Color is one of the most important quality characteristics in tomato, and the focus of numerous mapping studies. The attention to fruit color has recently increased as

Table 4. Summary of important characteristics for which genes or QTLs have been mapped in tomato chromosomes

Trait ^a	Wild species used	Mapping population ^b	Genes (G) or QTLs (Q)	Chromosome	References
Carotenoid candidate genes Corolla indentation	<i>L. pennellii</i>	ILs	23 G	Most chromosomes	(Liu et al. 2003b) (Bernacchi and Tanksley 1997)
	<i>L. hirsutum</i>	BC ₁	2 Q	2,8	
Flwr., exerted stigma Frt. antioxidant capacity Frt. ascorbic acid Frt. citric acid content Frt. color	<i>L. peruvianum</i>	BC ₃ , BC ₄	2 Q	2,9	(Fulton et al. 1997)
	<i>L. pennellii</i>	ILs	5 Q	3,6,7,10	(Rousseaux et al. 2005)
	<i>L. pennellii</i>	ILs	6 Q	3,5,10,12	(Rousseaux et al. 2005)
	<i>L. pennellii</i>	ILs	7 Q	4,5,8,9,10	(Causse et al. 2004)
	<i>L. pimpinellifolium</i>	BC ₁	2 Q	2,6	(Grandillo and Tanksley 1996b)
	<i>L. pimpinellifolium</i>	BC ₂ , BC ₃	5 Q	2,3,4,7,8	(Tanksley et al. 1996)
Frt. color (β-carotene)	<i>L. peruvianum</i>	BC ₃ , BC ₄	8 Q	1,6,7,8,9,10,12	(Fulton et al. 1997)
	<i>L. hirsutum</i>	BC ₂ , BC ₃	15 Q	1,2,4,5,7,8,9,10,11	(Bernacchi et al. 1998)
	<i>L. hirsutum</i>	subNILs	1 Q	1	(Monforte and Tanksley 2000a)
	<i>L. parviflorum</i>	BC ₃	13 Q	1,2,4,5,7,8,11,12	(Fulton et al. 2000)
	<i>L. chmielewskii</i>	NILs	1 Q	1	(Frary et al. 2003)
	<i>L. pennellii</i>	BC ₂ /BC ₂ F ₁	1 Q	12	(Frary et al. 2004b)
Frt. color (β-carotene)	<i>L. cheesmannii</i>	F ₂ , ILs	1 G (B)	6	(Ronen et al. 2000; Zhang and Stommel 2000)
	<i>L. parviflorum</i>	BC ₃	6 Q	2,4,8,9,10,11	(Fulton et al. 2000)
Frt. color (carotene)	<i>L. pennellii</i>	ILs	1 G (B)	6	(Ronen et al. 1999; Zhang and Stommel 2001)
	<i>L. esculentum</i>	RIL	2 Q 3 Q	6 2,3,8	(Rousseaux et al. 2005) (Saliba-Colombani et al. 2001)

(continued)

Table 4. (continued)

Trait ^a	Wild species used	Mapping population ^b	Genes (G) or QTLs (Q)	Chromosome	References
Fr. color (crimson)	<i>L. esculentum</i>	F ₂	1 G (<i>og^c</i> , <i>cr</i>)	6	(Ronen et al. 2000; Thompson et al. 1967)
Fr. color (external)	<i>L. parviflorum</i>	BC ₃	9 Q	1,2,4,5,7,8,11,12	(Fulton et al. 2000)
	<i>L. pimpinellifolium</i>	BC ₂ F ₆	2 Q	3,11	(Doganlar et al. 2002)
Fr. color (high-pigment-1)	<i>L. pennellii</i>	BC ₂ /BC ₂ F ₁	2 Q	5,12	(Fray et al. 2004b)
	<i>L. cheesmannii</i>	F ₂	1 G (<i>hp-1</i>)	2	(Peters et al. 1998; Yen et al. 1997)
Fr. color (high-pigment-2)	<i>L. pennellii</i>	BC ₁	1 G (<i>hp-2</i>)	1	(Mustilli et al. 1999; van Tuinen et al. 1997)
Fr. color (internal)	<i>L. parviflorum</i>	BC ₃	15 Q	1,2,4,5,7,8,9,10,11,12	(Fulton et al. 2000)
	<i>L. pimpinellifolium</i>	BC ₂ F ₆	3 Q	3,11	(Doganlar et al. 2002)
Fr. color (lycopene)	<i>L. pennellii</i>	BC ₂ /BC ₂ F ₁	1 Q	12	(Fray et al. 2004b)
	<i>L. pennellii</i>	ILs	16 Q	Most chromosomes	(Liu et al. 2003b)
	<i>L. pimpinellifolium</i>	BC ₁ S ₁	8 Q	1,4,5,6,7,10,12	(Chen et al. 1999)
	<i>L. parviflorum</i>	BC ₃	5 Q	2,3,5,8,12	(Fulton et al. 2000)
	<i>L. pennellii</i>	ILs	2 Q	3,6	(Rousseaux et al. 2005)
Fr. color (orange)	<i>L. pennellii</i>	BC ₂ /BC ₂ F ₁	2 Q	11,12	(Fray et al. 2004b)
Fr. color (yellow)	<i>L. parviflorum</i>	BC ₃	1 Q	12	(Fulton et al. 2000)
Fr. diameter	<i>L. pimpinellifolium</i>	BC ₁	3 Q	1,2,8	(Grandillo and Tanksley 1996b)
Fr. fructose content	<i>L. pimpinellifolium</i>	BC ₁ S ₁	8 Q	1,2,3,6,7,11	(Chen et al. 1999)
	<i>L. pimpinellifolium</i>	F ₂	7 Q	1,2,3,4,7,11	(Lippman and Tanksley 2001)
Fr. fructose content	<i>L. pimpinellifolium</i>	BC ₂ F ₆	12 Q	2	(Doganlar et al. 2002)
	<i>L. pennellii</i>	ILs	4 Q	4,5,7,9	(Causse et al. 2004)
Fr. fruc:gluc ratio	<i>L. hirsutum</i>	F _{2,3} , BC ₁	1 G (<i>Fgr</i>)	4	(Levin et al. 2000)
Fr. glucose content	<i>L. pennellii</i>	ILs	4 Q	4,5,9,12	(Causse et al. 2004)
Fr. length	<i>L. pimpinellifolium</i>	BC ₁ S ₁	9 Q	1,2,3,6,7,9,12	(Chen et al. 1999)

	<i>L. pimpinellifolium</i>	F ₂	7 Q	1,2,3,4,9,11	(Lippman and Tanksley 2001)
	<i>L. pimpinellifolium</i>	BC ₂ F ₆	5 Q	2,3,8,9,11	(Doganlar et al. 2002)
Fr. locule number	<i>L. pimpinellifolium</i>	BC ₁	2 Q	1,3	(Grandillo and Tanksley 1996b)
	<i>L. pimpinellifolium</i>	F ₂	3 Q	2,11	(Lippman and Tanksley 2001)
	<i>L. pimpinellifolium</i>	F ₂	5 Q	2,3,4,10,12	(Frary et al. 2004a)
Fr. malic acid content	<i>L. pennellii</i>	ILs	5 Q	3,4,8,12	(Causse et al. 2004)
	<i>L. esculentum</i>	RILs	Many Q	Various chromos.	(Causse et al. 2002)
Fr. organoleptic quality	<i>L. parviflorum</i>	BC ₃	1 Q	6	(Fulton et al. 2000)
Fr. ostwald	<i>L. chmielewskii</i>	BC ₁ , BC ₂	5 Q	3,6,7,8,10	(Paterson et al. 1988)
Fr. pH	<i>L. chmielewskii</i>	F ₂ , F ₃	9 Q	1,3,4,6,7,8,10	(Paterson et al. 1991)
	<i>L. chmielewskii</i>	BILs/BC ₂ F ₅	1 Q	7	(Azanza et al. 1995)
	<i>L. pimpinellifolium</i>	BC ₂ , BC ₃	5 Q	1,3,5,7,12	(Tanksley et al. 1996)
	<i>L. peruvianum</i>	BC ₃ , BC ₄	6 Q	2,3,9,10,12	(Fulton et al. 1997)
	<i>L. hirsutum</i>	BC ₂ , BC ₃	10 Q	1,2,3,4,6,8,9,10,12	(Bernacchi et al. 1998)
	<i>L. pimpinellifolium</i>	BC ₁ S ₁	6 Q	1,2,4,5,9,12	(Chen et al. 1999)
	<i>L. parviflorum</i>	BC ₃	10 Q	2,3,4,5,6,7,9,12	(Fulton et al. 2000)
	<i>L. esculentum</i>	RIL	2 Q	11,12	(Saliba-Colombani et al. 2001)
	<i>L. pennellii</i>	BC ₂ /BC ₂ F ₁	2 Q	3,12	(Frary et al. 2004b)
	<i>L. pennellii</i>	ILs	11 Q	2,4,5,8,9,10,11,12	(Causse et al. 2004)
Fr. phenolic content	<i>L. pennellii</i>	ILs	9 Q	3,5,6,7,8,9	(Rousseaux et al. 2005)
Fr. ripening	<i>L. esculentum</i>	F ₂	2 Q	5,12	(Doganlar et al. 2000)
	<i>L. pennellii</i>	F ₂	Many loci	All chromosomes	(Kinzer et al. 1990; Slater et al. 1985)
	<i>L. pimpinellifolium</i>	BC ₁	3 Q	2,8,9	(Grandillo and Tanksley 1996b)
	<i>L. pimpinellifolium</i>	BC ₂ , BC ₃	4 Q	2,4,7,8	(Tanksley et al. 1996)
	<i>L. peruvianum</i>	BC ₃ , BC ₄	4 Q	2,3,8,9	(Fulton et al. 1997)
Fr. rip. (Colorless non-rip.)	<i>L. chesmanii</i>	F ₂	Chr	2	(Tr et al. 2002)
Fr. ripening (never-ripe)	<i>L. chesmanii</i>	F ₂	Nr	9	(Yen et al. 1995)

(continued)

Table 4. (continued)

Trait ^a	Wild species used	Mapping population ^b	Genes (G) or QTLs (Q)	Chromosome	References
Frt ripening (non-ripening)	<i>L. pennellii</i> <i>L. cheesmanii</i>	F ₂	<i>nor</i> , <i>rin</i>	5,10	(Giovannoni et al. 1999; Moore et al. 2002; Vrebalov et al. 2002) (Kinzer et al. 1990)
Frt ripening (polygalacturonase)	<i>L. pimpinellifolium</i>	BC ₁	TOM6	10	(Kinzer et al. 1990)
Frt ripening (uniform rip.)	<i>L. pimpinellifolium</i>	BC ₁	<i>u</i>	10	(Chen et al. 1999)
Frt shape	<i>L. pimpinellifolium</i> <i>L. hirsutum</i>	BC ₁ S ₁ subNILs	4 Q 1 Q	1,9,10,12 1	(Monforte and Tanksley 2000a)
	<i>L. parviflorum</i> <i>L. pimpinellifolium</i>	BC ₃ F ₂	16 Q 1 Q	All 12 chromosomes 11	(Fulton et al. 2000) (Lippman and Tanksley 2001)
	<i>L. pimpinellifolium</i> <i>L. pimpinellifolium</i>	BC ₂ F ₆ F ₂	2 Q 4 Q	1,9 2,3,7,11	(Doganlar et al. 2002) (van der Knaap et al. 2000)
Frt shape (bumpiness)	<i>L. pennellii</i> <i>L. pimpinellifolium</i>	BC ₂ /BC ₂ F ₁ F ₂	4 Q 3 Q	2,8,10,12 8,9,11	(Frary et al. 2004b) (van der Knaap and Tanksley 2003)
Frt shape (bell-pepper)	<i>L. pimpinellifolium</i>	F ₂	3 Q	2,8	(van der Knaap and Tanksley 2003)
Frt shape (elongated)	<i>L. pennellii</i> <i>L. pimpinellifolium</i>	F ₂ F ₂	1 Q 2 Q	2 6,9	(Ku et al. 1999) (van der Knaap and Tanksley 2003)
Frt shape (heart)	<i>L. pimpinellifolium</i>	F ₂	4 Q	1,2,3,7	(van der Knaap and Tanksley 2003)
Frt shape (pear)	<i>L. pimpinellifolium</i>	F ₂	2 Q	2,10	(Ku et al. 1999)

Fr. shape (blockiness)	<i>L. pimpinellifolium</i>	F ₂	7 Q	1,2,3,7,8,12	(van der Knaap and Tanksley 2003)		
Fr. soluble solids (SS)	<i>L. pimpinellifolium</i>	BC ₂ , BC ₃	12 Q	2,3,4,5,6,7,8,11,12	(Tanksley et al. 1996)		
	<i>L. pimpinellifolium</i>	BC ₁	3 Q	3,6,9	(Grandillo and Tanksley 1996b)		
	<i>L. peruvianum</i>	BC ₃ , BC ₄	9 Q	1,2,7,8,9,10	(Fulton et al. 1997)		
	<i>L. hirsutum</i>	BC ₂ , BC ₃	5 Q	3,5,6,9	(Bernacchi et al. 1998)		
	<i>L. pimpinellifolium</i>	BC ₁ S ₁	13 Q	1,2,3,7,10,12	(Chen et al. 1999)		
	<i>L. hirsutum</i>	subNILs	1 Q	1	(Monforte and Tanksley 2000a)		
	<i>L. parviflorum</i>	BC ₃	5 Q	4,5,6,9	(Fulton et al. 2000)		
	<i>L. esculentum</i>	RIL	3 Q	2,9	(Saliba-Colombani et al. 2001)		
	<i>L. pimpinellifolium</i>	BC ₃ F ₆	2 Q	8,9	(Doganlar et al. 2002)		
	<i>L. pennelli</i>	BC ₂ /BC ₂ F ₁	3 Q	4,9,12	(Frary et al. 2004b)		
	<i>L. pennelli</i>	ILs	9 Q	1,3,4,5,7,9,10,12	(Causse et al. 2004)		
	Fr. SS × red yield	<i>L. pennelli</i>	ILs	14 Q	Most chromosomes	(Eshed and Zamir 1995)	
		<i>L. pimpinellifolium</i>	BC ₂ , BC ₃	4 Q	3,7,9	(Tanksley et al. 1996)	
		<i>L. peruvianum</i>	BC ₃ , BC ₄	9 Q	1,2,5,7,8,9,10,12	(Fulton et al. 1997)	
<i>L. hirsutum</i>		BC ₂ , BC ₃	9 Q	1,2,3,4,6,8,11,12	(Bernacchi et al. 1998)		
<i>L. parviflorum</i>		BC ₃	2 Q	5,8	(Fulton et al. 2000)		
<i>L. pennelli</i>		BC ₂ /BC ₂ F ₁	4 Q	3,5,12	(Frary et al. 2004b)		
<i>L. parviflorum</i>		BC ₃	4 Q	3,4,7,8	(Fulton et al. 2000)		
Fr. total acid		<i>L. pimpinellifolium</i>	BC ₂ , BC ₃	1 Q	9	(Tanksley et al. 1996)	
		Fr. viscosity	<i>L. peruvianum</i>	BC ₃ , BC ₄	4 Q	1,2,8,9	(Fulton et al. 1997)
			<i>L. parviflorum</i>	BC ₃	3 Q	2,9,10	(Fulton et al. 2000)
			<i>L. pennelli</i>	BC ₂ /BC ₂ F ₁	4 Q	2,3,9,12	(Frary et al. 2004b)
			<i>L. pimpinellifolium</i>	BC ₁	7 Q	1,2,8,11	(Grandillo and Tanksley 1996b)
			<i>L. pimpinellifolium</i>	BC ₂ , BC ₃	8 Q	2,3,4,5,7,9	(Tanksley et al. 1996)
			<i>L. peruvianum</i>	BC ₃ , BC ₄	10 Q	1,2,3,7,8,9,10,12	(Fulton et al. 1997)
	<i>L. hirsutum</i>		BC ₁	3 Q	1,3	(Bernacchi and Tanksley 1997)	
	<i>L. hirsutum</i>		BC ₂ , BC ₃	3 Q	2,3,4	(Bernacchi et al. 1998)	

(continued)

Table 4. (continued)

Trait ^a	Wild species used	Mapping population ^b	Genes (G) or QTLs (Q)	Chromosome	References
	<i>L. pimpinellifolium</i>	BC ₁ S ₁	12 Q	1,2,3,4,6,7,8,9,11,12	(Chen et al. 1999)
	<i>L. parviflorum</i>	BC ₃	8 Q	2,3,6,7,10,11,12	(Fulton et al. 2000)
	<i>L. esculentum</i>	F ₂	2 Q	4,6	(Doganlar et al. 2000)
	<i>L. pimpinellifolium</i>	F ₂	6 Q	1,2,3,11	(Lippman and Tanksley 2001)
	<i>L. esculentum</i>	RIL	5 Q	2,3,11,12	(Saliba-Colombani et al. 2001)
	<i>L. pimpinellifolium</i>	BC ₂ F ₆	2 Q	2,3	(Doganlar et al. 2002)
	<i>L. pimpinellifolium</i>	F ₂	7 Q	1,2,3,5,6,7,11	(van der Knaap and Tanksley 2003)
	<i>L. pennellii</i>	BC ₂ /BC ₂ F ₁	3 Q	3,10,12	(Frary et al. 2004b)
	<i>L. pimpinellifolium</i>	BC ₂ , BC ₃	6 Q	2,3,7,9	(Tanksley et al. 1996)
	<i>L. peruvianum</i>	BC ₃ , BC ₄	10 Q	1,2,6,7,8,9,10,12	(Fulton et al. 1997)
	<i>L. hirsutum</i>	BC ₂ , BC ₃	12 Q	1,2,3,4,5,6,7,8,12	(Bernacchi et al. 1998)
	<i>L. hirsutum</i>	subNILs	1 Q	1	(Monforte and Tanksley 2000a)
	<i>L. parviflorum</i>	BC ₃	5 Q	1,2,3,6,8	(Fulton et al. 2000)
	<i>L. pennellii</i>	BC ₂ /BC ₂ F ₁	6 Q	3,5,8,9,12	(Frary et al. 2004b)
	<i>L. pimpinellifolium</i>	BC ₂ , BC ₃	2 Q	2,7	(Tanksley et al. 1996)
	<i>L. peruvianum</i>	BC ₃ , BC ₄	12 Q	1,2,3,5,7,8,9,10,12	(Fulton et al. 1997)
	<i>L. hirsutum</i>	BC ₂ , BC ₃	11 Q	1,2,3,5,7,8,10,11,12	(Bernacchi et al. 1998)
	<i>L. parviflorum</i>	BC ₃	4 Q	2,5,8	(Fulton et al. 2000)
	<i>L. pennellii</i>	BC ₂ /BC ₂ F ₁	4 Q	3,5,12	(Frary et al. 2004b)
	<i>L. hirsutum</i>	BC ₂ , BC ₃	11 Q	2,3,7,8,11,12	(Bernacchi et al. 1998)
	<i>L. pennellii</i>	BC ₂ /BC ₂ F ₁	3 Q	8,9,12	(Frary et al. 2004b)
	<i>L. cheesmanii</i>	F ₂	<i>j</i>	11	(Wing et al. 1994; Zhang et al. 1994)
	<i>L. cheesmanii</i>	F ₂	<i>j-2</i>	12	(Zhang et al. 2000)
	<i>L. pimpinellifolium</i>	F ₂	ms-10	2	(Tanksley et al. 1984)
Fr. yield (total yield)					
Fr. yield (red yield)					
Fr. yield (green yield)					
Jointless					
Male sterility					

Plant growth habit	<i>L. peruvianum</i>	BC ₃ , BC ₄	6 Q	1,2,3,7,8,9	(Fulton et al. 1997)
	<i>L. pimpinellifolium</i>	BC ₂ F ₆	4 Q	2,3,9,11	(Doganlar et al. 2002)
Plant height	<i>L. pennellii</i>	ILs	16 Q	Many chromosomes	(Eshed and Zamir 1995)
	<i>L. pimpinellifolium</i>	BC ₁	1 Q	2	(Grandillo and Tanksley 1996b)
	<i>L. hirsutum</i>	BC ₁	4 Q	1,2,5,11	(Bernacchi and Tanksley 1997)
	<i>L. cheesmanii</i>	F ₈ RIL	7 Q	2,3,4,6,7	(Paran and Zamir 1997)
Phytochrome genes	<i>L. pennellii</i>	BC ₁	5 G	1, 2, 5, 7, 10	(van Tuinen et al. 1997)
Seed number	<i>L. pimpinellifolium</i>	BC ₁	4 Q	4,6,7,12	(Grandillo and Tanksley 1996b)
	<i>L. pimpinellifolium</i>	F ₂	2 Q	1,11	(Lippman and Tanksley 2001)
	<i>L. pimpinellifolium</i>	BC ₂ F ₆	4 Q	5,6,8	(Doganlar et al. 2002)
	<i>L. pennellii</i>	F ₂	10 Q	1,2,3,4,6,7,9,11,12	(van der Knaap et al. 2000)
Seed weight	<i>L. pennellii</i>	BC ₁	5 Q	1,2,4,7,8	(Tanksley et al. 1982)
	<i>L. cheesmanii</i>	F ₈ RILs	14 Q	1,2,3,4,6,7,9,11,12	(Goldman et al. 1995)
	<i>L. pimpinellifolium</i>	BC ₁	4 Q	2,4,10,12	(Grandillo and Tanksley 1996b)
	<i>L. pimpinellifolium</i>	F ₂	4 Q	1,2,4	(Lippman and Tanksley 2001)
	<i>L. pimpinellifolium</i>	BC ₂ F ₆	5 Q	1,4,5,7	(Doganlar et al. 2002)
Self-incompatibility	<i>L. peruvianum</i>	F ₁	S locus	1	(Tanksley and Loaiza-Figueroa 1985)
	<i>L. peruvianum</i>	F ₁	S locus	1	(Bernatzky 1993)
	<i>L. hirsutum</i>	BC ₁	S locus	1	(Bernacchi and Tanksley 1997)
Self-pruning	<i>L. esculentum</i>	F ₂	sp	6	(Ito and Currence 1964)
	<i>L. chmielewskii</i>	BC ₁	sp	6	(Paterson et al. 1988)
	<i>L. pimpinellifolium</i>	BC ₁	sp	6	(Grandillo and Tanksley 1996a)
Transgr. segregation	<i>L. pennellii</i>	F ₂	74 Q/8 traits	All 12 chromosomes	(de Vicente and Tanksley 1993)
Unilateral incongruity	<i>L. hirsutum</i>	BC ₁	6 Q	1,2,3,11,12	(Bernacchi and Tanksley 1997)

^a D = distance; Flwr. = flower; Frt. = fruit; L = length; W = width

^b NIL: near isogenic line; RIL: recombinant inbred line

the health benefits of lycopene, the major carotenoid responsible for the red color of tomato, has become more obvious (Gerster 1997). Previously, several major genes with significant effect on fruit lycopene (e.g. *hp-1*, *hp-2*, *dg* and *Og^c*) and other carotenoids contents (e.g. beta-carotene, *B*) had been identified and mapped onto the classical linkage maps of tomato (Wann and Jourdain 1985). During the past two decades, however, many studies identified numerous QTLs and candidate genes with significant effects on fruit color and/or carotenoids content Table 4. Although some QTLs were mapped to the chromosomal locations of known genes in the carotenoid biosynthesis pathway, many were mapped to new locations suggesting presence of additional genes affecting fruit color in tomato (Liu et al. 2003b). Currently, a few research groups around the world conduct research to identify, map and possibly clone new genes involved in determining fruit color in tomato. In addition, there are numerous programs attempting to improve tomato nutritional quality either through traditional breeding or transgenic approaches (Ronen et al. 1999; Zhang and Stommel 2000).

3.4.4. *Fruit soluble solids*

Fruit soluble solids content (SSC) has long been the focus of numerous tomato breeding programs worldwide. However, despite significant improvement in fruit size and yield, SSC has remained almost unchanged due in part to the negative relationship between fruit yield and SSC. Thus, breeders have sought alternative approaches to improve SSC of high yielding tomato cultivars. During the past two decades, considerable efforts have been devoted to identifying QTLs for SSC in order to facilitate improving fruit SSC without compromising the yield. The hope has been to identify QTLs that may not have any adverse effects on fruit size or yield. Currently, there are more than 20 published studies that have reported QTLs for fruit SSC in tomato (Table 4). Although these studies used different interspecific populations, there have been significant overlaps in the locations of QTLs. Furthermore, many studies have revealed that most QTLs that positively influence SSC are at the same locations as QTLs with negative impact on fruit weight (Chen et al. 1999), an unfortunate discovery. However, few studies have reported the identification of QTLs for SSC with little or no detrimental impact on fruit size (e.g. (Fridman et al. 2000)), though the effects of such QTLs are yet to be verified.

3.4.5. *Fruit yield*

Because of the complexity of yield, technically it is difficult to identify QTLs which are truly affecting this trait and could be utilized in marker-assisted breeding to improve crop yield. Nevertheless, many QTL mapping studies have tried to identify QTLs for fruit yield in tomato, including traits such as total yield, red yield and green yield, mostly using interspecific segregating populations (Table 4). Unlike QTLs for fruit weight and SSC that have been rather consistent across studies, there has been limited agreement in different studies with regard to yield QTLs (Table 4). This is not surprising considering the very complex nature of the trait and its low heritability. Also, there is no published report on the use or verification of fruit yield

QTLs for marker-assisted breeding in tomato, and it is not expected that such QTLs will have wide utility for improving tomato yield, at least in the near future. It is expected, however, that QTLs for components of fruit yield, such as fruit size, number and SSC, will be useful as indirect selection criteria to increase tomato fruit yield.

3.4.6. Fruit ripening

Traditional genetics and breeding research had resulted in the identification and manipulation of several ripening-related genes in tomato. During the past two decades, however, molecular biology techniques facilitated characterization of such genes and identification and mapping of many other ripening related genes, loci and QTLs (Table 4). Furthermore, a few ripening-related genes have been fine-mapped and cloned (Table 6). More detailed lists of genes with effects on fruit ripening in tomato can be found in Giovannoni (2001). Among the major ripening genes, at least one (*rin*) has been used in marker-assisted breeding (Table 5).

3.5. Mapped Genes and QTLs for Other Traits

In addition to the traits described in above, molecular markers have been employed to tag or map genes or QTLs for many other horticultural characters in tomato, as shown in Table 4. Many of these studies have contributed significantly to our better understanding of the genetic controls of different morphological and physiological characters in tomato. Examples include self-incompatibility, unilateral incongruity, transgressive segregation, self-pruning (determinate type plants), jointless pedicel, and seed size and number. The marker information can be useful for further basic research and for crop improvement through marker-assisted breeding.

3.6. Populations used for Gene and QTL Mapping

As in many other plant species, in tomato early filial and backcross populations have been used most widely for gene and QTL mapping (Tables 2–4). In some cases, however, advanced populations such as recombinant inbred lines (RILs), advanced backcross populations (AB; e.g. BC₂ and BC₃), backcross inbred lines (BILs, a.k.a. inbred backcross lines or IBC, e.g., BC₂S₃, BC₃S₂) and introgression lines (ILs) have been employed for genetic mapping. Currently a few tomato RIL populations are available, including one based on a *L. esculentum* × *L. cheesmanii* cross (Paran and Zamir 1997) and three based on different *L. esculentum* × *L. pimpinellifolium* crosses (Foolad et al. unpubl.; Graham et al. 2004;). RIL populations have been used for mapping QTLs for various traits, including fruit weight and SS (Goldman et al. 1995), morphological characters (Paran and Zamir 1997), abiotic stress tolerance (Foolad 2004), seed weight (Goldman et al. 1995) and disease resistance (Diwan et al. 1999; Foolad et al., unpubl). BILs have been used frequently to map QTLs for many traits (Tables 2–4), including fruit quality (Doganlar et al. 2002; Tanksley et al. 1996; Yang et al. 2005) and disease resistance (Coaker and Francis 2004; Foolad et al. 2002; Robert et al. 2001). Greater levels of homozygosity in the RIL

and BIL populations, compared with early segregating populations, have allowed more precise estimation of the location and effects of QTLs. Another type of population that has been extensively used for mapping in tomato is introgression lines (ILs). The first developed IL population of tomato consisted of 50 lines, each containing a single introgression from *L. pennellii* LA716 in the background of tomato cultivar M-82 (Eshed and Zamir 1995). Since then the number of ILs in this population has increased to 76, which together represent the entire genome of the *L. pennellii* LA716 in homozygous or heterozygous conditions and delimit 107 marker-defined mapping bins, each bin having an average length of 12 cM (Gur et al. 2004; Liu et al. 2003b). In addition to this IL population, a total of 99 NILs and BILs derived from a cross between cultivar E6203 and *L. hirsutum* accession LA1777 has been developed (Monforte and Tanksley 2000b). Most of the lines contain a single defined introgression from *L. hirsutum* in the *L. esculentum* genetic background, and together the lines cover more than 85% of the LA1777 genome. IL populations can be used for MAS pyramiding of important QTLs, as in the case of tomato yield and soluble solids QTLs (Gur and Zamir 2004). Furthermore, some ILs and BILs have been used to develop NILs for fine mapping and map-based cloning of genes and QTLs controlling traits such as self-pruning (Pnueli et al. 1998), color mutants (Isaacson et al. 2002; Ronen et al. 1999), SSC (Fridman et al. 2000; Yates et al. 2004), fruit weight (Frery et al. 2000) and shape (van der Knaap et al. 2004; Yates et al. 2004), stigma exertion (Chen and Tanksley 2004) and a few others (Table 6).

4. MARKER-ASSISTED SELECTION AND BREEDING

4.1. Current Use of MAS

The use of MAS in tomato breeding started in 1980s, when many seed companies took advantage of the reported linkage between *Mi* and *Aps-1*¹ and used the isozyme marker to select for nematode resistance. Recently, however, MAS has become a routine practice in many seed companies for improving tomatoes for many simple-inherited traits (Table 5). Unfortunately, most of these activities are not reported in public literature. However, a survey by the author indicated that many seed companies in the U.S. (e.g., Seminis Vegetable Seeds (now owned by Monsanto), Syngenta, Harris Moran, Sakata, Asgrow) and in Europe (e.g., Nunhems Zaden, Vilmorin, Seminis Vegetable Seeds Holland, ENZA, RijkZwaan, DeRuiter) routinely employ MAS for tomato disease resistance breeding. Examples include vertical resistance to corky root, fusarium wilt, late blight, nematodes, powdery mildew, bacterial speck, tobacco/tomato mosaic virus, tomato spotted wilt virus, and verticillium wilt (Table 5). MAS is also routinely practiced in seed companies for improvement of a few other simple traits in tomato, including, but not limited to, self-pruning (*sp* gene), jointless pedicel, ripening, and carotenoids contents (lycopene and β -carotene). However, there is little indication of the use of MAS for manipulating QTLs for complex traits, though MAS is being attempted to improve

Table 5. Known traits for which marker-assisted selection and breeding are done in tomato

Trait	Source species	Gene/QTL (Q)	Reference
Bacterial canker	<i>L. peruvianum</i>	3 Q	Seed companies
	<i>L. hirsutum</i>	<i>Rcm2.0, Rcm5.1(Q)</i>	(Coaker and Francis 2004)
Bacterial speck	<i>L. pimpinelli- folium</i>	Pto	Seed companies, (Yang and Francis 2005)
Bacterial spot	<i>L. esculentum</i>	<i>Rx-3 (Q)</i>	(Yang and Francis 2005)
Bacterial wilt	<i>L. esculentum</i>	2 Q	Seed companies
Blackmold	<i>L. cheesmanii</i>	Few Q	(Robert et al. 2001)
Corky root rot	<i>L. peruvianum</i>	<i>Py-1</i>	Seed companies
Fusarium wilt	<i>L. pimpinelli- folium</i>	<i>I-2C, I-3</i>	Seed companies
Jointless	<i>L. cheesmanii</i>		Seed companies
Late blight	<i>L. pimpinelli- folium</i>	<i>Ph-3</i>	Seed companies, University researchers
	<i>L. hirsutum</i>	4 Q	(Brouwer and St. Clair 2004)
Lycopene	<i>L. esculentum</i>	<i>Og^c, cr</i>	Seed companies
Powdery mildew	<i>L. chilense, L. hirsutum</i>	<i>Lv, Ol-1, Ol-2</i>	Seed companies
Ripening inhibitor	<i>L. cheesmanii</i>	<i>rin</i>	Seed companies
Root-knot nematode	<i>L. peruvianum</i>	<i>Mi</i>	Seed companies, public breeders
Self-pruning	<i>L. esculentum</i>	<i>sp</i>	Seed companies, public breeders and geneticists
Soluble solids	Not known	<i>Q</i>	Seed companies
Tomato spotted wilt	<i>L. peruvianum</i>	<i>Sw-5</i>	(Langella et al. 2004), seed companies
Tomato YLCV	Different species	Few Q	Seed companies
Tobacco mosaic virus	<i>L. peruvianum</i>	<i>Tm-2^a</i>	Seed companies
Verticillium wilt	<i>L. esculentum</i>	<i>Ve</i>	Seed companies

^aBrix as well as quantitative resistance to bacterial wilt, bacterial canker, bacterial wilt, powdery mildew and yellow leaf curl virus.

The use of MAS is less common in public tomato breeding programs, though it has been practiced to improve vertical resistance to a few diseases (Barone 2005) such as late blight (M Mutschler, Cornell University; MR Foolad, Penn State University), bacterial canker, bacterial speck and bacterial spot (Coaker et al. 2004), and horizontal resistance to blackmold (Robert et al. 2001) and late blight (Brouwer and St. Clair 2004). It also has been employed for simple traits such as self-pruning (many tomato genetics and breeding programs) and some complex fruit quality characters (Lecomte et al. 2004). However, based on the published results, in most cases where MAS was employed to transfer QTLs there were significant linkage drag problems. For example, in case of late blight where three near-isogenic lines were developed each containing one resistance QTL from *L. hirsutum*, while all lines showed expected level of resistance they also suffered from undesirable horticultural characteristics

Table 6. Fine-mapped and/or cloned genes and QTLs in tomato

Trait	Gene/QTL	Chromosome	Source species	Fine mapping population	Nature/activity/function	Reference
Aphid (potato) res.	<i>Meu</i>	6	<i>L. peruvianum</i>	NIL F ₂	NBS-LRR	(Rossi et al. 1998; Vos et al. 1998)
Bacterial speck res.	<i>I Pro</i>	5	<i>L. pimpinelli-folium</i>	NIL F ₂	Protein kinase	(Gu and Martin 1998; Martin et al. 1993a)
Bacterial spot res.	<i>Bs4</i>	5	<i>L. pennellii</i>	NIL F ₂	TIR-NB-LRR	(Schornack et al. 2004)
Fenthion resistance	<i>Prf</i>	5	<i>L. pimpinelli-folium</i>	NIL F ₂	NBS-LRR	(Martin et al. 1994; Salmeron et al. 1996)
Flower, exerted stigma	<i>se2.1 (Q)</i>	2	<i>L. pennellii</i>	NIL F ₂ and F ₃	Resistance gene Affects floral morphology	(Chen and Tanksley 2004)
Fr. color (β-carotene)	<i>B</i>	6	<i>L. pennellii</i>	NIL F ₂	Lycopene β-cyclase	(Ronen et al. 2000; Ronen et al. 1999)
Fr. color (crimson)	<i>og^c, cr</i>	6	<i>L. esculentum</i>	NIL F ₂	Lycopene cyclase null allele	(Ronen et al. 2000; Ronen et al. 1999)
Fr. color (high-pigment-2)	<i>hp-2</i>	1	<i>L. esculentum</i>	N/A	Homologue of DEETIO-LATED1	(Mustilli et al. 1999)
Fr. color (old gold)	<i>og</i>	6	<i>L. esculentum</i>	NIL F ₂	Lycopene cyclase null allele	(Ronen et al. 2000)
Fr. color (tangerine)	<i>CRISTO</i>	10	<i>L. esculentum</i>	NIL F ₂	Carotenoid isomerase	(Isaacson et al. 2002)
Fr. rip. (Colorless non-rip.)	<i>Cnr</i>	2	<i>L. esculentum</i>	F ₂ and FISH	Not determined	(Tør et al. 2002)
Fr. ripening (never-ripe)	<i>Nr</i>	9	<i>L. cheesmanii</i> <i>L. esculentum</i>	NIL F ₂	Blocks ethylene perception	(Lanahan et al. 1994; Wilkinson et al. 1995; Yen et al. 1995)
Fruit ripening (non-ripening)	<i>nor</i>	10	<i>L. esculentum</i>	NIL F ₂	MADS-box	(Giovannoni et al. 1995; Moore et al. 2002; Vrebalov et al. 2002)

Fr. ripening (polygalacturonase)	TOM6	10	<i>L. esculentum</i>	N/A	Pectin hydrolyzing	(Dellapenna et al. 1986; Grierson et al. 1986; Slater et al. 1985) (Giovannoni et al. 1995; Moore et al. 2002; Vrebalov et al. 2002)
Fruit ripening (ripening-inhibitor)	rin	5	<i>L. cheesmanii</i> <i>L. pennellii</i>	NIL F ₂	MADS-box	(Ku et al. 2000; Tanksley 2004) (van der Knaap et al. 2004) (Ku et al. 1999; Liu et al. 2002) (Alpert et al. 1995; Alpert and Tanksley 1996; Frary et al. 2000; Liu et al. 2003a) (Simons et al. 1998) (Fridman et al. 2002)
Fruit shape	<i>fs8.1</i> (<i>Q</i>) <i>Sun</i> (<i>Q</i>) <i>ovate</i> (<i>Q</i>) <i>fw2.2</i> (<i>Q</i>)	8 7 2 2	<i>L. pimpinelli-</i> <i>folium</i> <i>L. esculentum</i> <i>L. pimpinelli-</i> <i>folium</i> <i>L. pennellii</i>	NIL F ₂ NIL F ₂ and F ₃ NIL F ₂ NIL F ₂	Imparts blocky shape Imparts oval shape Plant-growth suppressor Controls carpel cell number	
Fruit weight						
Fusarium wilt resistance	<i>I2</i>	11	<i>L. esculentum</i>	NIL F ₂	Leucine zipper and LRR-NBS	(Liu et al. 2003a) (Simons et al. 1998)
Growth habit	<i>PW9-</i> <i>2-5</i> (<i>Q</i>)	9	<i>L. pennellii</i>	F ₂	Semi-det. growth	(Fridman et al. 2002)
Iron uptake	<i>chloronerva</i>	1	<i>L. pennellii</i>	NIL F ₂	Nicotianamine synthase	(Ling et al. 1999)
Jointless	<i>j</i>	11	<i>L. esculentum</i>	F ₂ , NIL F ₂	Suppresses form. abscission zone	(Mao et al. 2001; Wing et al. 1994; Zhang et al. 1994)
	<i>j-2</i>	12	<i>L. cheesmanii</i>	F ₂	Suppresses form. abscission zone	(Budiman et al. 2004; Zhang et al. 2000)

(continued)

Table 6. (continued)

Trait	Gene/QTL	Chromosome	Source species	Fine mapping population	Nature/activity/function	Reference
Leaf mould resistance	<i>Cf-2,4,5,9</i>	1,6	<i>L. peruvianum</i>	NIL F ₂	LRR	(Dixon et al. 1996)
Nematode (root-knot) resistance	<i>Mi-1, IMI-1.2</i>	6	<i>L. peruvianum</i>	NIL F ₂	NBS-LRR	(Kaloshian et al. 1998; Rossi et al. 1998; Vos et al. 1998)
Self-pruning (SP)	<i>sp</i>	6	<i>L. esculentum</i>	NIL F ₂	Regulates cycle veg./repr. growth	(Pnueli et al. 1998)
SP gene family	<i>sp21, 3D, 5G, 6A, 9D</i>	2,3,5,6,9	<i>L. pennellii</i>	NIL F ₂	Not determined	(Carmel-Goren et al. 2003)
Self-incompatibility	<i>S</i>	1	<i>L. peruvianum</i>	N/A	RNase activity	(Rivers et al. 1993)
Soluble solids	<i>Brix9-2-5, Lin5 (Q)</i>	9	<i>L. pennellii</i>	NIL F ₂	Apoplastic invertase	(Fridman et al. 2004; Fridman et al. 2000)
Tomato spotted wilt virus	<i>Sw-5</i>	9	<i>L. peruvianum</i>	NIL F ₂	NBS-LRR Resistance gene	(Adkins 2000; Brommenschkel et al. 2000; Langella et al. 2004)
Verticillium wilt	<i>Ve1, Ve2</i>	9	<i>L. esculentum</i>	N/AcDNA cloning	Glycoproteins w/ leucine zipper or Pro-Glu-Ser-Thr	(Kawchuk et al. 2001)

(Brouwer and St. Clair 2004). Further inspections of the NILs resulted in the detection of QTLs for other characters such as plant shape, canopy density, maturity, fruit yield and fruit size in the same introgressed regions. The results prompted the investigators to suggest further refining of the late blight QTLs before transferring to adapted genetic backgrounds. Similar conclusions were reached as to MAS transfer of QTLs for blackmold resistance from *L. cheesmanii* to the cultivated tomato, where predominantly negative associations were found between introgressed QTL alleles and horticultural characteristics (Robert et al. 2001). Such undesirable associations have been reported also for many other complex traits in tomato, and in most cases it was not determined whether they were due to genetic linkage or pleiotropic effects. However, with the current rough state of QTL detection, it is not unexpected that similar negative associations be experienced when practicing MAS for other complex traits. It is prudent that issues surrounding the utility of MAS should be addressed before it can become a routine practice for crop improvement.

4.2. Issues in Using MAS

Practical utility of MAS for manipulating simple traits has been well documented. However, despite tremendous investment in finding markers associated with important genes and QTLs, MAS has not become a routine practice in most plant breeding programs, in particular for improving complex traits. The issues associated with the use of MAS include: (1) elevated cost of high-throughput marker genotyping, which is not affordable by most breeding programs considering the large size of populations need to be screened; (2) unavailability of reliable PCR-based markers for many simple and complex traits; (3) lack of QTL validation across genetic backgrounds and environments; (4) large size of QTL intervals and association with undesirable characteristics; (5) unfamiliarity of many traditional plant breeders, who in fact release most of the modern cultivars, with the use of markers or their limited access to molecular marker laboratories; and (6) identification and mapping of genes and QTLs mainly by researchers who are not breeders or who may not have inherent interest in crop improvement. A better cooperation between basic scientists and plant breeders is needed to coordinate meaningful identification and use of gene/QTL-linked markers. Another significant limiting factor is lack of sufficient marker polymorphism in the cultivated species. Many tomato breeders focus on exploitation of genetic variation within the cultivated species, where MAS cannot be easily employed using traditional molecular markers. However, recent efforts in discovering SNPs within the cultivated tomato is expected to reduce this problem and facilitate the use of markers when exploiting intraspecific genetic variations.

5. FINE MAPPING AND MAP-BASED CLONING

5.1. Fine Mapping and Map-Based Cloning of Major Genes

Tomato was the first plant species in which a disease resistance gene, *Pto*, conferring resistance to bacterial speck caused by *Pseudomonas syringae* pv. *Tomato* (*Pst*), was

cloned using map-based cloning approach (Martin et al. 1993a). Further analysis indicated similarity of the open reading frame of *Pto* to serine-threonine protein kinases (Table 6) (Martin et al. 1994). Subsequently, similar map-based cloning strategy was employed and several other tomato genes were cloned, including *Prf*, which is required for *Pto* activity and tomato resistance to *Pst* and which also confers tomato susceptibility to organophosphate insecticide Fenthion (Salmeron et al. 1996), *Sw-5*, conferring resistance to tospovirus (Brommenschenkel and Tanksley 1997), *sp* (self pruning), conferring determinate growth habit (Pnueli et al. 1998), members of *sp* gene family (Carmel-Goren et al. 2003), and *j* and *j-2*, controlling jointless pedicel (Mao et al. 2000). It should be noted that both *j* and *j-2* are recessive mutants that completely suppresses the formation of pedicel abscission zone (Budiman et al. 2004; Mao et al. 2000; Zhang et al. 2000; Zhang et al. 1994), which is an essential character and widely used in both processing and fresh-market tomato cultivars. Several other major genes that have been fine-mapped and/or cloned via map-based cloning approach in tomato are listed in Table 6.

5.2. Fine Mapping and Map-Based Cloning of QTLs

Most QTL mapping studies detect QTLs within large marker intervals, usually 10 cM or greater, and often cannot determine whether a detected QTL contains one or more genes. Also, detected QTLs often affect more than one trait and it is unknown whether such effects are due to pleiotropic effects of the same genes/QTLs or genetic linkage of independent loci. In tomato, for example, the lower part of chromosome 1 has been identified to affect many agriculturally important traits, including morphological and fruit quality characteristics as well as resistance to many biotic and abiotic stresses (Frary et al. 2003; Monforte and Tanksley 2000a). Similarly, the long arm of chromosome 4 contains QTLs for many horticulturally important traits such as fruit SSC, shape and lycopene content (Yates et al. 2004). Furthermore, it has been demonstrated that introduction of a small segment of DNA from a wild into the cultivated species of tomato often results in significant changes in several characteristics, and it is unknown whether the same genes/QTLs affect different traits. During the past several years, however, advances in marker technology have facilitated physical characterization of QTL introgressions. For example, a NIL of tomato containing a 40-cM introgression from *L. hirsutum* accession LA1777 at the bottom of chromosome 1 was dissected by developing sub-NILs containing smaller segments (Monforte and Tanksley 2000a). In a similar study, a series of sub-NILs were developed containing introgressions from chromosome 1 of *L. chmielewskii* to fine map loci controlling several fruit quality characteristics important to processing tomato cultivars (Frary et al. 2003). It was determined that independent loci in the lower part of chromosome 1 affected fruit °Brix, yield and shape, whereas genetic factors affecting fruit weight, shoulder pigmentation and external color coincided with the location of a °Brix locus. These results, combined with results of other studies, prompted the investigators to conclude that the base of tomato chromosome 1 contains multiple QTLs whose effects cannot be attributed to the pleiotropic effects

of a single locus (Frery et al. 2003; Monforte and Tanksley 2000a). In another study, NILs containing the lower part of chromosome 4 from either *L. peruvianum* or *L. hirsutum* were dissected by developing series of sub-NILs containing small introgressions from either wild species (Yates et al. 2004). Results indicated presence of multiple, non-allelic loci controlling SSC and fruit weight in addition to other loci controlling fruit shape, weight and epidermal reticulation. In addition to these studies, many other QTL coincidences have been reported in tomato and other crop species, however, in most cases the nature of such coincidences has not been determined.

During the past decade, efforts have been made to clone QTLs and assert that QTLs have the same molecular bases as regular Mendelian genes. In fact, much of such efforts have been made in tomato. For example, the first map-based cloning of a QTL in plants was done for a fruit size QTL (*fw2.2*) in tomato (Frery et al. 2000). While tomato improvement for fruit size has been easy due to its high heritability, both inheritance studies and QTL mapping experiments revealed that this trait was controlled by several loci (Chen et al. 1999). To date, most, if not all, QTLs involved in the evolution of tomato fruit size (from small to large) have been identified and mapped (Table 4) (Lippman and Tanksley 2001). In many studies, one major QTL (*fw2.2*) was found to be associated with large phenotypic variation for fruit size (Chen et al. 1999; Grandillo et al. 1999). While the modern tomato cultivars carry large-fruit alleles at this locus, all wild *Lycopersicon* species examined contain small-fruit alleles (Alpert et al. 1995). Because of its large, consistently-detectable effects, a map-based cloning approach was pursued, which resulted in cloning and sequencing of *fw2.2* (Frery et al. 2000; Liu et al. 2003a). It was determined that *fw2.2* was expressed early in floral development and controlled carpel cell number. Subsequently, similar strategy was employed to fine-map and/or clone a few other QTLs in tomato affecting traits such as SSC, fruit shape and exerted stigma (Table 4). It is expected that with the advances in marker technology and QTL identification, more QTLs will be cloned using map-based cloning approach.

6. TOMATO GENOME ORGANIZATION AND SEQUENCING

The tomato nuclear genome comprises 12 chromosomes and approximately 950 Mb of DNA, containing 59% non-coding sequences, 28% coding sequences, 11% transposons, and 2% organellar sequences (Wang et al. 2005). Approximately 77% of the chromosomal DNA is comprised of centromeric heterochromatic regions, which are devoid of genes. The majority of genes are populated at distal euchromatic regions of the chromosomes, with an approximate gene density of 6.7 kb/gene, similar to that of *Arabidopsis* and rice. This study also indicates that a significant portion of the tomato euchromatin is methylated in the intergenic spacer regions. Currently the 12 tomato chromosomes are being sequenced by an international consortium of 10 countries, including China (chr. 3), France (chr. 7), India (chr. 5), Italy (chr. 12), Japan (chr. 8), Korea (chr. 2), The Netherlands (chr. 6), Spain (chr. 9), England (chr. 4), and the U. S. (chrs. 1, 10 and 11)

(http://www.sgn.cornell.edu/help/about/tomato_sequencing.pl). This effort is part of a larger initiative known as the International Solanaceae Genome Project (SOL): Systems Approach to Diversity and Adaptation. Lunched in 2003, this project has set research goals for the next 10 years (<http://www.sgn.cornell.edu/solanaceae-project/index.pl>). The major emphasis is on physical, evolutionary and functional genomics of the family Solanaceae. The first cornerstone of the project, however, is to generate a high-quality sequence for the euchromatic portions of the tomato chromosomes, as a reference for the Solanaceae. Concomitantly, however, other genome organizations studies are being conducted in tomato. For example, extensive efforts are being made to expand EST database of tomato, and to date more than 162,000 ESTs developed (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato). Although the EST-derived unigene sets of tomato do not represent the entire gene repertoire of this species, analysis of the tomato EST database and several sequenced BAC libraries have led to the prediction that tomato genome encodes ~35,000 genes, largely sequestered in euchromatic regions of the 12 tomato chromosomes, which correspond to less than 25% of the total nuclear DNA in tomato (van der Hoeven et al. 2002; Wang et al. 2005).

7. REFLECTION AND PROSPECTS

Remarkable progress has been made in generation of molecular markers and maps, mapping of genes and QTLs, and fine-mapping, map-based cloning and characterization of genes and QTLs in tomato. Comparatively, much less has been accomplished as to the actual use of marker information in tomato crop improvement, in particular for complex traits. There are several reasons for this discrepancy, as indicated in above. However, as judged based on most recent discoveries and progresses, the prospect for future use of markers in tomato improvement is good. Perhaps the most important element is development of low-cost, high-throughput PCR-based markers that are easy-to-assay and have high resolution. In particular markers that can detect polymorphism within the cultivated species or between the cultivated and closely-related wild species such as *L. pimpinellifolium* and *L. cheesmanii*. As sequencing of the tomato genome progresses, it is expected that the information will be utilized to develop more informative markers. It is also expected that a greater emphasis will be placed on development of functional markers, such as ESTs and candidate gene markers, which will be highly useful in both applied and basic research programs. Because of these and other advancements, it is not unexpected that in a near future MAS becomes a routine procedure in most tomato-breeding programs. Many breeders are convinced that even for many simple traits with high heritability MAS does have an edge over PS because of various potential limitations in phenotypic screening.

Currently, there is little evidence of actual use of markers for improving complex traits in tomato. A major limiting factor is uncertainties about the reliability of detected QTLs, as discussed in above. For many complex traits, such as yield and tolerance to abiotic stresses, obtaining reliable phenotypic data for QTL detection

is not straightforward, often leading to identification of QTLs which may not be trustworthy. Improvements must be made in our techniques to identify more tractable QTLs. One approach is to seek QTLs which control components of the genetic variation, rather than QTLs detected based on screening the ultimate trait itself. For example, partitioning of the total variation for a complex trait into contributing components and identification of QTLs for each component may lead to the detection of more reliable and useful QTLs. A subsequent and complementary step is to further refine and fine-map the identified QTLs by developing NILs and sub-NILs. Such refining may not only determine the actual value of each QTL, but also may establish whether any potential negative association due to linkage drag can be broken before transferring the QTLs. In other words, fine-mapping would allow detection of QTLs that are useful for marker-assisted breeding. The significance of such refinements is well recognized among tomato geneticists and breeders, and many research programs have initiated such activities. Another reason for optimism regarding the application of marker technology in tomato improvement is the increasing use of F_1 hybrid cultivars for commercial production. When developing hybrids, the use of MAS will not only be more practical but also more economical. However, in spite of all expected advancements and the optimism, MAS may not be a “silver bullet” solution to every breeding problem in tomato. Most likely, a combination of traditional protocols and marker-assisted breeding will become a routine procedure in many tomato improvement programs. I expect a long time before traditional breeding protocols become totally obsolete, if they ever become.

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CHAPTER 14

GENOMICS FOR IMPROVEMENT OF ROSACEAE TEMPERATE TREE FRUIT

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Abstract: Genomic studies of Rosaceous fruit trees have concentrated on two species: peach (*Prunus persica*), which has served as a model for other species of the same genus, such as the stone fruits (apricot, cherry and plum) and almond; and apple (*Malus x domestica*), which itself is a model for other close species such as pear, quince and loquat. High density or saturated maps exist in both peach and apple, and sets of microsatellite markers spaced across the genome of both species are used for gene tagging and mapping in other populations. Efficient methods for mapping new markers and genes have been developed, such as “bin mapping” and the “genome scanning approach”. Tens of major genes and QTLs have been located on the maps of both species, and some of them are close to markers routinely used for selection in plant breeding. Comparative mapping has shown that all members of the *Prunus* genus share the same genome structure and that apple and pear genomes have a highly similar genetic organization. There are chromosomal rearrangements between the genomes of apple and *Prunus*, but extensive regions of synteny and collinearity are maintained. Several genes of apple and peach have been cloned using map-based techniques or are in the process of being cloned. A physical map is in an advanced stage of construction for peach and one has recently been started in apple. Large EST collections have been developed, particularly in apple and *Prunus* providing tens of thousands of new markers and gene sequences useful for functional analysis and map construction. Microarrays are proving to be valuable tools for identifying candidate genes for characters of interest. This information is stored in several databases with varying degrees of public access.

1. INTRODUCTION

The Rosaceae are divided into four subfamilies. Two of them, Prunoideae and Maloideae, include the fruit tree crops that are the subject of this chapter. The other two subfamilies are the Rosoideae, with rose (*Rosa*), strawberry (*Fragaria*),

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blackberry and raspberry (*Rubus*) as the most important crops, and the Spiraeoideae, which has some ornamental trees and bushes of minor economic importance. The Pruniodeae are characterized by their fruit, the drupe, where the fleshy mesocarp surrounds a stony endocarp usually protecting a single large seed. All cultivated members of the Prunoideae subfamily belong to the genus *Prunus*, which encompasses almond (*P. dulcis*) and all stone fruits: peach (*P. persica*), apricot (*P. armeniaca*), European (*P. domestica*) and Japanese (*P. salicina*) plum, and sweet (*P. avium*) and sour (*P. cerasus*) cherry. The characteristic fruit of the Maloideae is the pome, formed by a fleshy hypanthium tissue surrounding the smaller ovary core where the seeds are located. The main cultivated species of this subfamily are apple (*Malus x domestica*), pear (*Pyrus communis* and *P. pyrifolia*), quince (*Cydonia oblonga*) and loquat (*Eriobotrya japonica*).

All *Prunus* species have the same basic chromosome number ($2n = 2x = 8$) and are diploids, except *P. domestica* ($6x$) and *P. cerasus* ($4x$). Their genome size is small, of approximately 300 Mbp for the diploid species (Arumuganathan and Earle 1991). The Maloideae have a tetraploid genome ($2n = 34$), thought to contain either a genome of the Spiraeoideae ($x = 9$) and one of the Prunoideae ($x = 8$), or two different genomes of the Spiraeoideae with further loss of one chromosome pair (Evans and Campbell 2002). The size of the apple and pear genomes is 600–800 Mbp (Arumuganathan and Earle 1991), approximately twice that of *Prunus*, suggesting that the two constituent genomes are small and of similar size to that of *Prunus*. In this chapter, we will review the progress in genomics of this group of crops, which has rapidly advanced over the last decade and has already resulted in relevant breeding applications.

2. MAPPING IN ROSACEOUS FRUIT TREES

2.1. *Prunus*

One of the first maps to be constructed with RAPD (random amplified polymorphic DNA) markers in plants was that of peach (Chaparro et al. 1994). RAPDs were the first markers based on the polymerase chain reaction (PCR) and their use opened a new era of map construction, where maps could be obtained with moderate investment of both time and money. RAPDs were useful but proved to be difficult to reproduce, and more robust markers have been used to construct maps since then. The first saturated map constructed with transferable markers, mostly restriction fragment length polymorphisms (RFLPs) plus a few isozyme genes, was based on an F2 almond ('Texas') x peach ('Earlygold') (TxE) population (Joobeur et al. 1998). The TxE map has since been improved (Aranzana et al. 2003; Dirlwanger et al. 2004a; Howad et al. 2005). It now has 826 markers: 449 SSRs (simple sequence repeat or microsatellite markers), 361 RFLPs, 11 isozymes and 5 STSs (sequence tagged sites), detecting the expected eight linkage groups (G1 to G8), spanning a total distance of 524 cM (average density 0.63 cM/marker) and with a maximum gap of 7 cM. This map, and most maps involving *Prunus* species, can

be viewed and compared with other maps at the Genetic Database for Rosaceae (GDR; <http://www.mainlab.clemson.edu/gdr/>).

The TxE map has been used by *Prunus* geneticists as the reference map to establish linkage group terminology and orientation. Many of the markers used for map construction in other populations have also been selected from TxE to allow good coverage of the *Prunus* chromosomes. Sixteen of these maps, with at least 28 anchor markers with TxE, have already been published (Arús et al. 2005a). A “consensus” map, containing the markers and major genes that have been mapped in different segregating populations, with TxE as the backbone, has been created, allowing the position of major genes and quantitative trait loci (QTLs) to be established on a single map. Twenty-eight major genes (Dirlewanger et al. 2004a) and 28 QTLs (Arús et al. 2005b), responsible for flower, fruit and leaf characteristics, plant architecture, disease resistance, and other features, have been located on this consensus map, allowing the identification of many transferable molecular markers (RFLPs, SSRs, etc.) in the genomic regions where these genes or QTLs are found.

A recent development in *Prunus* is the use of the “bin mapping” strategy proposed by Vision et al. (2000) to place new markers on the TxE map, with reasonable precision, using only a few plants. A set of six plants of the TxE population were selected by Howad et al. (2005), which allowed mapping of any marker to 67 unique chromosome fragments (bins) of 7.8 cM average size, covering the entire map length. Using this approach, Howad et al. (2005) mapped 264 new SSRs with about 1/14 of the effort needed to map all the 82 plants of the population and with only a moderate loss of accuracy. The TxE high density reference map is based on a highly polymorphic interspecific F2 population, which makes bin mapping a very efficient strategy. An F2 population is easier to use and more suitable for mapping purposes than the F1 segregating populations, as it allows selection of a smaller set of plants than other population structures, e.g. a set of 14–20 plants of a backcross would be needed to achieve a similar accuracy to 6 plants of an F2. The implementation of bin mapping in apple, pear and *Prunus* F1 populations is clearly desirable, and possible, but it will be more complex as they include loci that segregate in all possible ratios, and require the construction of two maps, one for each parent of the cross.

2.2. *Malus*

Apple possesses a rich resource of simply inherited resistance genes with major effects. Molecular mapping in apple therefore initially focused on identifying genetic markers for pest and disease resistances (Durham et al. 1994; Manganaris et al. 1994) that could be used for marker assisted selection by breeders. The emphasis on pathogen resistance has continued, but is now coupled to construction of full genetic framework maps (Maliepaard et al. 1998; Liebhard et al. 2003b). Such maps with a high density of transferable markers are needed for construction of QTL maps of complex traits (Durel et al. 2003; Liebhard et al. 2003a; Liebhard et al. 2003c),

for whole genome scans using the Genome Scanning Approach (GSA) (Erdivin et al. 2006; Patocchi et al. 2004; Patocchi et al. 2005) and for pedigree genotyping (van de Weg et al. 2004). The map positions of at least 59 major genes, including 24 major gene resistances and 25 genes potentially involved in fruit allergenicity, are summarized in a recent review (Gardiner et al. 2006b).

The earliest genetic maps in apple utilized non-transferable RAPD markers with a small number of transferable isoenzyme markers (Conner et al. 1997; Hemmat et al. 1994). The first apple reference map was developed in a 'Prima' x 'Fiesta' progeny and included a number of co-dominant transferable markers (microsatellites, isoenzymes and RFLPs as well as a small number of RAPDs and AFLPs (amplified fragment length polymorphisms) distributed over all 17 linkage groups (Maliepaard et al. 1998). This cross has been employed as the basis for maps of QTLs determining fruit texture (King et al. 2000; King et al. 2001) as well as resistance to apple scab (Durel et al. 2003) and fireblight (Calenge et al. 2005a).

The second reference genetic map, constructed in a 'Fiesta' x 'Discovery' progeny (Liebhard et al. 2003b) initially consisted of 129 SSRs plus 710 dominant AFLPs and RAPDs. This map has recently been enlarged to encompass a further 149 SSRs (Silfverberg-Dilworth et al. 2006), is the most detailed apple framework map available, and is the current reference for linkage group number (LG1-LG17) and orientation. The new markers enabled selection of an initial set of 86 highly polymorphic SSRs that will eventually allow genome wide genotyping of apple with 100 markers at an average distance of 15cM. The earlier version of this map was used as the framework to map both QTLs determining fireblight and scab resistance, and physiological traits (Calenge et al. 2005a; Calenge et al. 2005b; Liebhard et al. 2003a; Liebhard et al. 2003c), and 18 RGAs (resistance gene analogues) (Baldi et al. 2004) that are homologs of NBS/LRRs (nucleotide binding site/leucine rich repeat resistance genes). A map constructed in a 'Telamon' x 'Braeburn' progeny that shares some markers with the 'Fiesta' x 'Discovery' map has been used to map QTLs for fruit vitamin C content (Davey et al. 2006).

A third reference map, in 'Discovery' x TN10-8 has been used to locate 23 markers with homology to apple NBS/LRRs (Calenge et al. 2005b) in positions close to major scab and powdery mildew resistances, as well as QTLs for resistance to these diseases identified previously in the same progeny (Calenge et al. 2004a; Calenge et al. 2004b). It has also been used for more detailed mapping of QTLs for powdery mildew resistance (Calenge and Durel 2006) and to map markers for woolly apple aphid resistance *Er3* to LG8 (Gardiner et al. 2006b).

2.3. *Pyrus*

Genetic mapping in *Pyrus* was initiated later than in *Malus*, using a progeny resulting from a cross between a European (*P. communis*) and a Japanese pear (*P. pyrifolia*) (Yamamoto et al. 2004; Yamamoto et al. 2006) and the latest maps comprise 76 and 64 SSRs in 'Bartlett' (*P. communis*) and 'Hosui' (*P. pyrifolia*) respectively, as

well as several hundred AFLPs (Yamamoto et al. 2004). A recent conference report raises the number of SSRs mapped in these two varieties to 157 and 112 respectively (Yamamoto et al. 2006). A map of 'La France' (*P. communis*) has 176 SSRs, with the number of linkage groups consistent with the chromosome number of 17 and almost complete genome coverage (Yamamoto et al. 2006). The self incompatibility (SI) locus maps to LG17 in Japanese and European pears (Yamamoto et al. 2004; Yamamoto et al. 2002), and *Vnk* resistance to *Venturia nashicola* on LG1 (Terakami et al. 2006). Maps constructed in *P. communis* progenies of 'Passe Crassane' x 'Harrow Sweet' and 'Abbé Fetel' x 'Max Red Bartlett' are less complete, containing 41 and 31 SSRs respectively (Pierantoni et al. 2004). QTLs for resistance to fireblight have been identified in 'Harrow Sweet' (Dondini et al. 2004).

3. COMPARATIVE GENOMICS

3.1. Within the *Prunus* Genus

Prunus maps have been obtained with intra- and interspecific segregating populations, including individuals from seven species: peach, almond, apricot, cherry, *P. cerasifera*, *P. davidiana*, and *P. ferganensis*. In all of them the maps obtained with common markers were essentially syntenic and collinear (Arús et al. 2005a). In the rare occasions where marker location was not that predicted, it is probable that they occurred in duplicated genome regions and only one of the two possible markers could be mapped. These results strongly suggest that the *Prunus* genome is in common over all the species of the genus, in agreement with the pattern of frequent inter-crossability and interspecific hybrid fertility known to occur between many species of this genus (Scorza and Sherman 1996).

Only two major chromosomal rearrangements, both reciprocal translocations, have been reported in *Prunus*. The first was found by Jáuregui et al. (2001) in the F2 of a cross between 'Garfi' almond and the red leaf peach rootstock 'Nemared'. The translocated chromosomal segments occurred in G6 and G8. This reciprocal translocation has been confirmed in the three-way cross *P. cerasifera* x GN22, where GN22 is a progeny of 'Garfi' x 'Nemared' (Dirlewanger et al. 2004b). The same translocation was found by Yamamoto et al. (2005) in the cross 'Akame' x 'Juseitou', where 'Akame' is also a red-leafed genotype. Based on the current information, it seems likely that this mutation occurs only within the group of red-leafed peaches. The fact that the breakpoint of the mutation was located at the same genomic region of the gene determining red leaf colour (*Gr*) suggests a relationship of cause and effect between these two characteristics. The other reciprocal translocation was reported by Lambert et al. (2004) in a genotype of myrobalan plum (*P. cerasifera*) and this affects G3 and G5. These results suggest that reciprocal translocations may occur and be maintained within a given *Prunus* species, characterizing monophyletic transects, although the majority of the individuals of the species are within the general *Prunus* genome configuration.

3.2. *Malus and Pyrus*

Comparative genome mapping between *Malus* and *Pyrus* has occurred simultaneously with the construction of *Pyrus* genetic linkage maps, as they have all included a proportion of microsatellite markers developed for apple map construction. The latest pear maps (Dondini et al. 2004; Pierantoni et al. 2004; Yamamoto et al. 2006) each contain between 31 and 79 apple SSRs, enabling alignment of all pear and apple linkage groups. This suggests that genome organization is conserved between apple and pear, and that more detailed comparative mapping could assist transfer of genomic information from resource-rich apple to the less well studied pear and other members of the Maloideae (Arús et al. 2005a). Since 2004, the linkage groups of pear have been assigned the same numbers as in apple and comparative studies have indicated that at least half the apple SSRs are polymorphic in pear (Hemmat et al. 2003; Pierantoni et al. 2004; Wang et al. 2005) and the reverse holds also true for pear SSRs (S. Gardiner and J-M Celton, unpublished).

3.3. *Prunus and Malus*

An incomplete comparison between *Malus* and *Prunus* is possible based on 30 common loci (24 RFLPs and 6 isozymes) between the TxE *Prunus* map and the 'Prima' x 'Fiesta' apple map (Dirlewanger et al. 2004a). Two *Prunus* linkage groups (G3 and G4) had three or more markers in common with the apple map, each one detecting a pair of homeologous apple groups with the same marker order. *Prunus* G1 had eight markers in common with apple. G1 is the longest and most populated with markers in TxE and most other *Prunus* maps, suggesting that it corresponds with chromosome 1, as this is clearly longer than the rest (Salesses and Mouras 1977). Such a long chromosome does not exist in apple (Bouvier et al. 2000) and four of the markers located in the upper part of G1 correspond to two homeologous groups of apple, and the four remaining markers map to another apple linkage group. These results indicate that the long *Prunus* chromosome may be split into two in apple or in the original species that formed the *Malus* amphydiploid. Unpublished results (E. van de Weg, pers. comm.) detect additional syntenic regions between apple and *Prunus*, which suggest an overall high similarity between these two genomes.

3.4. *Prunus and Pyrus*

Comparative studies between *Pyrus* and *Prunus* have been initiated. Seven out of 65 *Prunus* microsatellite markers screened segregated in *Pyrus* (Yamamoto et al. 2004; Yamamoto et al. 2002) compared with 14 out of 31 apple microsatellite markers, which is in line with the relative genetic distances of *Malus* and *Prunus* from *Pyrus*.

3.5. *Prunus* and *Arabidopsis*

The *Arabidopsis* sequence has been compared with the map positions of: a) 227 RFLP loci mapped in TxE, most of them detected with probes based on Rosaceae or *Arabidopsis* EST sequences (Dominguez et al. 2003), b) 475 peach ESTs anchored to the *Prunus* consensus map (Jung et al. 2006), c) 1097 peach ESTs physically located in 431 *Prunus* BAC contigs (Jung et al. 2006), d) the complete sequence of one, and the partial sequence of two, peach BAC clones (Georgi et al. 2003), and e) the complete sequence of one myrobolan plum (*P. cerasifera*) BAC clone (Claverie 2004). In all cases it was possible to find syntenic regions, but they were limited to small DNA fragments indicating an overall picture of fragmentary genome conservation. The largest *Prunus-Arabidopsis* conserved fragment was found by Dominguez et al. (2003), where a region of 25 cM in G2 of *Prunus* corresponded to one of 5.4 Mbp in chromosome 5 of *Arabidopsis*.

4. LARGE INSERT LIBRARIES AND PHYSICAL MAPS

BAC (bacterial artificial chromosome) libraries have been constructed in apple and *Prunus* as tools for physical mapping and map-based cloning. The first BAC library in peach was developed by Wang et al. (2001) using the Chinese cultivar Jingyu and consists of 20,736 clones of average size 95 kb and estimated coverage of 6.7x the peach haploid component. Georgi et al. (2002) produced a 'Nemared' library (44,160 clones; average size 50–70 kb; 8x genome coverage) that has been used for construction of the peach physical map (Zhebentyayeva et al. 2006), the physical map around the evergrowing gene (Bielenberg et al. 2004), and to find SSRs for specific genome regions (Georgi et al. 2002). Vilanova et al. (2003) developed the first BAC library in apricot (101,376 clones; average size 64 kb; 22x genome coverage), using the plum pox virus (sharka) resistant cultivar Goldrich, and employed it to find clones linked to the self-incompatibility locus. A library of myrobolan plum was constructed in the nematode resistant accession P.2175 by Claverie et al. (2004) for map-based cloning of the *Ma* gene for resistance to root-knot nematodes (*Meloidogyne* spp.). The library has 30,720 clones of average size 145 kb, with an estimated 14–15x coverage of the genome.

A *Prunus* physical map is under construction by a consortium of research groups led by Dr. Albert G. Abbott at Clemson University (South Carolina, USA), using BAC fingerprinting in two peach BAC libraries ('Nemared' and one more recently constructed in haploid 'Lovell'). The current physical map (April 2006) contains fingerprints of more than 18,387 BACs assembled into 1,367 contigs (including 11,193 BACs) and 7,194 singletons covering more than 60% of the *Prunus* genome (Zhebentyayeva et al. 2006). One of the most interesting features of this map is that it is anchored in the *Prunus* genetic map (Horn et al. 2005). The position of 290 genetically mapped markers was established on BACs used to construct the physical map, 150 of them using RFLP probes mapped in TxE covering the whole genome. Moreover, a transcript map was created by hybridizing more than 3,000

EST putative unigenes to the 'Nemared' BAC library. With this approach it has been possible to determine the genetic map position of ESTs that are either in the same BAC or in the same BAC contig as one of the markers of the genetic map. In total, 749 EST unigenes that connect the *Prunus* genetic and physical maps have been identified so far.

The first apple BAC library of 37,000 clones was developed in 'Florina', which carries the *Vf* resistance to scab. Genome coverage is 5 haploid genomes and average insert size is 120kb (Vinatzer et al. 1998). The original source of *Vf*, *M. floribunda* 821, was used to construct a library of 31,000 clones covering 5 haploid genomes with an average insert size of 125kb (Xu et al. 2001). 'Goldrush', also carrying *Vf*, was used to construct a 35,712 clone library of similar genome coverage and average insert size of 110kb with initial *Bam*HI digestion (Xu et al. 2002). For the purposes of developing a genome-wide physical map (Han et al. 2006a), a complementary 'Goldrush' BAC library has been developed using *Hind*III digestion. This library consists of 46,791 clones with an average genome size of 115kb and represents 8 haploid genome equivalents. Primers have been designed for 1,050 SSRs to develop markers for BAC fingerprinting. A 56,000 clone BAC library and a 168,000 clone cosmid library have been constructed in 'Pinkie', a selection containing *Vf* and the powdery mildew resistance gene *Pl2*. Each library should cover nearly seven haploid genome equivalents (E. Rikkerink, pers. comm.).

Limited physical maps around resistance genes that have been targets for map-based cloning have been published. These include contigs around *Vf* on LG1 (Patoocchi et al. 1999b; Vinatzer et al. 2001b; Xu and Korban 2002; Xu et al. 2001) and *Sd1* on LG7 (Cevik and King 2002b) and *SBE* (starch branching enzyme) (Han et al. 2006b).

5. TRANSCRIPTOMICS

A range of complementary tools is available to determine functionality of candidate genes, ranging from annotation in EST (expressed sequence tag) databases to *in vivo* expression. Generally, a broad first cut is made using bioinformatics annotation of ESTs and microarray analysis, and subsequently the number of candidates for mapping to QTLs is narrowed down using RT-PCR and / or *in vivo* expression.

5.1. EST Collections

Several collections of ESTs have been developed by different research groups and in various fruit crops of this family. Some of them are public and others partially or completely private. The private EST collections are being progressively released to the public. The descriptions of the current EST collections have been divided into three parts, one concerning the publicly available data for the whole family, followed by the descriptions of EST developments of individual research groups in *Prunus* and in apple. The major EST sequencing projects usually involve the

development of databases for EST handling and analysis. These databases will be described in Section 6 of this chapter.

5.1.1. ESTs in public databases

More than 370,000 Rosaceae ESTs are currently available in public databases, most of them apple (~260,000) and *Prunus* (~85,000), mainly peach and apricot. The number of unigenes deduced from ~260,000 of these EST sequences, held in the Genome Database for Rosaceae (GDR) at a clustering threshold of 90%, is ~72,000 for the Rosaceae, ~68,000 in *Malus* and 13,000 in *Prunus*. ESTs are a source of high-quality molecular markers such as SSRs that are particularly abundant in these sequences (Morgante et al. 2002; Jung et al. 2005). In *Prunus*, 14,292 (19.8%) were detected after analysis of 72,114 EST sequences. In *Malus*, 19% of the HortResearch EST collection (see below) unigene sequences contain di- or tri-nucleotide repeats. Dinucleotide repeats were most frequently identified in the 5' untranslated region of the genes and AG repeats made up 88.3% of this class. In contrast, trinucleotide repeats are most common in the predicted coding regions and exhibited a lesser degree of sequence bias in their representation. Fifty-seven percent of di-, tri- and tetra-nucleotide repeats were 12 to 14 bases long, and only 17% were longer than 20 bases. This abundance of SSRs makes apple and *Prunus* ESTs a rich source of molecular markers for construction of framework maps (117 have already been mapped in the *Prunus* reference map and at least as many in *Malus*) and worth screening for when mapping candidate genes, as the markers are generally simple to develop and use.

EST collections are also a source of another important class of markers, single nucleotide polymorphisms (SNPs). Their frequency is usually high, so it is generally possible to identify SNPs between the parents of mapping populations when one is seeking to map candidate genes (S. Gardiner, H. Bassett, unpublished). SNP markers, although sometimes difficult to develop, are extremely useful for QTL mapping and eventually for MAS. Results available on SNPs in various Rosaceae species has been extracted from the GDR and are summarized in Table 1.

5.1.2. *Prunus*

With the objective of detecting and cloning genes coding for characters of tree and fruit development, Horn et al. (2005) created an EST collection from a cDNA

Table 1. Number of single nucleotide polymorphisms (SNPs) found in expressed sequence tag (EST) contigs of different Rosaceae species (source: Genome Database for Rosaceae)

	N° of contigs	N° SNPs	N° SNPs/kb
<i>Malus</i>	22,435	10,426	0.5
<i>Pyrus</i>	35	1	0.0
<i>Prunus</i>	5,047	3,776	0.9
<i>Fragaria</i>	372	283	0.3
<i>Rosa</i>	705	316	0.7

library from developing fruit mesocarp of peach doubled haploid line P-21-5-2N. A total of 9,984 ESTs, 5'-sequenced and with an average size of 502 bp, were assembled in 3,842 putative unigenes (1,309 contigs and 2,533 singletons). Of these unigenes, 24.3% had no significant homology to the NCBI nr protein database. These unigenes were used for hybridisation with the 'Nemared' BAC library for the peach transcript map mentioned in Section 4.

The Chilean Functional Genomics Consortium (Meisel et al. 2006), involved in the study of the postharvest behaviour of peach fruits, developed 50,625 cDNA sequences from four libraries of peach 'O'Henry' in four postharvest stages (mature fruits and mature fruits plus 15 days at 4°C, and both treatments after a shelf life period). The ESTs had an average length of 673 bp and were sequenced from the 5' end. A total of 45,809 good quality ESTs revealed 10,830 unigenes (6,661 singletons and 4,169 contigs). Only 636 (5.8%) of these unigenes had no homology to *Arabidopsis* genes. Most ESTs of this collection (32,535) have recently been publicly released.

The IBMCP of València (Spain) and the University of California (Davis) are collaborating to analyse gene expression associated with chilling injury in peach fruit. In this project, CHILLPEACH, a collection of full-length enriched cDNAs from fruit mesocarp has been developed using two genotypes, one sensitive and one tolerant to chilling injury (Granell et al. 2006). Sequences of high quality (3,049) were clustered as 495 contigs and 1,595 singletons for a total of 2,090 putative unigenes. About half the unigenes were previously unreported in *Prunus* and 23.6% of the sequences were not present in *Arabidopsis*. A subtractive hybridisation library enriched with cDNAs regulated during cold storage has also been developed by these groups.

Three cDNA libraries of 'Bergeron' apricot pericarp at three different stages of development (89, 107 and 117 days after anthesis, corresponding to green, half-ripe and ripe fruit, respectively) were used by Grimplet et al. (2005) to obtain 13,006 good quality sequences in approximately equal amounts from each library, with an average length of 527 bp, the majority sequenced from the 3' end. These ESTs were assembled in 5,219 unigenes (3,426 singletons and 1,793 contigs). Twenty-four per cent of these unigenes did not show any similarity with the non-redundant protein public database. Fifteen transcripts were found to be strongly differentially expressed based on the redundancy of each unigene at each stage of fruit maturation. Twelve of them have been validated with quantitative PCR (Q-PCR) (Chevalier et al. 1999; Grimplet et al. 2005), but some of the genes classified as differentially expressed with lower degrees of stringency were not validated with Q-PCR.

5.1.3. *Malus*

The first large-scale EST sequencing of apple resulted in the collection and analysis of 151,687 cDNA sequences by HortResearch (Newcomb et al. 2006). The sequences are predominantly from 'Royal Gala' (almost 120,000), with lesser numbers from 'Pinkie', 'Pacific Rose', 'M.9', 'Aotea 1', 'Braeburn' and 'Northern Spy'. The 43 source libraries represent a range of tissue types, with a bias towards

fruit (53,620 ESTs). The fruit libraries include a series from developing and then ripening fruit with samples from flower, whole fruit, fruit cortex and seeds. This series was developed to provide a resource of genes for research on processes involved in fruit development (e.g. early cell proliferation, cell expansion and ripening). Other tissues sampled include buds, shoots, leaves, roots, phloem and xylem (76,472 ESTs). Other ESTs were expressed in tissues exposed to biotic and abiotic stresses, for example leaves infected with *V. inaequalis*, or exposed to temperature stress, fruit stored at low temperature and altered atmospheric conditions after harvest, and fruit cell lines exposed to boron (21,595 ESTs). cDNA sequencing was predominantly from the 5' end, and average edited length of the sequences was 468 bases. Sequence clustering yielded 43,938 putative unigene sequences at a clustering threshold of 95% (a more stringent threshold than used by GDR above), broken down into 17,460 contigs and 25,478 singletons. The number of unigene sequences is expected to be an overestimate of the number of protein-coding transcripts represented in apple and the authors recommended further sequencing to reduce the number of unigene sequences. Comparison of *Arabidopsis* unigenes from all ESTs with protein coding genes suggests that the apple unigene set in this study represents approximately half the genes in apple.

Functional annotation is an important first step in identification of candidate genes for specific traits of economic importance. Predictive bioinformatics showed that only 5% of the apple unigene sequences failed to match a sequence in the genome of *Arabidopsis* (Newcomb et al. 2006). Candidates for pest and disease resistances constitute 0.25–3% of the ESTs and 0.55% of contigs in the dataset (Crowhurst et al. 2005; Rikkerink et al. 2003). Structural gene families that are potentially involved in the biosynthesis and maintenance of flavour and health promoting compounds in apple fruit are well represented. Over 1,000 transcription factors have been identified among the ESTs as a resource for candidates for regulation of biosynthetic pathways (Crowhurst et al. 2005; Newcomb et al. 2006).

The other major EST sequencing programme has delivered 109,824 sequences to the Rosaceae research community, with a further 50,000 scheduled (Gasic et al. 2006). The 34 source libraries were deliberately constructed from a diverse range of germplasm, to facilitate *in silico* SNP mining. Sources include: 'Goldrush', 'Jonagold', 'Granny Smith', 'Fuji', 'Braeburn', 'SunCrisp', 'M.9', 'M.111', 'Geneva 3041', and 'Geneva 3041' x *M. sieversii*. Both normalized and primary libraries have been constructed. Different developmental stages of 'Goldrush' are represented by libraries from leaf, bud, flower and shoot. Sequencing was from the 5' end, and sequence clustering yielded 13,538 contigs and 15,896 singletons. Bioinformatic analysis enabled assignment of putative functional category for 82.5% of sequences. When classified according to gene ontology, 38.3% of sequences were assigned to biological processes, 42.7% to cellular components and 33.3% to molecular function.

SSH (Suppression Subtractive Hybridization) is used to generate libraries enriched with transcripts that are differentially expressed and increases the likelihood of identifying weakly expressed genes (Diatchenko et al. 1999). It has

been used to identify 480 ESTs differentially expressed between uninfected leaves of the scab resistant cultivar 'Remo' and 'Elstar', which is susceptible to scab infection (Degenhardt et al. 2005). Levels of transcripts coding for proteins already known to be related to plant defence, such as B-1,3-glucanase, ribonuclease-like PR10, cysteine proteinase inhibitor, endochitinase, ferrocetalase and ADP-ribosylation factor were higher in 'Remo' than in 'Elstar'.

A recent study utilized EST frequency analysis of publicly available ESTs (150,000 sequences at the time), with the goal of identifying genes most likely to be highly expressed in apple fruit, expressed uniquely or preferentially in fruit, and/or temporarily or spatially regulated during fruit growth and development (Park et al. 2006). A subset of genes that may be related to the generation of flavour and aroma components in mature apple fruit was identified. It was concluded that although this technique offers an entry point into fruit molecular biology, there are a number of limitations and caveats. One is the sensitivity of identification of the most abundant ESTs to artefacts, including differential amplification of cDNAs during library preparation, and another is the contamination of libraries with abundant organellar DNAs, highly repetitive DNA in the nuclear genome and microbial nucleic acids.

cDNA-AFLP provides an expression profile of bands that can be sequenced for identification of candidate genes. It has been used in apple, but it tends to be cumbersome in application and is expected to be superseded by microarray technology. A study by Jensen et al. (2003) compared expression of genes in 'Gala' grafted onto apple rootstocks 'M.7' and 'M.9' and challenged with fireblight (*Erwinia amylovora*). Another study was performed to identify genes with expression changes during fruit maturation and ripening (Lin 2005). One hundred and fifty-five of the 204 differentially expressed fragments were confirmed by microarray analysis after sequencing, and 127 assigned to functional classes. A study with similar goals is yielding candidate genes (Dreesen et al. 2006) that will be mapped on a QTL map for fruit maturation and ripening (Kenis and Keulemans 2005).

Another form of transcript profiling, mRNA differential display, coupled with RT-PCR to eliminate false positives, has been employed to detect 14 cDNAs representing transcripts up-regulated during ripening of 'Modial Gala' fruit ripening (Goulão and Oliveira 2006).

5.2. Microarrays

Microarray analysis is becoming a significant tool for characterizing transcription profiles of collections of thousands of genes simultaneously, providing an initial selection of several hundred potential candidate genes that could be associated with a specific physiological process.

A peach microarray containing about 4,800 oligonucleotide probes obtained from EST sequences corresponding to different unigenes selected for their expression in the last stages of fruit development (μ PEACH1.0) was developed by Trainotti

et al. (2006) and used to study gene expression in the transition from the pre-climacteric to the post-climacteric phase in peach fruits. Transcriptome analysis of this microarray detected 267 up-regulated and 109 down-regulated genes. These include genes for ethylene biosynthesis and perception directly involved in the maturation process, genes responsible for quality factors such as flesh firmness and carotenoid content, and transcription factors involved in different aspects of fruit development and maturation. Microarray development and analysis is also being carried out by the groups dealing with peach fruit postharvest behaviour (Orellana et al. 2006; Granell et al. 2006) and by those targeting apricot fruit development (Grimplet et al. 2005).

The power of microarray technology has been demonstrated in apple by two detailed studies of fruit development (Janssen et al. 2006) and of ripening (Schaffer et al. 2006) that utilize a 15,760 50mer array developed by HortResearch (New Zealand). In the first study, nearly 2,000 of 13,000 unigenes demonstrated a significant change during development over 150 days from pollination to full tree ripeness (Janssen et al. 2006). Cluster analysis enabled grouping of genes associated with specific stages, such as cell division during early development, starch accumulation during mid development and starch decline as fruit begin to ripen. Quantitative PCR has been used to examine expression patterns for over 50 genes, confirming the expression patterns found by microarray for 70% of these, and identifying specific genes for further study. Key stages in ripening of fruit have been studied using transgenic apples transformed with an antisense copy of ACC oxidase. Fruit from these trees do not ripen unless they are treated with exogenous ethylene, allowing ethylene-induced ripening to be studied. Genes identified in this study are involved in the increase in volatiles related to flavour perception, increase in sweetness, softening of the flesh, and reduction of acidity (Schaffer et al. 2006). Candidate genes responding slowly to ethylene stimulus included those involved in flavour biosynthesis and ripening. By combining data from both of the above studies, it has been possible to identify the 500 genes that respond to ethylene, and distinguish between genes involved specifically in ethylene-induced ripening and genes induced by ethylene that are not specifically related to ripening. In addition it is possible to identify ripening associated genes that are not ethylene-induced (Schaffer et al. 2006). This type of approach that combines data from multiple experiments in the Rosaceae is likely to yield further significant results, particularly in the area of fruit development. The HortResearch microarray is available to the research community on a commercial and collaborative basis.

A 60-mer oligo array representing 55,000 apple contigs from the NCBI database and developed in collaboration with NimbleGen (<http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GPL3715>) is being used in a study of gene expression patterns of 'Gala' scions grafted on eight different apple rootstocks to identify a set of rootstock regulated genes that may be associated with resistance to disease and drought (Jensen et al. 2006). This microarray is commercially available. Another oligo-based array is being planned at INRA, Angers, France (Wattebled et al. 2006). Variations in gene expression related to apple texture will be confirmed

by quantitative PCR, enzymatic assays, or by transgenic validation in tomato. Candidate genes will then be mapped to QTLs in populations segregating for these traits and the data obtained will be applied in apple breeding programmes.

A specialized 5,000 clone cDNA microarray (MSU MDAZ5k) has been developed for the purpose of characterizing gene expression in natural and chemical-mediated apple fruit abscission, and developing improved strategies for regulating flower and fruit abscission, and hence crop load (van Nocker et al. 2006).

The European project HiDRAS (High-Quality Disease Resistant Apples for a Sustainable Agriculture) (Gianfranceschi 2006) has developed a small (1,600 clone), specialized cDNA microarray that targets genes controlling fruit development and maturation. Subtractive DNA libraries were used to enrich for fruit specific genes. Genes showing modulated development during fruit development (about 10% of those on the slides) have been sequenced and annotated prior to marker development and mapping to QTLs controlling fruit quality traits on the 'Fiesta' x 'Discovery' apple reference map (Soglio et al. 2006). A Japanese group has developed a similarly sized cDNA microarray (1,431 clones) from shoot and fruit libraries, and used it to examine gene expression changes following treatment with an *Alternaria alternata* host specific toxin (Hatsuyama et al. 2003).

In apple, there has been a focus on development of *in vivo* expression systems for verification of candidate genes identified from annotation and microarray experiments. The precise strategy will depend on the predicted function of the gene product, and can involve expression in yeast or *E. coli* followed by headspace analysis (for volatile products), transient expression in tobacco following *Agrobacterium* infiltration (for transcription factors that modify the expression levels of enzymes), stable expression in *Arabidopsis* or apple cell lines / whole plants (for other genes affecting complex phenotype) (Souleyre et al. 2005; Lee and Lee 2005; Hellens et al. 2005; Chagné et al. 2006a; Chagné et al. 2006b). The elucidation of the function of a gene product using a combination of these systems is clearly illustrated for the study of *M. pumila* alcohol acyl transferase (MpAAT1) (Souleyre et al. 2005), which used headspace analysis of a range of substrates by an *E. coli* expression system and a transient expression system in tobacco. It was demonstrated that this gene is responsible for the final step in the synthesis of a range of the esters that characterize the flavour of 'Royal Gala' fruit. In another case, the identification of a gene controlling red flesh in apple was assisted by expression of apple candidate genes in tobacco and apple (Chagné et al. 2006a; Chagné et al. 2006b; Espley et al. 2006).

Gene knock-down RNA interference (RNAi) technology (Wesley et al. 2001) utilizes the expression of gene transcripts in the whole plant in a sense or antisense fashion, and observation of the effect on phenotype to infer gene function. However, this requires an efficient transformation system and regulatory conditions that permit growth of transgenic plants, preferably under field conditions. A good transformation system exists for apple, but for *Prunus* the efficiency is low (Petri and Burgos 2005). For *Prunus* transformation is more easily performed in juvenile materials and is extremely dependent on species and genotypes, meaning that these

experiments often have to be done in other organisms. RNAi technology has been used in a number of studies on the role of ethylene in controlling development of apple fruit quality attributes by suppressing ethylene production in transgenic plants to study its effects on e.g. sugar, acid and ester level in fruit (Defilippi et al. 2004), levels of sorbitol, sucrose and starch in leaves (Cheng et al. 2005), flavour, aroma and texture in fruit (Dandekar et al. 2004; Defilippi et al. 2005), and is being used in a HortResearch study involving microarray analysis of genes involved in fruit ripening (Schaffer et al. 2006).

6. DATABASES AND ASSOCIATED BIOINFORMATICS SYSTEMS

There are a number of databases that have been developed by members of the Rosaceae research community, with varying degrees of public access. Only one of them, the GDR, is public, covers diverse topics (mainly structural and comparative genomics and transcriptomics) and includes all species of the family. The remaining databases are usually oriented towards one species, correspond to projects of research organizations, consortiums or individual research groups, and are mainly focused on the area of transcriptomics. This diversity of databases has led to a duplication of effort as well as to lack of availability of particular features developed in-house in individual systems, to the community as a whole.

The GDR is a curated and integrated web-based relational database that includes genomics and genetics data for members of the genus Rosaceae (Jung et al. 2004). The GDR contains comprehensive data of the genetically anchored peach physical map, Rosaceae maps and markers, and an annotated EST database of peach, almond, strawberry, and all publicly available Rosaceae sequences. The integrated map viewer provides a graphical interface to the genetic, transcriptome and physical mapping information. ESTs, BACs and markers can be queried by various categories and the search result sites are linked to the integrated map viewer or to the WebFPC physical map sites. CMap, the comparative map viewer, allows users to compare various Rosaceae genetic maps and the transcriptome map. In addition to browsing and querying the database, users can compare their sequences with the annotated GDR sequences via a dedicated sequence similarity server running either the BLAST or FASTA algorithm. GDR can be accessed at <http://www.rosaceae.org> and provides free EST processing services to the Rosaceae community.

ESTree is a public database (<http://www.itb.cnr.it/estree/>), developed by Lazzari et al. (2005), with a collection of 18,630 ESTs (6,155 from four *P. persica* mesocarp cDNA libraries obtained by this group and the rest from public databases) and is intended as a resource for peach functional genomics. These sequences can be processed through an automated pipeline including public software and in-house PERL scripts that allow these ESTs to be processed and provide information on contig assembly, SNP detection, gene annotation with Blastx and assignment to Gene Ontology classifications. Contig assembly has allowed 9,219 putative unigenes from these sequences to be detected. A total of 166 of these unigenes containing

putative SNPs have been identified and are currently being validated and bin mapped.

Two databases, CHILLPEACH (Granell et al. 2006) developed by the US-Spanish project and JUICE (Meisel et al. 2006) developed by the Chilean consortium mentioned in Section 4, have been created for the processing and assembly of the cDNAs obtained in these projects and are for the moment only for internal use. CHILLPEACH also includes devices for functional annotation, such as GO terms, BLAST results, EC numbers, protein family (PFAM) domains and sequence analysis to identify SNPs or SSRs.

The HiDRAS AppleBreed database provides access for members of the HiDRAS programme to genotypic and phenotypic data derived from plant populations with different pedigrees (progenies, cultivars used as progenitors, breeding selections) (Antofie et al. 2006). Its goal is to link molecular information to phenotypic data, and to ensure linkage between information on cultivars and on crosses (families). The core of the database model is based on individual trees and individual DNA samples. This structure allows multiple annual observations to be stored individually by genotype, even when the nature of these observations is very different (e.g. molecular data, physical or chemical measurements of fruit quality traits, evaluation of disease susceptibility, etc.) Phenotypic data, molecular marker data and links to the individual trees and DNA samples from which these data were derived, pedigrees, marker descriptions, primer sequences, molecular marker linkage maps and synonyms of the cultivar names are included, as well as validation procedures for phenotypic and marker data. Basic statistical overviews on the data are provided. The information on apple microsatellites is available online at <http://www.hidras.unimi.it>, including facilities to encourage interaction between research groups in this area (Silfverberg-Dilworth et al. 2006).

Tree Fruit Technology (TFT), a database developed at Michigan State University, which contains sequences from public databases for Rosaceous fruit crops grown in temperate regions of the USA, provides another resource for analysis of DNA sequence information. TFT is available online at <http://genomics.msu.edu/fruitdb>.

The HortResearch enterprise databases contain at least 270,000 EST, PCR, and full cDNA sequences from *Malus*. The database includes all HortResearch sequences merged with *Malus* sequences extracted from GenBank (Crowhurst et al. 2006). HortResearch has developed an automated analysis and visualization programme for the annotation of non-genomic DNA (BioView™ Sequence Analysis and Annotation System). The system is primarily written in PERL and employs MySQL as a relational database management system for persisting data and job scheduling. The automated pipeline assembles sequences into tentative consensus sequences, and implements modules for performing BLAST comparisons, identifying SSRs and motifs, predicting SNPs and microRNA targets, designing microarray oligonucleotides, compiling keyword dictionaries, and defining gene families. Visualizing relationships captured within the database is achieved via a web viewer. Administering the system, tracking and sharing mining efforts, initiating follow-on sequencing and vector construction, performing virtual subtraction,

and augmenting automated annotations by capturing end user knowledge is also undertaken via the web viewer (Crowhurst et al. 2006). The BioView system will incorporate a database for markers and support GMOD/CMap modules (<http://www.gmod.org/?q=node/77>) for comparative map display in its next release. This in-house system is acknowledged to be comprehensive and user-friendly.

7. CURRENT APPLICATIONS

Genomics provides a ‘tool box’ of technologies that have started to be used for development of new, improved varieties of *Prunus* and *Malus*. Genetic markers are used directly by breeders for germplasm assessment, breeders’ rights protection and marker-assisted selection from breeding populations. Other ‘tools’, e.g. bioinformatics, microarrays, quantitative PCR and *in vivo* expression, are providing insight into the function of gene products that determine characters of interest to breeders.

7.1. *Prunus*

7.1.1. *Marker assisted selection (MAS)*

Most of the major genes that have been placed on the *Prunus* consensus map (Dirlewanger et al. 2004a) are important in breeding programmes. They affect fruit quality (peach v. nectarine; flat v. round fruit; clingstone v. freestone; subacid v. acid flavour; yellow v. white or melting v. non-melting flesh), kernel (bitter v. sweet) or shell (hard v. soft) traits in almond; tree characteristics (columnar v. normal), disease resistance (root-knot nematode, plum pox virus, powdery mildew) or other characters (self-incompatibility, blooming time). Given the marker density of the current *Prunus* map, markers (usually SSRs) are available sufficiently close to most of these genes for use in marker assisted selection.

However, markers tightly linked or derived from the sequence of three genes are the only ones routinely used for selection in breeding programmes. The two independent genes (*Mi* and *Ma*) that confer root-knot nematode resistance are currently being applied for pyramiding these genes into a new wave of rootstocks (Dirlewanger et al. 2005). Markers obtained from the sequence of the stylar RNase involved in the self-incompatible reaction are being used to assign genotypes to self-incompatibility classes or to select for self-compatible individuals in almond, apricot and cherry progeny (López et al. 2005; Vilanova et al. 2005; Sonneveld et al. 2006). Other genes with potential for MAS are those of the major factors involved in plum pox virus resistance on G1 (Vilanova et al. 2003), and two more affecting fruit quality: the acid/subacid (*D*) gene and the melting/non-melting (*M*) gene. In addition, microsatellite markers are extensively used for cultivar fingerprinting, mainly for the enforcement of breeders’ rights and for applications such as parentage tests, or the management of germplasm collections (Arús et al. 2005c).

7.1.2. *Research on characters of value to breeders*

One of the targets of plant breeding is the incorporation of disease resistance genes in elite varieties. The ever-increasing amount of information on the sequence and function of genes involved in plant resistance to pests and diseases in many organisms is being used to complement the data on the known positions of major genes and QTLs to plum pox virus (Vilanova et al. 2003; Decroocq et al. 2005), powdery mildew (Foulongne et al. 2003; Verde et al. 2005), root-knot nematodes (Dirlewanger et al. 2004b), leaf curl (Viruel et al. 1998) and green leaf aphid (Foulongne 2002) in the search for candidate genes or the locations of disease resistance gene clusters. The strategy of resistance gene analogs (RGA) to map resistance gene clusters developed by Leister et al. (1996) in potato has been applied by Bliss et al. (2002) in peach and by Soriano et al. (2005) in apricot to map various RGAs in the genomes of these species. More extensive work has been done by Lalli et al. (2005) using the physical and genetic *Prunus* maps, the EST sequences kept at the GDR, and sequence data from RGAs obtained from degenerate primers in various *Prunus* species. Fifty-eight DNA fragments representing putative RGAs and/or genes involved in host defence or resistance were hybridised to the 'Nemared' BAC library used for physical map construction. Thirty of them identified 42 positions in the TxE map distributed in all linkage groups but one (G3), without the use of segregating populations. The positions detected by the RGAs often coincided with the locations of resistance genes or QTLs mapped previously, providing a new source of markers for MAS of seedlings with favourable resistance genotypes.

One of the key characters for the selection of new stone fruit cultivars is fruit quality, evolving from traits related to the external appearance (size, shape and skin colour) to aspects related to their organoleptic qualities, such as flavour, flesh consistency and aroma. Some of these characters were analysed in peach (Dirlewanger et al. 1999; Etienne et al. 2002) and peach x *P. davidiana* (Quilot et al. 2004; Quilot et al. 2005) progenies, and major genes or QTLs for fruit size, fruit shape, contents of different organic acids (citric, malic, quinic and shikimic) and sugars (sucrose, fructose, glucose and sorbitol) were identified. One of the candidate genes studied by Etienne et al. (2002) corresponded to a proton pump-encoding gene and co-located with the position of a QTL for sucrose and soluble solid content. Interestingly, some of these major genes or QTLs are located within the same genomic regions across different progenies and species, suggesting that gene position and gene action are conserved across species. For example, QTLs for fruit weight and dry matter weight mapped in both of these populations to the end of G5, close to the gene *G* that determines the skin hairiness of the fruit (peach v. nectarine).

The postharvest behaviour of fruit has become a major aspect of fruit breeding programmes. The stone fruit sold out of season in the main consumption centres of the Northern hemisphere is produced in the Southern hemisphere (mainly New Zealand, Chile and South Africa), implying a requirement for long periods (more than 30 days) between harvest and consumption. Research is being directed towards finding alleles of genes involved in fruit maturation, which may allow, alone or

in combination, the development of new varieties that maintain their high quality flavour after reaching the consumer. One of these is the previously mentioned *M* gene that determines melting (soft) v. non-melting (hard) flesh consistency (Peace et al. 2005). Another interesting gene (*Hd*) confers the stonyhard characteristic, where the fruit keeps its texture for a long time but, unlike fruit with the *M* gene, does not produce ethylene, and a postharvest ethylene treatment is required to soften the fruit (Haji et al. 2005). Tatsuki et al. (2006) showed that the stonyhard genotype occurs as a consequence of the suppressed expression during the ripening stage of a gene encoding one of the enzymes of ethylene biosynthesis, an ACC synthase (*Pp-ACS1*) gene that is normally expressed in tissues subjected to wounding. These results suggest that the *Hd* gene is part of the regulatory mechanism of *Pp-ACS1*. Mapping the position of the *Hd* gene with respect to that of candidate genes may help to clarify its nature. The fruit softness of peaches is currently being analysed with the candidate gene approach by Peace et al. (2006a) and a transcriptomics-based search of genes involved in fruit post-harvest life is being used by Granell et al. (2006) and Orellana et al. (2006).

Blooming and fruit maturity times are also important characters in *Prunus* breeding programmes (Scorza and Sherman 1996). Early blooming may expose flowers or recently formed fruits to late frosts, and late blooming cultivars are sought in almond and stone fruits to increase production and crop stability. The chilling requirement to overcome dormancy is associated with blooming time and is an important factor for genotype adaptation of most species, where the number of chill hours has to be sufficient to allow for regular flowering and fruit development in the region where the cultivar is grown. The production in regions with mild climates can be extended for early or late maturing cultivars, usually leading to higher market prices. Both bloom and maturity times have been described as polygenic and highly heritable characters (de Souza et al. 1998). QTLs for blooming time have been identified in three regions of the genetic map (Joobeur 1998; Dirlewanger et al. 1999; Quilot et al. 2004; Verde et al. 2005): in the central region of G4 that includes also a major gene (*Lb*) for blooming time of almond (Ballester et al. 2001), in the middle of G7, and in the southern region of G1, close to the evergrowing (*Evg*) gene that determines a continuous terminal meristematic growth in peach in winter dormancy-inducing conditions (Wang et al. 2002). Twelve candidate genes with sequences highly homologous to those involved in vernalization and flowering of *Arabidopsis* were mapped in TxE by Silva et al. (2005), and one of them, a MADS box transcription factor, was located near the *Evg* locus.

7.1.3. Map-based cloning

The root-knot nematode resistance gene *Ma* from myrobolan plum has been fine mapped and a BAC including two flanking markers identified (Claverie et al. 2004). This BAC has been fully sequenced and candidate genes for resistance identified by Claverie (2004). The *Evg* gene on G1 has been fine-mapped and a contig of three BACs spanning a region of 132 kb containing the gene was obtained by Bielenberg et al. (2004). Seven candidate genes have been identified with the wild

type sequence of this genomic region (Bielenberg et al. 2006), six of which are highly similar copies of a MICK structural class of MADS-box genes, and the seventh is a calcium-binding protein. The mutant genotype has a deletion in this region of 40-45 kb, which affects up to four of the MADS-box candidate genes. The gene *D* that determines subacid v. acid taste is in the process of being map-based cloned (E. Dirlwanger, pers. comm.). Other important genes have also been genetically characterized in depth. The two major components (stylar and pollen) of the self-incompatibility locus have been sequenced (Ushijima et al. 2003) and their variation has been associated with the known self-incompatibility alleles in different *Prunus* species. Strong evidence exists that an endopolygalacturonase gene corresponds to the gene *M* that determines the melting v. non-melting flesh consistency of the peach fruit (Lester et al. 1996; Peace et al. 2005).

7.2. *Malus*

7.2.1. *Marker assisted selection (MAS)*

Genetic markers have been utilized to varying degrees for at least 8 years to increase the efficiency of apple breeding programmes (Bus et al. 2000; Bus et al. 2002; Cheng et al. 1998; Kellerhals et al. 2000). Applications include screening of germplasm with markers for *Vm* (Cheng et al. 1998; Mattison and Nybom 2005) as an alternative to cumbersome allelism tests (MacHardy 1996); selection of parents with desirable alleles for a range of characters, including desired combinations of different resistance genes against the same pathogen (Bus et al. 2002); early selection from large breeding populations for adult traits at the seedling stage, including traits that are expensive to phenotype and for gene pyramids for resistances that cannot be selected by phenotype (Bus et al. 2002; Costa et al. 2005; Itai et al. 2003). MAS is being used internationally for at least nine major gene resistances against apple scab, five major resistances against powdery mildew, two against woolly apple aphid, and one against rosy apple aphid (Gardiner et al. 2006a). Selecting for desirable QTL traits with markers is less advanced although it has been initiated at INRA, France for scab resistance (C.E. Durel, pers. comm.). The value of MAS in a breeding programme is greater than the cost of replacing phenotypic screens, and includes the potential value of durable resistances in horticultural production systems (Luby and Shaw 2001). The introduction of automated systems for DNA extraction, setting up reactions and gel loading, as well as the use of marker multiplexes (Cook and Gardiner 2004; Frey et al. 2004) is reducing the costs of labour and consumables for MAS to breeding programmes.

High density saturated genetic framework maps are essential for the determination of marker-trait associations in apple using the Genome Scanning Approach (Patoocchi et al. 2004; Patoocchi et al. 2005; Silfverberg-Dilworth et al. 2006). Because of the highly polymorphic and co-dominant nature of microsatellite markers, framework maps are also a practical source of alternate markers for genes in introgression programmes incorporating new 'quality parents' in successive

generations (Gardiner et al. 2006a). Genetic framework maps that utilize multi-locus markers, such as microsatellites, RFLPs or SNPs developed from paralogous genes, are also invaluable for identifying regions that are homeologous between linkage groups (Liebhard et al. 2002; Maliepaard et al. 1998; Gardiner et al. 2006b). Genetic markers can be used to elucidate the segregation of traits subject to segregation distortions, such as the *Er3* resistance to woolly apple aphid (Gardiner et al. 2006b), as well as the number of genes involved in determining a trait such as the *Pl-1* powdery mildew resistance (Dunemann et al. 2005).

7.2.2. *Research on characters of value to breeders*

Analysis of the function of a gene product using application of a combination of genomic technologies is shown by the case of *MpAAT* (Souleyre et al. 2005). Gene mining in the apple EST database had identified 20 acyl transferase-encoding genes that were candidates for determining the ester profile behind the fruit flavour of 'Royal Gala' apples. One of these was chosen for study, because it was derived from fruit libraries, is closely related in sequence to acyl transferases that use alcohol as an acceptor, microarray analysis showed it is up-regulated in developing 'Royal Gala' fruit and ethylene treated fruit and it is expressed in apple flowers and fruit. The protein expressed by recombinant *E. coli* included the predicted fusion protein containing the expected MpAAT1 polypeptide, confirmed by LC-MS (Liquid Chromatography Mass Spectrometry). Headspace analysis by GC-MS (Gas Chromatography Mass Spectrometry) of *E. coli* cultures expressing *MpAAT1* that had been provided with a range of alcohol substrates and CoA donors defined the range of activities for MpAAT1. A transient expression system utilizing *Nicotiana benthamiana* (tobacco) produced a smaller range of esters compared with those from *E. coli*, not all found in apple fruit. It was concluded that the substrate pool in apple fruit is likely to determine the range of esters produced. The final step will be to map *MpAAT1* on a genetic map to determine whether this candidate gene co-segregates with a QTL for apple 'fruit flavour'.

The ultimate goal of the candidate gene approach is to identify markers with a gene directly controlling the trait of interest. An example of how the application of genomic technologies makes this possible is the identification and mapping of a gene controlling red colour in apple flesh and foliage (Chagné et al. 2006a; Chagné et al. 2006b; Espley et al. 2006). Candidate genes were identified in the apple EST databases on the basis of homology to genes controlling red colour in model plants, either genes involved in anthocyanin biosynthetic pathways or transcription factors (TFs) regulating those pathways. The candidates that represented biosynthetic genes were excluded on the basis of expression data from Q-PCR and microarray experiments that demonstrated that biosynthetic genes are all switched on or off in red and white fruits, respectively. A further candidate TF sequence was obtained, following PCR amplification with degenerate primers from the parents of a mapping population segregating for red flesh. The gene candidates were then reduced to the 13 that were differentially expressed between extreme phenotypes in Q-PCR and microarray analyses. One of these, a MYB transcription factor (MdMYB10)

expressed red pigment patches following transient transformation in tobacco, particularly when co-transformed with another apple transcription factor (Espley et al. 2006). Molecular markers developed from SNPs and microsatellites identified in the sequence alignments were screened over mapping populations to identify co-segregation with phenotype (Chagné et al. 2006a; Chagné et al. 2006b). One of the candidates, MdMYB10, exhibited an allele closely associated with red core colour and was mapped on a 'M.9' x R5 reference map (Celton et al. 2006a; Celton et al. 2006b). The linkage between MdMYB10 and the red core trait was confirmed in an association study by screening several markers developed from different SNPs in the gene sequence over a set of unrelated red and white fleshed individuals from international germplasm collections (S. Gardiner, D. Chagné, unpublished). Overexpression of MdMYB10 resulted in generation of highly pigmented apple plants that contained enhanced levels of anthocyanin. This suggests that differences in the activity of MdMYB10 determine the red core colour trait, but confirmation awaits fruiting of the transformants.

Eighteen resistance gene analogues (RGAs) with homology to NBS/LRR genes have been mapped as cleaved amplified polymorphism sequences (CAPS) or single-strand conformation polymorphism (SSCP) markers (Baldi et al. 2004) and 23 more were mapped by NBS profiling (Calenge et al. 2005b). Work in progress using candidates derived from EST sequences has resulted in mapping of over 40 more candidates, either as microsatellites or SNPs (Gardiner et al. 2003; S. Gardiner, unpublished). Clustering of RGAs and association with major gene and QTL loci conferring pathogen resistance has been noted in all three studies.

7.2.3. Map-based gene cloning

Map-based cloning in apple focused initially on major genes for resistance, with a more recent project targeting a gene involved in starch metabolism. Most progress has been made in identifying the *Vf* gene. The first BAC contig containing the *Vf* gene covered a 550 kb minimal tiling path and was developed by Patocchi et al. (1999b) on the basis of fine mapping of markers around the *Vf* gene and on new markers generated from BAC clones. Identification of candidate genes for *Vf*, named *HcrVf1* to *HcrVf4* (Vinatzer et al. 2001a) involved the use of BAC inserts to probe a large cDNA library and built on further work by Vinatzer et al. (1998) and Patocchi et al. (1999a). The candidate genes showed homology to the *Cf* resistance genes cloned from tomato. When one of the candidate genes (*HcrVf2*) was expressed in 'Gala' under a 35S promoter, it conferred resistance to apple scab (Belfanti et al. 2004), which was overcome by a scab race that specifically overcomes *Vf* resistance (Silfverberg-Dilworth et al. 2005). Screening of BAC subclones with labelled total cDNA (Xu and Korban 2002b) yielded an almost identical set of candidate genes (*Vfa1* to *Vfa4*). As transformation of cultivars 'Galaxy' and 'McIntosh' has demonstrated that *Vfa1* and *Vfa2* each independently confer resistance to apple scab (Malnoy et al. 2006b), it is not clear which candidates (or combination of candidates) are responsible for the *Vf* resistance. Work is at an advanced stage on map-based cloning of both *Sd-1* which confers resistance

to the aphid *Dysaphis devectora* (Cevik and King 2002), and the powdery mildew (*Podosphaera leucotricha*) resistance *PI2* (E. Rikkerink, pers. comm.). There is a brief report on the map-based cloning and characterization of a gene encoding starch branching enzyme (*SBE1*) from apple (Han et al. 2006b).

7.2.4. Genetic transformation

Transformation has been employed in apple to enhance resistance to pathogens, particularly where endogenous monogenic resistances have not been available in the germplasm (Malnoy and Aldwinckle 2006). Introduction of heterologous antibacterial or antifungal genes enhanced resistance to fireblight in apple (Ko et al. 2002; Liu et al. 2001; Norelli et al. 1994; Schneider et al. 2006) and pear (Lebedev et al. 2002; Malnoy et al. 2003), as well as to apple scab (Bolar et al. 2000; Bolar et al. 2001; Chevreau et al. 2004). Resistance to light brown apple moth was enhanced by introduction of heterologous genes coding for biotin binding proteins (Markwick et al. 2003). More recently, transformation with the endogenous NPR1 (Non-expressor of Pathogenesis Related 1) gene was used to increase fireblight resistance under some conditions (Malnoy et al. 2004; Malnoy et al. 2006a). Silencing of DspE (Disease Specific E) interacting proteins (DIPMs) (Borejsza-Wysocka et al. 2004; Borejsza-Wysocka et al. 2006) increased resistance to fireblight in some transgenic lines of 'Galaxy'. The development of promoters that are activated specifically by exposure to pathogens at the site of infection will reduce accumulation of transgenic proteins in the plant and has been demonstrated in apple for the *Pgst1* promoter from potato (Malnoy et al. 2006c).

8. FUTURE PROSPECTS

8.1. Resource Integration

Rapid progress in Rosaceae tree fruit research depends on the integration of research resources internationally within and across genera, as adequate funding is not available in any one country for any of these second or third tier crops to make significant progress alone in genomics. At the International Rosaceae Genomics Conferences, the third of which was held in Napier, New Zealand in 2006, such interaction was initiated on a broad scale (<http://www.mainlab.clemson.edu/gdr/>), and enabled the setting up of the International Rosaceae Genomics Initiative (RosIGI). A national programme has been initiated in the USA (Norelli et al. 2006; Peace et al. 2006b).

Links between databases and integration of bioinformatics systems are critical, and there should be a common portal for accessing databases. The most comprehensive of these databases is currently the GDR, which has already proven helpful to many research groups and could become a central resource for the whole Rosaceae scientific community. There needs to be a way that other databases that have developed special features can be used to support the wider Rosaceae community, while protecting any special features relating to intellectual property

for the developer. Substantial new resources should be developed in collaboration, e.g. microarray analysis systems, genome comparison analyses and whole genome sequences.

Sharing of significant mapping populations at the plant and/or DNA level according to individual countries' regulations has been initiated on a regional scale in Europe, and if extended across the world, would aid in correlation of mapping datasets and reduce duplication of effort. An international consortium of research groups (the RosPOP consortium), supported by the RosIGI, is currently being launched and will have its base in the GDR. The aim is for all members to have access and share populations, DNA and data. This is likely to facilitate interactions between different research groups in diverse activities related to major gene, QTL and candidate gene analysis as well as expression profiling, enabling studies to be conducted with the same genes or markers over several populations in common, but grown in different environments. Sharing of DNA samples from the parents of the mapping populations will enable polymorphism assessment of markers developed by different groups within RosPOP. Additional phenotyped populations will be linked into this network by the mapping of common markers.

The very large (>260,000) set of apple EST sequences available in public databases provides the resource needed for construction of the first internationally available microarray containing all available apple features. Areas under discussion internationally include the type of platform, length of the oligos and whether chips should be designed for individual genera, or a 'family chip' could cover all the Rosaceae.

The comparison of the apple and *Prunus* genomes is only beginning. It requires a more detailed analysis to establish with precision the major rearrangements that have occurred between these genomes. This has the additional interest of comparing two close genomes, one of which (apple) has recently undergone a process of polyploidization, and may help to understand how this event took place and some of its genetic consequences. Research on the comparison between the genome of other Rosaceous crops such as *Prunus* and *Fragaria* or *Fragaria* and *Rosa* is underway (Rousseau et al. 2006; P. Arús, unpublished). One of the challenges ahead is to work with the Rosaceae genome as a whole, particularly in the development of certain resources (microarrays, whole genome sequence etc.) and for this, knowledge of both the gross and finer differences between genomes is crucial.

8.2. Mapping and Sequencing

There are already almost complete sets of *Prunus* and *Malus* microsatellite markers spaced across the genome (Howad et al. 2005; Silfverberg-Dilworth et al. 2006) that are highly useful tools for breeding. The *Prunus* set is extensive (an average density of 1.05 cM/SSR), and highly transferable across the genus, but additional coverage of some regions is still needed. For apple, a set of alternatives needs to be developed at each approximate map position. A similar set of markers is needed for pear, and a spaced set of universal apple/pear primers would be invaluable

for comparative mapping. Information on alleles across a cultivar set would aid in choice of framework markers for new mapping projects.

New approaches to mapping have increased the efficiency of locating new markers and mapping traits. Bin mapping, developed in *Prunus*, will be an efficient strategy for mapping large numbers of new markers, e.g. microsatellites or candidate gene markers quickly and cheaply (Howad et al. 2005). The Genome Scanning Approach using sets of spaced markers provides an efficient method of mapping major genes to existing framework maps (Erdin et al. 2006; Patocchi et al. 2004; Patocchi et al. 2005) and apple mapping teams internationally are using this technology as a replacement for bulked segregant analysis using RAPD markers.

Pedigree genotyping (Bink et al. 2002; van de Weg et al. 2004) offers a new approach to QTL mapping in fruit trees by tracing the QTLs across multiple generations of related breeding material derived from a set of common ancestors. This means that the majority of alleles in a breeding programme can be identified and exploited, and that QTLs are identified that otherwise would remain undetected in a single cross because of epistasis.

The extent of linkage disequilibrium (LD), the non-random association of alleles, in Rosaceae fruit crops is unknown. Most Rosaceae are cross-pollinating because of their self-incompatibility system and, as for other allogamous species such as maize (Rafalski and Morgante 2004) and loblolly pine (Brown et al. 2004), they are expected to have a low level of LD conservation. But there are exceptions: peach is self-compatible, and other species, such as apricot, almond and cherry have self-compatibility alleles that are extensively selected for in breeding programmes. Self-pollinating species genomes become nearly fixed after a few generations making most recombination events ineffective, resulting in a slower LD decay. Higher levels of LD have been found in selfers such as *Arabidopsis* (Nordborg et al. 2002) and barley (Caldwell et al. 2006). Another factor that increases LD is the occurrence of a recent bottleneck, as happened in most of the peach cultivars currently growing in North America and Europe (Scorza et al. 1985). Vegetative reproduction, the usual way to propagate fruit trees, may also favour the maintenance of LD, because the genotype of each cultivar is fixed and the number of generations from a common ancestor may be fewer than in species with annual sexual reproduction. In fact, the highest level of LD described so far was in sugarcane (Jannoo et al. 1999), a clonally reproducing species. The analysis of LD in the Rosaceae is likely to uncover different levels of LD in different species, and within different origins of the same species, depending on their reproductive characteristics and their history and evolution as cultivated species.

Detailed knowledge of the genetic structure of fruit tree variability and the LD will be useful for many applications in genetics and plant breeding. Association analysis in selected unrelated genotype collections of morphological variability and markers will detect genome positions containing genes of interest and will be useful for fine mapping of genes involved in quantitative traits and for identification of multiple alleles and allele combinations. Association between markers obtained

with candidate gene sequences and phenotypic data in genotype collections with low LD is a test that allows candidate genes that are in the region of interest but do not affect the character to be discarded, providing further evidence of cause and effect relationships in those that remain associated. Variability analysis of whole genome scans with markers in selected unrelated populations may detect the presence of regions of the genome that exhibit low variability, which may correspond to selective "sweeps" caused by directional selection. In some cases it is possible to make hypotheses on selective sweeps in certain genome locations, as for the self-incompatibility locus in peach, which has been fixed for self-compatibility alleles, or for the *M* locus in non-melting peaches because of fixation of the recessive allele that determines the non-melting behaviour. Unexpected results may question the current knowledge about the recent evolution of these characters. Additional selective sweeps may detect interesting regions, possibly corresponding to positions of genes selected during the process of fruit tree domestication. The selection of populations with different levels of LD based on their different histories may also allow a two-tiered strategy for gene analysis (Nordborg et al. 2002; Caldwell et al. 2006), where high LD collections are used for whole genome scans to search for the positions of major genes or QTLs, and lower LD collections allow the validation of specific candidate genes or the study of intra-genic variability in connection with the observed phenotypic variability. Given the strong similarity between genomes within Rosaceae subfamilies, this approach can be attempted using genotype collections from different species (i.e. apple-pear or peach-almond).

Association analysis, particularly when performing whole genome scans, requires high throughput technology for SNP genotyping, as well as a large number of independent SNP markers. Now that there are hundreds of thousands of ESTs available from a broader base of cultivars, the way is open either for joint projects to develop and map thousands of SNP markers, or at a minimum, registration of interest in marker development at a central website to minimize duplication of effort.

The TILLING (targeting local lesions in genomes) method for genotyping (McCallum et al. 2000) was originally developed for detection of mutations induced by chemical agents such as ethylmethanesulphonate (EMS), but it may also be employed to genotype SNPs in natural populations ('ecoTILLING'), and may be used for association analysis in plants (Comai et al. 2004; Gilchrist and Haughn 2005).

Sequencing at least one of the Rosaceae genomes is of highest priority and given the high degree of similarity between species of the same subfamily (probably also between Prunoideae and Maloideae, although results so far are only partial), sequence information from one species should be useful for studies in others. Integrated genetic and physical maps will serve as sources for map-based cloning of genes controlling important traits. In-depth comparative mapping will reveal highly and poorly conserved genomic regions that respectively underlie similarities and differences between different species or genera. These will point to key areas for

targeted re-sequencing to reveal key associations involving important traits and to enable development of markers for MAS. New high capacity methods for targeted, in depth re-sequencing are becoming available and will enable microsynteny studies at the sequence level.

8.3. Regulation of Gene Expression

Much of the functionality of any genome is defined by the complex interactions among transcription factors (TFs) and genomic DNA (Gong et al. 2004). An initial study on the MYB family of TFs has indicated the value of investigating the functions of individual TFs. Full length sequencing and *in vivo* and *in vitro* expression analysis of 37 apple MYB transcription factor genes enabled speedy identification of a marker for red core in apple (see Section 7 above and Espley et al. 2006; Chagné et al. 2006a; Chagné et al. 2006b) and also indicated involvement in response to abiotic stress, pathogen response, temperature tolerance and branching (Allan et al. 2006).

Genomic approaches have also begun to advance the study of the classical plant growth regulators. Cloning of hormone receptors and identification of their mechanism of action has started to identify how these hormones regulate gene expression. For example, the auxin receptor has been shown to lead directly to degradation of transcriptional repressors by the SCF-mediated protein degradation pathway (Dharmasiri et al. 2005; Kepinski and Leyser 2005), indicating that plant hormones are directly involved in the regulation of gene expression. Recent work on the gibberellin receptor reviewed by Hartweck and Olszewski (2006) indicates degradation of DELLA proteins that in turn regulate gene expression. These close links between plant growth hormone and regulators of gene expression show that the classical plant hormones are an integral part of the regulatory networks. Genomic approaches that integrate manipulation of the hormone receptors and their downstream transcriptional regulators are likely to lead to control of key developmental traits in Rosaceae.

It has become clear that RNA is a key regulatory molecule in eukaryote cells and may contribute to the integration of expression of sets of genes (Baulcombe 2004). Small RNAs regulate target genes through sequence-specific mechanisms that may involve either gene silencing or the microRNA pathway. MicroRNA action may be through inhibition of transcription by methylation, or via gene silencing (Baulcombe 2004; MacDiarmid 2005). An understanding of which genes are regulated by small RNAs and the types of mechanism involved will be important for the ultimate development of systems to alter expression patterns in intact plants. An indication of the significance of the microRNA pathway is provided by the range of targets, which includes both transcription factors and protein degradation regulators, as well as genes involved in response to the environment, such as disease resistance genes (Baulcombe 2004). Bioinformatic analysis of the HortResearch EST database has enabled identification of several conserved primary small RNAs and their homologous target mRNAs. Direct isolation and cloning

has identified novel 19–25mers with many having putative target mRNAs in the database (C. A-Dwamena pers. comm.). Seven small RNA-related genes have been mapped on an apple framework map (S. Gardiner, D. Chagné, unpublished).

8.4. Microarrays

New microarray-based marker systems, such as single feature polymorphisms (SFPs) (Borevitz et al. 2003) and gene expression markers (GEMs) (West et al. 2006) that enable simultaneous genotyping and genome wide mapping of expression QTLs (eQTLs) in several systems, including higher plants, could provide a new tool for identifying candidate genes and analysing novel genetic interactions (Schadt et al. 2003; Kirst et al. 2005) as well as revolutionizing the scale of genetic mapping and MAS. The effectiveness of microarray technology has been demonstrated in principle for monitoring decrease in postharvest quality attributes in apple during storage (van Wordragen et al. 2003), and monitoring of gene expression appears to offer real possibilities for supporting decisions that need to be made during fruit storage and transport. Similar systems that could assist in making decisions during production, such as application of sprays to induce thinning, could be envisaged. A further application of microarray technology for border control purposes and for nurserymen, would be for the simultaneous detection of hundreds of viruses, viroids and phytoplasmas (Hadidi et al. 2004).

8.5. Introduction of Technologies into Breeding Programmes

The markers and technologies to introduce large scale MAS in fruit trees for some characters, are now available. An example is MAS for resistance genes into apple breeding programmes in order to reduce grower dependence on chemical control of pests and diseases, i.e. screening of breeding populations of tens of thousands of seedlings (see Sections 2 and 7 above). DNA can be extracted from 1,180 apple leaf samples overnight (Cook and Gardiner 2004; Cook et al. 2002) and the same number of PCR reactions processed and products analysed within the next 24 hours in a laboratory working with the HortResearch apple breeding programme (S. Gardiner, M. Cook, unpublished). As more markers are identified for QTLs and used for screening by breeders, targeted breeding for complex traits determining fruit quality will also become a reality.

The logical progression from simple MAS is “whole genome selection” (WGS), i.e. the use of markers to select the seedlings in a breeding population that possess a genome most like the quality grandparent(s), as well as the desired new character (Gardiner et al. 2006b; Pradhan et al. 2003). Fine mapping of resistance genes will provide markers to enable the simultaneous reduction of linkage drag in a very precise area around the introgressed genes. WGS is being investigated at HortResearch in combination with technology to decrease the time to first flowering. Preliminary trials have demonstrated that a seed-to-seed period of 18 months is possible in some genetic backgrounds when seedlings are grown under

a controlled environment regime with the timed application of a growth inhibitor (Austin et al. 2006). A transgenic approach to reducing the juvenile phase (Kotoda et al. 2006) may also be useful if applied to breeding parents.

8.6. Genetically Modified Food Products

In many countries the use of transgenic plants in food production is not currently accepted by regulatory authorities, and in any case is mistrusted by the consumer, with the result that transformation cannot be used for new variety development. For this reason, efforts have been made to introduce technologies that 'avoid or minimize the inclusion of superfluous transgenes or sequences' and marker-free transgenic apple plants have been obtained using a model vector that employed excision by recombination (Krens et al. 2004). In another study in apple, acceptable rates of transformation with a model construct (12–25% in preliminary experiments) have been achieved in the absence of a selectable marker (Malnoy et al. 2006d), and this technology may offer a route to future transgenic variety development utilizing endogenous apple genes. In addition, it has been proposed that cisgenic plants should be treated like traditionally bred plants by regulatory authorities (Schouten et al. 2006).

Some believe that transgenic rootstocks may be more acceptable to the consumer than transgenic scions and would provide a quicker route to new rootstock varieties than traditional breeding. Transformation with the *rolB* gene from *Agrobacterium rhizogenes* has been reported to enhance the rooting of pear and apple rootstocks (Zhu et al. 2001a; Zhu et al. 2001b; Zhu et al. 2003; Welander et al. 2004). However, the effect of such rootstocks on scion growth and fruit characters is yet to be reported. Other rootstock traits that could be manipulated with benefit to growers would be enhanced resistance to woolly apple aphid, *Phytophthora* and fireblight. An interesting possibility for developing apple cultivars with reduced allergenicity by using RNAi to silence allergen genes has been demonstrated in principle (Gilissen et al. 2005).

9. CONCLUSIONS

We have discussed ways in which genomics has started to support breeding of new varieties of Rosaceous tree crops, and how the applications of genomic technologies could be extended. Plans are being drawn up internationally, e.g. the Strategic Planning Document of the United States Rosaceae Genomics, Genetics and Breeding Initiative (http://www.mainlab.clemson.edu/gdr/community/rosexec/RosWP_March_2006.doc), for collective research strategies that will extend the impact of genomics on breeding for enhanced quality, shelf life and health enhancing attributes, lowered chemical pesticide inputs during production, as well as decreased labour and energy costs, and reduction of losses due to environmental stressors. In addition, the comparative studies across the wider Rosaceae family may prove a useful model system for answering fundamental questions for fruit crops, e.g. how

does the genome define the formation of a tree rather than a herbaceous plant?, and what determines the formation of a fleshy rather than a dehiscent fruit, or an aggregate versus a single fruit?

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CHAPTER 15

DNA MARKERS: DEVELOPMENT AND APPLICATION FOR GENETIC IMPROVEMENT OF COFFEE

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Abstract: Coffee tree belongs to the genus *Coffea*, comprising of two main cultivated species *C. arabica* L. (the only tetraploid species with $2n = 4x = 44$) and *C. canephora* Pierre ex A. Forehner (diploid, $2n = 2x = 22$), yielding arabica and robusta types of coffee, respectively. In addition, there are ~100 diploid, wild, coffee species many of which hybridize readily with each other. These provide a rich and valuable source of desirable genetic variability for improvement of cultivated coffee germplasm. Arabica coffee is known for excellent cup quality but suffers from a narrow genetic base due to its domestication history and susceptibility for diseases and pests. In contrast, robusta coffee though poor in quality has better adaptability to various biotic/abiotic stresses. To meet the ever-changing demands of the environment and also of the sensibilities of market, there is a continuous need for genetic improvement of coffee, which unfortunately is severely constrained owing to inherently slow pace of tree breeding using conventional methods compounded with a general lack of genetic markers, screening and selection tools. The overall experience in India and worldwide from onerous conventional breeding efforts to develop new evolved coffee varieties has been rather frustrating with only few successes. The situation warrants recourse to newer, easy and efficient practical alternatives/technologies that can surmount the above problems and provide acceleration, reliability and directionality to the breeding efforts. In this context, development/utilization of: genomic variations based DNA markers, molecular linkage maps and markers-assisted breeding approaches that provide high-genetic resolution and new hopes and possibilities for genetic improvement of difficult species like coffee has become essential, a realization dawned on coffee research community rather recently. This chapter gives a brief overview of the worldwide efforts undertaken in recent years to develop, use and integrate DNA marker tools/technologies in coffee genetics research. It also provides few thoughts about future needs and perspectives to fully harness the potential of DNA marker based applications in managing and utilizing the available germplasm resources, construction of linkage maps, QTL mapping and genetic improvement of coffee.

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1. INTRODUCTION

Coffee drink has become exceedingly popular since its age-old discovery as a drink in Arabia (ICO, London, 2006, <http://dev.ico.org/>). On a rough estimate, ~1.4 billion cups of this exotic beverage are consumed globally everyday for its distinctive flavour and stimulatory qualities. Though the discovery of coffee as a drink before 15th century is attributed to Arabs, coffee tree is supposed to have originated in the highlands of Ethiopia (Lashermes et al., 1996c). The Arabs, who got acquainted with coffee plant through their African slaves, cultivated coffee and safeguarded its cultivation by exporting only processed and nonviable coffee seeds thereby controlling the world coffee trade until the mid of 17th century (ICO, London, 2006). In order to break the Arab monopoly over coffee trade, in 1616, some Dutch people smuggled few coffee beans from Yemen and cultivated them in green houses in Holland. Mainly from this source, the Dutch and British people started cultivating coffee in their Asian, African and Caribbean/Latin American colonies. Thus coffee cultivation got established in its present places giving rise to different ecotypes, locally adopted forms, mutants and hybrids. As expected from this history and facts, today's cultivated coffee owes its origin to a handful of parental material introduced in the beginning to the present places of coffee cultivation thus exhibiting a very narrow genetic base (Ferwanda, 1976; Lashermes et al., 1996d). Today coffee is cultivated in about 80 countries in South and Central America, the Caribbean, Africa and Asia, in the areas generally lying between the tropics of Cancer and Capricorn with 70% of the coffee production coming from arabica and remaining from robusta coffee. By far, two biggest producers of coffee are Brazil and Colombia followed by Vietnam, Indonesia, Ethiopia, India and Mexico (ICO, London, 2006).

1.1. The Coffee Plant

Coffee tree belongs to the genus *Coffea*, comprising of two main cultivated species *C. arabica* L. (the only tetraploid species with $2n = 4x = 44$) and *C. canephora* Pierre ex A. Forehner (diploid species having $2n = 2x = 22$), and a third sparsely grown species *C. liberica* (or tree coffee) in some parts of Africa. In addition, there are >100 diploid, wild, coffee species and many species belonging to the related genus *Psilanthus* in the tribe *Coffeae* of *Rubiaceae* family that differ greatly in their size, morphology, adaptation and geographical distribution. Importantly, many of these diverse species hybridize readily with each other and thus are a rich and valuable source of desirable genetic variability for improvement of cultivated coffee germplasm.

C. arabica L., is supposed to have originated in the highlands of South-Western Ethiopia (Lashermes et al., 1996c) and *C. canephora* P. being originated in Central and West Africa in lowland forests of Eastern Liberia to Southern Kenya in Congo basin (Berthaud and Charrier, 1988). *C. arabica* is well adapted to highlands, whereas *C. canephora* and *C. liberica* thrives in lowland tropical areas. General plant features of two cultivated species of coffee are listed in Table 1.

Table 1. Plant features of *C. arabica* and *C. canephora*

	<i>C. arabica</i>	<i>C. canephora</i>
Growth habit	Shrubby, moderate vigour	Small tree, vigorous
Root system	Deep	Shallow
Ploidy level	Tetraploid (2n = 44)	Diploid (2n = 22)
Flowering time	Beginning of rainy season	Sporadic
Pollination	Autogamous (Carvalho, 1988)	Allogamous
Self incompatibility	Absent	Present (gametophytic)
Fruit maturation time	6–9 months	9–11 months
Fruit abscission at maturity	Yes	No
Yield potential	Lower	Higher
Optimal temperature	15–25°C	25–30°C
Optimal rainfall	1200–1500 mm/year	1500–3000 mm/year
Altitude grown	1000–2000 m	0–800 m
Disease and pest resistance	Low	High
Coffee flavour	Rich, acid	Bland, lacking acidity
Caffeine content	0.8 to 1.4%	1.7 to 4.0%
Price in global market	Higher	Lower

1.2. Economic Importance of Coffee and the World Coffee Crisis

Total global export of coffee exceeds US\$ 9 billion and the sector employs more than 25 million people globally at different stages of production, processing and marketing of coffee (Kaplinsky, 2004). Although, the final consumption of coffee accounts for around US\$55 billion annually, the coffee producing countries receive only ~US\$8 billion (only around 15 percent of the total coffee trade). Coffee economy, nevertheless, remains one major export earner for undeveloped and developing countries (Cardenas, 2001) and is rather backbone of economy for many of them. It accounts for over half of the export earnings in countries like Burundi and Uganda and for some of the other coffee-producing third world countries, major share of revenue is derived from coffee exports (Wasserman, 2002; Table 2). Coffee was second largest commodity export after oil till late 1990s, when coffee prices started falling and reaching its 30 year lowest in 2001–2002 and now stands after oil, aluminium, wheat and coal (Ponte, 2001) and dragged the coffee world into the ‘*World Coffee Crisis*’ (Cardenas, 2001; Osorio, 2004), which was a great economical, social and environmental loss and disaster for the coffee producing countries. The causes of crisis are deeply rooted into contemporary policies of world economy which involves globalisation/ liberalisation of trade coinciding with overproduction and imbalance in demand-supply (Cardenas, 2001) and of course in the laboratories and fields of coffee researchers including economists & policy makers, agronomists and geneticists & breeders as well.

Table 2. Importance of coffee in the economy of poor and underdeveloped countries (modified from Wasserman, 2002)

Country Name	Coffee as % of total export	Export % of GDP	Global position for coffee production (2004)
Burundi	56	8	28
Uganda	52	12	11
Rwanda	27	6	26
Ethiopia	26	16	5
Honduras	16	42	10
Nicaragua	15	37	19
Guatemala	15	20	9
Ecuador	11	42	18
Papua New Guinea	10	47	17
El Salvador	9	28	14

2. COFFEE GENETIC IMPROVEMENT

Domestication of the species has been characterized by successive reduction in genetic diversity (Etienne et al., 2002), which is further constrained especially in arabica by its primarily self-fertilizing reproduction behaviour leading to genetic homogenization. However, by using traditional breeding tools like introductions/explorations, selections, hybridisations, back crossing and hybrids, breeders have been able to create new and improved varieties for both arabica and robusta coffee.

Two botanical types are recognised each in *C. arabica* (Typica and Bourbon) and *C. canephora* (Robusta and Ngnda). Typica was the original arabica coffee taken to Asia and then to Latin America/Caribbean by the Dutch people. Bourbon coffee was identified as a coffee plant with smaller bean size found on the Bourbon island (now renamed as Reunion) in the Indian Ocean, and was later taken to South Americas and from there to East Africa to be cultivated as Bourbon variety. Bourbon is high yielding, slender variety lacking the bronze cast of the new foliage with smaller bean size and better resistance to berry disease than Typica. On the other hand, two botanical types of *C. canephora* are: 'Robusta or Erecta' (up right form) and 'Nganda' (spreading form).

Breeding efforts in early to mid nineties led to development of many improved arabica selections. Some prominent local selections (SI) for arabica coffee are Blue Mountain in Jamaica, Vila Sarchi, Columnaris, Coorg, Kent and S 795, French Mission, K- 7, SI 6, SI 14, SI 28, SI 34 etc. Apart from this, many explorations (FAO, 1968; Guillaumet and Hallé, 1978) undertaken in Africa to search for novel diversity have added sources of disease, pest, nematode and abiotic resistance. Inter-varietal crosses have yielded hybrids such as Mundo novo, Catuai etc. Similarly, several spontaneous natural mutants showing desirable characteristics like Caturra, Purpurascens, San Ramon, Pache comum, Maragotype are identified and now being cultivated as well as used for varietal improvement. *C. arabica* being self pollinated, these varieties tend to be genetically more stable. Although, now a number

of high yielding arabica varieties are available, but in general these are highly susceptible to various diseases like leaf rust (*Hemileia vastatrix* Berk & Br.), Coffee Berry Disease (CBD caused by *Colletotrichum kahawae*), stem borer (*Xylotrechus quadripes* Chevrolat) and nematodes (*Meloidogyne* spp. and *Pratylenchus* spp.).

In comparison to arabica coffee, there have been limited improvement efforts for robusta coffee. As this coffee plant possesses gametophytic self-incompatibility, it produces many forms and varieties in wild. Modern robusta cultivars are BR series in India, SA and BP selections in Indonesia, IF clones in Ivory Coast and IAC lines and Apoata (IAC 2258) in Brazil (Vossen, 2004). Recently, some programmes have been initiated for genetic improvement of robusta coffee (Montagnon et al., 2004; Anim-Kwapong and Adu-Ampomah, 2004). Apart from an example of the C×R hybrid developed in India between *C. congensis* and *C. canephora*, diploid interspecific hybrids were not a great success (Vossen, 2004). But none the less, robustas remain important as they provide the most needed genetic diversity including high yield potential, plant stature, hardy nature and resistance to various diseases, pests and abiotic stresses.

For incorporating disease resistance, sturdy stature and high yielding quality of robusta into arabicas with excellent cup quality, several arabica x robusta hybrids were also attempted (Carvalho, 1988; Fazuoli et al., 2001). A natural tetraploid hybrid of *C. arabica* and *C. canephora* termed as Hybrido-de-Timor (HdeT) identified from the erstwhile Portuguese colony of East Timor Island (Bettencourt, 1973), till today is probably the only best known source of resistance to leaf rust, some species of nematodes and coffee berry disease in arabica. Finding natural hybrids is a common phenomenon in many coffee plantations as well as in research stations (Cramer, 1957) and similar to HdeT, S-26 is another source of rust resistance that was found in India. Such natural hybrids provide important sources for desirable gene(s) and accordingly have been employed in breeding with varying success. Recently a sufficiently fertile intergeneric hybrid was produced between *Psilanthus ebracteolatus* Hiern and *C. arabica* L. suggesting that generic barriers could also be overcome in coffee for introgression purpose (Coutoron et al, 1998).

2.1. Present Status of Conventional Coffee Breeding Efforts and Need for Newer Molecular Tools

In today's scenario, thrust areas for coffee improvement programmes include enlarging the genetic base of arabica coffee, incorporation of disease, pest and nematode resistance (due to growing incidences of new diseases, pathotypes and lesser known diseases in new areas), abiotic stress tolerance (due to global climatic changes, deteriorating soil conditions and changing patterns of rainfall), encouragement for development of specialty, certified and branded coffees (like organic coffees, fair trade coffee, estate coffees etc.) and diversification of coffee plantations including quality, environmental stability and sustainable yield improvement (Herrera et al., 2001; Vossen, 2004). To meet these ever-changing demands of the environment and also of the sensibilities of market, a number of breeding and

selection programs have been undertaken worldwide including India since early 20th century. Despite few successes, overall experience from these long years of conventional breeding efforts worldwide has been rather frustrating, mainly because the pace of progress in coffee improvement has been abysmally slow. This dismal situation have been the outcome of inherently slow conventional breeding methods that are further compounded in case of coffee being a tree species that has its own problems. Some of the major constrains in coffee breeding, thus, have been:

1. Very long generation cycle; 4–6 years for seed to seed generation.
2. Narrow genetic base for arabicas.
3. Unavailability of true-to-breed type inbreds or homozygous lines for the diploid genotypes due to difficulty in selfing of plants.
4. Ploidy level variation (arabicas are tetraploids and robustas are diploids) and incompatibility barriers (robustas possess gametophytic self incompatibility system).
5. Reproductive barriers for interspecific crossing due to infertility, low frequency of intergenome crossing over and gametic or zygotie counter selection (Herrera et al., 2001, 2002a).
6. Poor knowledge base of genetics of coffee traits, unavailability of genetic markers/genetic maps, dense DNA marker based linkage maps with transferable landmarks across laboratories.
7. Unavailability of suitable screening tools/diagnostic markers for diseases and pathogens in correlation to continuous evolution of new pathotypes.
8. Relatively poor research base/resources in most of the producing countries.
9. Treatment as orphan species compared to other crop plants, leading to a delayed (relatively recent) efforts to integrate modern biology genomic tools/technologies for coffee research and improvement.

The situation called for newer, easy and efficient practical alternatives that could surmount the above problems faced in coffee improvement, a realization dawned on coffee research community rather recently. The advent of DNA variations based genetic markers and breeding approaches like MAS provided new hopes and possibilities for genetic improvement programs of difficult species like coffee. The great potential of DNA marker based technologies is now well demonstrated, and these are being utilized for crop improvement programmes through out the world for genotyping, varietal identification (and claiming intellectual property rights), germplasm finger printing, construction of linkage maps, Quantitative Trait Loci (QTL) identification and mapping in concert with Marker Assisted Selection (MAS) breeding to finally develop genetically improved crops with desirable traits. The DNA marker technologies were found equally useful in helping decipher many of the evolutionary puzzles about origin, spread, taxonomic relationships of many crop species. In recent years, therefore, conscious efforts have began globally to integrate such DNA/molecular markers based technologies, which can provide impetus, dependability and directionality to the efforts on genetic improvement of coffee. Accordingly, the coffee research is now experiencing many of these technologies evolve, flourish and to be utilized in future, but yet to go a long way before visible

gains become a reality. There are now large coffee genomics programs underway in many countries, few prominent being, Brazil, France, Italy, Columbia, and more recently India (where a large Coffee Genomic initiative has been started in 1999 under a National Network mode supported by Department of Biotechnology, Govt. of India).

In the following sections of this paper, an essence of global efforts put on for exploring and development of DNA markers for coffee, applications of DNA markers for deciphering genetic make up of coffee plant, deciphering origin of coffee plant, coffee germplasm assessment, construction of linkage maps, and QTL mapping are discussed.

3. DEVELOPMENT OF DNA MARKERS FOR GENETIC STUDIES IN COFFEE

With the advancement of molecular biology technologies, initial efforts with biochemical markers such as isozymes were soon replaced by DNA based approaches due to their apparent advantages for genetic studies in plants and animals. In the beginning, southern-based RFLP markers (Wyman and White, 1980) were widely used but were found to be difficult to adopt for large-scale studies that are hallmark of any worthwhile genetic study on genetic improvement. Therefore, soon with the advent of PCR technology in 1980s, a suite of PCR based marker approaches such as RAPD (Williams et al., 1990), AFLP (Vos et al., 1995), SSR (Weber and May, 1989; Litt and Luty, 1989; Tautz, 1989) and more recently SNP (Sachidanandam et al., 2001) based markers, are being developed and adopted for genetic studies worldwide. In case of coffee research, a similar (but modest) trend is seen in using the molecular markers to complement the conventional low-efficiency biometrical tools for genetic studies. Some of the initial studies in this regard include coffee germplasm assessment studies using markers like isozymes (Berthou and Trouslot, 1977; Moreno, 1990), RFLPs (Lashermes et al., 1999, 2000b; Herrera et al., 2002a) followed by multilocus RAPD (Lashermes et al., 1993, 1996a, 1996d; Agwanda et al., 1997; Anthony et al., 2001) and AFLP (Steiger et al., 2002; Anthony et al., 2002; Lashermes et al., 2000a). In the last decade, few efforts have been initiated to develop coffee specific genomic SSR markers that have resulted in a small repertoire of such markers comprising 183 publicly available SSRs (Table 3). In addition, there are 306 SSRs having limited access only through registration (www.coffeedna.org), and 170 SSRs developed by us (Aggarwal et al., 2004a) under the first Indian initiative on coffee genomics and are available on request. Here it is noteworthy to mention that our studies done to evaluate the occurrence and relative abundance of different SSRs have shown that coffee genome (across its different species), in general, is relatively very poor in SSRs with abundance of AT rich repeats (Figure 1, our unpublished data), thus suggesting it to be a difficult system for development of species-specific microsatellite markers. Recently, attempts have also been initiated to develop EST-SSRs (genic/functional SSR markers)

Table 3. Summary of publicly accessible coffee specific SSR primer pairs

Source reference	No. of working sets of primers published	Source species	Comments
Combes et al. (2000)	11	<i>C. arabica</i> (Caturra)	From library enriched for TG motif
Rovelli et al. (2000)	13	<i>C. arabica</i> (Caturra)	From two libraries each enriched with ATC and TG motif
Coulbaly et al. (2003b)	20	<i>C. arabica</i>	From available SSR containing sequences
Baruah et al. (2003)	9	<i>C. arabica</i> (HdeT)	From normal small-insert partial genomic library
Moncada and McCouch (2004)	34	<i>C. arabica</i> (Caturra)	From library enriched for GA motif
Poncet et al. (2004)	78	<i>C. arabica</i>	From available database sequences
Bhat et al. (2005)	9	CxR hybrid	From <i>in-house</i> EST library
Aggarwal et al. (2007)	16	<i>C. canephora</i> varieties (CxR hybrid and S-12)	From <i>in-house</i> EST library and available EST database
Total publicly available SSR primer pairs till date	(+ 200 designed primer sets)		183* (+ 200 designed primer sets)
Total SSRs mapped on different linkage maps			18 (Lashermes et al., 2001)
(Many of these markers are not in public domain but are available through mutual agreement)			29 (Coulbaly et al., 2003b)
			71 (Hendre, 2006)
			Total = 138

* In addition we have developed 170 coffee SSRs that are available on request (Aggarwal et al., 2004a)

utilizing coffee EST sequences (Bhat et al., 2005; Aggarwal et al., 2007). These new generation EST-SSR markers require availability of large EST databases as a prerequisite, which were severely limited for coffee till late 2005 (only ~400 ESTs were in public domain NCBI database). Utilizing the limited EST resources at hand, we have now designed 224 EST-SSR primer pairs, and have demonstrated

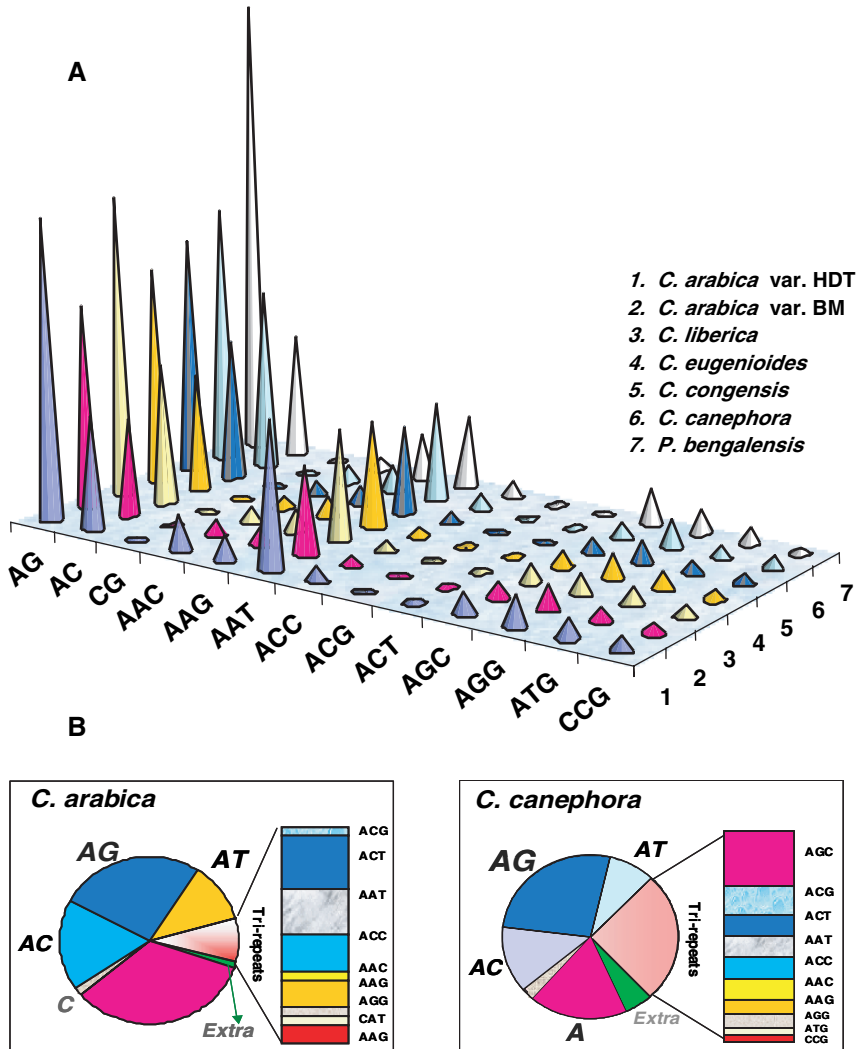


Figure 1. Relative abundance of different SSR motifs in genomes of different coffee species as revealed by: A) semi-quantitative Southern hybridization based slot-blot analysis, and B) *in-silico* sequence analysis of >1000 SSR positive clones from *C. arabica* and *C. canephora* (robusta) small-insert genomic libraries. Note almost similar comparable pattern/relative frequencies of different SSRs across coffee species and also between the two approaches of evaluation (our unpublished data)

their scope and utility in characterization of *C. arabica*, *C. canephora* germplasm and broad cross-species transferability (Aggarwal et al., 2007).

Despite these random successes with marker development, it will be important rather essential to expand the repertoire of coffee specific PCR based efficient SSR/microsatellite markers before we can really hope to integrate and realize the great potential of marker based approaches in research on coffee genetics and plant breeding/improvement programs. In this context, the recent addition of ~47,000 coffee ESTs with more than 13,000 unigenes (Lin et al., 2005) to NCBI database is a very fortunate development, which promises possibilities of many more coffee SSR markers. Similarly, the concept proposal to undertake low coverage genome sequencing of arabica coffee as a potential source to develop large number of SSR markers under the preview of recently enacted 'International Coffee Genomic Network' (2nd meeting at Trieste, Italy; www.coffeegenome.org) is a welcome step that if successful, would immensely help to hasten the realization of markers based molecular breeding in coffee.

4. APPLICATIONS OF DNA MARKERS FOR GENETIC DIVERSITY STUDIES IN COFFEE GENEPOOL

The coffee gene pool consists of primary germplasm comprising wild and cultivated varieties of *C. arabica* and *C. canephora* and secondary gene pool comprising >100 related diploid species of *Coffea* and *Psilanthus*. Cultivated arabica varieties have excellent cup quality but possess a narrow genetic base and susceptibility for diseases and pests. In contrast *C. canephora* varieties (robustas), generally are poor in quality. The secondary gene pool including robustas provides an important source for incorporating disease resistance, sturdy stature and high yielding quality. The diploid *Coffea* varieties are known to interbreed freely with each other and produce relatively fertile progeny (Anthony, 1992; Louarn, 1993) as well as fertile intergeneric hybrids can be produced (Coutoron et al., 1998). These attributes thus, demonstrate the importance of primary/secondary gene pool in coffee improvement but also suggest the need for thorough exploration of the available germplasm resources for useful variability using high resolution genetic approaches for any meaningful utilization. Some of the major studies carried out using different types of DNA markers to ascertain the genetic diversity in the available coffee germplasm are briefly summarized in the Tables 4 and 5, and are discussed ahead.

4.1. Genetic Diversity in Cultivated Gene pool of Coffee

The cultivated gene pool of arabica is believed to have been derived from few seeds, which survived the efforts to bring coffee out of Yemen to its present places of cultivation as become apparent from the historical evidences and facts (Vossen, 1985; Anthony et al., 2001, 2002). The genetic variability in arabica populations is expected to have reduced further owing to its autogamous behaviour, leading

Table 4. DNA markers based molecular studies performed for germplasm assessment and species status of *Coffea* and related taxa

Study source/ Reference	Techniques used	Germplasm studied	Important results	Phylogeny grouping and relationship
Lashermes et al. (1993)	RAPD (23 decamers)	Arabica (6 acc.), HdeT robusta (5 acc.), <i>C. eugenioides</i> , <i>C. congensis</i> , <i>C. liberica</i> (2 acc.), <i>C. pseudozanguebariae</i> , <i>C. resinosa</i> , <i>C. sp.</i> and <i>C. stenophylla</i> (1 acc. each) <i>Total = 19</i>	Very low variation in arabicas, high intraspecific variation in robusta and liberica, extensive genetic variation between <i>Coffea</i> spp.	I = <i>C. canephora</i> , <i>C. congensis</i> , <i>C. liberica</i> and <i>C. stenophylla</i> II = <i>C. arabica</i> , <i>C. eugenioides</i> III = <i>C. resinosa</i> IV = <i>C. pseudozanguebariae</i> Clusters according to geographical locations.
Orozco-Castillo et al. (1994)	RAPD (30 decamers)	Cultivated arabica (10 acc.), wild arabica (7 acc.), robusta (3 acc.), HdeT and its derivatives (6 acc.), <i>C. liberica</i> (1 acc.) <i>Total = 27</i>	High diversity exists in the assessed germplasm, species-specific markers observed.	I = Bourbon and its deriva- tives II = Typica and its deriva- tives III = Ethiopian collections IV = robustas V = <i>C. liberica</i>
Orozco-Castillo et al. (1996)	RAPD (15 decamers)	Arabica (4 acc.), robusta (3 acc.), <i>C. eugenioides</i> (2 acc.), <i>C. liberica</i> (2 acc.), <i>C. stenophylla</i> , <i>C. racemosa</i> , <i>C. pseudozanguebariae</i> , <i>C. humilis</i> , <i>C. congensis</i> , <i>C. sessiflora</i> , <i>C. breviceps</i> (1 acc. each) <i>Total = 18</i>	Intraspecific variation in arabica, robusta, liberica. Extensive variation among <i>Coffea</i> spp.	A = <i>C. pseudozanguebariae</i> , <i>C. sessiflora</i> , <i>C. racemosa</i> B = <i>C. canephora</i> , <i>C. liberica</i> , <i>C. breviceps</i> , <i>C. congensis</i> C = <i>C. humilis</i> , <i>C. arabica</i> , <i>C. eugenioides</i> , <i>C. stenophylla</i>
Lashermes et al. (1996d)	RAPD (140 decamers)	Cultivated arabica (7 acc.), Wild arabicas (13 acc.) <i>Total = 20</i>	Very low variation (Only 15 polymorphic bands from 12 primers).	I = cultivated arabicas. Kenyan wild arabicas II = wild arabicas, high diversity among wild than cultivated Clusters according to geographical location.

(Continued)

Table 4. (Continued)

Study source/ Reference	Techniques used	Germplasm studied	Important results	Phylogeny grouping and relationship
Agwanda et al. (1997)	RAPD (272 decamers)	Coffee Berry Disease resistant (high, medium and normal) and susceptible (high and normal) arabica genotypes <i>Total = 45</i>	Very low polymorphism (5.2% bands are polymorphic) and 3 markers linked to CBD resistance.	Cultivars specific groupings.
Lashermes et al. (1999)	RFLP (23 probes)	Arabicas (3 acc.), robustas (3 acc.), <i>C. congensis</i> (2 acc.), <i>C. eugenioides</i> (1 acc.), <i>C. sp.</i> Moloundou (1 acc.) <i>Total = 10</i>	Extremely low diversity in arabicas with some fixed heterozygosity. Many alleles in arabica were shared by canephoroïd and/or eugenioides species.	I = canephoras II = arabicas III = <i>C. congensis</i> IV = <i>C. eugenioides</i>
	GISH (fluorescence probes: <i>C. canphora</i> + <i>C. eugenioides</i>)	<i>C. arabica</i> , <i>C. canephora</i> , <i>C. eugenioides</i>	Detected 22 chromosomes each belonging to each constituent genomes <i>C. canphora</i> and <i>C. eugenioides</i> .	–
Lashermes et al. (2000a)	AFLP (42 primer pairs) canephora gene introgression into HdeT and its derivatives	Cultivated arabica (17 acc.), wild arabicas (6 acc.), introgressed arabicas (19 acc.), HdeT (2 acc.) cultivated robusta (3 acc.), wild robusta (5 acc.) <i>Total = 52</i>	No. of polymorphic bands in robustas (945) 9 times higher than arabicas (109), 50% introgression from robusta into HdeT.	Introgressed arabicas weakly grouped together, strong clustering of cultivated and wild robustas.
Lashermes et al. (2000b)	RFLP (16 single copy probes)	Segregating population of 14 F2 from F1 arabica hybrid plants and 70 BC plants generated from arabusta and arabica hybrid	3/16 probes polymorphic for arabicas. 11/16 probes polymorphic for arabusta. Confirm diploidised behaviour of arabica.	–

Combes et al. (2000)	SSR (11 primer pairs)	Arabica (32 acc.), robusta (10 acc.), 13 <i>Coffea</i> taxa and 2 <i>Psilanthus</i> taxa	Very low diversity and heterozygosity in arabicas, low to moderate diversity and heterozygosity in robustas, moderate to high transferability	-
Rajkumar et al. (2000)	RAPD (41 primers), ISSR (6 primers)	32 putative different accessions of <i>C. eugenioides</i> , <i>C. salvarrix</i> , <i>C. stenophylla</i> , <i>C. dewevreii</i>	All tested primers polymorphic between species, but resulted in almost monomorphic patterns between accessions of a species.	High inter-species but negligible intra-species (between accessions) diversity.
Anthony et al. (2001)	RAPD (150 decamers)	Wild and semi wild cultivars of arabica <i>Total = 118</i>	16 polymorphic primers with 29 polymorphic amplicons out of a total of 106.	6 clusters based upon origin and pedigree.
Steiger et al. (2002)	AFLP (6 primer pairs)	Arabicas: Bourbon (4 acc.), Catimor (8 acc.), Red Catui (6 acc.), Yellow Catui (3 acc.), Catui (1 acc.), Caturra (2 acc.), Mokka hybrid (MH, 8 acc.), Typica (26 acc.), Diploid: robusta (1 acc.), <i>C. liberica</i> (2 acc.) <i>Total = 58 arabicas, 3 diploids</i>	Very high genetic similarity within the arabica groups. Typica least diverse (97% sharing among accs.), mean genetic similarity 0.935 (range 0.767 to 1.0).	1 = 22 Typica acc., 2 Bourbon, 1 Catui II = 5 MH, 1 Typica III = 5 Catui, 2 Caturra IV = 2 Typica, 1 MH V = 4 Catimor VI = 2 Ibericas 12 accessions could not be grouped.
Anthony et al. (2002)	AFLP (37 primer pairs), SSR (6 primer pairs)	Confirmatory samples (1 each of Typica and Bourbon), Commercial cultivars (6 acc.), arabica mutants (2 acc.), arabica di-haploid (1 acc.), cultivars from collections (4 acc.), wild arabicas (11 acc.) <i>Total = 26</i>	High diversity among wild arabicas (90% of all) as compared to cultivated. High similarity between Typica and Bourbon.	Except for Typica and Bourbon as clusters, other accessions were grouped very weakly.

(Continued)

Table 4. (Continued)

Study source/ Reference	Techniques used	Germplasm studied	Important results	Phylogeny grouping and relationship
Prakash et al. (2002)	AFLP (36 primer pairs) gene introgression into S-26 derived tetraploid arabicas	17 introgressed genotypes (S- 288 x Kent arabica), <i>C. arabica</i> (5 acc.), S-288, Kent arabica <i>C. liberica</i> (5 acc.) Total = 29	Very low polymorphism in arabicas (only 35 bands), less alien genome introgression into the introgressed arabicas .	No evident relationship between introgressed cultivars.
Herrera et al. (2002a)	AFLP (30 primer pairs), SSR (19 Primer pairs)	From 6 triploid hybrids (<i>C. arabica</i> x <i>C. canephora</i>), 6 BC progenies were produced @ 10 plants per progeny Total = 60	Low introgression from canephora genome into arabica genotypes (13.8% of all the genotypes had canephora specific alleles.	–
Herrera et al. (2002b)	RFLP (11 probes), SSR (13 primer pairs)	1 tetraploid hybrid plant (arabica x tetraploid canephora i.e. IF18IT) crossed with <i>C.</i> <i>arabica</i> (Et 30) to produce F1 hybrid. 28 P1 BC plants and 45 P2 BC plants	Very high level of gene flow from canephora to arabicas.	–
Bardini et al. (2003)	Tubulin based polymorphism (TBP: β -tubulin intron 1 specific primer pair)	Arabica (5 acc.), robusta (9 acc.)	No polymorphism in arabica and some polymorphism in robustas.	–
Silviera et al. (2003)	RAPD (18 primers/ restriction enzyme combinations)	90 individuals belonging to 6 Sarchimor progenies, 2 cultivars and a progeny (all arabicas)	38.5% and 68.5% variations within and between populations. Low to moderate diversity detected.	Clustering according to progenitors.

Baruah et al. (2003)	SSR (9 primer pairs)	Arabicas (45 acc.), robustas (6 acc.), 13 <i>Coffea</i> spp., 4 <i>Psilanthus</i> spp.	Diversity and PIC values High in robustas, and low in arabicas, robust cross species transferability.	Fixed heterozygosity (ca. 100%) in arabicas proposed due to presence of duplicate loci.
Bhat et al. (2004)	EST-SSR (9 primer pairs)	Arabicas (15 acc.), robustas (8 acc.), 14 <i>Coffea</i> spp., 4 <i>Psilanthus</i> spp.	Low diversity/ very low PIC for arabicas, Low-high diversity/ PIC values for robustas, robust cross species transferability with moderate diversity.	Fixed heterozygosity (ca. 100%) in arabicas proposed due to presence of duplicate loci.
Chaparro et al. (2004)	RAPD (42 decamers)	47 explored arabica accessions	24 (57%) primers produced moderate polymorphism. 34.2% bands are polymorphic. High diversity in wild arabicas.	One loose cluster containing 22 accessions and other dispersed. No defined basis for clustering.
Poncet et al. (2004)	SSR (110 primer pairs)	<i>C. canephora</i> (11 acc.), <i>C. eugenioides</i> (4 acc.), <i>C. liberica</i> (4 acc.), <i>C. heterocalyx</i> (1 acc.), <i>C. sp.</i> Moloundou (2 acc.), <i>C. pseudozanguebariae</i> (10 acc.) Total = 32	High transferability among species (81.4%), high genetic diversity and PIC in canephora and pseudozanguebariae,	-
Moncada and McCouch (2004)	SSR (34 primer pairs)	Arabica cultivars (10 acc.), HdeT (2 acc.), wild arabicas (11 acc.), robusta (1 acc.), <i>C. eugenioides</i> (2 acc.), <i>C. liberica</i> (2 acc.), <i>C. congensis</i> (1 acc.), <i>C. kapakata</i> (1 acc.) Total = 30	More alleles/high PIC value in diploids, followed by wild arabicas, cultivated arabicas. But SSRs unearthed more diversity in cultivated arabicas than by RFLP or RAPDs.	I = cultivated arabicas, few wild arabicas, HdeT II = wild arabicas Others: <i>C. eugenioides</i> closer to arabicas, <i>C. kapakata</i> different from all; <i>C. liberica</i> , <i>C. canephora</i> and <i>C. congensis</i> closer to each other.

(Continued)

Table 4. (Continued)

Study source/ Reference	Techniques used	Germplasm studied	Important results	Phylogeny grouping and relationship
Aggarwal et al. (2004b)	RAPD (37 primers), AFLP (7 6-bp selective primer pair sets), SSR (150 primer pairs)	16 superior selections of coffee (14 arabica and 2 robusta) developed in India	Considerably more alleles/ high PIC value in diploid robustas compared to arabicas. SSRs unearthed more diversity in cultivated arabicas than by AFLP or RAPDs.	Clear separation between robusta and arabica selections. Reference panels of discriminating, polymorphic markers could be generated for individualization of each selection.
Prakash et al. (2005)	AFLP (15 primer pairs), SSR (12 primer pairs)	Indian collection of robusta genotypes (60 acc.), core collection robusta (14 acc.), <i>C. congensis</i> (3 acc.)	72 alleles with 12 SSRs, 205 polymorphic bands with AFLP, i.e. high level of polymorphism.	Clusters according to five diversity groups of robusta coffee.
Aga et al. (2005)	ISSR (15 primers)	160 genotypes belonging to 16 wild populations of arabica coffee	Moderate level of polymorphism (25% polymorphic bands).	Clustering of genotypes based on geographical location.
Masumbuko and Bryngelsson (2006)	ISSR (6 primers)	Arabica (100 acc. from 5 regions), <i>C. eugenioides</i> (2 acc.), <i>C. zanguiherae</i> (5 acc.), <i>C. mufindiensis</i> (3 acc.)	Low variability for arabicas.	Grouping based on species and geographical location.
Aggarwal et al. (2007)	EST-SSR (18 primer pairs)	Arabica (15 acc.), robusta (8 acc.), 13 <i>Coffea</i> spp., 4 <i>Psilanthus</i> spp.	16 polymorphic pairs, comparable PIC (0.42) and diversity in both arabica and robusta. High cross species transferability/sequence conservation across the species.	Clustering based on pedigree and geographical origin.

Table 5. DNA markers based studies for deciphering species relationship among *Coffea* taxa

Study source/ Reference	Technique	Species assessed	Important results	Groupings and relationship
Berthou et al. (1983)	Chloroplast (cp) and mitochondrial (mt) DNA RFLP polymorphism using <i>Hpa</i> II enzyme and <i>Sal</i> I enzyme respectively	Arabica (2 acc.), robusta (2 acc.), <i>C. eugeniooides</i> (2 acc.), <i>C. congensis</i> (2 acc.), 'nana' taxon, <i>C. excelsa</i> , <i>C. liberica</i> , <i>Paracoffea ebracteolata</i> , <i>C. arabusta</i>	No difference in cp-DNA of (arabica and eugeniooides) and (canephora and congensis). Similarity between mt-DNA of (arabica, eugeniooides, congensis) and (canephora and 'nana' taxon), unique patterns for other species	cp – DNA Group A: <i>C. arabica</i> , <i>C. eugeniooides</i> , <i>C. congensis</i> Group A'; <i>C. canephora</i> , 'nana' taxon mt – DNA Group 1: <i>C. arabica</i> , <i>C. eugeniooides</i> Group 2: 'nana' <i>C. arabica</i> has an ancestor similar to <i>C. eugeniooides</i> .
Lashermes et al. (1996c)	Chloroplast DNA RFLP in <i>atpB-rbcL</i> intergenic region	52 trees from 25 <i>Coffea</i> taxa Total = 52	Low divergence, 12 different plastomes were detected. Maternal inheritance of chloroplast confirmed.	– <i>Coffea</i> is monophyletic and recent in origin
Orozco-Castillo et al. (1996)	Chloroplast PCR-RFLP (<i>trnT-L</i> , <i>trnL</i> , <i>trnL-F</i>)	Arabica (4 acc.), robusta (3 acc.), <i>C. eugeniooides</i> (2 acc.), <i>C. liberica</i> (2 acc.), <i>C. stenophylla</i> , <i>C. racemosa</i> , <i>C. humilis</i> , <i>C. pseudozanguebariatae</i> , <i>C. congensis</i> , <i>C. sessiflora</i> , <i>C. breviceps</i> (1 acc. each); Total = 18	Amplicons from <i>trnL</i> , <i>trnL-F</i> shows enzyme site polymorphism.	I = <i>C. arabica</i> , <i>C. eugeniooides</i> , <i>C. humilis</i> , <i>C. stenophylla</i> II = <i>C. canephora</i> , <i>C. liberica</i> , <i>C. breviceps</i> , <i>C. congensis</i> III = <i>C. pseudozanguebariatae</i> , <i>C. sessiflora</i> , <i>C. racemosa</i>
Lashermes et al. (1997)	Mitochondria PCR-RFLP (V7 rDNA) ITS 1 and 2 region of nuclear ribosomal DNA	26 <i>Coffea</i> taxa, 3 <i>Psilanthus</i> taxa Total = 37	Monomorphic in all the species. ITS 2 region informative. Intra-species sequence variants were observed.	– Strong geographical correspondence among the clusters.

(Continued)

Table 5. (Continued)

Study source/ Reference	Technique	Species assessed	Important results	Groupings and relationship
Cros et al. (1998)	<i>TrnL-trnF</i> intergenic spacer of chloroplast DNA	23 <i>Coffea</i> taxa and 2 <i>Psilanthus</i> taxa	Low sequence divergence (only 20 types obtained). No difference between <i>C. canephora</i> , <i>C. congensis</i> and <i>C. breviceps</i> and among <i>C. engenioides</i> , <i>C. arabica</i> and <i>C. sp.</i> Moloundou.	Strong geographical correspondence among the clusters. Supports radial mode and recent origin of <i>Coffea</i> taxa in Africa.
Saini et al. (2000)	RAPD (20 primers), ISSR (10 primers)	15 <i>Coffea</i> spp., 4 <i>Psilanthus</i> spp. Individual as well as pooled samples	High diversity across different species	Well resolved species clusters showing correspondence to their botanical types, as well as geographical origin; <i>C. kapakata</i> was indicated to be a <i>Coffea</i> sp. rather than belonging to <i>Psilanthus</i> or <i>Psilanthopsis</i> genera. Four endemic paracoffea species appeared as a distinct cluster under related genus <i>Psilanthus</i> .

to genetic uniformity (Lashermes et al., 1996d). In addition, many of the present day cultivars are suggested to have originated from single mutations having greater effect (Krug and Carvalho, 1951). All these factors suggest a narrow genetic base of arabica, which is largely vindicated by DNA markers based assessment of coffee germplasm. The first ever study to this end (Lashermes et al., 1993) using PCR based RAPD markers revealed that while there were sufficient inter-specific variations between arabica and canephora, there was almost no detectable variation within the few arabica accessions analysed in the study. Subsequently, a large number of studies (Table 4) carried out using molecular markers viz., isozymes, RFLP, RAPD, AFLP to detect variability in arabica and robusta coffee germplasm revealed:

- a) Invariably a very low level of variability in cultivated arabica, which could be detected only when a very large number of markers were used for analysis;
- b) Suitability of molecular approaches like RAPD and AFLP (that provided relatively high multiplex ratios) to resolve the subtle variation in arabica germplasm compared to approaches like isozymes and RFLP that generally failed to detect the same (Berthou and Trouslot, 1977; Lashermes et al., 1996a; Lashermes et al., 1999);
- c) Considerably high genetic variability in the *C. canephora* and *C. congensis* germplasm compared to arabicas (Lashermes et al., 1993, Prakash et al., 2005), which could be detected rather easily even by RFLP analysis (Lashermes et al., 1999);
- d) Relatively higher genetic diversity in the wild collections of arabica than cultivated ones, but much less than that seen for the diploid robusta germplasm (Lashermes et al., 1996d; Orozco-Castillo et al., 1996; Lashermes et al., 2000a; Anthony et al., 2001, 2002; Chaparro et al., 2004; Moncada and McCouch, 2004; Aga et al., 2005);
- e) Arabica cultivar like HdeT or its derivatives exhibit more diversity than cultivated as well as wild arabica type (Lashermes et al., 2000a); it is possibly due to the genetic introgression that probably happened during natural hybridisation event underlying HdeT evolution;
- f) Multiplex markers like AFLPs could distinguish and group different accessions belonging to six arabica cultivars (Bourbon, Catimor, Catuai, Caturra, Mokka hybrid, Typica) based on their origin and pedigree but without any cultivar specific signatures (Steiger et al., 2002);

In recent years, with the development and availability of coffee specific SSR markers (Table 3), the same are now also being tried for coffee germplasm characterization. The few studies carried out using a limited number of SSR markers while in line with the earlier observations of low variability in cultivated arabica compared to wild arabica accessions and diploid coffee germplasm, but expectedly and desirably demonstrate the SSRs utility over other marker approaches in their ability to more efficiently detect the inherent low variability of arabicas (Aggarwal et al., 2004a, 2004b, 2007; Baruah et al., 2003; Bhat et al., 2004; Moncada and McCouch, 2004).

The efforts to date, thus, demonstrate that PCR based DNA markers can be used reliably for identification and ascertaining the genetic diversity in the available cultivated and wild exotic germplasm of coffee, and that the microsatellites are the most efficient and desirable markers for the purpose. Nevertheless, the experience from these studies also highlights the overall low efficiency of the molecular marker approaches for coffee genepool characterization. The data clearly establish that despite their high-genetic resolution, DNA typing need to be carried out at large scale to resolve the low variation inherent in the coffee genepool especially of arabica, thus making the whole exercise resource intensive and practically non-viable. The later constrain warrants development of ways and means to increase the efficiency of DNA markers based expensive but unavoidable approaches for coffee genetic analysis. An easy way to achieve this can be creation of molecular data banks enumerating DNA marker (approach) efficiencies and more importantly, DNA polymorphism status (using defined markers/guidelines) for the analyzed germplasm, for use as reference resource (Aggarwal et al., 2004b). Based on our experience, we propose construction of 'Reference DNA polymorphism databases' of elite coffee germplasm available world-wide using standard repeatable markers (such as microsatellites) that can then be used by the coffee geneticists/breeders community for various advantages namely, for: a) better management, utilization, registration and IPR protection of elite coffee germplasm; b) selection of suitable material/genotypes and informative DNA markers for breeding and linkage analysis; c) exchange of germplasm, etc.

4.2. Genetic Diversity in Secondary Genepool of Coffee

Secondary genepool of coffee comprises >100 related diploid species of *Coffea* and *Psilanthus*, which represents an immensely valuable storehouse for the desirable genetic variability/gene(s) of interest needed for genetic improvement of the cultivated coffee. This potential assumes even more significance, especially considering that: different coffee species interbreed freely and relatively easily with high levels of gene flow (Berthaud and Charrier, 1988; Louarn, 1993), and there exists considerable genetic diversity in this pool (Berthaud and Charrier, 1988; Lashermes et al., 1993; Orozco-Castillo et al., 1996; Saini et al., 2004, our unpublished data). But to exploit this enormous secondary genepool for genetic improvement of coffee, there is a need for comprehensive studies on genetic characterization that can reveal its genetic base as well as broad generic affinities among/across populations and species. This task can best be accomplished using DNA marker approaches that provide high-genetic resolution and thus have become central to the germplasm diversity studies. Accordingly, even in case of coffee the DNA markers have now been used increasingly to characterize the related wild genepool.

4.3. Phylogenetic Studies for Species Relationship using DNA Markers

More than 100 species have been described under the genus *Coffea* mainly based on differences in morphology, size and ecological adaptations. Interestingly, most of these species can interbreed freely suggesting them to be of relatively recent origin, and also the possibility of many of these purported species may actually be natural hybrids rather than true type species. The situation warrants systematic, comprehensive studies using high-resolution genetic approaches as possible by DNA marker analysis for deciphering interspecific relationships and redefine some of the coffee species/taxonomy. Some such studies carried out in recent years are summarized in Table 5 (few also in Table 4). As becomes evident from these studies, a number of DNA marker approaches (viz., evolutionary informative DNA signatures of organelle genomes (cp-DNA, mt-DNA) and/or ITS (Internally Transcribed Spacer) region of the nuclear 5S rDNA, and nuclear length polymorphism based markers like RAPDs, AFLPs and ISSRs, have been tried (generally on a limited number of coffee species/accessions) with varying success. Briefly, the results from these studies show:

1. Low polymorphism in organelle DNA providing poor resolution of species relationships, but providing support for monophyly of *Coffea* genus, and also suggesting *C. eugionoides* as possible maternal progenitor of *C. arabica* (Berthou et al., 1983; Lashermes et al., 1996c, Orozco-Castillo et al., 1996, Cros et al., 1998).
2. ITS region is phylogenetically more informative than organelle DNA signatures for deciphering species level divergence among *Coffea* species (Lashermes et al., 1997, Aggarwal, 2005), but needs to be evaluated more thoroughly for its overall potential. Lashermes et al. (1997) using only its sub-domain ITS2 specific polymorphism could resolve relationships of 26 coffee species, but also encountered confounding intra-species specific variation (almost similar to seen in the inter-species comparisons). Contrary to this, our detailed studies suggest that to obtain confident assessment of coffee species relationships one need to consider the complete ITS region polymorphism rather than a part of variation across its sub-domains ITS1 or ITS2 (Aggarwal, 2005).
3. Compared to the domain specific sequence signatures, length polymorphism based DNA markers like RAPD, AFLP, SSR and ISSR (which generally mirror the nuclear genomic variation), provide more resolution and relatively consistent results about the species relationship between different *Coffea* species. Most of these studies broadly support the conventional coffee taxonomic relationships and/or strong indicate geographical correspondance (Lashermes et al., 1993, 2000b; Orozco-Castillo et al., 1996; Saini et al., 2000; Steiger et al., 2002; Moncada and McCouch, 2004; Aggarwal, 2005; Aggarwal et al., 2007; see Tables 4 and 5).
4. In addition, our empirical findings involving comparisons of taxonomic interpretations of 19 species of *Coffea* and *Psilanthus*, based on DNA polymorphisms (across evolutionarily informative sequences viz., intergenic *trnL* regions of cp-DNA, 16S rDNA of mt-DNA, and nuclear ITS region, as well as, mobility

based nuclear markers- RAPD, ISSR and genomic-/EST-SSRs), representing each of the 3 genomic compartments of the cell (chloroplast, mitochondrion, nucleus) strongly suggest that organelle genomes may not be ideal indicators of evolutionary/taxonomic relationships of coffee species (Aggarwal, 2005). We expect this apparent failure of the organelle DNA to be due to the relatively easy inter-species crossing which is known to happen in natural conditions rather frequently across diploid coffee species.

The above studies amply demonstrate the potential of DNA polymorphism based analysis for understanding coffee systematics and phylogeography, but also brings in focus the need for devising standard guidelines and choice of suitable DNA markers/signatures for robust and reliable inferences. Some of the major considerations that need to be addressed are: a) whether organelle DNA/genomes provide suitable targets for coffee taxonomic studies; b) what and how many samples should be used to represent a given coffee species; c) among different nuclear markers (RAPD, AFLP, ISSR, SSR), which are the most suited; and d) finally how many markers should one use to obtain high level of confidence in resulting interpretations.

4.4. DNA Markers to Assess Gene Flow and Introgression into Cultivated Coffee

Inter-specific hybridisation and production of natural fertile hybrids are well documented in coffee (Louarn, 1993; Charrier, 1978) between and across ploidy barriers. Many of such natural hybrids, especially of arabica types, contain desirable traits that are expected to have resulted as a result of gene flow from the diploid coffee species surpassing ploidy barriers. Some such hybrids like HdeT and S-26 have been widely used in rust resistance breeding with successful development of few improved introgressed arabica cultivars showing some rust resistance, like Catimor, Sarchimor, S-26 (Bettencourt, 1973; Sreenivasan, 1986; Sreenivasan et al., 1993; Charrier and Eskes, 1998). However, identification of the original coffee sources of desirable rust resistance gene(s) and their introgression in *arabica* coffee has remained a matter of speculation and interest for long. Only recently using DNA markers based analysis, it has become possible to answer some of these important questions. AFLP analysis of HdeT and introgressed arabica cultivars (Catimor, Sarchimor) has suggested *C. canephora* as the putative source (Lashermes et al., 2000b). Similarly, AFLP typing of S-26 (putative natural spontaneous hybrid identified in India), its derived resistant Indian arabica variety S 795 and related breeding materials have indicated *C. liberica* as the source for putative resistance factor SH-3 (Prakash et al., 2002). Both these studies provide support to the theory that gene flow/introgressions from diploid coffee species to tetraploid *C arabica* might have happened quite often leading to successful gene flow to the cultivated coffee genepool, which in turn also suggest the practical feasibility and scope for attempting systematic interspecific hybridisation program for gene introgression and coffee improvement.

4.5. Origin and Evolution of *Coffea arabica* as Deciphered from DNA Markers

The evolution of arabica coffee has always been an intriguing puzzle for the researchers because it is the only autogamous and tetraploid species in the *Coffea* genus. The classical cytogenetic studies suggest that it might have originated from coupling of unreduced gametes from a cross involving *C. eugenioides* and any one of the *C. canephora*, *C. congensis*, and *C. liberica* (Cramer, 1957; Carvalho, 1952; Narasimhaswamy, 1962), followed by genetic regulation of synapsis helping in the progressive diploidisation and evolution of present day amphidiploid *C. arabica* from the archetype tetraploid intermediate (Charrier and Berthaud, 1985). However, meiotic pairing in interspecific hybrids generated from *C. eugenioides* and *C. canephora* was found better (Louarn, 1976) than in dihaploid plants of *C. arabica* (Mendes and Bachhi, 1940; Berthaud, 1976), suggesting possibility of chromosomal re-patterning after the polyploidization event. The recent DNA based studies using conserved regions from nuclear and/or chloroplast genome have not only substantiated some of the above conclusions but also provided better understanding of the evolution and origin of arabica coffee. The trnL-trnF intergenic spacer of cp-DNA of *C. arabica* was found similar to that of *C. eugenioides* and *C. sp.* Moloundou suggesting that the maternal species of *C. arabica* could be any one of these or a similar taxon (Cros et al., 1998). On the other hand, analysis of the ITS2 spacer sequences of nuclear 5S rDNA domain (Lashermes et al., 1996a; Lashermes et al., 1997) suggested one of the canephoroid species (*C. canephora*, *C. congensis* and *C. brevipeps*) as the other progenitor species, and possibility of genomic repatterning (rDNA homogenisation via elimination of sequence specific to the other putative maternal progenitor). Subsequently, using RFLP markers and GISH (genomic *in-situ* hybridisation) analysis Lashermes et al. (1999) meticulously demonstrated the presence of 22 chromosomes belonging each to canephoroid (C) and eugenioides (E) type genomes, and amphidiploid nature of *C. arabica*. Moreover, the observed RFLP allelic diversity across different genera (considering a divergence timeline of 5-25 MYA for the most common ancestor of coffee) suggested a relatively recent speciation of *C. arabica* over 1 MYA (Lashermes et al., 1999).

Despite the above progress in our understanding of the arabica evolution, there are many questions/speculations that need to be answered and/or validated. Some of these are: a) the extent and pattern of genomic reorganization, and whether the arabica genome is like a segmental allotetraploid; b) the underlying mechanism responsible for diploid behaviour- pairing specific gene(s) and/or structural differentiation of chromosomes; and c) the possibility of duplicate loci (one specific to each constituent genome) as indicated in some recent SSR based diversity analysis of arabica germplasm (Baruah et al., 2003; Bhat et al., 2005; Hendre, 2006). It is hoped that availability/use of the advance high genetic-resolution capable DNA based comparative genomic resources/tools (linkage maps, sequence databases, microarray chips etc.) would help provide answers to these intricate questions in near future.

4.6. Molecular Linkage Maps of Coffee

The utility of DNA marker based genetic maps in linkage studies and genetic improvement of crop species is now well established. The potential of such maps is expected to be even more in case of difficult plant species like, trees including coffee, where conventional breeding efforts are severely constrained due to lack of genetic markers, screening tools and long generation cycles (outlined in earlier section). It is expected that availability of dense DNA marker based linkage maps would greatly help in identifying marker tags/QTLs for gene(s) controlling different qualitative and quantitative traits of agronomical importance and of interest to coffee breeders, which in turn would promise MAS based accelerated breeding of improved coffee genotypes.

In recent years, a modest beginning has been made to generate molecular linkage maps of coffee with limited but promising success. Till date, five DNA marker based linkage maps have been developed using RAPD, AFLP, RFLP and SSR markers in different genetic backgrounds (Table 6). Four of these maps are of diploid coffee genome, represented by two *C. canephora* and other two interspecific maps. The first DNA marker based linkage map was constructed using RAPD and RFLP markers and double-haploid mapping population of a *C. canephora* clone IF-200 (Paillard et al., 1996). Subsequently, the same mapping population was used to develop another map wherein AFLP and SSR markers were also used (Lashermes, 2001). In general the studies revealed relatively low levels of mappable polymorphism, which seemingly led to the attempts involving the use of interspecific backcross (BC1) populations that were expected to be more informative, and/or use of more SSR markers. The first such interspecific map was developed by Ky et al. (2000) based on AFLP and RFLP marker analysis of the BC1 progeny $\{(C. pseudozanguebariae \times C. liberica \text{ var. dewevrei}) \times C. liberica \text{ var. dewevrei}\}$, and more recently a similar map has been developed using another interspecific BC1 population $\{(Coffea heterocalyx \times C. canephora) \times C. canephora\}$ and AFLP and SSR markers (Coulibaly et al., 2003b). These maps had more mapped markers with relatively better genome coverage than of *C. canephora*, and thus suggested the utility of the approach (inter-specific population). The studies also reinforce the potential and significance of the secondary genepool for basic, as well as, applied research on coffee genetics and improvement.

We at CCMB, have now developed a relatively well populated framework linkage map for cultivated diploid robusta, under the 1st 'Indian Initiative on Coffee Genomics', using a more practical alternative of using a pseudo-testcross population (Hendre, 2006). This first generation map (Figure 2) developed using a trait-specific (drought tolerance) mapping population has a total of 374 mapped markers (comprising 185 RAPDs, 118 AFLPs, 71 SSRs, and a moderate marker density of 3.3 cM centimorgans) spread over 11 major and 5 minor linkage groups. This is the most elaborate map of diploid coffee genome till to date, and also has a very large number of mapped SSRs. Thus, it is expected to be of great value in future studies on identification of QTLs for drought tolerance (one most important

Table 6. DNA marker based linkage maps available till date for coffee genome

Study Reference	Paillard et al. (1996)	Ky et al. (2000)	Lashermes et al. (2001)	Coulibaly et al. (2003b)	Pearl et al. (2004)
Species used for mapping	<i>C. canephora</i>	Interspecific	<i>C. canephora</i> P. ex Froehner	Interspecific	<i>C. arabica</i>
Parents	P. ex Froehner IF-200	{{(<i>C. pseudozanguebariae</i> X <i>C. liberica</i> var. <i>dewevrei</i>) X <i>C. liberica</i> var. <i>dewevrei</i> }	IF-200	{{ <i>Coffea heterocalyx</i> X <i>C. canephora</i> } X <i>C. canephora</i> }	Mokka Hybrid x Catimor Hybrid
Population	DH (n = 85)	BC (n = 62)	DH (n = 92), BC (n = 44)	BC (n = 74)	F1 (n = 60)
DNA Markers used	RFLP, RAPD	RFLP, AFLP	RFLP, RAPD, AFLP, SSR	AFLP, SSR	AFLP
No. of loci/ primers/ probes used to screen population	RAPD = 250 primers RFLP = 250 genomic probes and 75 cDNA probes	AFLP = 30 primer pairs RFLP = same as Paillard et al. (1996)	RFLP = same as Paillard et al. (1996) RAPD = over 90 primers AFLP = 6 primer pairs SSR = 18 primers	AFLP = 12 primer pairs SSR = 113 loci (Rovellie et al., 2000)+ 47 loci (Dufour et al., 2001)	AFLP = 288 primer pairs
No. of mapped loci	RAPD = 100 bands RFLP = 47 loci Total = 147	AFLP = 167 bands RFLP = 13 loci Phenotypic marker = 1 Total = 181	DH: RFLP = 36 loci RAPD = 11 bands AFLP = 97 bands SSR = 18 loci Total = 162 TC: RFLP = 32 loci RAPD = 6 bands AFLP = 53 bands SSR = 18 loci Total = 109 bands Total (DH) = 160	AFLP = 160 out of 188 polymorphic bands SSR = 29 out of 36 polymorphic loci Phenotypic marker = 1 Total = 190	AFLP = 464 bands Total = 464

(Continued)

Table 6. (Continued)

Study Reference	Paillard et al. (1996)	Ky et al. (2000)	Lashermes et al. (2001)	Coulibaly et al. (2003b)	Pearl et al. (2004)
Length of map (average marker density)	1402 cM (9.5 cM)	1,114 cM (6.15 cM)	1041 cM (6.5 cM)	1,360 cM (7.2)	1802.8 cM (3.9 cM)
%SD (bands and loci of polymorphic bands or loci)	RAPD = 20% RFLP = 12%	30%	DH = 44% TC = 14%	AFLP = 15.9% SSRs = 16.6%	25%
No. of linkage groups	15	14	11	15	31

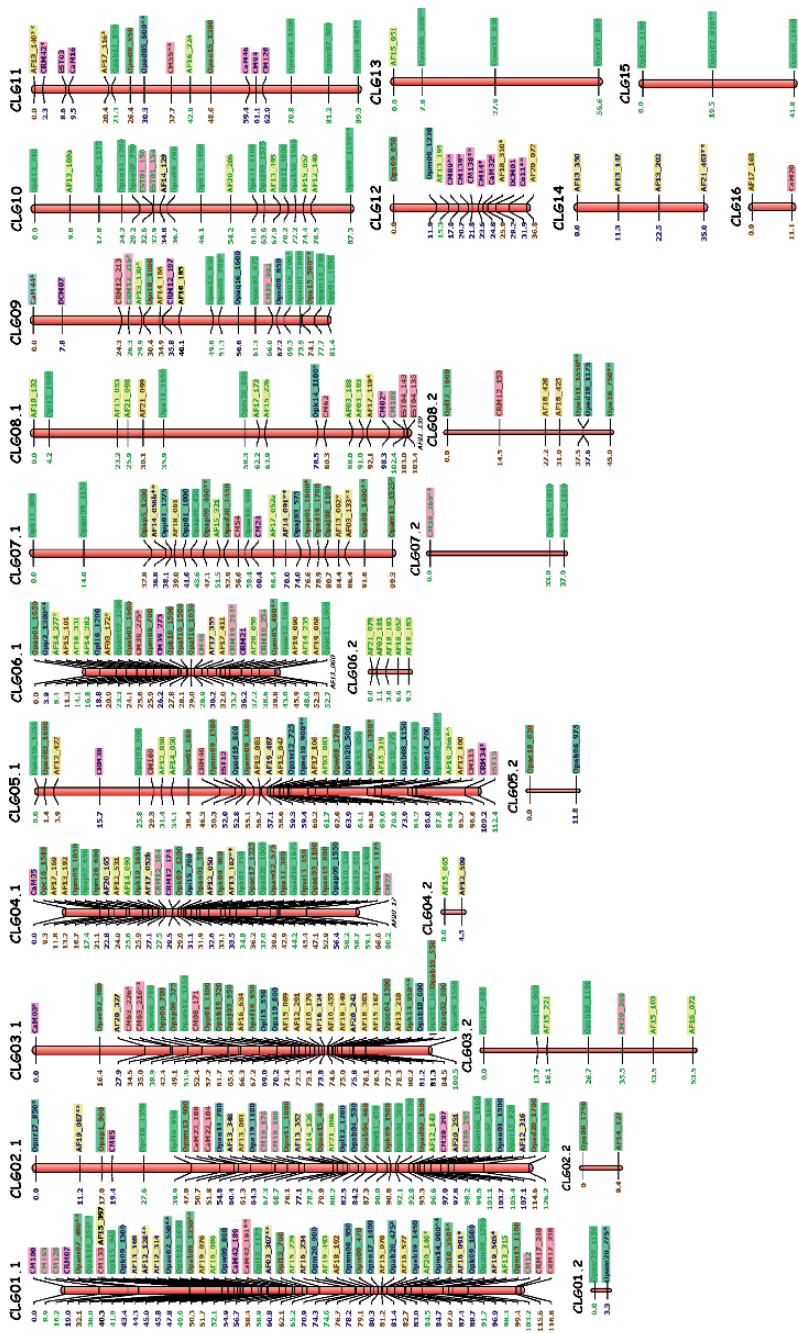


Figure 2. An integrated framework linkage map of robusta coffee (*C. canephora*) developed using a *pseudo-testcross* strategy and DNA markers (AFLP, RAPD, SSRs). The map comprises 11 major and 5 minor linkage groups, and has a total of 374 mapped markers. Prefixes Op-, AF- in the marker names indicates RAPD, AFLP respectively, while CM-/CR-/CaM- represents the mapped SSRs. The small groups shown below some of the major CLGs, represent the floating markers of the respective CLG placed in an 'Accessory group/map' (unpublished; Hendre, 2006)

trait of interest to breeders in India and elsewhere), and in realization of MAS based molecular breeding for accelerated robusta coffee improvement programs.

In contrast to diploid coffee, there had been very little progress in case of mapping of the tetraploid genome of arabica coffee. Till date only one AFLP based linkage map has been developed for arabica genome (Pearl et al., 2004) using a pseudo-F₂ population and pseudo-test cross approach (Grattapaglia and Sederoff, 1994). The length (ca. 1800 cM) and marker density (3.9 cM) of this map are significantly higher than observed for any of the diploid maps (that ranges from ca. 1000 cM to 1400 cM, with low to moderate marker density of 6.15–9.5 cM). But the map has a disadvantage that it is based on only AFLP markers, which although are good for map saturation, fine-gene mapping, but remain difficult to be uniquely characterized/identified and thus do not make ideal, stable, easily revisitable genomic landmarks like SSRs for use in subsequent studies/practical breeding schemes.

To our understanding, the most important hurdle in mapping arabica genome is its highly homogenous state with very little detectable variation at DNA level in the cultivated genepool (expected to be due to historical reasons mentioned in the beginning), which is further aggravated with it being a tetraploid. Thus, creating a useful reasonably saturated molecular/genetic reference linkage map of arabica coffee (which is of major interest to all coffee producing countries, including India) is going to be a mammoth task, which is not realistically feasible for any individual group/institute or probably even for any one country. Any worthwhile attempts to this end thus, warrant active interaction between global partners through international initiative like many other international programmes that have been started in recent years for generating mapping related resources for targeted crop plants, viz., International Tritiaceae Mapping Programme (ITMI), Genomic Network for Solanaceous crops (Sol; <http://www.sgn.cornell.edu/solanaceae-project/index.pl>), Loblolly Pines Genome Project (<http://dendrome.ucdavis.edu/lpgp/>), International Cotton Genome Initiative (<http://icgi.tamu.edu/>), International Grape Genome Project (<http://www.vitaceae.org/>), PROMUSA for banana improvement etc. The Department of Biotechnology, India along with the Coffee Board, India, initiated efforts to discuss the needs, feasibility, and possible mechanisms of extending the national initiative to a wider International platform for accelerating the coffee improvement efforts. These motivated efforts led to the 'Bangalore Declaration' in January 2004, followed by conceptualization and origin of a formal international group i.e. ICGI (International Coffee Genomic Initiative) in October 2004 in Bangalore during the ASIC2004 meet. ICGI had its first meeting in Paris, France in April 2005, which led to formalization of the International Coffee Genomic Network (ICGN; http://www.coffeegenome.org/about/launching_meeting.htm), wherein creation of saturated coffee maps with international participation have been identified as one of the major research priorities in immediate future.

4.7. Mapping of Quantitative and Qualitative Traits in Coffee

It is now well established that most of the plant traits of agronomical interest are quantitative, controlled by multiple genes that need to be judiciously manipulated to develop genetically improved germplasm, but hitherto were not accessible through conventional breeding approaches. With the development and availability of DNA marker based linkage maps for many animal and plant species, identification, mapping and selection of Quantitative Trait Loci (QTLs), have become practically feasible and achievable. The QTLs can be identified by monitoring mapped or unmapped DNA markers in conjunction with target trait using a segregating mapping population (Hackett, 2002), and/or diverse genotypes employing dis-equilibrium mapping (Gupta et al., 2005). Though having a molecular linkage map is not a prerequisite for linking a QTL, it is essential to map them for efficient selection and subsequent transfer of the trait. In case of coffee, QTL mapping is in its infancy with few successful efforts in recent years. In the first such study in coffee, few RAPD markers were linked with resistance to coffee berry disease (CBD) caused by *Colletotrichum kahawae* (Agwanda et al., 1997), using the indirect approach of diverse genotypes (5 susceptible and 8 resistant arabica cultivars/selections) and unmapped RAPD markers. The study revealed 24 candidate linked markers of which only three showed strong association with putative CBD resistance *T* gene (from Catimor) and proposed for use in marker assisted selection (MAS). On the other hand, Coulibaly et al. (2003a) could identify 3 fertility restorer QTLs using the partial AFLP based interspecific map. Similarly, QTL responsible for trigonelline content (an important compound responsible for the taste and flavour of coffee) was identified on the other interspecific linkage map developed by Ky et al. (2000). In addition, it is suggested that putative QTLs for caffeine, chlorogenic acid and sucrose content in diploid coffee (Ky et al., 2000), and for source-sink traits in arabica (Pearl et al., 2004), have been identified. In contrast to the above few reports on mapping of quantitative traits, there is one study wherein RFLP marker could be linked to self-incompatibility (validated to be a qualitative trait) on the robusta DH map (Lashermes et al., 1996b) and further validated on the intraspecific AFLP linkage map (Coulibaly et al., 2002).

Although few, the above reports are encouraging and give the hope that with the all increasing emphasis, excitement and international efforts on coffee genomics, elaborate coffee maps and markers would become available soon paving the way for identification of molecular tags/QTLs (for most of the important qualitative/quantitative traits of interest) and MAS based coffee breeding.

5. CONCLUSIONS

Coffee is a an important crop for the world socio-economy, which provides livelihood to the millions of people worldwide with sustenance of few third world countries solely dependent on coffee cultivation. Similar to other crop

plants, there is a continuous need for genetically improved coffee germplasm to meet the ever-changing demands of environment and markets, but it is also a difficult crop to conventional genetic improvement efforts. The problem is more acute in case of arabica coffee that for historical reasons suffers from a very narrow genetic base making genetic improvement more difficult to be realized. The constraints and barriers of conventional coffee improvement warrant newer powerful molecular approaches that can provide the requisite speed, efficiency, and directionality to breeding efforts. In this context, the DNA markers/technologies that provide high-genetic resolution becomes important and thus need to be integrated in research on genetics and improvement of coffee, which till recently was treated more like an orphaned plant species in context of molecular genomic work. It is fortunate that in recent years the realization has dawned on the coffee community worldwide, and modest but sincere efforts have been initiated that amply demonstrate the promise of the DNA markers/approaches in genetic studies on coffee. DNA analysis of the existing coffee germplasm resources indicate very low genetic diversity in cultivated genepool and highlight the need for exploitation of wild arabicas and the secondary gene pool (consisting of diploid species) for desired variability/gene(s) for coffee improvement. The potential of wild germplasm is exemplified recently by the discovery of 3 arabica plants devoid of caffeine in conventional breeding program that included explored arabica germplasm from Ethiopia (Silvarolla et al., 2004). Moreover, interspecies gene flow is widespread in coffee. Considered together, these suggest that exploration of extant germplasm/secondary genepool followed by conventional breeding approaches combined with advanced genotyping methods based on DNA markers, is a practically realistic proposition to boost the genetic improvement of coffee.

6. FUTURE PERSPECTIVES

Key for modern genetic improvement programmes includes application of DNA marker technology for characterization of desirable variability, construction of saturated molecular linkage maps with wider genome coverage and using robust, easily transferable markers like SSRs or SNPs. Availability of such resources would be the beginning for QTL mapping and application of Marker Assisted Selection in coffee. But to make this potential of DNA tools a reality, and also considering the major constraints and possibilities at global level the following activities need to be taken up on priority:

1. Development of good informative markers for genetic studies: Very few microsatellite markers, which are the most useful for genetic linkage studies, are available for coffee, and only a fraction of these are informative for elite arabica coffee germplasm (as evidenced in our work in India and similar studies worldwide). The situation thus necessitates the development of a very large number of microsatellite markers (many fold more than what have been developed till date) to have enough informative, usable markers for

rational deployment in genetic-linkage analysis/improvement programs of *Coffea arabica*.

2. Genetic diversity in coffee genepool and need for reference polymorphism panels: Enlarging the desirable genepool by concerted international efforts involving various research groups working on arabica coffee from different countries by: identification of sets of elite germplasm (being used or having potential for breeding programs) available with different groups worldwide to create a global resource of arabica expectedly defining the broadest genetic base; exchange of the identified germplasm resources (possibly in the form of DNA samples initially and later the shortlisted germplasm as per the need); DNA fingerprinting of germplasm using pre-defined standard protocols and robust DNA/SSR markers, by multiple labs to generate 'reference polymorphism panels', which can be shared to serve as a ready reference tool to identify most diverged, potentially useful germplasm for deployment in arabica breeding programs.
3. Molecular linkage mapping in coffee: This most important but also the most onerous activity, especially in case of arabica coffee, can best be achieved probably by: selecting/developing one or two common mapping population(s) using diverse elite parental genotypes, at mutually agreed center(s), followed by extensive DNA typing of the selected population(s) by multiple groups using different sets of markers (including coffee specific SSRs available at their end). This approach would ensure generation of enormous data in relatively short time, and expectedly would provide enough allelic polymorphism to develop a well spread molecular linkage map of arabica genome, which otherwise may remain a mirage.

It is hoped that the above would soon be feasible under the newly formalized International Coffee Genomics Network (ICGN), and would help create the wanting knowledge base and essential DNA resources paving a new and successful route to genetic improvement of coffee. Some immediate applications of these resources (information/tools) would be:

- Availability of rich repertoire of coffee specific PCR based efficient DNA markers would greatly facilitate genetic linkage analysis and germplasm characterization.
- Development of saturated reference molecular linkage maps would help fill the existing gaps in the coffee genetics, and tremendously help complement conventional genetic improvement efforts by the possibilities of MAS based molecular breeding. Availability of well-populated reference maps would also be useful for comparative genomic studies, involving comparisons with interspecific maps of coffee and many other species mainly of Solanaceous crops using anchor markers. Such studies can be useful to understand evolution of coffee gene(s)/genome, in finding synteny or even microsynteny relations with tomato or other Solanaceous (Cornell University) genomes, and utilizing genetic markers/tools developed under the international SOL Genomics network for coffee improvement.
- The success on the above fronts, in turn would make it much easier to identify DNA marker tags/QTLs linked to gene(s) of agronomic importance. Once estab-

lished, these would serve as efficient screening tools for difficult quantitative traits and selection of desirable segregating progenies in a breeding program, thus leading to considerable gains in time and less physical and financial resources.

As a result, coffee improvement may get impetus that will result in development of new coffee cultivars and varieties, which in turn may rejuvenate and lease a new life to the economics of world coffee. This will be helpful in providing proper livelihood and economic sustenance of the farmers as well as satisfaction to the coffee drinkers by having a good brew in their cup.

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CHAPTER 16

GENOMICS OF ROOT NODULATION IN SOYBEAN

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Abstract: Soybean is a suitable crop material for studying root nodulation and full genome sequencing because of its economic value. This review introduces the “nodulation” phenomenon that occurs in legume root systems such as the soybean. In addition, the paper identifies and discusses nodulation mutants (e.g., non-nodulation, ineffective nodulation, and super-/hypermodulation) and the genetic loci that control nodulation. The advent of genomics, proteomics, metabolomics, etc., has greatly contributed in improving our understanding of the symbiotic interactions between legume plants and Rhizobia, particularly for the identification of nodulation-related genes. Furthermore, molecular gene identification should be combined with biochemical pathways for nodulation in order to better understand the symbiotic interactions between legume and Rhizobia.

1. INTRODUCTION

The Leguminosae plant family is the third largest of the angiosperms, has spread to every continent, adapted to the tropics and the Arctic and comes in different varieties ranging from annual herbs to trees (Long 1989). *Rhizobium* and *Bradyrhizobium* are unique among microorganisms in their ability to develop nitrogen-fixing nodules on the roots of leguminous plants, which may be the most highly-evolved system for fixing nitrogen from the atmosphere.

Legume plants have two main sources of nitrogen including combined nitrate or ammonia nitrogen and biologically-fixed nitrogen from the atmosphere in the root nodule. As biological nitrogen fixation is of agronomic importance reducing the need for chemical nitrogen fertilizer for agriculturally-important crops such as

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soybean and alfalfa (Stacey et al. 2006), the utilization of nitrogen fixation in the root nodule is given considerable emphasis. Legume plants show a wide range of nodulation response: non-nodulation, ineffective nodulation, normal nodulation and super-/hypernodulation. In addition to the biological importance of these mutants for characterizing the molecular basis of symbiotic association, non-nodulating mutants can be used as a control to estimate the biological N_2 fixation. Furthermore, super-/hypernodulating mutants can reduce the nitrogen fertilizer requirements for crops, thereby reducing the impact of agricultural practices on the environment.

This paper deals with the genetic and molecular analysis of soybean symbiotic mutants. Emphasis is placed on the genetic and genomic components of the nodulating soybean mutant loci and the methods used in identifying the genes involved in nodulation.

2. NODULATION IN LEGUME AND SOYBEAN: PLANT-RHIZOBIUM INTERACTION

Many recent studies have focused on legumes rather than the model plant *Arabidopsis thaliana* because legumes show symbiotic relationships between plants and soil-borne bacteria and they have *Rhizobia* for nitrogen fixation (Sato and Tabata 2006; Shoemaker et al. 2006; Town 2006). In terms of genome full sequencing, the soybean draws more attention than other leguminous plants because of its economic importance in the symbiotic interactions between plants and *Bradyrhizobium* (Sutton 1983). During this symbiotic relationship, *Rhizobia* provide the host plant with nitrogen in the form of ammonia (as do the amides in the alfalfa and pea nodules and the ureides allantoin and allantoic acid in the soybean and cowpea nodules) inside the N_2 -fixing nodules on roots of leguminous plants; in turn, they receive carbohydrates and other compounds from the plant (Long 2001; Riely et al. 2004). This symbiotic nitrogen fixation is beneficial to the farmers because it leads to a significant decrease in the use of N-containing fertilizers (Riely et al. 2004; Stacey et al. 2006). External factors, such as light intensity, temperature, pH, soil moisture, etc., closely regulate the symbiotic formation of nodules (Lie 1974; Lee et al. 1998); in legume plants, the amount of nitrogen fertilizer also controls nodulation and nitrogen fixation. Externally-supplied nitrogen in the form of nitrate prevents symbiosis by inhibiting the infection process of the nitrogen-fixing bacteria (Carroll and Mathew 1990; Lee et al. 1998). Internally, this symbiosis is regulated by 'autoregulation' (Delves et al. 1986; Van et al. 2005; Lestari et al. 2005, 2006b), the primary functions of which are to control the number and growth of the nodules on the root; it functions by initiating the nodule primordial signal to the leaf, which produces a shoot-derived inhibitor for to restrict the progress of younger nodule primordia (Lee et al. 1991; Gresshoff 2003; Van et al. 2005).

The symbiosis between the soil bacteria and leguminous plants is characterized by a specific multi-step signal cascade (Israel et al. 1986); the first step is the release of flavonoids from the plant root to attract compatible *Rhizobia* (Schultze and Kondorosi 1998; Riely et al. 2004; Geurts et al. 2005; Stacey et al. 2006). Flavonoids

induce nodulation (*nod*) genes to encode enzymes for the synthesis of specific lipochitooligosaccharides nodulation signals (Nod factors) (Dénarié et al. 1996; Long 1996; Oldroyd et al. 2001). In turn, the nod factors trigger many initial steps of the signal transduction pathway for the root hair infection process after these factors are perceived by plant receptors (Riely et al. 2004; Stacey et al. 2006). Although flavonoid nod gene-induction is required for nodulation, precise nodulation can only occur if the nod genes are appropriately expressed quantitatively, spatially and temporally (Loh and Stacey 2003). The strong constitutive expression of the nodulation genes leads to defective, reduced nodulation phenotypes (Knight et al. 1986). These results indicate that the nod genes are involved in both positive activation and negative regulation of the *nod* genes (Cren et al. 1995; Fellay et al. 1998; Kondorosi et al. 1989). *Nol A* and *nod D2* are two key components in the negative regulation of the nodulation genes in *B. japonicum*. In addition, Loh and Stacey (2003) suggest that a key role it has multiple forms the *nol A* gene plays in allowing *B. japonicum* to nodulate its plant hosts. The Nod signal or components of the nod signal and the feedback regulated the nodulation genes of *B. japonicum* by the induction of *nol A* by chitin led to an increase of the repressor protein, *nod D2* (Loh and Stacey 2001). The nodulation genes are feedback-regulated by the nod signal, which contains either a tetrameric or a pentameric chitin backbone (Loh and Stacey 2003).

These nod factors initiate root hair curling and induce the formation of nodule primordia by activating the root cortical cell cycle; this is then followed by the bacterial infection into the root hairs by the infection threads (Schultze and Kondorosi, 1998; Stacey et al. 2006). When the infection thread reaches the cells of the developing primordium, the bacteria are released into symbiosomes and then are differentiated into bacterioids inside a plant cell (Schultze and Kondorosi 1998; Stacey et al. 2006). This microaerobic nodule environment is able to conduct nitrogen fixation because of the oxygen permeability carrier; inside the infected cell, an oxygen-binding protein, leghemoglobin, regulates and delivers oxygen (Lestari et al. 2005). Since differentiated structures of various nod factors and polysaccharides are present, host specificity is possible because of differences in the substrate specificity of the enzymes (Schultze and Kondorosi, 1998). Thus, high host specificity is a very important control for the interaction between plants and soil borne-bacteria, which include *Rhizobium*, *Mesorhizobium*, *Azorhizobium*, *Sinorhizobium*, and *Bradyrhizobium* (Schultze and Kondorosi 1998). Consequently, the formation of effective soybean nodules is a complex and highly-regulated process that requires the production and exchange of specific molecular signals between the host plant and the bacterial symbiont (Kosslak et al. 1987).

Among the signal components for symbiotic relationships, in nodule development ethylene is a negative regulator in the premature arrest of Rhizobial infection in pea (Lee and LaRue 1992), *Lotus*, and *Siratro* (Nukui et al. 2000). It has been suggested that aminoethoxyvinylglycine (AVG) or Ag⁺, which are inhibitors of ethylene formation and perception, might stimulate nodule production in *Medicago truncatula* roots (Schmidt et al. 1999); however, ethylene does not always act as a

nodule inhibitor in every legumes. Treatment with AVG and exogenous ethylene did not inhibit nodulation in soybeans although ethylene production was increased by infection by *B. japonicum* (Lee and LaRue 1992). In addition, Schmidt et al. (1999) compared the nodule development in soybean mutants that were either less responsive to ethylene or deficient in the usual number of nodules having Ag⁺. Neither the number of nodules nor ethylene sensitivity differed from the wild type, which indicates that ethylene is not significant in regulating soybean nodule formation. Thus, the feedback regulation is the other component in the signal cascade in the soybean nodule development by suppressing the development of younger nodules by the older nodules (Bhuvaneswari et al. 1981; Pierce and Bauer 1983).

3. NODULATION MUTANTS IN SOYBEAN

3.1. Isolation of Nodulation Mutant

The nodulation mutants of soybeans have been obtained either from the normal soybean population or from ethylmethane sulfonate (EMS), sodium azide or gamma irradiation mutagenesis (Carroll et al. 1985a, 1985b; Gresshoff and Delves 1986; Vance et al. 1988). Although both gamma rays and sodium azide mutagenesis increased the mutation frequency for chlorophyll deficiency, EMS treatment caused a 10-fold increase in the frequency of mutation. The M₂ generation of 'Bragg' seed that was mutagenized with EMS showed a chlorophyll deficiency of about 2.8% (Gresshoff and Delves 1986). The isolated mutants showed three major phenotypes: non-nodulation, ineffective nodulation and super-/hypernodulation.

Non-nodulating mutants have been isolated in several legume species including peas (Kneen and LaRue 1984, 1988; Borisov et al. 2003), chickpeas (Davis et al. 1985), common beans (Davis et al. 1988) and sweet clover (Kneen and LaRue 1988). In model legume plants, the *nodule inception* (*nin*) mutant of *L. japonicus* and *M. truncatula* hair curling (*hcl*) mutants were identified, and these mutants showed impaired nodule development or the upstream of signal transduction pathway for symbiotic interaction (Schauser et al. 1999; Catoira et al. 2001). Other non-nodulating model legume mutants were isolated by Stacey et al. (2006). A line with naturally-occurring, non-nodulation was also reported in soybeans (Williams and Lynch, 1954), and the non-nodulation trait was conditioned by a single recessive gene (*ry*). Root hair curling occurred, but infection threads did not develop in this mutant (Tanners and Anderson 1963). A soybean mutant, screened by Clark (1957) exhibited no nodulation in soil cultures but did exhibit occasional and irregular nodulation in sand-nutrient solution culture. Mathews et al. (1987) isolated three non-nodulation mutants (nod 49, nod 139 and nod 772) from Bragg after the mutagenesis of seeds with EMS and selected for non-nodulation in the M₂ in the presence of *B. japonicum* USDA 136. Mutants nod 49 and nod 139 lacked curled root hairs when inoculated with *B. japonicum* USDA 110; the mutant nod 772 showed occasional hair curling. Complementary tests indicated that nod 49 and nod 772 were allelic to *ry*₁, but nod 139 was not (Delves et al. 1988).

Three possible reasons for the occurrence of non-nodulation in these mutants were suggested by Gresshoff and Delves (1986). First, the non-nodulation mutants had altered production of substances in root exudates. These root exudates interact with the *nod D* gene product to behave as an activator of the nodulation genes in the nitrogen-fixing bacteria. Second, these mutants may be less sensitive to the bacterial signal during the infection processes. Third, there was a strong active defense response of these mutants against bacterial infection (Gresshoff and Delves 1986).

Ineffective nodulation of soybean was conditioned by dominant alleles. The *Rj₂*, *Rj₃*, and *Rj₄* genes showed ineffective nodulation with specific *Bradyrhizobium* strains. All of these dominant alleles condition the formation of small white nodules (Gresshoff et al. 1987). Non-nodulation and ineffective mutants that were defective in nodule formation are useful for understanding interactions between the host and bacteria. In addition to study the underlying mechanisms of symbiosis, these mutants can be also used as controls for measuring the contribution of symbiotic N₂ fixation to the total N economy of plant (Gresshoff et al. 1987).

Super- or hyper-nodulating mutants with enhanced nodulation and partial tolerance of nodulation in the presence of high levels of exogenous NO₃ have been developed in several legume crop plants such as peas (Jacobsen and Feenstra 1984), white beans (Park and Buttery 1988) and soybeans (Carroll et al. 1985a, 1985b). *L. japonicus* also has a hypernodulating mutant, *sym78* mutant *hypernodulation aberrant root1-1* (*har1-1*) encoded an LRR-RLK, which was characterized by high homology with CLAVATA1 (Krusell et al. 2002; Nishimura et al. 2002). *M. truncatula* super numeric nodules mutant also encoded SUNN proteins similar to HAR1 proteins (Schnabel et al. 2005). While limited information is available for peas and white beans, supernodulation has been well characterized in soybean (Carroll et al. 1985a, 1985b; Gresshoff and Delves 1986; Gresshoff et al. 1987; Searle et al. 2003).

Several groups of scientists have independently isolated super- or hypernodulating soybean mutants by using chemical mutagenesis from Bragg (Carroll et al. 1985a, 1985b), Williams (Gremaud and Harper 1989), Enrei (Akao and Kouchi 1992) and Sinpaldalkong 2 (Lee et al. 1997). The mutagen EMS has been commonly applied to produce several super-/hypernodulating mutants. More recently, an induced mutagenesis using fast neutron resulted in a supernodulating soybean mutant (Men et al. 2002). Supernodulating soybean mutants produce ten times the number of nodules as wild types, whereas hypernodulating mutants have two to five times as many nodules as wild types and exhibit a nodulation between supernodulation and wild-type nodulation (Kim et al. 2005). The contrast in nodulation responses between the super-/hypernodulating mutants and the wild type from which they are derived provides unique biological material for the study of host plant factors, which may be involved in the nodulation process.

Suppression of nodulation in the younger regions of the root caused by prior inoculation of more mature regions of the root has been referred to as an autoregulatory response (Pierce and Bauer 1983). However, the autoregulatory response governing nodulation was not present in supernodulating soybean mutants (Olsson

et al. 1989). Thus, the mutated gene of the *nts* mutant may be the same gene or closely linked to the gene that altered the autoregulatory signal. A general model for the autoregulation of nodulation was proposed based on the presence of a symbiotic nodulation inhibitor in the wild type (Gresshoff and Delves 1986). In wild type plants, the shoot produces a signal after it is stimulated by the root-derived compound, which is a product of nodule meristematic center. The shoot-derived signal acts as an inhibitor of symbiotic development at the continued cell division stage. The supernodulating mutant was thought to lack the ability to produce shoot-derived inhibitors.

Genetic studies and linkage mapping for these supernodulating mutants revealed that the mutated locus is located on linkage group (LG) H and contains separate alleles (Landau-Ellis et al. 1991; Kolchinsky et al. 1997). More recently, map-based cloning with markers pUTG-132 and UQC-IS1, which flank *NTS-1* on LG H, was performed to identify a gene conferring nodulation autoregulation (Searle et al. 2003). The expression of this gene in shoots is responsible for controlling the nodule meristem proliferation and communicating with distant nodules; thus is called the *Glycine max* nodule autoregulation receptor-like protein kinase (*GmNARK*) gene. Nonsense and missense mutations that comprise single-base changes on the gene were found to lead to supernodulation and hypernodulation phenotypes in the mutants (Searle et al. 2003).

3.2. Genetic Loci Controlling Nodulation in Soybean

Even though much is known about the bacterial contribution to the symbiotic nodulation of legumes, knowledge of the host contribution has thus far received less attention in the identification of the plant genes involved in nodulation. A few dozen of genes are assumed to be involved in nodulation and N_2 fixation, and range from host plant recognition to nodule formation. Since the nodulation trait has been recognized as governed by qualitative genes, classical genetic approaches have generally been applied to identify those genes. As mentioned above, several genetic loci for nodulation in soybean have been found by using the chemical mutants altered in the level of nodulation. William and Lynch (1954) first reported the recessive gene *ry*₁ in a spontaneous non-nodulating mutant that controls a restriction of nodulation with almost all *Bradyrhizobia* (Table 1). Since then, a serial of the *ry* loci from *ry*₂ to *ry*₇ have been identified as responsible for ineffective or redundant nodulation (Table 1). Three dominant genes, *Rj*₂ (Caldwell 1966), *Rj*₃ (Vest 1970), and *Rj*₄ (Vest and Caldwell 1972) controlled strain-specific restricted nodulation, which possessed genetic independence (Devine and O'Neill 1986). Mapping the genes that contribute to this phenotype change in nodule formation was conducted to discover which genes were altered resulting in non-nodulation (Matthews et al. 2001). The non-nodulation genes *ry*₁, *Rj*₂ and *Rj*₄ were mapped on separate linkage groups (LG D1b+CLG11, LG J and GmEST-BFM) depending on search results of the breeder's toolbox at SoyBase (<http://soybeanbreederstoolbox.org>), however, the *Rj*₃ gene has not yet been mapped. In the early stages of nodulation, the non-nodulation genes in soybeans may be involved in recognition of host plant to bacterial infection because

Table 1. List of the nodulation genes defined in soybean

Gene	Function	LG	References
<i>ry</i> ₁	Non-nodulation	D1b+CLG11	http://soybeanbreederstoolbox.org/
<i>Rj</i> ₂	Non-nodulation	LG J	Lohnes et al. 1993
<i>Rj</i> ₃	Non-nodulation	ND ¹⁾	–
<i>Rj</i> ₄	Non-nodulation	GmEST-BFM	Matthews et al. 2001
<i>Rj</i> ₅	Normal nodulation	ND ¹⁾	–
<i>ry</i> ₆	Non-nodulation	ND ¹⁾	–
<i>ry</i> (<i>nts</i>)	Super-/hypernodulation	LG H	Kolchinsky et al. 1997

¹⁾ Not determined

they condition specificity or susceptibility of certain strains of *B. japonicum*. The additional *ry*₆ non-nodulation gene has been proposed in the two mutants, nod 139 and NN 5, which were chemically mutagenized from William (Pracht et al. 1993), while the *Rj*₅ locus was symbolized as a normal nodulation of Harosoy 63. In contrast, the recently described locus *ry*₇ has been designated as super-/hypernodulation of nod-type mutant and the En 6500 mutant (Vuong and Harper 2000). This locus should be same as *nts* mutant locus derived from mutagenized Bragg. Nevertheless, few molecular studies on soybean mutants that are defective in nodule formation have been performed, which contrast with studies on the legumes, *L. japonicus*, *M. truncatula* and *P. sativum* (Stacey et al. 2006).

Unlike *ry*-type loci, extensive studies have been performed on the other locus *nts* involved in excessive nodule proliferation (Table 1). A remarkable achievement in positional cloning of the *nts* gene conferring super- or hypernodulation was accomplished by Gresshoff and his colleagues (2003); *nts* was the first gene that was isolated in the soybean. This gene, *GmNARK*, is responsible for autoregulation of nodule formation.

3.3. Application of Molecular Markers for Enhancing Nodulation

The evaluation of nonsense and missense mutations of the *GmNARK* gene led to supernodulation and hypernodulation mutants (Searle et al. 2003). The single nucleotide polymorphism (SNP) of *GmNARK*, which is between supernodulating mutant SS2-2 and wild-type Sinpaldalkong 2, was converted into specific PCR marker (Kim et al. 2005). The sequence mutation of A→T in *GmNARK* between SS2-2 and Sinpaldalkong 2 directly affect phenotypic variations of nodulation. SNP markers that are directly linked to a specific trait would be a very useful DNA marker and provide the advantages of complete linkage with no error in selection.

It is becoming clear that SNPs have great potential as genetic markers to directly map important genes and facilitate selecting desirable traits, or excluding deleterious traits in crop breeding (Kim et al. 2004). Nodulation traits in the soybean are difficult to assay phenotypically because of their subterranean nature and the requirement to inoculate with *Bradyrhizobia*. Thus, the single nucleotide amplified

polymorphic (SNAP) marker for supernodulation is able to distinguish between normal nodulation and supernodulation in both the cross populations of SS2-2 and soybean genotypes (Kim et al. 2005). Marker-assisted selection of this SNAP marker to improve the efficiency of nodule trait selection is widely applicable in soybean breeding activities including parent assessment, recovery of recurrent parent in backcrossing and trait selection in single seed descendents at early generation.

4. GENE EXPRESSION PROFILING FOR NODULATION

Classically, nodulating plants were biochemically and physiologically analyzed by examining the mechanisms of carbon supply, nitrogen fixation, ammonia assimilation and nitrogen transport, such as the expression of leghemoglobin (Gresshoff 2003). As molecular techniques were developed, differential screening was widely adopted to survey gene expression profiling with cDNA libraries (Table 2). Mylona et al. (1995) identified early nodulins and late functions that are associated with nitrogen fixation, such as leghemoglobin, glutamine synthetase and uricase. Nodule-specific cDNAs that are shown to be induced or enhanced during the first days of the symbiotic interaction were also identified by means of differential display (Goormachtig et al. 1995). Other methods of differential screening, such as differential hybridization, subtractive hybridization, differential display and EST and cold-plaque screening were used to study nodulation researches with various leguminous host plants. With the advantage for surveying tissue specificity, various plants could be screened for early nodulation-related genes (see Lievens et al. 2001). Gene expression patterns that were identified by differential screening were confirmed by using northern blot analysis, RT-PCR, RNase protection assay or quantitative RT-PCR for lower expression levels (Lievens et al. 2001; Gresshoff 2003).

The cDNA-amplified fragment length polymorphism (cDNA-AFLP) approach was used to study differential gene expression of nodulation (Simoes-Araujo et al. 2002; Kistner et al. 2005; Lestari et al. 2006a). Compared to differential display, this gel-based method could overcome problems such as low reproducibility, difficulty in detecting rare transcripts and generation of false positives (Bachem et al. 1998). The cDNA-AFLP approach was also used to differentially discriminate genes expressed on a large scale because direct side-by-side comparison among samples is possible, and prior sequence information is not required (Kistner et al. 2005; Lestari et al. 2006a). Simoes-Araujo et al. (2002) surveyed the expression profiles from cowpea nodules during heat stress. From about 600 bands, 55 transcripts-derived fragments (TDFs) were up-regulated and nine were down-regulated; they show similarities to wound-induced proteins, disease resistance protein, etc. Using cDNA-AFLP, 344 differentially-expressed genes were detected from about 7000 TDFs generated from *L. japonicus* roots (Kistner et al. 2005). Approximately 2% of the TDFs were up-regulated during the arbuscular mycorrhiza symbiosis, and a ser-protease and a cys-protease were activated during root nodule development. More specifically, 65 TDFs from Sinpaldalkong 2 (wild type), and 40 TDFs from SS2-2

Table 2. Lists of gene expression profiling in nodulation research

Methods	Species	References
Differential screening	<i>Sesbania rostrata</i> (Differential display)	Goormachtig et al. 1995
	<i>S. rostrata</i> (Differential display)	Lievens et al. 2001
	Various species	Lievens et al. 2001
cDNA-AFLP	Cowpea	Simoës-Araujo et al. 2002
	<i>L. japonicus</i>	Kistner et al. 2005
SAGE	Soybean	Lestari et al. 2006a
	<i>L. japonicus</i>	Asamizu et al. 2005
Microarray	<i>L. japonicus</i>	Colebatch et al. 2002, 2004
		Kouchi et al. 2004
		El Yahyaoui et al. 2004
		Hohnjec et al. 2005
		Lohar et al. 2006
		Küster et al. 2004, 2006
		Mitra and Long 2004
		Mitra et al. 2004
		Starker et al. 2006
		Maguire et al. 2002
		Lee et al. 2004
		Fedorova et al. 2002
		Tesfaye et al. 2006
In Silico transcriptome	<i>M. truncatula</i>	Morris and Djodjevic 2001
		Bestel-Corre et al. 2002
Proteomics	Clover	Mathesius et al. 2001, 2002, 2003
		Rolfe et al. 2003
	<i>Melilotus alba</i>	Mathesius et al. 2002
		Saalbach et al. 2002
	Pea	Hwang, C.H. (personal communication)
		Panter et al. 2000
	Soybean	Wan et al. 2005
		Winzer et al. 1999
	<i>Trifolium subterraneum</i>	Mathesius et al. 2002
		White sweetclover

(supernodulating mutant) were differentially expressed in response to *B. japonicum* symbiotic relationships (Lestari et al. 2006a). Interestingly, *GmNARK*, which was previously known as involved in autoregulation of nodulation, was detected by this study, thus confirming cDNA-AFLP as a powerful method for identifying interesting genes that had no prior assumptions.

Serial analysis of gene expression (SAGE) was also used to compare the gene expression patterns between roots and nodulating roots in *L. japonicus*; it should be mentioned that unequivocal gene identification through SAGE may be cumbersome because the technology is based on a 14-base pair tag sequence (Asamizu et al. 2005). Unlike cDNA-AFLP, SAGE was only deployed in the model

plants *Arabidopsis* and rice because neither EST nor genome sequences from these organisms were available. To model leguminous plants, *L. japonicus* was investigated for the transcript profiles because of the EST sequencing availability in the public databases as well as portions of the genome sequences. From SAGE libraries constructed from uninfected and nodulating roots, approximately 400 tag species from more than 80,000 SAGE tags were significantly expressed either in the uninfected or nodulating roots. This study also showed that transcriptional induction was different among leghemoglobin gene paralogs (Asamizu et al. 2005).

Molecular genetic technologies such as gene tagging and gene sequencing helped the understanding of the nodulation mechanism, the *Nod* and *Nol* genes for nodulation and the *fix* genes for nitrogen fixation (Gresshoff 2003). After that, microarray transcript analysis was developed to survey genes with an active role in nodulation (Table 2) as well as map-based the gene cloning method for specific genes (Endre et al. 2002; Stracke et al. 2002; Searle et al. 2003). Many studies on gene expression profiling by microarray were conducted with the model legume, *M. truncatula*. To identify genes expressed in the developing and mature nodules, and to detect differential expression of those genes, wild type and mutants were used to identify differentially-expressed genes in the symbiotic relationship between *M. truncatula* and *S. meliloti* (El Yahyaoui et al. 2004; Küster et al. 2004; Manthey et al. 2004). During nodule initiation, formation and function, El Yahyaoui et al. (2004) identified more than 750 differentially-expressed genes grouped into four categories: strongly up-regulated both in young and mature nodules, down-regulated during nodulation, activated mainly in mature nodules and genes that were transiently induced three to four days after inoculation. Additional genes were identified using a number of mutants of *M. truncatula* and *S. meliloti* (Mitra and Long 2004; Mitra et al. 2004; Starker et al. 2006). Among these studies, Mitra et al. (2004) and Starker et al. (2006) used an Affymetrix "symbiosis" gene chip to obtain about 10,000 *M. truncatula* tentative consensus sequences (TCs) that were available from TIGR and probed with the *S. meliloti* genome. The genes activated in the first 72 hours of nodule initiation were identified by a 6,000 unigene *M. truncatula* cDNA microarray (Lohar et al. 2005). In the other model legume, *L. japonicus*, cDNA microarray was used to survey the genes responsible for global changes in the development of root nodules and the metabolic differentiations toward nitrogen fixation (Colebatch et al. 2002, 2004). In addition, Kouchi et al. (2004) investigated gene expression profiling in early stages of root nodule development.

In silico-based gene discovery and tissue profiling with EST, collections have been performed for many plant species. Since EST databases of legume model plants were only available in limited numbers, an *in silico*-approach has been rarely adopted (Table 2). Fedorova et al. (2002) identified nodule-specific genes from *M. truncatula* EST databases (<http://www.tigr.org/tdb/mtgi>) that contained more than 140,000 sequences generated from 30 cDNA libraries. There were 340 putative TCs that were expressed only in root nodules; nodule-specific TCs were subdivided into nine groups by the predicted function, such as nodulins, plantacyanin, agglutinin, embryo-specific protein, calmodulin-like proteins, purine permease, etc. Both

studies used macroarrays and Northern blot analysis to experimentally confirm gene expression identified by *in silico* transcriptome profiling. Combined with targeted macroarrays, Tesfaye et al. (2006) used the same approach and identified 81 nodule-specific TCs that were involved in carbon and nitrogen metabolism under different conditions of nitrogen supply.

Proteomics that were carried out by protein separation by two-dimensional gel electrophoresis, multi-dimensional liquid chromatography, peptide sequencing by mass spectrometry, and bioinformatics were more developed (Trevaskis et al. 2002; Jorrín et al. 2006). Most research focused on *M. truncatula* and soybean because the EST and genome sequencings were intensively performed although various legume species were used for studying differential gene profiling in nodulation (Table 2). The proteome profiles were compared between three legumes (*M. truncatula*, *Melilotus alba* and *Trifolium subterraneum*) to evaluate cross-species similarities (Mathesius et al. 2002), but publicly available *M. truncatula* EST databases only identified the proteins and peptide mass fingerprinting. Therefore, Mathesius et al. (2001) were able to efficiently identify and analyze 485 proteins by peptide mass fingerprinting; 179 matched with the *M. truncatula* EST databases. *M. truncatula* root protein profiles were analyzed by time-course after inoculation with either *S. meliloti* or the arbuscular mycorrhizal *Glomus mosseae* (Bestel-Corre et al. 2002). Leghemoglobin that was identified by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry after trypsin digestion was induced in nodulated roots of *M. truncatula*. Mathesius et al. (2003) surveyed proteins in response to nanomolar to micromolar concentrations of bacterial N-acyl homoserine lactone (AHL), which play important roles in eukaryote-prokaryote interactions. More than 150 proteins were significantly changed, and 99 were identified by peptide mass fingerprinting.

Using proteomic analysis, soybean peribacteroid membrane (PBM) proteins possibly involved in protein processing were identified (Panter et al. 2002). These results suggested that these PBM homologs might play a role in protein translocation, folding, maturation or degradation in symbiosomes. A nodule-specific 53-kDa late nodulin from a soybean symbiosome membrane was also identified by proteomics, and its function and expression were confirmed by Northern blot analysis (Winzer et al. 1999). Wan et al. (2005) surveyed protein profiles of root hairs inoculated with *B. japonicum*. Of 37 protein spots, 27 were identified by database comparisons, and included both the proteins response to rhizobial inoculation and novel proteins. Comparative proteomics using a supernodulating mutant showed that malate dehydrogenase, peroxidase and the alcohol dehydrogenase in particular were up-regulated during nodulation in both the wild and supernodulating mutants (Hwang C.H. personal communication).

Other than *M. truncatula* and soybean, clover, pea and white sweet clover were used to investigate the *Rhizobium*-legume symbiotic relationship (Table 2). Natera et al. (2001) generated proteome maps after comparison between free-living bacterium grown in the culture and the bacteroid form isolated from root nodules. More than 250 proteins, such as leghemoglobin and NifH (nitrogenase H), were

induced or up-regulated in the nodule compared with the root, wherein about 350 proteins, such as glutamine synthetase, urease, a urea-amide binding protein and a PII (nitrogen regulatory protein) isoform were down-regulated in the bacteroid form. After isolating the PBM and peribacteroid space fractions from pea root nodules, 46 of 89 protein spots were identified through a database search (Saalbach et al. 2002). Endomembrane proteins, such as V-ATPase, BIP and an integral membrane protein, were detected in the PBM fraction, which suggests a role for the endomembrane system in PBM biogenesis. In clover, Morris and Djordjevic et al. (2001) identified 16 protein spots differentially expressed. Eight of 22 constitutively-expressed spots were similar to the putative protein homologies that were involved in several pathogenesis and stress-related proteins. With these results, the proteome maps were able to construct in a cultivar-specific legume-*Rhizobium* interaction.

All molecular technologies and knowledge, such as the transcriptome, proteome, metabolome and the study of mutations coupled with reverse genetics, can be used to identify genes that more intensely confer nodulation and symbiotic relationships. The characterized genes and proteins identified by these recently developed methods should be studied with the biochemical pathway for nodule formation to clarify the legume-*Rhizobium* interaction.

5. CONCLUSIONS AND PERSPECTIVES

Symbiotic N₂-fixation is a complex physiological process that is influenced by the interaction of genetic elements between legume plants and nitrogen-fixing bacteria. While substantial progress has been made in identifying bacterial genes and gene products that contribute to nodulation and N₂ fixation, relatively little progress has been made in understanding the contribution that host plant genes make to symbiosis. Host plant genetic control of symbiotic association has been well documented through classical genetic studies; however, physiological and biochemical manifestations of these genes in the host were poorly understood.

The genes identified in the nodulation soybean mutants conditioned four major phenotypes: 1) non-nodulating, 2) ineffective nodulation (little or no N₂ fixation), 3) hypernodulating and 4) supernodulating. These mutants are unique biological materials for characterizing the host plant regulation of nodulation and nitrogen fixation.

With the rapid development of functional plant genomics, the large numbers of expressed sequence tags (EST) are publicly available for soybean plants. In addition, recent advances in cDNA, oligonucleotide and Affymetrix microarray for large transcriptomic studies on nodulation, mass spectrometry (MS) techniques combined with two dimensional polyacrylamide gel electrophoresis (2-D PAGE) and the establishment of protein database are feasible for identifying novel candidate genes that might be implicated in nodulation.

The availability of nodulation mutants in soybeans in combination with the recent advances in omics approaches will accelerate our understanding of the molecular basis of symbiotic association by the functional characterization of nodule biology.

The integration of gene identifications and the basic biochemical pathway for nodulation would be helpful for a more complete understanding of the signaling cascade for the symbiotic relationships between legume plants and Rhizobia.

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CHAPTER 17

GENOMICS OF WHEAT DOMESTICATION

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Abstract: The review covers several issues concerning the state of molecular knowledge of the effects induced by domestication and breeding on the wheat crop. Genes at the root of the domestication syndrome are currently the focus of an active research which frequently uses comparative genomics approaches. Conclusions drawn on available data indicate that the domestication syndrome is originated by “sudden” genetic events, controlled by few major pleiotropic genes. These events were followed by the accumulation of a larger set of minor mutations, having a multifactorial mode of inheritance. Moreover the organization of nucleotide variability enables the detection of domestication-related molecular footprints, suggesting that the genomic regions more responsible for genetic variation and more related to domestication are reduced when compared to the whole genome size. The polyploidy history of the domesticated wheats is presented, making a specific mention to the origin of the wheat A, B, D and G genomes and to the molecular control of chromosome pairing in polyploids. A general presentation is also provided on the genomic changes which have accompanied the emergence of domesticated wheats. What follows is a molecular information on: i) the wheat adaptation to the environment (genomics of photoperiod, vernalization, heading date, plant height, and erect plant type); ii) the effect of domestication on seed-related yield components (genomics of seed size, grain hardness, and tillering); iii) modification of traits affecting harvestability (emergence of free-threshing seeds, rachis toughness, and presence of ear awns). Genetic bottlenecks which have been associated to wheat domestication and breeding are considered in a final section. The relatively young history of the wheat crop, the presumably small founder population of this gene pool, and the intensive long-term selection for agronomic traits did set the basis for a reduced genetic variability of the genus.

1. INTRODUCTION

The most important grass subfamilies, including *Pooideae*, radiated 50–80 million years ago (MYA). The *Triticeae* and *Poeae* (*Lolium rigidum*) tribes separated ca. 35 MYA, *Hordeum* diverged from the *Triticum/Aegilops* lineage ca. 11 MYA,

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and *Secale* from the same lineage 7 MYA. Several members of the grass family (*Poaceae*) – wheat (genus *Triticum*), rice (genus *Oryza*) and maize (genus *Zea*) – were domesticated in a time frame comprised between 8,000 and 12,000 years from present (BP). Agricultural systems based on these crops made possible, at the time, to support the civilization of South-West Asia, China, and South America (Diamond, 1997; summarized in Pozzi et al., 2002). Events leading to an increase of wheat ploidy to 4n should be moved back to some 300,000 years (Akhunov et al., 2003), and to approximately 8,000 years ago the synthesis of the 6n genome of bread wheat (Salamini et al., 2002).

Specific alleles of groups of tightly linked loci (genes known as domestication syndrome factors, DSFs), have been favoured during domestication (Le Thierry d'Ennequin et al., 1999; Lin et al., 1995). The noted strong linkage among DSFs is thought to facilitate the maintenance of the phenotypic identity of domesticated populations coexisting in the same area with wild lineages, a case observed for rice (Cai and Morishima, 2002). Alternatively, the clustering in specific linkage groups of domestication-related genes with relevance to plant adaptation has been explained by the effect of balancing selection in large natural populations: in this perspective, clustering of genes predisposed wild crops to be more rapidly domesticated, while the subsequent domestication process fixed useful mutations within the cluster (Lin et al., 1995). Fifty-six QTLs for 11 domestication-related traits underlying the difference between wild *T. dicoccoides* and *T. durum* are clustered in seven linkage intervals, mostly located on only 4 chromosomes of the tetraploid wheat (Peng et al., 2003). DSFs have been probably selected as a set of genetic factors common to different crops (Buckler et al., 2001), thus justifying the believe that domestication of the grasses was genetically convergent, as reported for maize, rice and sorghum (Paterson et al., 1995). This implies that homologous, if not orthologous, genes/alleles have been involved in the domestication of these particular plants.

When comparative genomics has been used to investigate the relative location of DSFs in sorghum, rice, and maize, three QTLs affecting seed size were found to occupy syntenic positions in the three species; for grain shattering, QTLs map to a single locus in sorghum, three loci in rice and 10 loci in maize, and the sorghum single locus corresponds to a single rice QTL and to two maize QTLs located on duplicated chromosomal regions (Doebley and Stec, 1993). Experiments were also carried out to map domestication QTLs in wheat: Peng et al. (2003) found 7 major wheat DSFs, and mapped them in gene-rich regions. They concluded that “*the high pleiotropy and/or tight linkage of most wheat domestication QTLs suggest an important role of recombination in either consolidation of positive mutations within the clusters and/or in reducing the antagonism between artificial and background selection.*” Several studies are currently addressing the molecular basis of domestication traits, and, interestingly, the picture emerging is similar across species. For example, plant height and flowering time are affected by mutations in the orthologous genes *RhtD1a* of wheat, *SLR1* of rice, and *D8* of maize (Ikeda et al., 2001).

Recent experiments approaching the possibility to map and clone QTLs (Doebley et al., 1997; Yano et al., 2000; reviewed in Salvi and Tuberosa, 2005) allow to draw some conclusions concerning the process of plant domestication: (i) the domestication syndrome originated by “sudden” genetic events, controlled by few major pleiotropic genes (Li et al. 1995; Lin et al., 1995). These events were followed by the accumulation of a larger set of minor mutations (Barton and Keightley, 2002); (ii) a multifactorial mode of inheritance characterizes domestication traits. It is likely that genetic changes in these traits are required to overcome a certain threshold if the developmental program of the plant has to shift towards the domesticated phenotype. This hypothesis relies on the observation that in natural populations genetic variation may exist without phenotypic variation (cryptic genetic variation). A consideration of the level and pattern of linkage disequilibrium and of the organization of nucleotide variability (reviewed in Morgante and Salamini, 2003) enables the detection of domestication-related molecular footprints, suggesting that the genomic regions more responsible for genetic variation and more related to domestication are reduced when compared to the whole genome size.

That selection acting on specific regions of the genome may be sufficient to explain the domestication syndrome is, to some extent, evident. In fact about 2% of maize genes investigated reveal a history of selection (Wright et al., 2005). This can be determined by Tajima's D statistics (Wright and Gaut, 2005), which detects deviations from neutrality by examining, in a set of genotypes, the average nucleotide differences and the number of segregating sites (Tajima, 1989). An alternative explanation for the genes found to be under selection may reside in the population history of the species under consideration. For example, the occurrence of bottlenecks, and how these have affected nucleotide variation, may lead to the same results induced by positive selection (selective sweeps; Tajima, 1989). Bottlenecks, as a matter of facts, contribute to decrease molecular polymorphism.

In conclusion, it is currently not easy to infer whether loci, existing today in a molecularly modified form, were fixed due to population history or were selected during the domestication process or during the subsequent breeding phase (Wright et al., 2005).

2. IMPORTANCE OF PLOIDY DURING DOMESTICATION

Polyploidy originates mainly from unreduced gametes which have twice the number of chromosomes expected from a species (Leitch and Bennet, 1997). When two unreduced gametes of the same species join in a zygote, the resulting progeny is autopolyploid. Unreduced gametes from different but interfertile species can also result in a zygote (allopolyploidy) with a double number of chromosomes compared to the average number present in the progenitor species. Allopolyploidization may involve intergenomic recombination (Soltis, 2005) and rapid loss of DNA (Ozkan et al., 2001), whereby subsequent diploidization restores disomic genetics (Levy and Feldman, 2002).

Often polyploid species are sterile owing to the formation of abnormal multivalents during meiosis. Allotetraploid and allohexaploid should have two or three different copies of each nuclear gene and thus polyploids can be viewed as a combination of genomes with independent past histories. Recent molecular genetic studies have made clear that even the simple genome of *Arabidopsis* is the result of non-random patterns of arrangement of duplicated genes, probably due to ancient duplication or polyploidization events (Kowalski et al., 1994; Vision et al., 2000; Ozkan et al., 2001; Li et al., 2004). In *Arabidopsis*, however, the diploidization process is advanced, with only approximately 50% of genes having a discernible paralog and 19% locating in a region consistent with a chromosomal or segmental duplication event (Paterson et al., 2000). The question is whether polyploid formation has a selective advantage. QTL mapping efforts suggest that there may exist a relationship between polyploidy and crop productivity (Freeling and Thomas, 2006), that is, that non linear changes in phenotype are made possible as a direct result of polyploidy.

Specific polyploids arose recurrently during flowering plant evolution (Soltis, 2005), accompanied by rapid genome restructuring (Leicht and Bennet, 1997).

2.1. Ploidy and Wheats Evolution

Ploidy changes are at the core of cereal evolution. These species can have 14 (diploid), 28 (tetraploid) or 42 (hexaploid) chromosomes. The diploid genomes that contributed to the formation of the modern hexaploids are several: genomes A (A^m and A^u respectively present today in *T. monococcum* and *T. urartu*), D, and S (S , S^s , S^b , S^l , S^{sh}). Two genomes found in polyploids wheats were given new names, B and G, because their diploid progenitors were at the time not known (Kilian et al., 2006). The genus *Triticum* includes cultivated species cytogenetically associated in 4 groups: einkorn (genome $A^m A^m$), emmer (genome $A^u A^u BB$), *T. timopheevii* ($A^u A^u GG$) and bread wheat ($A^u A^u BBDD$).

2.2. Origin of the Wheat Genomes

Monococcum (einkorn, genome $A^m A^m$) was probably the first wheat to be cultivated. The wheat was successively substituted, starting in the bronze age, predominantly by tetraploids and hexaploids with naked seeds. Wild emmer derives from a hybridization event dating back of 300,000 years (Akhunov et al., 2003). A diploid donor with the A genome (*T. urartu*, genome $A^u A^u$) hybridized with a second species, a representative of the *Aegilops* section *Sitopsis* (close to *Ae. speltooides*; Kilian et al., 2006), the donor of the B genome (Huang et al., 2002). The newly synthesized species (*T. turgidum* ssp. *dicoccoides*, genome $A^u A^u BB$, wild emmer) has given rise to the current tetraploid varieties of the domesticated 4n wheat *T. turgidum* ssp. *durum*, (free-threshing), and *T. turgidum* ssp. *dicoccum* (hulled). In comparison, the hexaploid wheat (*T. aestivum*, genome $A^u A^u BBDD$) is a young polyploid: the taxon exists only since 8,000 years (Nesbitt and Samuel, 1996).

Its origin is from an additional polyploidization event between an early domestication tetraploid *T. turgidum* and the diploid donor of the D genome, *Ae. tauschii* (genome DD).

2.2.1. The A genome

Several studies point to a high level of similarity between the chromosomes of *T. monococcum* (A^m) and chromosomes A^u of *T. aestivum* (Luo et al., 2000; Provan et al., 2004). This suggests that A^u and A^m genomes have not diverged significantly since their separation from a common progenitor. A quantification of their difference is provided by Brandolini et al. (2006), based on AFLP markers describing putative chromosome A-gene fragments (i.e. AFLP bands not deriving from the B genome, and not specific for the hexaploids, as it would be if they were derived from the D genome). The authors used 41 accessions of A-genome, 3 of AG-genome wheats (*T. timopheevii*), 19 of AB-genome wheats, 15 of ABD-genome wheats, and 1 of the D-genome donor *Ae. tauschii*. The data indicate that the A^u genome of *T. urartu* is more similar by around 20% to the A genome of tetraploid and hexaploid wheats, compared with the A^m genome of *T. monococcum*. The difference is smaller when the comparison of the A^u genome with the A genome of *T. timopheevi* is considered. The study supports the current belief that only a part of the variation existing in the wheat diploid gene pool is present in the polyploid wheats (Dvorak et al., 2006).

The domestication of the A genome has been followed by studying einkorn wheat which was domesticated from its wild progenitor, *T. boeoticum* (also genome A^mA^m). The two taxa differ mainly in plant and seed size and in seed dormancy. The site of einkorn domestication has been identified from the analysis of 288 amplified fragment length polymorphisms (AFLP) marker loci. *T. boeoticum* populations from the western foothills of the Karakadag mountains of southwest Turkey are more similar to einkorn than are other wild populations, which points to this area as the centre of its domestication (Heun et al., 1997).

2.2.2. The B genome

Wild emmer (genome AABB) has brittle ears, a character lost during the domestication that led to *Triticum dicoccum*. A study based on AFLP at 204 loci (Ozkan et al., 2002) demonstrated that domesticated tetraploid wheats are closely related to wild populations from south-eastern Turkey. In the Fertile Crescent at least two major races of wild emmer exist, and it was the one present in the south-eastern Turkish areas which has, apparently, been taken into cultivation. The same study proved that hulled emmer may have an origin independent from free-threshing 4n wheats. Mori et al. (2003) investigated chloroplast microsatellite variation in populations, from the Kartal Dagı Mountains and concluded that emmer was domesticated twice, once in the Kartal Dagı region and a second time in an unidentified site. Ozkan et al. (2005) excluded the Kartal Dagı as site and suggested that wild emmer from the Karaca Dag region and the Sulaimanyia region (Iran-Iraq border) were at the root of domesticated emmer. Luo et al. (2006; unpublished. results)

confirmed that wild emmer consists of two populations, with domesticated emmer deriving from the southern population. Moreover, they provide evidence for an extensive gene flow between wild and domesticated emmer, and conclude that either emmer was domesticated in south-eastern Turkey or that it was domesticated independently twice, and later on the Levantine gene pool was absorbed into the south-eastern domesticated gene pool. An early appearance of domesticated hulled and free-threshing emmer is consistent with early archaeological evidences concerning domesticated tetraploid free-threshing wheats (Salamini et al., 2002).

The origin of the B genome is still not definitively clear (Huang et al., 2002). The genome of *Ae. speltooides*, a probable progenitor, is designated S from the name of the *Aegilops* section *Sitopsis* (Huang et al., 2002; Slageren, 1994). A second point concerning the B genome origin is that this is highly related to the origin of the G genome of *T. timopheevi* (AAGG). The AABB and AAGG genome origins have been attributed to the same single hybridization event (Provan et al., 2004) or to separate allopoloidization events (Rodriguez et al., 2000). Huang et al. (2002) suggest that some loci of the S and of the G genome are closely related, leading to the conclusion that parts of their genomes may have the same origin in an *Ae. speltooides* progenitor. This evidence agrees with results of other studies implicating *Ae. speltooides* as the donor of the G genome. On the other hand, none of the loci of the *Aegilops* genome analyzed in the Huang's study (2002) closely resemble the hortologous locus of the B genome. Kilian et al. (2006) extensively evaluated if *A. speltooides* was the donor of the B and G genomes in tetraploids wheats. Five *Aegilops* species of the *Sitopsis* section were compared by AFLP fingerprinting to diploid, tetraploid, and nullitetrasonic *T. aestivum* wheat lines: the B genome could be attributed to S chromosomes of *Ae. speltooides*. The same study haplotyped eight genes in *Aegilops* and *Triticum* lines: B and G genomes share again the highest sequence similarity with *Ae. speltooides*, but they sequestered different samples of *Ae. speltooides* haplotype diversity. This evidence would prove that the hybridization events that led to AABB and AAGG genomes occurred independently.

In a recent study, Petersen et al. (2006) intended to overcome the limitation of most of the published wheat phylogenetic analyses, which are based on a small number of taxa, or are biased by the choice of polyploid wheat accessions having an *a priori* assumed role as progenitors of domesticated forms (Buchner et al., 2004), or consider few *Aegilops* and/or *Triticum* species (Zhang et al., 2002; Sallares and Brown, 2004). The authors studied two single-copy nuclear genes, *DMC1*, and *EF-G*, together with a plastid gene (*NADH* subunit F) from each of the A, B, and D genomes of hexaploid wheat and from the A and B genomes of the tetraploid progenitor of *T. turgidum*. The genes were amplified from accessions of diploid *Aegilops/Triticum* species, of tetraploid *T. turgidum* and from one accession of *T. aestivum*. According to these results, the D genome derives from *A. tauschii*, the A genome from *T. urartu* and the B genome from *Ae. speltooides*.

2.2.3. Hexaploid wheats

A wild hexaploid (AABBDD) genome lineage does not exist. In this sense, hexaploid wheat is the result of a hybridization between a domesticated form of emmer (*T. turgidum* most probably spp. *dicoccum*) and the diploid *Ae. tauschii* (genome DD; Kihara, 1944; McFadden and Sears, 1946; Cox, 1998; Feldman, 2001; Salamini et al., 2002). *Ae. tauschii* consists of the gene pools S and T, loosely related with the two subspecies *strangulata* and *tauschii* (Dvorak et al., 1998). RFLP studies indicated that the D genome is more closely related to the S than to the T gene pool. However, the finding of parallel polymorphisms in the wheat D genome and in the *Ae. tauschii* gene pools and the existence of wheat haplotypes closely related to the T gene pool negate the idea that 6n wheat originated via a single hybridization event (Caldwell et al., 2004; Talbert et al., 1998). Thus, the Chinese Spring D genome appears to be a conglomerate of contributions from both the T and S *Ae. tauschii* gene pools (Peng et al., 2004; Akhunov et al., 2003; Akhunov et al., 2006).

The seven (1A–7A) A chromosomes of hexaploid wheats are related, through common ancestors, to homeologous chromosomes B and D; that is, chromosomes 1A, 1B and 1D are syntenic as are 2A, 2B and 2D and so on. This is because the wheat A, B, and D genomes diverged between 2.5 and 4.5 MYA from their common ancestor (Huang et al., 2002). Hulled, brittle-rachis forms of *T. aestivum* have been frequently reported to be ancestral to the free-threshing forms. The emergence of free-threshing hexaploids is however still not completely clarified. Hulled forms of *T. aestivum* may derive from hybridization between free-threshing hexaploids and hulled emmer wheat (Dvorak et al., 2006). The molecular relationships of the putative progenitors - at 4n level several forms of *T. turgidum*, and the 6n hulled form of *T. spelta* - and the cultivated tetraploids remain thus elusive (Dvorak and Akhunov, 2005).

2.3. Molecular Control of Chromosome Pairing

The control of chromosome pairing provides insights on wheat evolution. *Ae. speltooides* forms are known that suppress pairing among homologous chromosomes (*Ph1* activity). If *Ph1* genotypes participate in allopolyploidization, the hybrids acquire a bivalent chromosome pairing, the case of *T. dicoccoides* (Sears, 1976). This was most probably a very important step in wheat polyploidization because *T. dicoccoides* is at the root of all polyploid wheats (with the exception of the wild AAGG genome; Kilian et al., 2006). Meiotic chromosome pairing in hexaploid wheats is in fact confined to strict homologous chromosomes, despite the presence in the genome of homoeologous chromosomes. This strict control contributes to the integrity of the A, B, and D genomes, and the cytological outcome is a diploid-like behaviour of the species leading to 21 bivalents at meiotic metaphase I. The control of chromosome pairing depends on a group of suppressing and promoting pairing homoeologous (*Ph*) genes (Sears, 1976), with *Ph1* located on the long arm of chromosome 5B. *Ph2* maps to the short arm of chromosome

3D (Mello-Sampayo, 1971; Sutton et al., 2003), and a further locus on the short arm of chromosome 3A (Driscoll, 1972). In the absence of *Ph1*, homoeologous recombination takes place among A, B, and D chromosomes, included also those of related species or genera (Koebner and Shepherd, 1985).

The *Ph1* locus has recently been cloned in *T. aestivum* (Griffiths et al., 2006) and found to have a complex structure consisting of a segment of subtelomeric heterochromatin inserted in a cluster of *cell division control 2* (*cdc2*) genes. Rearrangements of the region which seems responsible for pairing control may have arose on polyploidization or pre-existed in the diploid genome donors.

The molecular bases for chromosomal recognition and pairing during early meiosis in hexaploid wheat are still unknown. An hypothesis states that *Ph1* affects basic components of chromosome structure. Mutants at *Ph1* show an altered chromosome/chromatin organization and compaction, in meiotic and in somatic cells, but also premature separation of sister chromatids (Aragon-Alcaide et al., 1997; Vega and Feldman, 1998). In addition, *Ph1* mutant have alterations in the relative arrangement of homologous chromosomes in both meiotic and somatic cell (Mikahilova et al., 1998). Some lines of *Ae. speltoides* do not show *Ph1*-like activity and allow chromosome pairing, leading to tetravalent formation and, possibly, intergenomic translocations. The AAGG genome *T. araraticum* has extensive DNA loss and chromosomal rearrangements relative to *T. dicoccoides* (Ozkan et al., 2001) which are, in part, the result of intergenomic G-A translocations. This observation, combined with the fact that today *T. araraticum* – the supposed wild progenitor of the AAGG wheats - has an active *Ph1* allele, may suggest that *Ph1* was suppressed during the formation of the AAGG genome and later on restored via genetic segregation.

2.4. Genomic Changes During Domestication

Allopolyploidization was responsible for a rapid wheats genome evolution, including the elimination of single copy DNA, activation and silencing of specific genes and activation of retrotransposons (Ozkan et al., 2001; Kashkush et al., 2002; He et al., 2003; Gu et al., 2004). Feldman et al. (1997) conclude that the physical basis for a diploid-like meiotic behaviour of polyploidy wheat is under the control of two molecular processes: I) the non-random elimination of DNA sequences; II) the homoeologous pairing suppression by the *Ph1* locus. The first phenomenon increased the differentiation between homoeologous chromosomes, thus preventing intergenomic recombinations.

A gene sequence comparison approach has been frequently followed to investigate wheat evolution. A comparison of two large orthologous HMW-glutenin regions located on chromosome 1AS of *T. monococcum* and *T. turgidum* sbsp. *durum* indicates that sequence conservation is restricted to relatively small regions containing orthologous genes (Wicker et al., 2003). In this context, it is surprising that more than 90% of DNA sequences are not or are loosely conserved, even in the recently diverged orthologous glutenin regions of the tetraploid wheats. The

genome-specific differential amplification of the glutenin loci has most probably contributed to the genome divergence noted in the regions compared. A second study, dealing with the high molecular weight (HMW) glutenin loci (Gu et al., 2004), and based on sequencing of 307 kb region, has considered the *T. turgidum* A genome compared to the orthologous regions of the B genome of the same wheat and the D genome of *Ae. tauschii*. Several inactive genes were detected and, curiously, this was found more frequently in the A genome. In addition, a large number of retroelements was recognised (Li et al., 2004). The explanation proposed for this finding may indicate that polyploidization acts as a buffer against the accumulation of deleterious mutations (Wendel, 2000). The presence and activity of retroelements contributes to rapid genome expansion, as pointed out by Kong et al. (2004). The authors provide a comparison between orthologous regions including the high-molecular weight (HMW) glutenin of the wheat B and D genomes. The differences they observe between the B genomes of *T. turgidum* and *T. aestivum* could be due to events occurred since the appearance of the hexaploid wheats.

Belyavev et al. (2000), using genomic *in situ* hybridization, revealed a chromosome pattern interpreted as a process of enrichment of the A genome with repetitive sequences deriving from the B genome. Similar genomic changes may provide a physical basis for phenomena noted in polyploid wheats, such as gene silencing, or non random distribution of markers (typical of B, but not of A chromosomes; Peng et al., 1999). When synthetic allohexaploids were analysed, a fast non-random sequence elimination was observed in the first hybrid generations (Ozkan et al., 2001; Shaked et al., 2001). When synthetic genomes were compared in *Aegilops-Triticum* lines and in their diploid progenitors, it was noted that allopolyploidy induced a very early DNA sequence elimination (Feldman et al., 1997). Both expansion and reduction of wheat genomes have been described by Chantret et al. (2005).

In this context it is evident that transposable elements have been active in these genomes since the divergence of the A, B, and D chromosomes, as well as after the allopolyploidization event. For example, the high rate of genomic rearrangements at the *Ha* locus supports a plastic and dynamic nature of genomes. Issues raised by grass genomes studies are important when the “stabilization” of other allopolyploids genomes is under discussion, particularly when considering the possibility of bringing new species of synthetic origin into agricultural systems.

3. GENOMICS OF PHOTOPERIOD, VERNALIZATION, HEADING DATE, PLANT HEIGHT, AND ERECT PLANT TYPE

Modern plant breeding is supposed to have utilized, and to continue to use, novel variants of genes which have been fixed as “domestication-related alleles” (Paterson et al., 1995; Salamini et al., 2003). In barley, for example, domestication resulted in the elimination of *Btr* (*brittle rachis*) alleles causing brittleness of the rachis; later on, additional loci conditioning rachis weakness were identified and thus also became a target for breeding (reviewed in Wanlong and Gill, 2006).

Flowering time, plant height, and other yield components, like tillering capacity, are additional domestication traits currently under phenotypical, and molecular selection. In cereals, comparative genomics has progressively clarified that domestication and breeding operate, at least in part, on the same genes and metabolic pathways controlling, across different species, some of the mentioned traits (Buckler et al., 2001).

In wheat, spike development and date of heading are controlled by three major groups of genes: photoperiod response genes (located on 5A and 5D); vernalization response genes (5A, 5B, and 5D); and earliness *per se* genes (Bullrich et al., 2002; Shindo et al., 2003).

3.1. Photoperiod

Modern breeding has largely exploited the genetic variability at genes controlling flowering time and photoperiod sensitivity. The modification of time of flowering is important for regional adaptation and, eventually, for dry matter accumulation in all cereals. In this sense, short-day flowering wild grasses have been transformed into domesticated varieties in which flowering time is now unaffected by day length (Buckler et al., 2001). The photoperiodic and vernalization genes respond to environmental changes by delaying or accelerating flowering, thus avoiding the exposure of flower primordia to deleterious extremes of temperature. In winter wheat, which requires vernalization when grown in Northern latitudes, specific genes delay ear initiation after the establishment of the seedling in autumn. As a result, the temperature-sensitive stages of ear development do not occur during the winter season. Where summers are too hot for wheat growing, on the other hand, the dominant photoperiod-insensitive alleles *Ppd1*, *Ppd2*, and *Ppd3* accelerate the onset of ear initiation and development as in the early spring day length begins to increase. This allows the plant to complete the reproductive cycle before the start of the dry season (Worland, 1996; Law and Worland, 1997).

The spreading of photoperiodism-supportive wheat alleles reflects the adaptation achieved by breeders operating under varied environmental conditions all around the globe. A particular allele may have been used in preference to other equally potent, only as a consequence of its availability. For example, most European and North American spring wheat depend upon the dominant *Vrn-1A* allele on 5A for their spring habit, and this only reflects their common parentage. In fact, *Vrn-1D* and *5D*, frequent in Chinese and Japanese spring wheat (Stelmack, 1990), would have been also available and are equally suitable, but due to stochastic choices they were not used in Europe. Alleles controlling wheat flowering time are, thus, good markers to follow the world colonization by wheats.

One of the first breeding development was the introduction, early in the XX century, of the gene *Ppd1* from the Japanese variety Akagomugi into Italian breeding programs. This was important for the development of Italian early maturing wheats. This allele moved, around 1930, to Brazil, where it was used in the breeding of the early Brazilian wheats. The *Ppd1* allele was successively picked up by N. Borlaug

in his Mexican wheat programme (Borlaug, 1968), based on the transfer of the short plant height from the variety Norin 10 to modern wheats. The wheat breeding achievement of N. Borlaug and CIMMYT is now known as “green revolution”. A third movement of genes was to Australia, where the allele *Ppd2* arrived from England and *Ppd3* from Indian wheats. Both were necessary for the Australian wheat industry to develop new varieties more adapted to dry weather conditions.

The *Ppd1* and *Ppd2* genes are located on chromosomes 2D and 2B respectively, and *Ppd3* on 2A. *Ppd1*, while shortening life cycle, reduces plant height together with tillering, but also spikelet number. The two effects are opposite, in agronomic terms, even if the increase in spikelet fertility compensates for reduction in spikelet number.

3.2. Vernalization

The vernalization process requires a plant to be exposed to a long period of low temperatures to become competent to flower, while preventing the winter damage to the cold-sensitive flowering meristems. In winter wheats, the promotion of flowering is conditioned by long exposure to low temperature. These varieties are sown in the fall, contrary to spring wheats which do not require vernalization and can be planted both in spring and fall. Two are the genes involved in this response: *VRN1* and *VRN2* (Tranquilli and Dubcovsky, 1999). The former provides most of the variation in vernalization requirement of polyploids wheats. In these wheats, the gene is critical for adaptation to autumn sowing: the presence of *VRN1* alternative alleles divides wheat varieties into the winter and spring market classes. This allelic variation has to be related to a strong selection pressure carried out in contrasting environments during the last 8,000 years of wheat domestication and adaptation. Most of the *Triticeae* have a winter habit, a trait recessive to spring habit, where the recessive allele *vrn1* is the ancestral form. The *VRN2* of grasses is a dominant repressor of flowering down-regulated by both vernalization and short day.

The genes *VRN1* and *VRN2* have been cloned (Yan et al., 2003; Yan et al., 2004). The *VRN1* gene is similar to the Arabidopsis meristem identity gene *APETALA 1* (*API*), which initiates the transition of the apical meristem from the vegetative to the reproductive state. In diploid wheats and in the A genome of hexaploid wheats, the molecular nature of the dominant *Vrn1-A1* allele, which supports the spring growth habit, has been associated to mutations located in the promoter region, especially in the 50 bp immediately upstream of the ATG, where the level of variation is higher. Exceptions are several dominant alleles like *Vrn-A1*, *Vrn-B1*, *Vrn-D1* of hexaploid wheat, and *Vrn-H1* of barley which are not polymorphic in the promoter region relative to alleles with a wild type (non mutated) promoter. In a recent experiment, Fu et al. (2005) have sequenced the latter *VRN1* alleles and found a large deletion within the first intron of the gene. It has been hypothesized that a 2.8 kb region of intron I includes putative regulatory elements important for vernalization. Two further vernalization genes affect the vernalization response (Yan et al., 2006), barley *SVRN-H3* and wheat *VRN-B4*, which are located respectively to chromosomes

7HS and 7b . Both genes are now referred to as *VRN3*. The two genes are the orthologous of the Arabidopsis *FT* gene, which promotes flowering. These genes are expressed at high level in *Vrn3* plants, and support early flowering which is dominant to delayed. The wheat *Vrn3* dominant allele is characterized by an insertion of a retroelement in the promoter, while the barley gene has a mutation in the first intron, a mutation which differentiates dominant from recessive alleles (Yan et al., 2006).

VRN2 encodes a dominant repressor of flowering that controls negatively *VRN1* (Yan et al., 2004). The gene belongs to the zinc finger transcription factors class. The molecular nature of *VRN2* and the significant epistatic interactions observed for the gene products of *VRN2* towards the *VRN1* promoter, both support the conclusion that the two genes are acting in the same pathway. As the vernalization process reduces the abundance of the *VRN2* protein, the *VRN1* gene transcript increases, leading to the competence to flower. Vernalization requirement can be replaced by interrupting long day treatment with a change to short days in the photoperiod, which is associated with a down-regulation of the *VRN2* flowering repressor (Dubcovsky et al., 2006). In hexaploid winter wheat, the downregulation of the *VRN2* gene anticipates flowering for more than one month, demonstrating that the gene plays a role in the regulation of vernalization.

Spring wheats are double recessive at the two loci: the *vrn2* allele encodes an inactive repressor that cannot interact with the *VRN1* promoter. As a consequence, no effect on flowering time can be associated in this background to the molecular variation present in the promoter. The available model proposes that independent mutations in the promoter regions of genes of winter wheat, and barley have resulted in the loss of the recognition site of the *VRN2* repressor, and therefore, in a dominant spring growth habit (*Vrn1* allele).

3.3. Heading Time

Flowering time is regulated by day length (intercepted by photoperiod responsive genes) or by exposure to cold temperatures (mediated by vernalization responsive genes). Other genes exist controlling “earliness per se”, i.e. genes which regulate flowering time independently of the previously mentioned environmental signals. These are responsible for the fine-tuning of wheat flowering time. Bullrich et al. (2002) have mapped to chromosome 1A mL the “earliness per se” gene *Eps-Aml* in *T. monococcum*.

QTL mapping for loci controlling flowering time has been carried out by Peng et al. (2003) in a cross between the wild *T. dicoccoides* and the domesticated *T. durum*. The wild parent had typical traits: short stature, late flowering, small seeds, poor yield, and yield components. The authors individuate, by single- and linked-QTL analyses, up to 76 QTL effects. Allelic interactions supporting QTL factors followed dominance, recessive, additive, and heterotic models of inheritance (20% followed the latter). The map position of the QTLs controlling different traits and mapping to the same chromosomes were frequently overlapping. The

most significant QTLs were clustered in limited intervals of chromosomes 1B, 2A, 3A and 5A. The traits scored in the experiment were: spike number/plant; spike weight/plant; single spike weight; kernel number/plant; kernel number/spike; kernel number/spikelet; 100-grain weight; grain yield/plant; and spikelet number/spike. Flowering time QTLs (four) mapped on chromosomes 2A, 4B, 5A and 6B. The wild parent was sensitive to day length and flowered later compared to the cultivar. The authors conclude that the wild allele for the QTL on 5A increases the value of heading date and is responsible for late flowering of *T. dicoccoides*, whereas the wild alleles on 2A, 4B, and 6B accelerate flowering date.

Traditional rice cultivars can be sensitive to photoperiod; because of this, their cultivation can be practiced only once in one season. In most tropical and subtropical countries modern varieties having a photoperiod-insensitive allele of the *Se1/Hd-1* gene can be repeatedly grown in the same year (Khush, 2001). *Se1/Hd-1* is an orthologous of the closely related *CONSTANS* gene which controls photoperiod response in Arabidopsis (Yano et al., 2000). The *Hd3a* rice gene is controlling the transition to flowering. The gene has been isolated and reveals orthologous to the flowering promoting gene *FLOWERING LOCUS T (FT)* of Arabidopsis (Kojima et al., 2002). Breeding for early maturity in rice has also involved the *ef (early flowering)* genes (Khush, 2001).

The sowing-harvesting period of wheat can be similarly reduced exploiting the already mentioned *Ppd1* and *Ppd2* genes, that cause photoperiod insensitivity (Börner et al., 1998; reviewed in Griffiths et al., 2006).

3.4. Plant Height

Lodging is a detrimental phenomenon which accentuated its negative role in the 1960s, when the amount of fertilizers supplied to wheat and rice increased significantly. The problem was overcome by the deployment of new semi-dwarf varieties that were lodging-resistant. The success of the new genotypes was also depending on their capacity of partitioning a higher proportion of photosynthates into the grain, leading to a dramatic yield increase: the “green revolution” (reviewed in Hedden, 2003). The “green revolution” in rice is based on the use of mutations in genes controlling a key-step in gibberellin biosynthesis (for example, the *Semidwarf1 Sd1* gene encodes for a GA20-oxidase enzyme; reviewed in Pozzi et al., 2004). In wheat, semi-dominant mutations of the homoeologous/duplicated *Rht-B1a* and *Rht-D1a* genes conferring dwarfism characterize recent high yielding cultivars (Börner et al., 1996; Peng et al., 1999; reviewed in Hedden, 2003). *Rht-B1b* or *Rht-D1b* genes, present today in most wheat varieties, have been transferred from the Japanese variety Norin 10 into CIMMYT germplasm under the direction of N. Borlaug. Starting with CIMMYT germplasm, the same genes spread world-wide to wheat breeding programs. *Rht8*, also capable to reduce wheat plant height by 10 cm, derives from the Japanese variety Akagomugi. Probably the Korean peninsula was the center of origin of all these mutations which go back as early as in the third and the fourth centuries (Cho et al., 1993).

Other height reducing genes are known: the group of *Rht* genes may include up to 20 genes. In addition, genes were identified that reduce plant height without affecting early growth, or coleoptile length and vigour as do the *Rht* genes (Ellis et al., 2004). In a recent study these genes were mapped to different wheat chromosomes, thus widening their exploitation in plant breeding (Ellis et al., 2005). Wheat breeders have already looked at mutants of the duplicated gibberellin insensitivity loci to design varieties with specific heights (Peng et al., 1999).

The *Rht* (of the subgroups B and D) gene encodes a repressor of GA signalling. The gene is the orthologous of the Arabidopsis *GAI*, maize *dwarf8* (*d8*), and barley *Slender1* (*Sln1*) genes. Mutations in these genes, which result in GA-insensitive dwarfs are commonly observed (Peng et al., 1997; Peng et al., 1999; Chandler et al., 2002). Genes belonging to this class -also present in rice (Ikeda et al., 2001)-encode GRAS transcription factors characteristic for the presence of the DELLA domain in their encoded protein product. Deletions of this domain render the protein insensitive to GA-dependent mRNA degradation and result in dwarfing alleles (Peng et al., 1997; Peng et al., 1999). In wheat, the *Rht* genes have pleiotropic effects on flowering time and tillering (Khush, 2001). Interestingly, *d8* of maize is a target of selection and adaptation to various flowering times (Thornsberry *et al.*, 2001). Pleiotropic effects are not surprising for genes controlling hormone action and may be a common occurrence for the traits targeted by domestication and breeding (Cai and Morishima, 2002; Salamini, 2002).

4. GENOMICS OF SEED-RELATED YIELD COMPONENTS

The first steps in domestication concentrated on genes that facilitate harvesting and enable colonization of new environments. Soon after, yield must have assumed priority, as to minimize labour input and land needs (Buckler et al., 2001).

4.1. Seed Size

A transition from small-seeded plants with natural seed dispersal to larger-seeded non-shattering plants is evident for all seed crops. Seed size was positively selected in all domesticated cereals. The genetic control of seed size in domesticated versus wild tetraploid wheats was studied by generating *T. dicoccoides* substitution lines in *T. durum* (Elias et al., 1996). Kernel size is under complex polygenic control, and alleles contributing to an increase and to a decrease in kernel size have been mapped on chromosomes 1A, 2A, 3A, 4A, 7A, 5B, and 7B. In a recent experiment (Peng et al., 2003) eight QTL for grain weight were mapped. Tetraploid wheat and rice were compared based on functional genomics. The study indicates that all detected seed-size QTLs in *T. dicoccoides* correspond to their rice counterparts. QTLs for *T. aestivum* yield and for other important agronomic traits were also mapped to chromosomes 3A, 4A, and 5A (Shah et al., 1999; Campbell et al., 2003; Araki et al., 1999; Kato et al., 2000). Additional QTL controlling plant adaptation and morphology traits were reported, including heading date, plant height (Cadalen

et al., 1998), lodging, lead rust reaction, and spike morphology (Sourdille et al., 2002). In the work of Marza et al. (2006), in addition to grain yield, QTLs for physiological maturity date, plant height, shattering, and lodging were assigned to linkage groups 4B, 5A, 6A, 6B, 7A, and 7DL (inconsistency among environments and moderate QTL effect were, nevertheless, noted). Grain yield QTLs were assigned to chromosomes 5A and 4B, while shattering to 6B. Lodging QTLs were mapped to 1B, 4AL, and 5A. In a recent study (Bresghehlo and Sorrells, 2006) the association of 36 unlinked simple-sequence repeat markers with kernel size and milling quality was analyzed in a collection of modern cultivars of soft winter wheat. Authors concentrated on chromosomes 2D and 5A/5B, where they suspected the presence of QTLs for kernel size, and evaluated linkage disequilibrium (LD) observed among linked markers. A consistent LD for markers of chromosome 2D extended on linkage intervals shorter than 1 cM, while in the centromeric region of 5A LD was significant for ca. 5 cM. A refinement of their mapping, based on further 62 linked SSS₂, revealed on the three chromosomes tested markers with a significant LD linked to kernel size. Candidate genes mapping in the regions hosting the QTLs are described elsewhere in this text: *Rht8* (reduced height); *Ppd1* (response to photoperiod), *BI* (awnedness inhibitor), *Rht12* (reduced height), and *Vrn1* (response to vernalization).

4.2. Genomics of Grain Hardness

Grain hardness (i.e. whether the endosperm is physically hard or soft) is a major property of the grain of *T. aestivum* (for example, it controls the susceptibility to damage during milling and, thus, the amount of water uptake during baking). Compared to *T. aestivum*, *T. durum* is mainly used for pasta production because of its hard endosperm.

Already in the '60s, Symes (1965), using the particle size index as a measure for grain hardness, demonstrated the segregation of a major gene for this trait in the progenies derived from the cross of the wheat cultivars Heron and Falcon. Later, he confirmed this result using 7 additional crosses between hard and soft wheats. The gene influencing hardness (i.e. conferring the "soft" phenotype) named *Ha* was subsequently mapped to the short arm of chromosome 5D (Sourdille et al., 1996). The *Ha* locus encodes friabilins, consisting of three lipid binding proteins: puroindoline a (encoded by *Pina*), puroindoline b (encoded by *Pinb*) and Grain Softness Protein (encoded by *Gsp-1*) (Gautier et al., 1994; Rahman et al., 1994). The three genes are tightly linked and define the *Ha* locus on chromosome 5D (Sourdille et al. 1996; Giroux and Morris, 1998; Giroux et al. 2000).

To understand the molecular nature of the locus, a bacterial artificial chromosome (BAC) covering that region was analysed. The three genes were found in the order (from 5' to 3' ends) *Pinb*, *Pina*, and *Gsp-1* within a 60-kb fragment of the D genome deriving from *Ae. tauschii* (Turnbull et al. 2003). Interestingly, the same gene arrangement has been observed in *T. monococcum* (Chantret et al. 2005), a wheat with a genome closely related to the A^u genome donor of the A genome

of wheat. Hard wheats have a mutation in either *Pina* or *Pinb* (but not in *Gsp-1*). Transformation experiments (Beecher et al., 2002) proved the direct functional role of the *Pin* genes: transformed rice and wheat had an endosperm more soft than untransformed controls. These results still do not contribute to explain the biochemical basis of the action of the *PIN* proteins.

Transcript abundance of *Pinb* do not vary significantly between soft and hard seeds in any of the wheats tested (Beecher et al., 2002). In addition, other genetic factors unlinked to the *Ha* locus may contribute to modify grain hardness (mapping on chromosomes 1A and 6D; Perretant et al. 2000). In general it can be concluded that the *Pin* genes are the primary cause of grain softness and that at least two puroindoline genes should be present for the grain texture to be soft (Giroux and Morris, 1998). In rice the expression of *Pinb* alone is sufficient to increase grain softness (Krishnamurthy and Giroux 2001). A more recent study (Capparelli et al., 2003) indicates that the level of friabilin associated with starch is regulated by the level of expression of the *Pina* transcripts, and that for a fully functional friabilin by both *PINA* and *PINB* gene products are required. Studies conducted using a microarray based approach demonstrate that the major difference in *Pina* gene expression is necessary to determine the hardness of the grain (Clarke and Rahman, 2005).

The *Ha* locus (ca. 80 kb) represents a classical example of a locus controlling a trait whose variation arose as a result of gene elimination after polyploidization. The domestication history of the locus is complex: it arose in diploid wheats, it was lost in tetraploids, and was later on introduced in the hexaploids via the D genome. The D-version of the locus present in the wild ancestor *Ae. tauschii* is 29 kb larger than in hexaploid wheats (Giroux and Morris, 1998). *Pina* and *Pinb* gene loss from the *Ha* locus of tetraploids was caused by a large genomic deletion that probably occurred independently in the A and B progenitor genomes of the tetraploid wheats. Duplication of puroindoline genes in the *Pooideae* has shaped the *Ha* locus (Chantret et al., 2005). After the acquisition of *Ha* by hexaploid wheats via the D genome (at the time of the emergence of allohexaploid bread wheat), the spreading of the species to northern cultivation areas has forced breeders to select hard wheat hexaploid cultivars, and thus the selection pressure on *Ha* has increased. This resulted in mutations or genomic rearrangements at the locus.

4.3. Tillering

When studied, grasses and particularly cereals have revealed the existence of several mutants with an altered pattern of axillary bud development. In maize, the *teosinte branched1* (*tb1*) mutation causes a complete loss of apical dominance, associated to axillary buds grow, a phenotype mimicking the teosinte phenotype (Doebley et al., 1997). Tillering and branching mutations have been described also in barley, where the recessive *uniculm2* mutation on chromosome 6H fail to produce tillers (Babb and Muehlbauer 2003). The *moc1* mutation of rice, on chromosome 6, causes lack of axillary buds (Li et al., 2003). Additional recessive tillering mutants

with reduced culm number (*rcn1* to *rcn5*) have been reported in rice (Gramene, <http://www.gramene.org/index.html>). The genetic variation for the capacity of tillers to exist has been assessed for the wheat gene pool (Atsmon and Jacobs 1977): low tillering genotypes frequently have a unicum phenotype, enlarged spike and modified leaf morphology. Some wild diploid grasses belonging to the *Aegilops* section *Sitopsis* (like *Ae. speltoides*, the progenitor of wild tetraploid emmer), have an ear morphology mimicking the teosinte ear. This specific ear shape is even more similar to teosinte than current domesticated wheats. It is concluded that current wheats have already undergone changes leading to a maize-cob like morphology. In wheat, the unicum line “492” was studied by Richards (1988), and a single recessive gene (*tin*) located on chromosome 1AS was found to control tiller number (Spielmeyer and Richards, 2004). The absence, in *tin*, of tiller development is due to an altered pattern of axillary bud formation and outgrowth (W. Spielmeyer, unpublished). In *tin* populations, low-tillering lines have a greater harvest index, fewer and sterile tillers, and a larger grain size, demonstrating the agronomic potential of the *tin* gene in wheat (Richards 1988; Duggan et al., 2002). Comparative genomics analyses lead to the conclusion that *tin* and rice reduced tillering mutations and the barley *uniculm2* mutant map to non-syntenic chromosomes. A wheat sequence that was closely related to the gene that encodes the *tb1* mutation in maize hybridises to a single RFLP band on chromosome group 4 of wheat (W. Spielmeyer, unpublished).

5. GENOMICS OF SEED TRAITS RELATED TO HARVESTABILITY

5.1. Free-Threshing

At the start of wheat cultivation, varieties were characterized by hulled seeds. The harvested spikelets were stored in barns and, when needed, they were parched to liberate the seeds from the chaff. This operation of parching is often reported in the literature on hulled wheats. Nesbitt and Samuel (1995) list additional reasons for the use of heat in wheat harvest processing. Whatever was the significance of spikelet parching, the end results is that in excavated archaeological layer charred seeds are present which allow paleobotanical conclusions.

A relevant agricultural improvement was the introduction of free-threshing varieties. Species characterized by a low degree of glume tenacity and by fragile rachis and free-threshing habit were selected by the farmers and the harvest of grains became efficient. Free-threshing wheats have thinner glumes and pales which allow an early release of naked kernels. After threshing, free grains are winnowed and stored ready for milling. Free-threshing varieties – tetraploid hard wheat and hexaploid bread wheat – represent the final steps of *Triticum* domestication.

The appearance of the free-threshing phenotype can be discussed considering the comparative genetics of two hexaploid wheat types, one hulled (called spelt), and the other free-threshing. Both have the AABBDD hexaploid genome. Major

and minor mutations have been considered to explain the evolution of the free-threshing habit in this type of wheat (McKey, 1966). The major gene components is usually discussed starting with the gene or gene complex *Q*, located on the arm of chromosome 5A. In *T. vulgare*, the *Q* allele supports the formation of square-headed ears with good threshability, besides inducing softening of the glumes, reduction of ear length, more spikelets per ear, and toughness of the rachis (Snape et al., 1985; Kato et al., 1998; Kato et al., 2003). Mutagenic disruption of the *Q* gene in *QQ* hexaploid free-threshing wheat generates a *q* mutant phenotype, known as speltoid because *q* mutants have a morphology similar to that of spelt (i.e. tenacious glumes), with a *qq* genotype. Bread wheat lines which are *Qq* have intermediate phenotypes. Because spelt has the *q* allele, Sears was confident that the large *Q* allele of free-threshing wheats derives from a form of *q* that retains some activity. As a matter of fact, Muramatsu (1963) showed that *q* is active by creating genotypes with 1–5 doses of either *Q* or *q* alleles. He showed that a square-headed hexaploid ear derives from either two doses of *Q* or five doses of *q*. A second major gene (*Tg*) is associated with the D genome. Two further major genes are known: *C* on the long arm of chromosome 2D (Rao 1972) and *S-D1* on the long arm of chromosome 3D (Rao 1977). In hexaploid wheats the polygenic component is scattered throughout all three A, B, and D genomes. In tetraploid wheats, QTL studies identified a total of four putative loci for threshability (Simonetti et al., 1999), located on chromosomes 2B, 5A and 6A. Two of these QTLs correspond, in position, to the already mentioned genes *Q* and *Tg*. Sourdille et al. (2000) explored the mapping of QTL involved in ear morphology (spike length, number of spikelets and compactness). For each trait, 4 to 6 QTLs were identified with individual effects ranging between 7% and 22% of total phenotypic variation. Several QTLs were detected that affect more than one trait. In general, these studies revealed the existence of additional QTLs, but their number and location varied according to the population under study.

5.2. Brittle-Rachis

In a tough, non-brittle rachis the formation of fracture rachis zones is suppressed, or the collapse of the rachis is delayed until mature spikes are harvested. In virtue of this characteristic *T. dicoccum*, the domesticated emmer wheat, diffused also in Mediterranean countries during the Bronze Age. The same is true for einkorn (*T. monococcum*, non-brittle) compared to its wild progenitor *T. boeoticum*. Thus the brittle rachis trait is at the origins of agriculture and sedentary societies.

In *T. turgidum*, two dominant genes, mapping to chromosomes 3A and 3B are responsible for the brittle trait (Watanabe et al., 2002; Watanabe et al., 2005; summary of genes involved and on the related terminology in Salamini et al., 2002). The trait, in einkorn, is under the control of two genes that segregate 15 brittle to 1 tough rachis in the F2 progeny of wild x domesticated crosses (Sharma and Waines, 1980). In *T. aestivum* the group of Riera-Lizarazu (Jantasuriyarat et al., 2004) detected several QTL involved in free-threshing and spike compactness. Four

QTLs for glume tenacity were detected; six for threshability, which is related to non-brittle rachis; four for spike length; five for number of spikelets per spike and five for spike compactness. Some of the identified QTLs probably coincide with known genes, like *Tg*, or *Q*. Cao et al. (1997) identified a single dominant gene, *Br-A1*, as the determinant of rachis fragility in a feral form of *T. aestivum* from Tibet. The gene was later localized on chromosome 3D (Chen et al., 1998), as supported also by studies on *T. dicoccoides* x *T. aestivum* crosses (Rong et al., 2000).

To summarize, studies like those cited point to the existence of different loci controlling rachis characteristics but also glumes and ear rachis traits (Salamini et al., 2002), suggesting that: i) multiple genetic pathways are involved in controlling the trait(s); ii) the donors of the genomes that made up polyploid wheats were different at the locus controlling shattering, a conclusion that would support a diphyletic origin of tetraploid wheats. These considerations, combined with the mapping of shattering QTLs, allows the study of microsyntenous relationship of the trait in barley, rice, *T. turgidum* and *T. aestivum*, sorghum, and maize, pointing to a conservation of the identified region.

The rice shattering QTL (*qSHT-1*) is located in region syntenous with regions on chromosomes 3A, 3B and 3H (respectively of wheat genomes A and B, and of barley), even if comparative mapping indicates that the genes may not be orthologous (Nalam et al., 2006; Li et al., 2006). The *qSH1* gene has been recently cloned and shown to encode a BELL1-type homeobox gene (Konishi et al., 2006). Similar consideration can be made in the case of barley: the *Btr1* and *Btr2* barley loci controlling shattering (Komatsuda et al., 2004) are mapping in a region not syntenous to wheat. In *T. dicoccoides*, Peng et al. (2003) identified a gene controlling shattering and mapped it on the long arm of chromosome 2A. In the same region maps one of the QTL involved in 4n wheat domestication. Additional QTLs controlling the domestication syndrome are tightly linked in the same species to the *Br* gene and/or were the result of a pleiotropic effect of *Br*.

The *Q* and *Tg* genes affect, besides free-threshing, also the toughness of the rachis. For this reason, the *Q* gene is agronomically important. Upon cloning, *Q* and *q* alleles were found to differ both in structure and in the level of transcription, and this can be associated to phenotypic differences due to dosage effects observed in transgenic plants. High resolution mapping of the gene (Faris and Gill, 2002) was instrumental for the molecular identification of this gene which shows DNA sequence similarity to the Arabidopsis *APETALA2* gene (Faris et al., 2003). Further studies confirmed the association (Simons et al., 2006) and demonstrated that ectopic expression of *Q* in transgenic plants mimicked dosage and pleiotropic effects of *Q*. Increased transcription of *Q* was associated with spike compactness and reduced plant height. Previous research suggested that *Q* might have arisen from a duplication of *q* (Kuckuck, 1959), as it was observed that 5 doses of *q* conferred the same phenotype as 2 doses of *Q*. Simons et al. (2006) contradict this hypothesis, showing that most probably *Q* arose through a gain of function mutation.

Synthetic hexaploids deriving from free-threshing tetraploids crossed to hulled *Ae. tauschii* allowed the identification of a second genetic system that controls the

speltoid phenotype. The hybrids were hulled, even in the presence of the dominant *Q* allele. The suppression of the free-threshing character was thought to be due to a partially dominant *Tg* allele, on chromosome 2D of *Ae. tauschii*, with *Tg* inhibiting *Q* and thus leading to tenacious glumes. The conclusion is that free-threshing hexaploids have the genotype *tgtg, QQ*, whereas all tested *Ae. tauschii* lines have the dominant *Tg* allele (Kerber and Rowland, 1974). Villareal et al. (1996) found that, in synthetic hexaploids, two genes (rather than *Tg* alone) affect glume tenacity, both of which are contributed by the D genome. Rachis fragility and square-headed ears in free-threshing hexaploid wheats can assort independently. For instance, in experimental crosses with *T. spelta*, three genes segregate for rachis fragility, but only one, *q*, for tenacious glumes. Moreover, *q* and *Q* interact with genes on other chromosomes that also govern glume tenacity and fragility (Cao et al., 1997; Luo et al., 2000). A further gene, located at 32 cM from *Tg*, on chromosome 2D, belongs to the system.

When the free-threshing habit was studied in a *T. durum* x *T. dicoccoides* cross, the F₂ population showed a continuous distribution for the trait (Simonetti et al., 1999). F₃ lines were fingerprinted and four major QTL were located on three chromosomes. Two of these corresponds to the homologous genes *Tg* and *Q* of hexaploid wheats, whereby the *Tg*-like gene *Tg2* is located on chromosome 2. It is concluded that the polygenic control of the free-threshing character in tetraploid wheats is based on the selection on new loci in addition to *Q* and *Tg*.

5.3. Control of Awning

Awns are important for their contribution to yield under drought conditions; as such the trait was most probably subjected to domestication. The genetic control of awn presence/absence is simple: few genes control the differences between awned and awnless varieties. Three dominant inhibitors are described: *Hd* (Hooded), *B1* and *B2* (*tipped 1* and *2*) (McIntosh et al., 1998). Wheat genotypes homozygous for three recessive alleles are fully awned. Using aneuploid lines, the loci were mapped using on chromosome 4A, 5Am and 6B respectively, as recently reported also by Sourdille et al. (2002) in a QTL experiment.

6. WHEAT GENETIC DIVERSITY TRENDS DURING DOMESTICATION AND BREEDING

Crops seem molecularly less variably than wild relatives. This is explained by the bottlenecks that a species has experienced during domestication and breeding (Tanksley and McCouch, 1997; Wright and Gaut, 2005; Dvorak and Akhunov, 2005; Kilian et al., 2006). When nucleotide diversity was evaluated at 21 loci in 4 taxa, representing key steps in the evolution of wheats, the results indicated a single domestication event of the wild tetraploid and a possible diphyletic origin of bread wheat. Loss of molecular diversity in *T. dicoccum* (durum wheat), compared to *T. dicoccoides* was 84%, and 69% in *T. aestivum* (David J., pers. comm.). Similar

data have been provided by a study of barley, carried out analyzing seven genes (Kilian et al., 2006) and comparing wild to domesticated barley, where the number of haplotypes (70 vs. 17), Π values (7.7 vs. 2.8×10^{-3}) and Watterson's θ (10 vs. 2.8×10^{-3}) were significantly reduced in domesticated lines.

It has to be noted that in the cited cases the loss of diversity was calculated from data concerning several genes, and each of them had a different degree of nucleotide variation. For instance, Ravel et al. (2006) noted that in 26 bread wheat lines SNPs frequency was relatively low (1 every 335 bp), and essentially represented by the variation at only 2 out of 21 genes. This and similar findings may explain the contrasting data available from literature survey. For example Thuillet et al. (2005) found that the molecular diversity in domesticated tetraploid wheats was similar to the one of wilds. Dvorak et al. (2006) have reasoned that mutations-supported nucleotide diversity in domesticated 4n wheats had no relevance in the generation of present diversity. They concluded that the diversity in today cultivated 4n wheats is due to gene flow from wild lines (Luo et al., 2006). Gene flow has been reported also from wild emmer to *T. aestivum* in the case of the ABCT-A1 locus (Dvorak et al., 2006).

In general, it seems safe to summarize that the relatively young history of the wheat crop, the presumably small founder population of this gene pool, and the intensive long-term selection for agronomic traits did set the basis for a reduced genetic variability in the genus (Reif et al., 2005). Part of the reduction in genetic diversity in modern wheats (Lubbers et al., 1991; Lelley et al., 2000) is probably the product of genetic drift and selection, both unintentional and intentional, carried out by early farmers. Ninety-seven percent of land races were rapidly replaced, during the last century, by new wheat cultivars (Smale et al., 2002), which have been bred starting with a limited number of land races. The extended period of the last 50 years of intensive selection may have further reduced genetic diversity among cultivars, narrowing the germplasm base available for future breeding (Tanksley and McCouch, 1997). A risk is that mutation in pest populations, the widespread use of monocultures and the changes in environmental conditions introduce new biotic and abiotic stresses that the current cultivars can not cope with and, therefore, lead to severe crop losses. For example, in India during the 70ies a severe epidemics of shoot fly and karnal bunt have heavily affected wheat yield.

Where and how do we find a safe and sustainable approach to modern wheat breeding? Introgression of novel germplasm is one option. Reif et al. (2005) used CYMMIT germplasm and reported a loss of genetic diversity in spring wheat during domestication followed by a change from traditional land races to modern breeding varieties during the last 50 years of intentional breeding. The second step accounted for most of the reduction in genetic variability. This trend is supported by other and various surveys, pointing to the potential of land races stored in gene banks as a source of useful allelic variation (Cox et al., 1992; Villareal et al., 1995). In modern varieties, moreover, intraspecific relatedness of germplasm increased in time periods, as also evident for the stabilization and fixation of certain well defined wheat plant ideotypes, a kind of strong selective drift. Of course, the

green revolution has contributed (Evenson and Gollin, 2003): the high-yielding new semi-dwarf cultivars introduced by the green revolution were based on a limited number of key parents and their genes are now dominating the wheat germplasm base. Roussel et al. (2004) studied 559 French bread wheat accessions and found a significant reduction of genetic diversity. However, they also observed a recent increase in diversity which could be explained by a change in the late 70ies in the breeding strategy at CIMMYT. The CIMMYT's wheat breeding program is now increasing wheat genetic diversity on a large scale to respond to the need for biological diversification, environmental sustainability, and durable resistance to pathogens.

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CHAPTER 18

TRANSCRIPTOME ANALYSIS OF THE SUGARCANE GENOME FOR CROP IMPROVEMENT

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Abstract: Sugarcane is being considered one of the most important crops to meet the demand of the world bioenergy needs. However the productivity in commercial plantations around the world is far away from its potential of about 300 tons/ha. Sugarcane breeding did not take advantage yet of the best plant breeding technologies, mainly because constraints imposed by the high polyploid nature of its genome. Even transgenic technologies would face difficulties because of the complex behaviour of introduced genes regarding the chromosome where the gene is inserted. One important resource to overcome at least part of these difficulties is the availability of a large collection of sugarcane Expressed Sequence Tags. The transcriptome information has allowed the identification of genes involved in biotic and abiotic stress response, disease resistance and sucrose accumulation. In addition comparative mapping has allowed the identification in sugarcane disease resistance genes already mapped in sorghum and maize. In this chapter we discuss the use of transcriptome resources for sugarcane improvement.

1. INTRODUCTION

1.1. The Sugarcane Crop

Sugarcane is one of the most photosynthetically efficient cultivated crops, with a potential productivity of 300 tons of biomass per hectare under optimized agricultural conditions (Alexander, 1985). However, the average productivity of commercial plantations around the world is far below the full crop potential. Two factors affect sugarcane productivity, namely, the quality of agricultural

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management, and fertilization conditions and irrigation, and the availability of genetically improved varieties able to withstand the limited growing conditions imposed by biotic and abiotic stress. With regard to genetic improvement, a major constraint is imposed by the highly polyploid nature of the sugarcane genome, which is not amenable to most of the breeding techniques developed for diploid species. Despite this limitation, improved varieties with yields of over 120 ton/ha on a few commercial plantations in some countries have been developed in the last 30 years (: <http://www.fao.org/>). However, such a high yield is not the reality for most of the 20 million hectares scattered throughout the 90 sugarcane-cultivating countries, for which the average productivity is around 40–70 tons/ha (: <http://www.fao.org/>).

The first cultivated sugarcane varieties were basically clones of *Saccharum officinarum*, a species with a high sugar content domesticated from the wild species *S. robustum*. The yield increased substantially a century ago when breeders crossed *S. officinarum* with *S. spontaneum*, a disease-resistant and vigorous relative (Berding and Roach, 1987). Yields were subsequently improved by backcrossing the hybrids to *S. officinarum* to produce the basic germplasm source of the modern sugarcane cultivars. Over the centuries, sugarcane has been cultivated almost solely as a source of sucrose, which accumulates at high concentrations in the stem internodes. Sucrose is extracted and purified in large mill factories, and is widely used as a raw material in the food industry. A major breakthrough in the last 30 years in Brazil has been the development of technology for the large-scale fermentation of sucrose to produce fuel ethanol. In the last few years, more than 17 billion liters of sugarcane ethanol have been used as car fuel each year, with an unprecedented environmental benefit in reducing CO₂ emission. Based on the Brazilian example, sugarcane is believed to become one of the most important crops for renewable energy production in the near future.

In this scenario, genomics will provide important contributions for improving the sugarcane crop. The most obvious genomic tool is the transcriptome data produced by sequencing Expressed Sequence Tags (ESTs). Large EST collections have been produced in recent years and are available in public databases (Carson and Botha, 2000; Arruda, 2001; Vettore et al. 2003; Casu et al. 2004). ESTs are the primary source material for accessing the expression of thousands of genes through technologies such as microarrays (Figure 1). However, to fully explore these tools it is necessary to understand the structure of the sugarcane genome.

1.2. The Sugarcane Genome

Sugarcane has a complex genomic architecture characterized by a high level of ploidy (Sreenivasan et al. 1987). The cultivated sugarcane varieties have $2n = 100$ to 130 chromosomes derived from crosses between *S. officinarum* ($2n = 80$ chromosomes) and *S. spontaneum* ($2n = 40$ to 128 chromosomes) (Butterfield et al. 2001; D'Hont and Glaszmann, 2001). *Saccharum officinarum* has a basic chromosome number of $x=10$, whereas *S. spontaneum* has a basic chromosome number of $x=8$ (Grivet and Arruda, 2002). The different chromosome numbers of

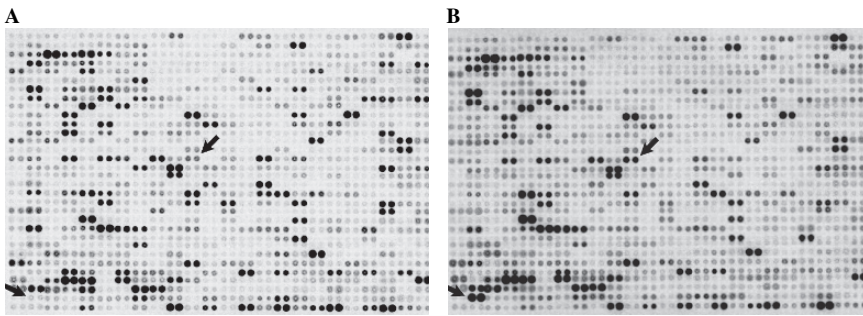


Figure 1. Example of a high-density membrane array used to analyze gene expression. Filters containing sugarcane cDNAs were probed with [^{33}P]cDNA reverse-transcribed from total RNA of control (A) and cold-treated (48 h at 4°C) (B) sugarcane plantlets. The arrows indicate examples of cold-inducible sugarcane ESTs

these two species allow the coexistence of distinct chromosomal organizations in the hybrid, with the possibility of some of the chromosomes being inherited entirely from *S. spontaneum* or from *S. officinarum* and some being recombination products between chromosomes of the two ancestral species (Grivet and Arruda, 2002). Despite the high level of ploidy, the monoploid genome sizes of *S. officinarum* and *S. spontaneum* are estimated to be around 930 Mbp and 750 Mbp, respectively, which are comparable to that of sorghum (~760 Mbp) and about twice that of rice (~390 Mbp). The modern sugarcane cultivars have around 120 chromosomes and a genome size of 10,000 Mb (D'Hont, 2005).

As in other grasses, the sugarcane genome contains an array of transposable elements (TEs), some of which are located between coding genes (Janoo et al. 2003). A large number of TEs is differentially expressed in sugarcane tissues and may affect the genomic stability (Araujo et al. 2005). These expressed TEs include homologs of the maize mutator (Mu) and the mudrA-like transposase. Sequences encoding the TEs found in the sugarcane EST project (SUCEST) database (Vettore et al. 2001) have also been found in *S. officinarum* and *S. spontaneum*, which suggests that these TEs were present in the ancestral Andropogoneae species common to sorghum, maize and sugarcane (Rossi et al. 2004a).

1.3. Synteny and Co-Linearity in Sugarcane and Other Grasses

The chromosome organization in grasses is highly conserved (Bennetzen and Freeling, 1993, 1997; Ming et al. 1998), possibly as a consequence of the short period that the species belonging to this family have evolved independently from a common ancestor. Comparative mapping has shown that the sugarcane and sorghum chromosomes are highly syntenic, which suggests that the order of the DNA sequences in their chromosomes has been conserved (Dufour et al. 1997; Glaszmann et al. 1997, Guimarães et al. 1997).

Sequence analysis of genomic DNA surrounding the alcohol dehydrogenase genes in sugarcane and sorghum has revealed a high colinearity in gene order, a conserved gene structure and a nucleotide similarity close to 95% (Janoo et al. 2003). These findings agree with those for comparative genetic mapping and reinforce the notion that sorghum has the simplest syntenic relationship with sugarcane, and makes sorghum the species of choice for modelling sugarcane (Dufour et al. 1997; Glaszmann et al. 1997; Guimarães et al. 1997; Ming et al. 1998; Asnaghi et al. 2000). Consequently, access to the sorghum genome and to the largest dataset of the sugarcane transcriptome will be of considerable use in the genetic improvement of sugarcane.

2. THE SUGARCANE TRANSCRIPTOME

2.1. Transcriptome Sequence Information

Large-scale EST sequencing has been used to quickly access gene sequence information. Currently, ESTs from over 1,000 species comprise the EST (dbEST) database at NCBI (<http://www.ncbi.nlm.nih.gov/dbEST>). Plants represent ~18% of all ESTs in the dbEST, with half of these sequences being from rice, wheat, maize, sugarcane, sorghum and barley. This huge amount of transcript information from grasses, especially sugarcane, sorghum and maize, provides a good opportunity for comparison of the expression profiles among these species. Traits associated with metabolism, development and responses to biotic and abiotic stress can be assessed by extensive comparative analyses of the gene expression among these related species.

The functional categorization of the transcriptome in these species has mapped the expression of genes implicated in major aspects of plant biology. When ESTs are derived from non-normalized cDNA libraries, as in the case of the SUCEST project (Vettore et al. 2001; <http://sucest.lad.ic.unicamp.br/en/>), a snapshot of the processes and pathways that are active in plant growth, development and metabolism can be inferred (Vettore et al. 2003). The expression analysis of SUCEST libraries by virtual northern blotting and array hybridization has shown that EST abundance can be used to assess transcript expression levels among tissues (Arruda, 2001; Nogueira et al. 2003; Vettore et al. 2003; de Rosa et al. 2005).

Analysis of large EST dataset depends on the accurate clustering of the set of messages arising from the same transcribed gene. This allows estimating the number and the expression profile of the genes in the collection. Clustering of sugarcane ESTs in the SUCEST project was performed using the CAP3 fragment assembler (Huang and Madan, 1999) for the whole set of ESTs (Telles and da Silva, 2001). The assembly produced was based on the result of the internal and external consistency tests, where the CAP3 assembly outperformed both the phrap-hs and phrap-d assemblies. This resulted in an overall EST clustering with low incidence of discrepant reads and few redundant clusters, *i.e.* two or more clusters that probably should be condensed to a single cluster.

2.2. Functional Profiling of the Sugarcane Transcriptome

The sugarcane EST project, SUCEST (Arruda, 2001), analyzed a collection of ~240,000 ESTs generated from 26 cDNA libraries constructed from different sugarcane organs/tissues sampled at various developmental stages (Vettore et al. 2001). These ESTs were assembled into contigs, denominated sugarcane assembled sequences (SAS), that allowed the targeting of over 30,000 sugarcane genes (Vettore et al. 2003). Comparison of the sugarcane transcriptome with monocots and dicots revealed that genes shared by these plants have been highly conserved, despite the long period of independent evolution (Vincentz et al. 2004). Thus, around 71% of the sugarcane and *Arabidopsis* proteins are similar, whereas 82% of the sugarcane proteins have a match in the rice genome. Likewise, approximately 11% of the SASs may correspond to genes restricted to monocots while around 18% of the SASs may represent proteins restricted to sugarcane (Vincentz et al. 2004).

Analysis of the sugarcane SUCEST dataset has revealed genes encoding a vast array of functions associated with useful agronomic traits (Vettore et al. 2003). SASs were functionally categorized and grouped into 18 broad categories of biological roles assigned according to their involvement in a cellular process or pathway. Almost 50% of all SASs was associated with five broad categories: cellular dynamics, stress response, protein metabolism, bioenergetics, and cellular communication/signal transduction. An “unable to classify” category comprising 17% of all categorized SASs correspond to genes whose roles were unknown or could not be assigned with confidence. Proteins were also categorized according to the presence and number of conserved domains. Most of the SASs identified in the SUCEST database coded for proteins containing one domain, but a significant number contained two or three domains. The most repeated domain found was the nucleoporin FG domain of the nuclear pore complex protein. The next most repeated domains were the leucine rich repeat (LRR) and the HEAT repeat (related to armadillo/_-catenin-like repeats), both of which are involved in protein–protein interactions. Also common were the M repeats, commonly found in bacterial M proteins, and the PPR repeat, of unknown function but frequently found in plants.

The SUCEST dataset allowed a preliminary analysis of the tissue-enriched expression profile. A SAS was considered tissue-enriched if it contained at least three ESTs originating exclusively from a single sugarcane tissue (Vettore et al. 2003). Around 2,8% of all SASs presented a tissue-enriched expression. Most of the SASs presenting tissue-enriched expression was found in developing seeds that contained 33% of the total tissue-enriched SASs. Among these SASs it were found six putative new genes with a high level of expression in developing seeds.

Another interesting feature of the SUCEST database was the presence of a large group of SASs encoding proteins containing the C3H-type zinc-finger domains C-x8-C-x5-C-x3-H, which are not as numerous in *Arabidopsis* (Riechmann et al. 2000). In general, 60% of the transcription factors found in *Arabidopsis* or rice were also present in the SUCEST database (Vettore et al. 2003).

Additional information was obtained by analyzing the expression profile of genes in plants submitted to biotic and/or abiotic stress. Figure 1 illustrates the use of

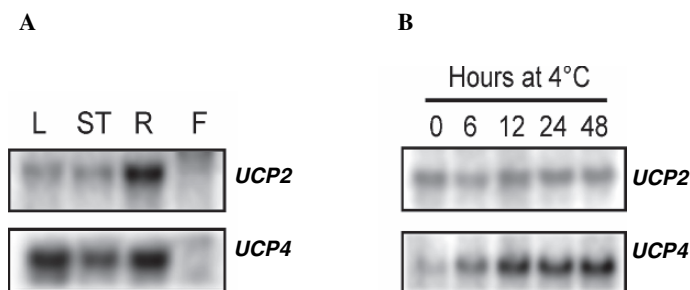


Figure 2. Accumulation of sugarcane UCP transcripts in plant organs. (A) RNA-blot analysis of *UCP2* and *UCP4* in control plants. F - flowers, L - leaves, ST- stem and R - roots. (B) Effect of exposure to cold on the accumulation of *UCP2* and *UCP4* transcripts

high-density membranes spotted with random sugarcane cDNA clones and probed with ^{33}P -labeled total RNA samples from plants submitted to cold-stress. Comparing the membranes hybridized with RNA probes from plants submitted to 4°C for 48 hrs with membranes hybridized with RNA probes from control plants it is possible to identify the cDNA clones that are up- or down-regulated by the cold treatment. The up-regulation can be further confirmed by conventional RNA gel blot analysis as exemplified in Figure 2. Using this approach, the expression profiling of sugarcane in response to low temperature revealed 34 cold-inducible genes, including cellulose synthase, ABI3-interacting protein 2, a negative transcription regulator, a phosphate transporter, and several genes encoding proteins of unknown function (Nogueira et al. 2003). Of particular interest was the transcription factor known as SsNAC23. This protein belongs to the NAC family and, in addition to be strongly induced by chilling stress, is also induced by water stress and herbivory (Nogueira et al. 2005). SsNAC23 may be involved in a general responsive process that protects sugarcane plants against biotic and abiotic stress. This process may be linked to a general regulatory network that can be activated to protect plants against oxidative stress. This hypothesis was raised by the finding that genes encoding plant mitochondrial uncoupling proteins (UCPs) were also induced by exposure to cold (Figure 2) (Nogueira et al. 2003). UCPs are a family of membrane proteins that mediate purine nucleotide-sensitive free fatty acid-activated H^+ flux through the inner mitochondrial membrane (Vercesi et al. 2006). Five members of the UCP family were identified in sugarcane (Borecký et al. 2006). Tissue-enriched expression profiling revealed that these UCPs were ubiquitously expressed, although differential expression patterns were observed during chilling stress. UCPs may help sugarcane plants respond more flexibly to biotic and abiotic stress by modulating the electrochemical proton potential ($\Delta\mu\text{H}^+$) that is directly involved in regulating the production of reactive oxygen species (Vercesi et al. 2006). Other stress-associated genes have also been identified by expression profiling analysis of sugarcane plants treated with methyl jasmonate (Bower et al. 2005; de Rosa et al. 2005).

The reaction to stress involves signalling pathways that mediate short-to-middle term responses. Over 3,500 genes involved in several aspects of signal transduction, transcription, development, cell cycle, stress responses and interaction with pathogens were identified in the SUCEST database (Souza et al. 2001). Microarray expression profiling of these signalling components in field cultivated plants revealed the expression pattern of distinct signalling proteins in flowers, roots, leaves, lateral buds, and internodes (Papini-Terzi et al. 2005). These genes may be candidate targets for controlling traits associated with the responses to biotic and abiotic stress.

Other stress related proteins identified in the SUCEST database were phyto-cystatins, a class of cysteine proteinase inhibitors implicated in the endogenous regulation of protein turnover, programmed cell death, and in defense mechanisms against pathogens. Two cystatin proteins were identified in the SUCEST database and named CaneCPI-2 and CaneCPI-3. These canecystatins were shown to inhibit papain, a cysteine proteinase from *Carica papaya* and shown to be equally expressed in leaf, meristem and root. (Gianotti et al. 2006).

Other sequencing projects have contributed to unraveling the sugarcane transcriptome. Ma et al. (2004) sequenced 9,216 ESTs from three cDNA libraries (apex, leaf and mature internode). About 57% of these sequences have significant similarity to previously characterized proteins while 28% corresponded matched proteins with unknown function. Sequence comparisons of this sugarcane ESTs with sorghum ESTs revealed similar compositions of expressed genes between tissues. Further, some of this new set of sugarcane ESTs showed no matches in SUCEST database, suggesting possible transcriptome differences between these two projects that may be attributed to germplasm and/or environmental factors affecting gene expression.

2.3. Expression Profiling of Genes Related to Sugar Accumulation

Enhancing the accumulation of sucrose is one of the major priorities of sugarcane breeders. In many sugarcane varieties, sucrose can account for up to 50–60% of the stem dry weight (Bull and Glasziou, 1963), with the limiting step in sucrose accumulation occurring during the elongation phase. This phase involves complex metabolic and physiological processes partitioned along the internodes of the cane (Whittaker and Botha, 1997; Botha and Black, 2000). Sucrose accumulation also depends on processes involved in stem maturation (Moore, 1995; Walsh et al. 2005).

The pattern of gene expression associated with sugar metabolism has been assessed by analyzing the transcriptome expression profile of stems during the elongation and/or maturation phase (Casu et al. 2003, 2004, 2005). Sugar transporters and enzymes involved in sucrose metabolism were differentially expressed in stem internodes during the elongation phase. Genes associated with stem maturation were also identified, including enzymes involved in sucrose synthesis and cleavage, in glycolysis and in the pentose phosphate pathway (Casu et al. 2003). Genes

involved in fiber and lignin biosynthesis and stress-induced responses were also up-regulated during the elongation phase (Casu et al. 2003). Of particular interest was the finding of a sugar transporter designated as PST type 2a that was abundantly expressed in the maturing stem. PST type 2a may be involved in sugar translocation in the phloem companion cells and associated parenchyma in the maturing stem (Casu et al. 2003).

3. MOLECULAR MARKERS FROM THE TRANSCRIPTOME

3.1. ESTs as a Source for Detecting Polymorphisms

Polymorphism analysis of 178 cDNAs encoding alcohol dehydrogenases (Adh) in four sugarcane cultivars revealed 37 SNPs (single nucleotide polymorphisms) in the coding and untranslated regions of three Adh genes (Grivet et al. 2003). Overall analysis revealed one SNP occurring for every 122 base pairs in the coding regions whereas insertions and deletions were limited to untranslated regions. There is considerable variation in base compositions at SNP loci between sugarcane genotypes, which can be used to fingerprint and identify individual genotypes (Cordeiro et al. 2006). This base composition variation segregates in progeny of mapping populations. Single dose SNPs appear to occur in sugarcane ESTs in low frequency.

A survey of the SUCEST database revealed over 2,000 SASs containing dinucleotide, trinucleotide and tetranucleotide simple sequence repeats (SSRs) (Pinto et al. 2004). Twenty-three out of 30 tested SSRs produced scorable polymorphisms in 18 commercial sugarcane clones. Testing of these SSRs in F_1 individuals from a cross between two commercial sugarcane cultivars produced 52 segregating markers that were potentially useful for mapping important traits since these EST-derived SSRs encoded proteins involved in sugar biosynthesis (Pinto et al. 2004). EST-derived RFLP markers have also been developed to target the sugar content (Da Silva and Bressiani, 2005).

Although the data are still limited, the EST collection now available for sugarcane is very rich in information on sequence polymorphisms. Polymorphism revealed by ESTs is particularly easy to detect in genes that are highly expressed, i.e., those for which several tens of ESTs are available.

3.2. Genes Associated with Disease Resistance

Analysis of the SUCEST database revealed a vast array of genes encoding proteins similar to the NBARC domain and NBS/LRR, LRR or S/T KINASE domains which are characteristic of the major classes of disease resistance genes (known as resistance gene analogs or RGAs) (Rossi et al. 2003). Since RGAs have been associated with disease resistance loci or quantitative trait loci (QTL) (Graham et al. 2000; Wang et al. 2001), this is potentially useful for sugarcane since these RGAs could be used to search the sorghum genome for genes or QTLs associated with disease resistance.

Fifty sugarcane RGAs revealed restriction fragment length polymorphisms (RFLP) that could be located on the sugarcane reference genetic map. One cluster

of two LRR-like loci mapped close to the gene conferring resistance to common rust (Rossi et al. 2004b). Similar results were obtained by McIntyre et al. (2005), who identified RGAs linked to major disease-resistance loci in sugarcane. The mapping of 31 of these RGAs revealed a significant association with QTLs for brown rust resistance. The presence of a brown rust resistance gene (*Bru1*) in sugarcane was confirmed by analyzing segregation of the disease in a population of 658 individuals, derived from selfing of clone R570 (Asnagui et al. 2004). Several of these RGAs were also mapped in sorghum in a region previously shown to contain a major rust-resistance QTL (McIntyre et al. 2004).

Resistance genes frequently occur in clusters (Michelmore and Meyers, 1998). In sugarcane, 16 of the 50 mapped RGA loci are organized in four clusters containing 3–6 RGAs, while 12 are in pairs (Rossi et al. 2004b). Sequence comparisons of these RGAs with the respective references in rice or maize revealed that the members of a given sugarcane cluster were more similar to the alien reference (*RPR1* or *Rp1-D*) than to members of the other sugarcane NBS/LRR locus. This finding suggested the existence of a common ancestral gene for rice *RPR1* and the sugarcane *RPR1*-like cluster, and for maize *Rp1-D* and the sugarcane *Rp1-D*-like cluster (Rossi et al. 2004b).

4. CONCLUDING REMARKS

Improvements in sugarcane productivity have not accompanied the progress observed for other grasses. This can be attributed to the highly polyploid nature of its genome that makes sugarcane not amenable to most of the breeding techniques developed for diploid species. Despite this limitation, recent progress in sugarcane genomics has provided a better understanding of its evolutionary origin and genomic structure. An important achievement in this regard was the sequencing of a large collection of sugarcane ESTs. The creation of large sugarcane transcriptome databases has allowed the identification of genes involved in sucrose metabolism and accumulation, disease resistance and responses to biotic and abiotic stress. Because of the high synteny of sugarcane and sorghum, genes discovered and mapped in sorghum have been useful in identifying their orthologs in sugarcane. In addition, the expression profiling of thousands of genes in plants subjected to biotic and abiotic stress has shed light on how sugarcane responds and adapts to different conditions. However, exploration of the information contained in the sugarcane transcriptome is still at an initial stage. Since the complete genome sequence of sugarcane has yet to be determined, the large collection of ESTs remains one of the most valuable resources for gene discovery and functional analyses in sugarcane.

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