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The application of biotechnology to wheat improvement

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Today, the world's population is increasing at the most rapid rate ever. Two hundred people are being added to the planet every minute. It is forecast that by the year 2050, the world's population will double to nearly 12 billion people. To feed this population, these people will require a staggering increase in food production. In fact, it has been estimated that the world will need to produce more than twice as much food during the next 50 years as was produced since the beginning of agriculture 10 000 years ago.

How will researchers continue to develop improved wheat varieties to feed the world in the future? At least for the foreseeable future, plant breeding as it is known today will play a primary role. What will change are the tools that can be employed. This chapter focuses on current approaches for the use of modern molecular-based technologies to develop improved varieties and discusses areas for future applications.

Biotechnology can be defined in many different ways, but for the purpose of this chapter, all areas that use molecular approaches to understand and manipulate a plant genome will be considered. However, for the sake of discussion, the techniques are divided between those that make use of molecular markers for studying the genetic material already present within the wheat plant and genetic engineering aimed at the introduction of novel genetic material. It is the latter that often raises concern and that many believe represents 'modern biotechnology'.

WHEAT MOLECULAR GENETICS

Molecular genetics, or the use of molecular techniques for detecting differences in the DNA of individual plants, has many applications of value to crop improvement. The differences are called molecular markers because they are often associated with specific genes and act as 'signposts' to those genes. Such markers, when very tightly linked to genes of interest, can be used to select indirectly for the desirable allele, and this represents the simplest form of marker-selection (MAS), whether used to accelerate the back-crossing of such an allele or in pyramiding several desirable alleles. Markers can also be used for dissecting polygenic traits into their Mendelian components or quantitative trait loci (QTL), thus increasing understanding of the inheritance and gene action for such traits and allowing the use of MAS as a complement to conventional selection procedures. Molecular markers are also used to probe the level of genetic diversity among different cultivars, within populations, among related species, etc. The applications of such evaluations are many, including varietal fingerprinting for identification and protection, understanding relationships among the units under study, efficiently managing genetic resources, facilitating introgression of chromosomal segments from alien species, and even tagging of specific genes. In addition, markers and comparative mapping of various species have been very valuable for improving the understanding of genome structure and function and have allowed the isolation of genes of interest via map-based cloning.

Several molecular marker types are available and they each have their advantages and disadvantages. In Table 10.1, the characteristics and usefulness of the most commonly used marker systems are shown. Restriction fragment length polymorphisms (RFLPs) were the first to be developed (some 15 years and have been widely and successfully used to construct linkage maps of various species, including wheat. With the development of the polymerase chain reaction (PCR) technology, several marker types emerged. The first of those were random amplified polymorphic DNA (RAPD), which quickly gained popularity over RFLPs due to the simplicity and decreased costs of the assay. However, most researchers now realize the weaknesses of RAPDs and use them with much less frequency. Microsatellite markers, or simple sequence repeats (SSRs), combine the power of RFLPs (codominant markers, reliable, specific genome location) with the ease of RAPDs and have the advantage of detecting higher

levels of polymorphism. The amplified fragment length polymorphism (AFLP) approach takes advantage of the PCR technique to selectively amplify DNA fragments previously digested with one or two restriction enzymes. Playing with the number of selective bases of the primers and considering the number of amplification products per primer pair, this approach is certainly the most powerful in terms of polymorphisms identified per reaction. For more details of the marker types discussed, refer to Hoisington *et al*. (1998).

TABLE 10.1

Characteristics and usefulness of molecular marker types for wheat molecular genetics[a]

Use[b]	RFLPs	RAPDs	SSRs	AFLPs
Varietal fingerprinting	++	-/+	+++	+++
Genetic diversity	++	-	++	+
Qualitative gene tagging	++	++	++	+++
QTL mapping	++	-/+	++	++
MAS	+	-	++	+/++
Comparative mapping	++	-	-	-
Principle	Endonuclease restriction; Southern blot; hybridization	DNA amplification with random primers	Amplification of simple sequence repeats using specific primers	Endonuclease restriction; use of adapters and specific primers
Types of probe/primers	gDNA, cDNA	Random 9- or 10-mer oligo-nucleotides	Specific 16- to 30-mer primers	Specific adapters and selective primers
Type of polymorphism	Single base changes; insertions/deletions	Single base changes; insertions/deletions	Changes in length of repeats	Single base changes; insertions/deletions
Genomic abundance	High	Very high	Medium	Very high
Level of polymorphism	Medium	Medium	High	High
Inheritance	Co-dominant	Dominant	Co-dominant	Dominant
Number of loci detected	3-9	1-10	1-3	70-140
Need for	No	No	Yes	No
sequence information				
Technical difficulty	Medium	Low	Low	Medium/High
Reliability	High	Intermediate	High	Medium/High
Quantity of DNA required	10-15 mg	10-50 ng	50-100 ng	100-1 000 ng
Use of radioisotopes	Yes/No	No	Yes/No	Yes/No
Start-up costs	Medium	Low	Medium	High
Development costs	Medium	Low	High	Medium/High

[a]RFLP = restriction fragment length polymorphism; RAPD = random amplified polymorphic DNA; SSR = simple sequence repeat; AFLP = amplified fragment length polymorphism; - = not useful; +/- = somewhat useful; ++ = useful; +++ = very useful.

[b]QTL = quantitative trait locus; MAS = marker-assisted selection.

Source: Adapted from Rafalski and Tingey, 1993.

The developments in molecular genetics in wheat have been relatively slow, especially when compared to other crops, such as maize, rice or tomato, due to wheat's ploidy level, the size and complexity of its genome, the very high percentage of repetitive sequences and the low level of polymorphism (Table 10.2). Much fewer maps exist in wheat and far fewer QTL studies have been reported when compared to other grass species. However, due to the large number of disease and pest resistances controlled by major genes, the mapping of such genes has

dominated the research activities in wheat molecular genetics. On the other hand, the hexaploid nature of wheat and its amenity to cytogenetic manipulation have offered unique tools for molecular geneticists of wheat. These include the use of various aneuploid stocks, such as nullitetrasomic and ditelosomic lines, to assign molecular markers to specific chromosome arms (Anderson *et al*., 1992; Plaschke *et al*., 1996), of chromosomal deletion stocks (Endo and Gill, 1996) for the physical mapping of markers (Röder *et al*., 1998a) and of single chromosome substitution lines to map genes of known chromosomal location (e.g. Galiba *et al*., 1995; de la Peña *et al*., 1997).

TABLE 10.2

Characteristics of the bread wheat genome that explain the slow progress in mapping as compared to a diploid, highly polymorphic species such as maize

Characteristic[a]	Wheat	Maize
Ploidy level	6x	2x
Number of chromosomes	21	10
Genome size *(number of base pairs x 10^6)*	16 000	4 500
Polymorphism level	Low	High
· RFLPs: probe x enzyme combinations (%)	20-30	80-85
· SSRs: primer pairs (%)	40-50	50-60
Repetitive sequences (%)	>80	60
To construct linkage maps of same density (15 markers/chromosome):		
Number of loci needed	315	150
Number of RFLP probes needed	1 000-1 500	200-250
Number of SSR primer pairs needed	700-800	250-300

[a]RFLP = restriction fragment length polymorphism; SSR = simple sequence repeat.

Wheat molecular linkage maps

The establishment of genetic linkage maps provides the basis for mapping the gene(s) responsible for the expression of traits of interest. In wheat, such maps have also corroborated cytological evidence of major chromosome rearrangements (Devos *et al*., 1995; Nelson *et al*., 1995a) and have allowed the comparative mapping among related species (e.g. Ahn *et al*., 1993; Börner *et al*., 1998; Devos *et al*., 1994).

The first RFLP maps were reported by Chao *et al*. (1989) for the group 7 homoeologous chromosomes. Using mapping populations developed at the John Innes Centre, Norwich, England, Devos *et al*. (1992) published the group 3 maps. These were followed by group 2 (Devos *et al*., 1993), group 5 (Xie *et al*., 1993), groups 4, 5 and 7 (Devos *et al*., 1995) and group 6 (Jia *et al*., 1996) maps. The Norwich wheat RFLP linkage map has also been published altogether (Gale *et al*., 1995) and now contains over 500 loci.

Another important mapping population was developed at the International Maize and Wheat Improvement Center (CIMMYT) by crossing a synthetic (amphihexaploid) wheat (*Aegilops tauschii* [syn. *Triticum tauschii*] x Altar 84 durum) to a spring bread wheat cultivar Opata 85 and was genotyped at Cornell University in the United States. The use of such a non-intervarietal cross resulted in a very dense map (about 1 000 RFLP loci) due to the higher polymorphism level. Maps of group 1 (Van Deynze *et al*., 1995), group 2 (Nelson *et al*., 1995b), group 3 (Nelson *et al*., 1995c), groups 4, 5 and 7 (Nelson *et al*., 1995a) and group 6 (Marino *et al*., 1996) have been published. Recently, Röder *et al*. (1998b) placed 279 SSR loci on the map also referred to as the ITMI (International Triticeae Mapping Initiative) map.

In addition to two other linkage maps in wheat (Liu and Tsunewaki, 1991; Cadalen *et al*., 1997), a number of RFLP physical maps have been constructed using Chinese Spring deletion lines (e.g. Kota *et al*., 1993; Hohmann *et al*., 1994; Gill *et al*., 1996). These deletion lines were also used to construct group 2 SSR physical maps (Röder *et al*., 1998a). In general, genetic maps have revealed a lower level of polymorphism in the D genome (Liu and Tsunewaki, 1991; Cadalen *et al*., 1997).

Furthermore, a large number of RFLP loci (Anderson *et al*., 1992; Devey and Hart, 1993) and a fair number of microsatellite loci (Plas-chke *et al*., 1996; Bryan *et al*., 1997) have been assigned to chromosome arm locations using nullisomic-tetrasomic and ditelosomic lines.

Mapping of single or major genes

In the last five years, a large number of genes of various functions have been mapped to specific wheat chromosomal regions. Table 10.3 includes a vast majority of those genes controlling disease and pest resistance, stress tolerance, quality and other traits. Several mapping/tagging strategies using mostly RFLPs and RAPDs have lead to these results. As seen in Table 10.3, several RFLP and RAPD linked markers were then converted to PCR-based, more robust markers, such as sequence tagged sites (STSs), sequence characterized amplified regions (SCARs) or allele specific amplicons (ASAs).

The existence of numerous sets of wheat near-isogenic lines (NILs) differing in the presence or absence of a resistance allele has facilitated the mapping of genes for which such lines exist (e.g. Hartl *et al*., 1993, 1995 for *Pm1*, *Pm2* and *Pm3*; Schachermayr *et al*., 1994, 1995 for *Lr9* and *Lr24*; Demeke *et al*., 1996 for *Bt-10*; Sun *et al*., 1997 for *Yr15*). Dweikat *et al*. (1997) screened a series of NILs in Newton for Hessian fly resistance alleles using 1 600 random 10-mer primers. One to three RAPD markers were identified for each of the 11 genes being tagged, and linkage determined by screening F2 populations segre-gating for each individual gene. On the other hand, Feuillet *et al*. (1995) screened Thatcher NILs for *Lr1* (on 5DL) with 37 RFLP probes mapping to group 5 chromosomes and found three to be linked to the gene after testing on F2 populations between Thatcher and *Lr1*/Thatcher. The same approach was used by Williams *et al*. (1994) who found two RFLP markers flanking the *Cre* (*Cre1*) gene on the long arm of 2B.

When the chromosomal location of a particular gene is known from previous genetic studies but no NILs are available, one can still utilize the markers mapped to that chromosome (Anderson *et al*., 1992) to score the parental lines for polymorphisms, construct a single chromosome map and determine which marker is closest to the gene of interest. This strategy was followed by Dubcovsky *et al*. (1996) to tag the *Kna1* locus in wheat responsible for higher potassium/sodium (K+/Na+) accumulation in leaves, a trait correlated with higher salt tolerance. Single chromosome maps and markers for genes on these chromosomes have also been developed using single-chromosome recombinant lines (Galiba *et al*., 1995 for *Vrn1* and *Fr1*; de la Peña *et al*., 1996, 1997 for *Pch2*; Korzun *et al*., 1998 for *Rht8*). These mapping populations were derived according to Law (1966) by crossing lines of the same background but differing for a single chromosome, back-crossing to a monosomic line for the chromosome under study, identifying the monosomic plants with a hemizygous recombinant chromosome, selfing those and detecting disomic recombinants. Despite the difficulties of producing such mapping populations, the main advantage they offer is that they allow the scoring of the phenotypic effect of the gene of interest without the confounding effects of other genes (on other chromosomes) involved in the expression of the same trait.

Bulk segregant analysis (BSA), developed by Michelmore *et al*. (1991) to tag disease resistance genes in lettuce, has been successfully applied in wheat. This approach has been mostly used with RAPDs (e.g. Hartl *et al*., 1995 for *Pm1* and *Pm2*; Hu *et al*., 1997 for *Pm1*) although it is now being used with AFLPs (Goodwin *et al*., 1998; Hartl *et al*., 1998). Either marker technique is used to screen two bulks of DNA samples from individuals identified in the two opposite tails of a segregating population for a target trait. For a major gene, all loci in the genome should appear to be in linkage equilibrium, except in the region of the genome linked to the target gene. To overcome the problems of limited repeatability of RAPDs, and the fact that repetitive sequences are often amplified (Devos and Gale, 1992), Eastwood *et al*. (1994) and William *et al*. (1997) used BSA and RAPDs on DNA enriched for low-copy sequences. In both cases, there was a noted increase in repeatability and levels of polymorphism detected compared with non-enriched DNA. The AFLP technology offers the advantage of the high number of DNA fragments amplified with one primer combination, and the problem of highly repetitive DNA is overcome by using methylation sensitive endonucleases, such as *Pst*I and *Sse*I.

The fact that several of the resistance genes mapped in wheat have been introgressed from alien species explains the success of tagging them since a higher level of polymorphism is detected compared to segments where no alien DNA is transferred.

Quantitative trait mapping

The low number of quantitative traits dissected into their QTL in wheat is a reflection of the focus given to simply inherited traits and the difficulty of building comprehensive genetic linkage maps. In addition, more work is involved to generate good quality, reliable phenotypic data from replicated field (or greenhouse) evaluations of the trait under study.

TABLE 10.3

Published markers for important genes in wheat

Trait[a]	Locus[b]	Source[c]	Marker[d]	Chromosome	Reference

Disease resistance					
Leaf rust	*Lr1*	*Triticum aestivum*	RFLP/STS	5DL	Feuillet et al., 1995
	Lr3	*T. aestivum*	RFLP	6BL	Parker *et a*/., 1998
	Lr9	*Aegilops*	RAPD/STS	6BL	Schachermayr et a/., 1994
		umbellulata	RFLP		Autrique et a/., 1995
	Lr10	*T. aestivum*	RFLP/STS	1 AS	Schachermayr *et a*/., 1997
	Lr13	*T. aestivum*	RFLP	2 BS	Seyfarth *et a*/., 1998
	Lr18	*T. timopheevii*	N-band	5BL	Yamamori, 1994
	Lr19	*Thinopyrum*	RFLP	7DL	Autrique et a/., 1995
			Isozyme		Winzeler et al., 1995
	Lr20	*T. aestivum*	RFLP	5 AL	Parker *et a*/., 1998
	Lr23	*T. turgidum*	RFLP	2BS	Nelson et a/., 1997
	Lr24	*Agropyron*	RFLP	3DL	Autrique et a/., 1995
		elongatum	RAPD/STS		Schachermayr *et a*/., 1995
			RAPD/SCAR		Dedryver et a/., 1996
	Lr25	*Secale cereale*	RAPD	4BL	Procunier et a/., 1995
	Lr27	*T. aestivum*	RFLP	3BS	Nelson et a/., 1997
	Lr29	*Ag. elongatum*	RAPD	7DS	Procunier et a/., 1995
	Lr31	-	RFLP	4BL	Nelson et a/., 1997
	Lr32	*Ae. tauschii*	RFLP	3DS	Autrique et a/., 1995
	Lr34	*T. aestivum*	RFLP	7DS	Nelson et a/., 1997
	QTL	*T. aestivum*	RAPD/RFLP	7BL, 1BS, 1DS	William et a/., 1997
Suppressor	*SuLr23*	-	RFLP	2DS	Nelson et a/., 1997
Stem rust	*Sr2*	*T. turgidum*	RFLP/STS	3BS	Johnston et a/., 1998
	Sr5	*T. aestivum*	RFLP	6DS	Parker et a/., 1998
	Sr9e	*T. aestivum*	RFLP	2BL	Parker et a/., 1998
	Sr22	*T. monococcum*	RFLP	7AL	Paull et a/., 1995
	Sr36	*T. timopheevii*	RFLP	2BS	Parker et a/., 1998
Stripe rust	*Yr15*	*T. dicoccoides*	RFLP/RAPD	1BS	Sun et a/., 1997
Powdery mildew	*Pm1*	-	RFLP	7AS	Ma et a/., 1994
			RFLP		Hartl et a/., 1995
			RAPD-STS		Hu et al., 1997
	Pm2	-	RFLP	5D	Ma et al., 1994; Hartl et a/., 1995
			RFLP, STS		Mohler and Jahoor, 1996
	Pm3	-	RFLP	1A	Ma et a/., 1994;
			RFLP		Hartl et a/., 1993
	Pm4a	-	RAPD	.	Li et a/., 1995
	Pm4b	-	AFLP	-	Hartl et a/., 1998
	Pm12	*Ae. speltoides*	RFLP	6B/6S	Jia et a/., 1994
	Pm18	-	RFLP	7AL	Hartl et a/., 1995
	Pm21	*Haynaldia villosa*	RAPD	6VS, 6AL	Qi et a/., 1996
	Pm25	*T. monococcum*	RAPD	1A	Shi et a/., 1998
Suppressor	SuPm8	-	Storage protein	1AS	Ren et a/., 1996
Wheat streak mosaic virus	*Wsm1*	*Ag. elongatum*	STS	-	Talbert et a/., 1996
Common bunt	*Bt-10*	-	RAPD		Demeke et a/., 1996
Loose smut	Ut-X	-	RFLP/RAPD-	-	Procunier et a/., 1997

	(T10)				
			STS		
	T19	-	Antibody	6A	Knox and Howes, 1994
Eyespot	*Pch1*	-	RFLP/Isozyme	7DL	Chao et *al*., 1989
	Pch2	*T. aestivum*	RFLP	7AL	de la Pena et *al*., 1997
Tan spot	QTL	-	RFLP	1AS, 4AL, 2DL	Faris et *al*., 1997
Fusarium	QTL	*T. aestivum*	AFLP/RFLP	3BS, 2AL,	Anderson et *al*., 1998
scab				6BS, 4BL	
Karnal bunt	QTL	*T. turgidum*	RFLP	3BS, 5AL	Nelson et *al*., 1998
Pest resistance					
Hessian fly	*H3,5,6,9,*	-	RAPD	1A, 5A	Dweikat *et al.,* 1997
	10, 11, 12,				
	13, 14, 16, 17				
	H9	-	RAPD	-	Dweikat et *al*., 1994
	H21	S. *cereale*	RAPD	2RL	*Seo et al.,* 1997
	H23, H24	*Ae. tauschii*	RFLP	6D, 3DL	*Ma et al*., 1993
	H27	*Ae. ventricosa*	Isozyme	$4M^V$	Delibes *et al.,* 1997
Cereal cyst nematode	*Cre1*	*T. aestivum*	RFLP-STS	2BL	Williams *et al.,* 1994, 1996
	Cre2	-	RFLP	6BL	Paull *et al.,* 1998
	Cre3	*Ae. tauschii*	RAPD	2DL	Eastwood *et al.,* 1994
	(Ccn-D1)				
Stres tolerance					
Cadmium uptake	-	-	RAPD	-	Penner et al., 1995
Aluminium	*Alt2*	-	RFLP	4D	Luo and Dvorak, 1996
tolerance			RFLP	4DL	Riede and Anderson, 1996
Drought	-	-	RFLP	5A	Quarrie *et al.,* 1994
induced ABA					
Na^+/K^+	*Kna1*	*T. aestivum*	RFLP	4D	Alien *et al.,* 1995
discrimination			RFLP	4DL	Dubcovsky *et al.,* 1996
Qualit traits					
Kernel	*Ha*		RFLP	5D	Nelson *et al.,* 1995a
hardness	*Hn,* QTL	.	RFLP	5DS, 2A, 2D,	Sourdille *et al,* 1996
				5B, 6D	
Grain protein	QTL	*T. turgidum*	RFLP	4BS, 5AL, 6AS,	Blanco et al., 1996
				6BS, 7BS	
High protein	-	*T. dicoccoides*	ASA	6B	Humphreys et *al*., 1998
LMW glutenin	-	*T. turgidum*	-	1B	D'Ovidio and Porceddu, 1996
HMW glutenin	*Glu-D1-1*	*T. aestivum*	ASA	1DL	D'Ovidio and Anderson, 1994
Flour colour	-	-	RFLP/AFLP	7A	Parker *et* al., 1998
Other trait					
Pre-harvest sprouting	QTL	*T. aestivum*	RFLP	-	Anderson *et al.,* 1993
Vernalization	*Vrn1*	.	RFLP	5AS	Galiba et *al*., 1995; Nelson *et al.,* 1995a; Korzun *et al.,* 1997; Kato et al., 1998
	Vrn3	-	RFLP	5DS	Nelson *et al.,* 1995a
Photoperiod	*Ppd1*	*T. aestivum*	RFLP	2DS	Worland *et al.,* 1997
	Ppd2	*T. aestivum*	RFLP	2BS	Worland *et al.,* 1997
Dwarfing	*Rht8*	-	SSR	2DS	Korzun *et al.,* 1998

	Rht 12	-	SSR	5AL	Korzun *et al.,* 1997
Fertility	*Rf1, Rf3*	-	RFLP	6BS, 1BS	Ma and Sorrells, 1995
restoration	*Rf4*				
Meiotic	*ph1b*	-	RFLP/STS	5BL, 5BL	Gill and Gill, 1996
pairing	Deletion	-	AFLP/STS		Qu et al., 1998

[a]ABA = abscisic acid; K^+/Na^+ = potassium/sodium; LMW = low molecular weight; HMW = high molecular weight.

[b]QTL = quality trait locus.

[c]*Ae. tauschii = T. tauschii.*

[d]RFLP = restriction fragment length polymorphism; STS = sequence tagged site; RAPD = random amplified polymorphic DNA; SCAR = sequence characterized amplified region: AFLP = amplified fragment length polymorphism; ASA = allele specific amplicon; SSR = simple sequence repeat.

Source: Modified from Langridge and Chalmers, 1998.

The ITMI map is one of the densest available, and the population from which it was developed is segregating for a number of traits. It has therefore been used to map some important traits in addition to several major genes. Known genes include vernalization (*Vrn1* and *Vrn3*), red-coleoptile (*Rc1*), kernel hardness (*Ha*) and powdery mildew (*Pm1* and *Pm2*) genes (Nelson *et al*., 1995a), as well as genes conferring and suppressing leaf rust resistance (Nelson *et al*., 1997). QTL mapped for kernel hardness (Sourdille *et al*., 1996), resistance to tan spot (Faris *et al*., 1997) and Karnal bunt resistance (Nelson *et al*., 1998) are included in Table 10.3.

Aside from the ITMI population, Anderson *et al*. (1993) reported on QTL for pre-harvest sprouting (PHS) after using around 40 RFLP markers on two segregating populations of recombinant inbred lines (RILs), which were evaluated for PHS in up to seven environments. Using a combination of RFLP markers on most of a RIL population and selective genotyping with AFLPs on a sub-set of the population, Anderson *et al*. (1998) identified five putative QTL associated with Fusarium scab resistance (see Table 10.3).

At CIMMYT, efforts in breeding for disease resistance in general, and leaf rust resistance in particular, have focused on the use of durable resistance (van Ginkel and Rajaram, 1993). Such resistance is controlled by a number of minor genes also referred to as adult plant resistance (APR) genes. In order to determine the number and location of these genes, and find tightly linked markers that will enhance the breeding efforts for such resistance, CIM-MYT has been involved in mapping APR loci in the leaf rust resistant cultivars Parula and Frontana. William *et al*. (1997), using BSA on RILs from a cross between Parula and Siete Cerros, identified three RAPD markers associated with two leaf rust resistance loci. Nullisomic-tetrasomic analysis showed that these are located on 7BL and 1BS or 1DS. CIMMYT has also constructed a genetic linkage map using RFLP, SSR and AFLP markers in a segregating population of Frontana x INIA66 in order to map primarily durable leaf rust resistance but also other important traits that are segregating in the same population. Although the map now includes about 450 marker loci, some gaps still exist and efforts are focused on filling those with the SSR markers that are becoming available (e.g. Röder *et al*., 1998b). With the current map and using composite interval mapping, CIMMYT has identified five and seven QTL for leaf rust resistance and barley yellow dwarf virus (BYDV) tolerance, respectively (Khairallah *et al*., 1998).

Marker-assisted selection

Three factors are required for the effective implementation of molecular markers in breeding programmes: (i) the availability of 'user-friendly' markers (cheap, easy and reliable); (ii) the validation of markers across different genetic backgrounds; and (iii) the possibility of implementing them within a breeding programme (Langridge and Chalmers, 1998).

RFLPs, RAPDs and AFLPs do not fit the first requirement. However, techniques are available to turn them into user-friendly markers. RFLP clones can be sequenced, and primers designed to amplify the DNA fragments are shown by hybridization to be polymorphic. However, the resulting STS or SCAR does not always turn out to be polymorphic, and further manipulations are needed if this is the case. The amplified fragment is usually digested with one or two restriction endonucleases to detect small length differences, or the fragment from two or more cultivars is cloned and sequenced again to create ASAs. ASAs are usually based on single

nucleotide differences. RAPD and AFLP fragments can be isolated from the gel, cloned and sequenced to generate STSs or SCARs, and if needed, ASAs. Attempts to generate such markers for wheat are neither always successful nor easily achieved. However, when they are, they represent very robust markers. SSRs, on the other hand, if tightly linked to genes of interest are probably the most attractive markers since no further manipulations are needed for implementation.

Despite the large number of markers for wheat genes listed in Table 10.3, few of those markers are close enough to the genes of interest to be useful in breeding applications. Some markers have been tested across a number of cultivars as a first step towards marker validation (e.g. Feuillet *et al*., 1995 for *Lr1*; Hartl *et al*., 1995 for *Pm1* and *Pm2*; Demeke *et al*., 1996 for *Bt-10*; Dweikat *et al*., 1997 for Hessian fly resistance genes; Ogbonnaya *et al*., 1998 for *Cre1* and *Cre3*). Although, in general, not a large enough number of varieties are tested, some markers seem to be promising, such as *Lr9* (Schacher-mayr *et al*., 1994), *Lr 10* (Schachermayr *et al*., 1997) and *Lr19* (Winzeler *et al*., 1995). Another factor contributes, though to a lesser extent, to the scarcity of markers used in breeding programmes. Often the scientists developing the markers are not directly connected with breeding activities and/or their laboratories are not set up to handle the numbers that would come out of a breeding programme.

The authors are aware of very few me presently using markers as additional selection tools. Examples of those include the use of ASAs or SCARs for cadmium uptake, high protein (HP) content and the 1B/1R translocation at Agriculture and Agri-Food Canada's Cereal Research Centre at Winnipeg in Canada (G.A. Penner, D.G. Humphreys and J.D. Procunier, personal communication, 1997). Ogbonnaya *et al*. (1998) report on very robust markers for both *Cre1* and *Cre3* and will be able to use those to pyramid the two resistance alleles in Australian material at the Victorian Institute for Dryland Agriculture, Horsham, Australia.

CIMMYT is presently in the process of validating a number of markers for genes of interest (e.g. *Ph1*, *Sr2*, *Lr1*, *9*, *10*, *24* and HP) across CIMMYT's germplasm and of designing a marker service facility for the easy and reliable implementation of validated markers in the breeding programmes. The facility is being designed to implement only PCR-based markers and will be equipped with a DNA sequencer and an automated pipetting station in order to be able to handle the large number of samples coming from the breeding activities.

The future of wheat molecular genetics

There is little doubt that wheat has been a difficult species for the application of molecular genetics. The low level of polymorphism between elite varieties coupled with the hexaploid nature of the crop provide significant challenges for those attempting to develop molecular markers and to use then in genetic studies. With the development of AFLP and microsatellite marker systems, renewed studies are underway to analyse the genetic basis of many important traits in wheat.

What does the future hold? While always difficult to predict, there are some significant developments in marker technologies and functional genomics worth mentioning. While the PCR-based marker systems have allowed more effective and efficient genotyping, DNA-array technology offers to increase substantially the number of genes that can be analysed (Shalon 1995; Schena *et al*., 1995; Shalon *et al*., 1996). Currently, the cost of the arrayer (to develop the chips containing the desired genes), the array reader (to detect the presence of each gene) and a set of gene sequences (to develop primers to be arrayed) have limited the application of this new technology to wheat. Both the arrayer and reader are decreasing in price and this will make this technology available to many laboratories in the near future. Efforts are also underway to develop complete expressed sequence tag (EST) databases for wheat and related species. If this data can remain in the public sector (such databases for wheat are currently available in the private sector), chips containing a significant number of wheat genes will be produced and used in the not too distant future.

Perhaps the next challenge facing wheat researchers will be gene isolation. Examples of transposon-based cloning and even map-based cloning are available in many species. A few researchers have had success in wheat, although the approaches possible are by no means routine. What is promising is the availability of large DNA libraries (yeast artificial chromosomes and bacterial artificial chromosomes) for *Ae. tauschii* (D genome, E. Lagudah, personal communication, 1997) and *T. monoccocum* (A genome, R. Wing, J. Dubcowsky and B. Keller, personal communication, 1997). These represent valuable resources for the identification and isolation of genes from wheat. The use of degenerate primers and probes from other species can readily provide candidate sequences. The only components lacking, or at least limiting, are a reverse-genetic system for wheat and a reliable and efficient genetic engineering system. The later is becoming more effective, while the former will require significant work to develop. The insertion of Ac/Ds into rice indicates that it is feasible, but the hexaploid nature of bread wheat will make it more complex. It may be more practical to develop the system in a diploid

species, such as *Ae. tauschii* or *T. monoccocum*.

Wheat has been and will continue to be a difficult species to investigate at the molecular level; however, recent innovations in technology have opened the door for renewed efforts to use wheat for molecular genetic investigations. While *Arabidopsis* and rice may provide interesting model systems, each plant species will require a certain level of study, hopefully utilizing what is known in other species. One can predict that researchers will have in their hands all the genes from most of the major crop species, including wheat, in the near future. The challenge then will be to determine the function of each and how to use this information to develop improved wheat varieties for feeding the world's growing population.

WHEAT GENETIC ENGINEERING

Cereals, including wheat, have been prime targets for genetic manipulation since the first reports of successful production of transgenic plants. However, the progress towards efficient cereal transformation has been slow, mainly due to difficulties encountered in the establishment of embryogenic cell culture methods and a lack of efficient DNA delivery systems.

Several different methods have been attempted for transforming wheat. Direct transfer of DNA into protoplasts mediated by polyethylene glycol (Mass and Werr, 1989; Potrykus, 1990) and electroporation (Larkin *et al*., 1990; Zhou *et al*., 1993) have proven to be ineffective. A more recent and versatile method for cereal transformation is microprojectile bombardment or biolistics. In this method, the DNA transfer process is genotype and tissue independent, although the regeneration of transformed cells still requires competent cells, which do demonstrate genotype differences. The biolistics method involves shooting cells with micro-particles coated with the desired DNA. By a still somewhat unknown process, the DNA is removed from the particles and ultimately inserts itself into the cell's (usually nuclear) genome. Usually, the insertion events are random and characterized by multiple copies and a certain degree of rearrangements (Jenes *et al*., 1993).

The most recent cereal transformation method involves the use of a naturally occurring bacterium, *Agrobacterium tumefaciens*. For several years, cereals were classified as non-hosts for *Agrobacterium*, as they were not infected *in vivo* or *in vitro*. Recent investigations have shown that *Agrobacterium* can attach to cereal cells, that these cells produce factors that induce *Agrobacterium* virulence genes and that the bacterium can transfer its T-DNA into the cell (Tinland, 1996).

The first reports of successful *Agrobacterium*-mediated transformation of wheat were those of Hess *et al*. (1990), which involved pipetting *Agrobacterium* onto the spikelets of wheat. Mooney *et al*. (1991) reported the first transformed cells from wheat embryos co-cultivated with *Agrobacterium tumefaciens*. These reports were considered promising, but *Agrobacterium*-mediated transformation of wheat was not considered practical until the recent reports by Cheng *et al*. (1997). *Agrobacterium*-mediated transformation has now been demonstrated for rice, maize and barley (Hiei *et al*., 1994; Rhodera and Hodges, 1996; Ishida *et al*., 1996; Tingay *et al*., 1997).

Whatever the method used, an effective selection regime is required for the isolation of transformed cells. Often, cereal tissue culture cells show a high level of natural resistance to the antibiotics or herbicides commonly used for selection (Hauptmann *et al*., 1988; Vasil *et al*., 1991). Currently, the most common selectable markers systems in use are PPT (phosphinothrycin) and bialaphos for the *bar* gene, G418 and paromomycin for the *npt*II gene, and hygromycin for the *aph*III-IV (or *hpt*) gene. None of these are as selective as required, and usually large numbers of regenerants must be screened to identify the few that are transformed. Even so, fertile transgenic wheat plants presenting herbicide resistance have been produced in several laboratories (Vasil *et al*., 1992; Weeks *et al*., 1993; Nehra *et al*., 1994; Becker *et al*., 1994; Zhou *et al*., 1995; Takumi and Shimada, 1996). Hygro-mycin-resistant wheat plants have also been produced (Sautter *et al*., 1991; Ortiz *et al*., 1996).

The availability of strong promoters, such as rice actin or maize ubiquitin, active in most cell types, are providing useful alternatives to the less active cauliflower mosaic virus 35S promoters (McElroy *et al*., 1991; Christensen and Quail, 1996; Taylor *et al*., 1993). Chamberlain *et al*. (1994) reported a new promoter (Emu) which drives very high levels of gene expression in wheat. Attention is also given to promoters that can regulate the spatial and/or temporal expression of a gene (McElroy *et al*., 1994). The modification of various characters, such as grain quality and disease resistance, will depend also on the availability of promoters to regulate gene expression in specific tissues. Recent reports have demonstrated that the use of a native wheat glutenin promoter effectively controls the expression of an introduced high molecular weight wheat glutenin gene (Shewry *et al*., 1995; Blechl and Anderson, 1996; Alt-

peter *et al*., 1996; Barro *et al*., 1997; Vasil and Anderson, 1997). Expression of the *barnase* gene under the control of a tapetum specific promoter resulted in male sterile wheat plants (De Block *et al*., 1997).

For practical application of genetic engineering technology, it is essential that transformed plants have continued expression and stable inheritance of the inserted transgene(s). There are a number of reports describing non-Mendelian inheritance and inactivation of the foreign genes (McElroy *et al*., 1994; Flavell, 1994). Such non-Mendelian inheritance and loss of expression appear to be independent of the cereal species transformed and the nature of the introduced genes. Current evidence indicates that the copy number and insertion position in the genome influence the level of stability and expression (Brettel and Murray, 1995). A better understanding of transgene inactivation is needed to improve the efficiency of the transformation process and the stability of the transgenes under field conditions. In addition, the development of site-specific recombination and transposon-mediated delivery systems may provide for improved transgene stability (Bretell and Murray, 1995).

Candidate genes for wheat genetic engineering

Genetic engineering has opened up new opportunities for plant breeders by enabling them to incorporate genes isolated from organisms outside the gene pools to which they usually have access. This broadens the possibilities they have for overcoming a number of biotic and abiotic stresses. This section describes some of the gene strategies that are being considered to provide useful products for breeders in a relatively short period of time.

TABLE 10.4
Candidate genes and targetted traits for wheat genetic engineering

Target trait	Candidate gene(s)[a]	Effects
Quality		
Bread-making quality	HMW-GS 1Ax1 HMW-GS Dx5B HMW-GS Dy10A	Increased levels of HWM-GS proteins in the endosperm of those varieties lacking these alleles; an increase in dough elasticity and strength
Nutritional quality	a, b and g zeins	Increased levels of proteins in the endosperm providing enhanced nutritional quality
Biotic stresses		
Fungal diseases	b1,3-glucanase, chitinase	Degradation of fungal cell walls
	Osmotin	Disruption of fungal membranes
	Ribosome-inhibiting protein	Disruption of fungal protein synthesis
Insects	Lectins	Induction of plant defence responses; enhanced resistance to certain grain weevils and aphids
	a-amylase inhibitors	Prevention of weevil growth and development
Nematodes	Cysteine proteinase inhibitors	Resistance to cereal cyst and root knot nematodes
Viruses	Coat protein genes	Prevention of disassembly and movement of viruses
Abiotic stresses		
Drought, heat, cold Aluminium	Peroxidases Citrate synthase	Protection against oxidative stress Binding to aluminium leading to prevention of aluminium from entering the roots

[a]HMW-GS = high molecular weight glutenin subunits.

Table 10.4 highlights a few of the genes and respective traits that are available or that have already demonstrated usefulness in wheat or other crop plants. The list is by no means exhaustive, as there is an increasingly large number of genes being cloned that will provide new opportunities in the future.

Quality traits

Other than reporter genes, perhaps the most targetted trait for genetic engineering in wheat is quality. Seed storage proteins (SSP) are contained in the seed of higher plants. These proteins have been classified as albumins, globulins and glutenins on the basis of their solubility in solvents. The high molecular weight glutenin subunits (HMW-GS) genes in wheat are located

on the long arm of the homeologous chromosomes 1A, 1B and 1D. Bread-making properties are particularly associated with variation at the *Glu-D1* and *Glu-A1* loci. The HMW-GS 1Ax1, 1Ax2, 1Dx5 and 1Dx10 have been shown to be associated with stronger dough, better elasticity and, hence, improved bread-making quality. Many elite wheat varieties lack the desired studies have demonstrated that the introduction of one or two HMW-GS genes results in a stepwise increase in dough elasticity. The transgenic lines produced so far have also demonstrated a very high level of expression and stability over several generations. This may imply that native genes are more tolerated by a plant genome.subunits and, thus, many research groups are attempting to introduce these via genetic engineering (Shewry *et al*., 1995; Blechl and Anderson, 1996; Altpeter *et al*., 1996; Barro *et al*., 1997; Vasil and Anderson, 1997). These -

In addition to increasing the bread-making quality, altered amino acid composition of the SSP is feasible and could result in improved nutritional properties. For example, the in-sertion of genes for proteins, such as zeins or albumins, could lead to an increase in the desired amino acid. Other approaches are also being considered, such as reducing the level of anti-nutritional factors and modifying starch and oil composition and content.

Induced fungal resistance

The fungal infection process is a complex mechanism that usually includes three stages: (i) prior-to-entry relationships; (ii) penetration; and (iii) establishment of the pathogen in the host. The plant resistance mechanisms involve the interaction of several factors, such as the environment, morphological peculiarities of the host and, in particular, biochemical defence genes. The later factors can be improved by the introduction of genes that modify the reaction of the plant metabolism to the infection. Examples are the introduction of the â1,3-glucanase gene that may stop the penetration of the fungus or the introduction of chitinase and osmotin genes that interact with the development of the fungal haustorium by changing the chitin structure and the osmolarity of the membrane.

Since single genes encode many of the active anti-microbial/anti-fungal factors, these defence systems are amenable to manipulation by gene transfer. The first report of success with such a strategy was the expression of a bean vacuolar chitinase gene in tobacco and *Brassica napus* that resulted in decreased symptom formation of *Rhizoctonia solani* (Broglie *et al*., 1991). Since this initial study, several other research groups have found similar results by transforming various anti-fungal genes into a range of crop plants including tobacco, tomato, canola and rice. These have resulted in enhanced resistance to a range of fungal pathogens, including *R. solani*, *Fusarium oxysporum*, *Cercospora nicotianae* and *Cladosporium fulvum* (Broglie *et al*., 1991; Logemann *et al*., 1992; Zhu *et al*., 1994; Jach *et al*., 1995; Jongedijk *et al*., 1995; Lin *et al*., 1995; Wubben *et al*., 1996).

An interesting observation is that the combination of different anti-fungal proteins can lead to synergistic protection against a broad range of phytopathogenic fungi (Zhu *et al*., 1994). Zhu's study demonstrated that coexpression of a rice chitinase and a â1,3-glucanase derived from alfalfa gave substantially higher protection against the pathogen *C. nicotianae* than either transgene alone. Similar results have been reported with tomato (Jongedijk *et al*., 1995) and tobacco (Jach *et al*., 1995). It is likely that this battery of inducible defences represents a series of complementary mechanisms for protection against both the initial attack and possibly secondary, opportunistic infections.

Other possible targets

Many other targets exist for improving wheat via genetic engineering. Several strategies are available for engineering resistance to insects, for example, the use of lectins for resistance to aphids and lepidopteran pests (Down *et al*., 1996; Gatehouse *et al*., 1996, 1997) and á-amylases for resistance to various coleopteran pests (Ishimoto *et al*., 1996). In addition, proteinase inhibitors have been used to engineer both insect (Duan *et al*., 1996) and nematode (Vain *et al*., 1998) resistance. Viral coat protein genes for enhanced resistance to viruses (Grumet, 1994), citrate synthase for tolerance to aluminium (de la Fuente *et al*., 1997) and even various options for improving the tolerance of wheat to drought, heat salinity and waterlogging are also being investigated. Often the most advanced studies are in the private sector and, thus, current information regarding their success or failure remains confidential. Hopefully, the reports, or at least the products, will be made available for use in developing new approaches for the multitude of stresses that wheat is subjected to in its growing environment.

CIMMYT's efforts in wheat genetic engineering

CIMMYT's initial activity in wheat genetic engineering has been to identify a range of elite

wheat cultivars that are not only regenerable, but also transformable. From these studies, the bread wheat genotypes Attila, Kauz, Baviacora and Bobwhite and the durum wheat genotypes Minimus, Ariza, Altar and Bajio were found to be highly regenerable and excellent candidates for transformation efforts (Bohorova *et al*., 1995). Efforts are now focused on the production of transgenic plants via biolistics and *Agrobacterium*-mediated methods. Current target traits are enhanced fungal resistance via various pathogen-related proteins, including chitinase, glucanase and ribosome-inhibiting proteins, and enhanced quality via HMW-GS genes. The first putative transgenic plants are being produced and investigated in a biosafety greenhouse. As these are confirmed, progeny are produced and analysed for the inheritance of each transgene. Ultimately, the best events will be taken to the field under appropriate biosafety regulations for proper evaluation of the expression of the inserted transgene and for effects on other agronomic characteristics.

CONCLUDING REMARKS

Although the potential of biotechnology has often been exaggerated, a high level of optimism is clearly justified for its use in the improvement of wheat. Undoubtedly, functional genomics, as it is now termed, will revolutionize the way in which plant breeding is undertaken in the future. Basic research is leading to an improved understanding of the genetic mechanisms operating within a plant in response to the diverse stresses that it is exposed to, as well as the overall production of biomass and grain. New methodology, such as automated marker systems and array technology, will allow the large number of samples to be processed that are encountered in a typical breeding programme. In fact, perhaps the biggest challenge facing scientists is how to store, process and access the vast amounts of information generated using these new marker systems. This knowledge base offers promise for making germplasm improvement faster, cheaper and more effective.

Emerging genetic engineering techniques are providing breeders with the never-be-fore-seen capability to create novel plants by combining genetic material from a wide array of sources. Although not without controversy, the options seem limitless and, with the proper oversight and understanding, should provide extremely powerful options to develop durable and highly productive plant varieties for almost any production environment.

The challenge for developing countries is to tap as much of this emerging technology as possible. This does not necessarily mean that countries must establish inhouse capabilities. What is required is that nations recognize the importance of the new approaches and ensure that appropriate legislation and regulations are enacted to allow the country to acquire, evaluate and most importantly deploy the new plant varieties produced via biotechnology. All available tools to ensure an adequate supply of food for the world must be employed.

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