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Molecular Breeding for Maize Improvement: An Overview

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The maize genome is one of the most extensively analyzed among the plant genomes. Consequently, maize has been at the forefront in development and evaluation of an array of molecular markers for varied purposes in genetics and breeding. Besides the well-demonstrated utility of molecular markers in genotype differentiation and analysis of genetic diversity in maize germplasm, application of DNA-based markers is also of considerable significance to tropical/sub-tropical maize production systems, such as in India, for mapping and marker-assisted selection for resistance to major biotic/abiotic stresses affecting production and productivity. Significant impetus in this direction has been provided in recent years through the Asian Maize Biotechnology Network (AMBIONET). This article provides an overview of the recent efforts under AMBIONET in relation to: (i) the molecular characterization of inbred lines developed by various public sector institutions in India; (ii) the analysis of genetic diversity in the Indian maize germplasm using microsatellite markers; and (iii) the mapping of quantitative trait loci conferring resistance to different downy mildews affecting maize in tropical Asia. Judicious integration of conventional and molecular approaches in maize breeding programmes is vital for efficient utilization of genetic resources, and improving the production and post-harvest characteristics of the elite germplasm. This shall, in turn, require further strengthening of synergistic linkages and partnerships among national and international research institutions to harness the rapidly emerging information and technologies related to molecular breeding in maize.

Keywords: ***Zea mays*, markers, fingerprinting, genome mapping, marker-assisted selection**

Introduction

Maize (*Zea mays* Linn.) holds a unique position in world agriculture as a food, feed and industrial crop *par excellence*. Although the developed countries, particularly USA, contribute predominantly to the maize production, demand for maize in developing countries is expected to surpass the demand for both wheat and rice by the year 2020 (Pingali & Pandey, 2001). However, average productivity of maize in several developing countries is still considerably low. About 45 million hectares of maize is grown in the lowland tropics, where a range of climatic, biotic and abiotic constraints severely affect productivity. The challenges are diverse and complex, and there is no single technological solution. While significant progress has been made in relation to maize improvement in India using traditional breeding strategies (Dhillon & Prasanna, 2001), considerable scope exists to further enhance maize productivity. Modern molecular tools and techniques can complement conventional approaches to allow breeders to effectively address priority research areas.

The term 'molecular breeding' is now popularly used for the utilization of molecular (DNA-based) tools, including markers, to enhance the efficiency of the breeding process. DNA markers have the potential to aid plant breeding programmes through diverse ways, such as fingerprinting of elite genetic stocks, analysis of genetic diversity, and increasing the efficiency of selection for difficult traits. Among the array of DNA-based markers available to plant scientists, the ones most commonly used are RFLPs, RAPDs, SSRs and AFLPs. Excellent reviews are available discussing the genetic bases of various DNA-based markers, the means for detecting molecular polymorphism, and the strengths and constraints associated with different markers for

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Abbreviations:
AFLP: Amplified fragment length polymorphism; EST: Expressed sequence tag; GS: Genetic similarity; MAS: Marker-assisted selection; PAGE: Polyacrylamide gel electrophoresis; PCR: Polymerase chain reaction; PIC: Polymorphism information content; QPM: Quality protein maize; QTL: Quantitative trait loci; RAPD: Random amplified polymorphic DNA; RFLP: Restriction fragment length polymorphism; RILs: Recombinant inbred lines; SNP: Single nucleotide polymorphism; SSR: Simple sequence repeats.

Table 1—Characteristics and utility of different molecular markers for applied molecular genetics in crop plants

	RFLPs	RAPDs	SSRs	AFLPs	SNPs
Fingerprinting	++	–/+	++	++	+++
Genetic diversity	++	–	++	+	?
Tagging qualitative genes	++	++	+	+++	++
Mapping polygenic traits	++	–/+	+	++	++
Marker-assisted selection	++	–	++	+/++	++
Comparative genome mapping	++	–	–	–	–
Principle	Endonuclease restriction; Southern blotting	DNA amplification with random primers	PCR of simple sequence repeats	Endonuclease restriction; amplification using adapters and specific primers	DNA sequencing
Type of polymorphism	Single base changes; Insertions/Deletions	Single base changes; Insertions/Deletions	Changes in length of repeats	Single base changes; Insertions/Deletions	Single base differences
Genomic abundance	High	Very high	Medium	Very high	Very high
Level of polymorphism	Medium	Medium	High	High	Very high
Inheritance	Codominant	Dominant	Codominant	Dominant..?	Codominant
Detection of allelic variants	Yes	No	Yes	No	Yes
No. of loci detected	1-5	1-10	1	30-100	1
Need for sequence information	No	No	Yes	No	Yes
Technical difficulty	Medium	Low	Low	Medium/High	Medium/High
Reliability	High	Intermediate	High	Medium/High	High
Quantity of DNA required	2-15µg	10-50 ng	2-15 ng	2-15 ng	2-15 ng
Use of radioisotopes	Yes/No	No	Yes/No	Yes/No	No
Probes/primers required	gDNA/cDNA	Random 9- or 10-mer oligonucleotides	Specific 16-30-mer primers	Specific adapters and primers	Specific primers
Start-up costs	Medium	Low	Medium	High	High
Development costs	Medium	Low	High	Medium/High	Medium/High

various applications (Karp *et al*, 1997; Liu, 2002). The most appropriate marker(s) for a particular application will depend on the target crop, it's breeding behaviour, specific objectives of the experiment, the resolution required, and the operational/financial constraints, if any. A comparison of the various marker systems in relation to their characteristics and applicability is provided in Table 1. For example, for genetic linkage map development, any type of molecular marker may be used. However, codominant markers (e.g., RFLPs, SSRs or SNPs), provide more genetic information in F_2 and backcross generations than markers detecting predominantly presence/absence or dominant polymorphisms (e.g., RAPDs or AFLPs). For comparative mapping within and across crop species, the use of RFLP as anchor loci are the best choice as they detect evolutionarily conserved loci in a more predictable manner than loci detected by hypervariable SSRs and AFLPs.

Among the different types of PCR-based DNA markers available for diverse applications in maize breeding, SSR markers are often preferred for reasons of cost, simplicity and effectiveness. SSR markers are robust, codominant, hypervariable, abundant, and uniformly dispersed in plant genomes

(Powell *et al*, 1996a,b). In maize, more than 1000 mapped SSR markers are available in the public domain (MaizeDB; http://www.agron.missouri.edu). Mogg *et al* (1999) showed that by sequencing the flanking regions of maize microsatellites, a SNP could be found every 40 bp. Given that the maize genome is estimated to be 2.5×10^9 bp in size, there is a potential for up to 62 million SNPs in maize. With the recent initiation of a large-scale EST sequencing programme in maize (http://www.zmdb.iastate.edu/zmdb/EST_project.html), a new and potentially rich source of SNPs has been uncovered (Edwards & Mogg, 2001). While both SSRs and SNPs can be reliably applied on a large scale with only small quantities of DNA required for PCR amplification, SNPs are highly amenable for automation, and therefore, offer significant advantages for plant breeding purposes. SSRs, however, are the preferred choice when codominant, multiallelic information is required, or when the infrastructure and resources are limited.

Applications of Molecular Markers

Molecular markers are increasingly being adopted by researchers involved in crop improvement as an effective and appropriate tool for addressing several basic and applied research areas relevant to agricultural production systems (Mohan *et al*, 1997; Prioul *et al*, 1997). DNA fingerprinting and genetic diversity analysis using molecular markers is of significant utility in effective management of germplasm collections (Warburton & Hoisington, 2001). Increasing emphasis is also being placed on comprehensive analysis of genetic diversity in breeding materials of major crops (Mohammadi & Prasanna, 2002). Accurate assessment of the levels and patterns of genetic diversity using molecular markers is particularly helpful in maize breeding for (i) maintenance and broadening of the genetic base of the elite germplasm; (ii) assignment of lines to heterotic groups; (iii) selection of appropriate parental lines for hybrid combinations; and (iv) generation of segregating progenies with maximum genetic variability for further selection.

DNA-based markers are also being used to discover and exploit the evolutionary relationships between various genera within a family (e.g., the grass family, Poaceae), and various species within a genus. Genetic mapping of members of the agriculturally-important grasses, including rice, wheat, maize, sorghum and sugarcane, with common DNA probes has revealed remarkable conservation of gene content and gene order (Devos & Gale, 2000), reinforcing the paradigm of the "grasses as a single genetic system" (Bennetzen & Freeling, 1993; Freeling, 2001). Comparative genomics has significant implications for the application of genetic information generated in one member of the grass family (such as rice or maize or sorghum) to the potential improvement of agronomically important traits in other members.

This article provides a brief overview of (i) the application of molecular markers to characterize maize germplasm and analyze genetic diversity; and (ii) the mapping and marker-assisted selection for specific agronomically important traits in maize. In discussing the above, the focus will be on the recent studies undertaken under the AMBIONET programme in India, and the work being carried out at CIMMYT's Applied Biotechnology Center in Mexico.

Molecular Profiling of Maize Germplasm

Maize breeders in India, as in most developing countries, have differentiated inbred lines mainly on the basis of major morphological characters such as plant height, anthocyanin colouration of various plant parts, tassel type, tassel branching, days to flowering, ear characters, cob colouration, grain colour and grain type (Virk & Witcombe, 1997). Although morphological descriptions are important for ascertaining the agronomic utility of germplasm, such descriptions are not very reliable because of complex 'genotype × environment' interactions that require assessment in multiple locations/environments (Smith & Smith, 1989). Detailed studies in various crop species, particularly in maize, have established that methods that solely depend on morphological data are neither consistent nor effective in unambiguous differentiation of elite breeding materials (Smith & Smith, 1988, 1989; Bar-Hen *et al*, 1995). Genetic heterogeneity, different combinations of alleles producing similar phenotypes, and environmental influence on genotypes, result in morphological similarities or differences that may not be proportional to the underlying genetic differences.

In the past, isozyme and zein chromatographic data (Stuber & Goodman, 1983; Smith, 1988) have been used to characterize elite inbred lines and commercial hybrids of maize (Bar-Hen *et al*, 1995). Isozyme analysis is relatively simple and less costly in comparison with molecular marker analysis; however,

inadequate genomic coverage, relatively low levels of polymorphism, developmental regulation and pleiotropic effects impose major constraints in effectively using these markers in genotype differentiation and analysis of genetic diversity (Smith & Smith, 1986; Dubreuil *et al*, 1996). In recent years, PCR-based SSR markers have been effectively used for differentiation of US and European maize germplasm, as they are particularly suited for genotype discrimination (Smith *et al*, 1997; Warburton *et al*, 2002).

In India, no systematic efforts were made to effectively apply molecular markers for genetic fingerprinting or analysis of genetic diversity in the maize inbred lines developed by public sector institutions, including those that are commonly used for hybrid maize breeding. Recently, however, studies have been carried out to profile a selected set of Indian maize genotypes, including inbred lines and single-cross hybrids, using both morphological and microsatellite markers, and to analyze the genetic diversity in the maize inbred lines that are commonly used in the public sector institutions (Pushpavalli *et al*, 2001, 2002; Mohammadi *et al*, 2002a). Observations were recorded on 20 'categorical descriptors' (qualitative or visually assessed quantitative characters) in 47 Indian inbred lines to ascertain their utility in effective differentiation of genotypes. The 'categorical' descriptors, however, revealed very low polymorphism in the Indian lines analyzed, with a total of only 55 variants, highlighting the severe limitations of utilizing only morphological data for establishing the identity or distinctness of genotypes. In contrast, molecular profiling of 69 inbred lines, comprising 58 Indian lines, 6 CIMMYT lines developed at Mexico (used as 'reference genotypes'), and 5 lines from the CIMMYT-Asian Regional Maize Programme (CIMMYT-ARMP), Thailand, using 58 polymorphic SSR markers revealed high levels of polymorphism (435 alleles) (Fig. 1A). Identification of 109 unique/rare SSR alleles (found in not more than 1 or 2 of the genotypes analyzed) facilitated effective discrimination of the genotypes analyzed. The high level of polymorphism displayed by the SSR loci was also reflected by the average PIC value (0.70). On the basis of high PIC values (>0.75) and distinct allelic size ranges, SSR markers such as *dupssr17*, *bnlg1647*, and *bnlg198*, could be effectively used in differentiating the Indian maize inbred lines. Distinct and non-overlapping size ranges of the amplification products of SSR loci with high PIC would also facilitate multiplexing for improving the assay efficiency (Fig. 1B), as suggested by Mitchell *et al* (1997).

The AMBIONET study also revealed high level of SSR heterozygosity in some of the Indian maize inbred lines. There could be various reasons including residual heterozygosity due to inadequate cycles of inbreeding, improper pollination control during seed multiplication, seed stock contamination or accumulation of mutations at diverse SSR loci. Amplification of similar sequences in different genomic regions due to duplications is another possible reason for occurrence of double-band phenotypes. High levels of heterozygosity for some of the inbred lines such as CM115, CM117, CM123, CM124, CM205 and CM208 were revealed earlier through isozyme analysis (Mauria *et al*, 2000). SSR profiling of these inbreds confirmed the above observation. However, some inbreds such as CM111 and CM116, which were considered as 'genetically pure' based on isozyme analysis (Mauria *et al*, 2000),

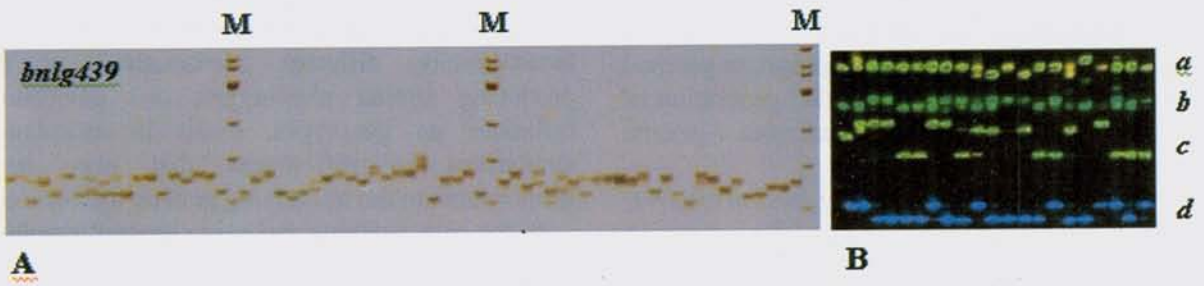

Fig. 1—(A) SSR polymorphism revealed by *bnlg439* (A) in Indian maize inbred lines using PAGE and silver staining technology. Φx-174/Hinf1 digest (size range of 66-726 bp) was used as molecular weight standard (M). (B) Polymorphism in selected Indian maize inbreds revealed by multiplexed, fluorescent-labeled SSR primers, *ph084* (a), *nc130* (b), *phi308707* (c), and *phi089* (d); the methodology, using semi-automated DNA sequencers, facilitates accurate sizing of SSR alleles, besides enhancing the assay efficiency.

showed high levels of heterozygosity in SSR analysis. Such incongruities in data derived from biochemical versus DNA-based markers could result due to the low level of isozyme polymorphisms and limited genomic coverage.

A sequential method combining marker information and agro-morphological description, proposed by Smith *et al* (1991) suggests: (i) comparison of two lines at marker loci and declaring them distinct only if their genetic similarity (GS) value is below a predetermined threshold; (ii) comparison of the two lines for agro-morphological traits only if their GS value is beyond this threshold. In the second step, the environmental variation for the morphological traits allows construction of statistical tests to determine the 'minimum distance' between the two inbreds, which assumes considerable significance in the context of plant variety protection. It would be interesting to ascertain the broader applicability and effectiveness of this proposal for routine profiling of elite breeding materials.

With the availability of high throughput technologies that can make use of fluorescent-labeled SSR markers through multiplexing, fingerprinting has been extended to classification of genetically diverse materials such as landraces, populations, open-pollinated varieties, and germplasm accessions. Earlier studies on characterization of populations have relied on only a few individuals per population, as the cost and time required to characterize each line tends to be the limiting factor. The efficiency and accuracy of population fingerprinting can be enhanced by using a bulking strategy for individuals of a specific population, followed by analysis of the bulks using multiplexed SSR primers and semi-automated DNA sequencing technology. A set of 7 tropical maize populations and 57 inbred lines at CIMMYT were recently fingerprinted using 85 multiplexed SSR primers, leading to identification of 53 highly discriminatory SSR markers (Warburton *et al*, 2002).

Analysis of Genetic Diversity in Indian Maize Germplasm using Molecular Markers

Pedigree information provides a broad estimate of the expected genetic relatedness among lines, but for allogamous crops such as maize, such information is often unobtainable or unreliable especially when inbred lines were derived from a broad base population (Melchinger *et al*, 1991; Messmer *et al*, 1993). DNA-based markers, particularly SSRs and AFLPs, have provided powerful tools for analyzing genetic diversity (Pejic *et al*, 1998; Vuylsteke *et al*, 2000). AMBIONET studies on molecular profiling of Indian maize germplasm provided valuable information about genetic relationships in the breeding materials (Pushpavalli *et al*, 2001, 2002; Mohammadi *et al*, 2002a,b). Analysis of SSR allele frequencies revealed a reasonably broad genetic base in the Indian maize germplasm. However, much of the SSR allelic variation in the inbred lines analyzed was contributed by the inbred lines developed at Punjab Agricultural University (PAU), Ludhiana. Cluster analysis of the genetic dissimilarity matrix for the genotypes under study using Ward's method, besides application of 'pattern-finding methods' such as principal coordinate analysis, aided in determining genetic relationships, which were broadly in agreement with the available pedigree data.

The cluster pattern based on the AMBIONET study revealed essentially three main groups having two sub-clusters each. The majority of the Indian inbred lines that were derived earlier from the Colombian germplasm (CM111, CM114, CM120, CM300) were clustered in Group I, while some Colombian lines (CM104, CM105 and CM115) were placed in Group II. Almost all of the early-maturing inbred lines developed at IARI, New Delhi, such as CM135, CM136, CM137 and CM138 clustered in Group II. Some of the inbreds developed at PAU, Ludhiana, and analyzed in this study (CM122, CM123, CM124, CM125, CM140) clustered in Group II. The validity of the clusters was reflected when the patterns were analyzed in relation to the well-known pedigree information for some inbred lines, particularly those developed by PAU, Ludhiana (Dhillon *et al*, 1998; Saxena *et al*, 2000). For instance, based on pedigree data, CM122 must be highly related to CM140, and both these genotypes should also show close genetic relationship with CM124 and CM125. These expected genetic relationships were clearly validated by the results of various clustering procedures that formed the basis for the consensus cluster pattern. Group II also included CM202 and its close derivatives, CM211 and CM208. These inbred lines also show close genetic association primarily with CM122 and CM140. This could be due to the fact that CM202 was extensively utilized in the derivation of Makki Safed Pool C4, which also comprises CM122 and CM140.

The study indicated the genetic distinctness of some of the Ludhiana inbred lines (LM5, LM6 and

CM139), as they were placed in a distinct cluster (Group III). LM5 and LM6, parental lines of the single-cross hybrid 'Paras', were derived from Tuxpeno Pool and Makki Safed Pool C2, respectively. Suwan-1, developed by Kasetsart University, Thailand, had also contributed to the development of Tuxpeno Pool (Dhillon *et al*, 1998; Saxena *et al*, 2000). AMB109, a line developed from Thailand germplasm (AMATL line) has shown close relationship with LM5, while other AMB lines (which mainly includes lines from Thailand and Philippines) displayed closer relationship with LM6 and CM139. The semi-exotic Pools A and B have close correspondence with Makki Safed and Tuxpeno Pools, respectively. This was also reflected in the placement of CM139 between LM5 (from Tuxpeno Pool) and LM6 (from Makki Safed Pool). The accuracy of the cluster pattern derived from this study was reflected by the grouping of some of the BIO lines, which served as 'controls'. For instance, BIO5 (an advanced line from LM5) grouped closely with LM5; similarly, BIO7 (an advanced line from LM6) with LM6, and BIO2 (an advanced line from CM211) with CM211.

The AMBIONET study in India also indicated close genetic associations among some downy mildew-resistant lines such as AMB112, AMB119, AMB115 and AMB109 (obtained from the CIMMYT-ARMP, Thailand) and NAI116, an unreleased Indian inbred that is highly resistant to sorghum downy mildew (SDM; *Peronosclerospora sorghi*) and Rajasthan downy mildew (*P. heteropogoni*) (Nair *et al*, 2001). This can be attributed to the utilization of SDM-resistant germplasm from Thailand in the development of NAI116 and some AMB lines (e.g., AMB112). The close clustering of the CIMMYT-ARMP lines and their genetic distinctness from a majority of the Indian maize lines highlights the possibility for further expansion of the genetic base of Indian maize germplasm through efficient use of these genotypes. In contrast to the CIMMYT-ARMP lines, the six inbred lines from CIMMYT, Mexico, showed dispersion in various clusters. The AMBIONET study serves as an effective foundation for further analysis of genetic relationships of the inbred lines being developed by the National Agricultural Research System (NARS).

In another recent study at IARI, a set of 23 QPM lines, including 13 inbreds developed by the national programme as well as 10 selected tropical/sub-tropical QPM lines developed by CIMMYT were analyzed for their grain quality, agronomic performance and molecular polymorphism using SSR markers. Polymorphic profiles for 36 SSR loci have aided in effectively differentiating the QPM inbred lines. The study resulted in identification of SSR markers, such as *bnlg439*, *phi037*, *bnlg125*, *dupssr34* and *bnlg105*, with high polymorphism information content in the selected QPM genotypes. Analysis using SSR markers indicated high levels of heterozygosity in majority of the Indian QPM lines and in one CIMMYT QPM inbred, CML188. Cluster analysis using SSR data, followed by canonical discriminant analysis, clearly distinguished the Indian QPM inbreds from those developed at CIMMYT (Kassahun & Prasanna, 2002). The cluster patterns were largely in congruence with the available pedigree information of the QPM inbreds studied. The study demonstrated the utility of SSR markers in analysis of genetic relationships among QPM lines, and shall aid in planned utilization of CIMMYT QPM lines in QPM breeding programmes being undertaken in India.

One of the potential applications of molecular marker data of inbred lines is to identify parents useful for developing or improving single-cross hybrid performance. Although it is unlikely that the markers such as SSRs affect the phenotypic expression of the targeted quantitative trait(s) directly, they can serve to identify adjacent (linked) genomic segments. In such a case, marker divergence of inbred lines can be useful to predict hybrid performance. This is of particular value in crops like maize where significant effort and resources are devoted to field-testing of newly created lines in various single-cross combinations to identify lines with superior combining ability. A number of studies have been carried out in maize to ascertain the association between molecular marker divergence and hybrid performance, leading to different results (Stuber *et al*, 1999). Majority of the studies, however, indicate that genotypic differences may be useful for preliminary selection of loci/alleles for possible improvement of hybrids (Mohammadi *et al*, 2002b), but probably will not accurately reflect performance of a hybrid. Field evaluation of nearly 92 hybrid combinations derived from 48 Indian maize inbred lines in three seasons/environments, recently carried out by the AMBIONET-India team, in conjunction with the SSR

allele data for these inbred lines, indicated that molecular marker divergence is not significantly correlated with the hybrid performance, reinforcing the conclusion made above. A consensus opinion is now emerging on this key issue that: (i) the genome should be well-saturated with uniformly spaced markers and/or high level of linkage equilibrium must exist for marker data to be reasonably successful in predicting hybrid performance; and (ii) marker data can be more useful for predicting hybrid performance of lines that are related and from a narrow genetic base than those derived from highly divergent genetic backgrounds.

QTL Mapping in Maize

The discovery of extensive, yet easily visualized, variability at the DNA level, coupled with the development of statistical packages that can help in analyzing variation in a quantitative trait in congruence with molecular marker data in a segregating population, led to mapping of QTL influencing an array of agronomically important traits in diverse crop plants including maize. A QTL may be defined as a *region* of the genome that is associated with an effect on a quantitative trait. Conceptually, a QTL can be a single gene, or a cluster of tightly linked genes that affect the trait. Excellent reviews dealing with various aspects of QTL mapping in crop plants are available (Beavis, 1998; Liu, 2002; Hackett, 2002). QTL mapping and identification of molecular markers closely linked to QTL with major effects on a target trait can permit MAS in backcross, pedigree, and population improvement programmes (Young, 1999; Ribaut *et al*, 2002a,b). This is especially useful for crop traits that are otherwise difficult or impossible to select for by conventional means.

Molecular markers have been used to identify and characterize QTL associated with diverse traits in maize including grain yield, characters concerned with domestication, environmental adaptation, disease and insect pest resistance, and drought and heat stress tolerance (Stuber, 1995; Stuber *et al*, 1999). Comprehensive information about such experiments can be obtained from the MaizeDB (http://agron.missouri.edu). A case study with potential utility in effective management of downy mildew diseases in maize in tropical Asian countries is discussed below.

Mapping QTL Influencing Resistance to Downy Mildews in Asia—A Case Study

Downy mildews occur predominantly in tropical/sub-tropical regions of China, India, Indonesia, Japan, Nepal, Pakistan, Philippines and Thailand in Asia, where the disease is an important factor limiting maize production (Pingali & Pandey, 2001). The major downy mildews that infect maize in the region include the sorghum downy mildew [*Peronosclerospora sorghi* (Weston & Uppal)], Philippine downy mildew (*P. philippinensis* [Weston] Shaw), Java downy mildew [*P. maydis* (Raciborski)], sugarcane downy mildew [*P. sacchari* (Miyabe) Shirai & Hara], and brown stripe downy mildew [*Sclerophthora rayssiae* var. *zeae* Payak & Renfro]. While *P. sorghi* causes downy mildew in both sorghum and maize, the maize strain of *P. sorghi* in Thailand, which is now reclassified as *P. zeae* rarely infects sorghum. Different types of downy mildew are reported in India, including sorghum downy mildew, brown stripe downy mildew and sugarcane downy mildew. In Rajasthan (India), the downy mildew pathogen which forms oospores in the wild grass, *Heteropogon contortus* (speargrass) was renamed *P. heteropogoni* (Siradhana *et al*, 1980) and the disease, caused when maize is infected by the conidial stage of the fungus, is now referred to as Rajasthan downy mildew (White, 1999). Despite the introduction of downy mildew resistant cultivars and the use of metalaxyl fungicide, severe incidence of the downy mildews still occurs in localized areas (Dalmacio, 2000). Cost concerns related to seed treatment with fungicide, and the emerging problem of chemical resistance build-up in the pathogen (Raymundo, 2000), point to the use of resistant varieties as a more cost-effective and environmentally-safe alternative in controlling this disease.

Identification of molecular markers linked to downy mildew resistance genes should have a major impact on maize breeding across the tropical Asian region. As a Network activity under the AMBIONET programme, four countries (India, Indonesia, Thailand and Philippines) undertook a QTL mapping project aimed at identifying downy mildew resistance genes (George *et al*, 2002). The mapping was based on evaluation of a set of recombinant inbred lines (RILs) derived from the cross of Ki3 (resistant) by CML139 (susceptible). The downy mildew resistant parent, Ki3, is a tropical yellow flint line with late maturity from Suwan-1, a cultivar developed in Thailand

against *P. zeae*. The susceptible parent, CML139, is a subtropical yellow-red semi-flint line with intermediate maturity, developed from CIMMYT materials for tropical corn borer resistance. Groh *et al* (1998) constructed a molecular map using 135 RILs developed from the Ki3 × CML139 cross and 143 RFLP markers, for QTL mapping of southwestern corn borer (SWCB) resistance. In the AMBIONET study, the same 135 RIL families were evaluated for downy mildew reaction (during 2000-2001) at Mandya in southern India against sorghum downy mildew (*P. sorghi*); at Udaipur in western India against Rajasthan downy mildew (*P. heteropogoni*); at Maros in Indonesia against Java downy mildew (*P. maydis*); at Farm Suwan in Thailand against sorghum downy mildew (*P. zeae*); and at Southern Mindanao in Philippines against Philippine downy mildew (*P. philippinensis*). The phenotypic data, thus, comprised downy mildew disease incidence data from individual environments as well as pooled data across environments. Composite interval mapping (Zeng, 1994) was carried out for joint analysis of data across environments to map QTL and to estimate their genetic effects.

The AMBIONET study led to the identification of QTL with significant effects on resistance to the five important downy mildew diseases affecting maize production in the Asian region. The QTL that were detected highlighted differences in the pathogen populations that characterize the four locations. Three QTL, two on chromosome 2 and one on chromosome 7, significantly influenced resistance only to particular pathogen populations. The first QTL on chromosome 2 (position 158 cM), with resistance due to alleles from the susceptible parent CML139, was specific to sorghum downy mildew at Mandya. The second QTL on chromosome 2 (position 234 cM) and the QTL on chromosome 7, with resistance due to alleles from the resistant parent Ki3, were found to influence specifically *P. heteropogoni* at Udaipur in India. The most important genomic region, having the highest LR values in the analysis of data from individual locations as well as in the joint analysis, and having a consistent expression against the different downy mildews, was found on chromosome 6. This QTL was consistently expressed across environments despite the significant effect of the environments having distinct pathogen populations.

The proportion of the phenotypic variance explained by each of the five QTL (R^2 values) varied across environments. Collectively, the five QTL identified in this study explained phenotypic variation in disease susceptibility ranging from 24% (Thailand) to 54% (Udaipur, India). Most significantly, the QTL on chromosome 6 had the largest contribution, accounting for nearly 20% and 31% of the phenotypic variance for *P. sorghi* and *P. heteropogoni* disease susceptibility at Mandya and Udaipur, respectively, and explaining more than half of the total phenotypic variance due to the five QTL in each of the four environments. Significant QTL × E interactions and large estimates of σ^2_{ge} observed across the locations indicated major influence of the environment, particularly the characteristic pathogen populations on the expression of downy mildew resistance.

Significantly, the major QTL on chromosome 6 (bin 6.05) is located in a region holding clusters of resistance genes in maize. Groh *et al* (1998) identified a QTL in Ki3 at an adjacent region on chromosome 6 conferring resistance to leaf feeding damage caused by south western corn borer. Other genes that have been located on chromosome 6 in bin 6.01 include *mdm1* which confers resistance to the maize dwarf mosaic virus (MDMV) (Simcox *et al*, 1995); *wsm1* which confers resistance to a related potyvirus, wheat streak mosaic virus (WSMV) (McMullen & Louie, 1991); *rhm1*, which confers resistance to the fungal pathogen *Cochliobus heterostrophus* (Zaitlin *et al*, 1993); and a QTL conferring resistance to sugarcane mosaic virus (SCMV) (Zhang & Li, personal communication).

Selection for QTL using genetic markers can be effective if a significant association is found between the quantitative trait and the genetic markers. The AMBIONET study identified three SSR markers *umc11*, *umc23a*, and *umc113* tightly linked to the QTL on chromosome 6 (George *et al*, 2002), indicating their possible use for MAS. Beyond their possible use in MAS, another potential application of these results would be the identification and analysis of candidate genes to deduce information about the nature and function of the detected gene(s) in determining resistance to downy mildews in Asia.

Chances of successful application of MAS for downy mildew resistance are better when QTL are identified in the germplasm used in the national breeding programme. For this purpose, the AMBIONET-India team also screened nearly 80

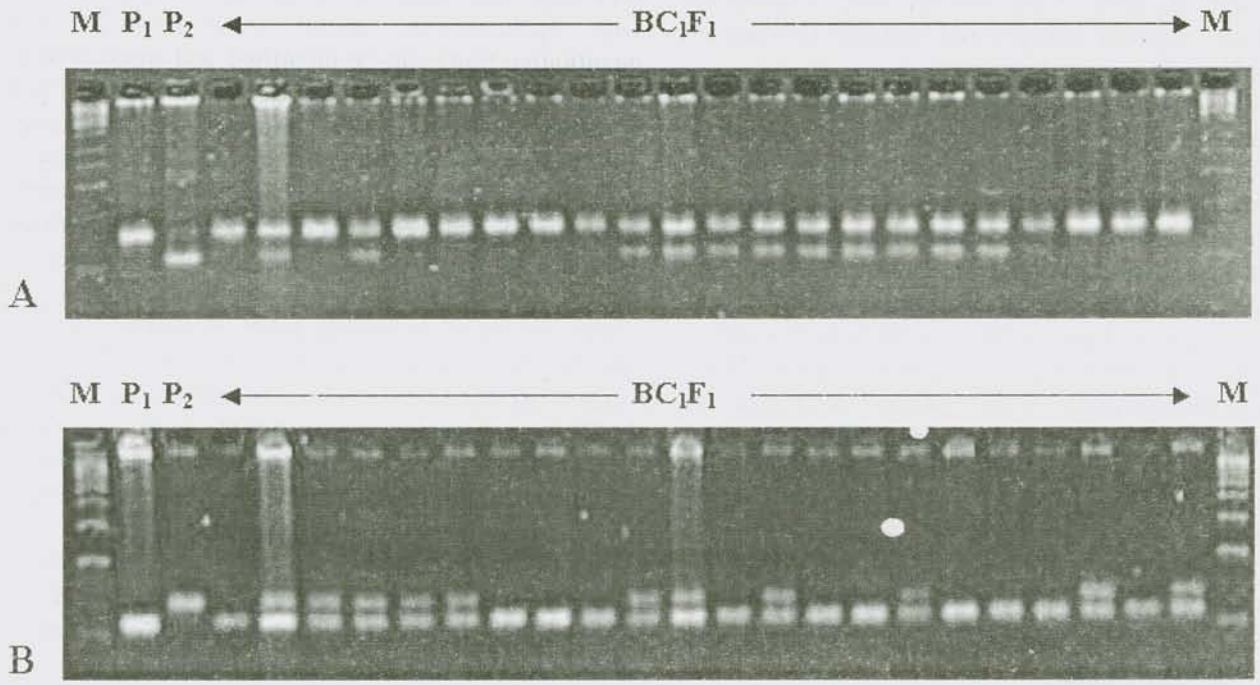

Fig. 2—Genotyping of a panel of BC_1F_1 mapping population (for QTL mapping of downy mildew resistance in maize), for two polymorphic SSR loci, *bnlg490* (A) and *bnlg1655* (B); the mapping population was derived using CM139 (P_1) and NAI116 (P_2) used as recurrent (susceptible) and donor (resistant) parents, respectively. A 100-bp ladder was used as the molecular size standard (M) for the gels run on a super-fine resolution 3.5% agarose.

inbred lines, including 50 Indian genotypes, against *P. sorghi* and *P. heteropogoni* at Mandya and Udaipur, respectively. The study led to the identification of NAI116, an excellent source of resistance against both the downy mildew diseases in India (Nair *et al*, 2001). A backcross mapping population was developed using CM139 (elite susceptible inbred) and NAI116. Analysis of the genotypic and phenotypic data from this mapping population (Fig. 2 A & B) confirmed the effect of the major QTL detected on chromosome 6 (bin location 6.05) by the earlier AMBIONET study of the RILs. Introgression of this major QTL governing resistance to downy mildews into CM139 using marker-assisted backcrossing is now ready to be undertaken.

Marker-assisted Selection (MAS) in Maize Breeding

MAS consists of identifying associations between markers and alleles of the gene/QTL of interest, and then using these associations to develop improved lines or populations (Ribaut & Hoisington, 1998; Knapp, 1998). Through marker-assisted backcrossing, individuals can be backcrossed until they contain the particular genomic segment in the genetic background of the recipient or recurrent parent. For MAS to be effective, recombination between the marker and the gene/QTL must be minimal. This is achieved using closely linked flanking markers.

The basic purpose of marker-assisted backcrossing is to speed up line conversion, and to reduce the linkage drag of the transferred gene(s). Through classical backcross (BC) breeding, the transfer of a single dominant gene would require six BC generations to recover 99% of the recurrent parent genome. This procedure is time-consuming and labour-intensive for breeding of crops such as maize, where turnover times of new lines and hybrids are fast. In a BC_1 generation the proportion of the recurrent parent genome would be distributed normally around a mean of 75% (in later BC generations, the distribution would become increasingly skewed) but given a sufficient sample size, it would contain plants with more than 85% recurrent parent genome. These plants can be identified with molecular markers to accelerate the breeding process (Tanksley *et al*, 1989). Without molecular markers flanking the target gene it is nearly

impossible to remove the linkage drag coming as "baggage" with the introgressed segment (Murray *et al*, 1988).

MAS is now being routinely applied in the breeding programmes of several crops, including maize, for (1) tracing favourable alleles in the genomic background of genotypes of interest; and (2) identifying individual plants in large segregating populations that carry the favourable alleles. For instance, two of the prominent examples of utilization of molecular markers in line conversions through a BC approach being practiced in maize at CIMMYT are: (1) introgression of the *opaque2* (*o2*) gene on chromosome 7 for the development of QPM lines, and (ii) transfer of a major QTL identified on the short arm of chromosome 1 that is associated with maize streak virus (MSV) resistance. The utility of MAS in QPM breeding is particularly worth discussing as QPM has considerable relevance to various maize-growing countries, particularly in the developing world, including India.

The maize grain accounts for about 15% to 56% of the total daily calories in diets of people in about 25 developing countries, particularly in Africa and Latin America (FAO Agrostat, 1992), where animal protein is scarce and expensive and consequently, unavailable to a vast sector of the population. Maize seed-protein quality can be improved by selecting for the homozygous recessive *o2* allele state (Mertz *et al*, 1964). The presence of the homozygous *o2* allele state is correlated with changes in the amino acid balance within the endosperm, and more specifically, a favourable increase in the proportion of lysine and tryptophan. Cloning and sequencing of the *o2* gene (Schmidt *et al*, 1990) allowed detection of three SSR markers (*phi057*, *phi112* and *umc1066*) within the sequence of the gene itself. CIMMYT has been routinely screening thousands of genotypes, using these three SSRs, in segregating populations to identify genotypes that have one copy of the *o2* mutant allele (BC strategy) and those that have two copies (self-pollination strategy). Selection is conducted before flowering to allow the pollination of only the selected plants. Integration of MAS for *o2* is a relatively simple and effective strategy for accelerating QPM development, and this strategy is currently being employed in various countries including India (Prasanna *et al*, 2001).

MAS can also be of great relevance to improvement of polygenic traits. By combining the QTL approach (selection for favourable QTL effects) with backcrossing, useful genes that control quantitative traits can be identified and transferred to advanced breeding lines (Lande & Thompson, 1990; Tanksley & Nelson, 1996; Hospital & Charcosset, 1997). In maize, Stuber *et al* (1992, 1999) mapped QTL associated with seven major traits (including grain yield), and were also able to generate improved versions of inbred lines using obsolete inbreds as donors. The efficiency of using molecular markers for improvement of polygenic traits in maize breeding programmes was also demonstrated in a few other studies (Ribaut *et al*, 2002a). Despite these examples, MAS for polygenic traits in maize, as in most other crop plants, is still in its infancy. Manipulating quantitative traits is difficult due to the involvement of a large number of genes involved in trait expression, often with varying effects, interactions between the genes (epistasis), and QTL × environment interactions (Beavis & Keim, 1996). This implies that several regions/QTL must be manipulated simultaneously to have a significant impact, and that the effect of individual regions is not easily identified.

CIMMYT researchers have devoted considerable efforts during the past three decades to improve pre- and post-flowering drought tolerance in maize. Although significant progress has been achieved for improving drought tolerance in CIMMYT maize germplasm through conventional breeding (Bänziger *et al*, 2000), the approach is slow and time-consuming. Use of molecular markers and QTL information based on carefully managed replicated tests has the potential to alleviate the problems associated with inconsistent and unpredictable onset of moisture stress or the confounding effect of other stresses such as heat. The approach was primarily based on breaking down the complex trait of drought tolerance into simpler components, and to manipulate genomic regions related to components like anthesis-silking interval (ASI) that are closely associated with drought tolerance. To this end, CIMMYT conducted several experiments on QTL analysis and MAS for transfer of drought tolerance to tropical maize, and obtained encouraging results. An integrated strategy of QTL mapping, MAS and functional genomics is now being explored to further provide useful information and tools to effectively complement conventional selection for drought tolerance in maize (Ribaut *et al*, 2002a).

Integrating MAS in Maize Breeding

Despite a wealth of published literature on QTL mapping, particularly in recent years, a number of constraints have imposed severe limitations on effective utilization of QTL information in plant breeding through MAS. Salient among these constraints are: (i) identification of a limited number of major QTLs controlling target traits; (ii) inadequacies/experimental deficiencies in QTL analysis leading to overestimation/underestimation of the number and effects of QTLs; (iii) lack of QTL/marker associations applicable over different sets of breeding material; (iv) strong QTL x environment interaction; and (v) difficulty in precisely evaluating epistatic effects. Recently, novel strategies have been proposed (Ribaut & Betran, 1999; Ribaut *et al*, 2002b), particularly using maize as a model system, to overcome some of these major constraints. The efficacy of such strategies in improving the efficiency of gene introgression using molecular markers, and reducing the cost of MAS experiments, is being analyzed at CIMMYT.

The cost-effectiveness of using molecular markers (SSRs) in MAS experiments in maize was also estimated (Dreher *et al*, 2000). The study revealed that when only a few SSR markers are used and when several hundred genotypes are screened, MAS is cost-effective. Using SSR markers to select for the *opaque2* gene during QPM development exemplifies the utility of MAS as an efficient substitute for phenotypic selection, considering the recessive nature of the gene, absence of obvious visual selection due to the interaction of this gene with modifiers involved in kernel vitreousness or hardness (an essential character in QPM), and greater cost per sample when the endosperm protein quality is analyzed through chemical analysis. The cost-effectiveness of MAS over phenotypic selection, particularly for complex polygenic traits, is also likely to improve in the future, with the availability of more efficient and high-throughput techniques for detection of molecular polymorphism.

Future Prospects

Understanding the complex web of interactions between genes and environmental factors, and effective application of this information for plant/animal improvement is a challenging endeavour for biologists. To obtain relevant information, it is imperative to exploit the tools of both classical and molecular genetics. The developments in the recent years in relation to molecular marker technology and QTL analysis have allowed identification of genomic regions involved in an array of agronomically important traits in diverse crop species, particularly maize. Such information is also providing clues to better understand genome organization as well as genetic phenomena such as epistasis, pleiotropy and heterosis. However, the impact of marker-based QTL analysis on varietal development has been less than expected, primarily due to two reasons: (i) experiments related to QTL discovery and varietal development have largely been independent, as pointed out by Tanksley & Nelson (1996); and (ii) for traits such as grain yield, QTL expression is usually dependent upon the genetic background, unlike traits such as disease or insect resistance which are usually less complex in comparison with grain yield (Stuber *et al*, 1999). Development of high-throughput, reproducible molecular marker technologies, coupled with advances in genomics research, are now promising to offer powerful tools to maize researchers for more effective integration and utilization of MAS for diverse applications in breeding programmes.

Besides some highly encouraging developments in molecular breeding, structural and functional genomics research in maize is progressing at a healthy pace (Stuber *et al*, 1999; Coe *et al*, 2002). From a time when the maize genome was considered to be too complex to consider large-scale physical mapping and sequencing, we have now reached a point where several research teams in the developed world are striving to generate contig maps for maize and determine the sequence and function of the several thousands of ESTs that are already identified (Coe *et al*, 2002). The advances in maize functional genomics, including gene expression profiling and proteomics (Lee *et al*, 2002), should allow us in the near future to identify the key genes as well as pathways involved in expression of traits such as biotic/abiotic stress tolerance (Cushman & Bohnert, 2000; Seki *et al*, 2001).

The NARS in India have demonstrated the commitment and capacity to effectively apply modern biotechnology, particularly molecular markers, for crop improvement. In maize, we should focus primarily on three areas: assessment of genetic diversity, application of marker-assisted selection using previously identified QTL and their flanking markers, and development of multiple trait-targeted

mapping populations based on parents that are adapted to the Indian agricultural production system(s). The data already developed under the AMBIONET programme in relation to molecular profiling of Indian maize inbred lines, besides agro-morphological data already available from the breeders, provide valuable information in clearly understanding the genetic diversity in the inbred genetic base, thereby permitting more efficient utilization of genetic resources, both indigenous and exotic, in breeding programmes. In addition, application of optimized molecular marker technology will reduce the costs of genetic resource conservation and further improve their utilization. Towards this goal, core collections of maize in India, that include maximum genetic diversity and best represent existing variation, must be developed.

Cost-effective application of molecular marker technology to agriculturally important problems in India cannot be done in isolation. Researchers in India can immensely gain by building effective linkages with partners elsewhere to harness the synergy of collective efforts in molecular breeding, as exemplified by AMBIONET. Networking can facilitate development of an integrated system for efficient application of molecular tools and techniques, including QTL mapping and MAS, in maize breeding programmes of the NARS. This would, in turn, significantly aid in development of improved germplasm, including cultivars, with greater yield potential and ability to overcome major biotic and abiotic constraints limiting maize productivity, in the minimum possible time and with minimal operational expenses. Collaborative research under AMBIONET is presently focused on the application of molecular marker technology to problems of national and regional importance, such as molecular characterization of locally important maize lines, mapping of QTLs for resistance to major diseases – downy mildews, SCMV and Banded leaf and sheath Blight – and tolerance to abiotic stresses (drought and low nitrogen conditions), and integration of MAS in the breeding programmes.

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