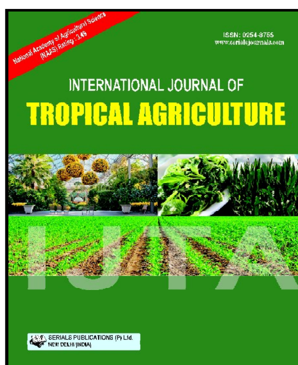

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Present Status and Future Prospectus for Applications of Genetic Engineering for Hybrid Development in Maize (*Zea mays*)

Sajad Hussain Dar*

Division of Genetics and Plant Breeding SKUAST- Kashmir, Srinagar, India, E-mail: Dar.sajad@rediffmail.com

Abstract: The process of plant breeding has been improved through improved biotechnological processes that allow the specific identification of heterotic groups, molecular approaches to screening, molecular marker assisted selection, embryo rescue, haploid breeding, and exploitation of somaclonal variation and through site directed mutagenesis or gene silencing approaches. The most fundamental aim in the study of intrinsic yield was to exploit the phenomenon of heterosis, the increased yield that can be obtained from the hybrid between two selected inbred parents. Many current projects, mostly on maize, are designed to understand the genetic basis of this process. Determination and measuring of genetic variation is the most important step in any breeding program and data obtained by the methods of molecular genetics could be best interpreted by correlation with the procedure already well developed in conventional genetics. The development of genetics and molecular biology has opened a new chapter in the field of describing agronomical important genotypes and providing their much more detailed characterization. The detection of genes or QTLs controlling traits is possible due to genetic linkage analysis, which is based on the principle of genetic recombination during meiosis. This permits the construction of linkage maps composed of genetic markers for a specific population.The main uses of DNA markers in plant breeding, with an emphasis on important MAS schemes have been classified into marker-assisted evaluation of breeding material; marker-assisted backcrossing; pyramiding; early generation selection; and combined MAS. Prior to crossing and line development, there are several applications in which DNA marker data may be useful for breeding, such as cultivar identity, assessment of genetic diversity and parent selection, and confirmation of hybrids. Backcrossing has been a widely used technique in plant breeding for almost a century. The most widespread application for pyramiding has been for combining multiple disease resistance genes.It seems clear that current breeding programmes continue to make progress through commonly used breeding approaches. Recombinant DNA technologies including MAS could greatly assist plant breeders in reaching this goal although, to date, the impact on variety development has been minimal. If the effectiveness of the new methods is validated and the equipment can be easily obtained, this should allow MAS to become more widely applicable for crop breeding programmes.

INTRODUCTION

An understanding of the scientific principles behind crop improvement practices has come only in the last hundred years. But the early, crude techniques, even without the benefit of sophisticated laboratories and automated equipment, were a true practice of biotechnology guiding natural processes to improve man's physical and economic well-being. More recently, using cross-breeding techniques, transgenic plants are being obtained. These plants are more tolerant of herbicides, resistant to insect or viral pests, or express modified versions of fruit or flowers have been grown and tested in outdoor test plots since 1987. The genes for these traits have been delivered to the plants from other unrelated plants, bacteria, or viruses by genetic engineering techniques. This is but one biotechnological application in crop improvement.

The biotechnology movement is a fledgling industry that has tremendous potential for development. It focuses on the use of fermentation and enzyme technologies, tissue culture and recombinant DNA (rDNA) technology and is more greatly applied to plant varieties rather than animal species. Tissue culture is by far the most developed type of technology but increasing attention is being paid to rDNA technology. There are also notable efforts on improved disease diagnostic and genetic resource management services and marker-assisted selection for economic traits, including disease resistance.

The process of plant breeding has been improved through improved biotechnological processes that allow the specific identification of heterotic groups, molecular approaches to screening, molecular marker assisted selection, embryo rescue, haploid breeding, and exploitation of somaclonal variation and through site directed mutagenesis or gene silencing approaches. After biological sciences development, particularly molecular biology, during last decade's molecular biotechnology, based on genetic engineering is widely used in genome structure and expression investigation. As consequence of the some results application**,** new varieties of commercially grown crops with higher yield are developed, as well as decreasing time to breed new genotypes having satisfactory resistance to different pathogens, tolerance to biotic and abiotic stress conditions etc. Inspitebnh of the fact that both information technology and molecular biotechnology are „strategic" technologies, it is out of the question that remarkable progress in maize genetics and breeding was gained during last few decades by the use of conventional genetics and breeding methods. Although modern maize breeding is based on the concepts of Shull (1910) methods of breeding and evaluation have changed due to utilisation of new or improved germplasm sources, more knowledge and information on the inheritance pathway of complex traits as well as information from theoretical and computer simulation studies.

Existing or induced variation that will enable the selection of superior genotypes is at the core of a successful breeding program. Kuckuk *et al.* (1991) classified breeding methods into three groups: (i) selection breeding, where breeder relies on existing variation in natural populations and genotype mixtures; (ii) combination breeding where combinatorial crosses are made and F1 is not used directly, but only to generate subsequent segregation material that will form the basis for selection and finally (iii) hybrid breeding where combinatorial crosses are made to create new genotypes that will be used as F1 seed. Crucial items to have effective breeding is the prediction of the best hybrid within a large set of possible genotype combinations based on genetic distance of the parental lines. According to Boppenmaier*et al.* (1992) this was not possible for line combinations only between heterotic pools of European material. It is important to survey and choose germplasm as pure lines, cultivars, populations, clones, genes, DNA sequences etc (Lee, 1995). Development of a large number of molecular

markers raises question whether only their application can further enhance the efficiency of maize breeding. It is important to collect more information on the reliable level of polymorphism in order to understand biological processes, including both genetic control of storage protein and oil biosynthesis as well as other important traits in different genome combinations.Crop improvement could also be achieved through tissue culture, improved diagnostics and other conventional biotechnological approaches such as N-fixation and biopesticides.

Analysis and exploitation of heterosis

The most fundamental aim in the study of intrinsic yield was to exploit the phenomenon of heterosis, the increased yield that can be obtained from the hybrid between two selected inbred parents. Many current projects, mostly on maize, are designed to understand the genetic basis of this process. For example, hybrids between the maize inbred lines B73 and Mo17 exhibit heterosis regardless of the direction of the cross. These reciprocal hybrids differ from each other phenotypically, and 0·30–0·50 of their genes are differentially expressed. Recently, a study described 4000 expression quantitative trait loci that allowed the identification of markers linked to variation in expression (Swanson- Wagner *et al.* 2009). Heterosis is not only observed in adult traits such as yield or plant height, but can be detected during embryo and seedling development. Hence, the maize primary root, which is the first organ that emerges after germination, is a suitable model to study heterosis manifestation (Paschold *et al.* 2009). Proteome profiling experiments of maize hybrid primary roots revealed non-additive accumulation patterns that were distinct from the corresponding RNA profiles and emphasized the importance of posttranscriptional processes such as protein modifications that might be related to heterosis. It is very likely that the underlying causes of heterosis will be revealed in the next few years and the existing methods for producing and exploiting hybrids will be greatly improved and extended beyond the existing crops such as maize and rice. Concurrent with the study of heterosis are investigations designed to improve the isolation of haploids that act as the source of homozygous lines required as parents for the production of F1 hybrids (Dunwell 2010a). Some of these novel methods, such as exploiting modified centromeric proteins (Ravi & Chan 2010), involve the use of transgenic plants.

Molecular breeding

Determination and measuring of genetic variation is the most important step in any breeding program and data obtained by the methods of molecular genetics could be best interpreted by correlation with the procedure already well developed in conventional genetics. Maize is extremely diverse genus, havingmorphological and biological differences. Despite the nearly unlimited diversity of germplasm, the main problem is the creation of suitable new crosses, arises from divergent parental lines. To find the best method, which provides discrimination according to the purpose of selection, will remain a challenge both for classical and molecular geneticists.Information on the genetic diversity and relationships of lines orpopulations is useful for choice of parents, crossing, and classification of germplasm into heterotic groups, prediction of heterosis and plant variety protection. Maize breeders are mainly concerned with the genetic diversity among and within breeding population and elite germplasm, because it largely determines the future prospects of success in breeding programs. Compressive studies of genetic diversity based on molecular markers has been reported in maize (Messmer*et al.* 1992; Melchineger*et al.* 1991;Srdic *et al.* 2006). Standard methods in maize breeding imply numerous crosses of inbred lines to different testers so as to gain information on genetic similarity, i.e. diversity of these inbreeds. According to this, inbreeds are allocated into specific heterotic groups.

Just these extensive field studies are the most expensive and the most time consuming part of contemporary maize breeding and selection, and at the same time this procedure is very restrictive due to the fact that only a few inbreeds can be crossed and estimated.

An alternative could be an allocation of inbred lines in heterotic groups based on molecular markers. As this approach would provide the use of a greater number of inbreeds it would significantly accelerate the process of development ofsuperior hybrids and decrease cots that burned the maize breeding programme. Commercial maize germplasm is divided into 12 groups based on patterns of heterotic behavior. Members of partially heterotic groups should be detectable by their genetic relatedness. About 125 maize inbred lines from different heterotic groups and miscellaneous origin have been examined using different molecular markers (protein, RAPD). Genetic distances between the genotypes were determined from the molecular marker data, and cluster analysis was used to find groups of related genotypes. The clusters found with molecular markers closelyresemble the heterotic groups to which the 125 genotypes belonged. Three main groups were distinguishable: a group of BSSS lines, a group of Lancaster lines, and a set of lines with European background. Similar groups were detected despite differences in marker type and genetic distance method used (Drinic*et al.* 2000; Drinic 2007;Srdic, 2006). Information on genetic diversity of commercially grown maize hybrids is very important for germplasm enhancement, hybrid breeding and in preventing environmental damage. Genetic uniformity implies risks of genetic vulnerability to stress factors, which may be reduced by use of unrelated single cross hybrids. It is important to choose among hybrids the one that will give highest yield and answer to environmental stress due to their existing genetic diversity (Troyer*et al.*1983). Three methods of determining genetic diversity among maize hybrids are being used: a method that is based on molecular markers (Melchineger*et al.*1991; Nagy and Marton, 2006), a method that is based on the genetics of inbreeding and heterosis for grain yield (Troyer *et al.* 1988;Williams and Halauer, 2000) and a method based on pedigree data (Smith*et al.* 2004). Hybrid maize breeding programs in Serbia as well as at Maize Research Institute were started in the 1950s. Five periods, each characterized by introduction of the new potentially higher yielding ZP hybrids with other agronomiccharacteristics improved, have determined maize breeding program at the Maize Research Institute "Zemun Polje" (Drinic*et al.* 2006).ZP hybrids from different periods have been study by RAPD markers (Eric, 2003;Bauer *et al.* 2005;Bauer*et al.* 2007). Cluster analysis showed distinctive grouping of hybrids from each period. Changes in genetic background of parental genotypes during the last 50 years have a major impact on genetic diversity among ZP maize hybrids.

Heterosis background investigation by molecular markers

Comparing to other crop species maize has probably the highest level of genetic polymorphism. The most significant practical consequence of the hugegenetic diversity between maize genotypes is the phenomenon of hybrid vigor or positive heterosis. Maize breeders have always been interested in choosing theparental lines which would result in positive heterotic combination withoutnecessarily making all possible crosses among the potential parental combination. The various methods are in use to predict heterosis and can be grouped into (i) per se performance, (ii) combining ability and (iii) genetic diversity as determined through geographic origin, morphological and agronomic traits as well as molecular markers. The experimental data indicate that heterosis is a function of heterozygosity in a higher number of loci and that the increase of the heterozygous loci number by crosses to genetically distant lines or populations increases the level of

heterosis in the crosses. Based on this hypothesisHallauer*et al.,* (1988) assumed that the magnitude of heterosis could be predicted on the basis of inbred lines differences obtained after use of molecular markers. Different classes of molecular markers have been used to analyze the genetic relationships among maize inbred lines and to examine the relationship between marker-based GD and heterosis in maize (Lee*et al.* 1989;Boppenmar*et al.* 1992). Correlation level varies depending on the analyzed material and various types of gene effects, pointing to the complexity of the genetic background of heterosis. The general conclusion of studies based on the results of molecular markers application is that heterosis is significantly related to heterozygosity of marker loci. The studies on the possibility to correlate heterosis and to make prediction of heterosis by the use of molecular marker data the breeders obtained high and significant correlation between GD from analyses of inbred lines with protein markers (Konstantinov*et al.* 1996, Drinic*et al.* 2006), RAPD (Srdic*et al.*2006; Drinic*et al.* 2006;Drinic*et al.* 2007), SSR and ALFP (Drinic*et al.* 2002). The results indicating that GD based on molecular markers at the level of gene expression – polypeptides pattern in dry seed, is correlated with heterosis and that markers could be used for prediction of heterotic effect.

Germplasm characterization

The development of genetics and molecular biology has opened a new chapter in the field of describing agronomical important genotypes and providing their much more detailed characterization, not only in terms of how distant their germplasms are from those of the existing one but also in the sense of monitoring the uniformity and stability of their characteristics relative to each other. Combining morphological, biochemical and molecular aspects in identification anddescription of agronomical important genotypes, it is possible to reveal theirunique genetic profiles e.g. fingerprints. The most useful markers for maize germplasm characterization are proteins markers, RFLP, SSR, RAPD, and AFLP. The SDS-PAGE method of protein profile sample mixture is included in ISTA international rules as a technique for distinguishing among and identifying commercial genotypes of different plant species. This method was used for genetic characterization of maize inbred lines as well as for distinguish sister lines at the Maize Research Institute, and for first screening of genetic purity of hybrid seed (Drinic*et al.* 2000;Eric, *et al.* 2003;Drinic*et al.* 2006). All analyzed genotypes have unique protein pattern and unique code - combination of numbers and letters - have been assigned to them.

Most polypeptides identified from the electrophoretic or chromatographic profiles are mostly products of the gene expression that are unevenly distributed in the genome and expressed at the developmental stage of the tissue, the sample has been taken from. This prevents providing a sample that would cover the entire genome (Galovic*et al,* 2006). The complete coverage of a genome can be achieved by the use of molecular markers for the variability identification at the level of DNA - DNA polymorphism. Genetic characterization of maize inbred lines with standard kernel type as well as popcorn and sweet corn inbreeds from MRI collection was done by RFLP (Konstantinov and Denic 1985; Kidric *et al.* 1987;Konstantinov *et al.*1988) RAPD, SSR and AFLP markers (Drinic *et al.* 2000, Drinic *et al.* 2004; Eric, 2003).

QTL mapping and MAS

The detection of genes or QTLs controlling traits is possible due to genetic linkage analysis, which is based on the principle of genetic recombination during meiosis (Tanksley 1993). This permits the construction of linkage maps composed of genetic markers for a specific population. Segregating populations such as F_2, F_3 or backcross (BC) populations are frequently used. However,

populations that can be maintained and produced permanently, such as recombinant inbreds and doubled haploids, are preferable because they allow replicated and repeated experiments. These types of populations may not be applicable to outbreeding cereals where inbreeding depression can cause non-random changes in gene frequency and loss of vigour of the lines. Using statistical methods such as single-marker analysis or interval mapping to detect associations between DNA markers and phenotypic data, genes or QTLs can be detected in relation to a linkage map (Kearsey 1998). The identification of QTLs using DNA markers was a major breakthrough in the characterization of quantitative traits (Paterson *et al.* 1988).

Reports have been numerous of DNA markers linked to genes or QTLs (Mohan *et al.* 1997; Francia *et al.* 2005). Previously, it was assumed that most markers associated with QTLs from preliminary mapping studies were directly useful in MAS. However, in recent years it has become widely accepted that QTL confirmation, QTL validation and/or fine (or high resolution) mapping may be required (Langridge *et al.* 2001). Although there are examples of highly accurate preliminary QTL mapping data as determined by subsequent QTL mapping research (Price 2006), ideally a confirmation step is preferable because QTL positions and effects can be inaccurate due to factors such as sampling bias (Melchinger *et al.* 1998). QTL validation generally refers to the verification that a QTL is effective in different genetic backgrounds (Langridge *et al.* 2001). Additional marker-testing steps may involve identifying a 'toolbox' or 'suite' of markers within a 10 cM 'window' spanning and flanking a QTL (due to a limited polymorphism of individual markers in different genotypes) and converting markers into a form that requires simpler methods of detection.

Once tightly linked markers that reliably predict a trait phenotype have been identified, they may be used for MAS. The fundamental advantages of MAS over conventional phenotypic selection are as follows.

a. *It may be simpler than phenotypic screening, which can save time, resources and effort.* Classical examples of traits that are difficult and laborious to measure are cereal cyst nematode and root lesion nematode resistance in wheat (Eastwood *et al.* 1991; Eagles *et al.* 2001; Zwart *et al.* 2004). Other examples are quality traits which generally require expensive screening procedures.

b. *Selection can be carried out at the seedling stage.* This may be useful for many traits, but especially for traits that are expressed at later developmental stages. Therefore, undesirable plant genotypes can be quickly eliminated. This may have tremendous benefits in rice breeding because typical rice production practices involve sowing pre-germinated seeds and transplanting seedlings into rice paddies, making it easy to transplant only selected seedlings to the main field.

c. *Single plants can be selected.* Using conventional screening methods for many traits, plant families or plots are grown because single-plant selection is unreliable due to environmental factors. With MAS, individual plants can be selected based on their genotype. For most traits, homozygous and heterozygous plants cannot be distinguished by conventional phenotypic screening.

These advantages can be exploited by breeders to accelerate the breeding process (Ribaut & Hoisington 1998; Morris *et al.* 2003). Target genotypes can be more effectively selected, which may enable certain traits to be 'fast-tracked', resulting in quicker line development and variety release. Markers can also be used as a replacement for phenotyping, which allows selection in off-season

nurseries making it more cost-effective to grow more generations per year (Ribaut & Hoisington 1998). Another benefit from using MAS is that the total number of lines that need to be tested can be reduced. Since many lines can be discarded after MAS early in a breeding scheme, this permits more efficient use of glasshouse and/or field space which is often limited because only important breeding material is maintained.

Considering the potential advantages of MAS over conventional breeding, one rarely discussed point is that markers will not necessarily be useful or more effective for every trait, despite the substantial investment in time, money and resources required for their development. For many traits, effective phenotypic screening methods already exist and these will often be less expensive for selection in large populations. However, when whole-genome scans are being used, even these traits can be selected for if the genetic control is understood.

Applications of MAS in plant breeding

The advantages described above may have a profound impact on plant breeding in the future and may alter the plant breeding paradigm (Koebner & Summers 2003). In this section, we describe the main uses of DNA markers in plant breeding, with an emphasis on important MAS schemes. We have classified these schemes into five broad areas: marker-assisted evaluation of breeding material; marker-assisted backcrossing; pyramiding; early generation selection; and combined MAS, although there may be overlap between these categories. Generally, for line development, DNA markers have been integrated in conventional schemes or used to substitute for conventional phenotypic selection.

A) Marker-assisted evaluation of breeding material: Prior to crossing (hybridization) and line development, there are several applications in which DNA marker data may be useful for breeding, such as cultivar identity, assessment of genetic diversity and parent selection, and confirmation of hybrids. Traditionally, these tasks have been done based on visual selection and analyzing data based on morphological characteristics.

(i) Cultivar identity/assessment of 'purity': In practice, seed of different strains is often mixed due to the difficulties of handling large numbers of seed samples used within and between crop breeding programmes. Markers can be used to confirm the true identity of individual plants. The maintenance of high levels of genetic purity is essential in cereal hybrid production in order to exploit heterosis. In hybrid rice, SSR and STS markers were used to confirm purity, which was considerably simpler than the standard 'grow-out tests' that involve growing the plant to maturity and assessing morphological and floral characteristics (Yashitola *et al.* 2002).

(ii) Assessment of genetic diversity and parental selection: Breeding programmes depend on a high level of genetic diversity for achieving progress from selection. Broadening the genetic base of core breeding material requires the identification of diverse strains for hybridization with elite cultivars (Xu *et al.* 2004; Reif *et al.* 2005). Numerous studies investigating the assessment of genetic diversity within breeding material for practically all crops have been reported. DNA markers have been an indispensable tool for characterizing genetic resources and providing breeders with more detailed information to assist in selecting parents. In some cases, information regarding a specific locus (e.g. a specific resistance gene or QTL) within breeding material is highly desirable. For example, the comparison of marker haplotypes has enabled different sources of resistance to *Fusarium* head blight, which is a major disease of wheat worldwide, to be predicted (Liu & Anderson 2003; McCartney *et al.* 2004).

(iii) Study of heterosis: For hybrid crop production, especially in maize and sorghum, DNA markers have been used to define heterotic groups

that can be used to exploit heterosis (hybrid vigour). The development of inbred lines for use in producing superior hybrids is a very time-consuming and expensive procedure. Unfortunately, it is not yet possible to predict the exact level of heterosis based on DNA marker data although there have been reports of assigning parental lines to the proper heterotic groups (Lee *et al.* 1989; Reif *et al.* 2003). The potential of using smaller subsets of DNA marker data in combination with phenotypic data to select heterotic hybrids has also been proposed (Jordan *et al.* 2003).

(iv) Identification of genomic regions under selection: The identification of shifts in allele frequencies within the genome can be important information for breeders since it alerts them to monitor specific alleles or haplotypes and can be used to design appropriate breeding strategies (Steele *et al.* 2004). Other applications of the identification of genomic regions under selection are for QTL mapping: the regions under selection can be targeted for QTL analysis or used to validate previously detected marker–trait associations (Jordan *et al.* 2004). Ultimately, data on genomic regions under selection can be used for the development of new varieties with specific allele combinations using MAS schemes such as marker-assisted backcrossing or early generation selection (described below; Ribaut *et al.* 2002; Steele *et al.* 2004).

B) Marker-assisted backcrossing: Backcrossing has been a widely used technique in plant breeding for almost a century. Backcrossing is a plant breeding method most commonly used to incorporate one or a few genes into an adapted or elite variety. In most cases, the parent used for backcrossing has a large number of desirable attributes but is deficient in only a few characteristics (Allard 1999). The method was first described in 1922 and was widely used between the 1930s and 1960s (Stoskopf *et al.* 1993).

The use of DNA markers in backcrossing greatly increases the efficiency of selection. Three general levels of marker-assisted backcrossing (MAB) can be described (Holland 2004). In the first level, markers can be used in combination with or to replace screening for the target gene or QTL. This is referred to as 'foreground selection' (Hospital & Charcosset 1997). This may be particularly useful for traits that have laborious or time-consuming phenotypic screening procedures. It can also be used to select for reproductive-stage traits in the seedling stage, allowing the best plants to be identified for backcrossing. Furthermore, recessive alleles can be selected, which is difficult to do using conventional methods.

Levels of selection during marker-assisted backcrossing

A hypothetical target locus is indicated on chromosome 4. (*a*) Foreground selection, (*b*) recombinant selection and (*c*) background selection.

The second level involves selecting BC progeny with the target gene and recombination events between the target locus and linked flanking markers (recombinant selection). The purpose of recombinant selection is to reduce the size of the donor chromosome segment containing the target locus (i.e. size of the introgression). This is important because the rate of decrease of this donor fragment is slower than for unlinked regions and many undesirable genes that negatively affect crop performance may be linked to the target gene from the donor parentthis is referred to as 'linkage drag' (Hospital 2005). Using conventional breeding methods, the donor segment can remain very large even with many BC generations (Ribaut & Hoisington 1998; Salina *et al.* 2003). By using markers that flank a target gene (e.g. less than 5 cM on either side), linkage drag can be minimized. Since double recombination events occurring on both sides of a target locus are extremely rare, recombinant selection is usually performed using at least two BC generations (Frisch *et al.* 1999*b*).

The third level of MAB involves selecting BC progeny with the greatest proportion of recurrent parent (RP) genome, using markers that are unlinked to the target locus—we refer to this as 'background selection'. In the literature, background selection refers to the use of tightly linked flanking markers for recombinant selection and unlinked markers to select for the RP (Hospital & Charcosset 1997Frisch *et al.* 1999*b*). Background markers are markers that are unlinked to the target gene/QTL on all other chromosomes, in other words, markers that can be used to select against the donor genome. This is extremely useful because the RP recovery can be greatly accelerated. With conventional backcrossing, it takes a minimum of six BC generations to recover the RP and there may still be several donor chromosome fragments unlinked to the target gene. Using markers, it can be achieved by BC_4, BC_3 or even BC_2 (Hospital & Charcosset 1997; Frisch *et al.* 1999*b*), thus saving two to four BC generations. The use of background selection during MAB to accelerate the development of an RP with an additional (or a few) genes has been referred to as 'complete line conversion' (Ribaut *et al.* 2002).

MAB will probably become an increasingly more popular approach, largely for the same reasons that conventional backcrossing has been widely used (Mackill 2006). For practical reasons, farmers in developed and developing countries generally prefer to grow their 'tried and tested' varieties. Farmers have already determined the optimum sowing rates and date, fertilizer application rates and number and timing of irrigations for these varieties (Borlaug 1957). There may also be reluctance from millers or the marketing industry to dramatically change a variety since they have established protocols for testing flour characteristics. Furthermore, even with the latest developments in genetic engineering technology and plant tissue culture, some specific genotypes are still more amenable to transformation than others. Therefore, MAB must be used in order to trace the introgression of the transgene into elite cultivars during backcrossing.

C) Marker-assisted pyramiding: Pyramiding is the process of combining several genes together into a single genotype. Pyramiding may be possible through conventional breeding but it is usually not easy to identify the plants containing more than one gene. Using conventional phenotypic selection, individual plants must be evaluated for all traits tested. Therefore, it may be very difficult to assess plants from certain population types (e.g. F_2) or for traits with destructive bioassays. DNA markers can greatly facilitate selection because DNA marker assays are non-destructive and markers for multiple specific genes can be tested using a single DNA sample without phenotyping.

The most widespread application for pyramiding has been for combining multiple disease resistance genes (i.e. combining qualitative resistance genes together into a single genotype). The motive for this has been the development of 'durable' or stable disease resistance since pathogens frequently overcome single-gene host resistance over time due to the emergence of new plant pathogen races. Some evidence suggests that the combination of multiple genes (effective against specific races of a pathogen) can provide durable (broad spectrum) resistance (Shanti *et al.* 2001; Singh *et al.* 2001). The ability of a pathogen to overcome two or more effective genes by mutation is considered much lower compared with the 'conquering' of resistance controlled by a single gene. In the past, it has been difficult to pyramid multiple resistance genes because they generally show the same phenotype, necessitating a progeny test to determine which plants possess more than one gene. With linked DNA markers, the number of resistance genes in any plant can be easily determined. The incorporation of quantitative resistance controlled by QTLs offers another promising strategy to develop durable disease resistance. Castro *et al.* (2003) referred to quantitative resistance as an insurance

policy in case of the breakdown of qualitative resistance. A notable example of the combination of quantitative resistance was the pyramiding of a single stripe rust gene and two QTLs (Castro *et al.* 2003).

Pyramiding may involve combining genes from more than two parents. For example, Hittalmani *et al.* (2000) and Castro *et al.* (2003) combined genes originating from three parents for rice blast and stripe rust in barley, respectively. MAS pyramiding was also proposed as an effective approach to produce three-way F_1 cereal hybrids with durable resistance (Witcombe & Hash 2000). Strategies for MAS pyramiding of linked target genes have also been evaluated (Servin *et al.* 2004). For many linked target loci, pyramiding over successive generations is preferable in terms of minimizing marker genotyping.

In theory, MAS could be used to pyramid genes from multiple parents (i.e. populations derived from multiple crosses). In the future, MAS pyramiding could also facilitate the combination of QTLs for abiotic stress tolerances, especially QTLs effective at different growth stages. Another use could be to combine single QTLs that interact with other QTLs (i.e. epistatic QTLs). This was experimentally validated for two interacting resistance QTLs for rice yellow mottle virus (Ahmadi *et al.* 2001).

D) Early generation marker-assisted selection: Although markers can be used at any stage during a typical plant breeding programme, MAS is a great advantage in early generations because plants with undesirable gene combinations can be eliminated. This allows breeders to focus attention on a lesser number of high-priority lines in subsequent generations. When the linkage between the marker and the selected QTL is not very tight, the greatest efficiency of MAS is in early generations due to the increasing probability of recombination between the marker and QTL. The major disadvantage of applying MAS at early generations is the cost of genotyping a larger number of plants.

One strategy proposed by Ribaut & Betran (1999) involving MAS at an early generation was called single large-scale MAS (SLS–MAS). The authors proposed that a single MAS step could be performed on F_2 or F_3 populations derived from elite parents. This approach used flanking markers (less than 5 cM, on both sides of a target locus) for up to three QTLs in a single MAS step. Ideally, these QTLs should account for the largest proportion of phenotypic variance and be stable in different environments. The population sizes may soon become quite small due to the high selection pressure, thus providing an opportunity for genetic drift to occur at non-target loci, so it is recommended that large population sizes be used (Ribaut & Betran 1999). This problem can also be minimized by using F_3 rather than F_2 populations, because the selected proportion of an F_3 population is larger compared with that of an F_2 population (i.e. for a single target locus, 38% of the F_3 population will be selected compared with 25% of the F_2). Ribaut & Betran (1999) also proposed that, theoretically, linkage drag could be minimized by using additional flanking markers surrounding the target QTLs, much in the same way as in MAB.

For self-pollinated crops, an important aim may be to fix alleles in their homozygous state as early as possible. For example, in bulk and single-seed descent breeding methods, screening is often performed at the F_5 or F_6 generations when most loci are homozygous. Using co-dominant DNA markers, it is possible to fix specific alleles in their homozygous state as early as the F_2 generation. However, this may require large population sizes; thus, in practical terms, a small number of loci may be fixed at each generation. An alternative strategy is to ‘enrich’ rather than fix alleles by selecting homozygotes and heterozygotes for a target locus within a population in order to reduce the size of the breeding populations required (Bonnett *et al.* 2005).

Future prospectus

Genetically modified crop technology has revolutionized agriculture in the United States, Canada, China, and Argentina and in many other countries. It exhibits the potential to have much wider impact, solving many of the current problems in agriculture worldwide. The different types of hybrid varietiesof crops that may become available in the future could boost crop yields while enhancing the nutritional value of staple foods and eliminating the need for inputs that could be harmful to the environment. While the environmental, health, and economic risks of GM crops should be carefully studied before full-scale adoption, the types of GM crops that are already available have thus far largely proven to be beneficial to agriculture and even to the environment, without evidence of adverse health or environmental impacts. In 2002, 58.7 million hectares of GM crops were grown worldwide with two thirds in the US. Others countries growing GM crops are Argentina, Australia, Bulgaria, Canada, China, Columbia, Honduras, India, Indonesia, Mexico, Romania, South Africa, Spain and Uruguay. Globally, nearly 12 million hectares of GM maize were grown in 2002. In the US, around 25% of the maize harvest is genetically modified. In Europe, commercial growing of GM Bt maize is already underway in Spain.

Considering the enormous potential of MAS in plant breeding, achieving a tangible impact on crop improvement represents the great challenge of molecular breeding in the early part of the twenty-first century. Solutions to the above-mentioned obstacles of MAS need to be developed in order to achieve a greater impact. In the short term, the most important factors that should enable the impact of MAS to be realized include:

(a) a greater level of integration among conventional breeding, QTL mapping/ validation and MAS,

(b) careful planning and execution of QTL mapping studies (especially for complex quantitative traits) and an emphasis on validating results prior to MAS,

(c) optimization of methods used in MAS such as DNA extraction and marker genotyping, especially in terms of cost reduction and efficiency, and

(d) efficient systems for data storage (from in-house laboratory information management systems (LIMS) to publicly available databases).

For MAS to reach its full potential for crop improvement, the advantages of MAS over conventional breeding need to be fully exploited. This may depend on *ex ante* studies evaluating alternative schemes prior to experimentation. Computer simulations may indicate the most effective breeding schemes in order to maximize genetic gain and minimize costs (Kuchel *et al.* 2005). Based on the schemes of MAS reviewed in this paper, the most important areas to target include:

(a) use of markers for the selection of parents in breeding programmes,

(b) continued use of MAS for high-priority traits that are difficult, time consuming or expensive to measure,

(c) using markers to minimize linkage drag via recombinant selection,

(d) screening of multiple traits per line (i.e. per unit of DNA), especially populations derived from multiple F_1s for pyramiding,

(e) exploiting the ability to rapidly eliminate unsuitable lines after early generation selection or tandem selection in breeding programmes, thus allowing breeders to concentrate on the most promising materials, and

(f) exploiting the time savings for line development (especially using background selection) for accelerated variety release.

Generally, innovation—big and small—may play an important role in obtaining tangible benefits from MAS. Dekkers & Hospital (2002) stated that there is considerable scope for innovative plant/molecular breeding schemes that are tailor-made for using DNA markers; such schemes could lead to a completely new plant breeding paradigm.

Advances in functional genomics will lead to the rapid identification of gene functions in the major cereal crops. This strategy usually relies on fine mapping using molecular markers, as well as other methods such as gene-expression studies (microarray), mutants and gene knockouts, RNAi and association genetics. The identification of gene function will allow the development of allele-specific markers that will be more efficient than using linked DNA markers. In addition, the identified genes can be used for transformation studies as well as mining of gene banks to find more useful alleles. Even though we can expect far-reaching advances in the area of gene function identification, the complex genetic interactions that produce different phenotypes may remain unexplained for the most part. However, even in these cases, we may identify chromosome fragments that are conducive to improved phenotype.

Maize varieties with conventional resistance have been and continue to be developed and will not be discussed further apart from the possible option of pyramiding conventional resistance with Bt maize in order to deliver maize varieties resistant to *B. fusca* that combine single gene (Bt) and quantitative (conventionally breed maize varieties) resistance so that more than one mortality mechanism is employed is a resistant variety with the prospect of being less prone to developing insect resistant populations.

A breeding application resulting from the development of high-throughput genotyping equipment is the use of 'whole-genome scans' for determining allelic variation at many agronomically important loci in the genome (Langridge & Chalmers 2005; Langridge 2006). One recent approach called 'breeding by design' could enable breeders to exploit known allelic variation to design superior genotypes by combining multiple favourable alleles (Peleman & van der Voort 2003). This also means that plants with the desired combinations of genes can be pre-selected before extensive and expensive field testing. In many cases, the objective would be just to avoid advanced testing of a number of lines with similar genotypic constitutions. Current limitations to the application of breeding by design or similar approaches include the prohibitive cost, since thousands of marker loci need to be scored in breeding material and, perhaps more importantly, our current knowledge and understanding of the function of the majority of agronomically important genes and allelic interactions with respect to phenotype which remain unknown. Therefore, at least in the short term, such approaches will probably not have a great impact on crop improvement.

CONCLUSION

Plant breeding has made remarkable progress in crop improvement and it is critical that this continue. It seems clear that current breeding programmes continue to make progress through commonly used breeding approaches. Recombinant DNA technologies including MAS could greatly assist plant breeders in reaching this goal although, to date, the impact on variety development has been minimal. For the potential of MAS to be realized, it is imperative that there should be a greater integration with breeding programmes and that current barriers be well understood and appropriate solutions developed. The exploitation of the advantages of rDNAtechnology relative to conventional breeding could have a great impact on crop improvement. The

high cost of rDNA technology will continue to be a major obstacle for its adoption for some crop species and plant breeding in developing countries in the near future. Specific MAS strategies may need to be tailored to specific crops, traits and available budgets. New marker technology can potentially reduce the cost of MAS considerably. If the effectiveness of the new methods is validated and the equipment can be easily obtained, this should allow MAS to become more widely applicable for crop breeding programmes.

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