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Overview on application of marker assisted selection for crop improvement

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ABSTRACT

The aim of this overview was to assess markers use in the contemporary world to improve crops and to generate information for crop breeders for their future endeavor. Genetic markers used in genetics and plant breeding can be classified into classical markers and DNA markers. Classical markers include morphological markers, cytological markers and biochemical markers. DNA markers have developed into many systems based on different polymorphism-detecting techniques. The exploitation of the advantages of MAS relative to conventional breeding could have a great impact on crop improvement. 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.

Keywords: Biochemical marker, gene pyramiding, marker assisted selection, molecular marker, morphological marker.

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Introduction

To improve plant varieties, since the late 19th century plant breeders relied on phenotypic selection, achieving breeding progress through the assessment of external and internal traits such as plant habitus, disease resistances, yield, or quality traits. New, improved varieties were developed by solely selecting plants with desirable phenotypes. Plant breeding techniques became very sophisticated over the years but time demanding too. Developing a new, improved plant variety by means of phenotypic selection can easily exceed 10 years. Only with the advent of molecular markers in the late 1970s, it became possible to select desirable traits more directly. Easily detectable DNA markers can now be used in plant breeding. Marker-assisted selection (MAS) has turned into a tool which is – to varying degrees – utilized in breeding companies and research institutes for the development of improved varieties, allowing for a breeding approach based on the genotype of plants rather than assessing the phenotype only (Foolad & Sharma, 2005).

DNA markers are sections of the genome of the organisms in question which are used for recognition. They can be understood as naturally occurring tags attached to specific segments of a chromosome, which in turn are associated with specific phenotypes. A marker can either be located within the gene of interest or be linked to a gene determining a trait of interest, which is the most common case. Thus MAS can be defined as selection for a trait based on genotype using associated markers rather than the phenotype of the trait (Foolad&Sharma, 2005).

Sometimes the term "Smart Breeding", an acronym for "Selection with Markers and Advanced Reproductive Technologies", which was first used in animal breeding (Davis et al., 1997), is used to describe marker supported breeding strategies.

The idea of MAS or Smart Breeding was taken up with great enthusiasm, and several breeders expressed the hope to "skip several breeding cycles and condense timelines" (Mazur 1995) and to finally having found a tool, "to control all allelic variation for all genes of agronomic importance"(Peleman & Van Der Voort, 2003). According to Xu & Crouch (2008) the greatest benefits of MAS are the possibilities

to "achieve the same breeding progress in a much shorter time than through conventional breeding","pyramid combinations of genes that could not be readily combined through other means" and to "assemble target traits more precisely, with less unintentional losses".

History of Marker Assisted selection

Although the idea for marker-assisted selection dates back to 1923, it is a young field of science and breeding. Sax (1923) observed an association between seed color (monogenic trait) and seed weight (polygenic, quantitatively inherited trait) in beans (*Phaseolus vulgaris* L.) and drew the conclusion that the single gene controlling seed color must be linked to one or more of the polygenes controlling seed size. The concept of using linked genes to follow the inheritance of genes controlling other traits had come into being. It was put forth in 1961 by Thoday, who made a first attempt to map and characterize all polygenes affecting a trait with the help of monogenic markers. Working with morphological markers, the main practical limitation of his work was the fact that only few suitable markers were available.

By the early 1980s, allozyme markers were being employed as a tool for the discrimination of genotypes, replacing the previously used morphological markers.

Allozyme markers are based on protein polymorphisms; they are allelic forms of enzymes and can be separated on electrophoretic gels and detected by staining the gels. Advantages of this method are the low costs, technical simplicity and the co-dominant nature of the marker. Co-dominance means that alleles of both parents can be detected in the F1, thus homozygous and heterozygous genotypes can be distinguished. However, the limited number of suitable allozyme loci in the genome and the requirement of fresh tissue of the right developmental stage are clear that DNA markers hold great promise, but realizing that promise remains elusive." In 2001, Gupta et al. stated that "the lack of cost-effectiveness and non-availability of high throughput approaches for handling large segregating populations have limited the use of molecular marker technology for plant breeding" and that it "has yet to find its rightful place in plant breeding programs". Three years later Holland (2004) still articulated the opinion, that "it is not likely that markers will soon be generally useful for manipulating complex traits like yield".

The overview was made with the objective of assessing how markers are used in the contemporary world to improve crops and to generate information for crop breeders for their future endeavor.

Genetic markers are the biological features that are determined by allelic forms of genes or genetic loci and can be transmitted from one generation to another, and thus they can be used as experimental probes or tags to keep track of an individual, a tissue, a cell, a nucleus, a chromosome or a gene. Genetic markers used in genetics and plant breeding can be classified into two categories: classical markers and DNA markers (Xu, 2010). Classical markers include morphological markers, cytological markers and biochemical markers. DNA markers have developed into many systems based on different polymorphism-detecting techniques or methods (southern blotting – nuclear acid hybridization, PCR – polymerase chain reaction, and DNA sequencing) (Collard et al., 2005), such as RFLP, AFLP, RAPD, SSR, SNP, etc.

Classical Markers

Morphological markers: Use of markers as an assisting tool to select the plants with desired traits had started in breeding long time ago. During the early history of plant breeding, the markers used mainly included visible traits, such as leaf shape, flower color, pubescence color, pod color, seed color, seed shape, hilum color, awn type and length, fruit shape, rind (exocarp) color and stripe, flesh color, stem length, etc. These morphological markers generally represent genetic polymorphisms which are easily identified and manipulated. Therefore, they are usually used in construction of linkage maps by classical two- and/or three point tests. Some of these markers are linked with other agronomic traits and thus can be used as indirect selection criteria in practical breeding.

Cytological markers: In cytology, the structural features of chromosomes can be shown by chromosome karyotype and bands. The banding patterns, displayed in color, width, order and position, reveal the difference in distributions of euchromatin and heterochromatin. For instance, Q bands are produced by quinacrine hydrochloride, G bands are produced by Giemsa stain, and R bands are the reversed G bands. These chromosome landmarks are used not only

for characterization of normal chromosomes and detection of chromosome mutation, but also widely used in physical mapping and linkage group identification. The physical maps based on morphological and cytological markers lay a foundation for genetic linkage mapping with the aid of molecular techniques. However, direct use of cytological markers has been very limited in genetic mapping and plant breeding.

Biochemical/protein markers: Protein markers may also be categorized into molecular markers though the latter are more referred to DNA markers. Isozymes are alternative forms or structural variants of an enzyme that have different molecular weights and electrophoretic mobility but have the same catalytic activity or function. Isozymes reflect the products of different alleles rather than different genes because the difference in electrophoretic mobility is caused by point mutation as a result of amino acid substitution (Xu, 2010). Therefore, isozyme markers can be genetically mapped onto chromosomes and then used as genetic markers to map other genes. They are also used in seed purity test and occasionally in plant breeding. There are only a small number of isozymes in most crop species and some of them can be identified only with a specific strain. Therefore, the use of enzyme markers is limited.

Molecular Breeding

Molecular Markers and Marker-Assisted Selection

The process of developing new crop varieties requires many steps and can take 10 to 25 years depending on the crop. Now, however, applications of agricultural biotechnology have considerably shortened the time it takes to bring them to market. It currently takes 7-10 years for new crop varieties to be developed. One of the tools, which make it easier and faster for scientists to select plant traits, is called marker-assisted selection (MAS).The different traits and physical features of plants are encoded in the plant's genetic material, the deoxyribonucleic acid (DNA). The DNA occurs in pairs of chromosomes (strands of genetic material), one coming from each parent. The genes, which control the plant's characteristics, are specific segments of each chromosome. All of the plant's genes together make up its genome. Some traits like flower color may be controlled by only one gene. Other more complex characteristics, however, like crop yield or starch content, maybe influenced by many genes.

Traditionally, plant breeders have selected plants based on their visible or measurable traits, called the phenotype. But, this process can be difficult, slow, influenced by the environment, and costly – not only in the development itself, but also for the economy, as farmers suffer crop losses. As a shortcut, plant breeders now use molecular marker-assisted selection. To help identify specific genes, scientists use what are called molecular markers which are short strings or sequence of nucleic acid which makes up a segment of DNA. The markers are located near the DNA sequence of the desired gene. Since the markers and the genes are close together on the same chromosome, they tend to stay together as each generation of plants is produced. This is called genetic linkage. This linkage helps scientists to predict whether a plant will have the desired gene. If researchers can find the marker for the gene, it means the gene itself is present.

As scientists learn where each of the markers occurs on a chromosome, and how close it is to a specific gene, they can create a map of the markers and genes on specific chromosomes. This genetic linkage map shows the location of markers and genes, and their distance from other known genes. Scientists can produce detailed maps in only one generation of plant breeding. Previously, scientists produced very simple genetic maps using conventional techniques. It was observed long ago that as generations of plants were crossed, some traits consistently appeared together in the new generations (genetic linkage). However, since researchers could concentrate on only a few traits in each attempt at cross-breeding, it took many crosses to obtain even a very simple genetic map. Using very detailed genetic maps and better knowledge of the molecular structure of a plant's DNA, researchers can analyze a tiny bit of tissue from a newly germinated seedling. They don't have to wait for the seedling to grow into a mature plant to test for the presence of the specific trait. Once the tissue is analyzed through molecular techniques, scientists know whether that seedling contains the appropriate gene. If it doesn't, they can quickly move on and concentrate analysis on another seedling, eventually working only with the plants which contain the specific trait. Currently, molecular marker-assisted breeding, an agricultural biotechnology tool is already a routine step in breeding of most crops where the gene and the markers for a specific trait are

known. This technique is being used in the efficient introgression of important genes into various crops including bacterial blight resistance in rice, increased beta-carotene content in rice, cassava, and banana, and submergence tolerance in rice, to name a few.

Molecular markers are also used to determine the genetic profile of a line or variety. Random primers are used to scan the genomic constitution of the plant through molecular methods. The information is fed to a computer program that will analyze the relatedness of one line to another. The information on genetic diversity of the lines is utilized in selecting for extremely unrelated parents useful for hybrid seed technology. The information will also provide details on the parentage of the line, the possible traits, and the unique identity of the plant useful for germplasm collection database. It should be noted, however, that molecular breeding through marker assisted selection is somewhat limited in scope compared to genetic engineering or modification because:

1) It only works for traits already present in a crop;

2) It cannot be used effectively to breed crops which have long generation time (e.g. citrus); and

3) It cannot be used effectively with crops which are clonally propagated because they are sterile or their off springs do not resemble the parents. This includes many staples such as yams, bananas, plantain, sweet potato, and cassava.

Molecular markers in recent years have accelerated plant breeding methods significantly with an objective of crop improvement. Molecular markers can be of any kind of marker system that differentiates two individuals at the molecular level. Many types of molecular markers are presently available, but no single marker technique is generally applicable for all applications. As early as the 1970s, protein markers (enzymes) were mainly used as molecular markers. Enzymes can be visualized using specific stains to get a visible product as a band in an electrophoretic system and different forms of an enzyme (reflected in different colored bands), are called isozymes (Charles and Robert, 1989). Other than isozymes, proteins markers can also be resolved based on their charge and size through the separation by a two-dimensional gel electrophoreic system.

The major limiting factor for isozymes is their low number, and most of them are either tissue or developmental stage-specific. The advent of DNA recombinant technology opened the area of development and exploitation of DNA-based markers which was further tuned after the development of PCR technology.DNA markers can be classified into three broad categories based on the method of their use. These are: Marker-aided selection (MAS)

These enables plant breeders to select a piece of DNA that is associated with a particular trait, thereby avoiding time-consuming and expensive tests to select the ideal parent or offspring. MAS can significantly speed up the plant breeding process and a new variety can be produced in approximately four to six generations, rather than in ten. MAS are particularly useful for breeding crops with resistance to moisture-stress for environments with an irregular supply of water. To achieve this characteristic, a variety of different traits would have to be selected and MAS allows plants that express these different traits to be rapidly identified. The technique is also useful in research which aims to interbreed maize varieties that are already resistant to moisture-stress with African varieties of the crop, which are otherwise well adapted (Ribaut et al., 2002).

Hybridisation Based Markers

RFLPs: Restriction fragment length polymorphism (RFLP), the very first kind of DNA marker developed, is an example of hybridization based marker, which employs cloned DNA sequences to probe specific regions of the genome for variations that are seen as changes in the length of DNA fragments produced by digestion with restriction endonucleases (Landry et al. 1987). DNA of the parental genotypes to be surveyed for polymorphism are digested with different restriction enzymes (e.g., EcoRI, HindIII, DraI), separated by agarose gel electrophoresis and transferred onto a nylon membrane. Labeled probes (short known DNA sequences) are allowed to hybridize with the digested DNA fragments which are bound to a nylon membrane and then visualized by autoradiography. RFLPs were used for the first time in the human genome mapping (Botstein et al., 1980). In plants, RFLPs were first used in maize (Helentjaris et al., 1986), and rice to prepare the first generation of molecular genetic maps. Subsequently dense RFLP maps were generated for all the major crop species such as barley (Graner et al., 1991), wheat

(Gill et al., 1991), soybean (Shoemaker and Olson 1993), etc. Though RFLPs are the most reliable DNA polymorphism that can be used for accurate scoring of genotypes, due to its time-consuming, laborious protocol with the involvement of radioactivity, RFLPs lost their importance in large-scale mapping and marker assisted selection projects for plant breeding applications.

PCR Based Markers

PCR markers are based on amplification of sequences using the polymerase chain reaction (PCR). To amplify the targeted sequences, two primers, which should flank the target sequence, are needed. PCR based markers rely on sequence variation in annealing sites or DNA length differences between amplified products obtained from corresponding genotypes.

RAPDs: Random amplified polymorphic DNA (RAPD) is the simplest example of a PCR marker that involves the use of 10 bp random primers (Williams et al., 1990). RAPDs have been widely used for mapping and genetic diversity studies (Vierling and Nguyen 1992, Fernández et al., 2002), but due to their poor reproducibility and lack of locus specificity, scientist tried other possibilities to find reliable and reproducible markers.

AFLPs: Amplified fragment length polymorphism (AFLP) is the one such kind, which combines the merit of RFLP and PCR techniques. The AFLP approach involves generation of a genomic library, creation of smaller fragments after restriction digestion, amplification of the DNA fragments after adapter ligation and PCR amplification and their detection on polyacrylamide gel electrophoreses by using a radioactive assay (Vos *et al*., 1995). AFLP markers are abundant in nature and have been used for construction of genetic linkage maps, high density linkage map of a targeted region, identification of QTLs controlling complex traits and studies on genetic diversity (Ellis et al., 1997). AFLP requires no specific prior knowledge about the genome, but despite this merit, still remains inaccessible for locus-specific applications in large segregating populations. Yet another limitation of AFLP is its dominant nature. Methods for converting AFLP markers into co-dominant sequence-tagged sites (STSs) were developed and utilized in some mapping programs (Brugmans *et al*., 2003). However, this is not very user-friendly, routine and always successful. Another drawback of the approach includes the generation of AFLP fragments from the repetitive portion of the genome. The cDNA-AFLP technique was developed to overcome the problem of repetitive elements; which applies to the standard protocol on a cDNA template (Bachem *et al*., 1996). The methylation sensitive enzymes for restriction digestion in AFLP procedure may decrease the ratio of repetitive DNA sequence (Weersena *et al*., 2003) SSRs or Microsatellites: A major break-through in molecular marker technology, perhaps, happened with the introduction of microsatellites or simple sequence repeats (SSRs), a form of variable number of tandem repeats, which can be of two to many (generally up to six) repeated nucleotides. Since microsatellites are abundant in the genome, locus-specific, co-dominant, highly polymorphic and amenable for high throughput, these were extensively used to develop genetic maps in rice, wheat, barley, and maize (Röder *et al*., 1998, McCouch et al., 2002, Sharopova *et al*., 2002). The generation of microsatellites generally involves the creation of a genomic or SSR-enriched library, subsequent screening the library for identification of SSR containing clones and their sequencing for identification of microsatellites. Thus the generation of microsatellites in a traditional manner has been an expensive and time-consuming task. In recent years, due to the availability of an enormous amount of sequence data such as ESTs in the public domain, it has been possible to utilize the available sequence data to screen for microsatellites.

Sequence Based Markers

The significant progress in the area of genomics is to develop markers which can detect polymorphism at the single base pair level. The development and utilization of such markers, however, is still at an initial phase.

SNPs: The recent marker system for detection of polymorphism at a single base is single nucleotide polymorphism (SNP). Availability of extensive sequence databases made a new avenue, the 'SNP" to exploit as a high-throughput marker for genome mapping studies. The availability of abundant, high-throughput sequence-based markers is essential for detailed genome-wide trait analysis. SNPs are markers amenable for high-throughput techniques, which show greatest sequence variation; a significant amount of effort has been invested in re-

sequencing alleles to discover SNPs. There are techniques to detect SNPs such as allele-specific PCR, single base extension and array hybridization methods (Gupta *et al*., 2002). Since SNP discovery and moreover SNP genotyping require expensive and sophisticated platforms, the development and exploitation of SNP markers is still restricted to major crop species such as rice (Nasu*et al*., 2002), wheat (Somers *et al*., 2003), barley (Kota *et al*. 2001, Kanazin *et al*. 2002), maize (Tenallion et al., 2001), and soybean (Zhu *et al*., 2003).

Applications ofMAS in Plant Breeding to Improve Crops

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.

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 analysing data based on morphological characteristics.

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).

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 (McCartney *et al*., 2004).

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 (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).

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. 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 (Ribaut *et al.*, 2001).

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. 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 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.

The second level involves selecting BC progeny with the target gene and recombination events between the target loci and linked flanking markers—we refer to this as '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 parent—this is referred to as 'linkage drag' (Hospital, 2005). Using conventional breeding methods, the donor segment can remain very large even with many BC generations (e.g. more than 10; Salina *et al.* 2003). By using markers that flank a target gene (e.g. less than 5cM 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 (Frisch *et al.*, 1999b). 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 (Frisch *et al.* 1999*a*,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.

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 (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, (Castro *et al.*, 2003) combined genes originating from three parents for rice blast and stripe rust in barley, respectively. MAS pyramiding were 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).

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 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 5cM, 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 (Koebner & Summers, 2003). 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.

Combined Marker-Assisted Selection

There are several instances when phenotypic screening can be strategically combined with MAS. In the first instance, 'combined MAS' (coined by Moreau *et al.,* 2004) may have advantages over phenotypic screening or MAS alone in order to maximize genetic gain. This approach could be adopted when additional QTLs controlling a trait remain unidentified or when a large number of QTLs need to be manipulated. Simulation studies indicate that this approach is more efficient than phenotypic screening alone, especially when large population sizes are used and trait heritability is low (Hospital *et al.,* 1997). Bohn *et al.* (2001) investigated the prospect of MAS for improving insect resistance in tropical maize and found that MAS alone was less efficient than conventional phenotypic selection. However, there was a slight increase in relative efficiency when MAS and phenotypic screening were combined. In an example in wheat, MAS combined with phenotypic screening was more effective than phenotypic screening alone for a major QTL on chromosome 3BS for *Fusarium* head blight resistance (Zhou *et al.,* 2003b). In practice, all MAS schemes will be used in the context of the overall breeding programme, and this will involve phenotypic selection at various stages. This will be necessary to confirm the results of MAS as well as select for traits or genes for which the map location is unknown.

In some (possibly many) situations, there is a low level of recombination between a marker and QTL, unless markers flanking the QTL are used (Sanchez *et al.,* 2000). In other words, a marker assay may not predict phenotype with 100% reliability. However, plant selection using such markers may still be useful for breeders in order to select a subset of plants using the markers to reduce the number of plants that need to be phenotypically evaluated. This may be particularly advantageous when the cost of marker genotyping is cheaper than phenotypic screening, such as for quality traits. This was referred to as 'tandem selection' and 'stepwise selection'.

In addition to complementing conventional breeding methods, mapping QTLs for important traits may have an indirect benefit in a conventional breeding programme. In many cases, this occurs when traits which were thought to be under the complex genetic control are found to be under the influence of one or a few major QTLs. For example, in pearl millet downy mildew resistance was found to be under the control of genes of major effect. Likewise, submergence tolerance of rice was found to be under the control of the major QTL *Sub1*, which helped simplify the breeding for this trait (Mackill *et al.,* 2006).

Advantages of Marker-Assisted Breeding

The use of DNA markers for screening and selecting of plants in a breeding programme provides several advantages and is therefore very attractive to plant breeders.

(i) As DNA marker based genotypes can be obtained from almost any plant tissue, plants can be screened already at the seedling stage or even as seeds, thus allowing early selection for traits which may be expressed in adult plants only (i.e. grain or fruit quality, male sterility, photoperiod sensitivity). With the availability of preflowering genotypic information MAS allows for controlled pollination, e.g. in marker-assisted recurrent selection.

(ii) Target alleles that are difficult, expensive and/or time consuming to score phenotypically can be selected with the assistance of markers (e.g. environmentally sensitive traits, as DNA markers are mostly neutral to environmental variation).

(iii) Selections can be made on a single plant basis where this would not be possible by phenotypic selection. Poor heritability does not pose a problem if selection is based on marker information.

(iv) For traits with complex inheritance every individual genetic component contributing to the trait can be selected separately. Also, multiple characters that would normally be epistatic (i.e. they show a certain positive or negative effect

only in combination with each other) can be maintained and ultimately fixed.

(v) Recessive genes can be maintained without the need for progeny tests in each generation, as homozygous and heterozygous plants can be distinguished with the aid of (co-dominant) markers. In backcrossing, DNA markers can help to minimize linkage drag around the target gene and reduce the generations required to recover a recurrent parent's genetic background.

(vi) In the choice of parents in crossing programs markers can be applied too. Here they can either help to maximize diversity, and in this way support the exploitation of heterosis, or they can minimize diversity, if gene complexes built up in elite inbred germplasm are to be preserved (YOUNG 1999; Koebner & Summers 2003; Koebner 2004; Edwards &Mccouch 2007; Jena &Mackill 2008).

Limitations of MAS

(a) Still at the early stages of DNA marker technology development

Although DNA markers were first developed in the late 1980s, more user-friendly PCR-based markers such as SSRs were not developed until the mid- to late 1990s. Although currently large numbers of SSRs are publicly available for major cereals, this number was initially very low. It is only during the last 5–7 years that these markers could have been widely used, and tangible results may not yet have been produced.If this is the case, there should be a notable increase in the number of published papers describing MAS in the next 10 years and beyond.

(b) Marker-assisted selection results may not be published

Although QTL mapping has many potential practical outcomes, it is considered to be a basic research process, and results are typically published in scientific journals. However, for plant breeding, the final 'product' is a new variety. Although these varieties are registered, explicit details regarding the use of DNA markers during breeding may not be provided. Another reason for the limited number of published reports could be that private seed companies typically do not disclose details of methodology due to competition with other seed companies. In general, the problem of publishing also extends to QTL validation and QTL mapping. New QTLs are frequently reported in scientific journals, but reconfirmation of these QTLs in other germplasm and identification of more useful markers are usually not considered novel enough to warrant new publications. This is unfortunate because it is exactly this type of information that is needed for MAS. Some of this information can be found in symposia abstracts or web sites, but often this information is not very informative.

(c) Reliability and accuracy of quantitative trait loci mapping studies

The accuracy of the QTL mapping study is critical to the success of MAS. This is particularly important when QTL mapping is undertaken for more complex traits, such as yield, that are controlled by many QTLs with small effects compared with simple traits. Many factors may affect the accuracy of a QTL mapping study such as the level of replication used to generate phenotypic data and population size (Young, 1999). Simulation and experimental studies have indicated that the power of QTL detection is low with the typical populations (less than 200) that are used (Beavis, 1998). As a result, confidence intervals for regions containing QTLs may be large, even for QTLs with large effects. Furthermore, sampling bias can lead to a large bias in estimates of QTL effects, especially in relatively small population sizes. These factors have important implications for MAS, since the basis for selecting markers depends on the accurate determination of the position and effect of a QTL.

(d) Insufficient linkage between marker and gene/ quantitative trait locus

In some cases, recombination occurs between the marker and gene/QTL due to loose linkage (Sharp *et al*., 2001). This may occur even if genetic distances from a preliminary QTL mapping study indicated tight linkage, because data from a single QTL mapping experiment may not be accurate (Sharp *et al*., 2001). The process of marker validation is required to determine the reliability of a marker to predict phenotype and this point out the advantages of using flanking markers.

(e) Limited markers and limited polymorphism of markers in breeding material

Ideally, markers should be 'diagnostic' for traits in a wide range of breeding material. In other words, markers should clearly discriminate between varieties that do and do not express the trait. Unfortunately, in practice, DNA markers are not always diagnostic. For example, a wheat SSR marker was diagnostic for the *Sr2* gene

(controlling stem rust resistance) for all except four susceptible Australian cultivars, in which the same marker allele was detected as for the source of resistance. This would preclude the use of this SSR marker for the introgression of resistance in the four susceptible cultivars, requiring that additional markers be developed. Even with the large numbers of available markers in some crops, there can be specific chromosome regions containing an important gene or QTL for which it is difficult to find polymorphic markers.

(f) Effects of genetic background

It has been observed that QTLs identified in a particular mapping population may not be effective in different backgrounds. For example, Steele *et al.* (2006) found that only one of four root-length QTLs were effective when transferred by backcrossing into a new rice variety. In some cases, this is due to the small effect of an allele transferred into elite varieties (Charcosset& Moreau, 2004). Often for QTL mapping experiments, parents that represent the extreme ends of a trait phenotype are selected. This increases the chance of detecting QTLs because QTL mapping is based on statistically different means of marker groups. The main disadvantage with this approach is that one (or even both) parent(s) may possess QTL alleles that are similar or even identical to the elite germplasm used in breeding programmes. QTL effect may be insignificant when used for introgression into elite varieties and/or the effect of a QTL may differ in different genetic backgrounds due to interactions with loci or epistasis (Holland, 2001).

(g) Quantitative trait loci environment effects

While the effects of many QTLs appear to be consistent across environments, the magnitude of effect and even direction of QTLs may vary depending on environmental conditions due to QTL×environment interactions. This often occurs for QTLs with smaller effects. The extent of QTL×environment interactions is often unknown because QTL mapping studies have been limited to only afor few years (replications) or locations. The existence of QTL×environment interactions must be carefully considered in order to develop an effective MAS scheme.

(h) High cost of marker-assisted selection

The cost of using MAS compared with conventional phenotypic selection may vary considerably, although only a relatively small number of studies have addressed this topic. Landmark papers showed that the cost–benefit ratio of MAS will depend on several factors, such as the inheritance of the trait, the method of phenotypic evaluation, the cost of field and glasshouse trials and labour costs. It is also worth noting that large initial capital investments are required for the purchase of equipment, and regular expenses will be incurred for maintenance. Intellectual property rights, for example, licensing costs due to patents, may also affect the cost of MAS. One approach to this problem is to contract the marker work out to larger laboratories that can benefit from economies of scale and high-throughput equipment.

(i) 'Application gap' between research laboratories and plant breeding institutes

In many cases, QTL mapping research is undertaken at universities whereas breeding is generally undertaken at different locations such as research stations or private companies. Consequently, there may be difficulties in the transfer of markers and relevant information to breeders in situations where the two groups do not work closely together. More importantly, also transfer problems may be related to the culture of the scientific community. Given the emphasis on conducting innovative research, and on the publication of research results within academic institutions, scientists do not have much motivation to ensure that markers are developed into breeder-friendly ones and that they are actually applied in breeding programmes. This is even truer for activities in the private sector where publication of results is generally discouraged.

(j) 'Knowledge gap' among molecular biologists, plant breeders and other disciplines

DNA marker technology, QTL theory and statistical methodology for QTL analysis have undergone rapid developments in the past two decades. These concepts and the jargon used by molecular biologists may not be clearly understood by plant breeders and other plant scientists (Collard *et al.* 2005). In addition to this, many highly specialized pieces of equipment are based on sophisticated techniques used for molecular genotyping. Similarly, fundamental concepts in plant breeding may not be well understood by molecular biologists. This restricts the level of integration between conventional plant and molecular breeding and ultimately affects the development of new breeding lines.

Result and Discussion

Despite the relatively small impact that MAS has had on variety development to date, there has been a 'cautious optimism' for the future (Young, 1999). It is good to predict six main factors that will give rise to a much greater level of adoption of MAS in plant breeding in the early part of the twenty-first century in many breeding programmes.

First, the extent to which DNA marker technology has already spread to plant breeding institutes coupled with the enormous amount of data from previous QTL mapping and MAS studies should lead to the greater adoption of MAS. Many such institutes now possess the essential equipment and expertise required for marker genotyping. Of course, the frequency of use will depend on available funding.

Second, since the landmark concept of 'advanced BC QTL analysis' directly integrated QTL mapping with plant breeding by combining QTL mapping with simultaneous variety development (Tanksley& Nelson, 1996), there have been several encouraging examples of an efficient merging of plant and molecular breeding. Ideally, QTL mapping and marker-assisted line development should now always be conceived together, in a holistic scheme.

Third, the increasing use of genetic transformation technology means that MAS can be used to directly select for progeny that possess transgenes via target gene selection. As discussed earlier, specific genotypes often with poor agronomic characteristics are routinely used for transformation. Therefore, MAS can be used to track the transgenes during elite line development.

Fourth, a rapid growth in genomics research has taken place within the last decade. Data generated from functional genomics studies have led to the identification of many candidate genes for numerous traits. SNPs within candidate genes could be extremely useful for 'association mapping' and ultimately MAS (Breseghello&Sorrells 2006). This approach also circumvents the requirement for constructing linkage maps and performing QTL analysis for new genotypes that have not been previously mapped, although genotyping and phenotyping of segregating populations (e.g. F_2 or F_3) is recommended for marker validation (Breseghello&Sorrells 2006). Furthermore, genome sequencing projects in rice and other crop species will provide considerable data that could be used for QTL mapping and marker development in other cereals (Varshney *et al*., 2005).

Fifth, many new high-throughput methods for DNA extraction and especially new high-throughput marker genotyping platforms have been developed (Syvanen 2001. A current trend in some crops is the adoption of high-throughput genotyping equipment for SSR and SNP markers, although the cost of these new platforms may be higher than for standard genotyping methods. Some of these genotyping platforms use fluorescently labelled primers that permit high levels of multiplexing. Some authors have predicted that SNP markers, due to their widespread abundance and potentially high levels of polymorphism, and the development of SNP genotyping platforms will have a great impact on MAS in the future (Koebner & Summers 2003). Numerous SNP genotyping platforms have been recently developed, usually for medical applications; however, at present no superior platform has been universally adopted.

Finally, the availability of large numbers of publicly available markers and the parallel development of user-friendly databases for the storage of marker and QTL data will undoubtedly encourage the more widespread use of MAS.

Today, the optimism of a decade ago has been tempered somewhat by constraints encountered by some current marker-assisted selection approaches. However, considering the potential for the development of new strategies, the future for polygenic trait improvement through DNA markers, and the contribution of this to plant breeding efforts worldwide, appears bright.

In the future, improved molecular kits such as microarray-based systems might enable surveillance to be carried out more cost effectively and extensively, possibly by larger teams of non-experts supervised by smaller numbers of experts. By their nature, new threats are unknown, but the more the relationships between crops and pests/disease organisms in general are understood, the better are the prospects to mount rapid and effective responses.

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. 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 those current barriersis well understood and appropriate solutions developed. The exploitation of the advantages of MAS relative to conventional breeding could have a great impact on crop improvement. The high cost of MAS 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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