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REGULAR ARTICLE

MARKER ASSISTED SELECTION IN RICE

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SUMMARY

A rising global population requires increased crop production and some research suggests that the rate of increase in crop yields is currently declining and traits related to yield, stability and sustainability should be a major focus of plant breeding efforts. These traits include durable disease resistance, abiotic stress tolerance and nutrient and water use efficiency. The use of DNA markers in plant breeding is called marker assisted selection (MAS). So far, about 40 major blast genes have been identified, about 30 genes have been mapped on different rice chromosomes, and tightly linked DNA markers have been developed. Eight blast resistance genes have been cloned and the genes have been used for their selective introgression into susceptible rice cultivars. Recently Genetic and physical mapping of blast resistance gene Pi-42(t) located on the short arm of rice chromosome 12 in a resistant genotype 'DHR9' has been achieved. The PCR-based allele specific and in Del marker sets are available for nine blast resistance genes and they provide an efficient marker system for MAS for blast resistance breeding. Recently a novel resistance gene Pi40 derived from wild Oryza species (*O. australiensis*), have been located on chromosomes 6 and it shows promise for broad spectrum resistance. Tetep, the likely donor of Pi5(t) confers broad-spectrum resistance to *Magnaporthe grisea*. Additionally, several blast resistance genes could be combined using MAS in a single genetic background to develop rice cultivars with broad-spectrum durable resistance to blast. In future combination of conventional and marker assisted selection approach will provide opportunities for breeders to develop high yielding, stress tolerant and better quality rice cultivars. No doubt the cost of using DNA markers is expensive but it is worth the investment.

Key words: Rice, Molecular markers, Polymorphism, Foreground and background selection

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1. Introduction

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population. It is cultivated on all the continents except Antarctica, over an area of more than 150 million ha, but most rice production takes place in Asia. The Green Revolution technology developed at the International Rice Research Institute (IRRI) in the 1960's increased world rice production. However, during the past decade, production potential of modern cultivars has remained stagnant. Advances in cellular and molecular biology have made cultivated rice, a model monocot species because of several landmark achievements such as:-

i) Successful production of transgenic plants and genetic transformation potential in *indica* and *japonica* cultivars;
ii) Cultivar development through anther and pollen culture;
iii) Construction of a comprehensive genetic and physical map of the rice

genome;

iv) Development of the genetic maps of chloroplast and mitochondrial genomes;

v) Construction of a high density molecular map for gene mapping and map-based gene cloning;

vi) Development of BAC and YAC libraries and development of the *Oryza* MAP Alignment Project (OMAP);

vii) Small genome size (around 289 Mb) and synteny with other cereals like wheat (*Triticium aestivum* L.), maize (*Zea mays* L.), and barley (*Hordeum vulgare* L.), and

viii) Complete rice cultivars and annotation of gene sequences.

Several biotic and abiotic stresses, as well as narrow genetic diversity in modern cultivars of rice, are the major constraints to further increases in productivity. With the development of a comprehensive molecular genetic map of rice 1488 genes (Jena and Mackill, 2008) have been identified corresponding to several traits of economic importance. In addition to several genes of morphological and physiological traits, 28 genes for bacterial blight, 40 for blast, 3 for virus diseases and about 30 genes for resistance to insects such as brown plant hopper (*Nilaparvata lugens*), green rice leafhopper (*Nephotettix cincticeps*), and gall midge (*Orseolia oryzae*) have been identified. Several genes and quantitative traits loci (QTL) have been identified for abiotic stresses such as drought, salinity, submergence and cold.

2. Molecular marker technology

Conventional cereal breeding is time consuming and very depended on environmental conditions. Breeding a new variety takes between eight and twelve years and even then the release of an improved variety cannot be guaranteed. Hence, breeders are extremely interested in new technologies that could make this procedure more efficient. Molecular marker technology offers such a possibility by adopting a wide range of novel approaches to improving the selection strategies.

Types of markers

Morphological markers

These are the traditional markers .Morphological mutant traits in a population are mapped and linkage to a desirable or undesirable trait is determined and indirect selection is carried out using the physically identifiable mutant for the trait. There are several undesirable factors that are associated with morphological markers. The first is there high dependency on environmental factors. Often the conditions that a plant is grown in can influence the expression of these markers and lead to false determination. Second, these mutant traits often have undesirable features such as dwarfism or albinism. And lastly, performing breeding experiments with the morphological markers is time consuming, labour intensive and the large populations of plants required need large plots of land and/or greenhouse space in which to be grown (Stuber *et al.*, 1999).

Biochemical markers

Isozymes are used as biochemical markers in plant breeding. Isozymes are common enzymes expressed in the cells of plants. The enymes are extracted, and run on denaturing electrophoresis gels. The denaturing component in the gels (usually SDS) unravels the secondary and tertiary structure of the enzymes and they are then separated on the basis of net charge and mass. Polymorphic differences occur on the amino acid level allowing singular peptide polymorphism to be detected and utilized as a polymorphic biochemical marker.

Biochemical markers are superior to morphological markers in that they are generally independent of environmental growth conditions. The only problem with isozymes in MAS is that most cultivars (commercial breeds of plants) are genetically very similar and isozymes do not produce a great amount of polymorphism and polymorphism in the protein primary structure may still cause an alteration in protein function or expression.

Molecular markers (DNA- based Markers)

Molecular markers have become important tools for genetic analysis and crop

improvement. DNA-Markers, which are phenotypically neutral and literally unlimited in number, have allowed scanning of the whole genome and assigning landmarks in high density on every chromosome in many plant species. Different types of molecular markers have been developed and evolved, including, but not limited to, Restriction Fragment Length Polymorphism(RFLP),Amplified Length Polymorphism(AFLP) ,Simple Sequence Repeat (SSR) or microsatellites, Cleaved Amplified Polymorphic Sequences (CAPS), Sequence Characterized Amplified Regions (SCARS), Expressed Sequence Tags (EST_S) ,Single Nucleotide Polymorhism (SNP_S), etc.

Five conditions that characterize a suitable molecular marker are:

1) Must be polymorphic

2) Co-dominant inheritance

3) Randomly and frequently distributed throughout the genome

4) Easy and cheap to detect

5) Reproducible

Qtl and polygenic traits

Most of the important agronomic traits of rice are complex and polygenic in nature, controlled by QTL. Several parameters, such as heritability of the target trait, population size, and possibility of false QTL detection (type I error), should be taken into consideration for the efficiency of QTL for MAS. A simulation study conducted by Moreau *et al* (1998) revealed the following relationships between QTL and MAS:

- If the heritability is high, the genotypic values are well estimated by the phenotype, and the weight given to markers is equivalent to phenotypic selection.
- MAS is not effective at an (selection index) of 5% and heritability < 0.15.
- The efficiency of MAS decreases as the number of QTL increases.
- The efficiency of MAS increases when individual QTL explain a large part of the genetic variance.
- The relative efficiency of MAS increases with population size (the population should be larger than 100 or 200 individuals) and if the distance between markers and QTL decreases.

The advantages of using MAS in rice improvement have been well documented (Jeana *et al*.,2003;Mackill and McNally,2004;Xu *et al*.,2004; Toojinda *et al*.,2005;Liu *et al*.,2006;Dwivedi *et al*.,2007;Mackill,2007)

The success of MAS is influenced by the relationship between the markers and the genes of interest. Dekkers distinguished three kinds of relationship (Dekkers, 2004)

1. The molecular marker is located within the gene of interest (i.e. within the gene Q, using the example above). In this situation, one can refer to gene assisted selection (GAS). This is the most favourable situation for MAS since, by following inheritance of M alleles, inheritance of the Q alleles is followed directly and are thus most difficult to find.

2. The marker is in linkage disequilibrium (LD) with Q throughout the whole population. LD is the tendency of certain combinations of alleles (e.g. M and Q) to be inherited together. Population wide LD can be found when markers and genes of interest are physically very close to each other and/or when lines or breeds have been crossed in recent generations. Selection using these markers can be called LD-MAS.

3. The marker is not in linkage disequilibrium (i.e. it is in linkage equilibrium [LE]) with Q throughout the whole population. Selection using these markers can be called LE-MAS. This is the most difficult situation for applying MAS.

QTL mapping and marker-assisted selection

(a) Features of cereal breeding

The fundamental basis of plant breeding is the selection of specific plants with desirable traits. Selection typically involves evaluating a breeding population for one or

more traits in field or glasshouse trials (e.g. agronomic traits, disease resistance or stress tolerance), or with chemical tests (e.g. grain quality). The goal of plant breeding is to assemble more desirable combinations of genes in new varieties. Standard breeding techniques for inbreeding cereal crops have been outlined in various textbooks. In the commonly used pedigree breeding method, selecting desirable plants begins in early generations for traits of higher heritability. However, for traits of low heritability, selection is often postponed until the lines become more homozygous in later generations (F_5 or F_6). Selection of superior plants involves visual assessment for agronomic traits or resistance to stresses, as well as laboratory tests for quality or other traits. When the breeding lines become homozygous (F_5 or later), they can be harvested in bulk and evaluated in replicated field trials. The entire process involves considerable time (5-10 years for elite lines to be identified) and expense. The size and composition of a plant population is an important consideration for a breeding programme. The larger the number of genes segregating in a population, the larger the population size required in order to identify specific gene combinations. Typical breeding programmes usually grow hundreds or even thousands of populations, and many thousands or millions of individual plants. Given the extent and complexity of selection required in breeding programmes, and the number and size of populations, one can easily appreciate the usefulness of new tools that may assist breeders in plant selection. The scale of breeding programmes also underlines the challenges of incorporating a relatively expensive technology such as MAS.

(b) There are five main considerations for the use of DNA markers in MAS

Reliability; quantity and quality of DNA required; technical procedure for marker assay; level of polymorphism; and cost (Mohler and Singrun, 2004).

Reliability

Markers should be tightly linked to target loci, preferably less than 5 cM genetic distance. The use of flanking markers or intragenic markers will greatly increase the reliability of the markers to predict phenotype.

DNA quantity and quality

Some marker techniques require large amounts and high quality of DNA, which may sometimes be difficult to obtain in practice, and this adds to the cost of the procedures.

Technical procedure

The level of simplicity and the time required for the technique are critical considerations. High-throughput simple and quick methods are highly desirable.

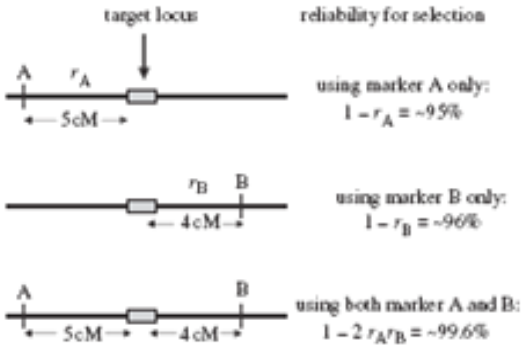
Level of polymorphism

Ideally, the marker should be highly polymorphic in breeding material (i.e. it should discriminate between different genotypes), especially in core breeding material.

Cost

The marker assay must be cost-effective in order for MAS to be feasible.

Fig.1: Reliability of selection using single and flanking markers (adapted from Tanksley (1983), assuming no crossover interference). The recombination frequency between the target locus and marker A is approximately 5% (5 cM). Therefore, recombination may occur between the target locus and marker in approximately 5% of the progeny. The recombination frequency between the target locus and marker B is approximately 4% (4 cM). The chance of recombination occurring between both marker A and marker B (i.e. double crossover) is much lower than for single markers (approx. 0.4%). Therefore, the reliability of selection is much greater when flanking markers are used



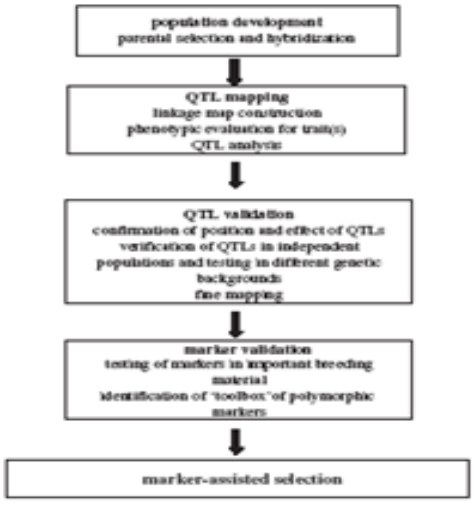
The most widely used markers in major cereals are called simple sequence repeats (SSRs) or microsatellites (Gupta and Varshney, 2000). They are highly reliable (i.e. reproducible), co-dominant in inheritance ,relatively simple and cheap to use and generally highly polymorphic. The only disadvantages of SSRs are that they typically require polyacrylamide gel electrophoresis and generally give information only about a single locus per assay, although multiplexing of several markers is possible. These problems have been overcome in many cases by selecting SSR markers that have large enough size differences for detection in agarose gels, as well as multiplexing several markers in a single reaction. SSR markers also require a substantial investment of time and money to develop, and adequate numbers for high-density mapping are not available in some orphan crop species. Sequence tagged site (STS), sequence characterized amplified region (SCAR) or single nucleotide polymorphism (SNP) markers that are derived from specific DNA sequences of markers (e.g. restriction fragment length polymorphisms: RFLPs) that are linked to a gene or quantitative trait locus (QTL) are also extremely useful for MAS (Sanchez *et al.* 2000).

(c) 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 out breeding 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 (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 ideally a confirmation step is preferable because QTL positions and effects can be inaccurate due to factors such as sampling bias. QTL validation generally refers to the verification that a QTL is effective in different genetic backgrounds. 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.

Fig.2: Marker development 'pipeline'



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.

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 (Zwart *et al.* 2004). Other examples are quality traits which generally require expensive screening procedures.

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.

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 (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 and 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 and 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 population development parental selection and hybridization QTL mapping linkage map construction phenotypic evaluation for trait(s) QTL analysis QTL validation confirmation of position and effect of QTLs verification of QTLs in independent populations and testing in different genetic backgrounds fine mapping marker-assisted selection testing of markers in important breeding material identification of 'toolbox'of polymorphic markers marker validation. Marker-assisted selection in plant breeding (Collard and Mackill, 2008) 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 analysing data based on morphological characteristics.

(i) Cultivar identity / assessment of 'purity'

1. 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.

(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 (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. 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.

(b) Marker-assisted backcrossing

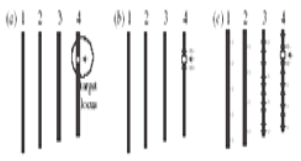
Backcrossing has been a widely used technique in plant breeding for almost a century. Backcrossing is a plant breeding method most commonly used to incorporate one or a few genes into an adapted or elite variety. In most cases, the parent used for backcrossing has a large number of desirable attributes 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.

The use of DNA markers in backcrossing greatly increases the efficiency of selection. Three general levels of marker assisted backcrossing (MAB) can be described. 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 and 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.

The second level involves selecting BC progeny with the target gene and recombination events between the target locus 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' . Using conventional breeding methods, the donor segment can remain very large even with many BC generations. 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.

Fig.3: 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



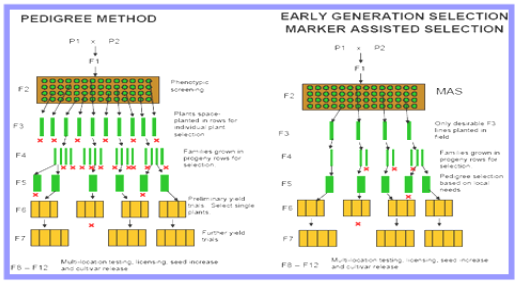
The third level of MAB involves selecting BC progeny with the greatest proportion of recurrent parent (RP) genome, using markers that are unlinked to the target locus — we refer to this as 'background selection'. In the literature, background selection refers to the use of tightly linked flanking markers for recombinant selection and unlinked markers to select for the RP (Frisch *et al.*, 1999). 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 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'

(b) 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 and 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 and 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 per cent of the F_3 population will be selected compared with 25% of the F_2). Ribaut and 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 and 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.

Fig.4: Early generation selection scheme (proposed by Ribaut and Betran (1999). Note that many lines can be discarded in an early generation which permits the evaluation of fewer lines in later generations



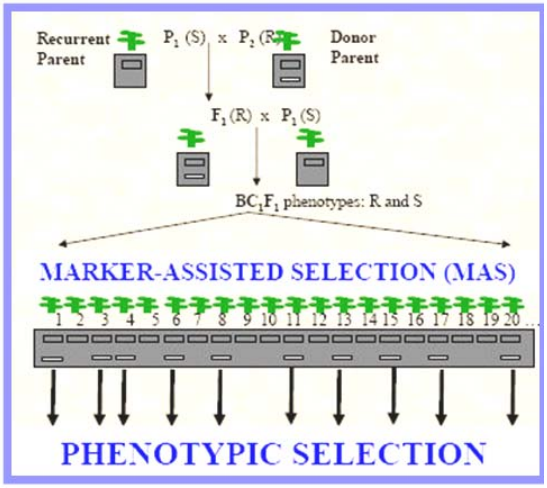
There are several instances when phenotypic screening can be strategically combined with MAS. In the first instance, 'combined MAS' (Moreau *et al.*, 2004) may have advantages over phenotypic screening or MAS alone in order to maximize genetic gain . This approach could be adopted when additional QTLs 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. 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 . 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. In other words, a marker assay may not predict phenotype with 100 per cent 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 (Han *et al.*, 1997). This was referred to as 'tandem selection' by Han *et al.* (1997) and 'stepwise selection' by Langridge and Chalmers (2005).

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.

Fig.5: Marker-directed phenotyping or 'tandem selection' can be used when markers are not 100% accurate or when phenotypic screening is more expensive compared to marker genotyping



(d) Marker assisted selection in control of blast disease

Blast disease caused by the fungal pathogen *Magnaprothe grisea* Cav. [anamorph *Pyricularia grisea* (Cooke) Sacc.] is one of the most serious diseases of rice worldwide Blast fungus infects both temperate and tropical rice grown under different ecosystems (i.e., upland, lowland, irrigated and rainfed) and causes more damage in areas where high-input rice cultivation is practiced. Infection normally begins when a blast spore germinates and penetrates the rice leaf, resulting in lesions of the rice plant, and ends when the fungus sporulates repeatedly for an extended time, dispersing many new airborne spores. Disease control worldwide has relied on the combination of chemical fungicides and integrated culture practice. For the last several years, plant breeding has achieved significant progress towards the enhancement of host resistance to this disease but the development of durably resistant cultivar is yet to be realized. The use of resistant rice cultivars is a powerful tool to reduce the use of environmentally destructive pesticides. Using classical plant breeding techniques, plant breeders have developed a number of blast resistant cultivars adapted to different rice growing regions worldwide. However, the rice industry remains threatened by blast disease because of the instability of the rice blast fungus. Recent advances in rice genomics provides additional tools for plant breeders to develop rice production systems that could be environmentally benign.

Fig.6: Leaf blast lesions

MAS is extremely powerful in blast resistance breeding because resistance phenotypes are often simple or encoded by single or few genes (Young, 1996). Blast resistance is governed by a specific interaction of a particular resistance (R) gene in rice with a particular avirulence gene in the pathogen. Since the initial definition of the plant resistance (R) genes by Flor (1942), many R genes have been identified. The vast majority of the known R genes is composed of proteins carrying nucleotide-binding sites and leucine-rich repeat motifs (NBS-LRR; Jones and Dangl, 2006). Most R genes recognize pathogen effectors developed by pathogens to inhibit defense, although there are a few exceptions (e.g.,Lee et al. 2009). Some of these effectors thus correspond to the initial definition by Flor of the avirulence gene. Depending on the presence/absence of these R genes and of the matching avirulence product, the interaction will be incompatible (plant is resistant) or compatible (plant is susceptible).Many R genes have been identified in rice and most code for NBS-LRR genes . After recognition mediated by the R gene, signal transduction occurs and requires regulators such as MAP kinases. Finally,

transcription factors like WKRYs activate a deep transcriptional reprogramming of the cell , leading to the activation of defense responses per se. These include production of antimicrobial secondary metabolites, pathogenesis-related (PR) proteins (e.g., chitinases, glucanases, PBZ1 in rice;Jwa et al. 2006), cell wall strengthening (Huckelhoven 2007), and programmed cell death, leading to hypersensitive response . The genes that act downstream of the disease resistance pathway are collectively called defense genes.

Most of the elements involved (receptors, regulators,transcription factors, and defense genes) are well conserved across species. For example, the NPR1 gene is a central regulator in both monocots and dicots. The NPR1 gene was successfully used in several plant species like Arabidopsis, rice , tomato, and wheat . One single R gene can trigger a complete resistance, thus masking the presence of other R genes in any given cultivar. Second, quarantine restrictions often prohibit the exchange of blast isolates, particularly in rice-growing areas that prevent verification of the existence of a particular R gene in a given germplasm. Third, blast pathogens can be classified into hundreds of physiological races, or pathotypes, that differ in their ability to cause disease on different rice cultivars. The blast avirulence genes are highly unstable and thus not easily tractable. R-genes are more stable for tagging the resistance. Blast R genes are one important component of many plant factors acting as sophisticated and multifaceted defense systems against pathogens. A number of blast R genes in different combinations have been incorporated into diverse cultivars by classical plant breeding. Incorporating stable combination of blast R genes into improved rice germplasm continues to be a major focus of rice breeding programs worldwide. Rice containing a Pi gene confers resistance to a fungal *M. grisea* race in a gene for gene manner. So far ,about 40 major blast genes have been identified, about 30 genes have been mapped on different rice chromosomes, and tightly linked DNA markers have been developed. The DNA markers have been used effectively to identify resistance genes, and MAS has been applied for integrating different resistance genes into rice cultivars lacking the desired traits. The PCR-based allele-specific and InDel marker sets are available for nine blast resistance genes, and they provide an efficient marker system for MAS for blast resistance breeding (Hayashi et al.,2006). Eight blast resistance genes have been cloned and the genes have been used for their selective introgression into susceptible rice cultivars (Lin et al.,2007). DNA markers for Pi-ta has been used to follow its introgression into advanced breeding lines (Johnson *et al.*, 2003). Pi-ta is very important for the US rice industry because Pi-ta confers resistance to predominant major field races of the rice blast fungus in the southern US. A US cultivar, Katy containing Pi-ta released a decade ago has been used as a blast resistant breeding parent (Moldenhauer *et al.*, 1990). Recently Genetic and physical mapping of blast resistance gene *Pi-42(t)* on the short arm of rice chromosome 12 in a broad spectrum resistant genotype 'DHR9' has been achieved. (Kumar et al.2009) .A novel resistance gene, *Pi40* derived from the EE genome wild *Oryza* species (*O. australiensis*), has been localized on chromosome 6 and fine mapped using the *e*-landing approach(Jeung et al.,2007). The R gene locus, tentatively designated as Pi36(t) was roughly mapped on rice chromosome 8 in the indica cultivar (cv.) Q61 by comparison of the BA1126550 sequence with rice sequences in the databases (liu et al.,2005) .The DNA marker (9871.T7E2b) linked to the blast resistance phenotype in the presence of the *Pi40* gene in a 70-kb chromosomal region was obtained from NBS-LRR disease resistance motif sequences (Jeung et al.,2007). It is imperative to use DNA markers identified within the gene or from the flanking region of the gene as a tool for an efficient MAS strategy in rice improvement. Tetep, the likely donor of Pi5(t) confers broad-spectrum resistance to *Magnaporthe grisea* (Yi *et al.*, 2004). Additionally, several blast resistance genes could be combined using MAS in a single genetic background to develop rice cultivars

with broad-spectrum durable resistance to blast.

(e) 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. 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) pyramided three major genes(Pi1, Piz-5 and Pita) using RFLP markers 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. 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.

(f) Marker assisted selection in rice breeding for bacterial leaf blight.

Bacterial blight is one of the most destructive rice diseases and can reduce yield by 20 to 30% (Singh *et al*., 1977). However, this approach is difficult through conventional breeding due to masking effects of genes such as Xa21, which convey resistance to many BB races. It is impossible to distinguish between plants having Xa21 alone and those having Xa21 and other genes. Marker-assisted selection allows the identification of plants with multiple resistance genes. Major genes conferring disease resistance in several crop species have been mapped with linked DNA markers, facilitating MAS for disease resistance in these crops . Marker-assisted selection has been successfully used in selecting for resistance in the absence of pathogens (Melchinger,1990), pyramiding

multiple genes for durable resistance against rice bacterial blight (Huang *et al*., 1997), and for development of multiple disease-resistant germplasm (Kelly, 1995;swamy *et al*.,2008) Twenty-eight genes conferring resistance to bacterial leaf blight (BB) have been reported in rice (Nino-liu *et al*.,2006).Several genes have been associated with tightly linked DNA markers, and some of them have been cloned (*Xa1*, *xa5*, *xa13*, *Xa21*, *Xa26*, *Xa27*) and used for breeding BB-resistant rice cultivars. With the exception of *xa5* and *xa13*, the BB resistance genes are dominant in nature and the markers are developed from the sequencing information of these genes, which are widely used in MAS (Chu et al.2006,Gu *et al*.). Because of the availability of DNA markers derived from the resistance genes, it is now possible to pyramid several resistance genes into susceptible elite rice cultivars. Using the gene pyramiding approach, improved indica rice cultivars with broad-spectrum durable BB resistance have been developed by combining *Xa4* and *Xa21*. The pyramided BB resistance genes, *Xa4*+*xa5*+*Xa21*, expressed strong resistance to virulent BB isolates of Korea compared with individual resistance genes that are moderately to completely susceptible (Jeung *et al* .,2006). The resistance genes *xa5*, *xa13*, and *Xa21* have been pyramided into an indica rice cultivar (PR106) using MAS that expressed strong resistance to BB races of India (Singh *et al* .,2001). Two commercially cultivated rice cultivars (Angke and Conde) were released in 2002 for cultivation in Indonesia. They possess gene pyramids *Xa4*+*xa5* and *Xa4*+*Xa7*, respectively. In the Philippines, two rice cultivars (NSIC Rc142 and NSIC Rc154) have the gene combination *Xa4*+*xa5*+*Xa21*. These genes have been integrated into the susceptible cultivar IR64 genetic background using MAS (Toenniessen *et al*.,2003). Genes for basmati quality from PB-1 and bacterial blight resistance from IRBB55 *xa13+Xa21* have been pyramided

3. Conclusions

Molecular marker maps, the necessary framework for any MAS programme, have been constructed for the majority of agriculturally important species but the density of the maps varies considerably among species. Currently, MAS does not play a major role in genetic improvement programmes in any of the agricultural sectors. Enthusiasm and optimism remain concerning the potential contributions that MAS offers for genetic improvement. However, this seems to be tempered by the realization that it may be more difficult and take longer than originally thought before genetic improvement of quantitative traits using MAS is realized. Further advances in molecular technology and genome programmes will soon create a wealth of information that can be exploited for the genetic improvement of plants and animals.

High throughput genotyping, for example, will allow direct Resolution of selection on marker information based on population wide Linkage Disequilibrium. Methods to effectively analyse and use this information in selection are still to be developed. The eventual application of these technologies in practical breeding programmes will be on the basis of economic grounds, which, along with cost-effective technology, will require further evidence of predictable and sustainable genetic advances using MAS. Until complex traits can be fully dissected, the application of MAS will be limited to genes of moderate-to large effect and to applications that do not endanger the response to conventional selection. Until then, observable phenotype will remain an important component of genetic improvement programmes, because it takes account of the collective effect of all genes.

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