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Marker-assisted Gene Pyramiding for Inbred Line Development: Practical Applications

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ABSTRACT

Aimed at assembling multiple desirable genes into a single genotype, gene pyramiding is a commonly used breeding strategy in self-pollinated crops. The use of this strategy has been greatly facilitated and widened by the rapid development of plant molecular biology and genomics. Marker-assisted gene pyramiding including introgression may be so far the most productive application of marker-assisted selection (MAS) in plant breeding. As part of a comprehensive review, the current literature in the practical applications of gene pyramiding is summarised in this paper. The basic principles of gene pyramiding, the process and useful guidelines for designing an efficient strategy, and the integration of gene discovery and pyramiding are discussed in a companion paper. Marker-assisted gene introgression as an intermediate step towards pyramiding multiple genes is also discussed. By targeting few genes with large effects, improved (converted) versions of some elite cultivars have been successfully developed in various crops. Experimental results in the improvement of quantitative trait by combining quantitative trait loci (QTL) varied from disappointing to promising with few successful examples. It is important that QTL should be precisely mapped and validated in the target genetic backgrounds before introgresion starts. It is anticipated that QTL pyramiding will be more fruitful if near isogenic lines in the same elite background are used as parents. Genetic transformation, which can introduce one or a few extraneous genes into crop species, can be used alone or in combination with sexual crossing to pyramid genes.

Keywords: genetic transformation, molecular markers, marker-assisted selection, quantitative trait loci, recurrent backcrossing

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INTRODUCTION

Gene pyramiding is a breeding method aimed at assembling multiple genes with known effects on target traits. It is mainly used in improving an existing elite cultivar for a few unsatisfactory traits, for which genes with large positive effects are identified. Traditionally, the identification of the sources of useful genes is very slow and a breeder's capability to trace the presence or absence of the target genes is limited. This limits the number of genes to be incorporated into elite cultivars at any time. The development of modern molecular and genomics technology has not only accelerated the discovery of favourable genes but also widened the sources of useful genes (Tanksley and McCouch 1997; Dekkers and Hospital 2002; Dubcovsky 2004). Therefore, the number of target genes can be many and more efficient gene pyramiding strategies are needed. Recently, gene pyramiding has been extended to QTL pyramiding to capitalise on the outputs of QTL mapping studies (Ashikari and Matsuoka 2006). Although in principle QTL pyramiding is no different from pyramiding major genes for qualitative traits, the fact that the effects of QTL are more environment specific (genetic and physical environments) does present more challenges. The basic principles of gene pyramiding, the process and useful guidelines for designing an efficient strategy, the integration of gene discovery and pyramiding has been discussed in a companion paper (Ye and Smith 2008). This paper summarises the current literature in the practical applications of gene (QTL) pyramiding for inbred line development. Also summarised are results of some QTL introgression experiments, which provide important insights of QTL pyramiding though not directly aimed at the development of marketable inbreds. A gene pyramiding program starting with a set of elite parental lines containing

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Invited Review

the target genes is likely to be more efficient than one where the parental lines have very diverse backgrounds. The development of such parental lines can be achieved by recurrent backcross (Zamir 2001). Therefore, recurrent backcrossing aimed at transferring one or two genes to an elite recipient line can be regarded as an intermediate step towards gene pyramiding of multiple target genes. The progress of marker-assisted recurrent backcrossing is discussed in this review as well.

RECURRENT BACKCROSSING

Aimed at transferring one or two genes to an elite recipient line, recurrent backcrossing has long been used by breeders as an efficient method for improving unsatisfactory traits within an existing elite cultivar (Allard 1999). In recurrent backcrossing individuals with desirable characteristics are selected and then backcrossed to one of the parents (see an example scheme in **Fig. 1**). The cultivar carrying the target gene is usually called the donor parent, while the one used as a parent in all the backcrossing generations is called the recipient or recurrent parent. Recurrent backcrossing is done to reduce the contribution of the donor genome. The number of backcross generations depends on the performances of the donor parent for other agronomic traits. When the donor parent is an elite cultivar with acceptable performance for other traits, one or two backcross generation may be sufficient. When the donor parent is poor for other traits, which is usually the case, several backcross generations are needed. If selection is applied for the desired characteristics only, the proportion of donor genome for all chromosomes except the one carrying the target gene is expected to be reduced by one-half at each backcross generation. Selection against donor can be used to speed up the reduction of donor genome. In practice, selection on phenotypic resemblance to the recurrent parent for other traits and the presence of the characteristics of interest are conducted simultaneously. On the chromosome carrying the target gene, the reduction of the donor genome is slower due to the selection for the presence of the target gene. This is particularly true for the chromosome region surrounding the target gene. Theoretical results (Stam and Zeven 1981) show that the donor segment attached to the target allele remains surprisingly large even after many generations of conventional backcrossing. Young and Tanksley (1989) found that lengths up to 51 centimorgans (cM) of the segment attached to a resistance gene after six backcross generations in tomato. This may result in a phenomenon known as linkage drag, that is, a negative trait is closely associated with the target gene being introgressed. In fact, linkage drag is identified as the main cause for the differences between the recipient line and the converted line (Zeven *et al*. 1983). Obviously, minimizing the size of the introgressed segment from the donor parent can be an effective way to eliminate/reduce linkage drag. Therefore the reduction of the size of introgressed segment size may be critical to the successful backcross breeding of a new cultivar, especially if the donor parent is a wild relative or exotic germplasm source. In the following sections the use of molecular markers in the recurrent backcrossing program are discussed.

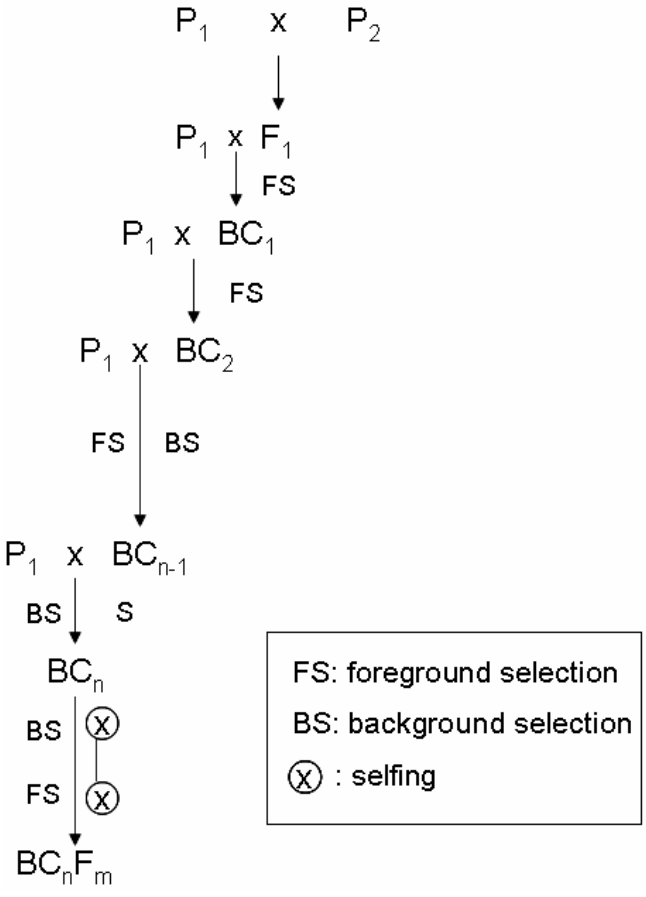

Fig. 1 An example recurrent backcrossing scheme with P1 and P2 as the recurrent and donor parent, respectively.

Marker-assisted foreground selection

Marker-assisted foreground selection was proposed by Tanksley (1983). The presence of a target allele in an individual is diagnosed by monitoring the genotype at markers linked to the gene for alleles of the donor parent. This is a powerful tool not only for manipulation of oligogenic traits under numerous situations in plant breeding (Melchinger 1990), but also for manipulation of quantitative trait loci (QTL) (Stuber 1995). Melchinger (1990) presented *a priori* approach for calculating the minimum number of individuals and family size required in recurrent backcrossing. Marker-assisted foreground selection is mainly used when the effects of target alleles are difficult or impossible to measure phenotypically. For instance, when the target is inherited recessively, the presence or absence of the target gene in a backcross individual cannot be known by observing its phenotypic performance. Traditionally, a phenotypic assay of progeny generated either by selfing or by crossing to the donor parent is used to determine whether an individual is retsined or discarded (Allard 1999). This is not only costly but also time-consuming. Suppose breeders identify a new resistance gene and would like to transfer it into an existing resistant cultivar containing other resistance genes to increase the durability. The individuals with the resistance phenotype do not show the presence of the target resistance gene, since the effect of the target gene is totally or partially masked by other resistance genes. This is one of the reasons why the application of pyramiding multiple resistance genes for the same disease is not very successful although its potential as a strategy for the development of cultivars with durable resistance has long been recognised. Suppose on the other hand, a breeder wants to transfer a gene conferring resistance to a devastating disease which is not yet present in his/her testing environments as a precaution or to increase the adoptability of cultivars by growers in disease hotspot areas. The breeder will not be able to observe the resistance phenotypically due to obvious reasons and phenotyping has to be done by a collaborator in a diseased environment. In situations like these, tightly linked molecular markers if available can be used as a diagnostic tool to trace the presence of the target gene in successive backcross generations. In the case of transferring recessive gene it removes the costly and time-consuming progeny testing and the presence of the target gene needs to be tested either by selfing or crossing to the donor only at the end of the breeding program. Marker assisted backcrossing will also be advantageous if phenotypic assays are more expensive than marker assays. For instance, the phenotyping resistance to nematodes in soybean (Young 1999), tomato (Tanksley 1983) and wheat (Eagles *et al*. 2001) are expen-

sive and unreliable and breeding for resistance has been very slow. However, cultivars with resistance have been developed by using the markers tightly linked to the resistance genes in all three crops. These may be among the best examples of the practical use of markers in crop breeding. Another important class of traits which are expensive to measure relate to end-product quality. Testing for quality characteristics of end product of wheat and barley is very expensive and requires large samples. The high cost involved makes it impossible to screen large populations and as a result the genetic progress is very slow since the applicable selection pressure is very low. The requirement of large samples effectively eliminates the possibility of individual selection in early generations. In wheat and barley, known genes such as glutenin (which can be regarded genes with perfect markers) with large effects on end quality traits have been used in Australia for cross selection (Eagles *et al*. 2001; Ye *et al*. 2004). However, genotyping for glutenin genes using electrophoresis is still too expensive to allow its use in large populations. Markers tightly linked to genes controlling these traits if developed will enable the early generation selection for these traits and greatly enhance the genetic progress.

Marker-assisted background selection

Marker-assisted background selection (MABS) was proposed by Young and Tanksley (1989) to accelerate recovery of the recurrent parent genome (RPG). Individuals are selected which are homozygous for the alleles of the recurrent parent at a number of marker loci covering the entire genome. MABS has been established as a standard tool in plant breeding. MSBS has been investigated by various authors (Hospital *et al*. 1992; Openshaw *et al*. 1994; Visscher *et al*. 1996; Frisch *et al*. 1999a, 1999b). Young and Tanksley (1989) stated that a sufficiently high proportion of the RPG is recovered after three generations of marker-assisted backcrossing. Hospital *et al*. (1992) showed a saving of two backcross generations because of MABS. Frisch *et al*. (1999b) demonstrated that the number of backcross generations required for the introgression of one target gene was reduced by two to four backcross generations. Frisch and Melchinger (2001a) showed that a saving of three backcross generations due to background selection is also a realistic goal for simultaneous introgression of two genes. Several useful points for practical breeders from these theoretical studies are: (1) Four impendent markers per chromosome not carrying the target gene are enough for background selection; 2) The use of equally spaced markers reduces the population size required; (3) Background selection is more efficient if it is applied in an advanced generation. This is because the selected individual only contributes half of the genome of the progeny of the next backcross generation and as a result the reduced donor genome from background selection has a carry-over rate of one-half to the next backcross generation; (4) Using a larger population size in advanced generation is advantageous if genotyping cost is high. With the advance of backcross generations many marker loci become fixed for the recurrent allele and do not need to be genotyped; and (5) Multiple-stage selection at each generation by exploring different types of marker genotypes can be used to reduce the number of marker genotyping. However, considering the cost of genotyping nowadays is largely the cost of DNA isolation as opposed to the cost of additional marker assays this point might be less important.

Reduction of linkage drag

Tightly linked markers flanking the target gene can be used to reduce the length of donor chromosome segment attached to the target gene and potential linkage drag (Frisch and Melchinger 2001b; Hospital 2001). The reduction of segment length depends on the flanking marker distance, population size and the number of generations (breeding duration). Frisch *et al*. (1999a) developed equations for calculating the minimal population size for obtaining at least one carrier of the target allele homozygous for the recurrent parent allele at one or both flanking markers. Hospital (2001) and Frisch and Melchinger (2001b) derived the probability distribution of the size of donor chromosome segments around the introgressed gene. The probability distribution function was then used to derive the expected segment length and variance, which can be used to investigate the effect of marker distance. The general conclusions in the context of practical breeding are: 1) in general a small flanking marker distance is advantageous. Heterozygosity at tightly linked foreground selection markers results in a high probability that an individual carries the target gene. Moreover, homozygosity at tightly linked background selection markers results in a short donor chromosome segment around the target gene. However, the population size needed to obtain recombinant genotypes increases rapidly with the reduction of marker distance and thus the genotyping cost is increased by using closer markers. 2) When flanking makers are used, symmetric marker brackets (i.e. the flanking markers are equally distant from the target gene) are preferable. This not only reduces the required population size but also reduces the probability that a selected recombinant has a relatively large intact donor chromosome segment. 3) When the distance between the flanking markers is short (<20 cM), the number of backcross generations performed has little impact on the reduction of donor segment length. 4) The probability of having a smaller intact segment is greater with selection in an early generation than with selection in an advanced generation, because crossover events in subsequent generations after selection may result in the reduction of the intact chromosome segment. However, the required population size to obtain the desired recombinant genotype may be prohibitive. 5) To reduce the population size required it is generally more profitable to allow three or more successive backcrosses. For close markers the probability of double recombination is much lower than the probability of single recombination, and thus the population size needed to obtain a double recombinant in a single backcross generation is much lower than twice the population size needed to obtain a single recombinant. Therefore, total population size can be drastically reduced if selection is conducted in two generations, selecting in the first generation a single recombinant on one side of the target gene and then selecting in the second generation for a single recombinant on the other side. Allowing more than two generations permits an even further reduction of the total number of individuals needed.

Foreground for the target genes and background selection for the reduction of the contribution of donor genome and linkage drag must be combined to obtain acceptable cultivar using recurrent backcrossing. Using the results of the theoretical studies mentioned above as guidelines, the logical steps of marker assisted selection in a backcross generation are as follows: (1) selecting individuals carrying the target allele. Perfect markers or the closest flanking markers are most useful; (2) selecting individuals homozygous for recurrent parent genotype at loci close the target gene or markers linked to it; (3) selecting individuals homozygous for recurrent parent genotype at few (i.e. 2) marker loci on the chromosome carrying the target allele; and (4) selecting individuals that are homozygous for recurrent parent genotype at other marker loci of the other chromosomes (four independent markers per chromosome). It is clear from these studies that it is easier to reduce the contribution of donor genome from chromosome regions rather than that surrounding the target gene. Moreover, although it makes sense to control linkage drag by reducing the introgressed segment size, segment size does not necessarily correspond to the presence or absence of linkage drag. In other words, a shorter segment may cause serious linkage drag in some cases while no obvious linkage drag was caused by a fairly long segment in other cases. Therefore, it might be easier to separate the background control and the

reduction of segment size. This is to say lines with the target gene are developed first by foreground selection and background selection for chromosomes not carrying the target gene and then phenotypically tested for key agronomic trait to see whether there is significant undesirable linkage drag. Nevertheless, it is always beneficial to identify the recombinants between the target gene and its close flanking markers if they arise. Therefore, it is advisable that markers flanking to the target gene should always be screened although the presence of recombination is not used as a condition to select against donor genome. The best line can be used as donor to reduce the segment size around the target gene by recurrent backcrossing if the undesirable linkage drag proves significant. This strategy will avoid spending time and resources in trying to reduce/eliminate non-existing or unimportant linkage drag. It also has the potential to utilise the possible beneficial alleles linked to the target allele. When the program is set up to reduce linkage drag through reducing the size of the segment containing the target allele, the closest available flanking markers should be used. Several generations of backcrossing are required to reduce the total number of individuals to be screened. Background selection for reducing the contribution of donor genome from regions other than that surrounding the target allele is not required. Continuous selection on the flanking markers should be applied to identify the recombinant individuals as soon as possible, since the exact generation at which the recombination took place is difficult if not impossible to ascertain. Decoux and Hospital (2001) developed a computer program for optimising a multi-generation backcross program aimed at reducing segment size attached to a target gene by finding the minimal total population size (across generations) given the specified overall probability of success.

SUCCESSFUL APPLICATIONS OF GENE PYRAMIDING IN PRACTICE

Marker-assisted gene introgression of a single major gene

Ragot *et al*. (1994) provided a good example of gene introgression using a MAS for foreground selection. By using marker to monitor the *Bt* (*Bacillus thuringiensis*) gene in the transgenic parent, and random markers for selecting for the recurrent parent genome, they not only transferred the *Bt* gene into elite maize lines but also confirmed the theoretical prediction that the use of markers to speed up the recovery of the recipient genome provides a gain in time equivalent to two generations of backcrossing. In this study the target gene was inserted into the genome of the transgenic line by genetic transformation and linkage drag was not of concern and the marker used for foreground selection was in the transgenic construct (no recombination).

Peleman and van der Voort (2003) provided a comprehensive example of the removal of undesirable linkage drag by MAS to develop a novel lettuce variety resistant to the aphid *Nasonovia ribisnigri*. This aphid is a major problem in field grown lettuce areas in Europe and California causing reduced and abnormal growth in addition to spread of viral diseases. Resistance could be introgressed from a wild relative, *Lactuca virosa*, by recurrent backcrossing. However, the new product from many rounds backcrossing was of very poor quality, bearing yellow leaves and greatly reduced yield. Markers flanking to the introgression were used to select individuals that are recombinant in the vicinity of the gene among more than 2000 F_2 plants. By testing the selected individuals for resistance and the absence of the negative characteristics at F_3, an individual was identified bearing recombination events very close to each side of the gene, which did not have the linkage drag. The linkage drag was caused by recessive genes at both sides of the resistance gene, which resulted in the failure of classical selection methods.

The development of a converted version of rice restorer line 'Minghui 63' is an example of marker-assisted gene introgression using both of foreground selection for the target gene and background selection for reducing chromosome segment length attached to the target gene and the recovery of recurrent genome (Chen *et al*. 2000). 'Minghui 63' is a very popular rice restorer line for hybrid production in China. However, it is susceptible to rice bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), one of the most devastating rice diseases. Using the isogenic line 'IRBB21', developed by the International Rice Research Institute (IRRI), as donor Chen *et al*. (2000) successfully introgressed *Xa21*, a gene that confers wide spectrum resistance to BB into 'Minghui 63'. The MAS system consisted of a marker that is a part of *Xa21*, a marker located at 0.8 cM from the *Xa21* locus on one side, and a marker at 3.0 cM from the gene on the other side. A total of 128 restriction fragment length polymorphism (RFLP) markers, evenly distributed on the 12 chromosomes, were used to recover the genetic background of 'Minghui 63'. The entire scheme took three generations of backcrosses and one generation of selfing to complete. In this scheme, the progeny of each backcross was first selected for the presence of the *Xa21* gene (foreground selection) by means of both PCR and disease inoculation. The *Xa21*-containing individuals in the BC_1F_1 were selected for recombination between *Xa21* and either of the flanking marker loci (Background selection for reducing attached segment length). In BC_2F_1, the *Xa21*-containing individuals were selected for recombination between *Xa21* and the other marker locus (Background selection for reducing attached segment length). The *Xa21*-containing plants in the BC_3F_1 were assayed with a large number of molecular markers covering the entire rice genome to identify individuals that were homozygous for the 'Minghui 63' genotypes at all marker loci, except the *Xa21* locus (Background selection for the recovery of recurrent genome). The selected individuals were then self-fertilized to produce individuals that were homozygous for the *Xa21* gene at this locus, thus completing the breeding procedure. The resulting improved version of 'Minghui 63' was at molecular level exactly the same as the original except for a fragment of less than 3.8 cM in length surrounding the *Xa21* locus. Both the new version 'Minghui 63(*Xa21*)' and its hybrid with 'Zhenshan 97A' showed the same spectrum of BB resistance as the donor parent. Field examination of a number of agronomic traits showed that the improved version was identical to 'Minghui 63', when there was no disease stress. Under heavily diseased conditions, the improved version showed significantly higher grain weight and spikelet fertility than 'Minghui 63'.

Another example is the incorporation of the *opaque2* gene along with phenotypic selection for kernel modification in the background of an early maturing normal maize inbred line, V25 (Babu *et al*. 2005). The normal maize protein is of poor nutritional quality due to a deficiency in two essential amino acids (lysine and tryptophan) and high leucine–isoleucine ratio. The maize mutant *opaque2* has enhanced nutritional quality but very poor for yield and agronomic performance. Great efforts have been made by breeders world-wide to combine the yield and agronomic performance of the elite inbreds and the good quality of *opaque2.* Babu *et al*. (2005) achieved this goal by a two generation marker-based backcross breeding program. Foreground selection was conducted using the *opaque2* specific SSR marker, *umc1066*. Flanking markers *bnlg2160* and *bnlg1200* (4.2 and 3.8 cM from the *opaque2* locus) were used to identify recombinants to reduce the attached chromosome segment length. Whole genome background selection was conducted using 77 SSR markers spanning all the bin locations in a maize SSR consensus map. The tryptophan concentration in endosperm protein was significantly enhanced in all the three classes of kernel modification i.e., less than 25%, 25–50% and more than 50% opaqueness. BC_2F_3 lines developed from the hard endosperm kernels were evaluated for desirable agronomic and biochemical traits in replicated trials and the best line was chosen to represent the quality

protein maize (QPM) version of V25, with tryptophan concentration of 0.85% in protein. In addition to getting the converted line this study also demonstrated the use of the prediction equations proposed by Frisch *et al*. (1990a, 1990b) and Hospital *et al*. (1992) for the determination of optimum population size of BC generations. It also highlighted the importance of phenotypic selection among the lines with target genes for other agronomic traits. Recently, Neeraja *et al*. (2007) reported the successful introgression of a major QTL for rice submergence stress (*Sub1*) to widely grown cultivars. In this study, markers that were tightly linked with *Sub1*, flanking *Sub1*, and unlinked to *Sub1* were used to apply foreground, recombinant, and background selection, respectively.

Pyramiding major genes for disease/insect resistance

Gene pyramiding has long been used by breeders to develop cultivars with multiple insect or pathogen resistance genes. It is hoped that a variety with multiple resistance genes is more durable since it is unlikely for the insect or pathogen to overcome all the resistance genes simultaneously. Pyramiding multiple qualitative alleles in single genotypes may also be an approach to increasing the level of resistance relative to that conferred by a single qualitative resistance locus and multigenic qualitative resistance may also lead to greater durability. Experimental results are inconclusive with regard to the effectiveness of pyramiding major resistance genes. For instance, Klopper and Pretorius (1997) investigated the effects of wheat leaf rust gene combinations in lines *Lr13* + *Lr34* (T34-13), *Lr13* + *Lr37* (T13-37) and *Lr34* + *Lr37* (T34-37). They found higher levels of resistance in the combination lines T13-37 and T34-37 than in the lines with the individual genes. In the T34-13 line, no increased resistance to pathotype UVPrt13 was apparent from assessment of the infection types in the glasshouse. Precise measurements of its resistance components showed, however, that it had a longer latent period and smaller uredinia and its resistance was highly effective in the field. Significant restriction of fungal growth during early post-infection stages occurred in the gene combination lines T34–13, T13–37 and T34–37. Colony size in these lines was also significantly reduced compared with that in the single gene lines and the leaf rust-susceptible line, when either or both pathotypes possessed avirulence for one of the *Lr* genes. However, Porter *et al*. (2000) tested the effectiveness of several greenbug resistance genes and their combinations against biotypes E, F, G, H, and I and found that pyramiding provided no additional protection over that conferred by the single resistance genes. Nevertheless, the most successful application of gene pyramiding is found in breeding for pest and disease resistance.

A good example of pyramiding major resistance genes with the aid of molecular markers is the development of rice lines with bacterial blight (BB) resistance. So far, roughly 29 BB resistance genes have been identified. None are effective individually against all the pathotypes, though some of the genes such as *Xa4* confer resistance to many pathotypes. Some of these genes have been incorporated into modern rice varieties and used for development of near-isogenic lines. Cultivars with one or more BB resistance genes have been developed by conventional backcrossing methods and used in different rice growing regions. With MAS lines with multiple BB resistance genes have been successfully developed.

Yoshimura *et al*. (1995) developed restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers for four BB resistance genes. Using these linked markers they selected lines homozygous for pairs of resistance genes, *Xa4* +*xa5* and *Xa4* +*Xa10*. Lines carrying *Xa4* +*xa5* and *Xa4* +*Xa10* were evaluated for reaction to eight strains of the BB pathogen, representing eight pathotypes and three genetic lineages. It was found that the lines carrying pairs of genes were resistant to more of the isolates than their single-gene parental lines. Lines carrying *Xa4* +*xa5* were more resistant to isolates of race 4 than either of the parental lines, while no such effects were seen for *Xa4* +*Xa10*. Thus, combinations of resistance genes may provide broader spectra of resistance. Huang *et al*. (1997) developed lines containing up to four BB resistance genes using markers. Four isogenic lines and their recurrent parent IR24, and a line containing two BB resistance genes (*Xa4 and xa5*) developed by Yoshimura *et al*. (1995) were used as parents. Intermediate lines with two resistance genes were also used in late crossing to accumulate more genes. Lines with three or four genes were developed by crossing between two-gene lines and one-gene or two- gene lines. The pyramided lines having three or four genes in combination also showed an increased and wider spectrum of resistance to bacterial blight than those having a single resistance gene. A three-gene line, IRBB59 (with *xa5*, *xa13*, and *Xa21*), was used as donor to transfer the three BB resistance genes into three new plant type lines with high yield potential, IR65598-112 and the two sister lines IR65600-42 and IR65600-96 (Sanchez *et al*. 2000). Sequence tagged site (STS) markers for all the three resistance genes from the previous identified RFLP and RAPD markers, developed by Huang *et al*. (1997) and Sanchez *et al*. (2000), were used for foreground selection. F_1 plants were obtained between the donor and the three recurrent parents and were advanced up to the BC_3 generation by MAS. Starting from the BC_1F_1, and in each of the following BCF_1 generations, approximately 50 plants were genotyped. From these, plants carrying resistant alleles of the three target resistance genes (based on their marker genotypes) and that were phenotypically similar to the recurrent parents were selected as the parents for the next backcross until BC_3F_1. The selected BC_3F_1 plants for each of the recurrent parents were selfed to produce BC_3F_2 seed. Based on phenotypic similarity to their recurrent parents, BC_3F_2 plants were selected for homozygosity at the STS marker genotypes and phenotyped for their reactions to the six Xoo races. The BC_3F_3 NILs having more than one BB resistance gene showed a wider resistance spectrum and manifested increased levels of resistance to the Xoo races, as compared with those having a single BB resistance gene. The resultant plants had very high degree of similarity to their respective recurrent parents, suggesting that the phenotypic selection in every backcross generation was effective. The results of these studies clearly demonstrated the usefulness of MAS in gene pyramiding for BB resistance, particularly for recessive genes, such as *xa-5* and *xa13*, which are difficult to select through conventional breeding in the presence of a dominant gene such as *Xa-21*.

Similarly, Singh *et al*. (2001) transferred the same three BB resistance genes, *xa5*, *xa13* and *Xa21*, into the elite cultivar 'PR106', which is widely grown in Punjab, India. IRBB22 with all three genes in IR24 background was used as donor parent. Lines of PR106 with pyramided genes were evaluated after inoculation with 17 isolates of the pathogen from Punjab and six races of Xoo from Philippines. Genes in combination were found to provide high levels of resistance to the predominant Xoo isolates from Punjab and six races from Philippines. Lines of PR106 with two and three BB resistance genes were also evaluated under natural conditions at 31 sites in commercial fields. The combination of genes provided a wider spectrum of resistance to the pathogen population prevalent in the region. Only 1 of the BB isolates, PX04, was virulent on the line carrying *Xa21* but avirulent on the lines having *xa5* and *xa13* genes in combination with *Xa21*. However, the performance of the pyramided lines on other agronomic traits, particularly in comparison with the recurrent parent was not reported. One of the pyramided lines, named SS113, containing all the three genes, was used by Sundaram *et al*. (2008) as donor to introgress the resistance genes into cv. 'Samba Mahsuri' (BPT5204), which is a medium slender grain indica rice variety and very popular with farmers and consumers across India because of its high yield and excellent cooking quality.

At each backcross generation, markers closely linked to the three genes were used to select plants possessing these resistance genes (foreground selection) and microsatellite markers polymorphic between donor and recurrent parent were used to select plants that have maximum contribution from the recurrent parent genome (background selection). A selected BC_4F_1 plant was selfed to generate homozygous BC_4F_2 plants with different combinations of BB resistance genes. The three-gene pyramid and two-gene pyramid lines exhibited high levels of resistance against the BB pathogen. Under conditions of BB infection, the three-gene pyramid lines exhibited a significant yield advantage over 'Samba Mahsuri'. Multi-location testing demonstrated that these lines retain the excellent grain and cooking qualities of 'Samba Mahsuri' without compromising the yield. One of these lines has been recommended for release as a commercial variety by the variety identification committee of the Indian Council of Agricultural Research. This study demonstrated that background selection with a limited number of polymorphic microsatellite markers (50), in conjunction with four backcrosses is sufficient to recover the yield and quality characteristics of the recurrent parent, which was consistent with the theoretical and simulation results. The markers used for background selection did not include any that are tightly linked to the target genes since the donor line had good agronomic performance.

Joseph *et al*. (2004) screened 13 NILs of rice with different BB resistance genes and gene combinations against four isolates of the pathogen from Basmati regions of India and identified *Xa4*, *xa8*, *xa13* and *Xa21* as effective against all the isolates tested. Two or more of these genes in combination imparted enhanced resistance as expressed by reduced average lesion length in comparison to individual genes. The two-gene pyramid line IRBB55 carrying *xa13* and *Xa21* was found equally effective as three/four gene pyramid lines. IRBB55 was then used as donor parent to transfer *xa13* and *Xa21* into Pusa Basmati-1, the most popular high yielding variety. Recombinants having enhanced resistance to BB, Basmati quality and desirable agronomic traits were identified. Unlike studies outlined above, this study used only a single backcross generation and extensive selection for agronomic traits was conducted in three selfing generations to recover the genome of the elite parent.

Cereal cyst nematode (CCN), *Heterodera avenae*, is a significant pathogen of wheat. Nine genes, designated *Cre1* to *Cre8* and *CreR*, in both hexaploid wheat and its relatives have been identified as sources of resistance to CCN (McIntosh *et al*. 2003). Diagnostic markers for *Cre1* and *Cre8* have been developed and are being employed successfully to pyramid the two resistance genes in Australia (Eagles *et al*. 2001; Ogbonnaya *et al*. 2001a, 2001b). Barloy *et al*. (2007) pyramided the two resistance genes *CreX* and *CreY* identified in *Aegilops variabilis* Accession No. 1 into a wheat background through MAS. CCN bioassays with the Ha12 pathotype showed that the level of resistance of the pyramided line was significantly higher than that of *CreX* and *CreY* single introgression lines, but lower than that of *Ae. variabilis*. The *CreY* gene, carried by line X8, seemed to confer a higher level of resistance to the Ha12 pathotype than the *CreY* gene carried by line D. The differences in the number of cysts between the pyramided lines and *Ae. variabilis* may be due to the lower expression of *CreX* and *CreY* genes in the wheat background or the possibility that more than two genes are involved in CCN resistance in *Ae. variabilis*.

Liu *et al*. (2000) developed three lines with two of the three powdery mildew resistance genes in the background of elite wheat cultivar 'Yang158'. In this study, near-isogenic lines (NILs) were first developed using 'Yang158' as recurrent parent and lines with different resistance genes as donor parents. They were then used as parents for pyramiding genes. Pyramided lines with two of the three powdery mildew resistance gene combinations, *Pm2* + *Pm4a*, *Pm2* + *Pm21*, *Pm4a* + *Pm21* were obtained by MAS in the F_2 generation. The pyramided lines showed good uniformity in morphological and other non-resistance agronomic traits. Since more than four backcross generations and intensive selection for recurrent characteristics were conducted in the development of the NILs used as parents in pyramiding, the process of developing pyramided lines is simplified.

Hittalmani *et al*. (2000) developed a rice line with two or three major blight resistance genes (*Pi*1, *Piz*-5 and *Pi*ta) using three NILs and RFLP markers. Each of the NILs carries the major genes *Pi*1, *Piz*-5 and *Pi*ta, respectively, in the background of the susceptible recurrent parent CO39. Three single-pair crosses were made between the NILs, and F_2 plants homozygous for resistance genes were identified based on the parental banding pattern of RFLP probes. Individual plants identified as carrying homozygous resistance genes *Pi*1+*Piz*-5 and *Pi*1+*Pi*ta were further crossed with each other and F_2 plants with all the three genes were identified. The plants carrying the two- and three-gene combinations that were tested for resistance to leaf blast in the Philippines and India indicated that combinations including *Piz*-5 have enhanced resistance compared to when it is present alone.

Asian rice gall midge (*Orseolia oryzae*) is a major pest across much of South and Southeast Asia. Seven genes conditioning resistance to gall midge larvae have been identified in rice (*Oryza sativa*) and are being used in cultivar improvement programmes. However, some of these genes are rendered ineffective by new gall midge biotypes. The two genes, *Gm-2* and *Gm-6(t)*, linked with a distance of ~16.3 cM, are known to confer resistance against a number of biotypes in India and China, respectively. Katiyar *et al*. (2001) successfully developed lines homozygous for the favourable alleles at both loci by conventional host-pest interaction method.

Sharma *et al*. (2004) constructed a gene-pyramided *japonica* line, in which two Brown planthopper (BPH) (*Nilaparvata lugens* Stål) resistance genes *Bph1* and *Bph2* on the long arm of chromosome 12 independently derived from two *indica* resistance lines were combined. The parent line containing *Bph1* and the one containing *Bph2* were both homozygous elite introgression lines developed by transferring the resistance genes from *indica* donor cultivars. A single F_1 plant heterozygous for the linked markers was selected. MAS was continued in F_2, F_3 and F_4 populations and a homozygous recombinant line was obtained. BPH bioassay showed that the resistance level of the pyramided line was equivalent to that of the *Bph1*-single introgression line, which showed a higher level of resistance than the *Bph2*-single introgression line.

He *et al*. (2004) reported the development of the improved versions of the two parental lines of 'Shanyou 63', an elite hybrid rice cultivar widely grown in China ('Zhenshan 97' and 'Minghui 63') using MAS and genetic transformation. (1) Two wide-spectrum BB resistance genes, *Xa21* and *Xa7*, were incorporated into the restorer line 'Minghui 63'. (2) 'Minghui 63' was transformed with a *Bt* δ-endotoxin gene to improve the stem borer resistance. (3) *Xa21* and *Bt* genes were combined into a line with the 'Minghui 63' background. (4) Two genes, *Pi1* and *Pi2*, showing broad-spectrum resistance to fungi blast, were introgressed into Zhenshan 97. (5) Two genes, for brown planthopper resistance, *Qbph1* and *Qbph2*, were introgressed into 'Zhenshan 97'. These versions of improved lines are being combined in various ways to make new hybrids to meet the needs of rice production.

Using four generations of MAS backcross breeding Toojinda *et al*. (2004) were successful in combining gene and major QTL for BB resistance, submergence tolerance (SUB), brown planthopper resistance (BPH) and blast resistance (BL) into KDML105. Selected backcross lines, introgressed with target gene/QTL, were tolerant to SUB and resistant to BB, BPH and BL. The agronomic performance and grain quality of these lines were as good as or better than KDML105.

Barone *et al*. (2005) pyramided several resistance genes in the same tomato variety. Two NILs, each possessing at

least 3 resistant genes (Momor, which was resistant to *Tobacco mosaic virus*, *Verticillium dahliae* and *Fusarium oxysporum* f.sp. *radicis-lycopersici*, and Motelle, which was resistant to *V. dahliae*, F. o*xysporum* f.sp. *lycopersici*, *Stemphylium* sp. and *Meloidogyne incognita*), were intercrossed and selfed for many generations. Selection for resistance was performed using molecular markers linked to the genes to fix them at the homozygous level in the same genotype. After the F_4 generation, various genotypes which carried all resistance genes at homozygous condition were obtained.

Shi *et al*. (2006) pyramided *Rsv*1, *Rsv*3, and *Rsv*4 for *Soybean mosaic virus* (SMV) resistance. A population of 84 lines derived from J05 (*Rsv*1, *Rsv*3) x V94-5152 (*Rsv*4) were developed, and six specific SSR markers were identified for SMV resistance genes. Two SSR markers Sat_154 and Satt510 were used for selecting lines having the *Rsv*1 gene, Satt560 and Satt726 for *Rsv*3, and Sat_254 and Satt542 for *Rsv*4. These SSR markers allowed for identification and selection of specific lines and individual plants containing different genes and for distinction of the homozygous and heterozygous lines or individual plants for all three resistance loci. Individual plants with homozygous alleles at three genetic loci (*Rsv*1*Rsv*1, *Rsv*3*Rsv*3 and *Rsv*4*Rsv*4) have been identified and the release of new soybean germplasm with three genes combined for SMV resistance is anticipated.

Werner *et al*. (2005, 2007) reported the pyramiding of three resistance genes for barley yellow mosaic virus. They compared the efficiencies of two pyramiding schemes, which were different in the use of DH.

Lee and Neate (2007) identified five RAPD markers, two in coupling ($OPAH5_{545C}$, and $OPBA12_{314C}$) and three in repulsion phase ($UBC285_{158R}$, $OPC2_{441R}$, and $OPB17_{451R}$), closely linked to *Rsp* genes conferring resistance to barley Septoria speckled leaf blotch (SSLB) using bulked segregant analysis in three F_2 populations, each containing a *Rsp* gene. These markers were then converted into the sequence tagged site (STS) markers SUBC285, SOPC2, SOPAH5, and SOPBA12 and used in MAS. The STS markers closely linked to *Rsp* genes also identified the SSLB resistance corresponding to *Rsp*1, *Rsp*2, or *Rsp*3 in gene pyramiding F_2 populations.

Pyramiding QTL

Most of the agronomic traits such as yield are quantitatively inherited. Manipulating these traits is difficult because of their intrinsic complexities: polygenic control, epistasis, and gene-by-environment interaction (G × E). Since QTL with major effects are easily manipulated by empirical breeding practices and may already be fixed in many breeding lines, it would be more productive to use marker technology as a means for placing greater emphasis on those QTL that show only relative minor effects (Stuber *et al*. 1999). When the effects of QTL are of small several QTL have to be manipulated simultaneously to achieve significant improvement. As discussed in the first part of this review (Ye and Smith 2008) this negatively affects the success and efficiency of a gene-pyramiding programs. Reported applications of QTL pyramiding for the improvement of quantitative traits are few, although introgression of multiple QTL have been used extensively for the validation of previous mapped QTL.

Toojinda *et al*. (1988) successfully introgressed two QTL for stripe rust resistance in barley into a genetic background different from the one used to map the QTL. The effects of both QTL were confirmed and additional QTL were detected in the new background, including some resistance alleles brought in by the susceptible parent.

Stuber and Sisco (1992) and Stuber *et al*. (1999) reported increased grain yield in maize by introgression using six parents containing favourable chromosome segments. Three backcross generations (two marker-facilitated) were used for the transfer of subsets of the identified chromosomal segments into the target lines, B73 and Mo17. This was followed by two generations of marker-facilitated selfing to fix the introgressed segments. However, all six target segments were not obtained in any given line. The "enhanced" lines were then crossed in appropriate combinations and the "enhanced" single crosses were evaluated in replicated yield tests. On the basis of 4 years of testing, yields of the best "enhanced" B73 × "enhanced" Mo17 hybrids exceeded the original B73 × Mo17 hybrid and high yielding commercial hybrids by 8 to 10% (628–1004 kg ha^{-1}). They found that there appears to be some indication that there may no advantage in transferring more than two to four segments. In fact, there is some indication that there could be a disadvantage. They offered several explanations for this observation: first increasing the number of transferred segments may be replacing the recipient genome with an excessive amount of linked donor chromosomal segments that could cause a deleterious effect. Second, epistatic interactions between a larger number of introgressed segments may result in a negative effect. Third, favourable epistatic complexes in coupling phase (e.g., between recurrent parent alleles) could be disrupted.

Four target chromosomal regions containing five QTL for pest resistance (ascysugar accumulation) were successfully introgressed from wild tomato into cultivated tomato (Lawson *et al*. 1997). However, the level of ascysugar accumulation resistance in the progeny introgressed for the five QTL was lower than expected and was also lower than the interspecific F_1 hybrid.

Sebolt *et al*. (2000) performed marker-assisted introgression of two QTL for seed protein concentration identified in *Glycine soja* accessions in cultivated soybean. Only one QTL was confirmed and the other QTL might have been lost during the backcross. When the confirmed QTL was transferred in three different backgrounds it had no effect in one background. Chee *et al*. (2001) transferred a QTL for grain protein concentration (GPC) in emmer wheat into an adapted durum wheat background. An inbred line with high GPC and other desirable agronomic characters selected from the recombinant inbred lines derived from LDN(DIC-6B)/VIC population was crossed to 'Renville', a good quality, high yielding durum cultivar. Recombinant inbred lines were developed by single seed descent and used to confirm the presence and location of this QTL.

Reyna and Sneller (2001) tested the effects of three beneficial yield QTL identified from the northern soybean cultivar 'Archer' in Southern background and testing environments. Four sets of NILs for each QTL were derived from heterozygous F_6 plants identified from the crosses of Archer × Asgrow A5403 and Archer × Pioneer 9641. None of the marker effects were significant for any of the three QTL for yield, height, and maturity, when averaged over all sets or for individual sets. Similarly, in barley Kandemir *et al*. (2000) evaluated the effects of three previously identified grain yield QTL on chromosomes 2S (2HS), 3C (3HC) and 5L (1HL) for their potential to increase yields of high-quality malting barley without disturbing their favourable malting quality profile. NILs were developed by introgressing QTL from the high-yielding cv. 'Steptoe' to the superior malting quality, moderate-yielding cv. 'Morex'. None of the 3 QTL studied altered the measured yield of the recipient genotype, per se, although QTL 2S and QTL-3 affected yield-related traits. However, QTL for plant height, head shattering, seed weight and number of rachis nodes/spike were detected in the QTL-3C region.

Ahmaid *et al*. (2001) introgressed two QTL for resistance to rice yellow mottle virus identified in a highland cultivar into a lowland rice cultivar. In total three backcross and three selfing generations were used. The donor was a double haploid (DH) line selected from the original mapping population. One marker per QTL was used for selecting the QTL. Background selection was conducted using markers on the chromosomes without the target QTL for the recovery of recurrent genome in BC_1 and BC_3 generations. Phenotypic screening for resistance segregation in the selfing generation after selection in the backcrossing generation was used to ensure the QTL were not lost.

Shen *et al*. (2001) developed near-iosgenic lines (NILs) containing QTL associated with rice root traits on rice chromosomes 1, 2, 7, and 9 (designated as targets 1, 2, 7, and 9) identified in previous mapping studies. The donor parents were 4 doubled haploid lines that had the desirable alleles at the target QTL and >50% of the recipient (IR64) genome. Several BC_3F_3 lines with one or two QTL were obtained by MAS. Among the four QTL, one exhibited the expected effect in the progeny, one was finally revealed as a false positive, one segment was shown to contain two QTL in repulsion phase that reduced its effect and one segment did not exhibit the expected effect. They also found the association of the NILs with some non-target traits. For instance, increased height and reduced tiller number per plant were detected for two of the three target-1 NILs. Three of the five target-2 NILs had increased height and reduced tiller number. Most target-7 NILs had significantly increased height; some of them had either more or less tillers. All target-9 NILs had significantly reduced tiller number. Three of the four NILs with introgressed targets 1 and 7 QTLs were significantly taller than IR64.

Yousef and Juvik (2002) successfully selected on three markers linked to QTL that enhanced seedling emergence in sweet corn. Three RFLP marker alleles linked to QTL that enhanced seedling emergence identified in an $F_{2:3}$ sweet corn mapping population were used to transfer these QTL into three elite commercial sweet corn inbreds. A recombinant inbred line derived from the original mapping population was used as a donor parent. The introgressed QTL alleles were observed to enhance seedling emergence in the BC_2F_1 generation as was observed in the original $F_{2:3}$ mapping population. In this study, a combination of QTL linked to umc139 and php200689 markers resulted in the highest seedling emergence compared with other combinations including all three of the beneficial marker-QTL alleles together.

Bouchez *et al*. (2002) reported the marker-assisted introgression of favourable alleles at three quantitative trait loci (QTL) for earliness and grain yield among maize elite lines. Introgression started from a selected RIL, which was crossed three times to one of the original parents and then self-fertilized, leading to BC_3S_1 progeny. Markers were used to assist both foreground and background selection at each generation. The marker-assisted introgression proved successful at the genotypic level. Also, QTL positions were generally sustained in the introgression background. For earliness, the magnitude and sign of the QTL effects were in good agreement with those expected from initial RIL analyses. Conversely, for yield, important discrepancies were observed in the magnitude and sign of the QTL effects observed after introgression. One high-yielding allele putatively detected from the low-yielding parent exhibited an negative effect on yield.

Castro *et al*. (2003a, 2003b) developed double haploid (DH) lines combining two barley strip rust (BSR) resistance QTL alleles from the accession "Calicuchima-sib" on chromosomes 4 (4H) and 7 (5H) (QTL4 and QTL7), and a BSR resistance QTL allele from cv. 'Shyri' on chromosome 5 (1H) (QTL5). Results on seedling resistance validated the effects and locations of QTL4 and QTL5, but the QTL7 did not have a significant effect on disease symptom expression. The presence of resistance alleles at both loci substantially increased the probability of recovering the resistant phenotype. In mapping populations, a resistance allele at a QTL on chromosome 6(6H) was necessary for resistance, in conjunction with a resistance allele at either QTL4 or QTL5. In the DH lines studied in this experiment, resistance alleles were also necessary at two QTL, but the two QTL are on chromosomes 4(4H) and 5(1H). In other words, an allele at QTL4 or QTL5 can substitute for an allele on chromosome 6(6H). Results on adult resistance validated the effects of resistance alleles at all the three QTL regions on disease severity and area under disease progress curve and these QTL explain 94% of the genetic variation of the trait expression. A comparison of QTL effects as estimated in the source mapping populations and in the derived lines reveals changes in magnitude of effect.

Using two doubled-haploid (DH) experimental lines (BCD47 containing resistance alleles at the QTL on chromosomes 4H and 5H and BCD12 on 1H), developed by Castro *et al*. (2003a), as donors of the resistance alleles, Richardson *et al*. (2006) developed a set of QTL introgression lines in a susceptible background. These disease resistance QTL was combined in one-, two-, and three-way combinations in a susceptible background. These lines were used to measure four components of disease resistance: latent period, infection efficiency, lesion size, and pustule density. Pyramiding multiple QTL alleles led to higher levels of resistance in terms of all components of quantitative resistance except latent period. There were linear reductions in infection efficiency, lesion size, and pustule density as more resistance alleles were added to individual genotypes, but resistance pyramiding did not increase latent period. The introgression of the same resistance alleles at the same QTL into different lines did not always lead to the same resistance. There was more variance among lines within the quantitative resistance allele introgression classes and no variance among lines within the qualitative resistance gene introgression group. Pyramids of multiple resistance QTL alleles where the 4H QTL was present led to lower infection efficiency, lesion size, and pustule density.

Gur and Zamir (2004) demonstrated that tomato yield can be increased dramatically by pyramiding three independent yield-promoting genomic regions from the drought-tolerant *Lycopersicon pennellii*. The yields of hybrids that were parented by the pyramided genotypes were more than 50% higher than that of a control market-leader variety under both wet and dry field conditions.

Lecomte *et al*. (2004) introgressed five chromosome regions strongly involved in organoleptic quality attributes of tomato were introgressed into three different recipient lines through marker-assisted selection. All the favourable alleles for quality traits were provided by the same parental tomato line. Three improved lines were obtained after three backcrossing and two selfing generations. Breeding efficiency strongly varied according to the recipient parent, and significant interactions between QTL and genetic backgrounds were shown for all of the traits studied. About 50% of the QTL were confirmed in each background and new QTL were detected. The QTL with the largest effect were the most stable.

Thabuis *et al*. (2004) successfully pyramided the favourable alleles of four QTL for resistance to *Phytophthora capsici*, which was identified in a small-fruited pepper line by three cycles of marker-assisted backcrossing using a bell pepper line as recipient. A DH line selected from the original mapping population was used as donor. Two populations, derived by selfing the plants selected after the first selection cycle, were genotyped and evaluated phenotypically for their resistance level. The additive and epistatic effects of the four resistance factors were re-detected and validated in these populations. A decrease of the effect for the moderate-effect QTL and of the epistatic interaction was observed.

Ashikari *et al*. (2005) identified major QTL for grain number (*Gn1*) and QTL for plant height (*Ph1*) using the progeny from the cross between the *japonica* rice 'Koshihikari' and the *indica* rice 'Habataki'. NILs in the Koshihikari' genetic background were developed and used to combine both beneficial traits. Two lines were crossed and a pyramiding line carrying *Gn1* and *Ph1(sd1)* was selected from the progeny using MAS. The pyramided line showed increased grain production (23%) and reduced plant height (20%) compared with 'Koshihikai'.

Steele *et al*. (2006) conducted a marker-assisted backcrossing breeding program to improve the root morphological traits, and thereby drought tolerance, of the Indian upland rice variety, 'Kalinga III', which had not previously been used for QTL mapping. The donor parent was Azucena, an upland *japonica* variety from Philippines. Five

segments on different chromosomes were targeted for introgression; four segments carried QTL for improved root morphological traits (root length and thickness) and the fifth carried a recessive QTL for aroma. Two crosses between BC_3 lines were used to stack the five targets. The target segment on chromosome 9 significantly increased root length under both irrigated and drought stress treatments, confirming that this root length QTL from Azucena functions in a novel genetic background. No significant effects on root length were found at the other four targets. Azucena alleles at the locus RM248 delayed flowering. Selection for the recurrent parent allele at this locus produced early-flowering NILs that were suited for upland environments in eastern India.

Ribaut and Ragot (2006) reported the results of a marker-assisted backcross (MABC) selection experiment aimed at improving grain yield under drought conditions in tropical maize. The introgression increased grain yield and reduced the asynchrony between male and female flowering under water-limited conditions. Eighty-five per cent of the recurrent parent's genotype at non-target loci was recovered in only four generations of backcross by screening large segregating populations (2200 individuals) for three of the four generations. Selected MABC-derived BC_2F_3 families were crossed with two testers and evaluated under different water regimes. Mean grain yield of MABC-derived hybrids was consistently higher than that of control hybrids (crosses from the recurrent parent to the same two testers as the MABC-derived families) under severe water stress conditions. Under those conditions, the best five MABC-derived hybrids yielded, on average, at least 50% more than control hybrids. Under mild water stress (defined as resulting in <50% yield reduction), no difference was observed between MABC-derived hybrids and the control plants.

The combined use of high-throughput genotypying and marker-assisted backcross was adopted by Bai *et al*. (2007) to transfer the major Fusarium head blight (FHB) QTL from Sumai 3 and its derivatives into locally adapted hard winter wheat with minor FHB-resistance QTL. Three crosses were made between Sumai 3 derived soft red wheat lines and three locally adapted hard winter wheat cultivars ('Harding', 'Wesley' and 'Trego'). About 80 BC_2F_2 plants homozygous for the 3BS QTL were selected from each backcross population based on closely linked markers. BC_2F_3 lines were evaluated in greenhouse for Type II resistance and 135 highly resistant and 87 moderately resistant lines were identified. These materials have the potential to develop marketable FHB resistant HWW cultivars and useful germplasm lines. Wilde *et al*. (2007) demonstrated that both phenotypic selection and MAS were effective for pyramiding three QTL for wheat FBH. MAS was faster and cheaper than phenotypic selection but had the risk of missing the QTL with small effect. They suggested that a phenotypic selection in the field should follow marker-based selection to bail out genetic variance that is caused by resistance genes that have so far been undetected in QTL-mapping studies by enhancing selection gain.

Pyramiding by transgenic approach

In addition to sexual hybridization, genetic transformation can be used to incorporate extraneous genes. The main advantages of genetic transformation are (1) the sources of genes to be transferred are much wider than sexual hybridization, and (2) the possibility of undesirable linkage drag is avoided. In the context of gene pyramiding, genetic transformation can be explored in two ways. Firstly, stable transgenic lines can be used as parental lines in the conventional pyramiding program. Secondly, co-transformation or repeated transformation can be used to pyramid multiple genes. Since the development of stable transformats with multiple genes is more difficult to obtain and the genotypes amenable to transformation may not be those to be improved, the first option is preferable. Co-transformation has been used to achieve the pyramiding of *Xa21* and *gna* (*Galanthus nivalis* agglutinin) genes in rice (Tang *et al*. 1999). Conventional crossing of two independent transgenic homozygous rice lines by Datta *et al*. (2002) pyramided *Xa21* gene, a chitinase gene, and a *Bt*-fusion gene into IR72 to confer multiple resistances against BB, Sheath Blight (ShB), and yellow stem borer. Kim *et al*. (2003) pyramided a maize ribosome-inactivating protein gene and a rice basic chitinase gene and the ShB resistance of the transgenic rice lines was enhanced. To obtain rice lines with multiple resistance against blast and blight Narayanan *et al*. (2004) developed lines with two major genes for blast (*Magnaporthe grisea*) resistance using MAS and NILs, and then transformed these blast-resistant pyramids with the cloned BB resistance gene *Xa21*. Bioassays with six independent transformants showed that transgenic CO39 plants were resistant to both pathogens, *M. grisea* and *Xoo*. Kalpana *et al*. (2006) showed co-expression of transgenic rice chitinase and thaumatin-like protein (TLP) in an elite *indica* rice line, which showed a significantly higher level of ShB resistance than the chitinase or TLP transformants. To pyramid SB and BB resistance Maruthasalam *et al*. (2007) co-transformed elite *indica* rice cultivars with genes expressing a rice chitinase (*chi11*) and a thaumatin-like protein (*tlp*) conferring resistance to fungal pathogens and a serine-threonine kinase (*Xa21*) conferring BB resistance through particle bombardment. Stable integration and expression of the transgenes in a few independent transgenic lines were obtained. Progeny analyses showed the stable inheritance of transgenes to their progeny. Co-expression of chitinase and thaumatin-like protein in the progeny of a transgenic Pusa Basmati1 line revealed an enhanced resistance to the SB pathogen, *Rhizoctonia solani*, as compared to that in the lines expressing the individual genes. A transgenic Pusa Basmati1 line pyramided with *chi11*, *tlp*, and *Xa21* showed an enhanced resistance to both SB and BB. Wei *et al*. (2008) described the breeding of a transgenic rice restorer line for multiple resistance against bacterial blight, striped stem borer (SSB) (*Chilo suppressalis*) and herbicide. Two stable transgenic rice lines, Zhongguo91 containing *cry1Ab* gene (for insect resistance) and *bar* gene (for tolerance of herbicide), and Yujing6 containing *Xa21* gene for BB resistance, were developed by PIG gene gun bombardment and *Agrobacterium*-mediated transformation, respectively. Two elite restorers, T773-1 expressing *cry1Ab* and *bar* genes and T773-2 expressing *Xa21* gene, were obtained by five successive backcrossing to the elite restorer line Hui773. The transgenic restorer line T773 with good agronomic traits and obvious multiple resistance to BB, SSB and herbicide were then developed by selection in four selfing generations of the cross between T773-1 and T773-2. Bioassay, selectable marker and reporter genes were used in the selection process. The hybrid (F_1 generation) produced from the cross between T773 and a corresponding male sterile line Zaohua2A maintained resistance to BB, leaffolder and SSB, and showed significant heterosis.

PERSPECTIVES

For traits for which there is convincing and unequivocal evidence for the presence of two or more additive or complementary genes, pyramiding these genes into a common genotype would help maximise the character expression or gains from selection. Pyramiding such genes could also broaden the genetic basis of cultivars. Pest resistance conferred by single or few genes are the most rewarding area of gene pyramiding application. To pyramid multiple resistance genes into a single cultivar, breeders must be able to monitor the effects of these genes, which is not always possible through phenotypic measurements since the effect of one gene may be affected by the present of other genes due to epistasis and/or the masking effect. The use of MAS makes it possible to identify plants with various numbers of resistance genes with very similar resistance performance. It also makes it possible to select for recessive genes without progeny testing. Therefore, marker-assisted pyramiding

is being actively used in breeding for qualitative resistance and is proven fruitful.

When QTL are to be pyramided, several difficulties arise. It is more difficult to select for the presence of QTL since QTL location is estimated with only a given precision (Visscher *et al.* 1996). This requires using more markers and optimizing the positions of these markers with respect to the uncertainty of the true QTL location (Hospital and Charcosset 1997). Once the introgression is achieved, it must be checked that the effect of the QTL in the new genetic background is the same as the effect estimated originally. It is unlikely that one single QTL for a quantitative trait could explain enough genetic variation to justify the economic effort corresponding to the marker-assisted introgression program. Several QTL should be introgressed simultaneously. This necessitates using larger population sizes of foreground selection and reduces the possibilities of background selection. Both theoretical and experimental studies showed that the introgression of up to five chromosome regions using linked markers was feasible (Hospital and Charcosset 1997; Koudande *et al.* 2000).

The pyramiding of QTL has been less successful in terms of achieving expected improvement. The possible reasons for the unexpected results of QTL introgression experiments suggested by Hospital (2005) among others are (1) the putative QTL is false positive; (2) QTL expression is testing environment specific (QTL-by-environment interaction (QEI); (3) QTL are interacting among each other or with genetic background effect (epistasis); and (4) The chromosomal segments detected as QTL hold not just one but several genes. QTL with small effects are more likely to be false positive. Therefore, pyramiding should target QTL with relatively large effects. The false positive rate in QTL identification should be kept low so that resources are not wasted in introgressing false QTL (Bernardo 2004). Genotype-by-environment interaction (GEI) is a well known phenomenon of quantitative traits and thus it is not surprising that QTL underlying these traits are also sensitive to testing environments. QTL-by-environment interaction is well documented for many traits in many crop species. For gene pyramiding to be effective it should aimed at QTL with good stability across the target population of environments. The interactions between QTL and between QTL and genetic background are more difficult to handle. Given that the effects of the genetic backgrounds on the trait of interest could vary independently of the introgressed regions it is necessary to introgress QTL in several recipient lines. If QTL are not precisely mapped, large regions of donor chromosomes can be transferred. This has at least two possible consequences. Firstly, the chromosomal segments transferred hold not just one but several genes. Recombination between those genes would then simply modify the effect of the introduced segments. There are many examples where fine-mapping of the detected QTL results in the establishment of more than two genes (Eshed and Zamir 1995; Monna *et al*. 2002; Steinmetz *et al*. 2002; Christian and Keightley 2004). Secondly, unfavourable linkage drag may be caused by the unintentional introduction of undesirable alleles. Therefore, QTL should to be precisely mapped before starting the introgression. The use of NILs for QTL confirmation and pyramiding is recommended.

Techniques exist for the delivery of isolated or modified single genes into almost all cultivated species by genetic transformation. Co-transformation techniques have also been well established for some crop species. Developments in molecular biology allowed the cloning of specific genes to be used for transformation as well as the control of their expression. The ability to isolate and transfer desirable genes eliminates the issue of retention of unwanted and genetically linked traits, an important problem associated with classical gene introgression/pyramiding. It is anticipated that transgenic lines with various desirable gene(s), which are not readily available for crossing based pyramiding, will become important parental materials for enhancing various attributes of plant varieties through gene pyramiding and other breeding methods.

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