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Review

A Review on crop improvement through marker-assisted recurrent backcrossing

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ABSTRACT

The combined use of marker-assisted selection (MAS) and recurrent backcrossing is a versatile method of plant breeding and is known as marker-assisted recurrent backcrossing (MARB). Markers are used during recurrent backcrossing to select for the presence of target gene (foreground selection), to select against donor genome contribution (background selection) and to reduce the introgressed segment size and thus linkage drag. MARB reduces the number of backcrossing for recurrent recovery by 3-4 generations if one or two genes are transferred. For foreground selection markers are most useful for traits that are expensive and/or difficult to measure. Linkage drag when present is difficult if not impossible to remove by phenotypic selection. The benefits of MARB were well demonstrated by theoretical and simulation studies, and confirmed by empirical applications. MARB has also been used in generating genetic materials for genetic studies of complex traits. Introgression lines (ILs) have many advantages for QTL mapping compared to other population types such as recombinant inbred lines (RILs) and double haploids (DH). Near isogenic lines (NILs) are commonly used in further study of identified quantitative trait loci (QTL). MARB also offers the possibility of fine mapping QTL by breaking down the QTL-containing segment into smaller pieces.

Key Words: *Background selection, Breeding, Foreground selection, Linkage drag, Marker assisted selection, Recurrent backcrossing.*

INTRODUCTION

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 desired characteristics are selected and then crossed to one of the parents in more than one generation (Figure 1). The parental line carrying the target gene is usually called the donor parent, while the one used as a parent in all the backcrossing generations is called as recipient or recurrent parent. An important objective of recurrent backcrossing is to reduce the contribution of the donor genome. Traditionally, recurrent backcrossing is mainly used to improve qualitatively inherited traits such as disease and insect resistance, since the presence of target trait genes must be confirmed by phenotyping mostly at the individual level and individual phenotypic performance is a good indicator of the genotype only if genes have a major effect on phenotypic performance and the error of phenotyping is minimal.

The development of modern plant molecular and quantitative genetics in the last two decades has the potential to revolutionise what has mostly been experienced-based empirical plant breeding. It provides enhanced knowledge of the genetics of the breeding traits and of the relative genomic location of functionally related as well as neutral markers associated with the genes responsible for the traits.

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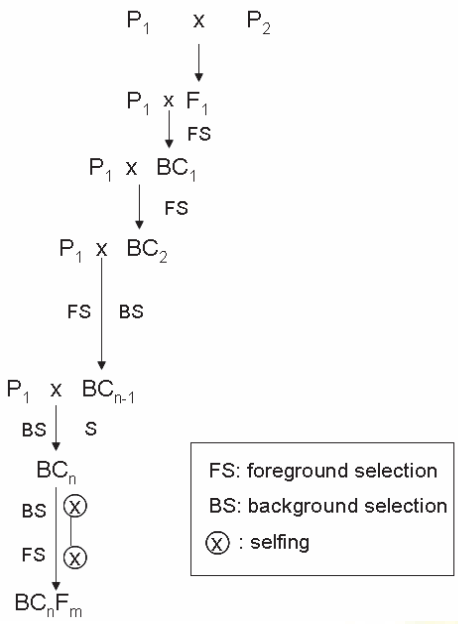

Figure 1 An example recurrent backcrossing scheme with P1 and P2 as the recurrent and donor parent, respectively.

It has dramatically widened the applicability of recurrent backcrossing at least in the following aspects. Firstly, for traits that are simply inherited, but that are difficult or expensive to measure phenotypically, and/or that do not have a consistent phenotypic expression under certain specific selection conditions, marker-based selection is more effective and /or economic than phenotypic selection (Paterson *et al* 1991, Stuber *et al* 1999, Dekkers and Hospital 2002, Dubcovsky 2004, Frisch and Melchinger 2005, Kuchel *et al* 2005). Secondly, traits which are traditionally regarded as quantitative and not targeted by recurrent backcrossing program can be improved using recurrent backcrossing if major QTL affecting the trait are identified. Thirdly, markers provide a more effective option to control linkage drag and speed up the recovery of recurrent genome and make the use of genes contained in unadapted resources easier. MARB now is the method of choice for inbred line development targeted at improving traits controlled by major genes. MARB has also been used in generating genetic materials for genetic studies of complex traits. For instance, near isogenic lines (NILs) are commonly used in further study of identified quantitative trait loci (QTL), while population of introgression lines (ILs) have many advantages for QTL mapping compared to other population types such as recombinant inbred lines (RILs), double haploids (DH) and F_2. In this review we summarised the theoretical and simulation results of MARB, provided comprehensive summary of the use of MARB in practical breeding and the dissection of genetic basis of complex traits, and discussed the prospective of MARB.

Marker-Assisted Foreground Selection (MAFS)

MAFS was proposed by Tanksley (1983). The resence 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 for manipulation of oligogenic traits under numerous situations in plant breeding (Melchinger 1990), but also for manipulation of QTL (Stuber 1995). Melchinger (1990) presented an a priori approach for calculating the minimum number of individuals and family size required in recurrent backcrossing. MAFS 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 kept or discarded (Allard 1999). This is not only costly but time-consuming as well. In MAFS the presence of the target gene needs to be tested only at the end of the breeding program. As another example, breeders identify a new resistant gene and would like to transfer it into an existing resistant cultivar containing other resistance genes to increase the durability. In this case, the individuals with resistant phenotype do not indicate the presence of the target resistant 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 fruitful although its

potential as a strategy for the development of cultivars with durable resistance has long been recognized. If a breeder wants to transfer a gene conferring resistance to a devastating disease which is not yet present in his/her testing environments just to prepare for the future or increase the adoptability of his/her cultivars by growers in areas with the disease. He/she 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. MAFS will also be advantageous if phenotypic assays are more expensive than marker assays. For instance, the bioassays for measuring resistance to nematodes in soybean (Young 1999), tomato (Tanksley 1983) and wheat (Eagles *et al* 2001) are expensive 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. The efficiency MAFS will decrease substantially if the markers are not perfectly or tightly linked with useful trait genes. Association of a marker with a trait allele and consequently the reliability of the MAS decreases with increasing cycles of meioses. With a recombination value of r between a marker and trait allele, the probability of this linkage being maintained unbroken across m cycles of meiosis is equal to (1 - r)m. To keep this probability higher than a certain critical value, say P, m must not exceed lnP/ ln(1 - r), suggesting that a phenotypic test should be performed every m generations of selection to confirm the persistence of the initial linkage. Put in another way, the probability of losing the target allele by recombination is 1 - (1- *r*)*t*. For example, if the marker locus exhibits 10% recombination with the target gene, there is a 10% chance of losing the target allele each generation, and a 27% chance of losing the target allele after three generations of meiosis. However, if the recombination frequency is 1%, there is only a 3% chance of losing the target allele after three generations of meiosis. When tightly linked markers are not available, election on a pair of markers flanking the target locus can be very effective. If two marker loci M_1 and M_2 flank the target locus, one would select progeny that have both M_1 and M_2 alleles. The probability of losing the target allele with flanking marker selection is equal to the probability of selecting a double recombinant progeny from among the doubly heterozygous backcross progeny. If the flanking loci have recombination frequencies r_1 and r_1, respectively, with the target locus, the probability of losing the target allele due to double crossovers within the selected region is $\frac{r1r2}{1-r1-r2+2r1r2}$. This probability can be much lower than the probability of losing the target allele based on selection for a single marker. For example, if the flanking markers each have 10% recombination frequency with the target locus, there is only a 1.2% chance of losing the target allele after a single generation. In any case, with tighter linkage, the chance of losing the target allele is reduced. However, this requires more plants to be tested and higher cost per plant. It is imperative to explore markers that are tightly linked with trait genes. Multiple marker loci closely linked to the target gene, permits discrimination on the basis of the haplotype of several markers rather than just the genotype at one marker. For example, Cregan *et al* (1999) developed two SSR markers tightly linked to the *rhg1* gene. Neither marker alone could distinguish all resistant from all susceptible genotypes, because of identical in state alleles shared by some resistant and susceptible lines, but the two markers together could discriminate almost all resistant and susceptible lines. One resistant cultivar carried the susceptible allele at both loci, presumably due to recombination between marker and resistance loci during line development. Thus, recombination can change the linkage phase between markers, but if MAS is used first to select putatively resistant lines, followed by phenotypic evaluation of resistance, the linkage phase will remain intact in all selected progeny. Therefore, MAS can be self-reinforcing, ensuring that the same set of markers will be effective in future crosses.

Marker-assisted background selection: It is well-known that if selection is applied for the desired characteristics only (foreground selection), 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. 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. Marker-assisted background selection (MABS) was proposed by Young and Tanksley (1989) to accelerate recovery of the recurrent parent genome (RPG). In MABS, 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* 1999 a & b). Tanksley *et al* (1989) stated that a sufficiently high proportion of the RPG is recovered after three generations of MABS. Hospital *et al* (1992) expected 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 saving of three backcross generations due to MABS is also a realistic goal for simultaneous introgression of two genes. Several useful points for practical breeders from these theoretical and simulation studies are (1) Four independent 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 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.

Linkage Drag: As discussed above, 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. This may result in the phenomenon known as linkage drag, that is, a negative trait is closely associated with the introgression of the target gene. 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. 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 cM of the segment attached to a resistance gene after six backcross generations in tomato. Tightly linked markers flanking the target gene can be used to reduce the length of donor chromosome segment attached to the target gene and potentially 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 arget 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. 2) When the distance between the flanking markers are short (<20 cM), the number of backcross generations performed has little impact on the reduction of donor segment length. 3) 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. 4) 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 probabilities 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 one the other side. Allowing more than two generations permits an even further reduction of the total number of individuals needed.

Foreground selection 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 (MAS) in a backcross generation are (1) select individuals carrying the target allele. Perfect markers or the closest flanking markers are most useful. (2) select individuals homozygous for recurrent parent genotype at loci close the target gene or markers linked to it, (3) select individuals homozygous for recurrent parent genotype at few (i.e. 2) marker loci on the chromosome carrying the target allele, and (4) select 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 in some cases while no obvious linkage drag was caused by a fair long segment in other cases. Therefore, it might be easier if we 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 is proven significant. This strategy will avoid spending time and resources in reducing/eliminating 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 predict. Decoux and Hospital (2002) 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.

Introgression of a Single Major Gene: Ragot *et al* (1994) provided a good example of gene introgression using marker-assisted selection for foreground selection. By using marker to monitor the Bt 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 is inserted into the genome of the transgenic line by genetic transformation and linkage drag is not of concern and the marker used for foreground selection is in the transgenic construct (no recombination). Pelemand and van der Voort (2003) provided a comprehensive example of the removal of undesirable linkage drag by MAS, the development of 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 round 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 bearing recombination events very close to each side of the gene was identified, 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 confers wide spectrum resistance to BB into ‘Minghui 63’. The MASsystem is consisted of a marker that is a part of *Xa21*, a marker located at 0.8 centimorgans (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 other agronomic performance. Great efforts have been made by breeders world-wide to combine the yield and other 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 recombinant 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 ie., 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* (1990 a & b) and Hospital *et al* (1992) for the determination of optimum population size of backcross generations. It also highlighted the importance of phenotypic selection among the lines with target genes for other agronomic traits.

Introgression of two or more major gene: The development of rice lines with bacterial blight (BB) resistance is a good example of pyramiding major resistance genes with the aid of molecular markers. 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 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 *Xa4*/*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 fromthe 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 BC3F3 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 MARB in pyramiding genes for BB resistance, particularly for recessive genes, such as *xa-5* and *xa13*, that 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 introgressed genes were evaluated after inoculation with 17 isolates of the pathogen from the Punjab and six races of Xoo from the Philippines. Genes in combination were found to provide high levels of resistance to the predominant Xoo isolates from the Punjab and six races from the 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 contained all the three genes, named as SS113, was used by Sundaram *et al* (2007) as donor

to introgress the resistance genes into cultivar '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 consistence with the theoretical and simulation results. The markers used for background selection did not include these 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 the 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 rice 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 were conducted in three selfing generations to recover the genome of the elite parent. Using a four generation of MAS backcross breeding Toojinda *et al* (2003) were successful in transferring genes 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, or resistant to BB, BPH or BL. The agronomic performance and grain quality of these lines were as good as or better than KDML105.

Introgression of 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 x 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. Reported applications of QTL introgression for the improvement of quantitative traits are few, although introgression of multiple QTL has 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 (1992, 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 markerfacilitated selfing to fix the introgressed segments. However, all six target segments were 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 yr of testing, yields of the best "enhanced" B73 x "enhanced" Mo17 hybrids exceeded the original B73 x 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 numbers 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 F1 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 be lost during the backcross. When the confirmed QTL was transferred in three different backgrounds it had no effect in one background. Chee *et al* (2000) transferred a QTL for grain protein concentration (GPC) in emmer wheat into an adapted durum wheat background. An inbredline 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 F6 plants identified from the crosses of Archer x Asgrow A5403 and Archer x 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 highland cultivar into a lowland rice cultivar. In total three backcrossing 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 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 1and 7 QTLs were significantly taller than IR64. Youelf 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 F2:3 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 observedin the original $F_{2:3}$ mapping population. In this study, a combination of QTL linked to umc139 and php 200689 markers resulted in the highest seedling emergence compared with other combinations including all three of thebeneficial marker-QTL alleles together. Bouchez *et al* (2002) reported that the 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 progenies. 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 finally exhibited an effect opposite to the expectation. Lecomte *et al* (2004) introgressed five chromosome regions strongly involved in organoleptic quality attributes of tomato 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 largest effect were the most stable. Thabuis *et al* (2004) successfully transferred 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. Steele *et al* (2006) conducted a MARB breeding programme 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 thisroot 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. Ragot *et al* (2006) reported the results of a MARB 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 screeninglarge segregating populations (2200 individuals) for three ofthe four generations. Selected MABC-derived BC_2F_3 families were crossed with two testers and evaluated under different water regimes. Mean grain yield of MARB-derived hybrids was consistently higher than that of control hybrids (crosses from the recurrent parent to the same two testers as the MARB-derived families) under severe water stress conditions. Under those conditions, the best five MARB-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 MARB-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 moderate resistant lines were identified. These materials have the potential to develop marketable FHB resistant HWW cultivars and useful germplasm lines.

Recurrent Backcrossing of complex traits: Conventional recurrent backcrossing has severed the plant breeding society well before the adventure of molecular markers. Although MARB could be efficient and effective as discussed above, the underlying genetics of the target trait does not need to be known for the success of recurrent backcrossing. A major contribution of the modern molecular biology to plant genetics and breeding is that it provides the toolkits for the genetic dissection of complex traits, which makes the manipulation of individual gene (QTL) a reality. It is relatively easier to identify genes and markers linked to them for qualitative traits, since the inheritance is usually very simple and the effect of gene is highly predictable. Most of the important agronomic traits such as yield, stress resistance and quality are quantitative traits. Genes for quantitative traits are more difficult to be identified. Quantitative trait loci mapping using purposely generated mapping population such as F_2 plants, backcross plants, Recombinant Inbred Lines (RIL), Backcross Inbred Lines (BIL) or Doubled Haploid Lines (DHL), as well as a linkage map constructed using molecular markers are currently the standard approach for identifying QTL controlling quantitative trait. Large population size is required to provide sufficient power to detect typical QTL. For example, nearly 300 F_2 progeny are required to detect a QTL responsible for at least 10% of the total variance. The QTL are localized with relatively poor resolution, typically approximately 20

cM. Since many QTL are segregating within the mapping population and contribute 'phenotypic noise', it is difficult to be certain whether a given plant has inherited a specific QTL allele. Moreover, the lines (plants) of the mapping population may be difficult to use directly as parents in practical program. Because backcrossing isolates a gene or chromosomal region in a different genetic background (the genetic background of the recurrent parent), it helps to dissect the genetic architecture of quantitative traits. MARB has been explored in different manners to aid the identification and utilization of QTL.

Advanced backcross QTL analysis (AB-QTL)

The advanced backcross QTL analysis (AB-QTL) proposed by Tanksley and Nelson (1996) simultaneously identify and introgress favourable alleles from unadapted donor into elite background. The general AB-QTL analysis is comprised of the following experimental phases 1) Generation of an elite by donor hybrid. 2) Backcrossed to the elite parent to produce BC_1 population which is subjected to marker/or phenotypic selection against undesirable donor alleles. 3) BC_2 or BC_3 population was genotyped with polymorphic molecular markers. 4) The segregating BC_2F_2 or BC_2F_3 population is then evaluated for traits of interest and anlysed for QTLs. 5) Selection of target genomic regions containing useful donor alleles for the production of NILs in the elite genetic background. 6) evaluation of the agronomic traits of the NILs and elite parent controls in replicated environments. The ABQTL approach has mainly been evaluated in many crop plant species to determine whether genomic regions derived from wild or unadapted germplasm have the potential to improve yield. A CAB search using the key words advanced backcross and QTL analyses (20/02/08) ended up with 640 publications. However, the donor genome may mask the magnitude of some favourable effects that were identified for certain introgressed alleles. Thus, the yield-promoting QTL did not make a substantial contribution to the phenotype and the best lines were inferior to commercial cultivars. A major limitation to AB-QTL is difficulty in maintaining an adequate population size in selected backcross populations so that useful alleles are not lost and the QTL can be accurately mapped (Varshneya *et al* 2005).

QTL mapping using introgression lines (ILs): Eshed and Zamir (1994a & b) proposed the use of introgression lines (ILs). ILs are produced by systematic backcrossing and introgression of marker-defined exotic segments in the background of elite varieties. An example ILs development scheme is given in Figure 2. These ILs can be considered similar to a genomic library with a huge genome insert. Phenotypic characterization of each line can reveal which chromosome fragment from the donor the gene has associated with an interesting trait. Multiple traits can be studied in one population using the same genotypic data. Because identifying QTL genes using ILs does not require linkage map construction or sophisticated statistical analysis for QTL, this is a more user-friendly method for practical breeding programs and also for biological science. They enable the phenotypic analysis of specific QTL, offering a common genetic background in which direct comparison of two lines can be used to evaluate the phenotype conditioned by a single introgressed exotic segment (Tanksley *et al* 1996). The statistical power of QTL mapping is increased, because excluding extra genetic factors reduces phenotypic variation. ILs facilitate fine mapping of QTLs, because the location of a QTL can be narrowed to a smaller genomic interval by evaluating a series of ILs that differ for overlapping regions of the genome (Paterson *et al* 1990). ILs are also valuable resource for the unravelling of gene function by expression profiling or map-based cloning. ILs can be easily evaluated for all the important traits to identify any undesirable traits linked to the target gene(s) due to the relatively large chromosome segment introgressed can be identified before introgression. If necessary, undesirable genes should and can be eliminated by chromosome recombination in progeny between the IL and the recurrent parent and screened by MAS. Since ILs only contain a low percentage of exotic germplasm and the

elimination of unfavourable exotic alleles can thus be easily and rapidly accomplished. This will speed up the transfer of the desirable alleles into the elite varieties (Ashikari and Matsuoka 2006). It should be emphasised that the number of replicated measurements has a larger impact on power of QTL mapping using ILs. In a study using abrodopsis, Keurentjes *et al* (2007) found that at least five replicated plants should be analysed to obtain enough statistical power.

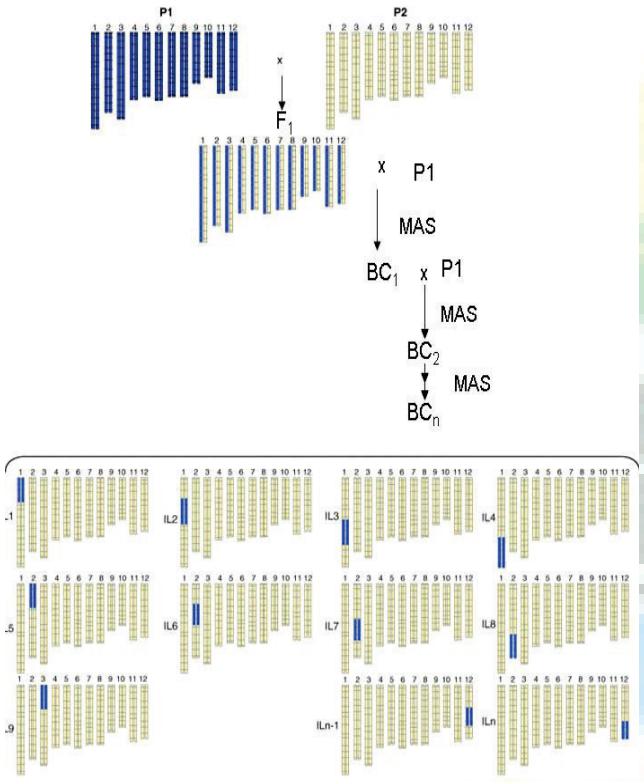

Figure 2 Production of Introgression lines (ILs). The donor plant is repeatedly backcrossed with a recurrent parent several times. The whole genome genotype of each backcrossed line is analyzed using molecular markers to identify the remaining chromosome segments from the donor plant. The backcrossed lines are arranged so that theyare successively overlapping and covering the whole donor genome from the top of chromosome 1 to the bottom of chromosome 12 (IL1 to ILn) (Modified from Ashikari and Matsuoka 2006).

However, fewer lines can be analysed as long as genome-wide coverage is maintained. The mapping resolution depends on the sizes of the introduced segments. Reducing the segment size increases resolution at the cost of increased the number of lines required to have good genome coverage and prolonged development time.

Fine mapping using near isogenic lines (NILs): Near isogenic lines (NILs) are sometimes also referred to as introgression lines in literature. However, there are subtle differences between NILs and ILs. A set of NILs are different only for a small part of genome (The part of the genome that differs can be a single gene or a chromosome segment), while a set of ILs are different for many smaller parts of genome. ILs are mainly used for the identification of genome segments that contain traitaffecting QTL and full donor genome coverage is required, while NILs are used to further investigate already identified gene/QTL and only the target chromosomal segment is monitored. NILs have been widely used in QTL confirmation, investigating QTL effects in different background (Christians *et al* 2004), check for dominance and/or espistatic relationships between QTL Eshed and Zamir 1995, 1996, Lin *et al* 2000, 2003, Yamamoto *et al* 2000), resolving linkage between QTL (Monna *et al* 2002, Takeuchi *et al* 2003, Christians and Keightley 2004). NILs are also ideal materials for QTL pyramiding (Ye and Smith 2008). Fin mapping using NILs have been reported for some QTL (Brouwer and Clair 2004)

Perspects: MARB, the combined use of MAS and recurrent backcrossing, greatly widens the applicability of recurrent backcrossing for cultivar improvement. It also severs as an important tool for genetic study of complex traits. For breeding much wider range of traits now can be improved using MARB. Similar to the conventional recurrent backcrossing, traits controlled by single or few genes are the most rewarding area of MARB application. When QTL are to be introgressed, several difficulties arise. It is more difficult to select for the presence of QTL since QTL location is estimated with only a given imprecision (Visscher *et al* 1996). This requires using more markers and optimizing the positions of this markerswith 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 reducesthe 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 introgression of QTL has been less success 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 surprised 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 were not precisely mapped, large regions of donor chromosomes were transferred. This has at least two possible consequences. First, 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 finding of more than two genes (Eshed and Zamir 1995, Monna *et al* 2002, Steinmetz *et al* 2002, Christian and Keightley 2004). Second, 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. The consistence of the linkage phase between the target gene and its linked markers across multiple populations presents a serious problem for selecting for the target gene using markers. Markers linked to the QTL identified by linkage mapping using one or a few populations may or may not be useful in gene introgression because different subsets of QTL will be polymorphic in each population, and the linkage phases between the marker and QTL alleles can differ even between closely related genotypes. The tighter the linkage the more consistent the linkage phrase across populations. With the rapid development of plant functional genomics functional markers will become available for more traits and crops and greatly facilitate the application of MARB in practical breeding. Linkage phrase also tends to be more consistent if the source of QTL is from a gene pool which is very distinct from the one used by the breeders. Thus, markers linked to novel alleles from exotic germplasm or wild relatives are more likely to be successfully implemented (Tanksley and McCouch 1997).

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