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Review Article

Application of molecular markers for biotic stresses

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ABSTRACT

Biotic stress is stress that occurs as a result of damage done to plants by other living organisms, such as bacteria, viruses, fungi, parasites, harmful insects, and weeds. It is a major focus of agricultural research, due to the vast economic losses caused by biotic stress to cash crops. The relationship between biotic stress and plant yield affects economic decisions as well as practical development. The impact of biotic injury on crop yield impacts population dynamics, plant-stressor coevolution, and ecosystem nutrient cycling (Peterson and Higley, 2001). Biotic stress causes serious losses in the yield and quality of crops, and its control is therefore very important. The use of fungicides and insecticides is limited in crop plants because of their cost and considerations of safety to humans and the environment.

Biotic stress and Molecular markers

One of the most efficient strategies for controlling diseases and pests is the development of resistant varieties. A potential grass variety must contain genes for resistance against biotic stress. Plants have many mechanisms for protecting themselves against biotic stress. Because plants cannot move to escape from the stress, they have developed defensive strategies, such as hypersensitive reaction and the production of phytoalexins. Much attention has been directed at the isolation of genes for resistance to biotic stress and the analysis of molecular mechanisms of resistance. It has become clear that common genes trigger resistance networks and control resistance to biotic stress (Takken and Joosten 2000).

Breeding for resistance is one of the major efforts in many breeding programmes. As in organic farming the use of pesticides is prohibited, resistant cultivars are of high importance. To select for resistant plants, screening assays are needed that often are laborious and require extensive knowledge on plant-pathogen interactions. To facilitate the introgression of resistance genes into adapted breeding material, molecular markers are a powerful tool. With the advent of DNA marker technology, several types of DNA markers and molecular breeding strategies are now available to plant breeders and geneticists, helping them to overcome many of the problems faced during conventional breeding. Markers that reveal polymorphisms at the DNA level is known as molecular markers. The last two decades have witnessed a remarkable activity in the development and use of molecular markers both in animal and plant systems. This activity started with low-throughput restriction fragment length polymorphisms and culminated in recent years with single nucleotide polymorphisms (SNPs), which are abundant and uniformly distributed. There have been several reports of the potential applications of molecular markers to plant improvement (Burr *et al*. 1983; Helentjaris *et al*. 1985; Beckman and Soller 1986). A molecular marker is defined as a particular segment of DNA that is representative of the differences at the genome level. Molecular markers may or may not correlate with phenotypic expression of a trait. Molecular markers offer numerous advantages over conventional phenotype based alternatives as they are stable and detectable in all tissues regardless of growth, differentiation, development, or defense status of the cell are not

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confounded by the environment, pleiotropic and epistatic effects. The identification/development of a new class of very useful DNA markers called genic molecular markers (GMMs) utilizing the ever-increasing archives of gene sequence information being accumulated under the EST sequencing projects on a large number of plant species in the recent years. These markers being part of the cDNA/EST-sequences, are expected to represent the functional component of the genome i.e., gene(s), in contrast to all other random DNA based markers (RDMs) that are developed/generated from the anonymous genomic DNA sequences/domains irrespective of their genic content/information. Therefore, identifying DNA sequences that demonstrate large effects on adaptive plant behavior remains fundamental to the development of GMMs (Varshney et. al, 2007).

Molecular markers are now widely used to track loci and genome regions in several crop breeding programmes, as molecular markers tightly linked with a large number of agronomic and disease resistance traits are available in major crop species (Phillips and Vasil 2001, Jain *et al*. 2002, Gupta and Varshney 2004). These molecular markers include: (i) hybridization-based markers such as restriction fragment length polymorphism (RFLP), (ii) PCR-based markers: random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellite or simple sequence repeat (SSR), and (iii) sequence-based markers: single nucleotide polymorphism (SNP). The majority of these molecular markers has been developed either from genomic DNA libraries (e.g. RFLPs and SSRs) or from random PCR amplification of genomic DNA (e.g. RAPDs) or both (e.g. AFLPs). These DNA markers can be generated in large numbers and can prove to be very useful for a variety of purposes relevant to crop improvement. For instance, these markers have been utilized extensively for the preparation of saturated molecular maps (genetical and physical). Their association with genes/QTLs controlling the traits of economic importance has also been utilized in some cases for indirect marker-assisted selection (MAS) (e.g. Koebner 2004, Korzun 2002). Other uses of molecular markers include gene introgression through backcrossing, germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization and phylogenetic analysis (see Jain *et al*. 2002). For plant breeding applications, SSR markers, among different classes of the existing markers, have been proven and recommended as markers of choice (Gupta and Varshney 2000). RFLP is not readily adapted to high sample throughput and RAPD assays are not sufficiently reproducible or transferable between laboratories. While both SSRs and AFLPs are efficient in identifying polymorphisms, SSRs are more readily automated (Shariflou *et al*. 2001). Although AFLPs can in principle be converted into simple PCR assays (e.g. STSs), this conversion can become cumbersome and complicated as individual bands are often composed of multiple fragments (Shan *et al*. 1999), particularly in large genome templates. An increasing number of monogenic, race-specific genes showing a gene-for-gene interaction have been mapped, and agronomically important genes have been correlated to molecular markers, as demonstrated for potato in Table 1. For wheat, such validated markers are available for resistance genes against powdery mildew (Pm1c, Pm17, Pm24, mlRD30), the yellow dwarf virus, the cyst nematodes (Cre1 and Cre3), and the rusts (Lr9, Lr21, Lr24, Lr38, Lr47; Sr38, Yr5, Yr17) and Fusarium head blight (Mohler and Singrun 2005). Presently, the most powerful application of such identified genes and molecular markers is opened up by MAS. It offers the opportunity of combining different genes for a given pathosystem in a single genotype (gene pyramiding). A prerequisite for gene pyramiding is that characters are not allelic. Furthermore, knowledge on the gene distances in genetic or better physical maps is very helpful. Using such information, it was possible to combine three race-specific powdery mildew genes (Pm) in a

single line which is now under variety test, hoping that such a pyramided resistance will be rather durable (Figure 2).

Simple sequence repeat for Marker assisted selection

SSRs are actually considered the most efficient markers, but their use is still limited because of the long and laborious steps to develop them. There are two general strategies to access these regions and create SSR markers: (1) searching for sequences containing microsatellites in the available data bases; or (2) constructing and screening the genomic (or other) library with probes complementary to microsatellite sequences. Exceptionally, some strategies without library construction n have been developed. The efficiency of SSRs for MAS of QTLs has been demonstrated by Li *et al*. (2001), in conditioning soybean resistance to southern root-knot nematode. The abundance and highly polymorphic nature of SSRs that have become publicly released allowed the researchers to quickly identify several markers tightly flanking the QTL. The comparison of published maps for markers near the region of interest accelerated the process. The linked markers were then used to rapidly breed the resistance genes into a productive soybean line. Similar procedures for MAS have also been successful with soybean mosaic virus (SMV) and soybean cyst nematode (SCN) (Dr. Buss and Dr. Tolin, unpublished). The use of SSRs for tagging disease resistance genes has been very successful in soybeans. Examples include resistance to soybean mosaic virus (SMV) (Gore *et al*., 2002; Jeong *et al*., 2002) and sudden death syndrome (SDS) (Iqbal *et al*., 2001).

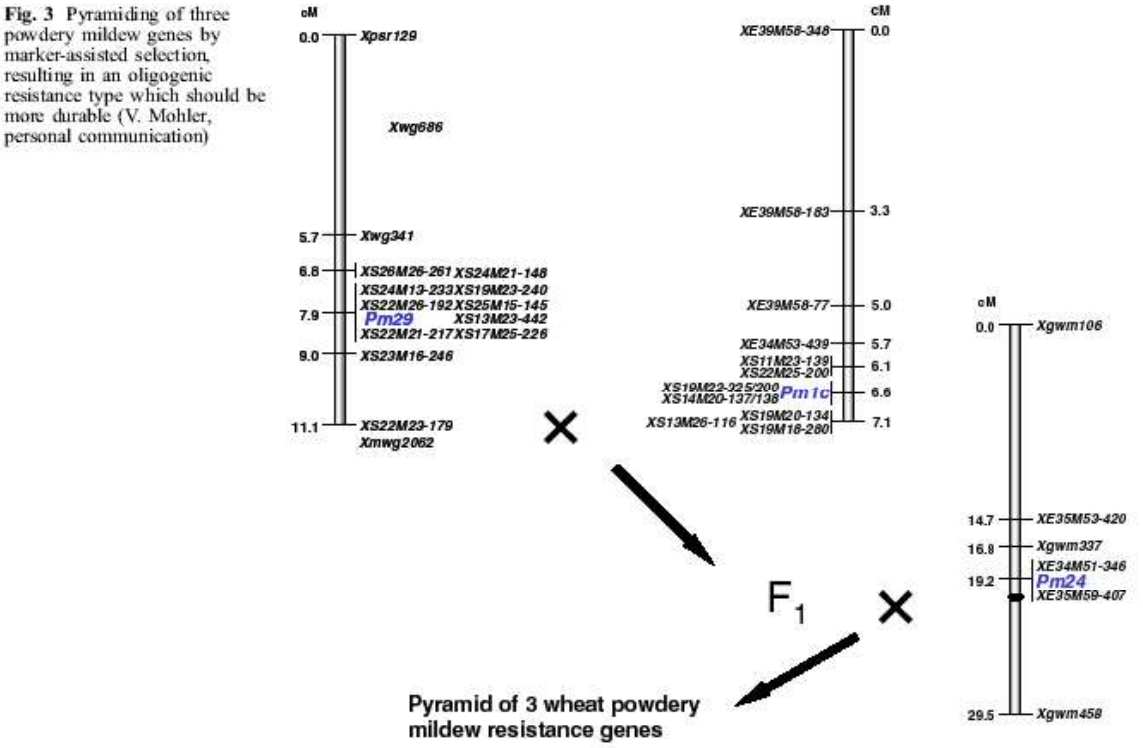
Identification of new SSR polymorphism

The positional cloning of many disease loci has been facilitated by high-resolution genetic maps. The precise localization of the DNA sequence responsible for a disease usually requires the

Table 1 Some important and mapped DNA markers on the example of potato (for a review of literature, see Wenzel 2006)

Trait	Gene	Chromosome
Potato virus Y	Ry_{adg}	XI
	N_{ytdr}	IV
	Rx1 ; Rx2	XII, V
	Na	XI
	Nb	V
	Nx	IX
Potato leaf roll virus	PLRV QTL	XI
Globadera rostochientsis	Gro	V
	H 1	
Globadera pallid	Gpa QTL	IV, V, IX, XII
	Gpa2	XII
Phytophthora infestants	RB	X
	R1	V
	R2	IV
	R3, R6, R7	XI
	Rbbc	VIII
	QTL	V
Synchytrium endobioticum	Sen1	XI
Erwinia carotovora	QTL	
Tuber starch, tuber yield	QTL	I-XII
Cold sweetening	QTL	V, IX
Skin colour	QTL	X
Tuber flesh color	QTL	III

Figure 2 Pyramiding of three powdery mildew genes by marker-assisted selection, resulting in an oligogenic resistance type which should be more durable (Wenzel, G. 2006).



development of very high-density physical and genetic maps. The availability of multiple polymorphic genetic markers is crucial to this effort (Dib *et al*, 1996). Current widely used methods for the identification of new simple sequence repeat polymorphisms involve PCR based and subcloning strategies (Dib *et al*, 1996; Chen *et al*, 1995). Subcloning, once the primary method of isolating ne microsatellite sequences (Malo *et al*. 1995), largely has been supplanted by PCR-based methods because of the relatively large amount of work and technical difficulties involved in subcloning strategies, including sublibrary construction and screening with oligonucleotide probes (Ostrander *et al*, 1992). PCR-based strategies, although generally faster and more successful than those using sub cloning, still suffer from several disadvantages.

Many RFLP markers for disease and insect resistance have been identified in wheat (Antique *et al*, 1995; Ma *et al*, 1994; Williams *et al*, 1994), but because of the low level of polymorphism among wheat cultivars (Chao *et al*, 1989), it is difficult to detect polymorphisms in new wheat mapping populations with some RFLP probes.

In wheat, RFLPs and random amplified polymorphic DNAs (RAPDs) have been used to study wheat scab, and several markers were identified for scab resistance QTL (Bai, 199521); however, all of these markers accounted for only a small portion of the variation. A low level of polymorphism of RAPDs and RFLPs within wheat may be a barrier to the identification of markers that are closely linked to major scab resistance genes (Bai, 1995, Chao *et al*, 1989).

In a study Bai *et al* (1999), 11 AFLP markers closely linked to the QTL region for scab resistance were identified. These results indicate that AFLPs are efficient for generating molecular markers for wheat scab resistance.

Molecular markers for disease resistance by NBS profiling

Many resistance genes have been identified and sequenced in plants. Analysis of these sequences has uncovered conserved motifs, such as nucleotide binding site (NBS) and leucine rich repeat (LRR) regions in the DNA, that confer resistance to a broad spectrum of pathogens (Yu *et al*., 1996; Collins *et al*., 2001). Both are signature regions in the DNA that encode for domains known to be involved in the majority of plant pathogen resistance pathways (Collins *et al*., 2001). This knowledge can be extrapolated across species to identify analogous sequences, resistance gene analogues (RGAs), which include these signature motifs and may be involved with some type of disease resistance. The insights provided by RGAs create an opportunity that can greatly facilitate disease resistance gene identification and cloning. RGAs have already been developed in rice (Mago *et al*., 1999), tomato (Foolad *et al*., 2002), *Brassica napus* (Fourmann *et al*., 2001), grapevine (Donald *et al*., 2002), wheat and barley (Collins *et al*., 2001), and soybean (Yu *et al*., 1996).

It has already been illustrated that marker technology can be applied to RGAs, and several RGA sequences have been mapped in soybean. Data from several soybean studies support clustering of RGA families that map in close proximity to known resistance genes (Yu *et al*., 1996; Kanazin *et al*., 1996; Graham *et al*., 2000). This provides a framework for cloning genes through a "candidate" gene approach. For example, RGAs identified by Hayes *et al* (2000) and Yu *et al* (1996) were pivotal in the rapid high-resolution mapping and cloning of soybean potyvirus resistance genes (Gore *et al*., 2002; Hayes *et al*., in press). NBS profiling is a new marker technology that improves the detection of molecular markers for disease resistance (Vander Linden *et al*., 2004). The advantage of NBS profiling compared to other marker technologies, such as AFLP and SSR, is its gene-targeting nature. The technology directs a PCR reaction to NBS domains which are part of the largest class of disease resistance genes. NBS profiling produces multiple markers in a variety of resistance genes in a single assay.

The NBS-R3 gene could be a candidate for resistance to downymildew in sunflower. The same approach was used in recent studies to map candidate disease-resistance genes in soybean (Kanazin *et al*. 1996; Yu *et al*. 1996) and in potato (Leister *et al*. 1996).

Resistance to Biotic Stress

Plants frequently encounter many potential pathogens. However, a limited number of these pathogens can infect plants and cause disease. A large amount of work has been done in this field. The gene-for-gene hypothesis has been demonstrated by molecular evidence (reviewed by Takken and Joosten 2000; Bonas and Lahaye 2002). A resistance gene (R gene) recognizes an elicitor produced by the Avr gene of the pathogen and activates the plant defense network, which includes oxidative burst, ion fluxes, cross-linking and strengthening of the plant cell wall, production of anti-microbial compounds, and induction of pathogenesis-related proteins (Hammond-Kosack and Jones 1996; Sudha and Ravishankar 2002).

Resistance Genes to Biotic Stress in Plants

More than 30 resistance genes have been isolated in several species, and these have been classified into 8 distinct structural categories by Hulbert *et al*. (2001). Recently, several novel types of resistance genes were reported, such as tomato Ve gene (Kawchuk *et al*., 2001), Arabidopsis RRS1-R gene (Lahaye, 2002), and barley Rpg1 gene (Brueggeman *et al*., 2002). The resistance genes consist of common motifs such as a nucleotide binding site (NBS), leucine-rich repeats (LRRs), kinase, coiled-coil domain (CC), Toll/interleukin-

1-receptor (TIR), and transmembrane domain (Hammond- Kosack and Jones 1997). The functions of these motifs are not completely clear. All R genes without Pto contain LRRs. The LRRs are hypervariable and probably act as receptors for Avr factors produced by pathogens. An extensive review of the LRR domain has been conducted by Jones and Jones (1997), and the model of specific recognition of R genes has been reviewed by Robert *et al*. (1998). The majority of R genes encode an N-terminal NBS and a C-terminal LRR region. This NBS–LRR of R genes confers resistance to bacteria, viruses, fungi, nematodes, and insects (Baker *et al*. 1997; Rossi *et al*. 1998), suggesting that a common mechanism in the form of a gene-forgene relationship is present.

Resistance Gene Loci in Plants

Genetic and molecular studies have demonstrated that R genes are frequently clustered in the genome (reviewed by Michelmore and Meyers 1998). The flax M locus (Anderson *et al*. 1997), the lettuce Dm3 locus (Anderson *et al*. 1996), the rice Xa21 locus (Song *et al*. 1997), the tomato I2 locus (Simons *et al*. 1998), and the tomato Cf 4/9 locus (Takken *et al*. 1999) contain multiple R genes. High rates of both unequal crossing-over and gene conversion occur in the Rp1 loci of maize (Sudupak *et al*. 1993; Richter *et al*. 1995). This is thought to facilitate the generation of novel R-gene specificity against pathogens. R loci consist of genetically separable recognition specificities, such as at least 14 specificities in the maize Rp1 locus (Hulbert 1993). The tomato Cf2 locus contains two R genes conferring resistance to tomato leaf mould (Dixon *et al*. 1996). The tomato CF4 locus confers resistance to tomato leaf mould, and this resistance is conferred by two distinct R genes, Cf-4 and Hcr9-4E, which recognize Avr4 and Avr4E, respectively (Takken *et al*. 1999). In these cases the genes at one R locus confer resistance to several races of pathogen. The Mi gene in tomato confers resistance to both aphids and nematodes (Rossi *et al*. 1998). Two R genes at a single R locus in potato confer resistance to distinct pathogens such as potato virus X and nematodes (Vander Vossen *et al*. 2000). This indicates that mapping of an R gene to one pathogen is potentially helpful for the mapping of R loci against other pathogens. Meyers *et al*. (1999) estimated that Arabidopsis and rice contain approximately 200 and 750–1550 R genes, respectively, and that the number of NBS sequences per cluster in Arabidopsis ranges from 2 to 18, with an average of 4.9. Bai *et al*. (2002) estimated that there are more than 600 NBS LRR-type genes in the rice genome.

Identification of Novel R Loci and R Genes

To pyramid R genes in target variety, identification of several major or minor genes is needed. In the first step, identification of several R loci is essential. Linkage analysis using DNA markers tightly linked to known R loci allows finding novel R loci. Markers linked to a novel R locus can be mapped to the reference map of perennial ryegrass described by Jones *et al*. (2002a) and to that of Italian ryegrass described by Fujimori *et al*. (2000) to assign the gene to the ryegrass linkage map. This map contains SSR markers developed by Hirata *et al*. (2000) and anchor probes developed by Inoue *et al*. (2002). Additionally, mapping of cleaved amplified polymorphic sequences (CAPS) and resistance gene analogue (RGA) to this map is in progress by Miura *et al*. (Japan Grassland Farming and Forage Seed Association). Mapping of major resistance genes is useful for increasing our knowledge of resistance in at least 3 ways. First, major genes are more easily scored and mapped precisely on linkage maps than are minor genes, because the resolution of QTL analysis is too low to locate minor genes precisely. Welz and Geiger (2000) found that major and minor genes could be mapped at identical chromosomal positions, indicating that the R locus of major genes is potentially useful for the mapping of minor genes. Second, it has been demonstrated that some

resistance genes to different pathogens can be mapped at a single locus or at several loci linked closely with each other (Rossi *et al*. 1998, vander Vossen *et al*. 2000). Thus, linkage analysis of major genes may allow us to increase our knowledge of the loci conferring resistance to diverse pathogens. Finally, linkage analysis of major genes is useful in the analysis of minor genes. The effects of minor genes may be masked by those of major genes in the linkage analysis. Therefore, major genes may have to be removed from a population for the linkage analysis of minor genes. In developing populations for linkage analysis, DNA markers tightly linked with major resistance genes are useful for selecting parents carrying only minor resistance genes. Identification of the specificities of R genes to pathogens is important in the development of varieties with durable resistance. To determine the specificities of R genes, lines carrying single resistance genes in a homozygous state are needed as test lines for race identification. Development of these lines by conventional methods is not easy, because of the out-crossing habit of Italian ryegrass. However, DNA markers tightly linked to major resistance genes facilitates the development of test lines. Lines with known R genes in the homozygous state may be useful not only as test lines, but also as breeding material.

Marker-Assisted Selection for resistance gene

To prevent inbreeding in plant breeding programs, many individuals have to be selected from a breeding population, so the population must be large. However, using markers can be helpful in small populations, for example, for introgression of a target gene to another species or for the development of lines carrying one R gene, as mentioned above. In these cases, we can easily use DNA markers to develop lines with target genes. The presence of DNA markers tightly linked to the R gene locus enables plant breeders to monitor the frequency of R genes and the genetic diversity of R loci in the breeding populations. Information about the percentage of useful genes in the breeding population acts as a good index for breeders. Although uniformity in the target locus is important in the majority of traits such as quality, heading date, and yield, genetic diversity in R loci is potentially useful for stabilizing resistance to diverse diseases. Because pathogens can evolve rapidly, they can overcome single R genes easily. Therefore, high diversity of the R locus may be useful in stabilizing resistance to various pathogens. Wolfe (1985) reported that heterogeneity for disease resistance is useful in disease control. SSR markers may be suitable for analyzing the genetic diversity of the R locus in a breeding population, because of its features of locus specificity and high allele number.

First Generation of Genetic Markers

Different technologies have been used to develop markers for *R* genes in wheat. A very limited number corresponds to morphological markers such as leaf chlorosis or pseudo black chaff for *Sr2* (Brown 1997) or leaf tip necrosis for *Lr34* and *Yr18* (Singh 1992a, b). This type of marker is usually of limited use as it is often affected by environmental conditions or developmental growth stages. However, in some cases phenotypic markers such as the leaf tip necrosis associated with *Lr34* and *Yr18* have been intensively used in selection for durable resistance in wheat (Rajaram *et al*. 1988). A number of biochemical markers, particularly isoenzymes, have also been developed as markers for leaf rust and eyespot disease resistance genes in wheat (McMillin *et al*. 1986; Winzeler *et al*. 1995). However, this type of marker requires protein extraction, is labor-intensive and not well adapted to automation and high-throughput analysis for breeding.

Molecular markers are based on the detection of polymorphisms in the DNA sequence. Their number is theoretically almost unlimited and, they are not affected by environmental conditions and plant growth stages. The first molecular markers

for disease resistance genes in wheat were developed at the beginning of the 1990s and they were mainly restriction fragment length polymorphism (RFLP) markers. A large number of RFLP probes has been generated from *T. aestivum* and *Ae. tauschii* libraries (Liu *et al*. 1990; Anderson *et al*. 1992; Devos and Gale 1993); for more details see the review of Gupta *et al*. (1999). They have been extensively used to establish genetic maps (for review, see Langridge *et al*. 2001) and the development of markers for agronomically important traits in wheat. For many years, marker development in wheat has relied on RFLP probes directly originating from wheat. This has resulted in low-density maps which did not always allow the efficient development of markers for target genes. The situation dramatically improved with the discovery of the conservation of the marker order (colinearity) at the genetic map level on homoeologous chromosomes of grass genomes (Moore *et al*. 1995; Keller and Feuillet 2000). As colinearity mainly concerns genes and most of the RFLP probes correspond to cDNAs, RFLP markers from one species have been successfully used for mapping in other grass species. This has allowed the increase of genetic map densities at resistance gene loci in wheat and the development of tightly linked markers. To date, more than 36 RFLP markers have been developed for monogenic pest and disease resistance genes in wheat and they still represent a large part of the markers used to identify QTLs for quantitative disease resistance However, RFLPs are not well suited for marker-assisted selection as they are labor-intensive, time-consuming, require large amounts of DNA and often have to be radioactively labeled. In addition, because they only rely on sequence differences in restrictions sites, they show a limited amount of polymorphism in wheat. For these reasons, they are not used routinely in marker assisted selection programs.

Polymerase Chain Reaction-Based Markers High-Throughput for Marker-Assisted Selection

The discovery of the polymerase chain reaction (PCR) has revolutionized the development of molecular markers because it only requires very low amounts of DNA which can be rapidly extracted from different plant material with high-throughput methods (Kang *et al*. 1998; Paris and Carter 2000; Stein *et al*. 2001). The first types of PCR-based markers were random amplified polymorphic DNA (RAPD) markers. Their main advantage is that RAPDs do not require any knowledge of the target sequence as single random primers of 9–10 mers are used for PCR. To date, 17 RAPDs have been developed for wheat disease *R* genes. However, the low temperature of amplification used in this technique makes RAPD markers not very robust and difficult to reproduce in different laboratories using different thermocyclers. For these reasons, in many cases sequence tagged site (STS) markers, which correspond to the specific amplification of a target DNA sequence at stringent temperature, have been derived either from low-copy RFLP or from RAPD markers.

The second generation of PCR-based markers consisted of microsatellites or simple sequence repeats (SSR) and amplified fragment length polymorphisms (AFLP). SSRs which comprise short repeat units of 1–6 nucleotides are very abundant and dispersed throughout the genome. AFLPs which combine the use of restriction site polymorphisms and specific PCR amplifications have a high multiplex ratio compared to the other marker systems. Both techniques benefit from the advantages of PCR and have a higher marker index (calculated on the information content and multiplex ratio of the marker) than RFLP and RAPD (Powell *et al*. 1996). Microsatellites, which are mainly codominant, are more robust than RAPD and easier to transfer between populations than AFLPs. The main disadvantage of SSRs

resides in their high developmental costs which cannot be supported by every laboratory.

Markers for Single Traits vs. Markers to Dissect Complex Traits

So far, most of the markers have been developed for monogenic *R* genes, which when used as single genes are not very durable in the field. Even if molecular markers allow the combination of several single *R* genes (pyramidization) to increase durability, breeders are most interested in targeting quantitative forms of resistance. Quantitative resistance can either be due to the combined action of several minor genes (QTLs) such as the resistance to *Fusarium* head blight or to single loci which are strongly influenced by the environment such as the slow rusting genes *Lr34* and *Lr46*. The increase in the type and number of molecular markers in wheat in the last decade has allowed a more efficient dissection of quantitative disease resistances into single QTLs. This is demonstrated by the release of more than 20 publications since 2000 for QTLs for disease resistance in wheat. Quantitative resistance is very difficult to select for in conventional breeding programs. With the comparison of QTLs obtained in different environments and populations, it is now possible to identify major QTLs which can be targeted by markers and integrated in MAS schemes.

Markers Derived by Homology with Known Plant Disease Resistance Genes

Since 1998, a number of plant *R* genes have been cloned (Richter and Ronald 2000; Hulbert *et al*. 2001). A majority of them belong to the NBS-LRR class and contain short conserved domains. So-called resistance gene analogs (RGAs) have been isolated from wheat and barley using degenerated primers corresponding to very conserved regions (Ploop, kinase2 and GLPLAL) within or close to the NBS domain (Leister *et al*. 1998; Seah *et al*. 1998; Spielmeyer *et al*. 1998; Collins *et al*. 2001), or using the resistance gene analog polymorphism (RGAP) technique (Chen *et al*. 1998; Shi *et al*. 2001; Yan *et al*. 2003). A number of these RGAs were mapped at known disease resistance loci and represent good markers for wheat disease resistance genes. In a few cases, RFLP or RAPD markers associated with wheat *R* genes were also found to correspond to RGAs (Lagudah *et al*. 1997; Frick *et al*. 1998; Huang and Gill 2001; Ling *et al*. 2002). So far, six of the RGAs are used as markers for *R* genes in wheat.

Third Marker Generation Derived from Large-Scale Analysis

Single nucleotide polymorphisms in allelic sequences have recently been investigated as a new source of markers in plant breeding, especially in maize (Rafalski 2002). SNPs can be discovered either by sequencing a number of PCR products amplified from specific target sequences in different genotypes Molecular Markers for Disease Resistance: The Example Wheat 361 or by in silico analysis of genomic or cDNA sequences. In wheat, a tremendous effort of cDNA sequencing has been undertaken and coordinated by the International Trititiceae EST Cooperative (ITEC; http://wheat.pw.usda.gov/ genome/) in the last 3 years. This has resulted in the best collection of expressed sequence tags (ESTs) among plants, with more than 549,000 ESTs in the public database to date (http://www.ncbi.nlm.nih.gov/dbEST/dbEST–summary.html).

These sequences represent a valuable resource for SNP detection as well as for SSR detection which are also found in noncoding regions of cDNAs. SNPs can be used as single genetic markers which may be identified in the vicinity of target genes, but there is also a great potential in using the association of SNP haplotypes with particular traits such as disease resistance. Association studies can be of particular help in analyzing quantitative traits with a much higher resolution than QTL analysis performed in F2 populations or

recombinant inbred lines (Buckler and Thornsberry 2002; Rafalski 2002). Different technologies are now available to assay SNPs (Langridge *et al*. 2001) and there is no doubt that in the future such markers will also be integrated in molecular breeding programs.

Use of Molecular Markers in Marker-Assisted Selection for Disease Resistance

To date, molecular markers have been developed for more than 85 different monogenic disease resistance genes and for five different quantitative disease resistances. A key question lies in the validity of these markers for MAS. Langridge *et al*. (2001) have distinguished different steps in the validation of a marker for MAS. First of all, markers which are often developed in one population have to be tested in other populations originating from crosses with one parent of the original mapping population. In many cases, the markers developed in one or two germplasms will not be found in others originating from different breeding programs. Therefore, it is very important to saturate the resistance locus with about ten markers within a genetic distance of less than 10 cM (ideally with markers located at less than 1 cM from the target genes). This increases the chance of finding at least one marker useful for any breeding population. For these reasons, the high level of SSR polymorphism makes them ideal for MAS. So far, very few markers have been thoroughly tested in practical breeding programs. Recently, Sharp *et al*. (2001) have evaluated the usefulness of four sets of markers for important rust resistance genes.

Conclusion and future prospects

Resistance to biotic stress is an essential trait in crops. Although many varieties resistant to disease have been developed, the analysis of biotic stress at a molecular level is still lacking. The use of molecular techniques would enable us to clarify the nature of resistance to biotic stress. The use of information from the genome sequence of Arabidopsis and rice opens up new strategies for developing markers and isolating useful genes in crop plants. In the near future we will be able to increase our knowledge of the number of R loci related to biotic stress, and this information will make it easier for us to analyze the mechanism of resistance to biotic stress.

The development of varieties with high and durable resistance to biotic stress will be possible using advanced technology. Clarification of the genetic variation within target species may be the essential process. If there are several R genes in the target species, the use of DNA markers will help us to develop resistant varieties with several resistance genes. If there is no R gene against the target pathogen within the target species, the introgression of resistance genes from a related species will be an efficient way of achieving this aim. If there is no material within a related species, transformation may provide useful breeding materials. Each laboratory will carry out linkage analysis of resistance to important diseases and will develop breeding materials and DNA markers linked to this resistance. In the international collaboration, each resistance gene against important diseases in each country will be mapped and assigned to a reference map by using common SSR markers.

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