Review

Molecular markers and transposon mutagenesis for genes identification

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Molecular markers now help plant breeders to track useful genes conferring agronomic and quality traits, including yield, drought and heat stress resistance, insect and disease resistance, and many others. Molecular markers are simple ways to detect the genetic differences between individuals or populations. There are many types of molecular markers such as: restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), sequence-tagged-sites (STSs), microsatellites or simple sequence repeats (SSRs), amplified fragment length polymorphisms (AFLPs), and single nucleotide polymorphisms (SNPs). They improve the efficiency of plant breeding by carrying out indirect selection through molecular markers linked to the trait of interest. Therefore, the most applications of molecular markers techniques in marker assisted breeding include genetic distance analysis, variety identification, isolation of markers linked to specific genes, and marker assisted backcrossing. Transposon-based approaches are very powerful for identification of essential and infection-related genes in the different plants and the context of microbial genomics. The development of techniques including PCR-based signature-tagged mutagenesis is now used to find genes.

Key words: Molecular markers, transposon mutagenesis, identification genes.

INTRODUCTION

Advances in molecular biology during the last decade have provided a new class of genetic markers, namely DNA markers. The use of DNA marker technology in marker-assisted selection to aid conventional plant breeding is now well established. Marker assisted selection (MAS) is an indirect selection method relying on markers outside the target gene. Selection is not done based on the phenotype but based on the genotype of a marker that is linked to the gene affecting the phenotype. In theory, MAS is more effective than phenotypic selection when correlation between the marker genotype scores and the phenotypic value is greater than the square root of heritability of the trait, assuming that the heritability of the marker is one (Dudley, 1993). MAS makes early selection before phenotypic evaluation possible and simplifies selection of traits that are difficult to score. The efficiency of MAS can be increased by using markers flanking the target gene instead of a single linked marker (Tanksley, 1983). General advantages of marker assisted breeding include its ability to reveal sites of variation in a DNA sequences, more common than phenotypic markers. Thus, selection of plants based on DNA markers before the phenotypic trait for resistance is expressed, holds promise for greatly accelerating the rate of development for superior crops.

The relative advantages of molecular markers over morphological markers for most genetic and breeding applications were discussed by Stuber (1992) and are summarized as follows: (1) For molecular markers, genotypes usually can be determined at the whole plant, tissue, or (sometimes) cellular levels. For most morphological trait markers, genotypes generally can be ascertained only at the whole plant level, and, frequently, the mature plant is required. (2) For many plant species, several naturally occurring alleles are available at most molecular marker loci. Thus, natural variation in existing populations can be used without the need to construct special genetic stocks, as may be required for many

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Table 1 .Comparison of different DNA-marker systems. Modified from Rafalski & Tingey (1993), and Ridout & Donini (1999).

	RFLP	RAPD	SSR	AFLP	STS
Type of polymorphism	Single base changes Insertions Deletions	Single base changes Insertions Deletions	Changes in number of repeats	Single base changes Insertions Deletions	Single base changes Insertions Deletions
Level of polymorphism	High	Medium	Very high	Medium	Low
Dominance	Codominant	Dominant	Codominant	Dominant	Co-Dominant
Number of loci analyzed per assay	1-2	5-10	1	100-150	1-3
DNA required per assay	2-10 g	20 ng	50 ng	0.5-1.0g	10-50ng
Sequence information required ?	No	No	Yes	No	Yes
Development costs	High	Low	High	Medium	Medium
Running costs per assay	Medium	Low	Medium	Medium	Medium
Repeatability	Very high	Fair	Very high	Very high	High
Ease of use	Labour intensive	Easy	Easy	Difficult initially	Easy

morphological traits. (3) Dominant-recessive interactions frequently prevent distinguishing all genotypes associated with morphological traits, whereas alleles at most molecular marker loci behave in a co dominant manner. (4) Different alleles at molecular marker loci rarely show pleiotropic effects. For morphological trait loci, pleiotropic and deleterious effects are frequently associated with certain alleles.

The development of molecular marker techniques now allows the breeder to discriminate among individual gene effects. This in turn allows the elucidation of the numbers and genomic organization of quantitative trait loci (QTLs) as well as an insight into the relative contribution of major and minor genes to the expression of quantitatively inherited traits (Stuber and Edwerds, 1986).

In recent year transposon mutagenesis has been used to study gene function and allows the generation of large numbers of independent mutations throughout the entire genome of an organism. Transposons, can jump within the genome and change the regulation or expression of genes, were first discovered in *Zea mays* by McClintock. Transposons have been developed to identify essential genes under various growth conditions, as well as bacterial genes essential for pathogenicity. Transposon can be used for genetic foot printing, and genomic analysis and mapping by in vitro transposition (GAMBIT) include the essential gene test (EGT) assay. Transposons can be classified into two major classes; firstly they can retroelements that transpose via an RNA intermediate that is synthesized by a reverse

transcriptase; secondly by directly from DNA to DNA.

CLASSES OF MOLECULAR MARKERS

A molecular marker is simply a short sequence of DNA that is so tightly linked to the desirable trait that selection for the presence of the molecular marker actually ends up selecting for the desirable trait.

Several different types of DNA markers are currently available for genetic analysis and new marker types are being developed continuously. Markers differ from each other in many respects: the initial workload and costs for building up the marker system, running costs and ease of use, level of polymorphisms, dominance, number of loci analyzed per assay, reproducibility and distribution on the chromosomes. Detection of polymorphism at the DNA level is usually based either on restriction patterns or differential amplification of DNA. The choice of the best marker system depends on whether it will be used in evolutionary or population studies, genetic mapping or fingerprinting (Rafalski and Tingey 1993; Kalendar et al., 1999; Ridout and Donini 1999). A comparison of different DNA marker systems is shown in Table 1.

Restriction fragment length polymorphism (RFLP) marker

RFLP was first used for creating a linkage map to the

humans by Botstein et al. (1980) and the first applications in plant breeding were proposed by Burr et al. (1983). RFLPs are visualized after Southern blotting (Southern, 1975) by hybridization to labeled DNA probes and subsequent autoradiography. Differences in the restriction patterns are caused by single nucleotide mutations at the restriction site or by longer deletions/insertions between restriction sites. RFLPs are exceedingly numerous and are expected to have genetic characteristics such as lack of dominance, multiple allelic forms and absence of pleiotropic effects on economic traits of particular usefulness in breeding programs. RFLP applications so far considered include: varietal identification and mapping of QTLs, screening genetic resource strains for useful quantitative trait alleles and their marker-assisted introgression into commercial varieties and marker-assisted early selection of recombinant inbred lines in plant breeding programs (Beckmann and Soller, 1983).

RFLP probes are useful as anchor markers for comparative studies within or between species and have been used for comparative mapping in the grass genera (Van Deynze et al., 1998; Devos and Gale, 1997). On the other hand, Raman and Read (1999) reported that RFLP analysis is costly, laborious, low frequency of desired polymorphisms in polyploid plants, and involves radioisotopes, and hence is not suitable for large scale screening of individuals. PCR based assays are efficient and easier.

PCR-based markers

Fragments of genomic DNA suitable as genetic markers can be produced by PCR amplification. This can be done by synthesizing PCR primers to uniquely amplify portions of the sequence of known genes or mapped RFLP markers. In the most favorable case, the PCR products form the two mapping parents will be of different sizes, will be inherited as codominant markers, and the resultant polymorphisms can be directly observed by running the products on an agarose gel. No southern blots, DNA hybridization, or autoradiography are necessary.

Random amplified polymorphic DNA (RAPDs)

The random amplified polymorphic DNA method is based on the polymerase chain reaction (PCR) using short (usually 10 nucleotides) primers of arbitrary sequences. Polymorphism of amplified fragments are caused by: (1) base substitutions or deletions in the priming sites, (2) insertions that render priming sites too distant to support amplification, or (3) insertions or deletions that change the size of the amplified fragment (Williams et al.,1990). Nearly all RAPD markers are dominant, so it is not

possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (one copy) or homozygous (two copy) with a dominant RAPD marker. Codominant RAPD markers, observed as different size DNA segments amplified from the same locus, are detected only rarely. Waugh and Powell (1993) concluded that RAPDs have the advantages of being technically simple, quick to perform, require only minute amounts of DNA and involve no radioactivity.

RAPDs have been used to determine genetic relationships in wheat, sorghum, and soybean (Barakat et al., 2000; Dahlberg et al., 2002; Li and Nelson, 2002). Each paper provides information that supported the use of RAPDs in the identification of unique cultivars or populations based on genetic diversity. Moreover, RAPDs have played a very important role in the selection process for desired genotypic characteristics. Hernandez et al. (2003) found that of the eighty decamer primers used, only fourteen were polymorphic when analyzed against the common bean parents (BV and BM). The polymorphic primers were screened in 34 individuals of the F7, showing the most extreme phenotypes (17 RILs of long and 17 RILs of short cooking time). Out of the primers screened, three generated polymorphic DNA fragments that were apparently associated with cooking time. These RAPDs were scored against 70 RILs from the BM x BV populations. One of these RAPDs, UNAM 16, 310 bp (generated by 5`GGCTGCAGAA 3` decamer), was found to be associated with the short cooking time phenotype ($R^2 = 0.21$, $P = 0.0001$).

Despite some obvious advantages, some problems have also been encountered with the use of RAPDs as molecular markers. First, reproducibility of results is inconsistent. Second, detection of polymorphisms is still limited. Third, the non-codominant phenotype devalues the use of RAPDs as a generally applicable marker system in wheat. There are, however, many applications where RAPD will find great utility in wheat genetics. The tagging of genes controlling quantitative or other agronomic characters in individual populations of chromosome recombinant lines, double haploid or recombinant inbred populations is likely to be facilitated by use of RAPD, providing the sensitivity of the experimental conditions is kept in mind (Devos and Gale, 1992).

Recently, sequence-characterized amplified regions (SCAR) analysis was developed to produce more specific and reproducible results (Paran and Michelmore, 1993; Jung et al., 1999). SCAR markers are created by using a longer primer (extended sequence of a RAPD primer) that has a specific sequence of aproximately 20 bases. By increasing the specificity, the results are less sensitive to changes in reaction conditions and are more reproducible (Hernandez et al., 1999). Reliable SCAR markers have already been successfully derived from RAPD fragments in wheat (Hernandez et al., 1999). Motawei and Abdalla (2003) showed that amplification of

specific primers (SCAR-85) with DNA extracts of stem bases of barley cultivars revealed the presence of *Fusarium graminearum* in barley cultivars except cultivar G124 under water stress.

Sequence-tagged-sites (STSs)

The conversion of mapped RFLP and randomly amplified polymorphic DNA (RAPD) markers to their sequence-tagged-site (STS) (Olson et al.,1989) counterparts has proven an effective method of obtaining easy-to-use reliable markers of genes valuable enough to merit marker- assisted-selection (Nieto-Lopez and Blake, 1994). An STS is a short, unique sequence, amplified by PCR, which identifies a known location on a chromosome. Tragoonrung et al. (1992) found that seven of the eight marker sequences tested could be easily manipulated to permit evaluation of segregation by PCR. As significant QTL loci are identified in crops, converting mapped RFLP markers which flank agronomically important loci to PCR-based detection systems will provide a user-friendly technology to the ultimate users of genetic maps, the plant breeder's. The author also found that two types of polymorphism were distinguished using these primer sets: (1) insertion/deletion event that could be read directly from agarose gels, and (2) point mutation events. The latter were identified using polyacrylamide-gel electrophoresis of products following digestion with restriction endonucleases. Talbert et al. (1994) indicated that 9 of 16 STS primer sets tested revealed polymorphisms among 20 hexaploid wheat genotypes when PCR products were digested with restriction enzymes. These results suggest that the STS-based PCR analysis will be useful for generation of informative molecular markers in hexaploid wheat. Moreover, Blake et al. (1996) described a series of 135 barley-specific markers amplified by 115 STS primers sets developed from sequences from previously mapped restriction fragment length polymorphism (RFLP) markers. These easily distinguish the cognate barley products from their wheat counterparts and should find ready use in the identification of lines, which contain wheat/barley translocation events.

Microsatellites or simple sequence repeats (SSRs)

Simple sequence repeats (SSRs) consist of no more than six repeating base pairs. They are useful when used for marker assisted selection because of the high levels of polymorphisms, and a frequent dispersion of related sequences throughout plant genome. Comparatively little work has been done using microsatellites since their advent in 1991 by Condit and Hubbel, but has proven to be an effective tool for selection of genetic traits.

A GGC microsatellite is widely distributed in the rice genome, and several alleles which differ in copy number of the basic repeat are present for one locus which has been characterized. (GT)n microsatellites are also found in rice, and when oligonucleotides of (GT)n are used as hybridization probes on Southern blots of rice genomic DNA, a large number of restriction fragments are detected, and the resultant patterns are useful as fingerprints (Kochert, 1994).

Saghai-Maroof et al. (1994) have surveyed microsatellite DNA polymorphisms at four loci in barley genotypes representing a wide range of the habitats in which wild and cultivated barley are found worldwide. They indicated that the chromosomal segments marked by the simple sequence repeats (SSRs) alleles are under the influence of natural selection. The SSR variants allow specific DNA sequences to be followed through generations. Thus, the great resolving power of SSR may provide clues regarding the precise targets of natural and man-direct selection.

SSR markers can detect a higher level of polymorphism among inbred lines than any other method (Xiao et al., 1996). Gethi et al. (2002) estimated the level of genetic diversity among and within maize inbred lines from different sources using SSR markers. They found that of the total variation observed in gene frequency, 87.8% was found among inbred lines, 7.6% among sources within inbred lines, and 4.6% within sources. Genotypes of identically named inbred lines from eight different sources differed slightly on the basis of 44 SSR loci.

SSR markers may cost less, use a small amount of DNA and require less time. All of these advantages would be reasons for using SSRs in the marker-assisted selection. Aghaee-Sarbarzen et al. (2001) reported that the use of sequence tagged microsatellite (STMS) markers already mapped to different wheat chromosomes unequivocally indicated that STMS marker *gwm368* of chromosome 4BS was tightly linked to the *Aegilops triuncialis* leaf rust resistance gene transferred to wheat. Moreover, Spielmeyer et al. (2003) found that flanking microsatellite markers were identified and one tightly linked marker (*gwm533*) was shown to be associated with presence of broad-spectrum stem rust resistance gene *Sr2* in wheat. This marker was validated in a wide rang of germplasm and can now be implemented in marker-assisted breeding to facilitate selection for this durable, broad-spectrum but difficult to score rust resistance gene.

Amplification restriction fragment length polymorphism (AFLPs)

Amplification restriction fragment length polymorphism (AFLP), has been described as a powerful technique to identify molecular markers for plant DNA (Vos et al., 1995). AFLP is performed by 1) restriction endonuclease

digestion of genomic DNA and ligation of specific adapters; 2) amplification of the subpopulation of genomic DNA by PCR using primer pairs containing common sequences of the adapter and one to three arbitrary nucleotides; and 3) gel electrophoresis analysis of the amplified fragments. The combination of different restriction endonucleases, the choice of selective nucleotides in the primers, and resolution of sequencing gel makes the results highly reproducible and able to detect multiple polymorphic DNA markers. Therefore, the AFLP technique has become a well-accepted DNA fingerprinting technique for the construction of genetic linkage maps in plants (Lin et al., 1997).

The most straightforward applications of the AFLP technique in marker assisted breeding include genetic distance analysis, variety identification, and isolation of marker tightly linked to specific genes. Genger et al. (1999) found that bulk segregant using the AFLP technique identified large numbers of polymorphisms between resistant and susceptible to *Rhynchosporium secalis* for both barley lines 240 and 245. Although many of these polymorphisms proved not to be linked to resistance, five polymorphisms closely linked to the line 240 resistance gene, and two polymorphisms loosely linked to the line 245 resistance gene, have been identified.

AFLP has the ability to detect large numbers of polymorphisms with single primer pairs, without any prior knowledge of the target genome. The AFLP markers have been successfully used to determine genetic diversity in many plant species including forage crops (Sharma et al., 1996; Pillay and Myers, 1999; Roldan-Ruiz et al., 2000). Mellish et al. (2002) concluded that on the basis of an analysis of AFLP markers, the wheatgrass cultivars Fairway, Parkway, and S9240 appear to be true *Agropyron cristatum*. The clustering of Nordan agrees with other evidence suggesting that it is an allotetraploid of *Agropyron cristatum* and *A. mongolicum*. The clustering of the hexaploid Douglas with Nordan suggests that this cultivar also contain germplasm from both *Agropyron cristatum* and *A. mongolicum*. Within-population variance accounted for 88% of the total AFLP variation in the six *Agropyron* populations assayed, while among-population variance accounted for only 12%.

Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a nucleotide (A, T, C, or C) in the genome sequence is changed. SNPs are the most abundant sequence variations encountered in most genomes (Cho et al., 1999; Griffin and Smith, 2000). Various large-scale discovery projects are currently aiming at identifying SNPs from a broad range of organisms, including crop plants. SNPs use as molecular markers is gaining interest because they have great potential for applications in plant breeding programs, for the identification of genetic loci affecting traits of interest, and for the characterization and exploitation of genetic resources. In addition, a dramatic increase in the number of DNA sequences submitted to databases is facilitating the identification of SNPs in many species, while the single most important factor preventing their widespread development and use appears to be the cost (Chiapparino et al., 2002).

Paris et al. (2001) reported the development of Single Nucleotide Primer Extension (SNuPE) assays enabling co-dominant genotyping of SNPs from small amounts of barley tissue. The method was used to select barley seedlings carrying the *mlo* powdery mildew resistance based on SNPs discovered within genes encoding these traits. Moreover, a high-throughput single nucleotide polymorphism (SNP) genotyping system was developed and used to select barley seedlings carrying superior alleles of beta-amylase (Paris et al., 2002).

Kanazin et al. (2002) evaluated the prevalence of DNA sequence polymorphisms (SNPs) at 54 loci from five barley (*Hordeum vulgare ssp. vulgare*) genotypes selected to represent the three major cultivated spring barley germplasm pools. Thirty-eight of these 54 loci contained single nucleotide polymorphisms. SNPs represent the most common class of genetic marker. As with other classes of markers, the usefulness of the SNPs revolves around their informativeness, transferability, stability and cost per data point. The most desirable type of genetic marker would be unambiguous, inexpensive to assay and would be assayable singly or in parallel with hundreds of other markers.

Transposon mutagenesis

Transposon mutagenesis has been widely exploited in different organisms to isolate genes that encode unidentified products (Sundaresan, 1996). A transposon tagging system in wheat has been developed by introducing the *Ac* transposes gene under the CaMV 35S promoter into cultured wheat embryos by particle bombardment (Takumi, 1996). Thus in future it could be expected traits of commercial importance to be tagged for a wider utility in important crop plants (Patnaik and Khurana, 2001). Also, this method has been used to create mutants libraries and to study of the biology and pathogenesis of a variety of microorganisms by using in vitro and in vivo analysis. Transposon was used as tools for studying whole-genome and single-gene in bacteria, yeast, and other microorganisms. A variety of applications were done by inserting transposon randomly such as genetic footprinting, gene transcriptional and translational fusion, signature tagged mutagenesis (Choi and Kim, 2009). Gene expression, protein localization, and gene disruption of some organisms were studied by tagged transposons. This can be identified of transposon

insertion sites in genome by direct genomic sequencing using a transposon-specific primer (Burns et al., 1994; Horecka and Jigami, 2000). Molecular genetic manipulation tools have been developed and improved after the sequence of the bacterial genome. These tools help scientists to study the genetic that occur naturally in prokaryotes and characterize the genes functions in different environments.

Transposons have been utilized to determine essential genes in the bacterial genome. This application is involved in the generation of random transposon mutants followed by the identification of genes that are depleted during outgrowth under certain circumstances. PCR with a pair of primers, one which anneals to the end of the transposon and the other to a nearby gene of interest, were used to determine the essential genes. This technology was initially exploited for the screening of essential genes in *S. cerevisiae* under different physiological conditions using the Ty1 transposon (Smith et al., 1995; Smith et al., 1996). Moreover, there have been some examples in which genetic footprinting systems have been utilized to identify the essentiality of genes in the genome and the potential functions of a large set of genes with unknown function, in *H. Influenzae* (Akerley et al., 1998; Akerley et al., 2002), *P. Aeruginosa* (Wong and Mekalanos, 2000), and *S. Pneumonia* (Akerley et al., 1998).

Transposons mapping for genes

A number of methods have been described for genetic screening based on randomly inserting modified bacterial transposon sequences into plasmid-based yeast genomic libraries and then transforming pools of the yeast DNA containing the artificial transposons into the yeast genome by recombination (Castano et al, 2003. Burns et al 1994). This result in each clone is marked by a different bacterial insertional event that could be selected phenotypically. For identifying the location of artificial transposon insertions in the yeast genome, it was first sequenced the insertion junctions of five independent URA3-marked Tn7-based artificial transposons present in a plasmid-based yeast genomic library (Kumar et al, 2004). In this way it was known the precise insertion site for each artificial transposon. The yeast DNA segments from the five plasmids were transformed into yeast strain FY3, and cells that had acquired uracil prototrophy by homologous recombination of the segments were chosen. The purified genomic DNA from the transformed strains, pooled the DNA, was digested with *StuI*, and extracted fragments using probes specific to either the 59 end or the 39 end of URA3. In addition, 137 lines with transposed Ds-bar elements have been identified in barley. Of those 137, flanking sequences have been determined for ~80 lines and currently 35 have been mapped (Cooper et al., 2004).

It was found that the modification of the transposon by

the BF638R restriction/modification system increased transposition efficiency six fold (Veeranagouda et al., 2012). They pointed that the EZ::TN5-based mutagenesis was more efficient than other transposon mutagenesis. Insertional mutagenesis is a powerful tool for determining gene function in crop plant species. Cui et al. (2012) found that the Tnt1 element was stably 10 transformed into soybean plants by A. tumefaciens-mediated transformation. Analysis of 99 Tnt1 flanking sequences revealed insertions into 62 (62%) annotated genes, indicating that the element preferentially inserts into protein coding regions. Tnt1 insertions were found in all 20 soybean chromosomes, indicating that Tnt1 transposed throughout the soybean genome (Cui et al. 2012). Also, they demonstrated that the Tnt1retrotransposon was a powerful system and could be used for effective large-scale insertional mutagenesis in soybean.

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