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Methodological Advancement in Molecular Markers to Delimit the Gene(s) for Crop Improvement

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ABSTRACT

Molecular markers, in recent years, have accelerated plant breeding methods significantly with an objective of crop improvement. At present a variety of molecular markers are available and the choice of using a particular type of marker depends on the user. With the advances in the area of genomics, new type and gene-derived markers as well as novel approaches such as genetical genomics, linkage disequilibrium (LD)-based association mapping, etc. have been developed for identification of "perfect" markers for their use in breeding practices. The present article provides an overview on presently available but main type of molecular markers and their use in trait mapping, map-based cloning, estimation of diversity in germplasm collection to understand the population structure as well as in the area of comparative genomics. While dealing the above topics, major emphasis have been given on modern genomics tools and approaches such as functional molecular markers (EST-SSRs, EST-SNPs, SFPs), expression genetics or genetical genomics, high throughput approaches and automation technologies, public databases, etc. Utilization of modern genomics approaches such as functional genomics coupled with molecular marker technologies have a great potential to facilitate plant breeding practices and thus marker-assisted breeding seems to be evolved to genomics-assisted breeding in the near future.

1. INTRODUCTION

For any crop improvement 'creation of variation' and 'effective use of available variation' are important. These variations can be obtained from either crossing two different parental genotypes or selecting existing variation from the enormously available germplasm in the plant kingdom. Ancient farmers were the first to start "plant breeding" by selecting the best plant lines for their needs and brought them into cultivation. There is enough archeological evidence to show how farmers employed selection pressure to meet their demands as early as 12,000 years ago. As knowledge continues to grow, plant breeding has evolved as a major discipline in plant biology. There were many landmarks in plant breeding after the re-discovery of Mendelian genetics. Crossing two morphologically different parental genotypes allowed plant breeders to study recombination and crossing-over events. Morphological markers played a major role in following genetics of the traits e.g. flower colour and shape, seed size and colour, plant height, etc. Morphological markers are not necessarily simple Medelian inherited genes, which caused a set-back for plant breeders in their breeding programs. With the limited availability of morphological markers, plant breeders were forced to find stable detectable variation among the parents in their crossing programs for the improvement of crop plants.

There is an enormous amount of genetic diversity at the DNA level in higher plants such that no two organisms are likely to be identical in DNA base sequence. There is also a tremendous amount of DNA variation present in natural populations of plants. Molecular biology has offered molecular markers to detect such DNA variation, which can be used to guide traditional plant breeding. Once linkage between a marker locus and the gene for an agronomic trait of interest has been established, DNA diagnostic tests can be used to guide plant breeding. This potential revolution leads to the understanding of, and ability to manipulate, oligogenic and quantitative traits with the recent advances in genetic marker technology. The development and availability of abundant, naturally occurring, molecular genetic markers during the last two decades (**Table 1**) has generated renewed interest in counting, locating and measuring the effects of gene or genes (polygenes or QTLs) controlling quantitative traits.

Table 1 List of DNA marker techniques, that have been evolved over the years.

Acronym	Technique	Reference
AFLP	amplified fragment length polymorphism	Vos *et al*. 1995
AM-PCR	anchored microsatellite primed PCR	Wolff *et al*. 1995
AP-PCR	arbitrarily primed PCR	Welsh and McClelland 1990
ASA	allele-specific amplification	Wu *et al*. 1989
ASSR	anchored simple sequence repeat	Wu *et al*. 1994
CAPS	cleaved amplified polymorphic sequence	Akopyanz *et al*. 1992
DAF	DNA amplification fingerprinting	Caetano-Annolles *et al*. 1991
DALP	direct amplification of length polymorphism	Desmarais *et al*. 1998
DAMD-PCR	direct amplification of microsatellite DNA by PCR	Heath *et al*. 1993
DFLP	DNA fragment length polymorphism	Hongtrakul *et al*. 1998
dRAMP	digested RAMP (see later for RAMP)	Becker and Heun 1995
IFLP	intron fragment length polymorphism	Hongtrakul *et al*. 1998
IM-PCR	inter-microsatellite PCR	Zietkiewicz *et al*. 1994
IRAP	inter-retrotransposon amplified polymorphism	Kalendar *et al*. 1999
ISA	inter-SSR amplification	Zietkiewicz *et al*. 1994
ISSR	inter-simple sequence repeats	Zietkiewicz *et al*. 1994
MAAP	multiple arbitrary amplicon profiling	Caetano-Annolles 1994
MP-PCR	microsatellite-primed PCR	Meyer *et al*. 1993
OLA	oligonucleotide ligation assay	Landegren *et al*. 1988
RAHM	randomly amplified hybridizing microsatellites	Cifarelli *et al*. 1995
RAMPO	randomly amplified microsatellite polymorphisms	Richardson *et al*. 1995
RAMP	randomly amplified microsatellite polymorphism	Wu *et al*. 1994
RAMS	randomly amplified microsatellites	Ender *et al*. 1996
RAPD	random amplified polymorphic DNA	Williams *et al*. 1990
RBIP	retrotransposon-based insertion polymorphism	Flavell *et al*. 1998
REMAP	retrotransposon-microsatellite amplified polymorphism	Kalendar *et al*. 1999
RFLP	restriction fragment length polymorphism	Botstein *et al*. 1980
SAMPL	selective amplification of microsatellite polymorphic loci	Morgante and Vogel 1994
SCAR	sequence characterised amplified regions	Paran and Michelmore 1993
SFP	single feature polymorphism	Borevitz *et al*. 2003
SNP	single nucleotide polymorphism	Landegren *et al*. 1988
SPAR	single primer amplification reactions	Gupta *et al*. 1994
S-SAP	sequence-specific amplification polymorphism	Waugh *et al*. 1997
SSCP	single strand conformation polymorphism	Hayashi 1992
SSLP	simple sequence length polymorphism	Tautz 1989
SSR	simple sequence repeat	Hearne *et al*. 1992
STAR	sequence tagged amplified region	Rafalaski and Tingey 1993
STMS	sequence-tagged microsatellite site	Beckmann and Soller 1990
STR	short tandem repeat	Edwards *et al*. 1991
STS	sequence tagged site	Olson *et al*. 1989
VNTR	variable number of tandem repeats	Nakamura *et al*. 1987

2. MOLECULAR MARKERS

Molecular markers can be of any kind of marker system that differentiates two individuals at the molecular level. Many types of molecular markers are presently available, but no single marker technique is generally applicable for all applications. As early as the 1970s, protein markers (enzymes) were mainly used as molecular markers. Enzymes (i.e. protein, or direct of gene products) can be visualized using specific stains to get a visible product as a band in an electrophoretic system and different forms of an enzyme (reflected in different colored bands), are called isozymes (Tanksley and Orton 1983). Other than isozymes, proteins markers can also be resolved based on their charge and size through the separation by a two-dimensional gel electrophoreic system. The major limiting factor for isozymes are their low number, and most of them are either tissue- or developmental stage-specific.

The advent of DNA recombinant technology opened the area of development and exploitation of DNA-based markers which was further tuned after the development of PCR technology. In fact, at present, the term molecular marker is meant only for DNA-based markers. Based on the methodology involved in use of DNA markers, they can be classified into three broad categories.

2.1. Hybridization based markers

***RFLPs**:* Restriction fragment length polymorphism (RFLP), the very first kind of DNA marker developed, is an example of hybridization based marker, which employs cloned DNA sequences to probe specific regions of the genome for variations that are seen as changes in the length of DNA fragments produced by digestion with restriction endonucleases (Landry *et al.* 1987). DNA of the parental genotypes to be surveyed for polymorphism are digested with different restriction enzymes (e.g., *Eco*RI, *Hin*dIII, *Dra*I), separated by agarose gel electrophoresis and transferred onto a nylon membrane. Labeled probes (short known DNA sequences) are allowed to hybridize with the digested DNA fragments which are bound to a nylon membrane and then visualized by autoradiography. RFLPs were used for the first time in the human genome mapping (Botstein *et al.* 1980). In plants, RFLPs were first used in maize (Helentjaris *et al.* 1986), tomato (Bernatzky and Tanksley 1986) and rice (McCouch *et al.* 1988) to prepare the first generation of molecular genetic maps. Subsequently dense RFLP maps were generated for all the major crop species such as barley (Graner *et al.* 1991), wheat (Gill *et al.* 1991), soybean (Shoemaker and Olson 1993), etc. Though RFLPs are the most reliable DNA polymorphism that can be used for accurate scoring of genotypes, due to its time-consuming, laborious protocol with the involvement of radioactivity, RFLPs lost their importance in large-scale mapping and marker assisted selection (MAS, see later) projects for plant breeding applications.

2.2. PCR based markers

PCR markers are based on amplification of sequences using the polymerase chain reaction (PCR). To amplify the targeted sequences, two primers, which should flank the target sequence, are needed. PCR based markers rely on sequence variation in annealing sites or DNA length differences between amplified products obtained from corresponding genotypes.

RAPDs*:* Random amplified polymorphic DNA (RAPD) is the simplest example of a PCR marker that involves the use of 10 bp random primers (Williams *et al.* 1990). RAPDs have been widely used for mapping and genetic diversity studies (e.g. Vierling and Nguyen 1992, Fernández *et al.* 2002), but due to their poor reproducibility and lack of locus specificity, scientist tried other possibilities to find reliable and reproducible markers.

AFLPs*:* Amplified fragment length polymorphism (AFLP) is the one such kind, which combines the merit of RFLP and PCR techniques. The AFLP approach involves generation of a genomic library, creation of smaller fragments after restriction digestion, amplification of the DNA fragments after adapter ligation and PCR amplification and their detection on polyacrylamide gel electrophoreses by using a radioactive assay (Vos *et al.* 1995). AFLP markers are abundant in nature and have been used for construction of genetic linkage maps (eg. Mano *et al.* 2001), high density linkage map of a targeted region (eg. Komatsuda *et al.* 2004), identification of QTLs controlling complex traits (eg. Yin *et al.* 1999) and studies on genetic diversity (Ellis *et al.* 1997, Schut *et al.* 2001). AFLP requires no specific prior knowledge about the genome, but despite this merit, still remains inaccessible for locus-specific applications in large segregating populations. Yet another limitation of AFLP is its dominant nature. Methods for converting AFLP markers into co-dominant sequence-tagged sites (STSs) were developed and utilized in some mapping programs (Brugmans *et al.* 2003). However, this is not very user-friendly, routine and always successful (Shan *et al.* 1999). Another drawback of the approach includes the generation of AFLP fragments from the repetitive portion of the genome. The cDNA-AFLP technique was developed to overcome the problem of repetitive elements; which applies to the standard protocol on a cDNA template (Bachem *et al.* 1996). The methylation sensitive enzymes for restriction digestion in AFLP procedure may decrease the ratio of repetitive DNA sequence (Weersena *et al.* 2003)

SSRs or Microsatellites: A major break-through in molecular marker technology, perhaps, happened with the introduction of microsatellites or simple sequence repeats (SSRs), a form of variable number of tandem repeats, which can be of two to many (generally up to six) repeated nucleotides. Since microsatellites are abundant in the genome, locus-specific, co-dominant, highly polymorphic and amenable for high-throughput, these were extensively used to develop genetic maps in rice, wheat, barley, and maize (Röder *et al.* 1998, McCouch *et al.* 2002, Sharopova *et al.* 2002). The generation of microsatellites generally involves the creation of a genomic or SSR-enriched library, subsequent screening the library for identification of SSR containing clones and their sequencing for identification of microsatellites. Thus the generation of microsatellites in a traditional manner has been an expensive and time-consuming task (Gupta and Varshney 2000). In recent years, due to the availability of an enormous amount of sequence data such as ESTs in the public domain, it has been possible to utilize the available sequence data to screen for microsatellites (see later).

2.3. Sequence based markers

The significant progress in the area of genomics, is to develop markers which can detect polymorphism at the single base pair level. The development and utilization of such markers, however, is still at an initial phase.

SNPs: The recent marker system for detection of polymorphism at a single base is single nucleotide polymorphism (SNP). In fact, the availability of extensive sequence databases made a new avenue, the 'SNP" to exploit as a high-throughput marker for genome mapping studies. The availability of abundant, high-throughput sequence-based markers is essential for detailed genome-wide trait analysis. SNPs are markers amenable for high-throughput techniques, which show greatest sequence variation; a significant amount of effort has been invested in resequencing alleles to discover SNPs. There are techniques to detect SNPs such as allele-specific PCR, single base extension and array hybridization methods (Gupta *et al.* 2002). Since SNP discovery and moreover SNP genotyping require expensive and sophisticated platforms, the development and exploitation of SNP markers is still restricted to major crop species such as rice (Nasu *et al.* 2002), wheat (Somers *et al.* 2003), barley (Kota *et al.* 2001, Kanazin *et al.* 2002), maize (Tenallion *et al.* 2001), and soybean (Zhu *et al.* 2003).

3. PRE-REQUISITE FOR LINKAGE MAPPING AND IDENTIFICATION OF GENE(S)

The most critical decision in constructing linkage maps with DNA markers is made in developing the mapping population. In making these decisions, several factors must be kept in mind, the most important of which is the goal of the mapping project. Young (1994) reviewed the important factors for a mapping project, whose success or failure is mainly dependent on which parents are chosen for crossing, the size of the population, how the cross is advanced and which generations are used for DNA and phenotypic analysis. Sufficient detectable DNA sequence polymorphisms between parents must be present. In the absence of detectable DNA polymorphism, segregation analysis and linkage mapping are impossible. Naturally outcrossing species tend to have high levels of DNA polymorphisms and virtually any cross that does not involve related individuals will provide sufficient polymorphism for mapping (Helentjaris 1987).

Once suitable parents have been identified, the type of genetic population to be used for linkage mapping must be considered. Several different kinds of genetic populations are suitable. The simplest are the F_2 population derived from true F_1 hybrids, and their backcross (BC) populations. For most plant species, F_2 populations are easy to construct, although sterility in the F_1 hybrid can limit some combinations of parents, particularly in wide crosses. In general any segregating population can serve the purpose of linkage mapping. This can be of F_2, recombinant inbred lines (RIL), doubled haploid (DH) lines, backcross from F_1 (BC_1), and backcross from F_2 (BC_2), etc. Usually 10-20 markers are required for each chromosome to give reasonable genome coverage. It is not difficult in populations of most crop species to identify and map 10-50 segregating molecular markers per chromosome pair (Kearsey 1998). Linkage mapping is putting marker loci in order, indicating the relative distances among them, and assigning them to their linkage group on the basis of their recombination values from all pair-wise and three-point combinations. The first map of the human genome based on molecular markers (Botstein *et al.* 1980) fuelled the development of molecular marker-based genome maps in other organisms. Presently we have linkage maps for most of the important plant species (Phillips and Vasil 2001).

4. IMPACT OF GENOMICS ON MOLECULAR MARKERS

4.1. ESTs, EST-SSRs and EST-SNPs

Expressed Sequence Tag (EST) is a short DNA sequence of 150-400 bp, from a cDNA clone that corresponds to an mRNA. ESTs are developed and publicly available for most crop plants (Rudd 2003). As high-throughput functional genomics approaches like Serial Analysis of Gene Expression (SAGE) have been developed, these lead to the generation of more numbers of ESTs. The cDNA clones corresponding to the ESTs of interest can be used as RFLP or CAPS based markers (Varshney *et al.* 2005a). This EST sequence data also serves the purpose of identifying SSRs and/or SNPs. Before the ESTs, development of SSRs, and SNP markers were expensive and required high resource laboratories, but presently any user could download from the database and can use some special bioinformatic programs like MISA for SSR detection (Thiel *et al.* 2003, Varshney *et al.* 2005a) and SNipPer for SNP discovery (Kota *et al.* 2003, Varshney *et al.* 2005c). EST-SSR markers have already been developed in many plants including crop and tree species (Varshney *et al.* 2005a), EST-SNP markers for some crop species such as barley (Kota *et al.* 2001 2003), wheat (Somers *et al.* 2003), maize (Batley *et al.* 2003), sugarbeet (Schneider *et al.* 2003), *inter alia*. There are good numbers of ESTs from ornamental plants publicly available (*Antirrhinum majus*-25,310; *Zinnia elegans*-17,975; *Petunia*-8,773; Rose-3,511; *Alstroemeria peruviana*-998; http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html), which are the useful resource to develop genic markers in ornamental plants also. For the majority of molecular markers developed from ESTs, a putative function can be deduced and therefore they are known as 'functional markers' (Anderson and Lübberstedt 2003, Varshney *et al.* 2005c) that provide an added value. Thus these markers are a good resource for assaying the functional genetic variation in germplasm collection (Kota *et al.* 2001, Eujayl *et al.* 2002). Development of such functional markers, however, is restricted to only those plant species where enough amount of sequence data is available. Nevertheless, functional markers (derived from the cDNA-conserved portion of genomic DNA) have the advantage of being used across related species (Yu *et al.* 2004b, Varshney *et al*. 2005d). Therefore, it is possible to utilize the functional markers from major crop species (rice, wheat, soybean, etc.) to minor or “orphan crops” such as rye and millets (Varshney *et al.* 2005c).

4.2. Microarrays and SFPs

In recent years, genomic research has paralleled the speed in progress in technological excellence in development of microarrays on DNA chips. In any organism, EST databases and/or cDNA libraries can be used for the preparation of cDNA microarrays for a study of the functions of all specific gene sequences and their expression in time and space (Sreenivasulu *et al.* 2002). Microarrays are being developed in many crop plants and are being utilized in a wide range of applications like expression profiles of genes in different organs of a plant system or in different genotypes for a given agronomic or physiological trait, in reverse genetic approaches and in proteomics (Ruan *et al.* 1998). These microarrays can also be used for detection of SNPs and development of molecular markers such as SFPs.

Single feature polymorphism (SFPs) are basically SNP-based markers which are identified in transcript profiling data by visualizing differences in hybridization signals in different cultivars. In fact, the polymorphisms present in DNA are transcribed into the mRNA and can potentially affect the hybridization to the microarrays/GeneChip probes, if present in a region complementary to the probe. Polymorphisms generated during mRNA processing, such as alternative splicing and polyadenylation could also affect hybridization of the target RNA. So far SFPs in plant species have been developed in *Arabidopsis* (Borevitz *et al.* 2003) and barley (Rostoks *et al.* 2004 2005, Cui *et al.* 2005).

4.3. BAC/YAC libraries

A perfect blend of molecular biology with cloning technology has revolutionized the cloning of large-sized DNA fragments. During the late 1980s the creation of yeast artificial chromosomes (YACs) for cloning of megabase-sized DNA fragments became possible, and library-based exploration of even the largest genomes appeared practical (Burke *et al.* 1987). There are some serious drawbacks of YACs as a cloning vector such as chimeric clones (Anderson 1993) or possessing insert rearrangements (Cai *et al.* 1998a 1998b). Such clones are unsuitable for sequencing and mapping research, and a great deal of time is devoted to remove chimeras and clones with rearranged inserts; additionally, manipulation and isolation of YAC inserts is difficult and time-consuming (Woo *et al.* 1994). With these disadvantages of YACs, "bacterial artificial chromosomes" (BACs) emerged as an alternative to YACs (Shizuya *et al.* 1992) to clone up to 500 kb in length. BACs are relatively immune to chimerism, insert rearrangements and easier to manipulate and propagate compared to viral- or yeast-based clones (Marra *et al.* 1997). BAC libraries in which each clone is stored and archived individually are rapidly becoming a central tool in modern genetics research. Such libraries have been made for a different plant species, like Arabidopsis (Choi *et al.* 1995), rice (Nakamura *et al.* 1997), barley (Yu *et al.* 2000), wheat (Lijavetzky *et al.* 1999, Chen *et al.* 2002), soybean (Salimath and Bhattacharyya 1999), apple (Vinatzer *et al.* 1998), Medicago (Nam *et al.* 1999), rose (Kaufmann *et al.* 2003), *Petunia* (McCubbin *et al*. 2000), etc. Recently, by using flow cytometry it has been possible to develop BAC libraries for individual chromosomes. Chromosome-specific BAC/YAC libraries for each of the 24 human chromosomes have been generated from sorted chromosomes (Kim *et al.* 1995). Chromosome or chromosome arm specific libraries will reduce the complexity in sequencing the gene space in polyploid species like wheat that has a large genome and many chromosomes (Safar *et al.* 2004).

Although large insert libraries mentioned above are mainly used in preparation of local and genome wide physical map after high-throughput DNA fingerprinting (Marra *et al.* 1997) and contig assembly (Ding *et al.* 1999) in map-based cloning and genome sequencing projects, markers (especially STS or CAPS) can also be developed from BAC- end sequences (Venter *et al.* 1998). Gene-rich BAC clones are a useful resource for development of genetic markers.

5. APPLICATIONS OF MOLECULAR MARKERS

5.1. QTL mapping and marker-assisted selection

A “QTL”, the acronym for Quantitative Trait Locus, is one of the genes or gene blocks that underlie a quantitative trait (Gelderman 1975). Before the discovery of molecular markers, QTLs were referred to as polygenes (Mather 1949). QTL analysis is predicated on associations between

phenotypic values for the quantitative trait and the marker alleles segregating in the mapping population. It has two essential stages; the mapping of markers and the association of the trait phenotype values with the marker genotypes. The basic theory underlying marker mapping has been available since 1920. Sax (1923) first reported association of simply inherited genetic markers with a quantitative trait in plants when he observed segregation for seed size associated with segregation for a seed coat colour marker in beans (*Phaseolus vulgaris* L.). Rasmusson (1935) demonstrated linkage of flowering time (a quantitative trait) in peas (*Pisum sativum* L.) with a simply inherited gene for flower colour.

After the advent of molecular marker techniques, genetic maps were developed or saturated with molecular markers for almost all crop plant species (Phillips and Vasil 2001). The phenotyping data obtained for the progeny lines in different environments together with marker data for the mapping population provided the molecular markers linked with gene(s) or QTLs for the corresponding phenotyping trait. Moreover, in the last few years, with the availability of a high number of common markers mapped in different mapping populations of major crops led to the possibility of construction of consensus maps (Varshney *et al.* 2004). So it is often useful to take the publicly available information for mapping into other mapping populations. A dense map with reliable quantitative data is essential to precisely locate QTLs of a trait of interested.

Molecular marker technology – after conducting QTL or gene mapping –has potential to maximize the rate and effectiveness of genetic gain using marker-assisted selection (MAS) in plant breeding. The idea behind MAS is that there could be genes with significant effects that could be targeted specifically in selection. The relative efficiency of MAS over phenotypic selection in plant breeding has been the subject of several theoretical studies (Gimelfarb and Lande 1994 1995, Whittaker *et al.* 1995 1997, Hospital *et al.* 1997, Luo *et al.* 1997, Moreau *et al.* 1998). Selection of individual plants based on an index had also been proposed by Lande and Thompson (1990) where index is derived by combining phenotype and a molecular value predicted using a marker. Few studies have also demonstrated the possibility of success via MAS in actual breeding programs (Young *et al.* 1995, Young 1999). Although there is a large body of information available on detection of QTLs, still very few efforts have been made towards introgression of these QTLs in to elite materials using MAS. There are only a handful of reports where marker-assisted introgressions lead to release of a commercial variety (Koebner 2004, Langridge 2005). For instance, recently, marker-assisted improvement of an existing commercial hybrid (HHB 67) in pearl millet led to the development and release of downy mildew resistant version of the hybrid at the national level in India (T. Hash, ICRISAT, pers. comm.). In several other crops such as common beans, maize, rice, and tomato, MAS is being used routinely for qualitative traits (controlled by major genes) mostly by the private sector (Koebner 2004). Whereas, MAS for quantitative traits (controlled by minor genes with small effects) still remains to be a matter of concern, no doubt, molecular marker technology is a powerful tool with the potential to manipulate plant breeding. MAS is now perceived as a relatively less expensive procedure to accomplish breeding tasks in a more effective manner than previously possible with conventional breeding approaches. Despite many advantages of the technology, the potential of MAS has yet to be fully realized in the hands of plant breeders. There are a number of factors behind slow integration of MAS into actual plant breeding programs such as: high costs of establishing a biotechnology laboratory, less usefulness of markers when the target trait is under the control of minor genes with small phenotypic effects (Koebner and Summers 2002). In spite of all the difficulties, the prospects of MAS continue to be bright. Speed with which the field of biotechnology is advancing will make MAS cheaper and more effective. Furthermore, as more and more associations of traits with markers are established, characterized and mapped, the power of MAS for developing more durable cultivars will increase substantially in the near future. Moreover, we should remember that MAS, like any other single technology/tool, will not be a "silver bullet" solution to all the breeding problems.

5.2. eQTL (expression QTL) and genetical genomics

In recent years, functional genomics approach has been combined with QTL analysis and the combined approach to identify the candidate genes for a trait of interest has been proved very promising and gained centre stage in genome analysis. In this approach, expression profiling of individual genes is conducted in a segregating (mapping) population and subjected to QTL analysis. The combined approach is called genetical genomics (Jansen and Nap 2001) or expression genetics (Varshney *et al.* 2005b) and QTLs by used expression data are called e(xpressed)QTLs.

Genetical genomics or expression genetics involves hybridizaton of total mRNA or cDNA of the organ/tissue from each individual of a mapping population onto a microarray carrying a large number of cDNA fragments representing the species/tissue of interest and quantitative data are recorded reflecting the level of expression of each gene on the filter. The expression data could be used as quantitative data and used for QTL mapping. There are softwares for visualization like Expressionview for combined visualization of gene expression data and QTL mapping (Fischer *et al.* 2003). From the results of expression pattern of segregating populations, eQTL analysis identifies gene products influencing the quantitative trait (level of mRNA expression) in *cis* or *trans*, which identify a regulated gene within the QTL and genes outside the QTL. The *trans*-acting is of specific interest because more than one QTL can be connected (Schadt *et al.* 2003). The mapping of eQTLs allows multifactorial dissection of the expression profile of a given mRNA/cDNA, protein or metabolite into its underlying genetic components, and also allows locating these components on the genetic map (Jansen and Nap 2001, Jansen 2003). For crops with the availability of genomic sequences with annotation of the genomic regions (such as rice), the identification of the genes and their regulatory sequences involved in the expression of an individual trait will be very straight forward (Sreenivasulu *et al.* 2004a 2004b). In mouse, humans, *Arabidopsis* and maize, this approach has been used for a genome-wide study to detect expression of individual genes/QTLs for specific traits (Schadt *et al.* 2003). More recently, Kirst *et al*. (2004) used this approach to map expression profiles associated with xylem growth in eucalyptus. Using 91 lines from an interspecific backcross between *Eucalyptus grandis* and *Eucalyptus globulus,* they identified numerous gene expression patterns correlated with differences in xylem growth. Many of the differentially expressed genes are known to be involved in the biosynthesis of lignin and lignin components and shared expression QTL with a wood growth QTL.

5.3. Physical mapping and Map-based cloning

While a genetic map shows the recombinational distance between markers or genes, the physical map is the map depicting the real (physical) distance between markers. Once we have a physical map of the region harboring the gene of interest, we can isolate it. Basically gene isolation could be made using two different approaches viz., forward and reverse genetics. In reverse genetics, the mutant phenotype will be induced by insertional mutagenesis through T-DNA or gene silencing and RNA interference (RNAi) (Matzke *et al.* 2001). Map-based cloning (MBC) is the

outcome of forward genetics, in which the natural or induced gene will be mapped together with a large number of markers for genetic mapping and then a physical contig will be made, that is eventually used for gene isolation. The MBC approach involves the utilization of molecular markers, preparation of a high density genetic map around the region of interest and ultimately the local physical map. The simplified stepwise scheme is given in **Fig. 1**.

Reasonable resolution power of genetic map required a large segregating population (*circa*. 2,000 F_2 plants). The targeted region should be saturated with more number of markers; this can be achieved by bulk segregant analysis (Michelmore *et al.* 1991). Chromosome walking is feasible once a high-resolution map has been made for the targeted gene. This can be started by screening a genomic library using flanking or co-segregating markers of the targeted gene. Most of the important crops plants are presently having large insert libraries (i.e. BAC/YAC, see **4.1.**). It is desirable to know if our gene of interest exists in the constructed genomic library. The identified positive clones of BAC/YAC library will be then used for sequencing (Kelley *et al.* 1999), subcloning (Ripoll 2000) or shot-gun sequencing (Stein *et al.* 2000) to identify low-copy sequences to generate a new marker. The newly developed marker resultant of chromosome walking will be used for the next round of screening. The positive clones supposed to have the gene of interest should be sequenced followed by annotation. There are many success stories for map-based cloning; being a model crop for gramineae 'rice' has been considered to be the best crop for map-based cloning (Stein and Graner 2004, Salvi and Tuberosa 2005). For instance in rice, resistance genes *Xa21* (Song *et al.* 1995) and *Pib* (Wang *et al.* 1999) were cloned. Advances in detection and precise identification of QTLs, allowed the feasibility to isolate QTLs as simple inherited monogenic traits.

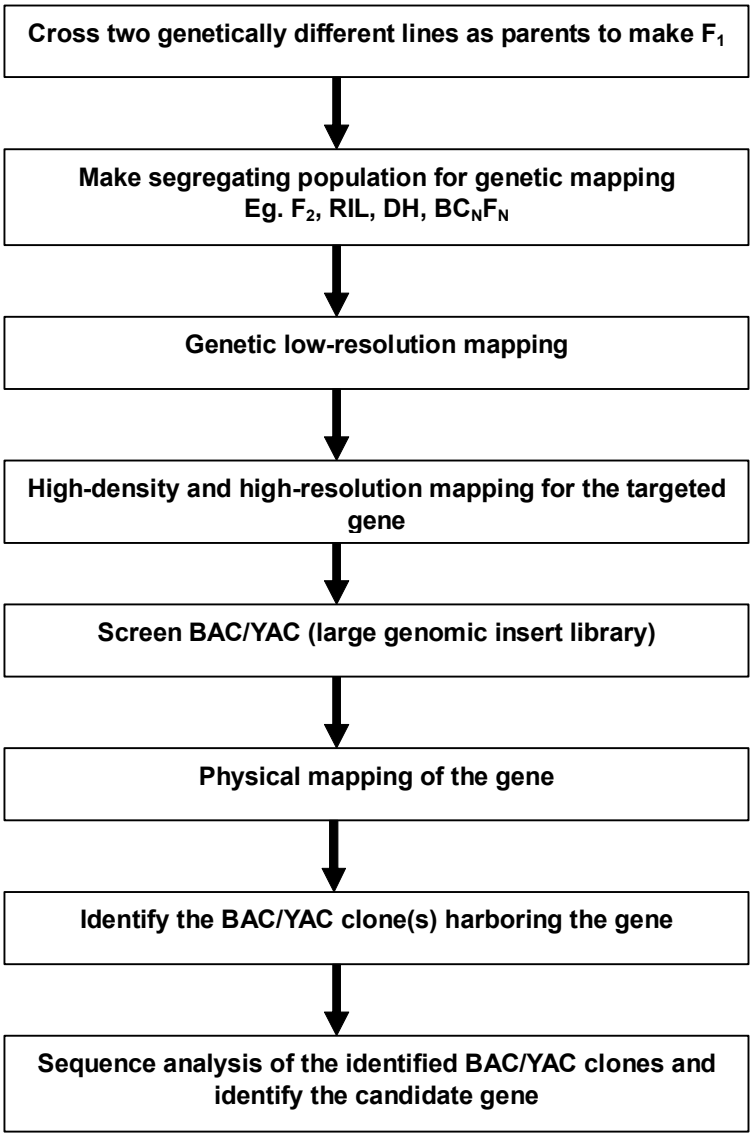

Fig. 1 Schematic outline of map-based gene isolation.

5.4. Diversity studies in plant genetic resources

Another important use of molecular markers is to identify superior alleles after mining the genetic resources for plant breeding. Genetic resources can be defined as all materials that are available for improvement of a cultivated plant species (Becker 1993). An understanding of germplasm diversity and genetic relationships among breeding materials is an invaluable aid for crop improvement strategies. In general diversity studies focused on inventory and establishing core collections, assessment of the mating system and the population structure and heterotic grouping of potential lines suitable for hybrid breeding. Conventional analyses of genetic diversity in germplasm accessions, breeding lines and populations have relied on pedigree information, and morphological and agronomic performance data. With the advent of DNA marker technologies, there is an improvement of the accuracy and number of lines that can be assessed in germplasm collections (Mohammadi and Prasanna 2003). The tools of genome research unleashed the genetic potential of wild and cultivated germplasm resources for the breeding program (Gur and Zamir 2004). Molecular markers are extensively used in diversity studies to identify genetically similar or distinct accessions, and to determine individual degrees of heterozygosity and heterogeneity within populations of plant genetic resources. Over the last 20 years, the number of diversity studies conducted increased almost linearly (Haussmann *et al.* 2004). There are a huge number of published diversity studies mainly based on isozymes, RAPDs and AFLPs. Haussmann *et al.* (2004) reviewed diversity studies from 1984-2003 and reported about 2,500 published works during 2003. Diversity studies during 2003 comprised a wide range of species of which surprisingly tree species accounted for the majority followed by cereals, legumes, fruits, vegetables and ornamental plants. In recent years genetic diversity in germplasm collection instead of using molecular markers has been shifted to sequence diversity. Sequence diversity in genetic resources has been exploited in novel approaches called 'association mapping' (Buckler and Thornsberry 2002) or 'allele mining' (Comai *et al.* 2004). While association mapping provides the perfect molecular marker(s) for the trait of interest, the allele mining approach provides the allelic series for a given gene in the germplasm collection that can be used for creating a useful haplotype to improve the breeding strategies.

5.5. Transferability and comparative mapping

Initially molecular markers (RFLPs) were developed for only a few crop species and the plant geneticists/plant breeders started to use molecular markers of one species in another. These results suggested that molecular markers can be used across species and genera. This also gives interesting insights of relationships across species and genera. It was postulated that grass genome be the 'Lego model" in 1995 (Moore 1995). Later a comparative map diagram, commonly called the circle diagram revealing the relationships among different cereal species was prepared in 1998 (Gale and Devos 1998) that kept on being updated (Devos *et al.* 1999 2000, Gale and Devos 2000). Based on colinearity/synteny,

molecular markers from wheat or barley were used to enhance the genetic maps of minor cereal species such as rye, or millets (Khlestkina *et al.* 2004). An extensive review on comparative mapping studies between Arabidopsis and Brassicaceae, within the species of Poaceae, Solanaceae and Fabaceae was done by Paterson *et al.* (2000). Comparative mapping has been proved very useful for using the information from one species to benefit that of another species.

In recent years, due to the availability of sequence data for a number of plant species (generally in the form of ESTs) the comparative mapping approach has been upgraded to comparative genomics. Comparative genomics implies the study of similarities and differences in structure and function of hereditary information across taxa, and uses molecular tools to investigate many notions that long preceded identification of DNA as the hereditary molecule. Release of the complete sequence of the *Arabiodopsis* genome (TAGI 2000) and rice genome sequences (Goff *et al.* 2002, Yu *et al.* 2002, Cheng *et al.* 2005, IRGSP 2005, http://rgp.dna.affrc.go.jp/IRGSP/), together with an enormous amount of ESTs for different plant species (Rudd 2003) has contributed substantially for the comparative mapping in cross-species or sometimes cross-genera comparisons in monocotyledonous and docotyledonous. The high genetic colinearity of the rice (*Oryza sativa*) genome with the larger genomes of maize, barley, and wheat (Gale and Devos 1998, Paterson *et al.* 2000) was reported; likewise there is also high colinearity between brassica and *Arabidopsis* (Paterson *et al.* 2000). It was demonstrated and model genomes were utilized for studying genome evolution and to support gene isolation. For instance, the colinearity based gene isolation of *Vrn*1 gene in wheat (Yan *et al.* 2003), and barley gene *ror*2 (Collins *et al.* 2003), *Rpg1* (Brueggeman *et al.* 2002), and *Rph*7 (Brunner *et al.* 2003) were successfully accomplished using rice as a model genome.

6. TECHNOLOGICAL ADVANCEMENT IN GENOMICS

6.1. Automation and High-throughput technologies

Generation of marker data for identifying gene(s) involves steps such as DNA isolation, primer development, electrophoresis, hybridization, visualization, scoring and interpretation. Automation should be necessary for most of these steps, if we need to reduce costs and errors. With the robotic assisted lazer technologies, it is possible to prepare several hundreds of DNA samples per day per person. Among the different marker systems described, the markers which are amenable for high-throughput has drawn more attentions than laborious markers. For genotyping of SSRs and/or AFLPs in a large number of entries of a segregating population high throughput approaches are absolutely necessary. The high-throughput starts from DNA extraction with Matrix Mill (Harvester Technology Inc., USA) or FastPrep System (Qbiogene, USA), followed by PCR amplification. Normally genotyping for MAS, the low quality and less quantity of DNA will serve the purpose. Presently available DNA extraction technologies allow one person to extract more than 500 samples per day. Alternatively there are semi-automatic systems mainly for pipetting would maximize the time efficiency. Fluorescent labeled primers with different dyes (FAM, HEX, TAMRA, NED, etc) and genotyping in automated sequencing machines allow to combine about 3-8 markers together in one experiment (multiplexing). To scale-up the automation, markers which are non-gel based would be the best marker system; SNPs are the optimal for automation, for instance MALDI-TPF MS can be utilized for discriminating between two alternative alleles of a SNP (Griffin and Smith 2000) and TMHA HPLC WAVETM DNA molecular platforms comprising HPLC/capillary electrophoresis system (Transgenomics, USA) are also used for automated screening involving SNP discovery and detection.

6.2. Public database and sequencing projects

With the continuous accumulation of genomic resources such as ESTs, clustering and annotation of gene indices, marker sequences, linkage/QTL maps, consensus and comparative maps, made the importance of bioinformatics to the laboratory scientists mainly doing wet-lab work. It is necessary to get to know the knowledge of available databases and the programs used to retrieve and manipulate and/or interpret result from these resources. For instance, scientists working with Triticeae species, must be in regular access with NCBI (www.ncbi.nlm.nih.gov), TIGR (www.tigr.org), Plant data base (www.plantgdb.org), Gramene (www.gramene.org), GrainGenes (http://wheat.pw.usda.gov), cerealDB (www.cerealsdb.uk.net) and HarvEST (harvest.ucr.edu). Likewise there are databases for specific crops or plant species, rice (http://rgp.dna.affrc.go.jp/), Arabiodopsis (http://www.arabidopsis.org/), tomato (http://www.sgn.cornell.edu/), Medicago (http://www.medicago.org/genome/). A recent update on use of bioinformatics in the field of plant genomics is available in Edwards and Batley (2005).

7. FUTURE DIRECTIONS OF MARKER RESEARCH

As evident, a variety of molecular markers and techniques have been developed for genome analysis. Linkage/QTL mapping on one hand provided the useful molecular markers linked with QTLs or genes for trait of interest for their use in MAS or MBC, genetic diversity studies on the other hand provided the means of exploiting the potential of genetic resources locked in the genebanks. In the present genomics or post-genomic era when the sequence data have already become available through genome or EST sequencing projects for some plant species and similar efforts are underway for many other plant species, it has been possible to develop the molecular markers (and novel markers like SFP, SNP) directly from genes. Development of such functional markers may speed up in coming years as these markers will prove promising in marker-assisted breeding and a useful resource for assessment of functional diversity in germplasm collection. Further the comparative genomics in coming years will not only assist us in using information from one species to the other but it will also enhance our knowledge about the genome relationship and evolution.

Functional genomics presently is gaining momentum and will be centre stage in plant genetics in coming years. When we map gene(s), we should know their functions and their network, and how their interaction results in a complex phenomenon. How these genes are regulated and interacted not only with each other but also by the environment? How this knowledge can help us to predict a phenotype for a given genotype in more efficient way? What are the factors present in a genome that mask the desired phenotype? Certainly answering such questions is difficult at present. However, functional genomics approaches such as transcriptomics, genetical genomics/expression genetics, TILLING (Targeting Induced Local Lessions In Genome), association mapping and allele mining possess the potential to answer the above questions to some extent

in the near future. Exploitation of genomics approaches to delimit the genes for a trait of interest may lead us towards genomics-assisted breeding for crop improvement (Varshney *et al.* 2005b). Without any doubt all these approaches will help and supplement the breeding practices, but they can not replace the conventional breeding.

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