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REVIEW ARTICLE

MOLECULAR MARKERS AND THEIR APPLICATION IN IMPROVEMENT OF VEGETABLE CROPS

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ABSTRACT

Marker is a tag which is conspicuous or apparent or which helps in identification of traits. There are three different types of markers viz., morphological, biochemical and molecular. Morphological markers have certain constraints i.e. narrow diversity, influenced by environment, problem with epistasis, pleiotropy, incomplete penetrance and variable expressivity. Biochemical markers reveal polymorphism of sequences of certain proteins. It has certain constraints like redundancy of genetic code, incomplete genomic coverage and also sensitive to environmental pressures. Molecular markers directly reveal the polymorphism at the level of DNA. These are tags that can be used to identify specific genes and locate them in relation to other genes.

INTRODUCTION

There are mainly two types of molecular markers i.e. Hybridization based or Non-PCR based marker for example Restriction Fragment Length Polymorphism (RFLP) (Botstein et al. 1980). Polymerase chain reaction (PCR) based markers example Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990), Sequence characterized amplified regions (SCARs) (Michelmore et al., 1991) and Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995). Molecular markers are used for development of saturated genetic maps; DNA fingerprinting; Phylogenetic and evolutionary studies; heterotic breeding; gene tagging and marker assisted selection (MAS). Identification of vegetable crop varieties by molecular markers in tomato (Noli et al., 1999), Potato (Ashkenazi et al., 2001), Onion, garlic and related species (Fischer and Bachmann 2000). Molecular markers are linked to major disease resistance in tomato like *Meloidogyne incognita* (Williamson et al., 1994) and Tomato mosaic virus (Sobir et al., 2000).

A molecular marker is a DNA sequence that is readily detected and whose inheritance can easily be monitored. The use of molecular markers is based on naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit for applied purposes.

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A marker must be polymorphic; that is, it must exist in differ forms so that chromosome carrying the mutant gene can be distinguished from the chromosome with the normal gene by a marker it also carries.

A molecular marker should have some desirable properties.

i) It must be polymorphic as it is the polymorphism that is measured for genetic diversity studies.
ii) Codominant inheritance: The different forms of a marker should be detectable in diploid organisms to allow discrimination of homo and heterozygotes.
iii) A marker should be evenly and frequently distributed throughout the genome.
iv) It should be easy, fast, and cheap to detect.
v) It should be reproducible.
vi) High exchange of data between laboratories.

Unfortunately, no single molecular marker meets all these requirements. A wide range of molecular techniques is available that detect polymorphism at the DNA level. These have been grouped into the following categories based on the basic strategy, and some major ones are described.

Non-PCR based approaches: Restriction fragment length polymorphism (RFLP)

PCR-based approaches: Random amplified polymorphic DNA (RAPD), microsatellite or simple sequence repeat polymorphism (SSRP), amplified fragment length polymorphism (AFLP), arbitarily primed PCR (AP-PCR) etc.

Targeted PCR and sequencing: Sequence tagged sites (STS), sequence characterized amplified region (SCARs), sequence tagged microsatellites (STMs), cleaved amplified polymorphic sequences (CAPS), etc.

NON-PCR-BASED APPROACHES

Restriction fragment length polymorphism (RFLP):

RFLP was the first technology that enabled the detection of polymorphism at the DNA sequence level. Genetic information, which makes up the genes of higher plants, is stored in the DNA sequences. Variation in this DNA sequence is the basis for the genetic diversity within a species.

In this method, DNA is digested with restriction enzyme(s), which cuts the DNA at specific sequences, electrophorised, blotted on a membrane, and probed with a labelled clone. Polymorphism in the hybridization pattern is revealed and attributed to sequence difference between individuals. The DNA sequence variation detected by this method was termed restriction fragment length polymorphism (Botstein *et al* 1980). By definition, RFLP variation is environmentally independent. Variation in one DNA fragment obtained with a specific enzyme is treated as one RFLP. Plants are able to replicate their DNA with high accuracy and rapidity, but many mechanisms causing changes in the DNA are operative. Simple base pair changes or large-scale changes as a result of inversions, translocations, deletions or transpositions may occur. This will result in loss or gain of a recognition site and in turn lead to restriction fragments of different lengths between genotypes can be detected on Southern blots and by a suitable probe.

Procedure

RFLP analysis comprises the following basic steps:

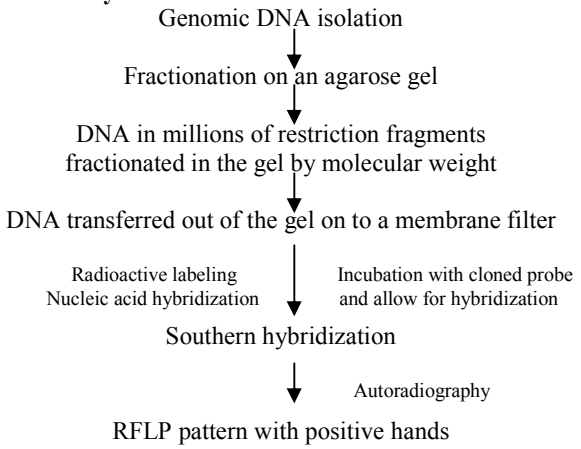
i) DNA isolation,
ii) Cutting DNA into smaller fragments using restriction enzyme(s),
iii) Separation of DNA fragments by gel electrophoresis.
iv) Transferring DNA fragments to a nylon or nitrocellulose membrane filter.
v) Visualization of specific DNA fragments using labelled probes
vi) Analysis of results

Genomic DNA of an organism is isolated and digested with a particular restriction endonuclease. It will cut the DNA into a large number of fragments. Since DNA has a large number of phosphate groups, which are negatively charged at neutral pH, it will migrate toward the anode in an electric field. In a porous medium of agarose or polyacrylamide, DNA fragments will migrate toward the anode at a rate that is proportional to their molecular weight.

Relatively small DNAs of chloroplasts will usually produce about 40 discrete restriction fragments when digested with *Eco* RI and can be separated on agarose gels. These are stained with ethidium bromide and the pattern can be seen under UV light. But digestion of nuclear DNA yield millions of DNA fragments in a continuous range of sizes, and the gel appears to run as a smear. However, individual restriction fragments are still well resolved in the gel and RFLPs are still present between DNAs from different organisms.

Since RFLPs of nuclear DNA cannot be directly visualized, the usual procedure is to use small pieces of chromosomal DNA as probes to detect individual restriction fragments. Using the high specificity of DNA-DNA hybridization, such probes can detect individual restriction fragments in the complex mixture of fragments of nuclear DNA present in a restriction digest. To use this technique, a set of chromosomal DNA fragments are prepared as probes. For preparation of probes, DNA is isolated from the species of interest, digested with a restriction enzyme to generate relatively small fragments, usually of 0.5 - 2.0 kb. Individual restriction fragments are ligated into a bacterial plasmid and the plasmid is transformed into a bacterial cell. By growing these transformed bacteria, one obtains a large supply of a single plant DNA restriction fragments, which are suitable for use as a hybridization probe. Such a set of probes is called a library. Southern blots are prepared from the digested DNA as explained earlier and then probed with one of the cloned probes from the library.

RFLP analysis



The potential of RFLPs as diagnostic markers became evident from studies of the human globin genes, where a direct correlation between the sickle cell mutation carried by a specific β-globin allele and the presence of certain RFLP fragments was evident. The molecular basis of sickle cell anaemia is now precisely known. This is not the case for most heritable traits, particularly for those showing quantitative inheritance. For such traits, genetically closely linked markers that do not influence the phenotypic expression of the trait can be identified and they can serve as diagnostic tools. When a genetic linkage map saturated with marker loci is available, the genome of a species can be systematically scanned for markers cosegregating with a specific trait of interest. Because of their abundance, RFLPs are the first class of genetic markers allowing the construction of highly saturated linkage maps. This was first suggested for the human genome by Botstein *et al* (1980). The potential of this approach in plant breeding was recognized few years later, and its feasibility was experimentally tested in maize and tomato. Since then, an impressive amount of RFLP data has been accumulated in several plant species including important crops. Extensive RFLP linkage maps have been constructed and several genes contributing to agronomically relevant traits have been mapped by RFLP markers.

Advantages of RFLP

1. It permits direct identification of a genotype or cultivar in any tissue at any developmental stage in an environment independent manner.
2. RFLPs are codominant markers, enabling heterozygotes to be distinguished from homozygotes.
3. It has a discriminating power that can be at the species / population (single locus probes) or individual level (multi locus probes).
4. The method is simple as no sequence-specific information is required.

Disadvantages of RFLPs

1. Conventional RFLP analysis requires relatively large amount of highly pure DNA.
2. A constant good supply of probes that can reliably detect variation are needed.
3. It is laborious and expensive to identify suitable marker / restriction enzyme combinations from genomic or cDNA libraries where no suitable single-locus probes are known to exist.
4. RFLPs are time consuming as they are not amenable to automation.
5. RFLP work is carried out using radioactively labelled probes and therefore requires expertise is autoradiography.

PCR based techniques

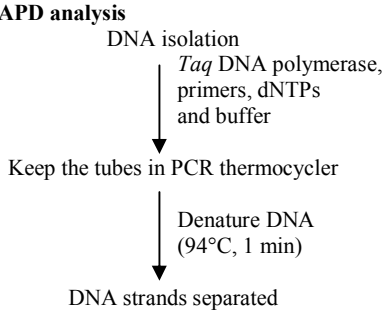
(a) Random amplified polymorphic DNA (RAPD) markers

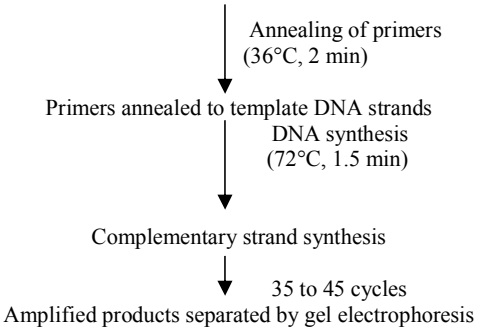
RAPD analysis is a PCR-based molecular marker technique. Here, single short oligonucleotide primers are arbitrarily selected to amplify a set of DNA segments distributed randomly throughout the genome. Williams *et al* (1990) showed that the differences as polymorphisms in the pattern of bands amplified from genetically distinct individuals behaved as Mendelian genetic markers.

RAPD amplification is performed in conditions resembling those of PCR, using genomic DNA from the species of interest and a single short oligonucleotide (usually a 10-base primer). The DNA amplification product is generated from a region that is flanked by a part of 10-bp priming sites in the appropriate orientation. Genomic DNA from two different individuals often produces different amplification patterns (randomly amplified polymorphic DNAs (RAPDs)). A particularly fragment generated for one individual but not for other represents DNA polymorphism and can be used as a genetic marker. Using different combinations of nucleotides, many random oligonucleotide primers have been designed and are commercially available. Such primers can be synthesized in an oligonucleotide synthesizing facility based on sequences chosen at random. No seperate information is required from the plant to be studied. The choice of single primers (or RAPD primers) to use is done operationally. Since each random primer will anneal to a different region of the DNA, theoretically many different loci can be analyzed. 1.

The PCR reaction typically requires cycling among three temperatures, the first to denature the template DNA stands, the second to anneal the primers, and the third to extend at temperatures optimal for *Taq* polymerase. This cycle is usually repeated 25 to 45 times.

Outline of RAPD analysis





Generally, an annealing temperature of 35-40°c is used in PCR reactions for RAPD analysis. Presence of a RAPD band corresponds to a dominant allele against absence of band that corresponds to a recessive allele. Thus, heterozygous and homozygous dominant individuals cannot be differentiated with RAPD markers.

Advantages of RAPD

1.Need for a small amount of DNA (15-25 ng) makes it possible to work with populations which are inaccessible for RFLP analysis.
2.It involves non radioactive assays.
3.It needs a simple experimental set-up requiring only a thermocycler and an agarose assembly.
4.It does not require species-specific probe libraries; thus, work can be conducted on a large variety of species where such probe libraries are not available.
5.It provides a quick and efficient screening for DNA sequence based polymorphism at many loci.
6.It does not involve blotting or hybridization steps.

Limitations

1. RAPD polymorphisms are inherited as dominant-recessive characters. This causes a loss of information relative to markers which show codominance.
2. RAPD primers are relatively short, a mis-match of even a single nucleotide can often prevent the primer from annealing; hence, there is loss of band.
4. RAPD is sensitive to changes in PCR conditions, resulting in changes to some of the amplified fragments.

Amplified fragment length polymorphism (AFLP):

This is a highly sensitive method for detecting polymorphism throughout the genome, and it is becoming increasingly popular. It is essentially a combination of RFLP and RAPD methods, and it is applicable universally and is highly reproducible. It is based on PCR amplification of genomic restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of few nucleotide bases (Vos *et al* 1995).

AFLP involves the following steps :

DNA is cut with restriction enzymes (generally by two enzymes), and double-stranded (ds) oligonucleotide adapters are ligated to the ends of the DNA fragments.

Selective amplification of sets of restriction fragments is usually carried with ^{32}P-labeled primers designed according to the sequence of adapters plus 1-3 additional nucleotides. Only fragments containing the restriction site sequence plus the additional nucleotide will be amplified. Gel analysis of the amplified fragments: The amplification products are seperated on highly resolving sequencing gels and visualized using autoradiography. Fluorescent or silver staining techniques can be used to visualize the products in cases where radio-labelled nucleotides are not used in the PCR.

Advantages

1. This technique is extremely sensitive.
2. It has high reproducibility, rendering it superior to RAPD.
3. It has widescale applicability, proving extremely proficient in revealing diversity.

4. It discriminates heterozygotes from homozygotes when a gel scanner is used.
6. It is not only a simple fingerprinting technique, but can also be used for mapping.

Disadvantages

1. It is highly expensive and requires more DNA than is needed in RAPD (1 mg per reaction).
2. It is technically more demanding than RAPDs, as it requires experience of sequencing gels.
3. AFLPs are expensive to generate as silver staining, fluorescent dye, or radioactivity detect the bands.

Simple sequence repeats (microsatellites):

The term microsatellites was coined by Litt and Lutty (1989). Simple sequence repeats (SSRs), also known as microsatellites, are present in the genomes of all eukaryotes. These are ideal DNA markers for genetic mapping and population studies because of their abundance. These SSR length polymorphisms at individual loci are detected by PCR, using locus-specific flanking region primers where the sequence is known. Thus, STMs require precise DNA sequence information for each marker locus from which a pair of identifying flanking markers are designed. This is impractical for many plant and animal species that are not well-characterized genetic systems. Some of these SSR-based methods have been collectively termed microsatellite-primed PCR (MP-PCR).

Steps of SSRs analysis

- Isolate the DNA of representative cultivar / line.
- Restrict with 4 base pair cutter.
- Size fractionation (0.5 - 0.7 kb)
- Ligate to a suitable vector and transform into *E.coli*.
- Following hybridization identify the desired transformation.
- Go for end sequencing of the selected clones
- Design the primers for amplification.

Advantages of SSRs

- Codominant markers
- Highly polymorphic
- Highly reproducible

Disadvantages

- Costly primer developing.

Sequence tagged sites:

STS is a short unique sequence (60-1000 bp) that can be amplified by PCR, which identifies a known location on a chromosome (Olsen *et al* 1989). Specific PCR markers that match the nucleotide sequence of the ends of DNA fragment can be derived from primers, for example, an RFLP probe or an expressed sequence tag. To date, all STSs that have been used in mapping projects have been derived from well-characterized probes or sequences. STSs are the physical DNA landmarks and PCR is the experimental method used to detect them. STS maps simply represent the relative order and spacing of STSs within a region of DNA. Using this technique, tedious hybridization procedures involved in RFLP analysis can be overcome. STSs have been extensively used for physical mapping of genome. Examples of STSs are:

Sequence tagged microsatellites (STMs)
Sequence characterized amplified regions (SCARs)
Cleaved amplified polymorphic sequence (CAPS).

Sequence tagged microsatellites (STMs):

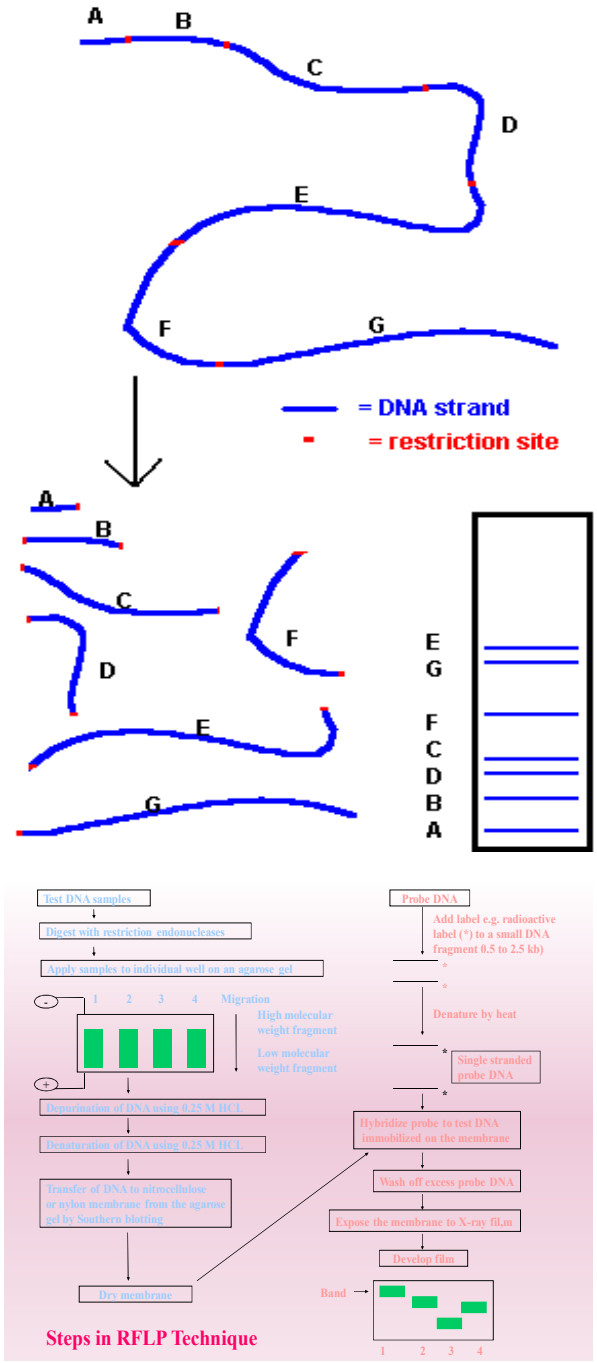
The term microsatellites was coined by Litt and Lutty (1989). Simple sequence repeats, also known as microsatellites, are present in the genomes of all eukaryotes. These are ideal DNA markers for genetic mapping and population studies because of their abundance. These are tandemly arranged repeats of mono-, di-, tri- and tetranucleotides with different lengths of repeat motifs (eg. A, T, AT, GA, AGG, AAC, etc.). A motis is A, AT, AGG etc. and repeat number is denoted by *n*. Thus a repeat $(AT)_9$ means AT nucleotides are tandemly arranged one after another nine times. In a genome of a particular species when this repeat is identified in a gene, which constitutes a microsatellite, the gene is sequenced with its flanking sequences to design primers for amplification of microsatellites. The regions flanking the microsatellite are generally conserved among genotypes of the same species. PCR primers to the flanking regions are used to amplify the SSR-containing DNA fragment. Length polymorphism is created when PCR products from different individuals vary in length as a result of variation in the number of repeat units in the SSR. Genebank sequence data have also been used for designing primers for amplification of microsatellites. Thus, SSR polymorphism (SSRP) reflects polymorphism based on the number of repeat units in a defined region of the genome.

Procedure

A specific microsatellite contained within a stretch of DNA can be amplified by PCR using flanking primer sequences, and then analyzed on Metaphor agarose or polyacrylamide gels. The gels are stained with ethidium bromide and seen under UV light. The variation in length of the PCR product is a function of the number of SSR units. This is a relatively new technique and is specially useful in inbreeding crops such as wheat and barley, which are characterized by low levels of RFLP variation.

Sequence characterized amplified regions (SCARs):

A SCAR is a genomic DNA fragment at a single genetically defined locus that is identified by PCR amplification using a pair of specific oligonucleotide primers. Williams *et al* (1991) converted RFLP markers into SCARs by sequencing two ends of genomic DNA clones and designing oligonucleotide primers based on the end sequences. These primers were used directly on genomic DNA in a PCR reaction to amplify the polymorphic region. If no amplified fragment length polymorphism is noticed, then the PCR fragments can be subjected to restriction digestion to detect RFLPs within the amplified fragment. SCARs are inherited in a codominant fashion in contrast to RAPDs, which are inherited in a dominant manner. Paran and Michelmore (1993) converted RAPD markers into SCARs. Amplified RAPD products are cloned and sequenced. The sequence of primers derived from the termini of a band is identified as a RAPD marker. Two 24-base oligonucleotide primers corresponding to the ends of the fragment (the 5' ten bases are the same as the original 10-mer used in the RAPD reaction and 14 internal bases from the end) have been synthesized. These primers with their increased specificity generally amplify a single highly repeatable band, not the 5-10 bands for the progenitor 10 base primers. SCARs are similar to STSs, but do not involve DNA hybridization for



detection and can therefore contain repeated DNA sequences.

SCARs have several advantages over RAPD markers. RAPDs show dominant nature, amplification of multiple loci, and are sensitive to reaction conditions. The mapping efficiency of RAPD markers in F_2 populations is decreased by their dominant nature. The conversion of dominant RAPDs to codominant SCARs increases the amount of information per F_2 individual. As the annealing conditions for SCARs are more stringent than for RAPDs, SCAR primers detect only one locus. Also, the use of longer oligonucleotide primers for SCARs allows a more reproducible assay than the one obtained with the short primers used for RAPD analysis. SCARs can readily be applied to commercial breeding programs as they do not require the use of radioactive isotopes.

Table 1: Molecular marker technique:

	Technique	Reference
RFLP	Restriction fragment length polymorphism	Botstein *et al* (1980)
SSCP	Single-strand conformation polymorphism	Orita *et al* (1989)
SSLP	Minisatellite simple sequence length polymorphism	Jarmen and Wells (1989)
AP-PCR	Arbitrarily-primed PCR	Welsh and McClelland (1990)
RAPD	Random amplified polymorphic DNA	Williams *et al* (1990)
AS-PCR	Allele specific PCR	Sarkar *et al* (1990)
SAP	Specific amplicon polymorphism	Williams *et al* (1991)
SCAR	Sequence characterized amplified region	Williams *et al* (1991)
DAF	DNA amplification finger printing	Caetano-Anolles *et al* (1991)
SSR	Simple sequence repeats	Hearne *et al* (1992)
CAPS	Cleaved amplified polymorphism sequence	Lyamichev *et al* (1993)
SSLP	Microsatellite simple sequence length	Saghai *et al* (1994)
STS	Sequence tagged site	Fukuoka *et al* (1994)
ALP	Amplicon length polymorphism	Ghareyazei *et al* (1995)
AFLP	Amplified fragment length polymorphism	Vos *et al* (1995) (Kumar *et al* 2003)

Uses of molecular marker in vegetable crops

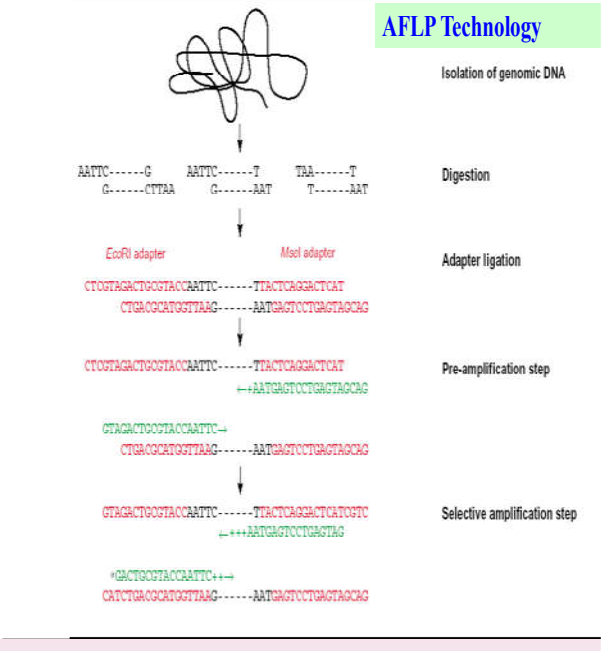
Development of saturated genetic maps

In the past genetic maps were based mainly on morphological and isozyme markers. But these markers are limited and are influenced by environment and developmental stage. Molecular marker on the other hand are large in number and are not influenced by environment and development stage. Saturated linkage maps are pre-requisite for gene tagging, marker assisted selection and map based gene cloning. Yayeh (2005) identified first genetic linkages in male fertile garlic accessions based on single nucleotide polymorphism simple sequence repeats and randomly amplified polymorphic DNAs. Thirty seven markers formed nine linkage groups covering 415 centrimorgans (cM) with average distance of 15 cM between loci. A male fertility locus was placed on the map. A 109 point linkage map consisting of three phenotypic loci (P_1, Y_2 and Rs), six restriction fragment length polymorphic DNA (RFLPs), two random amplified polymorphic DNAs (RAPDs), 96 amplified fragment length polymorphism (AFLPs) and two selective amplification of microsatellite polymorphic loci (SAMP) was constructed in carrot by Vivek and Simon (1999). A genetic map of an interspecific cross in Allium based on amplified length polymorphism markers constructed by VanHeusden *et al* (2000). The map based on *A. cepa* markers consisted of eight linkage groups whereas map based an *A. roylei* markers comprised 15 linkage groups. Zhang *et al* (2004) constructed linkage map for watermelon using recombinant inbred lines (RILs) from a cross between the high quality inbred line 97103 and the fusarium wilt resistant plant introduction using RAPD and SCAR markers. This map is useful for further development of quantilative trait loci (QTLs) affecting fruit quality and for identification of genes conferring resistance to fusarium wilt.

Assessment of genetic diversity

Molecular markers have proved to be excellent tools for assessment of genetic diversity in a wide range of plant species. The information is often of direct utility to plant breeders since it is indicative of the performance, adaptation or other agronomic qualities of the germplasm. Molecular markers have provided very useful information about the overall genetic range of crop germplasm. For breeders this information is important to take decisions regarding the utility of germplasm particularly in search for rare and unique genes. Germplasm of narrow genetic base is obviously unlikely to harbour novel genes e.g. those conferring resistance to biotic and abiotic stresses.

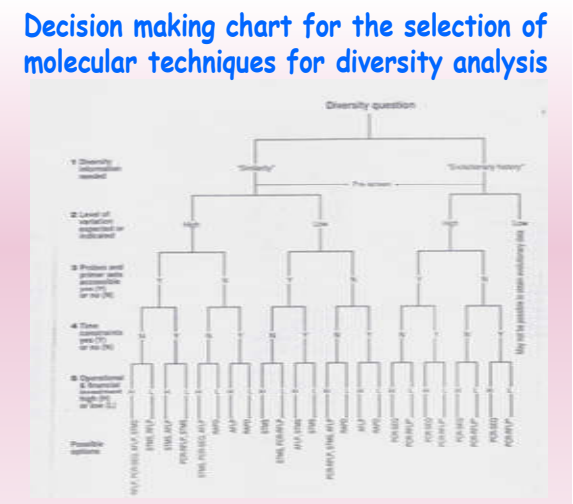
RAPD analysis of pepper breeding lines (Heras *et al* 1996) revealed very narrow genetic base with more than 50% of the DNA bands being common among all the lines. In an assessment of the world collections of tomato, Villand *et al* (1998) found South American accessions to have greater diversity than old world accessions. Shim and Jorgensen (2000) carried out AFLP analysis in wild and cultivated carrots and found that the old varieties released between 1974-76 were more heterogeneous than newly developed F_1 hybrids varieties. Archak *et al* (2002), using RAPD markers in tomato, found old introductions and locally developed varieties of 1970s exhibiting significantly greater variation than the ones released in 1990s.



Ruiz and Martinez (2005) studied the genetic variability of some traditional tomato cultivars of Spain using simple sequence repeats (SSR) and sequence related amplified polymorphism (SRMP) markers. They found that Mexican cultivar zapotec, a breeding line and virus resistant commercial hybrid 'Anastasia' were most distant of all the cultivar. Twelve amplified fragment length polymorphism (AFLP) and 10 inter-simple sequence repeat (ISSR) primers were applied to estimate genetic diversity in 68 varieties of cultivated radish by Muminovic *et al* (2005). They detected substantial level of genetic variability in germplasm of cultivated radish and within cultivated material, black radish and French breakfast radish types formed a separate clusters. AFLP marker analysis detected a greater genetic variability among American than among Spanish accessions of *Cucurbita maxima* (Ferriot *et al* 2004). Levi and Thomas (2004) identified 80.2 - 97.8% genetic similarity among hair loom cultivars of water melon using ISSR and AFLP markers. They also concluded that ISSRs and AFLPs are highly effective in differentiating among water melon cultivars of elite lines with limited genetic diversity than RAPD marks.

Gene tagging

The most interesting application of molecular markers at present time is the ability to facilitate the method of "conventional" gene transfer. Gene tagging refer to mapping of genes of economic importance close to known markers. Thus, a molecular marker very closely linked to gene act as a tag that can be used for indirect selection of gene in breeding programmes with the construction of molecular map, especially the RFLP maps, several genes of economic importance like disease resistance, stress tolerance, insect resistance, fertility restoration genes, yield attributing traits have been tagged. Gene tagging is a pre-requisite for marker assisted selection and map based gene cloning. In case of tomato TMV resistance *Tm-2* locus, nematode resistance, *Mi* gene, Fusarium oxysporum resistance gene, powdery mildew resistance gene, has been tagged. Huang *et al* (2000) tagged powdery mildew resistance gene *ol-1* on chromosome 6 of tomato using RAPD and SCAR markers.



DNA fingerprinting for varietal identification

DNA fingerprinting can be used for varietal identification as well as for ascertaining variability in the germplasm. Although any type of marker can be used but RAPDs, microsatellite and RFLPs are marker of choice for the purpose because all these are PCR based and does not require any prior information on nucleotides. The fingerprinting information is useful for quantification of genetic diversity, characterization of accessions in plant germplasm collections and for protection of property of germplasm especially the cms lines. Molecular marker has been used widely for DNA fingerprinting of cultivars and breeding lines in a number of vegetable crops like tomato (Kaemmer *et al* 1995), beans (Hamann *et al* 1995), pepper (Prince *et al* 1995), potato (Ford and Taylor 1997,

McGregor *et al* 2000). DNA technology has great potential for enhancing purity assessment in hybrids. Genetic purity of three F_1 chilli hybrids was determined using two molecular techniques RAPD and ISSR by Mongkolporn *et al* (2004). They found that RAPD analysis successfully detected all three F_1 hybridity while ISSR detected only two. This was due to the RAPD marker system producing a greater number of marker than the ISSR system.

All the available molecular tools from the simplest and readily useable RAPDs, ISSRs, DNA amplification fingerprinting (DAF) to the more precise, elaborate but robust AFLPs, microsatellites and RFLP based variable number of tandem repeats (VNTR) analyses have been utilised for discrimination of closely related lines as well as high yielding varieties.

Breeding lines and accession identification

Several situations during a breeding programme may require identification of breeding lines and accessions. Mislabelling is a common problem in breeding experiments due to the large number of lines that need to be handled. Breeding lines can get contaminated due to mixing of seed samples and cross contamination in field. Molecular markers are ideal for distinguishing closely related genotypes that differ in few morphological traits.

Use of human minisatellite probe 33.15 and the M13 repeat sequences for their ability to distinguish sister lines of two F6 backcrosses were demonstrated by Stockton and Gepts (1994). A comparison of the utility of 33.15 and M13 probes with (GACA) and ribosomal DNA sequences with respect to the polymorphism detected was made. The $(GACA)_4$ repeat was observed to be least efficient in discriminating the closely related lines of beans. Waycott and Fort (1994) could differential nearly identical germplasm lines of bitterhead letuce on the basis of RAPD markers. Kaemmer *et al* (1995) fingerprinted tomato accessions using microsatellite probes. The authors reported the utility of the technique in purity testing of breeding lines and in F_1 progeny testing. Using RAPD technique, Tivang *et al* (1996) revealed variation among and within artichoke breeding populations. Heterogeneity was observed within clonal cultivars. Roose and Stone (1996) reported the utility of RAPD and RFLP markers in distinguishing F_1 from F_2 seeds in asparagus and for evaluation of seed purity. Phippen *et al* (1997) used molecular markers to distinguish 14 phenotypically similar accessions of 'Golden Acre' variety of cabbage. Ten pairs of potential duplicate accessions in a total of 134 capsicum accessions were identified by Rodriguez *et al* (1999) on the basis of RAPD markers. Further, misclassified and unclassified accessions were placed in the correct groups. Using microsatellite markers, Fisher and Bachmann (2000) distinguished 83 accessions of onion.

Sex identification

Early identification of male and female plants can bring considerable efficiency in breeding programmes of dioecious species. Jiang and Sink (1997) developed SCAR markers in asparagus which were linked to the sex locus at a distance of 1.6 cM. Codominants STS markers enabling the differentiation of XY from YY males in asparagus were developed by Reamon Buttner and Jung (2002).

Cultivar identification

In crops like tomato, pepper, potato, alliums, cucurbits, lettuce and spinach, microsatellites have been developed to enable highly reliable identification of cultivars. Comparative assessment of different DNA fingerprinting techniques carried out in tetraploid potato revealed AFLP to have the highest discrimination power followed in decreasing order by multilocus SSR, RAPD, ASSR and single locus SSR. In pepper, Gaikwad *et al* (2001) found ISSR markers to most efficient in detecting polymorphism. However, due to very high number of markers generated per assay by AFLP, the marker index of AFLP markers was prominently higher than that of ISSR and RAPD. Broun *et al* (1992) identified two telomeric tendemly repeated sequences (7bp) and a closely linked 162 bp subtelomeric repeats in tomato that accounted for 2% of the total chromosomal DNA. These sequences have a very high mutation rate of 2% per generation. They have been shown to be extremely useful for distinguishing otherwise very similar tomato and melon varieties.

An important application of molecular identification is the support it is likely to provide to the new plant variety registration system. The Indian Plant Variety Protection and Farmers' Rights Act 2001 grants intellectual property rights to developers of new crop varieties in the form of plant breeders' rights. In order to be eligible for registration and protection under the act, a candidate variety must meet the criteria of distinctness, uniformity and staibility (DUS). Morphological data provide the basis for DUS testing. For determining distinctness, the variety is compared for a number of characters with the extent varieties. The PVPFR has also a provision of Essentially Derived Varieties (EDV) wherein the protection benefits are to be shared with breeders of the variety from which the EDV has been derived. For the present, DNA profiles alone as proof of unique identity of a plant variety is not acceptable. None the less, plant breeders may seek to strengthen their claim for protection of new varieties by including molecular profiles as supplementary information to establish the distinctness of their varieties. Molecular profiles may be particularly relevant in cases of biotechnologically developed varieties where only small apparent phenotypic differences exist between new variety and an extinct one.

MAP based gene cloning

One of the most serious limitations to the advance of plant molecular biology and biotechnology is the difficulty in isolating genes responsible for specific characters, yield, disease resistance, insect resistance and quality are just few of the important characters for which genetic variation exists within crop species, but for which the corresponding genes have not yet been cloned. The advent of genome mapping at the DNA level (especially RFLPs) has provided a method for localizing genes of economic importance to specific chromosomal positions. The ability to map any gene of economic importance to a defined chromosomal site opens the possibility of isolating genes via chromosome walking. This method is called map based gene cloning.

Map based cloning consists of four major step :

1. Development of a high resolution molecular linkage map in the region of interest.

Table 1: Identification of vegetable crop varieties by molecular markers

Sr. No.	Crop	Technique	Reference
1.	Tomato	Microsatellites, RAPD, RFLP	Kaemmer *et al* (1995), Bredemeijer *et al* (1998), Noli *et al* (1999)
2.	Potato	AFLP, microsatellites, ISSR, RAPD	McGregor *et al* (2000) Ashkenazi *et al* (2001)
3.	Onion, garlic and related species	AFLP, microsatellites, ISSR, RAPD	Arifin *et al* (2000), Fiseher and Bachmann (2000)
4.	Pepper	AFLP, RAPD	Prince *et al* (1995), Las Heras Vazquez *et al* (1996), Paran *et al* (1998)
5.	Brinjal	RAPD	Karihaloo *et al* (1995)
6.	Vegetable brassica	Microsatellites, RAPD	Margale *et al* (1995), Cansian and Echeverrigaray (2000)
7.	Cucurbits	ISSR, microsatellites, RAPD	Gwanama *et al* (2000), Danin *et al* (2001)
8.	Pea	RAPD	Samee and Nasinee (1996)
9.	Beans	RAPD, RFLP	Stockton and Gepts (1994)
10.	Spinach	Microsatellites	Groben and Wricke (1998)
11.	Asparagus	RAPD	Khandka *et al* (1996), Roose and Stone (1996)
12.	Artichoke	RAPD	Tivang *et al* (1996)
13.	Lettuce	AFLP, microsatellites	Hill *et al* (1996)
14.	Carrot sweet potato	AFLP, RAPD	Shim and Jorgensen (2000), He *et al* (1995)

Table. 2. Molecular markers linked to major resistant genes in vegetable crops

Crop	Pathogen	Gene	Marker(s)	Reference
Pepper	Tomato spotted wilt virus	*Tsw*	RAPD	Jahn *et al* 2000
	Tomato spotted wilt virus	*Tsw*	CAPS	Moury *et al* 2000
	Xanthomonas vesicatoria	*Bs2*	AFLP	Tai *et al* 1999
Bean	Common bean mosaic virus	I	RAPD	Melotto *et al* 1996
Pea	Pea common mosaic virus	Mo	RFLP	Dirlewanger *et al* (1994)
	Erysiphe polygone	Er	RAPD	Dirlewanger *et al* (1994)
Cucumber	*F. oxysporum f.* sp. melonis	Fo m2	SSP	Wechter *et al* (1998)
Melon	*F. oxysporum* f. sp. melonis	Fo m2	RAPD	Wechter *et al* (1995)

Table. 3. Molecular markers linked to major resistant genes in tomato

Pathogen	Gene	Marker(s)	Reference
Meloidogyne incognita	Mi	RAPD	Williamson *et al*, 1994
Meloidogyne javanica	Mi3	RAPD	Yaghoobi *et al*, 1995
Cladosporium fulvum	Cf2	RFLP	Dixon *et al*, 1995
Verticillium dahliae	Ve	RFLP	Diwan *et al* 1999
F. oxysporum f. sp. Radicislycopersici	Fr2	RAPD	Fazio *et al*, 1999
Cucumber mosaic virus	Cmr	RFLP	Stamova and Chetalat, 2000
Yellow leaf curl virus	Ty2	RFLP	Hanson *et al*, 2000
Tomato mosaic virus	*Tm2*	SCAR	Sobir *et al* 2000

achieved through generation of yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC).

3. Identification of appropriate YAC or BAC clones for isolating pitative clones harbouring the gene of interest.
4. Verification through transformation that the target gene is isolated.

In tomato, the availability of a high density molecular map and a yeast artificial chromosome library potentially provides the foundation on which to initiate map based gene cloning for genes underlying any trait that can be genetically mapped (Martin *et al* 1992).

Marker assisted selection (MAS)

In this technique, linkages are sought between DNA markers and agronomically important traits such as resistance to pathogens, insects and nematodes, tolerance to abiotic stresses, quality parameters, and quantitative traits. Instead of selecting for a trait, the breeder can select for a marker that can be detected very easily in the selection scheme. The essential requirements for marker assisted selection in a plant breeding program are as follows :

DNA marker based selection for disease resistant trait essentially requires following conditions:

- The identified DNA marker(s) should co-segregate or closely linked (1 cM or less) with the resistant trait. Alternatively, less tightly linked flanking markers should be available for the resistant gene(s).
- The availability of an efficient screening technique(s) for DNA markers, which can be practically feasible to handle large populations.
- The screening technique should have high reproductibility across laboratories.

The screening technique should be cost effective with high reproducibility.

A number of markers linked with monogenic disease resistance are available in vegetable crops (Table 2), especially in tomato (Table 3). Such mapping has been facilitated by the use of different kind of mapping populations like near isogenic lines (NILs) developed by repeated back crossing, recombinant inbred lines (RILs) developed by single seed decent or double haploid (DH) methods. Now a days, bulk segregant analysis (BSA) is increasingly being used to map monogenic resistance, because it allows rapid mapping of genes.

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