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Molecular Marker Technologies in Crop Authentication and Seed Purity Assessment: Advances and Applications

Ankita Thakur*[1], N. K. Bharat[1], Rohit Verma[1], Abhishek Tomar[1], Vinay[2] and Madhulika Thakur[3]

[1]*Department of Seed Science and Technology, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, 173230 HP, India*
[2]*School of Agriculture and Technology, Maya Devi University, Dehradun, 248011, Uttarakhand, India*
[3]*Department of Agricultural Economics, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, 173230 HP, India*

ABSTRACT

Molecular markers are crucial tools in modern plant genetics and breeding, enabling the detection of genetic polymorphisms at the DNA level. These markers, which include RFLP, RAPD, SSR, SNPs, and others, provide critical insights into genome structure, diversity, and gene-trait associations without being influenced by environmental factors. This review explores the classification, properties, and diverse applications of molecular markers in plant science. Key uses include DUS testing, seed health diagnostics, variety identification, genetic purity assessment, germplasm conservation, genetic diversity studies, QTL mapping, and marker-assisted selection (MAS). Advancements from hybridization-based to PCR-based and sequence-based markers have significantly improved marker specificity, throughput, and cost-efficiency. Techniques like next-generation sequencing and DNA chip technology have further revolutionized genotyping accuracy and speed. Despite their many benefits, molecular markers have not been widely used in regular seed testing because of challenges, including high implementation costs and the need for specialist knowledge. They should become more accessible, nevertheless, as a result of continuous attempts to standardize processes and falling prices. The development of more robust and affordable marker systems, as well as the integration of genetic data with phenotypic and environmental data for thorough variety identification, are the main goals of future research.

Keywords: *DNA Polymorphism, DUS Testing, Genetic Diversity, Marker-Assisted Selection (MAS), Molecular Markers, Next-Generation Sequencing, Plant Genome Analysis, Seed Quality Assurance*

INTRODUCTION

All eukaryotic creatures have the same fundamental organization of nuclear DNA strands into chromosomes and chromatin fiber. Nonetheless, it is frequently discovered that the structure of plant genomes is incredibly dynamic and complicated, and it influences the expression and function of genes [1]. A better understanding of the genomic structure may help one better understand the underlying genetic differences across plants from different taxonomic ranges and within the same species. These differences show up as DNA sequence alterations or polymorphisms, which may be identified using a variety of genetic markers [2][3]. Genetic markers serve as determinants or landmarks associated with specific genes or traits that aid in individual differentiation [4]. To understand the differences in plant genomes, several genetic markers have been developed and used over time. Genetic markers are classified into classical and molecular markers. Classical markers include morphological, cytological, and biochemical markers, which are easily influenced by the environment and lack specificity. On the other hand, Molecular markers are certain DNA sequences or nucleotide(s) that mostly do not change throughout the existence of an organism and may therefore provide a clear, succinct, and thorough picture of the genetic variants [5]. Numerous such markers have been created and applied over time to assess the genetic complexities among significant plant varieties. The development of molecular markers started with first-generation markers that were inefficient and continued with more recent markers that were more specific. All of this has made it possible to create high-throughput, potent techniques for sequencing whole transcriptomes and genomes, such as Next Generation Sequencing.

MOLECULAR MARKERS

A molecular or DNA marker is a variation in the nucleotide sequence of DNA that is close to or closely associated with a target gene that expresses a characteristic in various organisms or species. The target gene, expressed characteristic, or biological function, together with the closely related molecular marker, are usually inherited together [6]. The locus or loci denoting the precise genomic position of the molecular marker inside chromosomes may or may not be known. Notably, characteristics related to the expression or function of the connected gene or genes are unaffected by molecular or DNA markers. When molecular markers are closely linked to a trait or gene that has a specific biological function, they act as useful indicators or flags that indicate a specific gene locus and help identify or detect the traits that are linked to it, regardless of whether the genes involved are known or unknown and whether the gene or genes can be detected [7].

*Corresponding Author: ***Ankita Thakur***

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In practical terms, a molecular marker encompasses not just the related polymorphism but also all of the specific techniques or processes needed to discover or identify it. Most of the time, a molecular marker has only been thought of from the limited perspective of polymorphism or individual variations in DNA sequences. Notably, though, a molecular marker can sometimes be as simple as a primer, a set of primers, a restriction enzyme, a combination of primers and enzymes, or other pertinent elements, along with the steps needed to run the marker. This means that for a DNA segment to be classified as a molecular marker, it must contain the entire set of primers, restriction enzymes, or other pertinent components, as well as the established, comprehensive protocol for detecting that specific molecular marker. In the absence of such comprehensive marker-specific data, a sequence polymorphism cannot serve as a meaningful molecular marker. It is this comprehensive set of data that, in fact, practically constitutes a molecular marker.

Causes of DNA polymorphism

If variations in the marker nucleotide sequences known as polymorphisms occur across or among people or species, DNA markers can help identify individual genotypic variances within the same or different species. Nucleotide sequence variations within or across species are caused by a variety of DNA mutations, which result in molecular marker polymorphisms [8]. In general, point mutations resulting from single-nucleotide substitutions, rearrangements involving insertions or deletions, duplication of DNA sections, translocations and inversions, and errors in tandemly repeated DNA replication are the main causes of marker polymorphisms in organisms [9].

Key Properties of a Reliable DNA Marker

- The marker can be automated, multiplexed, easily assayable, inexpensive, and widely and uniformly distributed across the genome.
- An excellent molecular DNA marker should also be highly reproducible, multi-functional, genome-specific, and highly polymorphic to discriminate between homozygotes and heterozygotes effectively.
- It should also be co-dominant in expression to facilitate effective discrimination between homozygotes and heterozygotes [10].

APPLICATIONS OF MOLECULAR MARKERS

Molecular Markers in DUS Testing

The International Union for the Protection of New Varieties of Plants (UPOV) has been prompted to consider including molecular markers in the DUS testing method by research supporting its predicted efficacy. Since molecular markers have been demonstrated to be quicker and less expensive than morphological markers, DUS testing might profit from their application. For DUS testing or infringement cases, more trustworthy techniques should be used to find varieties with comparable morphological characteristics. Even though UPOV member states frequently combine molecular methods with phenotypic evaluation of varieties, recent research suggests that each country should validate this approach based on the species composition of botanical taxa used for DUS testing and the molecular markers employed [11][12]. Among the molecular markers, particularly, SSR (simple sequence repeat) and SNP (single-nucleotide polymorphism) markers are frequently employed.

Seed Health Testing

Advanced molecular techniques, such as DNA chips or microarray technology, have been employed for the rapid and precise detection of seed-borne pathogens. The special capacity of nucleic acid molecules to hybridize selectively with molecules of complementary sequences is the foundation of microarray technology. These methods enable the simultaneous identification of multiple pathogens within a short time frame (approximately six hours), demonstrating significant potential in seed health diagnostics.

Germplasm Conservation and Maintenance

Molecular markers serve as a precise tool for identifying duplicate accessions in germplasm collections, ensuring the efficient management and conservation of genetic resources by eliminating redundancy. In germplasm conservation, it is crucial to minimize the inclusion of duplicate accessions. An average of 50% is thought to be duplicated in various collections [13]. Duplicate accessions can be securely removed from the holding for improved maintenance by using molecular markers to identify them clearly. Nei's dissimilarity matrix may be used to calculate the genetic distance between the accessions; those with mean distances below the threshold value may be regarded as possible duplicates [14].

Variety Identification and Genetic Purity Analysis

Molecular markers provide an accurate and reliable approach for the identification of plant varieties [13]. They are employed to assess the genetic integrity of a sample by comparing its allele profile with that of an authenticated reference variety. For genetic purity testing, DNA markers such as RFLP, AFLP, RAPD, SSR, ISSR, and SNPs have also been utilized recently; SSRs and SNPs being the most commonly used of these. In a large range of cereal crops, including rice, oats, and wheat, genetic diversity and heterosis have been correlated using molecular markers [15]. SSR and STS markers were used to verify purity in hybrid rice in place of the traditional "grow-out tests," which involved maturing the plant and examining morphological and floral characteristics [16].

Assessment of Genetic Diversity

Recent developments in genome sequencing and molecular markers offer a fantastic opportunity to evaluate the genetic diversity of a substantial germplasm collection [17]. The extent and distribution of genetic diversity in a crop species must be analyzed to comprehend the developmental relationship between the sample and structured access to genetic resources for breeding purposes. Numerous techniques, such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellites, have proven effective in assessing genetic diversity in germplasm, cultivars, and advanced breeding material [18]. The most widely used markers for identifying genetic variation in different crops are DArT and SNP markers [4].

Marker-Assisted Selection (MAS) for Trait Improvement

One of the most promising markers is molecular genetic markers, which breeders employ as selection tools to determine whether particular alleles are present or absent in plants of interest. Through the use of marker-assisted selection, a recently developed method, certain issues with traditional breeding are avoided, and phenotypic selection criteria are improved through either direct or indirect gene selection.

The availability of several molecular markers and genetic maps has made MAS possible for both quantitative trait loci (QTLs) and traits that are influenced by considerable genetic variation. By making it easier to introduce single genes with the desired alleles into elite cultivars and eliminating the unwanted donor parent genome in a backcrossing program, MAS increases the effectiveness of Mendelian trait selection. It also makes it possible to identify and safeguard commercial cultivars using fingerprinting [19].

Quantitative Trait Loci (QTL) Detection

Molecular markers facilitate the detection of QTLs and their association with phenotypic traits, contributing to trait improvement. Measuring the distances between genes via gene continuity analysis should be done using genetic mapping and easily recognizable genes in the form of phenotypic features [20]. Developments in PCR-based techniques and DNA markers have simplified correlation analysis and allowed for the creation and use of QTL mapping and correlation maps in several plant species [4]. Map of Genetic Linkages Construction and QTL analysis of two interspecific reproductive isolation traits in sponge gourd [21]. In breeding programs, MAS for easily inherited characteristics is becoming more and more significant, enabling the breeding process to proceed more quickly.

CLASSIFICATION OF DNA MARKER

DNA markers can be categorized according to the method of inheritance and gene activity [3]:

A marker that enables heterozygous F_1 individuals to discriminate between the two alleles from two homozygous parents is known as a codominant marker. A marker that is only capable of identifying dominant alleles, it is unable to distinguish between genotypes that are homozygous and heterozygous, is known as a dominant marker.

Based on the method of detection, DNA markers are classified into several groups [6]:

a. Hybridization-based markers
b. Polymerase chain reaction (PCR) based markers
c. Sequence-based markers.

FIRST GENERATION/HYBRIDIZATION-BASED MARKERS

Restriction Fragment Length Polymorphism

The discovery of the human genome marked the beginning of the development of RFLP (Restriction Fragment Length Polymorphism) technology. Polymorphic DNA is detected during restriction digestion of genomic DNA from different plant sources. RFLPs rely on variations in DNA sequence length caused by restriction enzymes cleaving genomic DNA at random but particular restriction enzyme recognition sites in conjunction with DNA probe hybridization in Southern blotting [22]. After being separated, genomic DNA is hybridized to short, radioactively labeled probes and subjected to restriction digestion. A unique profile is produced for every locus by combining restriction endonucleases and probes. Autoradiography allows for the visualization of the DNA banding profiles.

DNA Chip /DNA Microarray Technology

Microarrays are molecules that are immobilized on a solid surface and are used in DNA chip technology, which is a hybridization-based approach. The DNA/RNA samples serve as probes after being immobilized in the microarray. On solid surfaces like glass slides, silicon chips, etc., the probes become immobile. The probe must be created before tests using the DNA chip approach may be carried out. After the probes are mounted on the chip, the target material is fluorescently tagged. The probes can be DNA, cDNA, or oligonucleotide probes of known sequence. The tagged samples must next be hybridized to the chip to be analyzed. The fluorescence level is then captured and evaluated by placing the microarray cartridge in a scanner [23].

Second Generation /PCR-Based Molecular Markers

Random Amplified Polymorphic DNA (RAPD)

The PCR method, known as Random Amplified Polymorphic DNA (RAPD), uses DNA sequences that bind randomly to the target DNA as a single primer. These sequences are typically 10 bases long and have a high GC content. RAPD creates a distinct band pattern for every individual by using short, arbitrary primers to amplify random genomic regions using PCR. According to established procedures, all genomic DNA is isolated and purified [24]. After that, a PCR reaction is prepared using a single primer that binds to genomic sequences at random to enable amplification. By splitting the amplicons in a 1–1.5% gel, the DNA banding profile is produced. This method effectively addresses the main drawbacks of RFLP since it can effectively amplify even smaller amounts (10–25 ng) of DNA samples and can be finished faster. Typically, a large number of markers are created and distributed across the genome.

Sequence Characterized Amplified Region (SCAR)

Sequence Characterized Amplified Region (SCAR) markers, which they developed from pre-existing RAPD markers in lettuce. The conversion of RAPDs into a co-dominant, more locus-specific, and repeatable SCAR marker improves the reliability of the marker [25]. By first cloning and then sequencing the necessary amplicons, it was derived from earlier multi-locus marker approaches. Following that, long primers that complement the ends of the amplicons are created for PCR amplifications. After multi-loci PCR amplifications, highly polymorphic and particular amplicons may be discovered and their terminal sequences sequenced. The target DNA sequence is amplified once again using the SCAR primers that were created. Long oligonucleotide primers (22–24 bases long) complementary to the sequences are developed for certain traits/loci. This method makes it simple to transform a dominant marker into a co-dominant one. Agarose gel electrophoresis is utilized to view the produced amplicons.

Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) was developed by integrating PCR and RFLP methods; the limitations of RAPD, ISSR, and RFLP were addressed, leading to a far more effective method that could identify a larger degree of polymorphism. AFLP combines selective PCR amplification of restriction fragments with restriction digestion of DNA [26]. Gel electrophoresis is then used to separate the fragments.

Before restriction digestion, intact genomic DNA is separated and purified. Either one or both of the uncommon and common cutter restriction endonucleases are used. After the fragments produced by restriction digestion are ligated to the proper adapters, PCR amplifications are conducted in successive rounds.

The amplicons are separated on the proper gel, ideally using Polyacrylamide Gel Electrophoresis (PAGE), and labeled with fluorescent or radioactive labeling.

Simple Sequence Repeats (SSR) or microsatellite

Microsatellite DNAs are tandemly repeating sequences of mono-, di-, tri-, and penta-nucleotides that are found all across the genome. These repeating nucleotide segments frequently differ among and between members of a similar species. SSRs are based on differences in the quantity of short DNA sequences that are tandemly repeated; these sequences are typically 1-6 base pairs in length. Using primers that surround the repeat region, PCR is used to find these variants. Following the isolation of the whole genomic DNA, a quality check and quantification are performed. In order to optimize the reaction and conditions, PCR reactions are then established using ISSR primers with different amounts of reaction buffer, MgCl2, and dNTP. Because it is essential for amplification, the annealing temperature must be carefully chosen. For ISSR reactions, the ideal annealing temperature is typically 5°C below the primer's Tm [2]. DNA amplification is produced when lengthy microsatellite primers attach to the DNA template more easily under ideal reaction circumstances. Following electrophoretic separation, the amplicons may be seen using polyacrylamide gel electrophoresis or on a 1–1.5% agarose gel.

ISSRs (Inter Simple Sequence Repeats)

The Inter Simple Sequence Repeats (ISSR) marker was noticeably better than the previously published RAPD marker. A single primer, typically a lengthy microsatellite sequence anchoring at both ends, is used in the Inter Simple Sequence Repeats (ISSR) technique, which amplifies DNA segments located at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite directions. The amplified DNA sequence is surrounded by two microsatellite sections that are oppositely oriented and identical.

ISSR analysis typically begins with PCR amplification using an ISSR primer and extracted genomic DNA as a template. ISSR-PCR is then followed by PCR product electrophoresis on agarose or polyacrylamide gel and visualization, ISSR band grading, and data analysis. The purpose of ISSR primers is to target genomic microsatellite portions with repeated, simple DNA sequences. These primers are typically lengthy, ranging in size from 15 to 30 bases. Primers that are anchored at the 30 or 50 end or unanchored may be utilized in ISSR analysis. Typically, the primary anchor consists of one to four defective nucleotides that cross over into the adjacent microsatellite sequences [27].

Single-Strand Conformation Polymorphism (SSCP)

SSCPs were created using the fundamental idea that a single DNA strand's mobility during gel electrophoresis depends only on its shape [28]. When single stranded DNA fragments undergo conformational changes due to sequence variances, SSCP can identify these alterations. The movement of the DNA in non-denaturing gel electrophoresis is impacted by these structural changes. Single-strand point mutations can change their conformation, which allows for efficient genotype and electrophoretic mobility differentiation.

One significant benefit of SSCP is its capacity to identify point mutations and polymorphisms without the need for previous genome sequencing. SSCPs are sections that range in length from 200 to 800 bp in the DNA sequence. Typically, 20–25 bp long, the primers are made to be complementary and specific to those sequences.

Cleaved Amplified Polymorphic Sequences (CAPS)

CAPS combines RFLP and PCR-based amplification; however, it differs from the two methods in that it uses this marker system to perform restriction digestion on amplified PCR products [29]. The CAPS marker is also known as PCR-RFLP. The existence of restriction sites in PCR-amplified genomic areas serves as the basis for CAPS markers. After being broken down by restriction enzymes, the amplified DNA is examined using gel electrophoresis.

The PCR amplicons are then digested using restriction endonucleases once the template DNA has been amplified using the proper primers. Agarose gel electrophoresis or polyacrylamide gel electrophoresis can be used to visualize amplicons. It is possible to design particular primers for the CAPS test by mining genome sequences from internet data banks, or by using cDNA sequences or cloned RAPD, ISSR, SCAR, and AFLP markers.

SCoT (Start Codon Targeted Polymorphism)

SCoT marker was mostly derived from conserved plant genome areas that were identified by the presence of the ATG start codon. It may possibly have been based on transcribed sections linked to a particular functional gene [28]. It is often considered to be better than ISSR and RAPD approaches, which both fall short of having marker resolutions as high as SCoT markers.

The SCoT marker employs a brief sequence of single primers that are flanked by the ATG start codon of the translation site and complementary to conserved areas. However, sequence information is not necessary before primer creation because these sequences are preserved in nature [30][31]. The PCR amplifications are then performed using these primers.

Third Generation/ Sequence-based markers

Single Nucleotide Polymorphisms (SNPs)

Individuals of a given species differ from one another at the genetic (DNA) level because of variations in a single DNA base pair inside their genome or genomes. We refer to these variations as single-nucleotide polymorphisms (SNPs) [4]. As a result, SNPs are quite specific and may guarantee the existence of several markers. It is intended that the forward PCR primer's 3′ end nucleotide complements just one SNP allele. The PCR amplicons will only be produced when the particular complementary allele attaches itself to the primer's 3′ end. If an SNP causes the allele to be replaced by another allele, no amplicons will be found.

Recently developed high-throughput genotyping methods, including next-generation sequencing (NGS), genotyping-by-sequencing (GBS), chip-based NGS, and allele-specific PCR, have been used to uncover highly informative SNPs.

Diversity Arrays Technology (DArT)

DNA polymorphisms at several loci can be concurrently detected by DART using a microarray platform. DNA probes are hybridized with genome-representative DNA fragments. A randomized library of fragments designed specifically to increase the likelihood of finding SNPs, mostly in the form of insertions or deletions throughout the genome, is screened as part of DArT. The creation of a genomic library, which serves as the genomic representation, is the fundamental process of DArT [32].

Following the digestion of genomic DNA by restriction enzymes and the ligation of restriction fragments to adapters, genomic representations are created for each unique DNA sample that is being typed. PCR is then used to minimize the genome complexity by using primers with selected overhangs and complementary sequences to the adaptor. To create a "discovery array," the representation fragments are cloned, the cloned inserts are amplified using vector-specific primers, purified, and arrayed onto a solid substrate (microarray).

Expressed Sequence Tags (EST)

EST is complementary DNA (cDNA), which is produced by converting messenger RNA (mRNA) into DNA using reverse transcriptase. The formation of ESTs is crucial for increasing the stability of mRNAs. ESTs are produced using cDNA sequencing [33]. The 50 or 30 ends of the cDNA are sequenced to produce 50 ESTs or 30 ESTs, respectively, using a few hundred nucleotides. Millions of ESTs from a wide variety of species are currently accessible in several databases. SNPs are created from ESTs that primarily consist of 30-untranslated regions (5'UTRs) of cDNA clones. This method maximizes the possibility of finding nucleotide variations associated with the genetic diversity that currently exists in organism species by designing primer pairs that complement the EST sequences, amplifying corresponding regions from multiple genotypes using PCR, and then comparing the sequences across genotypes to identify SNPs.

Sequence-Tagged Sites (STS)

STS markers are short, distinct DNA sequences that are readily recognized and associated with particular genomic regions. The exact STS sequence is frequently present only at a location and nowhere else in the genome. The exact STS is any genomic locus specified by its primer sequences.

Zhang et al. [34] were able to successfully create STS from individual RAPD markers in the form of SCARs. After cloning RAPD polymorphic bands, sequencing was used to ascertain the nucleotide composition of the terminal ends. Primers were designed using the discovered sequences to specifically amplify the required regions. As an alternative to overcoming the challenges posed by gel-based marking approaches, the current systems for high-throughput SNP genotyping, primarily use allele-specific hybridization, heteroduplex analysis, and mini-sequencing applications. Some of the most modern high-throughput genotyping methods have been used to uncover highly informative SNPs, including next-generation sequencing (NGS), genotyping-by-sequencing (GBS), chip-based NGS, and allele-specific PCR. As a result, these markers are now the most appealing among the several different molecular markers for genotyping that are accessible [35].

Retrotransposon-based markers

Long terminal repeats (LTRs), which are incredibly conserved sequences, are found at the ends of retrotransposons [36]. Numerous retrotransposons within and across plant species have been analyzed, and the results indicate that they are very heterogeneous and display insertion polymorphism [37]. Primers for retrotransposon-based marker analysis are designed using the LTR sequences as a reference. Since retrotransposon insertions are irreversible, they are especially suitable for phylogenetic analysis. The primary retrotransposon-based markers are Inter-retrotransposon amplified polymorphism (IRAP), Retrotransposon microsatellite amplification polymorphisms (REMAP), Retrotransposon-based insertion polymorphism (RBIP), and Inter-primer binding site (IPBS).

CONCLUSION

Molecular markers have become extremely useful tools for comprehending the intricate and ever-changing structure of plant genomes. These markers offer important insights into genetic diversity both within and between plant species by detecting DNA polymorphisms linked to certain genes or characteristics. As time has progressed, low-throughput, labor-intensive procedures have given way to high-throughput, highly specific, and economical approaches like next-generation sequencing technologies and SNPs. Their applications cover a wide range of crucial fields, such as marker-assisted selection, genetic purity analysis, variety identification, DUS testing, seed health diagnostics, and QTL mapping. Molecular markers reduce the need for conventional phenotypic assessments while simultaneously enabling accuracy in plant breeding and conservation. The accuracy and effectiveness of genetic research will be significantly improved by the integration of molecular markers with bioinformatics and sequencing platforms as genomic technologies advance. In the end, these tools are essential for promoting crop enhancement initiatives and guaranteeing food security in the face of international agricultural difficulties.

Future Scope of Study

The development of low-cost, high-throughput molecular marker systems appropriate for on-site seed quality assessment should be the goal of future research. Precision breeding will be improved by combining DNA data with phenotypic and environmental factors. Large-scale adoption in crop enhancement and variety authentication initiatives will be made easier by the standardization of marker procedures and developments in AI-driven bioinformatics. New technologies that promise more precise and trait-specific detection include CRISPR-based genotyping and the creation of functional markers. These developments will greatly increase the efficiency of plant breeding and seed quality assurance.

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

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