Molecular marker technology based on molecular hybridization

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Abstract: Molecular marker technology centered on molecular hybridization represents an important branch of early molecular technologies. This paper comprehensively analyzes and summarizes the categories, mechanisms and characteristics of molecular marker technologies based on molecular hybridization, with emphasis on their practical applications in rice.

Keywords: molecular hybridization, molecular marker, RFLP, minisatellite

1. Introduction

Molecular marker technologies centered on molecular hybridization mainly include RFLP markers and minisatellite markers. Their basic principle is as follows: after digesting DNA molecules from different organisms with restriction endonucleases, randomly selected genomic clones, microsatellites and other probes labeled with isotopes or non-isotopes are used for DNA hybridization. Finally, DNA polymorphism is detected by autoradiography or other chromogenic techniques. RFLP polymorphism is mainly caused by single-base substitution, insertion, deletion, translocation and inversion of DNA fragments, whereas minisatellite polymorphism arises from variations in the number of repeat units. As the earliest discovered DNA molecular marker technique, RFLP serves as a typical representative.

2. RFLP molecular markers

The RFLP molecular marker technique was first developed by Grozdicker et al. in 1974. Its full name is Restriction Fragment Length Polymorphism, abbreviated as RFLP. In 1980, Botstein constructed a genetic map using this marker [1].

2.1. Basic concept of RFLP

DNA carries and transmits genetic information in most organisms (excluding a small number of RNA viruses). It is a polymer formed by deoxyribomonophosphates linked via 3',5'-phosphodiester bonds. Restriction endonucleases are DNA endonucleases that recognize specific nucleotide sequences and cleave DNA molecules at these sites. For instance, restriction endonuclease EcoRI recognizes a six-nucleotide sequence: 5'-G↓AATTC-3', and cuts the double-stranded DNA at the position marked ↓. The more restriction recognition sites present in a DNA molecule, the more frequently the DNA will be digested, producing DNA fragments of varying lengths known as restriction fragments. DNA fragments of different lengths can be separated in gels by electrophoresis. The DNA fragments distributed on the gel are then transferred onto nitrocellulose or nylon membranes through Southern blotting. Subsequent hybridization of the membrane-bound DNA fragments with isotope- or non-isotope-labeled DNA probes reveals polymorphism of DNA fragments complementary to the probe sequence, namely restriction fragment length polymorphism (RFLP). It is currently the most widely used molecular marker based on Southern blotting. DNA sequences usable as probes in RFLP research include DNA fragments of specific genes, various complementary DNAs (cDNAs), random genomic DNA, and artificially synthesized DNA oligonucleotides [2].

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2.2. Basic principle of RFLP

RFLP essentially reflects differences in the nucleotide sequences of genetic materials among different organisms. The genetic variations among crop species, varieties, and even individuals originate from DNA sequence differences at the molecular level, which arise from changes in the nucleotide arrangement of chromosomal DNA during evolution and growth. Variations in DNA nucleotide sequences lead to distinct restriction enzyme recognition sites. After DNA molecules are digested by restriction endonucleases, the resulting fragments differ in size and length, thereby generating restriction fragment length polymorphism, namely RFLP.

RFLP are basically generated through two pathways. The first is single-base substitution at restriction endonuclease recognition sites, which leads to the loss or gain of such sites and produces RFLPs; this type is known as the single-base mutation type, or point polymorphism. The second pathway arises from extensive sequence variations within DNA and is termed the structural rearrangement type. The structural rearrangement type can be further divided into two categories. One category involves the relative shift of restriction sites caused by insertion, deletion or inversion of DNA fragments, resulting in RFLPs. The other category is induced by hypervariable regions composed of multiple tandem repeat sequences. These hypervariable regions are generally located adjacent to genes. The copy number of tandem repeats within hypervariable regions varies greatly among different individuals, which causes substantial length variation of hypervariable regions. Consequently, the fixed positions of restriction sites flanking the hypervariable regions shift relatively along with the hypervariable regions, giving rise to RFLPs [2].

2.3. Detection and analysis process of RFLP

Digestion of chromosomal DNA from higher plants with restriction endonucleases generates a large number of DNA fragments of varying lengths and sizes. Fragments with different lengths can be separated by gel electrophoresis. However, restriction digestion of higher plant DNA produces an excessive number of fragments of different sizes, forming a continuous smear on the gel. Although electrophoresis can separate these fragments, specific fragments cannot be distinguished. Small fragments of chromosomal DNA are therefore used as probes for DNA-DNA hybridization detection, and both the probe DNA and the DNA on the gel must be single-stranded to facilitate hybridization. All DNA is transferred from the gel to a solid support such as a nitrocellulose membrane or nylon membrane via Southern blotting. Subsequently, isotope-labeled probes are hybridized with the DNA immobilized on the membrane, and the hybridization signals are detected by autoradiography [2].

2.4. Application of RFLP technology in variety identification

2.4.1. Principle and operation flow of variety identification using RFLP markers

Theoretically, each variety represents a distinct genotype, and corresponding RFLP fingerprints (obtained from combinations of multiple restriction endonucleases or probes) can mostly be identified through analysis. Such RFLP fingerprints enable differentiation between this variety and other varieties or contaminated individual plants. Accordingly, DNA extracted from a single seed of a certain variety or from different varieties is digested with specific restriction endonucleases. DNA fragments of varying sizes are separated by gel electrophoresis, and the DNA within the gel is transferred onto nitrocellulose or nylon membranes. Hybridization is then performed using probes labeled with radioactive isotopes (such as ^{32}P) or non-radioactive substances (such as digoxigenin). After autoradiography, the RFLP fingerprint of the variety to be identified can be visualized on X-ray films. Comparative analysis with standard RFLP fingerprints facilitates impurity and authenticity testing of the variety. It is evident that RFLP analysis depends on the types and combinations of restriction endonucleases and probes. To date, more than 200 restriction endonucleases have been isolated, each with specific recognition sites spanning 4 to 10 bases, and over 3500 mapped RFLP markers have been identified in rice. Bases at any locus may be substituted by other bases, resulting in the loss of existing restriction sites or the generation of new ones for other restriction enzymes. Therefore, DNA restriction fragment length polymorphism of the variety can be detected provided appropriate restriction enzymes are selected [3].

2.4.2. Technical characteristics of RFLP markers

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The main characteristics of RFLP markers are as follows: they are distributed throughout the whole genome with an almost unlimited quantity; they have no phenotypic effects and are not restricted by developmental stages or organ specificity; they are codominant and capable of distinguishing homozygotes from heterozygotes; their detection results are stable and reliable. However, this technique requires a large amount of DNA and involves complicated detection procedures, making it difficult to apply in large-scale breeding practices.

2.4.3. Research applications and technical limitations of RFLP markers

Among commonly used DNA markers for rice, RFLP markers were the first molecular markers discovered and applied in genetic map construction and gene mapping. In 1988, Cornell University constructed the first rice molecular marker linkage map consisting of 135 loci using RFLP markers, marking the beginning of quantitative molecular genetics research in rice. In 1994, Cornell University and the Rice Genome Program of Japan separately reported high-density rice linkage maps based on RFLP markers, which greatly facilitated the rice genome sequencing project and gene mapping research [4]. Wang et al. (1989, 1992) performed RFLP analysis on 70 rice varieties using 150 probe combinations. Subsequently, they used 25 probes to genotype 93 accessions from 21 Oryza species and conducted Oryza classification and variety identification via RFLP molecular markers. Mao Long et al. analyzed RFLPs in progenies derived from crosses between tall wild rice and cultivated rice [5]. Wang Zhimin et al. (1996) carried out DNA fingerprinting analysis on 59 foxtail millet varieties (including landraces, improved varieties and lines) using the highly polymorphic probe gFM31, and successfully distinguished 58 genotypes [6].

Therefore, the RFLP marker method is regarded as one of the most reliable approaches for variety authenticity and purity identification at present. Nevertheless, the analytical procedure is cumbersome and costly, making it unsuitable for purity testing of large batches of hybrids. Fewer polymorphic RFLP loci exist among closely related cultivated hybrid rice varieties, and the information provided is particularly insufficient for pedigree analysis (Jeffreys et al., 1985). The RFLP polymorphism is even lower among closely related cultivated rice varieties, rendering variety identification by RFLP almost infeasible (Akagi et al., 1997; Rongwen et al., 1995). Furthermore, the requirement for isotope handling complicates its application in seed testing, which greatly restricts the practical application of RFLP technology [7].

3. Minisatellite DNA markers

3.1. Basic characteristics of minisatellite DNA markers

Minisatellite DNA is a type of Variable Number of Tandem Repeat (VNTR), which refers to short repetitive DNA sequences composed of tandemly repeated core motifs. It is mainly distributed in the subtelomeric and centromeric regions of chromosomes. Minisatellites generally consist of core sequences ranging from more than ten to dozens of base pairs (bp) with variable repeat numbers. The total length of an individual minisatellite DNA can vary from dozens of bp to several thousand bp. Minisatellites follow the Mendelian codominant inheritance pattern [8].

3.2. Discovery, polymorphism origin and related research of minisatellite markers

Minisatellites were first discovered in the human genome by human geneticists during 1980–1984. Wyman et al. (1980) proposed that hypervariable regions (HVRs) exist in the human genome and verified that these regions comprise tandem repeat sequences [9]. Jeffreys et al. (1985) found that the core motifs of repeat units are homologous within repetitive sequences, featuring multiple alleles, high abundance and strong conservation in eukaryotic genomes. Variations in tandem copy numbers occur among different individuals, and such polymorphism may arise during DNA replication or unequal crossing over. Extensive research on minisatellites in plants has been conducted since 1988. Dallas (1988) successfully obtained DNA fingerprints of six cultivated rice varieties using human minisatellite probes. The DNA fingerprints were identical among individual plants within the same variety, while the probability of different varieties sharing identical DNA fingerprints was merely 10×10^{-17} [10]. Regarding the origin of minisatellites, most scholars hold that strand slippage and base mispairing between the slipped strand and complementary strand

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during DNA replication or repair led to insertion or deletion of one or several repeat units, thereby generating length polymorphism among different individuals or populations [8].

3.3. Technical characteristics and application status of minisatellite markers

Minisatellite DNA exhibits high polymorphism, yet suffers from a shortage of available probes. Researchers have to synthesize probes independently or employ DNA fragments obtained from human genomic research for hybridization with crop DNA. Based on Southern blotting, these experiments require a long testing cycle [11]. Accordingly, minisatellite DNA is generally combined with RFLP technology to generate minisatellite DNA fingerprints, namely performing Southern hybridization with minisatellite DNA as the probe. Compared with RFLP markers, a single minisatellite probe can detect 10–20 highly polymorphic loci in one assay, raising the polymorphism information content (PIC) to 0.7–0.9. Nevertheless, drawbacks of minisatellite DNA fingerprints limit their application. For one thing, they share the same technical deficiencies as RFLP. For another, their uneven chromosomal distribution hampers linkage analysis. Furthermore, minisatellite probes are relatively long, difficult to acquire, and limited in quantity [8].

At present, minisatellite marker technology is mostly applied to paternity testing in medicine and animals. Its research and application in plants, especially in rice, are limited, and it has been largely replaced by microsatellite (SSR) technology.

4. Conclusion

In summary, RFLP markers and minisatellite markers centered on molecular hybridization are classical early DNA molecular marker technologies. RFLP markers feature wide genomic distribution, codominant inheritance, stable and reliable results, and can be applied to genetic map construction, gene mapping, as well as authenticity and purity identification of crop varieties. However, drawbacks including complicated operations, large DNA consumption, high costs, low polymorphism among closely related varieties, and the requirement of isotopes in partial detection restrict their use in large-scale sample screening. Minisatellite markers possess higher polymorphism information content, and a single probe can detect multiple loci simultaneously. Nevertheless, they suffer from limited probe resources, uneven chromosomal distribution, reliance on Southern hybridization, and long experimental cycles. Both markers have laid an important foundation for germplasm classification and genetic diversity research, and promoted the development of crop molecular genetics. Restricted by inherent technical limitations, they have been gradually replaced by simpler and more efficient markers such as SSR in plant research, especially in rice. Even so, their theoretical frameworks and experimental experience still provide valuable references for the development and application of subsequent molecular markers.

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