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Application of molecular markers in plant genome study

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Abstract
The development of molecular techniques for genetic analysis has led to a great increase in our knowledge of plant genetics and our understanding of the structure and behaviour of plant genome. During last three decades, several powerful DNA based marker technologies have been developed for the assessment of genetic diversities and molecular marker assisted breeding technology. In plant systems, the prospects of DNA profiling and fingerprinting is becoming indispensable in the context of establishment of molecular phylogeny, assessment of somaclonal variants, characterization of plant genomics, marker-based gene tags, map-based cloning of agronomically important genes, variability studies, synteny mapping, marker-assisted selection of desirable genotypes etc. In this review article, various molecular markers are reviewed with emphasis on specific areas of their application in higher plants.

Keywords: Molecular markers, Plant genome, DNA fingerprinting

Identification of different genotypes of crop species and varieties is important particularly when new crop varieties are to be released, different accession of wild species are to be characterized and purity of germplasm is to be determined. For a long time such identification has been based on the phenotypic differentiation and morphological features which are indicative of the genotype like maturity of grain in wheat (Hu *et al.*, 1996), morphology of leaves, flowers and grain quality in rice (Villareal *et al.*, 1999; Lee *et al.*, 1999; Mandal *et al.*, 2000). Though it can provide unique description of cultivated varieties, however, morphological characters are represented only by a few loci because there are not a large enough number of characters available. Moreover, these characters are also affected by the environment and growth practices. To have an accurate and reliable estimate of genetic relationship and diversity, a large number of polymorphic markers are essentially required. Similarly, introgression lines, which are sometimes different from the parent by only few base pair sequences, are difficult to differentiate from each other on morphological and phenotypic basis.

To circumvent such difficulties biochemical markers such as isozymes were developed and have been used successfully for the last few decades (Arus *et al.*, 1982; Sangwan *et al.*, 2003). Seed storage protein (Gupta and Robelen, 1986) and High Performance Liquid Chromatography (Buchler *et al.*, 1989) were also used for cultivar identification. Since the level of polymorphism detected through these techniques is low and tissue specific, therefore, their utilization has become limited.

With the advent of molecular markers, a new generation of markers has been introduced over the last two-three decades, which has revolutionized the entire scenario of biological sciences. DNA-based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, etc. In this article, DNA markers developed during the last three decades of molecular biology research and utilized for various applications in the area of plant genome analysis are reviewed.

1. Various types of markers

Polymorphism among the different genotypes of a species is the raw material for genome analysis. To be a genetic marker, the marker locus has to show experimentally detectable variations among the individuals in the test population. The variation can be observed at different biological levels, from the simple heritable phenotype to detection of variation of a single nucleotide. Once the variation is identified and the genotypes of all the individuals in the test population are known, the frequency of recombination events between loci is used to estimate the linkage distances between markers for the construction of a linkage map. Target traits or target genes in a segregating population can be identified with the assistance of linked genetic markers to accelerate traditional breeding programs. This process is known as marker assisted selection (Zheng *et al.*, 1995).

An ideal genetic marker should be highly polymorphic in nature, show co-dominant inheritance (which can be determined both the homozygous and heterozygous

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states of diploid organisms), occupy frequency in the genome, show selective neutral behavior (the DNA in the genome of any organism is neutral to environmental conditions or management practice), should be easily available, have an easy and fast assay with high reproducibility, show no effect of alternate alleles on the plant morphology and should have low or no interaction among markers allowing the use of many markers at the same time in a segregating population.

In genome analysis three types of markers have been used: Morphological markers, protein markers and DNA - based markers. High quality markers, based on the detection of polymorphism in proteins and DNA, have been developed during last three decades (Reviewed by Winter and Kahl, 1995; Mohan *et al.*, 1997; Joshi *et al.*, 1999). They have been termed as molecular markers (Tanksley, 1993). A molecular marker is confined to a discrete difference in the genetic information possessed by two individuals within a species that can be detected by molecular methods. Such markers can be detected by inferring the genotype on the basis of observed marker phenotype. The marker phenotype can be observed by assaying a primary to secondary product of a gene or by analyzing a specific DNA sequence.

The molecular markers have most or all the requisite properties of good genetic markers. For these reasons, their potential as tools for the plant breeding is much higher than that of morphological markers.

1.1 Morphological Markers

Early mapping studies concentrated on discrete traits with simple Mendelian inheritance, which often had one to one correspondence with the genes controlling the traits and phenotypic differences (polymorphisms) between parents were co-inherited. In such cases, morphological characters (phenotypes) can be used as reliable indicators for specific genes. Morphological characters have been the subject of numerous studies in population genetics and agriculture and used for the identification of species, families and genera. However, morphological markers are strongly influenced by environment and identification of morphological markers with each genotype is difficult. To distinguish true genotypic variation from phenotypic variation, special breeding programs and experimental designs are required. Besides these difficulties involved, a large number of morphological markers have been studied and mapped for maize, tomato, rice and many other plants. In the last two decades molecular markers have developed to circumvent some or all the problems associated with the morphologic markers.

1.2 Protein Markers

Protein polymorphisms are detected by separation through gel electrophoresis followed by specific staining of a discrete protein sub-class. Much of the detectable protein variations identify allelic sequence variations in the structural genes encoding the proteins. Alternately, some protein variation is due to post-translational modification. Two classes of proteins, isozymes and allozymes are used as markers. Isozymes are allelic variants of the some enzyme, generally encoded by different loci (Tanksley and Orton, 1983; Weeden *et al.*, 1988), while allozymes are different proteins encoded by different genes performing the same enzyme function.

Isozymes are the most commonly used of protein markers. Markert and Moller in 1959 introduced the term isozyme to define each one of the possibly many multiple forms of an enzyme existing in the same population of an organism. In isozyme analysis, a tissue extract is separated according to their net charge and size by electrophoresis using a starch or polyacrylamide gel. The gel is stained for a particular enzyme by a substrate and a dye under the appropriate reactions, resulting in bands (s) at the position to where the enzyme has migrated. Depending on number of loci, their stable of homo or heterozygosity in an individual, and the enzyme molecular configuration one to several bands are visualized. The positions of the bands are polymorphic and thus informative.

Isozyme linkage maps have also been established for several plant species, including important crops such as tomato (Tanksley and Rick, 1980), rye (Wehling *et al.*, 1985), rice (Wu *et al.*, 1988; Ranjhan *et al.*, 1988). In *Cymbopogon* isozyme marker was used to study genetic diversity (Sangwan *et al.*, 2003).Although the isozyme markers are limited and their expression is often restricted to specific developmental states or tissues, due to the ease of detection through electrophoresis and specific staining, they are used in conjugation with DNA markers.

1.3 DNA Markers

A DNA marker is a small segment of DNA showing sequence polymorphism in difference in individuals within a species. A wide variety of techniques have been developed in the past few years for visualizing the degree of polymorphism. Two basic marker systems have emerged for polymorphism detection in the desired segment, hybridization based markers and PCR based markers.

1.3.1 Hybridization based DNA markers

The realization, that length polymorphism in restriction fragments between individuals could be detected on DNA blots using radioactively labeled probes that hybridize to target sequence in the genome led to revolution in plant genetics. Such hybridization-based markers were applied to a wide range of plant species.

RFLP: In Restriction Fragment Length Polymorphism (RFLP), DNA is digested with restriction endonucleases, which cut genomic DNA at specific palindromic recognition sequences; the resulting fragments are separated by gel electrophoresis, blotted onto a filter and probes are hybridized to the separated

fragments. The polymorphism of fragment size, which depends on the presence and distances between the recognition sequences is detected by autoradiography. Variations in fragment length between individuals or species arise either when mutations alter restriction sites or as a result of insertions or detections in the recognition sites. RFLP markers are co-dominant, allowing detection and characterization of multiple alleles at a given RFLP locus among individuals in a population. They are reliable markers in linkage analysis and breeding and can easily determine if a linked trait is present in a homozygous or heterozygous state in individual, information that is highly desirable for recessive traits (Winter and Kahl, 1995). Bostein *et al.*, (1980) used for the first time RFLP marker to construct a genetic map. Later on, this hybridization-based marker was used widely for mapping of the genomes of many plants such as rice (Mc Couch *et al.*, 1988), tomato (Tanskley *et al.*, 1989; Saliba *et al.*, 2000), *Arabidopsis* (Reiter *et al.*, 1992), potato (Gebhardt *et al.*, 1989), wheat (Anderson *et al.*, 1992). RFLP markers have proved their importance in gene tagging and are very useful in locating and manipulating quantitative trait loci (QTL) in a number of crops. Besides their usefulness, generation and application of RFLP markers are time consuming and expensive. Firstly, one out of several markers provides a polymorphism. This Problem is serious, especially in crosses between closely related cultivated breeding lines. Secondly, for every polymorphic locus tested is cross, a single experiment has to be performed and this is a formidable task with saturated maps such as those of rice, tomato and maize, with hundred or more markers. In addition a large amount of DNA is required for restriction digestion and southern blotting. The requirement of radioactive isotope makes the analysis relatively expensive and hazardous. Finally, the inability to detect single base change restricts the use of RFLPs in detecting point mutations occurring within the regions at which they are detecting polymorphism. Information based on RFLP markers has been used to generate PCR based markers in crops.

Sequence Tagged sites (STS) Sequence tagged sites (STS) are short unique fragments of DNA (approx. 300bp). RFLP markers linked to desire traits are converted into PCR based STS markers from the nucleotide sequence of the probe polymorphic band pattern, or amplicons specific to different genotypes. Using this technique tedious hybridization procedures involved in RFLP analysis are avoided. This approach is extremely useful for studying the relationship between various species at a specific locus (Bustos *et al.*, 1999). STS marker was used in genome analysis of plants (Mazur and Tingy, 1995; Bustos *et al.*, 1999) and integrated into plant breeding programs for marker-assisted selection of the trait of interest.

Expressed Sequence Tags (EST) Theses are introduced by Adams *et al* (1991) and are obtained by partial sequencing or random c-DNA clones. In this approach, the sequence of 300-500bp or one or both ends of each of a large number of randomly chosen c-DNA clones are determined, which in most cases unambiguously identify the corresponding genes. Once generated, they are useful in cloning specific genes of interest and synteny mapping of function genes in related organisms. ESTs are popularly used in full genome mapping of functional genes in related organisms. Moreover, an EST that appears to be unique helps to isolate new genes. EST markers are identified to a large extent for rice, *Arabidopsis*, etc. wherein thousands of functional c-DNA clones are being converted into EST markers (Sasaki, 1994: Cook *et al.*, 1996).

Allele-Specific Associated Primers (ASAPs) To obtain an allele specific marker, a specific allele (either in homozygous or heterozygous state) is sequenced and primers are designed for amplification of a single DNA fragment from the specific allele at stringent annealing temperatures. These markers tag specific alleles in the genome and are more or less similar to SCARs (Mohler and Jahoor, 1996). ASAPs have exhibited their utility in genotyping of allelic variants of loci that results from both size differences and point mutations, e.g. waxy gene locus in maize (Stattuck-Eidens *et al.*, 1991)

Single Strand Conformational Polymorphism (SSCP) SSCP can detect DNA sequence alterations as small as a single nucleotide change (Orita *et al.*, 1989). This method exploits the tendency of single stranded DNA to form intramolecular base pairs, resulting in a sequence dependent conformation with a specific mobility in acrylamide gels. Changes in DNA sequences, even in a single base pair, can cause alterations in the conformation, which result in the changes in electrophoretic mobility. In practice, SSCPs are detected by digesting the DNA with restriction enzymes, denaturing the DNA, separating the DNA by conformation through polyacrylamide gel electrophoresis, followed by Southern blotting using a specific fragment as probe. In another method, PCR is used
to amplify a specific fragment separated on a high-resolution polyacrylamide gel. It was found useful in the detection of heritable human diseases. In plants, however, it is not well developed although its application in discriminating progenies can be exploited, once suitable primers are designed for agronomically important traits (Fukuoka *et al.*, 1994)

Restriction Land Mark Genomic Scanning (RLGS) Introduced by Hatada *et al* (1991) for genomic DNA analysis of higher organisms, this method is based on the principle that restriction enzyme sites can be used as landmarks. It employs direct labeling of genomic DNA at the restriction sites, and 2-dimensional (2D) electrophoresis to resolve and identify these landmarks. The technique has proven its utility in genome analysis of closely related cultivars and for obtaining polymorphic markers that can be cloned by the spot target method (Hirotsune *et al.*, 1993). It has been used to isolate DNA spots specific for a mutable slender-glume gene in rice (Teraishi *et al.*, 1995).

1.3.2 PCR Based DNA Markers

The development of the Polymerase Chain Reaction (PCR) for amplifying DNA sequences led to a revolution in the applicability of molecular methods and a range of new technologies were developed which would overcome the technical limitations of hybridization based markers. In PCR, arbitrary or known sequence primers are used to amplify one or several discrete DNA segments that can be separated in agarose or polyacrylamide gels. Each product is derived from a region of the genome containing two DNA sites with sequences complementary to the primer(s) on opposite strands and sufficiently closer for amplification to work.

Randomly Amplified Polymorphic DNA (RAPD) RAPD uses single short oligonucleotide primers of arbitrary sequence for the amplification of randomly distributed segments of genomic DNA (Williams *et al.*, 1990; Welsh and Mc Clelland, 1990). If the binding sites of primers are on the opposite strands of the DNA in inverted orientation, the segment of DNA flanked by the primer is amplified. Polymorphism in RAPD results from different types of changes in the genomic DNA: base pair substitution, insertion and deletions, which modify or eliminate the primer annealing sites; insertions in the genomic sequence that changes the intervening length of the DNA between the primer sites; and insertions which separate the primer sites to a distance that will not permit amplifications (Williams *et al.*, 1990). This procedure usually amplifies 1-15 DNA fragments from a single primer PCR reaction (Reiter *et al.*, 1992). The amplified DNA band is highly polymorphic between individuals in a population. The primers are usually 10bp length with GC content of at least 50% and have a low annealing temperature (36-40^{0}C). RAPD is an inexpensive and easy technology for fingerprinting, mapping and related research (Rafalski *et al.*, 1991; Waugh and Powell, 1992; Hadrys *et al.*, 1992; Tingey and Del-Tufo, 1993; Williams *et al.*, 1993). In *Cymbopogon*, RAPDs have been used to study genetic diversity among elite varieties (Sangwan *et al.*, 2001, 2003; Shasany *et al.*, 2000), identification of somaclonal variants (Nayak *et al.*, 2003), to establish species relationships and to study the genetic polymorphism of somatic embryo derived plantlets (Bhattacharya *et al.*, 2008).

The RAPD approach has had several modifications, the use of shorter primers (less than 10 nucleotides) in combination with PAGE and highly sensitive silver staining results in DNA amplification fingerprinting (DAF; Caetano-Anolles *et al.*, 1993) and AP-PCR that uses arbitrary primers (Welsh and Mc Clelland, 1990).

Paran and Michelmore (1993) introduced the technique of SCAR (Sequence characterized amplified regions for amplification of specific bands), in which the RAPD marker termini are sequenced. Longer primers, extending into the genomic DNA 3' to the initial RAPD primer, with higher and stringent annealing temperatures are designed for specific amplification of a particular locus; the presence or absence of the band indicating polymorphism. SCARs are dominant markers but may be converted to codominant markers by digesting them with restriction enzymes followed by separation in a denaturing gel. RAPD markers have successfully used for cultivar analysis of various plant species including rice (Ko *et al.*, 1994; Rana *et al.*, 1999), cauliflower (Hu and Quiros, 1991), banana (Kaemmer *et al.*, 1992), *Brassica* (Demeke *et al.*, 1992), cotton (Khan *et al.*,2000),wheat(Farooq *et al.*, 1994). Similarly, in *Cymbopogon* RAPD markers were also used to study genetic diversity, and to establish species relationship (Sangwan *et al.*, 2001, 2003; Khanuja *et al.*, 2005). RAPD markers are reliable and produce reproducible results for phylogenetic relationship. Various molecular markers are also widely used to detect and characterize somaclonal variation at the DNA level (Ford-Llod *et al.*, 1992). Of the available techniques, RAPD is mostly used to identify variant somaclones (Rani *et al.*, 1995; Rout *et al.*, 1998). In a number of plant species RAPD markers were applied to detect gross genetic changes of somaclones (Dey *et al.*, 1997; Hossain *et al.*, 2003; Patzack *et al.*, 2003; Godwin *et al.*, 1997).

In contrast to RFLP markers, RAPD markers not only require extremely small amounts of genomic DNA but also eliminate the need for blotting and use of radioactive probes. RAPD have considerable appeal for surveys of genomic variation and marker assisted selection since they are relatively inexpensive, randomly sample a potentially a large number of loci and prior sequence information is not needed for primer designing. However, the reproducibility of the process needs to be optimized.

Microsatellites and Minisallites Microsattelites and minisattelites are short tandem repetitive DNA sequences dispersed through out the eukaryotic genome. The term microsatellite was coined by Litt and Lutty (1989), while the term minisatellites was introduced by Jeffrey (1985). They were reported first in humans (Bell *et al.*, 1982) and now available in genomes of nearly all higher eukaryotes including plants (Tautz and Renz, 1984; Langercrantz *et al.*, 1993). These DNA elements frequently change their length by slipped-strand mispairing and other less understood process (Levinson and Gutman, 1987; Jeffreys *et al.*, 1988; Zischler *et al.*, 1992), which generate varying number of repeats at a given locus. However, the flanking single copy sequences are normally conserved, which provide a valuable source of polymorphisms for linkage analysis (Lathrop *et al.*, 1985; Jeffreys *et al.*, 1986; Nakamura *et al.*, 1987; Wells *et al.*, 1989), identification of species and cultivars (Weising *et al.*, 1992), and marker assisted selection (Beckman and Soller, 1990).

Minisatellites are tandem repeats of sequences ranging from 9-100bp in the genome; the number of repeats varies, but usually less than 1000. For minisattelites, the loci that vary in the number of repeat units between genotypes are referred to as variable number of tandem repeats (VNTRs; Nakamura *et al.*, 1987), or hypervariable regions (HVRs). These loci, containing tandem repeats within a genome, generate high level of

polymorphism between individuals in a population (Jeffreys *et al*., 1985).

Microsatellites are stretches of DNA, consisting of tandemly repeating mono-, di-, tri-, tetra- or penta nucleotide units, which are hypervariable and ubiquitously distributed through out the genomes of most eukaryotic species. Microsatellites are short tandem repeats (STRs) or simple sequence repeats (SSRs). The SSRs are favoured over minisattelites as the former are evenly distributed in the genome (while the later are usually confined to the telomeric region), found in abundance, and are easier to discover and employ using the PCR.

There are two commonly used ways to identify micro satellite loci suitable for use as genetic markers. For some plant species, such as *Arabidopsis*, rice, where a large amount of DNA sequence data has already been accumulated, microsatellites may be identified by searching through the DNA sequence data bases for sequences containing simple repeats. Primers are commonly designed directly from sequence data. However, for most plant and animal species, a large effort using hybridization and sequencing is needed to identify microsatellites suitable for use as genetic markers. Hybridization using simple repeats as probe to screen genomic clones can be used to identify clones containing microsatellite loci. The conserved DNA sequences flanking SSRs have been used for designing suitable primers for amplification of SSR loci. Any such pair of primers, when used to amplify a SSR locus of a number of genotypes, will reveal SSR polymorphism in the form of differences in the length of the amplified product; each length representing an allele at that locus. The length differences are attributed to the variation in the number of repeating units of a particular SSR locus. As polymorphism is based on differences in the number of repeats of simple sequences, polymorphism revealed by SSRs is also termed as simple sequence length polymorphism (SSLP) and sequence tagged microsatellite sites (STMS).

The uniqueness and value of the microsatellite arise from their multi-allelic nature, co-dominant transmission, ease of detection by PCR, relat.ve abundance, extensive genomic coverage and requirement for only a small amount of genomic DNA. In addition, primer sequence information of the markers may be distributed between laboratories, thus providing a common language for collaborative research and acting as universal genetic mapping tools.

Saturated microsatellite maps have been developed for several plant species including rice (Temnykh *et al*., 2000; Chen *et al*., 1997; Akagi *et al*., 1996), maize (Senior and Heun, 1993; Chin *et al*., 1996; Taramino and Tingey, 1996), barley (Becker and Heun, 1995), wheat (Roder *et al*., 1998), Arabidopsis (Bell and Ecker, 1994) and soybean (Akkaya *et al*., 1995).

Inter Simple Sequence Repeat markers (ISSR) This technique uses primers based on microsatellites to amplify regions between microsatellite loci (Zietkiewicz *et al*., 1994). The resulting bands are electrophoresed in agarose gels, stained with ethidium bromide and viewed under UV light. This technique is more reproducible and generates 3 to 5 times the variation of RAPD bands/ marker (Nagaoka and Ogihara, 1997). ISSR markers have been shown to be more reliable and conform closely to dominant Mendelian inheritance, which makes them useful for genotype analysis and genome mapping elucidate genetic relationships among blueberry cultivars (Levi and Rowland, 1997), peas (Lu *et al*., 1996), wheat (Nagaoka and Ogihara, 1997), and used as a probe linked to particular characters in rice (Akagi *et al*., 1996).

Randomly Amplified Microsatellite Polymorphism (RAMP) In this PCR- based strategy, genomic DNA is first amplified using arbitrary (RAPD) primers, the amplified products electrophoretically separated, and the dried gel is hybridized with microsatellite oligonucleotide probes. This technique combines the advantages of oligonucleotide fingerprinting (Epplen *et al*., 1991), RAPD (Williams *et al*., 1990), and microsatellite-primed PCR (Weising *et al*., 1995). The advantages are the speed of the assay, the high sensitivity and the high level of variability detected and the non-requirement of prior DNA sequence information (Richardson *et al*., 1995). RAMP has been successfully employed in the genetic fingerprinting of tomato, kiwi fruit and closely related genotypes of *Dioscorea bulbifera* (Richardson *et al*., 1995).

Amplified Fragment Length Polymorphism (AFLP): AFLP combines the reliability of the RFLP markers with the ease of PCR (Vos *et al*., 1995). The relatively high polymorphism inherent in the random placement of restriction sites between different genomes as detected by RFLP and the ease of PCR combined with the potential nucleotide sequence variability within a short stretch of DNA directly flanking these restriction sites makes AFLP a highly informative assay for both plant and animal genomes.

The AFLP procedure involves digestion of genomic DNA with two restriction enzymes, a rare cutter and a frequent cutter, ligation of double stranded adapters (corresponding to the restriction sites) to both ends of the digested fragments, pre-amplification of the ligated DNA fragments with primers complementary to the adapters, and selective amplification of a subset of the pre-amplified DNA fragments using primers with one to three arbitrary selective nucleotides at the 3' ends. By changing the selective nucleotides of the primers, different sub-sets of the genome can be amplified. The amplified fragments are detected through radioactive or non-radioactive labeling followed by electrophoresis on a polyacrylamide sequencing gel. AFLP assays are reproducible, require no sequence information and use a small number of generic primers.

AFLP analysis can be applied regardless of the origin and complexity of the genome. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited as landmarks in genetic and physical mapping (Vos *et al*., 1995). AFLPs are useful as tools for DNA fingerprinting (Hongtrakul *et al*., 1997) and for cloning and mapping of variety-specific genomic DNA

sequence (Yong *et al*., 1996). AFLPs are abundant in rice (Cho *et al*., 1996; Mackill *et al*., 1996) and a map consisting of 208 AFLP markers is available for rice (Maheswaran *et al*., 1997). Similar to RAPDs, the bands of interest obtained by AFLP can be converted into STSs (Paglia *et al*., 1998). Thus, AFLP provides a newly developed, important tool for a variety of applications.

Conclusion: The above review reveals that in recent times DNA fingerprinting technologies in plant systems have been a matter of immense interest among the plant scientists throughout the world. In the context of utilization and protection of plant genetic resources, molecular marker assisted breeding strategies and assessment of somaclonal variants has become the important issues. An ever-increasing number of reports are being published in each year directing towards new dimension of research for this promising technique. The different methods on DNA based markers of presently reviewed article revealed their utility in establishing genetic identity and estimating the extent of genetic relationships. Moreover, genome mapping with various molecular markers has tremendous potentialities to bring new dimensions in the field of plant molecular genetics and for understanding molecular evolution.

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