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## Molecular Markers: Assisted Selection in Soybeans

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### 1. Introduction

Modern agriculture seeks increasing gains in productivity, due to great demand for food and the reduction of new agricultural frontiers. A major concern relates to fungal diseases and pest damage, and productivity growth necessarily implies reducing losses caused by these organisms.

Genetic improvement provides plants with different degrees of resistance, which can be used by farmers, making the most economical and efficient management. The process of obtaining resistant cultivars is usually done by the transfer of resistance alleles from exotic sources, which need further evaluation. This strategy has been used successfully in breeding programs for many years.

The evaluation process in plants is an improvement methodology with high cost, complex and subject to environmental variations. Another problem encountered concerns the manipulation of plant pathogens in a place where they occur. As an alternative to overcome the problems mentioned above are used molecular markers. With the development of research in molecular biology, there was the possibility of having one more tool in breeding programs, using DNA as the basic material.

The markers can be classified according to the methodology used to identify them: hybridization - RFLP (Restriction Fragment Length Polymorphism) or amplification of DNA - RAPD (Random Amplified Polymorphic DNA); SCAR (Sequence Characterized Amplified Regions); microsatellites (or SSR - Simple Sequence Repeats) and AFLP (Amplified Fragment Length Polymorphism). The markers are based on natural variation in DNA sequence and have Mendelian segregation.

The use of molecular markers was initiated in the last century, when Bateson & Punnett (1905) indicated the possibility of linkage between genes controlling characteristics of petal color and shape of pollen grain. The strategy of using molecular markers requires basic knowledge about the genetic nature of the trait studied, classifying it as a qualitative or quantitative (Ferreira & Grattapaglia, 1995), whose difference is based on the magnitude of the effect of replacing one allele by another in a given locus.

Molecular markers can be a useful tool to monitor the transfer of alleles of interest. In the early stages of intermediate and improving the process is efficient, but final confirmation is essential in field conditions (Alzate-Marin et al., 2005).

This need for phenotypic analysis requires quality in the polls so that the marker may reflect the field conditions. The test is performed with molecular markers using only a small

sample of plant tissue (usually leaves). No damage occurs and the plant can be conducted normally.

Several breeding programs have used molecular biology techniques, aiming at the marker-assisted selection (Alzate-Marin et al., 2003; Benchimol et al., 2003; Maluf et al., 2008). Features such as disease resistance, pest resistance, genetic purity, gene pyramiding, are some areas with possibilities of action research. Phenotypic characteristics difficult to measure can also be evaluated using molecular markers.

The marker-assisted selection is a process of indirect selection in which the character in question has a high heritability, since not influenced by environmental factors. There is increased efficiency of plant breeding, reducing the number of progenies and the number of generations for the stabilization of the genotypes. The selection can be performed in early generations (Barbosa Neto, 1998; Federizzi, 1998).

This is a useful tool in the genetic improvement of plants introduced differential pricing depending on the type of bullet used. The procedures require specialized laboratories with sophisticated equipment and qualified personnel. There is need to integrate multidisciplinary involving researchers with backgrounds in classical plant breeding, chemistry, biochemistry, plant physiology, statistics, computer science, bioinformatics and others.

The chapter aims to describe the key molecular markers, showing the evolution over recent decades and its practical applications in soybean. It also aims to relate key findings and future possibilities of this tool that are joining forces for the development of soybean.

## **2. Molecular markers based on DNA hybridization**

### **2.1 RFLP (Restriction Fragment Length Polymorphism)**

Changes in DNA caused by changes in the nucleotides can be identified if they occur on a site of a restriction nuclease. If the DNA of plants, which differ in one or more of these nucleotides is digested by these enzymes, fragments of different sizes are generated and can be identified and subsequently cloned (Botstein et al., 1980). These fragments are called RFLP (Restriction Fragment Length Polymorphism).

The polymorphism revealed by restriction fragment analysis allows studies of biological phenomena and processes: characterization of germplasm, genetic map construction and assessment of genetic diversity.

In many studies were identified RFLP markers, which contributed to a broad coverage of the genome. Several genetic maps have been generated, allowing multiple applications within the plant breeding (Nodari et al., 1993). The first research related to RFLP markers were assigned to Helentjaris et al. (1986).

The principle of the technique is the extraction of DNA from a plant and its subsequent fragmentation through different restriction enzymes. Plants have, in most cases, more than one billion base pairs in its genome, and enzymatic digestion produces thousands of fragments that vary in length according to the distribution of restriction sites. The collective analysis is impossible, but the technique allows them to be separate and distinct.

The quality of DNA to be used is essential for the results obtained by RFLP technique. The genetic material is fragmented using restriction enzymes (Ferreira & Grattapaglia, 1995).

The DNA fragments obtained were separated by electrophoresis and subsequently transferred to a membrane using a technique called Southern blotting (Southern, 1975). The identification of the fragments is possible, using radioactive probes, which can be a

fragment of the plant itself, complementary to the fragment of interest. At the end of the process the membranes are exposed to an X-ray film, showing the hybridization due to emission of radiation by the probe. The polymorphism observed among the plants may be related to genetic differences. The marker behaves as co-dominant, where at each locus studied is possible to identify individuals homozygous or heterozygous. The amount of information produced is large and allows the analysis of gene action and interactions between alleles.

The restriction nucleases are enzymes capable of breaking the DNA strand cutting it systematically in specific locations. The first enzyme found in the bacterium *Haemophilus influenzae*, showed ability to cut the genetic material of *Escherichia coli* and was named *HindIII*. Enzymes have been isolated from bacterial strains differ by cleavage at specific restriction sites. The enzymes recognize sequences of four to eight bases. Enzymes that recognize restriction sites composed of four base pairs cleave DNA on average every 256 nucleotides ( $4^4 = 256$ ). Those who recognize sites with 6 and 8 bp cleave DNA on average every 4096 and 65536 bp, respectively. However, this average can vary significantly, depending mainly on the base composition of DNA analyzed.

Preliminary evaluations of the cleavage sites, the relationship between the amount of enzyme and DNA, and exposure time are factors that determine the success of this step of RFLP technique. After digestion the samples receiving the loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 25% ficoll type - 400 - in water) and are subjected to electrophoresis (Ferreira & Grattapaglia, 1995).

The concentration of agarose gel used varies between 0.5% and 2% depending on the size of the fragments generated by digestion. For larger fragments, we use a lower concentration and when the fragments generated have low molecular weight, is used gels with higher concentration. The dye ethidium bromide (0.5 ug/ml) is added to the gel heated in order to promote the visualization of the fragments under ultraviolet light.

At the end of electrophoresis, the fragments should be transferred to a nylon membrane or nitrocellulose filter, according to the methodology Southern blotting (Southern, 1975): 1. Place the gel in alkaline solution (0.5 N NaOH, 1.5 M NaCl) for thirty minutes to break the hydrogen bonds of the double helix and allow hybridization with probes; 2. Transfer the gel to a neutral buffer solution (0.5 M Tris-Cl, 1.5 M NaCl, pH 7.5) for thirty minutes; 3. Transfer the fragments to the membrane by capillary action, which could last more than six hours; 4. Rinse the membrane in 10X SSC solutions and set in oven for thirty seconds ultraviolet and 5. Place the membrane to dry at room temperature and then subjected to treatment in vacuum furnace for three hours at 90°C and 6. Use immediately or store the membrane at 4°C.

The last step of the RFLP technique is the process of hybridization between the fragments generated by enzymatic digestion and the probes and subsequent exposure to X-ray. According to Ferreira & Grattapaglia (1995), the probes can be clones obtained by reverse transcription of mRNA, fragments of genomic DNA, fragments generated by amplification of known sequences or RAPD bands. The selection of clones is critical to the success of the technique. Depending on the type of probe used RFLP markers can show good results within the genome.

The membrane and the probes are placed in a common solution to the homologous sequences may hybridizing. This step is conducted for about twelve hours at 60°C. After this period the membranes are washed in SSC solution, dried and exposed to X-ray.

Through RFLP markers can be generated linkage maps used for mapping other traits of agronomic importance. Through them it is possible to detect associations between markers

and genes of interest. When the model is defined, the selection criterion becomes an RFLP marker and not the phenotype. This procedure is called marker-assisted selection and can be used for difficult to assess characteristics: resistance to nematodes, protein production.

Some researchers have used RFLP technique (Apuy et al., 1988; Doyle, 1988; Doyle & Beachy, 1985) and reported at the time, the low level of polymorphism found in soybean plants. The correct choice of enzymes and determination of time of exposure of DNA to enzymatic action, can determine the degree of polymorphism.

Keim et al. (1990) developed a genetic map for soybean using RFLP marker associated with QTL (Quantitative Trait loci) and highlighted the quality of the technique. The same authors reported on the importance of maps for the improvement and understanding of plant evolution.

The RFLP technique has high consistency and repeatability of results. Several searches were performed for characterization of cultivars (Autrique et al., 1996; Gebhardt et al., 1989; O'Donoghue et al., 1994). Tozuka et al., (1990) conducted in 1097 collecting wild plants of soybean (*Glycine soja*) in Japan and applied the RFLP technique in mitochondrial DNA, in order to separate them into groups using two probes (*coxII* and *atp6*). The authors stressed the quality of technical and plants classified in 18 groups.

The resistance to cyst nematode (*Heterodera glycines* Ichinohe), due to the damage it causes in soybeans, is the subject of several studies. *Rhg4* gene was mapped on linkage group A2 by Weisemann et al. (1992) and Webb et al. (1995). Mahalingam & Skorupska (1995) obtained similar results working with RFLP and RAPD markers. Factors such as the large number of steps and use, in many cases, of radioactive probes, prevent the use of the technique on a large scale.

### 3. Molecular markers based on DNA amplification

#### 3.1 PCR (Polymerase Chain Reaction)

The technique of polymerase chain reaction (PCR) was developed in the late 80s (Mullis & Faloan, 1987; Saiki et al., 1988) and revolutionized the molecular genetic studies (Watson et al., 1997). The PCR allows the production of a large number of copies of specific DNA sequence without cloning. The impact of the PCR technique and methods derived from it Kary Mullis took to win the Nobel Prize in 1993. Many methods for cloning, sequencing and analysis of DNA polymorphism were accelerated or replaced by the use of the many derivations of the PCR technique (Ferreira & Grattapaglia, 1995).

The reaction has as its basic principle the natural replication of DNA molecules that occur in cells. After opening the double strand, obtained by heating, a primer specific base pairs is the region of interest, and then there is the amplification of a new single strand. The double-stranded DNA serves as template for the synthesis. The reaction is mediated by the action of Taq polymerase and results in amplification of a specific fragment.

According to Watson et al. (1997) PCR technique is relatively simple and versatile. The equipment required for initial reaction is the DNA that contains the sequence to be amplified, two oligonucleotides (primers) that direct the starting point of synthesis, DNA polymerase and the four deoxyribonucleotides (A, T, C, G). The mixture also contains a buffer solution, magnesium chloride and water.

The reaction is performed in an appropriate tube, heated (92-95°C) for 5 minutes, so that the double-stranded molecules are split to form single strands. These single strands serve as

templates for the primers. Then the temperature is reduced (35 to 60°C) providing suitable conditions for pairing between primers and complementary sequences. In the next step the temperature is high (72°C) for up to 5 minutes. At the end of the temperature is again raised to 94°C for 20 seconds, for the separation of short strips of DNA that serve as templates for new cycles. For most protocols, the number of cycles varies between thirty and sixty.

The discovery of the bacterium *Thermus aquaticus* which lives in water temperature of 75°C and isolation of its polymerase (Saiki et al., 1988), enabled the automation of PCR by using thermocyclers (Watson et al., 1997), which are heating blocks that can be programmed to control the time and reaction temperature. They are equipment with the capacity to change the temperature quickly and repeat the cycle according to the protocol and the number of amplified fragments doubles every cycle. The Taq polymerase of bacteria supports high temperatures without being destroyed.

The enzyme requires a double-stranded fragment in the case, provided by specific primers to initiate amplification of the complementary strand of DNA. If two primers complementary to both strands of DNA are used, the amplification occurs in both directions and two new single strands are generated. Each will have as starting point the sequence of an initiator and extend beyond the other's position in initiating complementary strand. The extension process is always from the position 3' of the initiator. Millions of copies of a given follow-up of DNA, typically up to 4000pb can be synthesized using Taq polymerase.

According to Watson et al. (1997) in 1985 there were three research reports with PCR, some years later the PCR was being used in thousands of labs around the world. The technique caused a major revolution in the practice of molecular genetics.

### 3.2 RAPD (Random Amplified Polymorphic DNA)

Molecular markers may characterize a plant from plant tissue samples. Welsh and McClelland (1990) and Williams et al. (1990) proposed a technique denominated RAPD (Random Amplified Polymorphic DNA) using primers of arbitrary sequence (10 nucleotides) and identifies polymorphic among different individuals. RAPD molecular markers can be used for studies related to genetic mapping, population genetics, molecular systematics, fingerprint genotypes and marker-assisted selection.

The technique allows the identification of numerous markers without prior notification, which may or may not be associated with traits of interest. If a particular marker is physically linked to a gene of agronomic interest, the selection of this results in indirect selection marker gene (Ferreira & Grattapaglia, 1995). The situation described is characterized as marker-assisted selection. If the distance between the marker and the gene is small, the efficiency of indirect selection will be higher.

The protocols used to show satisfactory results, but generally you should take steps to maintain the quality of DNA and reagents. The type of thermocycler can also be considered as a variable. The observation of the proposed action results in quality bands and the possibility of replication of results.

The principle of the technique is based on the PCR reaction: elevated temperature of the solution to the double-stranded DNA to open in their hydrogen bonds. Then the reduced temperature and allowing the pairing occurs between the primers and the DNA strand. Finally the temperature is again raised to the Taq DNA polymerase to amplify the fragments thereby generating bands that will be visualized in gel electrophoresis.

The temperature variations are possible using a thermal cycler that automatically performs the operations for each step: denaturation (92-95°C), pairing (35-60°C) and amplification

(72°C). The cycle is repeated about 40 times and the final product of amplification can be visualized in agarose gel with ethidium bromide dye under ultraviolet light. RAPD markers behave as dominant, characterized by amplification of fragments or not.

The low temperature necessary for linking the primer to the template makes the process very dependent on the conditions of amplification. Therefore, modifications in the thermocycler used, in the DNA polymerase and other reagents of the reaction mixture can change the pattern of amplification. This may not represent a major problem in a group of researchers who carefully standardize the conditions of amplification, but may prevent data from being compared between different laboratories (Alzate-Marín et al., 2005).

The successful use of RAPD molecular marker is directly linked to quality of genomic DNA, which can be extracted using CTAB protocol. A soybean leaf stage provides enough DNA for several reactions. The leaf collected should be placed in contact with liquid nitrogen and then macerated. DNA can be extracted immediately or the leaves can be stored at -80°C.

After extraction, the DNA must be quantified for standardization to occur in the reactions, which assures quality of the bands obtained. For RAPD reactions, quantities of DNA in the order of nanograms provide adequate conditions for the success of the process. DNA can be stored at low temperatures (-20°C to -80°C) depending on usage needs. Contaminant compounds such as proteins, polysaccharides and phenolic compounds can affect the quality of DNA and inhibit the action of Taq polymerase.

The observed differential to obtain RAPD markers, in relation to the PCR process is the use of arbitrary primers, and a large number of random amplification. Thus the bands obtained can not be directly related to a gene of interest. The primers are oligonucleotides of known sequence featuring 10bp. Currently a large number of primers is offered on the market, which allows an enormous amount of reactions with the same DNA.

To associate a characteristic phenotypic marker for a particular molecularly was proposed by Michelmore et al. (1991) methodology BSA (Bulked Segregant Analysis).

Once determined the phenotypic trait of interest, plant a segregating F<sub>2</sub> population are classified according to the established criteria, such as resistant or susceptible to a given disease.

The plant DNA can be extracted from a small leaf sample, taken at the beginning of plant development, individually stored refrigerated. All plants of the F<sub>2</sub> population provide DNA for further RAPD reactions.

After the end of the phenotypic evaluations plants should be classified into distinct groups, contrasting in relation to the characteristic under study. Each group is composed of a small number of plants following the theory. For a dominant RAPD marker segregating in an F<sub>2</sub> population, the probability of a bulk of  $n$  individuals having a band and a second bulk of equal size not having a band will be  $2(1-[1/4]^n)(1/4)^n$  when the locus is unlinked to the target gene. Therefore low individuals per bulk are required. For example, the probability of an unlinked locus being polymorphic between bulks of 10 such individuals is  $2 \times 10^{-6}$ . Even when many loci are screened, the chances of detecting an unlinked locus are small. As smaller bulks are utilized, the frequency of false positives will increase. However, as the linkage of all polymorphisms is confirmed by analysis of a segregating populations, bulked segregant analysis with only small numbers of individuals in one or both bulks will provide great enrichment for marker linked to target loci (Michelmore et al., 1991).

Two solutions containing DNA should be prepared in equimolar concentrations, for the continuation of the technique. Example: A group with DNA from plants resistant to disease and another group with the DNA of plants susceptible to disease. Initially the RAPD reactions are performed with the DNA of each group. Thus polymorphism in a group may be associated with the characteristic that distinguishes them.

Once polymorphism observed between the groups, the reactions are being carried out individually with DNA from plants of groups, with the objective to confirm the results.

A marker that shows Mendelian segregation can be considered a gene locus. Segregation of phenotypic characteristics assessed and polymorphic fragments should be individually assessed according to the 3:1 hypothesis.

Then the analysis must be performed by testing the hypothesis of joint 9:3:3:1 segregation. If the hypothesis is rejected, the marker and the allele that determines the desired trait can be on the same chromosome. The question is which the distance between them, which can be determined by the number of recombinant individuals

The smaller the genetic distance between the marker and the allele of interest, the greater the efficiency of molecular markers, to the point that if the distance is close to zero, the marker is the gene itself. So there is a need for an excellent mechanism for phenotypic evaluation and qualified people to apply it, for all subsequent work depends on this step.

Another point that needs to be analyzed is concerned for the fate of a marker allele is not in question but rather a region of chromosome near the gene of interest. The situation may lead to specific markers since the chromosomal region, classified as a molecular marker may not be present in different plants. The absence of amplification can be explained by mutations or rearrangements between the two sites, or on the site of hybridization of the initiator (Paran & Michelmore, 1993). Differences in only one base pair may be sufficient to inhibit amplification, especially at position 3' (Williams et al., 1990).

It is expected that with advances in functional genomics projects, some markers will lose its practical application, because knowing the sequence of the allele, the job is much more accurate.

Martins Filho et al. (2002) used the technique BSA, in a survey of three F<sub>2</sub> populations of soybean exhibiting resistance to the fungus *Cercospora sojina*. The authors have built on resistant and susceptible groups, with six plants in each one of them and were able to identify RAPD markers in three populations. The DNA of plants assessed individually confirmed the identification observed in the groups, validating the methodology BSA. The distances between the markers found by the authors and the locus studied ranged between 2.3 and 6.7 cM.

Carvalho et al. (2002) identified two RAPD markers linked to gene for resistance to stem canker in soybeans, which were amplified by the same initiator: OPAB19<sub>1320</sub> present in all plants classified as homozygous resistant (coupling phase) and OPAB19<sub>1150</sub> present in susceptible plants (repulsion phase), whereas heterozygous plants classified as resistant showed the two fragments. The distance between the gene and the markers, was estimated at 4.7 cm.

Gavioli et al. (2007) worked with F<sub>2</sub> populations of soybean plants resistant and susceptible to the fungus that causes stem canker, which were evaluated in a greenhouse. The authors applied the methodology BSA (seven plants in each group) and identified a polymorphic RAPD band, with 588 bp, within the group of resistant plants. There were two replications of observed distances of 6.0 and 7.4 cM from the RAPD marker and the locus of interest. DNA from plants of each group was analyzed individually and the band found previously was confirmed (Figure 1).

Recently Costa et al. (2008) identified RAPD markers linked to genes conferring resistance to rust caused by *Phakopsora pachyrhizi*. The authors applied the methodology BSA (ten plants in each group) and related markers: OPBB16<sub>650</sub>, OPAK04<sub>800</sub> OPR04<sub>850</sub> and distant to 5.1 cM and 6.3 cM and 14.7 cM, respectively, the locus of resistance *Rpp4*. The authors concluded

that the RAPD primers identified in the survey are indicated for assisted selection of soybean genotypes with the same source of resistance in this study. The selection can be performed in the early stages of development to occur without destroying the plants.

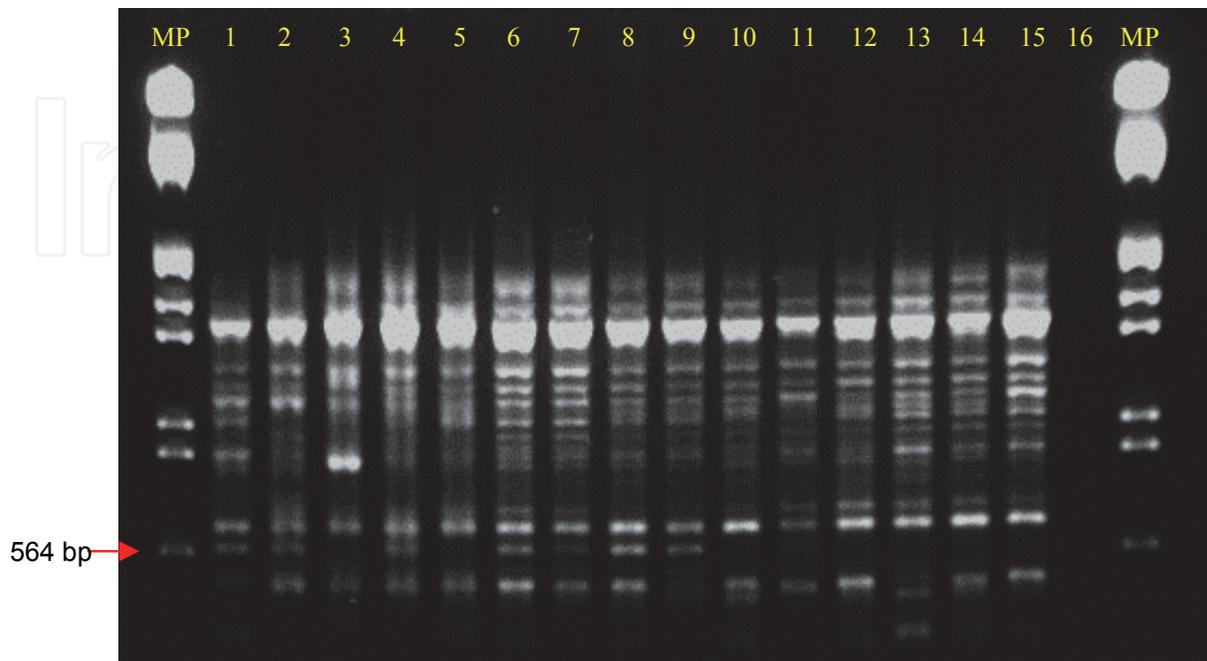


Fig. 1. RAPD polymorphic fragment of 588bp observed in: 1. resistant parent BR92-15454; 2. F<sub>1</sub> plant; 4. resistant bulk and 6-9. F<sub>2</sub> resistant progenies and absent in: 3. susceptible parent IAC-11; 5. susceptible bulk and 10-15. F<sub>2</sub> susceptible progenies. 16. Negative control. Molecular pattern (MP), originating from the digestion of  $\lambda$  with the enzymes *EcoRI* and *Hind III*.

On soybeans, other authors have worked with RAPD markers and were able to identify some of them linked to genes of interest. Heer et al. (1998) identified markers for genes that determine resistance to cyst nematode. Chowdhury et al. (2002) worked with plants resistant to downy mildew and reported the existence of two markers linked to the gene that determines resistance, respectively, 4.9 and 23.1 cM.

Segregating progeny may be evaluated for the presence or absence of a particular marker. The heterozygote cannot be distinguished and this represents a loss of information in relation to RFLP markers. Populations consisting of backcross progeny, recombinant strains and di-haploids do not undergo this loss of information, since the full information available can be obtained in the presence or absence of the marker (Reiter et al., 1992).

### 3.3 SCAR (Sequence Characterized Amplified Regions)

RAPD reactions can be performed easily, but problems related to standardization of equipment, reagents and protocols, often leads to difficulties for certain searches can be repeated. As referenced above, this may not be a big problem in a group of researchers careful (Alzate-Marin et al., 2005).

To overcome the inconsistent results with RAPD markers, polymorphic fragments obtained, have been cloned, sequenced and converted into SCAR markers (Sequence Characterized Amplified Regions) (Paran & Michelmore, 1992). According to the authors SCAR markers

have advantages: specificity for detecting a single locus and less sensitivity to variations of the reactions. SCAR markers have co-dominant nature and were defined as fragments of genomic DNA, located in a defined locus, which are identified by PCR amplification using a pair of specific oligonucleotides as primers (Ferreira & Grattapaglia, 1998; Nietzsche et al., 2000).

The RAPD polymorphic fragments have to be isolated, cloned and sequenced and then will be used for the synthesis of new primers. Usually the SCAR primers have at their 5' end, the initiator used in RAPD reactions and its initial 3' end, the additional bases that will characterize the new initiator.

The SCAR markers can be synthesized starting from a molecular marker RAPD. The isolation of the RAPD fragment is accomplished through a direct cut in the agarose gel containing the desired band. The isolated fragment must be inserted into a vector, usually a plasmid, which will be used in the process of bacterial transformation. Transformed colonies is necessary to separate the fragment that contains, those not containing the desired DNA fragment. After this step the selected colonies are grown for growth and subsequent multiplication of the fragment. Detailed protocols on bacterial transformation can be obtained in Sambrook et al. (1989).

The fragments should be extracted and purified from plasmids. Restriction enzymes are able to cut the plasmid at sites flanking the fragment. The insert should be sequenced to be known that the bases are among the RAPD primers.

For the synthesis of new initiators is considered some parameters such as GC percentage (minimum 50%) and pairing temperature (more than 56°C). Usually the new primers have between 16 and 24 base pairs. There are computer programs that help in developing new primers. Martins Filho et al. (2002) worked with two primers containing 18 nucleotides in each, and determined the temperature from 62°C as an ideal pairing.

At the end of the process, the new SCAR primers should be tested with the plants of the F<sub>2</sub> population and parents to prove the link between the marker and the locus of interest. The confirmation allows the use of marker assisted selection in the process.

Often there is loss of polymorphism of RAPD, when converted into SCAR markers. The problem results from the amplification of two alleles of that locus, which prevents the differentiation between plants. Paran & Michelmore (1992) reported that the polymorphism observed in the RAPD reaction, can be caused by differences in the nucleotide sequence of the site of annealing or rearrangement in the internal sequence of the amplification. A mis-pairing, mainly at the 3' primer, prevented the amplification of a fragment of the genotypes. In a SCAR primer, with the largest number of nucleotides, the end without pairing, is positioned in its middle region and may not interfere with the amplified fragments.

The loss of polymorphism can be solved through the use of restriction enzymes, which promote the cut at specific sites in one allele of a given locus. The technique was successfully used by several authors, among them: Weng et al. (1998), Lahogue et al. (1998), Dax et al. (1998) and Zhang & Stommel (2001).

The choice of restriction enzyme based on the sequencing of the fragments SCAR. The sequences are evaluated within and should be sought restriction sites that can differentiate them. Monomorphic fragments in molecular weight may differ in base sequence.

The fragments are then PCR amplified and digested individually. The result of electrophoresis may reveal again the initial polymorphism. Gavioli et al. (2007) converted a RAPD polymorphic in a SCAR marker. The process resulted in the loss of polymorphism,

which was resolved by digesting the fragments with the enzyme *HincII* (Figure 2). The technique was efficient because alleles from resistant parent were digested by *HincII* enzyme produced two fragments, one of 531 bp and another of 57 bp, while the susceptible parent stayed with the fragment of 588 bp. F<sub>1</sub> plants heterozygous for the locus, showed a pattern of three bands. The same result was observed in F<sub>2</sub> plants classified as heterozygous resistant.

The loss of polymorphism was recovered by enzymatic digestion and plants could be distinguished in homozygous recessive, homozygous dominant and heterozygous. The process becomes more expensive, but allows for the recovery of the polymorphism and the use of SCAR marker.

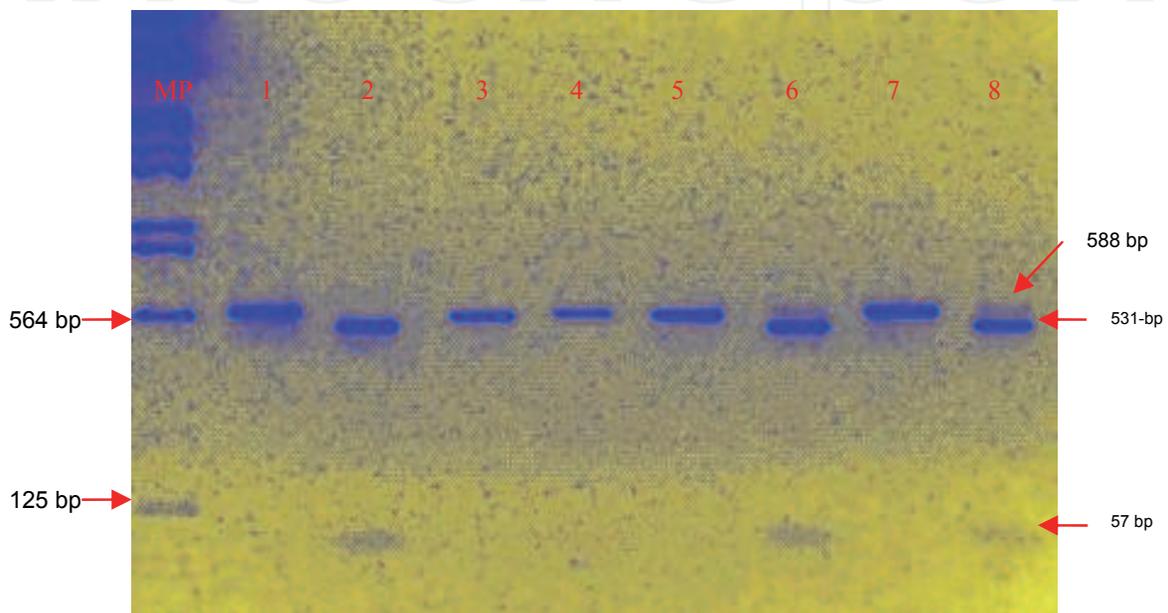


Fig. 2. SCAR monomorphic fragment of 588bp with and without enzymatic digestion (*HincII*): 1. resistant parent BR92-15454; 2. resistant parent BR92-15454 (digested); 3. susceptible parent IAC-11; 4. susceptible parent IAC-11 (digested); 5. F<sub>1</sub> plant, 6. F<sub>1</sub> plant (digested); 7. heterozygous resistant F<sub>2</sub> plants; 8. heterozygous resistant F<sub>2</sub> plants (digested). Molecular pattern (MP), originating from the digestion of  $\lambda$  with the enzymes *EcoRI* and *Hind III*.

There are numerous studies to obtain SCAR markers linked to disease resistance of crops. In soybean there are the surveys conducted by Heer et al. (1998) who worked with resistance to soybean cyst nematode; Martins Filho et al. (2002), in studies on resistance to the fungus *Cercospora sojina*; Zheng et al. (2003) in relation to the mosaic virus and Carvalho et al. (2002) in the evaluation of soybean plants resistant to stem canker.

### 3.4 Microsatellites – SSR (Simple Sequence Repeat)

Another type of molecular marker is the microsatellite or simple sequence repeat (SSR). Microsatellites or STR or SSRP (Simple Sequence Repeat Polymorphisms) or STMS (Sequence Tagged Microsatellite Sites) are some of the dominions assigned to these molecular markers. The use of these markers has been increasing, due to the fact that you use the PCR technique, co-dominant and present with a relatively high frequency within the plant genome (Akkaya et al., 1992).

Are sequences consisting of repetitions of one to four nucleotides, which occur naturally in the genome, such as repeated (AT)<sub>n</sub>, (ATT)<sub>n</sub>. The genome of plants has, on average, ten times less than the microsatellite genome (Powell et al., 1996). The repeats are more common in plants (AT)<sub>n</sub>, (GA)<sub>n</sub>, (AC)<sub>n</sub>, (AAT)<sub>n</sub> and (AAC)<sub>n</sub> (Gupta & Varshney, 2000; Wang et al., 1994). The DNA from organelles has a low frequency of SSRs (1 per 317 Kb) (Wang et al., 1994). Microsatellites are present in coding regions and non-coding (Zane et al., 2002).

The variation of n number of repeated elements generates a great amount of polymorphism. According Brondani et al. (1998), microsatellites have characteristics that result in benefits for the plant breeding: Nature co-dominant and multiallelic; highly polymorphic, allowing precise discrimination even of highly related; abundant and uniformly dispersed throughout the genome plants; can be analyzed by the PCR reaction. In plants one of the first findings was made by Nybom et al. (1992).

Another important point is that the DNA sequences flanking the SSRs are conserved within the same species, allowing the selection of specific primers that amplify via PCR. The amplification using a pair of primers complementary to unique sequences that flank, resulting in an enormous fragment length polymorphism. This size variation of PCR products is a consequence of the occurrence of different numbers of repeating units within structure of microsatellites (Ashkenazi et al., 2001; Cregan et al., 1999a; McCouch et al., 1997; Morgante & Olivieri, 1993). Thus, alleles may be determined for a given population. Homozygous individuals have the same number of repetitions in the chromosomes, while heterozygous individuals have different numbers of repeats in both chromosomes. Therefore, the locus is defined by the pair of primers and the various alleles by the size of amplified bands.

Some mistakes during DNA replication in different individuals of the same species, can provide a varying number of repeats within a microsatellite, which are different alleles. Currently, many species of plants already possess a set of microsatellite markers for use in genetic studies (Akkaya et al., 1992; Cregan et al., 1999a).

The practical use of microsatellite markers has occurred in human studies (Litt & Luty, 1989) and attracted the attention of plant breeders, since several studies have shown that microsatellites are widely distributed in the genome of the species (Brunel, 1994). According to Ferreira & Grattapaglia (1995), in eukaryotic genomes, these simple sequences are very frequent, randomly distributed, besides being highly polymorphic genetic locus.

The initial protocols identified the microsatellite locus in clones of total genomic libraries using probes complementary to regions of interest, such as (AC)<sub>10</sub> and (AG)<sub>20</sub> (Rassmann et al., 1991). The amplification products are separated by electrophoresis, which in most cases, should be done on polyacrylamide gel because of the small size difference between fragments. The attainment of the primers is the most expensive step of the process of using microsatellite markers in marker assisted selection in plants.

Each microsatellite locus can be analyzed individually or jointly with another, when the alleles of each locus have sizes sufficiently different to migrate into separate zones in the gel (Lanza et al., 2000).

Microsatellite markers are indicated to various kinds of analysis, because they are polymorphic in soybean, highly reproducible, co-dominant and by their low cost, considering that about 650 pairs of primers specific for soybeans are available on the market (Cregan et al., 1999a).

Registration and the granting of rights to new varieties is usually done based on morphological and physiological characteristics, uniformity and stability, necessitating the

distinction in at least one of them. However, most morphological traits are quantitative traits, with its expression being altered by environmental factors. Moreover, the registration number is increasing rapidly, making it impossible for authorities to compare the new varieties efficiently with existing ones. The marker has been proven useful for identifying varieties and tested for various crops such as soybeans.

The microsatellite markers have been used in soybean for mapping specific genes that determine agronomic traits, and also to identify QTLs (Quantitative Trait Loci) of economic importance, involved in grain yield and genetic resistance to pests and diseases, which are characteristics of complex inheritance (Yuan et al., 2002).

The development of genetic maps is considered one of the applications of greatest impact in the technology of molecular markers for genetic analysis of species and, potentially, in plant breeding. In this context, the genetic maps enable: Full coverage and analysis of genomes; decomposition of complex genetic traits into their Mendelian components; location of genomic regions that control traits of importance; quantification of the effect in these regions studied feature, directing all this information to use in breeding programs.

Some quantitative trait loci (QTL) for resistance to some important diseases of soybean, were mapped in the chromosomal region adjacent to the locus of resistance to leaf rust *Rpp5*. Near the marker Satt009, were mapped QTL for resistance to the pathogen *Sclerotinia sclerotiorum*, the fungus that causes white mold in soybean (Arahana et al., 2001), and an adjacent chromosomal region, is mapped a QTL for resistance to the cyst nematode, which causes the most serious threat to soybean production (Concibido et al., 1997).

Mekesem et al. (2000) constructed a map of high saturation of three genomic regions in soybean, which contains the *Rhg4* and *rhg1* alleles that confer resistance to SCN, and the region containing the *Rfs* allele, which confers resistance to *Fusarium solani* f. sp. *glycines*.

In linkage group N, 3.2 cM from Satt009 marker was mapped a locus of resistance to *Phytophthora sojae*, the pathogen that causes root rot in soybean and marker linked to Satt080, and a QTL for resistance to *Fusarium solani* f. sp. *glycines* (Njiti et al., 2002).

Other microsatellite markers linked to various diseases of soybean have been reported in the literature. Mudge et al. (1997) concluded that microsatellite flanking Satt038 and Satt130 allele *rhg1*. Cregan et al. (1999b) detected the SSR Sat-168 and Satt 309 delimiting *rhg1*. The marker Satt215 was found to be linked to the gene *Rbs1*, which confers resistance to black pod-of-staff, with selection efficiency of 88% (Bachman et al., 2001). A new gene, *RCS<sub>Peking</sub>*, which confers resistance to stain-frog-eye, was mapped to 1.1 cM of marker Satt244, on linkage group G (Yang et al., 2001). Funganti et al. (2004) identified the marker Satt114, linked to the nematode resistance locus of the root knot nematode (*Meloydogyne javanica*), in linkage group F.

Garcia et al. (2008) mapped the locus *Rpp5* in three different populations of soybean (PI 200456, PI 471904 and PI 200526), and obtained: six markers linked to this locus in population PI 200456 (Satt530, Sat\_208, Sat\_166, Sat\_275, and Sat\_280 Sat\_266 ) and five in PI471904 (Satt530, Sat\_208, Sat\_166, Sat\_275, Sat\_280) and three in PI 200526 (Sat\_166, and Sat\_275 Sat\_280).

Morceli et al. (2008) identified two new microsatellite markers potentially associated with resistance of soybean to soybean rust caused by *Phakopsora pachyrhizi*. The authors evaluated the markers on individual plants, and found the link to *Rpp5* gene and are present on linkage group N of soybean. The efficiency of selection was determined for all markers linked to gene *Rpp5*, and the combination of the markers Sat\_275 + Sat\_280 was 100%.

Schuster et al. (2004) used markers for determining genetic purity of seed lots of soybean. The authors presented a table listing the primers used more frequently in the determination

of genetic purity of seed lots of soybean: Sat\_128, Satt070, Sat\_085, Satt079, Sat\_110, Satt184, Satt005, Satt141, Satt186, Satt146, Sat\_064, Sat\_094, Satt163, Satt181, Sat\_105, Satt162, Satt183, Satt167, Sat\_099, Satt156, Satt150, Satt175, and Satt136 Sat\_127.

The following is related in detail, the protocol used by Schuster et al. (2004): Total volume of 10 or 25 mL, containing 12.5 mM Tris-HCl (pH 8.3), 62.5 mM KCl, MgCl<sub>2</sub>, 2.5 mM, 125 mM of each deoxynucleotidios (dATP, dTTP, dGTP and dCTP), 0.2 μM of each primer (sense and antisense), a unit of Taq DNA polymerase and 30 ng of DNA. The total volume of the reaction was 25 mL when the separation was performed in 3% agarose gel and 10 mL when the separation was performed on 10% polyacrylamide gel. The amplifications were performed in Perkin Elmer 9600 thermocycler, programmed for an initial step of 7 min at 72°C, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C. Finally, a step of 7 min at 72°C. The amplified fragments were separated by electrophoresis on 3% agarose gel containing ethidium bromide etídio (0,2 μg/mL) and 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA), or 10% polyacrylamide gel in TBE buffer. In this case, the gels were stained after the run in TBE containing ethidium bromide (2μg/mL).The gels were photographed under ultraviolet light.

The authors concluded that analysis of DNA extracted from seeds, considered atypical by the method of visual analysis with microsatellite markers is effective in determining the genetic purity of seed lots of soybean, and that the use of proteinase K and RNase in the process of extraction allows to obtain greater quantities and better quality of DNA for analysis.

Fuganti et al. (2004) conducted research towards the identification of microsatellite molecular markers for selection of soybean genotypes resistant to the nematode *Meloydogyne javanica*. The authors concluded at the time that the markers, SOYHSP 176 and Satt 114, the linkage group F of soybean, can be considered potential markers to be used in the process of marker assisted selection of soybean genotypes resistant to root knot nematode

Hoshino et al. (2002) performed an extensive review of the microsatellite markers and reported that despite its widespread popularity there are certain limitations: 1. Identification of genotypes: usually performed by determining the length of the PCR product. The polymorphism is attributed to changes in the number of repeating units, however, it is possible that small insertions or deletions occur in adjacent regions that did not necessarily alter the number of repetitions, but change the length of the fragment generated. In such cases, determining the number of repetitions from a cloned fragment and its subsequent use for amplification in other species without sequencing, can lead to mistakes in the estimation of genetic distances; 2. null alleles: the occurrence of point mutations, insertions or deletions at the site of pairing of the initiator can prevent the amplification of a given microsatellite locus. If changes are not fixed in the population, only a portion of the alleles will be amplified; 3. mutation rate: Positive correlations between the length of the microsatellite sequence and mutation rate are not always true, and can cause errors in the estimation of genetic distances; 4. Frequency of different organisms: The application of this marker in a growing number of organisms has revealed that the abundance of repetitive sequences varies significantly between species. While some species contain a sufficient number of microsatellites for population studies, others have few sequences of this kind; 5. Artifacts PCR: Theoretically, the PCR technique allows the amplification of microsatellites from a single cell, but the amplification of microsatellites from small amounts of DNA are associated with high frequency of errors. Two common types of mistakes in this case are: the amplification of the alleles with incorrect length, and no amplification of one allele in

heterozygotes. The authors asserted that this type of marker is a valuable tool for analysis of genetic variability in germplasm of plant species and therefore its maintenance.

Microsatellite markers have many advantages compared to other types of markers (RFLP, RAPD, AFLP) are highly polymorphic and informative; the co-dominant inheritance, which allows discrimination between homozygous and heterozygous; are multi-allelic; occurring abundantly in genomes of eukaryotes; are based on PCR and thus need small amounts of DNA; are highly reproducible; require no radioactivity; are well dispersed in the genome in coding regions and non-coding; loci are often conserved between related species.

The microsatellite markers have been used extensively to the major species of agronomic importance and have potential to occupy a prominent place among the markers of greatest use.

### 3.5 AFLP (Amplified Fragment Length Polymorphisms)

Since its development and publishing, this technique has been used to characterize genotypes, genetic mapping, especially in species with low DNA polymorphism (Zabeau, 1993). The use of this marker is based on the technique that combines DNA fragmentation with restriction enzymes type II, that cleave DNA at specific sites of rare cutting (recognize sites of 6-8 bases, ex: *Apal*, *EcoRI*, *HindIII* and *PstI*) and frequent cutting (recognize sites of four bases, ex: *MseI* and *TaqI*) and amplification of these fragments by PCR (Vos et al., 1995).

The use of specific restriction enzymes, allows the knowledge of the cohesive ends generated, in which they are linked adapters that serve as binding sites for primers in a PCR reaction. It is essential that the DNA digestion is complete, because the partial digestion can reveal false polymorphisms. The purity of DNA used is a fundamental requirement for obtaining good results.

In the process of digestion, the two enzymes (unusual cutting / frequent cutting) can be used simultaneously (double digestion) or in two steps, if there is a reaction buffer common to both enzymes. Three classes of fragments are generated: frequent/frequent; unusual/frequent and unusual/unusual.

From the digestion of genomic DNA with *EcoRI* and *MseI* is expected that most of the fragments are cut *MseI/MseI* (frequent/frequent). Fragments cut *EcoRI/MseI* (unusual/frequent) occur in approximately equal frequency to twice the number of restriction enzyme sites of *EcoRI* and fragments cut *EcoRI/EcoRI* (unusual/unusual) occur in low volume (Ferreira & Grattapaglia, 1995).

The fragments generated by enzymatic digestion should be linked to specific adapters that have additional terminals to the ends resulting from cleavage. The process of connecting the adapters involves using ligases that enables DNA fragments to bind to the adapters.

With this procedure, both the sequence of adapters, as the result of the restriction site are known, allowing the construction of specific primers to these sequences for pre-amplification restriction fragment through PCR reactions. The primers consist of a sequence complementary to the adapter, followed by another site-specific restriction enzyme, and an extension of selective nucleotides at the 3' end (Lopes et al., 2002).

In the first stage of amplification, called pre-amplification, a selective nucleotide is used in the 3' end of primers. In the second phase of amplification, called selective amplification, three selective nucleotides are used in the terminal 3' primers.

The second amplification is done with a sample of the first. Therefore, only the fragments that have complementary nucleotides to the selective nucleotides will be amplified. Thus, the alleles of AFLP loci (presence or absence of a specific fragment or band) are from the loss

or gain of a restriction site or the complementary bases selective or not used at the terminals 3' primers where one starts PCR with the region, which flanks the restriction site (Lopes et al., 2002). The authors presented a table with the restriction sites, sequences of adapters and primers used for six enzymes in AFLP analysis (Table 1).

Restriction enzyme	Restriction site	Adapter /Primer	Base sequence
<i>EcoRI</i>	5'...G↓A A T T C...3' 3'...C T T A A ↑G...5'	Adapter	5'-C T C G T A G A C T G C G T A C C-3' 3'-C A T C T G A C G C A T G G T T A A-5'
		Primer	5'-G A C T G C G T A C C A A T T C E-3'
<i>MseI</i>	5'...T↓T A A...3' 3'...A A T ↑T...5'	Adapter	5'-G A C G A T G A G T C C T G A G-3' 3'-T A C T C A G G A C T C A T- 5'
		Primer	5'-G A T G A G T C C T G A G T A A E-3'
<i>PstI</i>	5'...C T G C A ↓G...3' 3'...G ↑A C G T C...5'	Adapter	5'-C T C G T A G A C T G C G T A C A T G C A-3' 3'-C A T C T G A C G C A T G T- 5'
		Primer	5'-G A C T G C G T A C A T G C A G E-3'
<i>HindIII</i>	5'...A ↓A G C T T...3' 3'...T T C G A ↑A...5'	Adapter	5'-C T C G T A G A C T G C G T A C C-3' 3'-C T G A C G C A T G G T C G A-5'
		Primer	5'-G A C T G C G T A C C A G C T T E-3'
<i>ApaI</i>	5'...G G G C C ↓C...3' 3'...C ↑C C G G G...5'	Adapter	5'-T C G T A G A C T G C G T A C A G G C C-3' 3'-C A T C T G A C G C A T G T- 5'
		Primer	5'-G A C T G C G T A C A G G C C C E-3'
<i>TaqI</i>	5'...T ↓C G A...3' 3'...A G C ↑T...5'	Adapter	5'-G A C G A T G A G T C C T G A C-3' 3'-T A C T C A G G A C T G G C-5'
		Primer	5'-C G A T G A G T C C T G A C C G A E-3'

Table 1. Restriction sites, sequences of adapters and primers used for six enzymes in AFLP analysis (E: arbitrary nucleotide used in the pre-amplification).

Fragments cut *EcoRI/MseI* are preferentially amplified, and this is due to lower efficiency of hybridization of primers *MseI* compared to that with primers *EcoRI*, and also the fact that the fragments *MseI/MseI* have terminal inverted sequence being amplified by a single initiator, enhancing the formation of a loop structure that competes with the annealing of primers (Vos et al., 1995).

For separation and identification of AFLP amplification products, the best method is to electrophoresis in denaturing polyacrylamide gel, which provides a high level of resolution, being effective for detecting single nucleotide differences. The number of fragments visualized in a polyacrylamide gel is variable and can reach values higher than one hundred. Theoretically the larger the genome size, the greater the amount of fragments. The readings of the gels can be manual or automated. The manual reading is performed after the revelation of the banding pattern by staining with silver nitrate or revelation in autoradiographs, in this case using primers labeled with radioisotopes. The second type is performed by DNA analyzers and requires fluorescent labeling.

The AFLP technique detects a greater number of fragments compared to other techniques that reveal molecular markers and provides comprehensive coverage of the genome. The use of restriction enzymes combined with appropriate conditions for hybridization of primers for the amplification reactions combines the robustness of the RFLP technique with the practicality of PCR.

Just as the RAPD technique, the methodology requires no prior information of DNA sequences. AFLP markers present together, the exploratory capacity of RFLP polymorphisms (presence or absence of restriction sites) with the advantage of PCR. AFLP markers are dominant and heterozygous genotypes can not be directly discriminated against the homozygotes.

Malone et al. (2003), analyzing genetic contamination in soybean determined that, in addition to the cultivars presented a high degree of genetic similarity, analysis of the banding pattern obtained by AFLP revealed the presence of additional bands in all samples when compared with the strains pure (seed genetics). The variation in the fingerprinting of cultivars suggested to be related to genetic contamination occurred between the batches studied, and exogenous contamination.

Mertz et al. (2009) tried to verify the effectiveness of the technique of cDNA-AFLP in obtaining fragments of genes differentially expressed in soybean seed coats with contrasting permeability and have concluded that the technique could be a promising alternative for studies aimed at identifying genes related to seed quality. According to the authors, the cDNA-AFLP technique is effective in identifying genes expressed, because it allowed the taking of 47 differentially expressed cDNA fragments between the coats of soybean genotypes CD-202 and TP.

Colombari Filho et al. (2010) undertook a study to evaluate the heterosis for grain production in soybean and its relationship with genetic distances obtained with the AFLP molecular marker. The authors concluded that heterosis for grain production in soybean is correlated with genetic distances obtained with AFLP markers and it is possible to select from crosses from the molecular genetics distance between the parents.

The quality of DNA needed is an important factor for the success of the AFLP technique. During the extraction of a large number of samples of genomic DNA preparations can be varied in quantity and in quality. A DNA of high purity is required to ensure a complete digestion by restriction enzymes in all DNA samples. The quality of the enzymatic digestion can lead to errors of interpretations (Ferreira & Grattapaglia, 1995).

#### 4. Relationship between markers and use practices

The markers presented are some similarities between themselves regarding the level of polymorphism, random distribution in the genome stability, gene expression among others. The comparison between them is presented in Table 2 adapted from Ferreira & Grattapaglia (1995).

Characteristics	RFLP	RAPD	Microsatellites	AFLP
Level of polymorphism	Low - High	Low - High	Very High	Very High
Environmental stability	High	High	High	High
Number of locus	High	High	High	High
Gene expression	Co-dominant	Dominant	Co-dominant	Dominant
Number of alleles per locus	Multiallelic	Two	Multiallelic	Two
Distribution in genome	Multiple	Random	Random	Random
Accessibility Technology	Moderate	Very High	Very Low	Moderate
Identification of genotypes	High	Very High	Very High	Very High
Application in breeding	Mean cost	Low Cost	Expensive	Low Cost
Specific mapping	Moderate	Very High	Moderate	Very High
Evaluation of germplasm	High	High	High	Very High
Genetic mapping	High	High	Very High	High
Genetics autogamy	Moderate	High	Very High	Very High
Genetic allogamous	Moderate	High	Very High	Very High
Phylogenetic analysis	Very High	Moderate	High	Moderate

Table 2. Comparative analysis for some characteristics of molecular markers.

The molecular markers reported so far in the chapter became important tools in the genetic improvement of plants. The following is a brief account made of the possible uses for these markers.

Construction of genetic maps: The Mendelian segregation observed in molecular markers, allows them to be used to construct genetic maps of connection and enable the location of genes within the chromosomes. QTLs mapping;

Characterization of genetic variability: the polymorphism generated from DNA and observed in the form of bands, allows us to study and confirm the genetic variability among plants;

Monitoring of genes: Genes may be screened in breeding programs that use the technique of backcrossing. Some applications of molecular markers linked to breeding programs can be facilitated through the use of this tool. In programs of backcross markers can maximize the efficiency of programs by increasing the probability of conversion of individuals and

reducing the time required to obtain an acceptable recovery of the recurrent parent (Borém & Miranda, 2005). The proportion of recurrent parent obtained in the first backcross generation, after selection of a marker in each of the chromosomes of tomato, roughly equals the proportion in three backcross generations in the absence of selection (Tanksley & Rick, 1980);

**Genetic characterization:** Description of a plant gene in their DNA. Molecular methods allow the varietal characterization with high discrimination power. Several studies have shown the quality of information on the molecular identification of germplasm (Lee et al., 1989; Smith & Smith, 1991).

**Marker-assisted selection:** The possibility of identifying genes of interest, to be strongly linked to molecular markers allows the selection can be made indirectly. In cases where gene expression occurs only in the final phase of the cycle of the species, such as the lipoxygenase in soybeans, the possibility of indirect selection through molecular markers becomes attractive (Borém & Miranda, 2005). The same authors reported useful for screening individuals who have two or more genes whose expression produces similar phenotypes, as is the case of pyramiding genes conferring resistance to a pathogen.

Great prospects for the area of molecular markers have been discussed. The potential usefulness of these techniques will be achieved, for example, through indirect selection based on markers: when the desired character has low heritability or present difficult evaluation, in situations where there are undesirable genetic correlations between traits, and especially the practice of early selection in crops perennials. The isolation, cloning and manipulation of individual genes that control quantitative traits of economic importance is a goal, theoretically attainable.

## 5. New markers

The possibility of manipulation of genetic material, derived from discoveries as plasmids, restriction enzymes and the enzyme reverse transcriptase led to the development of RFLP markers. The PCR technique revolutionized the field of studies on molecular markers and allowed the studies that led to the development of RAPD, microsatellites and AFLP, described in the chapter.

The speed of evolution of knowledge, provided by the large number of studies, development of equipment, reagent delivery and qualification of researchers put it, every day, new tools available to the scientific community. No doubt the generation of knowledge is beneficial and desirable, but the constant changes lead to the need for updates, especially laboratories, which is not always possible. In this line of reasoning, are presented some of the newer molecular markers.

The development of markers in the studies reveals the possibility of treating areas with occasional changes, such as a molecular marker. A Single Nucleotide Polymorphism or SNP is a small change or variation that may occur in a DNA sequence in a significant portion (more than 1%) of a population.

SNPs are the most frequent forms of genetic variations. Approximately 90% of human genetic variations are SNPs that occur every 300 ~ 600 nucleotides and has become markers of choice for its high abundance and development of technologies for large-scale genotyping as microarray hybridization, primer extension, and connection. SNPs are changes in DNA that are maintained in future generations, the mutation was distinguished by being present with a range greater than 1%. When the mutation is set at a minimum frequency of 1% is considered a SNP (Kwok & Gu, 1999).

Variations in the sequences are the result of mutations and SNPs are mutations that have spread over generations and may occur either in coding regions as in non-coding genomes. They are classified as non-synonymous when they occur in coding regions and can result in an amino acid substitution in the protein sequence. Classification as synonymous SNPs is given the changes that occur in non-coding region. These markers can be used in genotyping and as a new strategy for identification of polymorphism in species with low degree of polymorphism.

Molecular markers are very important in structural and functional genomics of animal species, plants and microorganisms. The marker developed by Hu & Vick (2003) was named Region Amplification Polymorphism Target (TRAP). It's is a fast and efficient using bioinformatics tools and data from EST's to generate polymorphic markers around targeted candidate gene sequences based on PCR. It is considered a dominant marker, which combines the ease of the RAPD markers with polymorphism of AFLP markers.

The polymorphism is generated from a combination of a fixed primer designed from an EST sequence of interest, and an arbitrary primer. Recent studies have reported the use of TRAP markers to access genetic diversity in sugarcane and identification of possible candidate genes for cold tolerance and metabolic pathway involved in sucrose (Alwala et al., 2006). The technique is extremely simply practice involving the extraction of genomic DNA and a PCR reaction.

Möller (2010) evaluated 286 F<sub>2</sub> plants from crosses of IAC-100 and CD-215 in order to identify markers linked to genes conferring resistance to sucking bugs of soybean seeds. For the marker TRAP, 11 primer combinations fixed / arbitrary generated 230 brands, and 31 (13.48%) polymorphic, an average of 2.82 marks per polymorphic combination. Despite the lower number of different polymorphic by combining the percentage of polymorphism obtained with the marker TRAP (0.13) was higher than for AFLP (0.10).

Finally the marker Nucleotide Binding Site (NBS) which has been successfully used in potato, tomato, lettuce, barley (Van der Linden et al., 2004), allowing the identification of areas of resistance genes highly conserved between species. The technique consists of amplification of specific regions using degenerate primers homologous to conserved sequences of resistance genes (R-genes).

The visualization of fragments generated by molecular markers, is usually performed using gel electrophoresis, which can hinder the precise correlation between the bands and allelic variations. Technological developments put at the disposal of researchers, hybridization methods such as microarrays. The high cost restricts their use in most laboratories. Alternatively, we developed a methodology called: Diversity Arrays Technology (DArT), which allows analysis of genomic representations without the need for sequencing.

The technique is based on the construction of panels of diversity and hybridization microarray. Its use allows the visualization of the presence or absence of specific fragments in the genome.

## 6. Conclusion

Molecular markers have facilitated the studies of genetics, taxonomy and evolution of plants delivering breakthrough in scientific knowledge. New possibilities of genetic manipulation emerged, directly benefiting the plant breeding.

The selection methods were largely enhanced by the use of molecular markers, and the success of marker-assisted selection depends on the degree of association between him and

the characteristic of interest: the greater the association, the lower the chance of recombination between the marker and gene controlling the trait, with a higher selection efficiency. The use of molecular markers presents several advantages over morphological markers: lack of phenotypic analysis, which has high cost and difficulty in performing; no need for special environments (drought and inoculation with pathogens); allows for subjective evaluations (performance organoleptic); there is no destruction of the plant evaluated; allowing the early identification of features found only in advanced stages of plant development.

In plant breeding, there are some challenges, and obtain individuals with the gene combination desirable for the features of interest, presented as one. Two factors are involved in resolving the issue: the existence of variability and the selection process. The identification of individuals requires an efficient method and skill of the investigator.

Molecular markers may help in the selection process for individuals as well as in the process of reviewing the existing diversity. The applications follow some peculiarities and the challenge of reducing costs is a objective to be achieved.

The advantages and limitations of these tools were described in the chapter and serve as a basis for further studies and investigated. The speed in the generation of new knowledge requires much intellectual and financial investment from the professionals working in the field of molecular marker-assisted selection.

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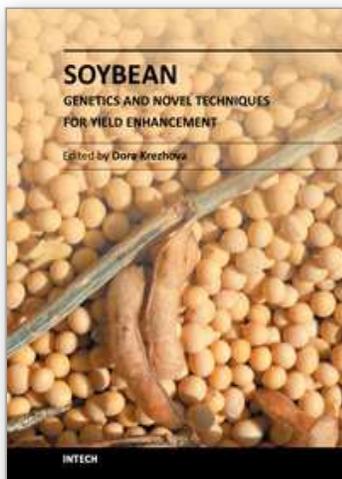
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This book presents the importance of applying of novel genetics and breeding technologies. The efficient genotype selections and gene transformations provide for generation of new and improved soybean cultivars, resistant to disease and environmental stresses. The book introduces also a few recent modern techniques and technologies for detection of plant stress and characterization of biomaterials as well as for processing of soybean food and oil products.

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