4

Genetic Improvement: Molecular-Based Strategies

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1. Introduction

Soybean (*Glycine max* (L.) Merr.) is the world's primary source of protein feed supplement for livestock and accounts for much of the world's vegetable oil supply. Additionally, healthy aspects of soyfoods go beyond the oil and protein and include minor compounds with nutraceutical properties such as isoflavones, saponins and tocopherols (Rajcan et al., 2005). Over the past three decades, world production of soybean has tripled, from 75 449 966 t in 1978 to 230 952 636 t in 2008 (www.fao.org), what is attributed to the scientific and technological developments in most regions as well as increasing world population, consumer acceptance and consumption of soybean in non-traditional regions of the world. All the sectors, involved with the entire soybean production and processing chain, have responded accordingly to comply with the demands of a globalize economy.

The genetic improvement of soybean, based on breeding strategies, contributes to advances in production and food processing industry by developing high-yielding and high-quality soybean cultivars, hereby enhancing value-added, healthy and safe properties of final soy products. Yield has been and remains the trait of greatest emphasis by breeders, as it is the trait with the greatest effect on a producer's net income. Studies of genetic progress reported that yields increased about 15 to 38 kg ha^{-1} annually over the period of seventy years (Specht et al., 1999; Wilcox, 2001; Ustun et al., 2001; Egli, 2008). Besides yield, progress has also been made in selecting for resistance to pathogens, insects and nematodes, tolerance to other production hazards, improvement in seed protein and oil, as well as other agronomic characteristics.

The genetic improvements have been accomplished mainly through the use of conventional (also termed empirical or traditional) breeding. The conventional breeding strategies are based on crossing, selection and fixation of superior phenotypes to develop improved cultivars and breeds suited to specific conditions with the aim to fulfill the needs of farmers and consumers. As the result of soybean self-pollinating reproductive behavior, conventional breeding procedures such as pedigree breeding, single pod descent, backcrossing and bulk population breeding are some of the more common procedures used to develop soybean cultivars. Although progress in soybean breeding accomplished only by

conventional breeding methods is significant, for further genetic advances in soybean germplasm the use of conventional breeding methods exclusively is no longer sufficient. There are multiple reasons for that. First of all, the development of new cultivar with conventional breeding methods requires at least ten generations. The length of this process is often in disproportion with rapid changes in market demands. In fact, on global level, changes in climate, soil structure and fertility, production technology, appearance of new phytopathogen races etc. became so rapid that the cultivar developed by conventional hybridization of parents with desired traits about ten years ago is no longer capable of accomplishing its genetic potential due to environmental stress factors. In addition, the burden of undesired genetic material (material incompatible with set breeding aims) constitutes a big problem in conventional breeding, because the elimination of undesired phenotypes requires more area, more time and thus bigger investments. In classical genetic improvement programs, selection is carried out based on observable phenotypes of the candidates for selection and/or their relatives but without knowing which genes are actually being selected. Plant scientists have made significant advances in understanding the agronomical, species-specific, breeding, biochemical and molecular processes that underlie important genetic, physiological and developmental traits, or that affect the ability of plants to cope with unfavorable environmental conditions for several decades (Gepts, 2002). The discoveries and implementations of biotechnology and molecular biology for selection purposes provide a stable background for generating of new knowledge and practical use in agricultural research and practice as well as to meet the growing demand for more and with better quality food and feed (Todorovska et al., 2010).

Main objectives of plant biotechnology are attempts to engineer metabolic pathways for the production of tailor-made plant polymer or low molecular weight compounds and the production of novel polypeptides for pharmaceutical or technical use. In general, goals of plant biotechnology are not much different from classical breeding goals. They can be divided into attempts to optimize input and output traits. Input traits refer to increased resistance towards abiotic and biotic stress, strategies to increase crop yield and to improve post-harvest characteristics. Attempts to improve output traits include production of foreign proteins for pharmaceutical and technical use, production of endogenous or novel polymers for food and non-food applications as well as synthesis of low molecular weight compounds including vitamins, essential aminoacids and pharmaceutically relevant secondary plant products (Sonnewald & Herbers, 2001). Scientists in the laboratory can genetically engineer soybean plants with unique genes, but plant breeding is necessary to put the new transgenes via sexual reproduction into the proper genetic background so that it is adapted to the intended areas of use. Recent developments in molecular biology and genomics are greatly accelerating the speed with which knowledge gained in basic plant science can be applied to species improvement (Dekkers & Hospital, 2002). Therefore, the molecular based plant breeding techniques are assuming an increasingly more important role in genetic improvement of soybean germplasm. Currently, conventional breeding strategies have priority, and in combination with molecular technologies have provided the possibility of broadening genetic variability of cultivated soybean as well as development of new germplasm that is better adapted to new market, production and environment demands (Verma & Shoemaker, 1996; Orf et al., 2004; Sudarić et al., 2008, 2010; Vratarić & Sudarić, 2008; Mladenović Drinić et al., 2008; Cober et al., 2009).

Modern biotechnology application in soybean breeding can be divided in two major categories:

- molecular genetics and,
- genetic transformation.

Molecular genetics studies how genetic information is encoded within the DNA and how biochemical processes of the cell translate the genetic information into the phenotype. Genetic transformation involves the alteration of the genetic constitution of cells or individuals by directed and selective modification, insertation of native or foreign gene, or deletion of an individual gene or genes. According to Shoemaker et al. (2004), soybean has emerged as a model crop system because of its densely saturated genetic map, a well-developed genetic transformation system and the growing number of genetic tools applicable to this biological system.

The emphasis in this chapter will be on selected information about new technological developments derived from molecular biology for soybean breeding purposes. Two main aspects will be considered: the use of genetic markers and transformation (genetic modification).

2. Genetic marker systems

The term genetic markers refers to genes with known locations in chromosomes, that are clearly visible on phenotypic level, and are easy to track in series of consecutive generations. Furthermore, it is defined as any DNA fragment that displays some form of observable polymorphism in analysed individuals (Hill et al., 1998; Liu, 1998; Konstantinov et al., 2004). Three types of genetic markers have been used in soybean genomic analysis:

- morphological markers,
- protein based markers (biochemical markers),
- DNA based markers (molecular markers).

According to Liu (1998), to be a genetic marker, the marker locus has to show experimentally detectable variation among the individuals in the test population. The variation can be considered at different biological levels, from the simple heritable phenotype to detection of variation at the single nucleotide. Once the variation is identified and the genotypes of all individuals in the test population are known, the frequency of recombination events between loci is used to estimate linkage distance between markers. Different types of markers may identify different polymorphisms. The utilisable value of the certain genetic marker system depends on several parameters such as: reliability determined by the analysis results, reproducibility, universality or independence from specific plant material in tests, high level of observed polymorphism, random distribution of marker in the genome and interesting location on genetic map, which implies the existence of gene–marker relationship for the trait of interest. Likewise, the genetic interpretation of markers strongly depends on the sequence complexity of the genome and the kind of variation the marker identifies.

2.1 Morphological markers

Morphological traits such as shape, colour, size or height often have one to one correspondence with the genes controlling the traits. In such cases, the morphological characters (the phenotypes) can be used as reliable indicators for specific genes and are useful as genetic markers on chromosomes. Since the number of morphological traits of certain species is limited, so is the number of morphological markers. Morphological markers are usually easy to observe, but it is difficult to have a large number of them

segregating in a single or few populations. To obtain a reasonable number of polymorphic morphological markers, many mapping populations are needed (Liu, 1998). Furthermore, many morphological traits are visible only on certain plant part and only in certain stage of plant development (Fig. 1) which further limits the time of use. Morphological markers are in selection process used mainly in early generations, for description and/or identification of genotypes in germplasm collections (gene banks) and in the procedure of variety's official released (lists of descriptors from the International Union for the Protection of New Varieties of Plants (UPOV).

Fig. 1. Flower colour of soybean – morphological marker *(photo: Vratarić, M.)*

2.2 Protein based markers

Proteins are result of gene expression. Different alleles of genes may result in proteins with different aminoacid compositions, size or modifications. Differences in charge and size can be easily detected using gel electrophoresis and can be used as genetic markers. Protein markers are divided in two groups:

- storage proteins;
- functional proteins or isozymes.

Seed proteins, as specific gene products, could indicate genetic specify of genotypes, and therefore could be used as markers for characterization of varieties, to resolve taxonomic relationships or for seed purity testing (Bushehri et al., 2000; Nikolic et al., 2004, 2005; Konstantinov et al., 2005; Malik et al., 2009). The SDS-PAGE is a practical and reliable method for species identification because seed storage proteins are largely independent of environmental fluctuation (Gepts, 1989). Genetic diversity and the pattern of variation in the soybean genotypes have been evaluated with seed protein (Alipour et al., 2002; Bushehri et al., 2000, Nikolic et al., 2005, Malik et al., 2009). In soybean, plant with a narrow genetic base in their pool, protein markers are not sufficient for characterization and study genetic diversity (Nikolic et al., 2005). Srebric et al (2010) identified Kunitz –free progeny of cross between common varieties *Kador* and *Kunitz* by electrophoresis of seed proteins (Fig. 2).

The isozymes are commonly used type of protein marker, which have been used for several decades. Isozymes are proteins with small differences in amino acid content, which catalyze the same chemical reaction, but are under the control of different genes.

Isozymes as markers are co-dominant, they don't undergo epistatic interactions with other molecular markers, and their expression does not stand under the influence of environment. They are limited in number and tissue and are developmental-stage dependent. Before DNA markers were discovered, isozymes were extensively used in many plant species and are still often used in conjunction with DNA markers.

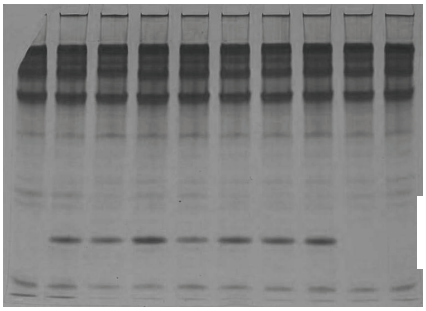


Fig. 2. SDS PAGE patterns of protein from soybean genotypes. KTI-Kunitz Trypsin Inhibitor. 1,9,10 genotypes without KTI; 2-8 genotypes with KTI *(photo: Srebric, M.)*

In soybean, isozyme polymorphism has been used for characterising and identifying genotypes and varieties (Cardy & Beversdorf, 1984; Doong & Kiang, 1987), for studying population genetics (Miroslav & Jiri, 1996), for examining geographical patterns of variation (Griffin & Palmer, 1995; Hirata et al., 1999), in seed production for determining uniformity and genetic purity of cultivars and identifying different varietals impurities in seed material. Bushehri et al. (2000) evaluated twenty one soybean (*Glycine max*) cultivars electrophoretically for the banding pattern of storage proteins and suggested that SDS-PAGE is a more powerful tool to characterize soybean cultivars compared to isozyme patterns.

2.3 DNA based markers (molecular markers)

Molecular marker refers to the DNA sequence with exactly defined nucleotide order and distribution, strictly specific for different organisms. According to Liu (1998) two basic approaches have been used to detect variation in the small region of DNA. The fragment can be detected by nucleic acid hybridization, which uses another fragment from the same locus which has been isolated and purified from the same or related species. The previously known segment must share considerable DNA sequence homology with the fragment of interest and can be labelled and used as a probe to detect the fragment of interest by complementary base pairing. The second approach is based on the amplification of sequences using polymerase chain reaction (PCR). To amplify a target segment, two primers designed using known sequences of the segment are needed.

In plant breeding, molecular markers have several advantages over the traditional phenotypic markers: accuracy, reliability, speed, indifference to the conditions under which the plants are grown and detectability in all stages of plant growth. Mode of action, level of polymorphism, informativeness, developmental cost, number of sample that could be run, level of skill, reliability are important considerations when selecting markers for specific applications. Although each marker system is associated with some advantages and disadvantages, the choice of the system is dictated by the intended application, equipment and the cost involved.

In soybean breeding, molecular marker applications are currently focused in four areas:

- germplasm characterization,
- marker-assisted selection (MAS),
- marker-assisted backcrossing and
- gene discovery.

Knowledge of genetic diversity in soybean elite breeding material has a significant impact on the improvement of plants, efficient utilization of eligible germplasm polymorphism and genotype selection for different breeding objectives. Genetic diversity can be assessed based on pedigree analysis, phenotypic data or molecular markers. Pedigree analysis and phenotypic evaluation of genetic diversity have a number of limitations, which have largely been exceeded by the development of molecular markers. Their advantage is based on fact that molecular markers are almost unlimited in number and are not influenced by environmental factors. Therefore, molecular markers have a significant role in estimating the diversity degree and genetic constitution of the existing germplasm, as well as, in the predicting of the heterotic effects based on the genetic distance between the parents in hybrid programmes. Genetic marker information in parental selection could have a favourable impact on breeding efficiency and contribute to the faster achievement of genetic improvement in soybean.

The implementation of molecular markers closely associated with desirable traits is being used to increase the efficiency and effectiveness of conventional breeding by indirect selection of the desirable plants in segregating population. Such selection approaches, based on the use of markers and called in general MAS has been used to increase the probability of identifying truly superior genotypes, by focusing on determination of genotypes with superior potential, and by enabling simultaneous improvement for traits that are negatively correlated (Knapp, 1998; Dekkers & Hospital, 2002; Todorovska et al., 2010). MAS is used more readily than the usual techniques to screen single traits, such as resistance or restorer genes: nematode resistance, insects resistance, pathogen resistance.

The combination of reliable phenotyping and MAS has been particularly important in transferring desirable alleles by simple backcrossing into elite germplasm. Application of markers of introgression programs can result in a reduction in the number of breeding cycles by improving selection efficiency, particularly at the early stage.

Also, molecular markers allow us to identify and map the Quantitative Trait Loci (QTLs), i.e. the relevant loci responsible for genetic variability of quantitatively-inherited traits. Because of the genetic by environment interactions on most quantitative traits, breeding for them requires replicated field trials conducted over 2 or more years in different locations. This is obviously time consuming and expensive. The ability to select for an easily identifiable marker that is a good predictor of the presence or absence of a QTL trait can save time and money in a breeding program. Discovery and tagging of QTL is a prerequisite of this type of MAS (Shoemaker et al., 2004).

In soybean, different DNA marker systems such as Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNAs (RAPD), DNA Amplification Fingerprinting Markers (DAF), Simple Sequence Repeats (SSR), Amplified Fragment Length Polymorphism (AFLP) and Single Nucleotide Polymorphism (SNP) have been developed and applied.

2.3.1 Restriction Fragment Length Polymorphism

Restriction Fragment Length Polymorphism (RFLP) represents the first generation of DNA markers used for plant genomes (Weber and Helentjaris, 1989). The basis of RFLP is using restriction enzymes (endonucleases), which recognise short DNA fragments (3-6 bases) and cut the DNA at sequence-specific sites. Polymorphism of genomic DNA detected through DNA fragment length after its digestion with restriction enzymes is due to variability in

number and array of restriction sites, which are recognised by restriction endonucleases. The next step is to separate the paired strands of the DNA fragments (denaturation) by putting the gel in alkali. The denatured single-strand DNA fragments are usually transferred to a nylon or nitrocellulose membrane filter such that the fragments retain the same pattern on the membrane as on the gel. This blotting procedure was first applied to DNA analysis by E. Southern and is still referred to as a Southern blot (Southern, 1975). The final step of the Southern blotting is to visualize specific DNA fragments using a radioactively labelled probe. Alternatively, non-radioactive probes can be used. In this case, the hybridized fragments are then detected by non-radioactive assays involving chemiluminescence of colorigenic detection. The result is ideally a series of bands on a gel which can then be scored for presence or absence of particular bands. Differences between genotypes are usually visualised as an altered pattern of DNA restriction fragments.

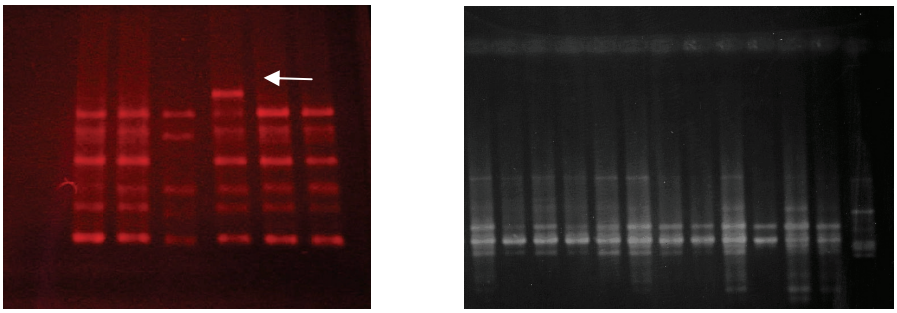
Application of RFLPs has advantages and disadvantages. The main advantages of RFLP methodology are: results are highly reproducible between laboratories, co-dominant markers and simplicity of the method. Disadvantages are: time consuming, expensive to perform and using radioactively labelled probes.

In soybean, the use of RFLPs started in the late 1980s (Apuya et al., 1988; Keim et al., 1990) which contributed the development of first genetic map of soybean genome which contained 150 RFLP markers with a total length of about 1200 recombination units (Keim et al., 1990). This map, developed jointly by the USDA-ARS (U.S. Department of Agriculture-American Research of Soybean), Iowa State University and ASA (American Soybean Association) and referred to as the ˝Public˝ map saw further expansion during the 1990s with the addition of RFLP loci (over 350) (Shoemaker and Olson, 1993). At the same time, the soybean genome map with over 600 identified loci was developed (Rafalski and Tingey, 1993). These initial maps were constructed using populations created from crosses among cultivated and wild soybean, because large proportion of the loci on these maps would not be expected to segregate in crosses among cultivated soybean genotypes. Despite the great utility of RFLP based molecular genetic linkage maps substantial effort was expended in the development of alternative markers that detected greater levels of molecular genetic variation. In addition, the duplicated nature of the soybean genome, to which RFLP probes will hybridize on an average of 2.55 times (Shoemaker, et al., 1995) created complications with the use of RFLP. Thus, in the early 1990's, PCR-based marker systems including RAPD, SSR and AFLP markers began to be developed and used by soybean geneticists (Cregan, 1999).

2.3.2 Randomly Amplified Polymorphic DNA

The basis of Randomly Amplified Polymorphic DNA **(**RAPD) markers is the polymerase chain reaction (PCR) amplification with arbitrarily chosen primers that initiate DNA synthesis from sites to which the primer is matched. So, RAPDs were designed to solve the problem of lack of pre-existing DNA sequence information. A single, arbitrarily chosen short oligonucleotide can be used as a primer to amplify genome segments flanked by two complementary primer-binding sites in inverted orientation (Williams et al., 1990). If the sites occur on opposite strands of a segment of DNA in inverted orientation and the distance between the sites is short enough for PCR, then the segment flanked by the sites can be amplified. Polymorphism of genomic DNA is detected through the length of synthesized DNA fragments. If polymorphism exists in the binding sites among different genotypes or the fragment length differs at the same site from genotype to genotype, then a RAPD marker is obtained.

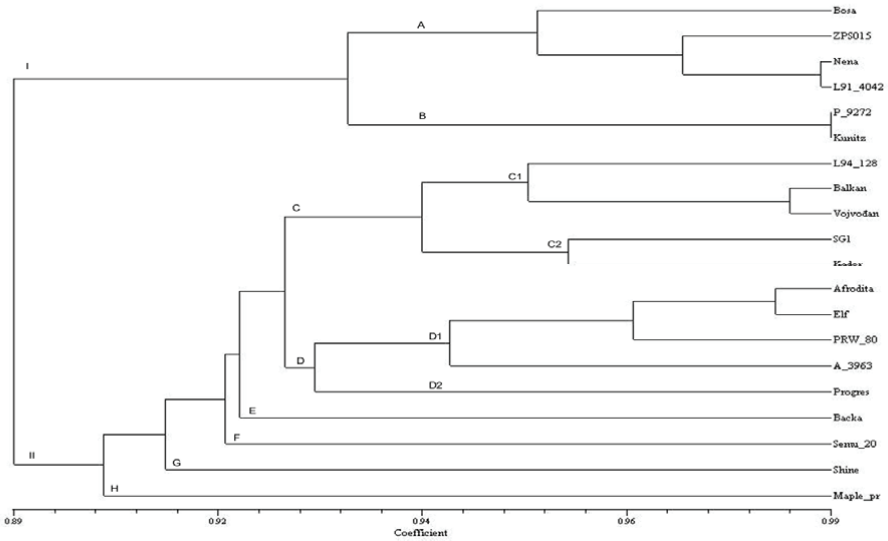
In practice, only small amounts of DNA from each genotype are needed as templates. PCR reaction can be carried out in a 96-well plate using a programmable thermocycler. The amplification products are separated on an agarose gel and visualized using ethidium bromide staining. The key point about this technique is that nothing is known about the identity of the amplification products. However, the amplification products are extremely useful as markers in genetic diversity studies. Other important features of the technique are: simplicity, unit costs per assay are low, RAPDs are dominant, and reproducible results can be obtained if care is taken to standardise the conditions used (Ford-Lloyd & Painting, 1996). RAPD markers for analysing plant genome were developed by Welsh & McClelland (1990), and by Williams et al. (1990). The use of RAPDs for analyzing soybean genome was started in the early 1990s. The RAPD markers have been widely used for genetic diversity study of soybean germplasm (Correa et al., 1999; Baranek et al., 2002; Nikolic et al., 2007; Peric et al., 2008a) (Fig. 3.).



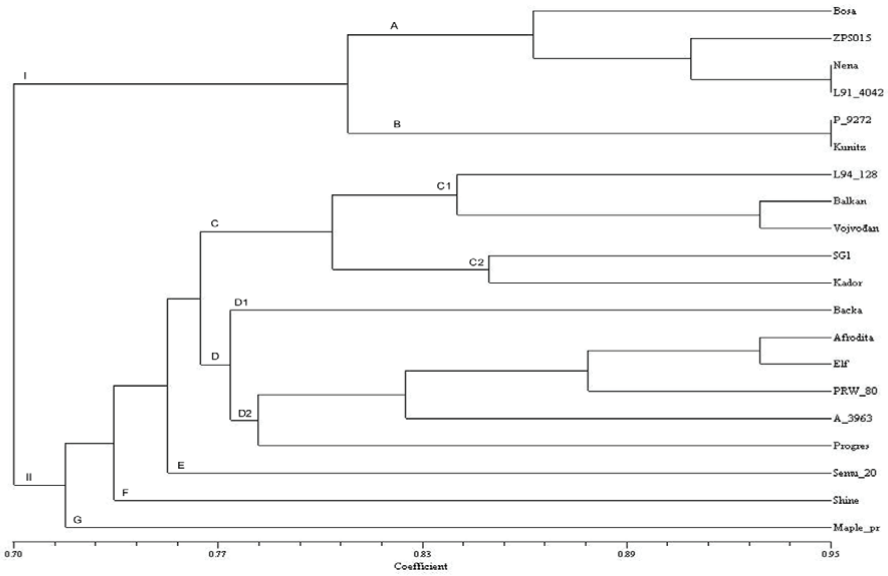
(a) (b)

Fig. 3. (a) RAPD polymorphism in 6 soybean genotypes with primer GEN 4-70-9 (arrow marked unique band in genotype 4) *photo: Mladenović Drinić, S.)* (b) RAPD polymorphism in 13 soybean genotypes *(photo: Mladenović Drinić, S.)*

In studies of genetic diversity, researchers are interested in clustering similar individuals, so that the greater difference occurs among the formed groups. Statistical methods, as cluster analysis, factor analysis and principal component analysis can be applied to help in this kind of study. Among them, cluster analysis stand out as it does not demand an initial hypothesis regarding the probability distribution of the data and as it provides easy interpretation. Considering that the results of clustering can be influenced by the similarity coefficient choice, these coefficients need to be better understood so that the most efficient ones in each specific situation can be employed. For example, Mladenovic Drinic et al. (2008) were investigated the influence of four similarity coefficients (Sorensen-Dice (D), Jaccard (J), Roger & Tanimoto (RT), Simple-matching (SM)) over the following cluster analysis, based on data from RAPD marker analysis of twenty soybean genotypes. PCR amplification of genomic DNA was performed using 27 RAPD primers. Eighteen RAPD primers showed clear and reproducible bands, while 9 primers didn't showed amplification at all, or bands were smeared and weak. Totally 86 RAPD fragments of different molecular weight were observed out of which 37.2% were polymorphic. Results were indicated on a low genetic diversity of soybean. Genetic similarity ranged from 0.845 to 0.986 (D); 0.607-0.954 (RT), 0721-0.977 (SM) and 0.7317-0.9718 (J). When dendograms were contrasted by the CI_c index, small differences among them were made evident (Fig. 4).



(a)



(b)

Fig. 4. (a) Dendogram of 20 soybean genotypes based on RAPD markers using Sorens-Dice coefficients (b) Dendogram of 20 soybean genotypes based on RAPD markers using Roger & Tanimoto coefficients

The dendogram obtained by Rogers & Tanimoto coefficient was identical to that of Simple matching as were Sorensen-Dice and Jaccard. Consequently, results of RAPD markers

analysis can provide the previous information on the genetic similarity of parents, and based on it, the performance of traits in the progeny can be predicted, as well as proportion of superior progenies generated by each cross in advanced generations of selfing (Barroso et al., 2003; Peric et al. 2006, 2008b).

2.3.3 DNA Amplification Fingerprinting Markers

Similar to RAPD markers, the basis of DNA Amplification Fingerprinting Markers **(**DAFs**)** is the use of PCR with arbitrarily chosen primers, i. e. DAF markers are amplified with the use of a single arbitrarily chosen primer. The procedure was described by Caetano-Anolles et al. (1992). The basic differences between RAPD and DAF technologies are: DAF has shorter arbitrarily chosen primers (usually 5-8 nucleotides), so for the electrophoresis of DAFs we use polyacrylamid gel with silver staining, and for RAPDs we use agarose gel.

The use of DAF markers in soybean genome analyses started during 1990s. A limited number of DAF-generated polymorphisms were mapped in the University of Utah, Minsoy x Noir 1 RIL population (Prabhu & Gresshoff, 1994). The authors noted that DAF-generated polymorphic markers occur frequently and reliably, that they are inherited as Mendelian dominant loci and that they can be used in genome mapping.

2.3.4 Simple Sequence Repeats (microsatellites)

The use of simple (short) sequence repeats (SSRs) (small DNA fragments, usually 2-5 bp long) is based on amplification of short DNA fragments with repeating core motif (repeats 9-30 times). Polymorphism of genomic DNA is detected through the number of short repeat units after amplification in polymerase chain reaction with the use of primers which limit the loci of satellite DNA.

Microsatellites have high level of variability in many plant and animal species. Most common forms of repeat units are simple di-nucleotides like $(CA)_n$:$(GT)_n$, $(GA)_n$:$(CT)_n$, $(CG)_n$:(CG):$(GC)_n$, and $(AT)_n$:$(TA)_n$ (n is number of repeats), while microsatellites with 3 or 4 nucleotides are rare. The most common motifs in soybean are: AT, ATT, TA, TAT, CT, CTT (Mohan et al., 1997).

First applications of SSRs in plant genome analyses were in soybean. In early 1990s, two scientific groups (Akkaya et al., 1992; Morgante & Olivieri, 1993) published similar results demonstrating high levels of polymorphism, co-dominance and locus specificity for SSR markers in soybean. Because of the numerous advantages (high level of polymorphism, single locus nature, random distribution in the genome), SSR markers are excellent complement to RFLP markers for soybean researches in the fields of molecular biology, genetics and plant breeding.

The development and mapping of a large set of soybean SSR markers was initiated in 1995 with the support of the United Soybean Board, resulting in development of more than 600 SSR loci. These loci were mapped in three different mapping populations (Cregan et al. 1999). One of these was the USDA/Iowa State population. Second was the 240 RIL University of Utah populations developed from a cross of the cultivated soybean genotypes Minsoy and Noir 1. The third was the University of Nebraska Clark x Harosoy isoline population consisting of 57 F2-derived lines. These three separate maps provided useful information relative to the consistency of marker order and genetic distance among the different populations. Song et al. (2004) reported that the 420 newly developed SSRs were

mapped in one or more of five soybean mapping populations: Minsoy x Noir 1, Minsoy × Archer, Archer × Noir 1, Clark × Harosoy, and A81-356022 × PI468916. The JoinMap software package was used to combine the five maps into an integrated genetic map spanning 2,523.6 cM of Kosambi map distance across 20 linkage groups that contained 1,849 markers, including 1,015 SSRs, 709 RFLPs, 73 RAPDs, 24 classical traits, six AFLPs, ten isozymes, and 12 others. The number of new SSR markers added to each linkage group ranged from 12 to 29. In the integrated map, the ratio of SSR marker number to linkage group map distance did not differ among 18 of the 20 linkage groups. However, the same authors noted that the SSRs were not uniformly spaced over a linkage group, clusters of SSRs with very limited recombination were frequently present. These clusters of SSRs may be indicative of gene-rich regions of soybean, indicating the significant association of genes and SSRs. The creation of a high-density, integrated soybean linkage map with more precisely positioned markers would permit a better overall assessment of the distribution of SSR loci in the soybean genome.

Genetic diversity of Asian soybean germplasm (Abe et al., 2003; Wang et al., 2006) as well as European soybean germplasm (Tavaud-Pirra et al., 2009) is studied by microsatellites. Sudaric et al (2008, 2009) evaluate the genetic diversity of the selected soybean germplasm using SSR markers, as well as to compare the effectiveness of breeding procedures with and without the use of genetic markers in parental selection. Based on SSR marker data and phenotypic data, an association was found between the agronomic performance of the derived lines and the genetic distance between the parental lines. Crosses between more diverse parents resulted in derived lines with greater values for grain yield (Table 1) and grain quality (Table 2) compared with the parents than crosses between similar parents. The results indicated on usefulness of genetic marker information in parental selection, contributing to breeding efficiency.

Code of derived line	Female component	Male component	Derived line (F_4)		
			Value	Deviation of	
				Female component	Male component
Small genetic distance between parental lines					
L-86-03	3.14	3.62	3.50	+ 0.36	- 0.12
L-104-03	3.14	4.20	3.65	+ 0.51*	- 0.55*
L-165-03	4.22	3.67	3.98	- 0.24	+ 0.31
L-224-03	4.22	4.14	4.20	- 0.02	0.06
Large genetic distance between parental lines					
L-23-03	3.28	3.66	3.92	+ 0.64*	+ 0.26
L-96-03	3.28	3.94	4.20	+ 0.92*	+ 0.26
L-115-03	4.09	3.89	4.52	+ 0.43*	+ 0.63*
L-193-03	4.09	3.67	4.38	+ 0.29	+ 0.71*

** signficant at P=0.05 according to F-test*

Table 1. Grain yield (t/ha) of tested soybean lines (2007, Osijek, Croatia)

Code of derived line	Female component	Male component	Derived line (F_4)		
			Value	Deviation of	
				Female component	Male component
Small genetic distance between parental lines					
L-48-03	38.51	39.96	39.40	+ 0.89*	- 0.56*
L-94-03	38.51	40.50	39.56	+ 1.05*	- 0.94*
L-136-03	40.88	39.27	40.35	- 0.53*	+ 1.08*
L-212-03	40.88	40.52	40.62	- 0.26	+ 0.10
L-11-03	38.87	39.28	39.43	+ 0.56*	+ 0.15
L-65-03	38.87	39.61	39.75	+ 0.88*	+ 0.14
L-170-03	40.06	39.79	40.18	+ 0.12	+ 0.39*
L-205-03	40.06	40.16	40.46	+ 0.40*	+ 0.30

** signficant at P=0.05 according to F-test*

Table 2. Protein content in grain (% in ADM) of tested soybean lines (2007, Osijek, Croatia)

The SSR markers linked to the major QTL will be useful for marker-assisted selection in soybean-breeding programs (Funatsuki et al., 2005; Panthee et al., 2006). A set of SSR markers have been subjected to continuous development and utilization for high throughput molecular mapping in soybean (Akkaya et al., 1992, 1995; Narvel et al., 2000; Burnham et al., 2003; Shultz et al., 2007).

2.3.5 Amplified Fragment Length Polymorphism

The use of Amplified Fragment Length Polymorphism (AFLP markers) is based on combining the use of restriction enzymes (endonucleases) and selective amplification with polymerase chain reaction. Polymorphism of genomic DNA is detected through the length of DNA fragments after its digestion with restriction enzymes and amplification in polymerase chain reaction. The amplification products are then separated on highly resolving sequencing gels and visualised using autoradiography. Where radio-labelled nucleotides are not used in the PCR step, fluorescent or silver staining techniques can be used to visualise the products (Ford-Lloyd & Painting, 1996). AFLP analysis is a highly sensitive, highly reproducible and widely applicable method for detecting polymorphisms throughout the genome. Disadvantages of AFLP system are: expensive, technically demanding, uses radioisotopes and problems in interpreting banding patterns.

In soybean, less attention was focused on the development of AFLP markers than in other plant species, mostly because of the successful application of SSRs. The use of AFLPs in soybean started as late as mid 1990s (Vos et al., 1995). However, one of the largest available AFLP maps of any plant species was developed in soybean (Keim et al., 1997). This map has the total of 840 loci from which 650 are AFLPs, while the USDA/Iowa State map has 1004 loci, most of which are RFLPs and SSRs. In the development of AFLP map, authors used somewhat altered protocol according to Travis et al. (1996). The AFLP has proven very useful to saturate specific genomic regions using bulked segregate analysis or the

comparison of near isogenic lines for a trait of interest because of the large amount of marker data that can be obtained with AFLP without the need for previous knowledge of DNA sequence (Muehlbauer et al., 1988, Cregan, 1999). Still, AFLP technologies are continuously being modified and perfected (Lin et al., 1999; Mano et al., 2001).

2.3.6 Single NucleotidePolymorphism

Differences in individual DNA bases between homologous DNA fragments along with small insertions and deletions are collectively referred to as single-nucleotide polymorphism (SNP). SNPs can serve as genetic markers that can complement and greatly augment existing genetic linkage maps and are therefore useful as genetic markers in QTL analysis or other DNA marker based genetic analysis (Cregan, 1999). SNP is the most abundant source of DNA polymorphism in humans (Collins et al., 1998), while in plants, SNPs nature and frequency researches have just started to acquire significance (Gupta et al., 2001; Rafalski, 2002; Shoemaker et al., 2004). Many SNP detection methods have been developed, such as oligonucleotide ligation, denaturing high-performance liquid chromatography (DHPLC) and primer extension (Wu & Wallance 1989; Hoogendoorn et al. 1999; Pastinen et al. 2000; Wolford et al. 2000).

In soybean, SNPs nature and frequency researches have intensified (Cahill, 2000, Zhu et al., 2003; Kim et al., 2004; Van et al., 2004, 2005), and thus are likely to have an important role in the future of soybean genome analyses and manipulation.

3. Genetic modification

Tremendous progress in plant molecular biology over the last three decades has enabled development and use of techniques for manipulation with genetical structure of organisms, with the aim to ˝transfer˝ adequate genes and acquire desired combinational properties. Unlike traditional plant breeding, which involves the crossing of hundreds or thousands of genes, genetic transformation allows transfer of only one or a few desirable genes. This more precise technique allows plant breeders to develop crops with specific beneficial traits and without undesirable traits. Through traditional breeding methods, genes have been transferred from one individual to another with the aim of production individuals which clearly exhibit particular desirable traits. These crossing are usually between individuals of the same, or closely related species. The gene pool available for use, in traditional crossing, is thus limited to those genes present in individuals which can be induced to breed using natural crossing methods. The use of recombinant DNA technologies enables the movement of a single or a few genes within or across species boundaries to produce plants with new traits, transgenic plants. Also, it is possible to get rid of an undesirable trait by shutting down the ability of the cell to make the product specified by the gene (Konstantinov et al., 2002; Drinic Mladenovic et al., 2004).

The processes involved in developing genetically modified plants are the following:

- identification and isolation of the desired gene,
- gene cloning,
- development of transgenes,
- gene transfer and
- introduction into breeding processes.

The identification of a single gene is not sufficient. It is necessary to be acquainted with regulatory mechanisms of gene actions, as well as, with their secondary effects and

interactions with other genes. Prior to this, it is necessary to identify an organism containing the desires gene in its genome (Konstantinov et al., 2002). Todorovska et al. (2010) has noted that the main limitation of this technology is the availability of preliminary knowledge about the role of a gene in determining a given trait and is, at present, only applicable for traits that are determined by one or a relatively small number of genes.

Transgenic plants represent completely new genotypes (plant with novel traits). Therefore, in order to confirm expected phenotypic expression of the new trait, selection after the gene transfer is necessary, the same after conventional hybridization.

Nevertheless, there still are many unknowns and disputes concerning transgenic plants from many different aspects: ethical, philosophical, religious, economic, ecological, sanitary, legal etc, and much time will still be needed to put transgenic plants in their rightful place with the help of scientific research (Keller & Huttter Carabias, 2001; Taylor, 2007).

Legislative framework that regulates cultivation and commercialisation of transgenic plants, and the use of their products, is made, exists, and is being changed under the influence of science, business and national interests. According to the Cartagena Protocol on Biosafety (http://bch.cbd.int/protocol/) entered into force 2003 signed by 103 world countries and with 160 parties to Protocol by 2010, each country has the right to protect its biodiversity. Researches show that transgenic plants can transfer some of their features by pollen to related species that grow in the same area. Natural crossing between transgenic and conventional cultivars of the same species can occur in the same way, creating progeny that, beside desired traits, has novel genetic combinations with insufficiently known outcomes. Furthermore, it still isn't completely known how transgenic plants would behave if they escaped controlled cultivation and entered into the ecosystem. Their presence can affect the genome of some native forms of the same species that are considered ˝gene bank˝ of the certain area and are of unique significance for breeding. Congruently, 28 European countries signed Berlin Manifesto for GMO-free Regions and Biodiversity in Europe (www.gmo-free-regions.org/gmo-free-conference-2005/berlin-manifesto.html) in 2005, which points out that European regions have the right to determine their own ways of farming, producing and selling food, thus protecting the environment, landscapes, culture and heritage, their seed, their rural development and their future. This includes the right to decide about the use of genetically modified plants in agriculture and ecosystems.

To date, genetic modifications were made on several important crops such as: soybean, corn, canola (rapeseed), sugar beet, potato, cotton, wheat, barley, rice and beans. One of the first important practical uses of genetic engineering in agriculture was development of glyphosate tolerant cultivars of soybean, corn, canola (rapeseed) and cotton (Moll, 1997). Glyphosate (N-(phosphonomethyl) glycine) is translocational, nonselective, post-emergence broad-spectrum herbicide. It's toxic effect on plants consists of inhibition of 5-enolpyruvyl shicimic acid-3-phosphate synthase (EPSPS) enzyme. EPSPS is very important in biosynthetic metabolism of aromatic amino acids – tryptophan, tyrosine and phenylalanine (Haslam, 1993). Glyphosate functions by occupying the binding site of phosphoenol pyruvate in EPSPS and blocking its activity, which in turn prevents aromatic amino acid production. Aromatic amino acids are very important for many biochemical processes such as: protein synthesis, cell membrane formation, protection against pathogens etc. Prevention of their biosynthesis leads to withering of plants. EPSPS is present in all plants (localised in chloroplasts or plastids), bacteria, fungi, but not in animals, which instead obtain aromatic amino acids from their diet. Researches done by Padgette et al. (1995, 1996) show that it is very difficult to accomplish significant endogenous tolerance to glyphosate in plant species

with conventional breeding and induced mutations. Nevertheless, in 1983 glyphosate resistant CP4 gene was identified in *Agrobacterium* sp. strain CP4 (Padgette et al., 1996). Using biotechnology, gene was cloned and transferred into plants. As a result, new transgenic plants contained two types of genes in their genetic material: native genes for glyphosat-intolerant form of EPSPS and foreign, bacterial CP4 genes for glyphosat-resistant form of EPSPS. Inserted CP4 genes enabled plants to normally biosynthesize aromatic amino acids, so even after glyphosat application plants wouldn't wilt.

Greatest commercial success with glyphosate-tolerant (GT) cultivars was made in soybean. First GT soybean cultivars were named Roundup Ready (RR), after the commercial herbicide Roundup, which contained glyphosate as an active ingredient. Development of RR soybean cultivars enabled broad use of Roundup herbicide in arable crops, and thus very successful wide spectrum weed control in soybean. Before RR soybean cultivars, producers applied adequate herbicide regime, which was sometimes phytotoxic for soybean plants and/or left residues in the soil that were harmful for the following crop (Gianessi and Carpenter, 2000). Introduction of RR soybean into production resulted in reduction of herbicide treatment of crops. Single treatment with Roundup was sufficient which added to decreasing of production costs, and increasing of productivity. Commercialization of RR soybean started in 1996 in the USA. Production of RR cultivars had advantage over production of conventional cultivars from economic aspect, and thus they were rapidly accepted by American farmers. From 1996 to 1999, RR cultivars have been sown on 7, 17, 44 and 57% of total field area under soybean in USA, and from year 2000 to 2005, on 54, 68, 75, 81 and 87% (USDA, National Agric. Statistics Service). In 2009, GT soybeans are planted in nine countries covering 69,2 million hectares (52% of global biotech area of 134 mill ha) or 77% of total area planted with soybean (90 mil ha) (James, 2009). Results of the researches conducted by Minor (1998), Oplinger et al. (1998) and Elmore et al. (2001) showed that GT soybean cultivars didn't have considerably better agronomic properties than conventional cultivars concerning seed yield, seed quality and resistance to diseases. The only advantage of GT cultivars is the cheaper production process because of the lower costs of weed control.

In general, herbicide tolerance is considered the first generation of soybean fears obtained by biotechnology. To date, glyphosat-tolerant cultivars have had the greatest commercial success. Further researches are made with the aim to discover genetic resistance to other active ingredients in herbicides. The main goal of these soybean cultivars is reducing the use of pesticides and decreasing the number of applications, which would result in decreasing of production costs and increasing profit. Unlike the first generation GT soybean which was developed with gene gun technology, second generation (RReady2Yield™) was developed with more efficient and precise *Agrobacterium* insertion technology. Genetic mapping of soybean allowed yield enhancing regions of soybean DNA to be identified and in conjunction with advanced insertion and selection technology allowed the RReady2Yield™ gene (MON 89788) to be precisely inserted in one of the high yielding zones (http://www.monsanto.com/pdf/features/mon89788.pdf). The second generation RReady2Yield™, as a result of the linkage established between yield and glyphosate tolerance, offered significant increases in yield of 7 to 11% over the first generation. In 2009, RReady2Yield™ varieties of selected maturity classes, represented the first commercially approved product from a new wave of a whole new class of second generation biotech crop products were commercialized for the first time in a controlled launch in the USA and Canada on approximately 0.5 million hectares (James, 2009).

The second generation fears put into soybean via biotechnology is increased oleic acid content (Kinney, 1996), increased lysine content (Falco et al., 1995) and achieved resistance to pests from Lepidoptera sp. by Bt (*Bacillus thurigiensis)* technology (Walker et al., 2002). The greatest commercial success in this generation was made by cultivars with high oleic acid content (HO cultivars). The first HO soybean lines (G94-1, G94-19, G168) were commercialized in 1998. The reasons behind their development are following. It is well known that soybean is the biggest resource of vegetable oil in the USA, and primary resource for production of biofuels. Average oil content in soybean seed is around 18%, and usual oil composition is: palmitic acid (11%), stearic acid (4%), oleic acid (22%), linoleic acid (53%) and linolenic acid (8%). Oils with high content of mono-unsaturated fatty acids (such as oleic acid) are especially important for processing industry, because they have bigger oxidative stability. This is why the researches were directed towards increasing oleic acid content in soybean oil. Mutagenesis increased oleic acid content to 30-65% (Takagi & Rahman, 1996), while genetic engineering increased it to more than 80% (Buhr et al., 2002).
Third generation of transgenic soybean lines is being created in laboratories and for now they still haven't been commercialized. Properties included in the researches are: enzymes (especially oxalate oxidase for the resistance to the disease *Sclerotinia sclerotiorum*), long-chain fatty acids, vitamins, pharmaceutical ingredients, bioplastics, increased yield, drought and cold tolerance and many other benefits. Furthermore, genetic engineering was successfully used to eliminate allergenic proteins from soybean seed. Transgenic soybean lines with inactivated genes for main allergenic proteins are already being tested, and if they pass the tests we can expect their soon commercialization.
Although on global level there are still controversies concerning transgenic plants, and researches demand large financial investments, further researches and technological development are continuous. Advance in functional genomics enables the discovery of increasing number of genes available for transformation, and with perfecting the methods of genetic engineering; new transgenic soybean cultivars with specific traits are being created.

4. Conclusion

Biotechnology can be defined broadly as a set of tools that allows scientists to genetically characterize or improve living organisms. Several emerging technologies, such as molecular characterization and genetic transformation, are already being used extensively for the purpose of plant improvement. Tools provided by biotechnology will not replace classical breeding methods, but rather will help provide new discoveries and contribute to improved nutritional value and yield enhancement through greater resistance to disease, herbicides and abiotic factors. Plant biotechnology depends upon a number of laboratory procedures that have been developed recently to manipulate DNA and provide new genes of interest to the plant breeder. These procedures have resulted in crop plants that have great commercial value, and many companies are marketing genetically engineered crop varieties. In addition, biotechnology has allowed scientists, as never before, to expand their visions of designing new crop plants to serve humankind. In order to continue the development of gene technology in agriculture, the risks must be carefully analyzed and the plants monitored to detect possible problems. *Glycine max* (L.) Merr has the genetic diversity for differentiation, produces a balanced combination of protein, fat and carbohydrate to serve as a valuable food, feed, and bio-feedstock, inhabits cropping systems as a valuable contributor of nitrogen, and possesses other agronomical complementary traits. Given the coming

advancements in biotechnology, the future of soybean will require the sound use of genetic resource within *Glycine*, adequate funding for research and development, and a clear vision of the opportunities that lie ahead. Scientific discoveries in the area of structural and functional plant genomics would lead to production of new soybean varieties with advanced nutritive and agronomic properties, created by combining conventional breeding methods and biotechnology tools. Based on the availability and combination of conventional and molecular technologies, a substantial increase in the rate of genetic gain for economically important soybean traits can be predicted in the next decade.

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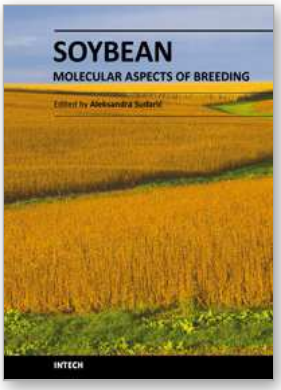

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The book Soybean: Molecular Aspects of Breeding focuses on recent progress in our understanding of the genetics and molecular biology of soybean and provides a broad review of the subject, from genome diversity to transformation and integration of desired genes using current technologies. This book is divided into four parts (Molecular Biology and Biotechnology, Breeding for Abiotic Stress, Breeding for Biotic Stress, Recent Technology) and contains 22 chapters.

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