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BIOTECHNOLOGY IN SOYBEAN BREEDING

Aleksandra SUDARIC[1], Marija VRATARIC[1], Snezana MLADENOVIC DRINIC[2], and Maja MATOSA[1]

[1]Agricultural Institute Osijek, Osijek, Croatia
[2] Maize Research Institute, Zemun Polje, Belgrade, Serbia

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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. Other emerging sciences, including genomics and proteomics, are also starting to impact 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. In soybeans, biotechnology has and will continue to play a valuable role in public and private soybean breeding

Corresponding author: Aleksandra Sudaric,Agricultural Institute Osijek,Južno predgrađe 17,31000 Osijek,Croatia,email: aleksandra.sudaric@poljinos.hr

programs. 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. In this paper, a short review of technologies for molecular markers analysis in soybean is given as well as achievements in the area of genetic transformation in soybean.

Key words: breeding, biotechnology, genetic transformation, molecular markers, soybean

INTRODUCTION

Soybean (*Glycine max* (L.) Merr), is the leading oil and protein crop of the world, which is used as a source of high quality edible oil, protein and livestock feed (RAJCAN *et al.*, 2005) In recent years, the scientific and technological developments in most regions have increased soybean production on the global level, and all the sectors, involved with the entire soybean production and processing chain, have responded accordingly to comply with the demands of a globalized economy.

From viewpoint of genetics, soybean is a self-pollinated species with natural outcrossing of 0.5 -1 %. As the result of its self-pollinating reproductive behavior, plant-breeding procedures such as backcrossing, single pod descent, pedigree breeding and bulk population breeding are some of the more common procedures used to develop high-yielding and high-quality soybean varieties. Conventional breeding strategies have been very successful in improving soybean productivity and quality. Today, the molecular based plant breeding techniques are assuming an increasingly more important role in genetic improvement of soybean germplasm. However, modern biotechnology in itself will never replace plant breeding research, but rather will enhance and improve upon the efficiency of plant breeding. 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. So, conventional breeding strategies have priority, and in combination with biotechnology have provided the possibility of broadening genetic variability of cultivated soybean and of creating new germplasm that is better adapted to new market, production and environment demands (VERMA and SHOEMAKER, 1996; ORF *et al*, 2004; ORF, 2008; SUDARIC *et al.*, 2005, 2008, 2009; VRATARIC and SUDARIC, 2008).

Modern biotechnology application in soybean breeding can be divided into 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.

TECHNOLOGIES FOR MOLECULAR MARKER ANALYSIS

Molecular markers refer to the DNA sequence with exactly defined nucleotide order and distribution, strictly specific for different organisms. These 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. In soybean breeding, molecular marker applications are currently focused in four primary areas: germplasm characterization, marker-assisted selection (MAS), marker-assisted backcrossing and gene discovery. MAS is used more readily than the usual techniques to screen single traits, such as resistance or restorer genes: nematode resistance (MEKSEM *et al*., 2001; DIERS and KIM, 2009), insects resistance (ZHU *et al*., 2006), pathogen resistance (ARAHANA *et al*., 2001; BACHMAN *et al*., 2001; NJITI *et al*., 2002; TOLIN, 2004). Molecular markers have a 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, contributing to soybean breeding efficiency (DOLDI *et al*., 1997; NARVEL *et al*., 2000a; SUDARIC *et al*., 2008; DRINIC MLADENOVIC *et al.,* 2008). Molecular markers are divided into two main groups: protein markers (biochemical markers) and DNA markers.

Protein markers - are divided into two groups: storage proteins and functional proteins or isozymes (most commonly used protein markers). 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 the environment. However, their use is limited due to their limited number. In soybean, protein markers are mostly used for identification of cultivars, testing F_1 hybrid seed, material divergence analysis, as well as, in seed production for determining uniformity and genetic purity of cultivars and identifying different varietal impurities in seed material (DOONG and KIANG, 1987; MIROSLAV and JIRI, 1996; NIKOLIC *et al.,* 2004*,* 2005; DRINIC MLADENOVIC *et al*., 2006, 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. 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.

RFLP markers (*Restriction Fragment Length Polymorphism)* – represent the first generation of DNA markers used for plant genomes (WEBER and HELENTJARIS, 1989). The basis of RFLPs is using restriction enzymes (endonucleases), which

recognize 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 recognized by restriction endonucleases. The use of RFLP markers in soybean started in the late 1980s (APUYA *et al.*, 1988) which contributed the development of first genetic map of soybean genome (KEIM *et al.,* 1990). This map had further expansion during the 1990s with the addition of over 350 RFLP loci (SHOEMAKER and OLSON, 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.

RAPD (AP-PCR) markers (*Randomly Amplified Polymorphic DNA - arbitrary primer PCR)* – The basis of 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. Polymorphism of genomic DNA is detected through the length of synthesized DNA fragments. 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; DRINIC MLADENOVIC *et al.*, 2008; PERIC *et al.*, 2008a). The utilization of RAPD markers 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).

DAF markers (*DNA Amplification Fingerprinting Markers)* – The basis of DAF markers is the use of polymerase chain reaction (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 8 nucleotide long), so for the electrophoresis of DAFs is used polyacrylamid gel with silver staining, and for RAPDs is used agarose gel. The use of DAF markers in soybean genome analyses started during 1990s (PRABHU and GRESSHOFF, 1994).

SSR markers (*Simple Sequence Repeats (microsatellites)* – The use of microsatellites (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 and 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, SSR markers are excellent complement to RFLP markers for soybean researches in the fields of molecular biology, genetics and plant breeding. 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) are 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 and grain quality compared with the parents than crosses between similar parents. The results indicated the usefulness of genetic marker information in parental selection, contributing to breeding efficiency. The SSR markers linked to the major QTL will be useful for marker-assisted selection in soybean-breeding programmes (FUNATSUKI *et al.*, 2005; PANTHEE *et al.*, 2006). A set of simple sequence repeat (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.* 2000b; BURNHAM *et al.* 2003, SHULTZ *et al.*, 2007).

AFLP markers (*Amplified Fragment Length Polymorphism)* – The use of 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. 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), and one of the largest available AFLP maps of any plant species was developed in soybean (KEIM *et al.*, 1997). AFLP technologies are continuously being modified and perfected (LIN *et al.*, 1999; MANO *et al.* 2001).

SNP markers (*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). According to the fact that SNP represents nucleotide variation (for example sequence ACGTATA instead of ACTTATA), they are potentially useful as genetic markers because they enable the distinction of one haplotype from another. In soybean, SNPs nature and frequency researches have intensified (CAHILL, 2000; ZHU *et al.,* 2003), and thus are likely to have an important role in the future of soybean genome analyses and manipulation.

In general, the ability to utilize molecular markers to identify the genomic location of plant genes has played an important role in revolutionizing the science of

plant breeding and genetics. In soybean, RFLPs and SSRs have been used extensively to map genomic location of quantitative trait loci for many agronomic, physiological and seed composition traits.

GENETIC TRANSFORMATION

In the last three decades, technological advance within plant biotechnology 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 producing individuals which clearly exhibit particular desirable traits. These crossings 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).

Transgenic plants represent completely new genotypes (recombinations) and therefore, in order to confirm expected phenotypic expression of the new trait, selection after the gene transfere is necessary, the same as 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.

The first genetically modified (GM) soybeans were planted in the United States in 1996. More than ten years later, GM soybeans are planted in nine countries covering 65,8 million hectares (53% of global GM area) or 72% of total area planted with soybean (91 mil ha) in 2008.

In soybean, the first generation traits created by biotechnology were herbicide resistance with glyphosate resistance. The primary outcome of the resistant soybean varieties has been reduced costs and increased production efficiency (OPLINGER *et al*., 1998; ELMORE *et al*., 2001). The second generation traits 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). 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: special enzymes (especially oxalate oxidase for the resistance to the disease *Sclerotinia*

sclerotiorum), long-chain fatty acids, vitamins, pharmaceuticals, drought and cold tolerance, bioplastics, increased yield, and many other benefits. Although on the global level there are still controversies concerning transgenic plants, and researches demand large financial investments, further researches and technological development are continuous.

CONCLUSION

Biotechnology implies a deliberate manipulation of the DNA of living organisms, usually through the use of genetic engineering, in which genes are transferred via a vector from one organism to another, bypassing sexual reproduction. The revolution in plant biotechnology is and will be an important contributor to plant breeding programs, including soybean. 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.

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.

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BIOTEHNOLOGIJ U OPLEMENJIVNJU SOJE

Aleksandra SUDARIC[1], Marija VRATARIC[1], Snezana MLADENOVIC DRINIC[2], i Maja MATOSA[1]

[1] Poljoprivredni institut Osijek, Osijek, Hrvatska
[2] Institut za kukuruz, Zemun Polje, Beograd, Srbija

I z v o d

Biotechnologija u širem smislu se može definisati kao skup tehnika koje omogučavaju naučnicima da genetički karkterišu ili poboljšaju živi organizam. Nekoliko tehnologija, kao što su molekularna karakterizacija i genetička transformacija se već široko primenjuju u oplemenjivnaju biljaka. Genomika i proteomika takođe imaju uticaj na pobošljanje biljaka. Metode biotehnologije neće zameniti konvecionalne metode oplemenjivanja već obezbediti nova saznanja i doprineti poboljšanju hranjive vrednosti i povećanju prinosa povećanjem rezistentnosti na izazivače bolesti, tolerantnosti na herbicide i abiotički stress. U oplemenjivanju soje, biotehnologija ima i imaće značajnu ulogu i u privatnim i javnim programima. Na osnovu dostupnosti i kombinacijom konvecionalnih i molekularnih tehnologija znatno povećanje genetičke dobiti za ekonomski važna svojstva može da se predvidi u narednim decenijama. U ovom radu je dat kratak prikaz primene tehnologije molekularnih markera u oplemenjivanju soje kao i dostignuća u oblasti genetičke transformacije.

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