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Methods of Transformation and Detection of Genetic Modifications in Field and Vegetable Crops

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Abstract: Genetically modified (GM) plants are organisms to which a gene or genes from unrelated species are introduced using methods of genetic engineering. The process of introducing genes into unrelated species and getting them to function is known as “genetic transformation”. Although there are many variations on the plant transformation topic, the main methods used include direct introduction of DNA into genomic DNA and the use of bacterial species (*Agrobacterium tumefaciens*) to deliver the gene of interest into the host plant.

Genetically modified plants have become an integral part of agricultural production, and increasingly commercialized GM plant species are available now (soybean, maize, potato, tomato, oil rape, cotton etc.). Since some of them are suspected of affecting human health, testing of GMO prescribed by a law regulative has become obligatory in most countries. GMO can be detected on the basis of differences between non-modified and transgenic plants, which can be on the level of DNA, proteins, or on the basis of newly obtained characteristics. The commonest method used to analyze proteins of transgenic plants is ELISA test, based on the use of specific antibodies for the protein in question. The methods based on DNA analysis use the reaction of chain polymerization of DNA (PCR) and can be divided into: the qualitative one - used for determination of the presence of genetic modifications, and the quantitative one - revealing GMO percentage (competitive PCR and Real Time).

Key words: Genetically modified organisms (GMOs), ELISA, PCR, testing, transformation.

Introduction

The term genetically modified organisms (GMOs) has been introduced to describe organisms whose genetic material has been modified in a way that does not occur under natural conditions of cross-breeding or natural recombination. Applied to crops, the term refers to plants whose gene or gene from different species has been stably introduced into the host genome using techniques of genetic engineering. In most cases, the introduced genes have been shown to produce a gene product (protein) in the plant that gives them a trait not previously possessed. The process of introducing genes into unrelated species and getting them to function is known as "genetic transformation".

Genetically modified plants have become an integral part of agricultural production, and an increasing number of GM plant species are commercially available now (soybean, maize, potato, tomato, oil rape, cotton etc.). Genetically modified plants are tolerant of herbicides, resistant to insects, viruses and fungi, or have transformed composition of nutrients and male sterility/fertility restoration.

Methods of transformation are relatively new, and their consequences still unknown. Scientific opinions on this subject are divided. It is suspected that some of them can affect environment and human health (Metcalf *et al.* 1996, Milosevic *et al.* 2003). To protect the consumer freedom of choice, many countries have issued GMO labelling regulation. GM food must be labelled at the threshold at 0.9% in European Union, 3% in Korea, 0.5% in Germany and 5% in Japan (Berdal and Holst-Jensen 2001). The law dealing with GMO in Serbia outlined according to the EU law forbids introduction of GMO into the environment and demands labelling of food containing more than 0.9% of GMO.

Methods of genetic engineering in plants

Development of genetically modified plants requires:

- Identification and isolation of gene with specific trait of interest.
- Developments of a gene construct which besides having gene of interest also contains promoter (functional element controlling gene expression) and terminator (functional element included in transcription termination). Other elements, which can also be introduced into the gene construct with the aim to control and stabilize gene functions, mark the presence of the construct in GMO, or make combination of different elements of the construct easier. In plants developed by use of biotechnological methods, the introduced genes are called *transgenes*.
- Insertion of the construct into plant cell using method of genetic engineering.
- Development of a plant from transformed cell.
- Testing of plant with the aim of determining the presence of the introduced gene.
- Multiplication of plants with desirable traits.
- Running field trials in order to determine if the introduced gene is stable and if its traits are satisfactory.

Methods of transformation

Genetic transformation can be achieved methodologically by direct introduction of gene construct or via a plant transformation vector containing the desired gene construct. *Agrobacterium* plasmids, DNA of plant viruses, DNA of organelles, and pollen can be used as vectors. The most common methods used in plant transformation include: *Agrobacterium* –mediated transformation and direct methods of transformation.

a) *Agrobacterium* –mediated transformation

The greatest number of plant species was transformed using bacterial species, *Agrobacterium tumefaciens* (Figure 1). It is capable of transferring DNA into plant cell via mobile Ti plasmid. In nature, this transfer causes plant tumor forming (crows galls). In laboratory, genes causing tumor growth are removed so bacterium can insert the gene of interest without causing tumor growth. Ti plasmid is cut by means of restriction enzymes at precisely determined place i.e. determined DNA sequence. The same enzyme is used to treat gene construct and in that way sticky ends are obtained enabling linkage between gene construct and plasmid.

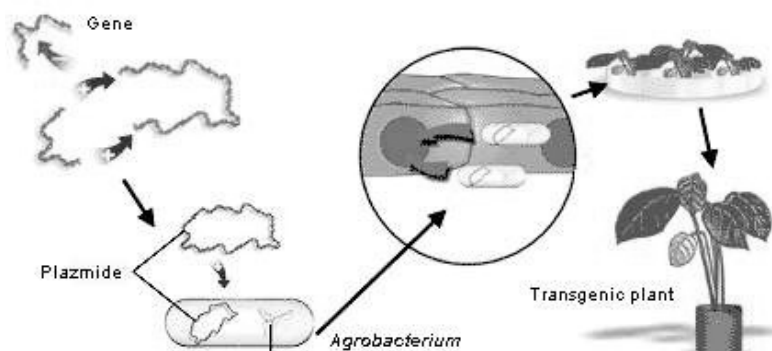


Fig. 1. *Agrobacterium* –mediated transformation of plants

The next step is the introduction of the newly formed plasmid into bacteria culture, most often *E. coli*, because the manipulation with recombinant DNA of Ti plasmid into *E. coli* is made significantly easier, and its transfer using the process of conjugation into *Agrobacterium* is also easily achieved (Van Haute *et al.* 1983). The transformed bacteria have, apart from the information on the new protein formation, genes resistant to antibiotics enabling control of successful plasmid transformation (by adding certain antibiotic into medium) prior to its introduction into *Agrobacterium*.

The disadvantage of this method is the impossibility to transform all plant species especially cereals.

b) Direct methods of transformation

Methods of direct introduction of DNA can be divided into four groups: chemical stimulation of endocytosis, electroporation, microinjecting, and biolistic method. With these methods (excepting the biolistic one) DNA is introduced using different physical and chemical agents into a cell with removed cell wall (protoplast). Protoplasts have great ability of accepting DNA when treated by chemical or physical agents. Once found in the protoplast, DNA is incorporated into genomic DNA. The disadvantage of this method is poor regeneration of a plant from protoplast.

The biolistic method is also known as a gene-pistol method. This method uses small metal particles, most often made of gold, coated with desirable DNA and "shot" into plant cell. Once DNA is found inside of the cell, it can be incorporated into genomic DNA (Becker *et al.* 1994). The advantage of this method is that it enables the introduction of DNA into deeper cell layers enabling the use of parts of tissue for transformation.

Methods of determination of plant genetic modification

As mentioned earlier, transgenic plants have a new gene (or a set of new genes) introduced into their genome. A new gene (genes) is prescribed into an informational RNA, on the basis of which a new protein is expressed. It gives plant a new trait such as resistance to insects, or tolerance to herbicides. Genetically modified plants can be detected on the basis of difference between unmodified variety and transgenic plant, which can be at the level of DNA, RNA, proteins, or on the basis of characteristics of the variety (Milosevic and Taski 2005).

Sometimes it can be proved that the plant was transgenic on the basis of a newly developed trait of the variety. Such is, for example, the case with transgenic oil rape whose composition of fatty acids had been changed. It can be proved whether the sample contains transgenic oil rape by measuring the content of fatty acids. Transgenic plants resistant to total herbicide can be proved by treating plants with this herbicide. However, much more reliable methods are based on detecting appropriate proteins and especially on detecting newly introduced DNA. Today, this is the commonest method used for detecting genetic modifications among field and vegetable crops. Methods based on RNA detection are not appropriate due to the instability and short duration of iRNA.

Method based on protein analysis

If methods based on protein analysis are to be used, it is necessary that the protein formed on the basis of the introduced DNA be detected. It can be determined only if it is not already naturally present in plant and if it is synthesized in the analyzed tissue.

The commonest method used for analyzing proteins of transgenic plants is ELISA test (Enzyme-Linked Immuno Sorbent Assay). It is based on using specific antibodies for the protein of interest. The antibody is linked to an

appropriate protein, and an appropriate secondary antibody with a conjugated enzyme enabling colour appearance is linked to that protein. The colour is easily visualized, and the concentration can be measured on the basis of the standard curve of the protein of interest. Protein tests are available in different forms such as micro-titer plates, tapes, or tubes.

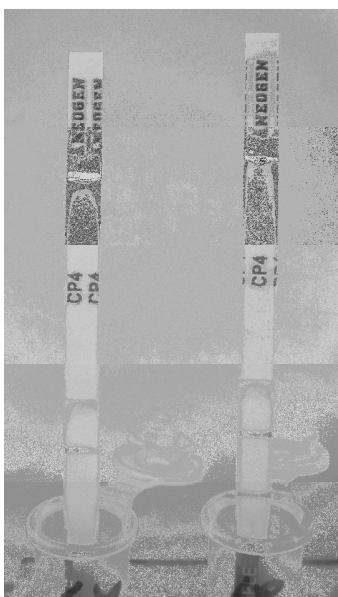


Fig. 2. GMO detection using test strips

test strips (Nikolic *et al.* 2009). As all soybean seeds were controlled for presence of GMO upon receipt at the Institute of Field and Vegetable Crops, test strips were used for preliminary testing and proved to be very economical and reliable.

The lack of identification of genetic modification on protein is that it requires a protein with an unchanged structure that is limited to fresh or partially processed food.

Methods based on DNA analysis

The analytical method based on PCR (Polymerase Chain Reaction) technology is most often used for detecting DNA sequences inserted into plants. PCR makes selective *in vitro* amplification of a certain DNA region possible by imitating *in vivo* phenomena of DNA replication (Mullis *et al.* 1987).

On the basis of a single chain DNA obtained by the denaturation of native DNA, a new complementary DNA chain is synthesized at appropriate conditions. Which part of DNA chain will be *de novo* synthesized depends on primers hybridized on the opposite sides of gene intended for multiplication. Small quantities of specific fragments of DNA can be multiplied more than million times by repetition of DNA

Strip tapes are suitable for testing raw plant material or seeds due to quickly obtained tests. On the test tape there are immobilized antibodies specific for transgenic protein, which are linked to coloured reactant. When a strip is placed in GM sample a sandwich is created between the antibody and the antigen of a transgenic protein. The obtained complex travels through the porous membrane, having two zones remained unlinked, one specific for transgenic protein sandwich, and another one for antibodies. The presence of one coloured strip suggests that the sample is not genetically modified, and the two lines reveal the presence of genetic modification (Figure 2). Test strips for GMO detection are very suitable for field testing, as the result can be obtained in 15-30 min. Due to the large number of samples collected during the 2006 and 2007 growing seasons for monitoring of the Roundup Ready soybean in the Vojvodina province, the presence of GMO in seeds sampled at trading spots was determined by

denaturation cycle, hybridization of primers for complementary sequences in DNA, and extension. Enzymes of DNA polymerase, used nowadays for PCR, are isolated from bacteria living in thermal springs, so they are thermo stable, and their activity is not lost at high temperatures necessary for DNA denaturation.

A great number of methods based on PCR have been developed for GMO detection. A prerequisite for GMO detection is a solid knowledge of the type of genetic modification in question, including design of inserted gene, as well as regulatory elements (promoter and terminator) used. The minimal quantity of sample containing intact DNA of appropriate gene is necessary for analysis. The method based on PCR was chosen due to its great sensitivity, high specificity and resistance of DNA to technological processes. The methods for GMO detection based on PCR were divided into: the qualitative method, used for determination of the presence of genetic modification, and the quantitative one, pointing out to GMO percentage.

a) Qualitative PCR

The qualitative PCR test is based on multiplication of parts of transgenic sequences such as: regulatory sequence (35S promoter and NOS terminator), resistance of markers to antibiotics or specific gene (EPSPS, cryIA). During PCR, many copies of regulatory elements or gene sequences, which could be separated by electrophoresis, are made, if present in the sample. The appearance of fragments of 195 bp (with primers for 35S promoter) proves that genetic modification is present in the sample (Figure 3) (Taski *et al.*, 2006b). Even if there is a great number of promoters and terminators, CaMV35S promoter (Roundup Ready soybean, maize Bt-176, T25 etc) or NOS (Roundup Ready soya, Bt-11 etc.) is the most commonly used (Hemmer 1997). Therefore, the above regulatory elements used for screening food and feed for the presence of GMO in Laboratory for seed testing (Taski *et al.* 2006a). These elements also were used in monitoring food products in two projects funded by the Department of Novi Sad in the programme of environmental protection entitled "Novi Sad-City without GMO" and "Health Validity of the Food at the Green Markets of Novi Sad" (subproject „Check of Residues Genetically Modified Organisms“). No products containing GM were found in the analyzed food samples taken in the municipality of Novi Sad by the random sampling method.

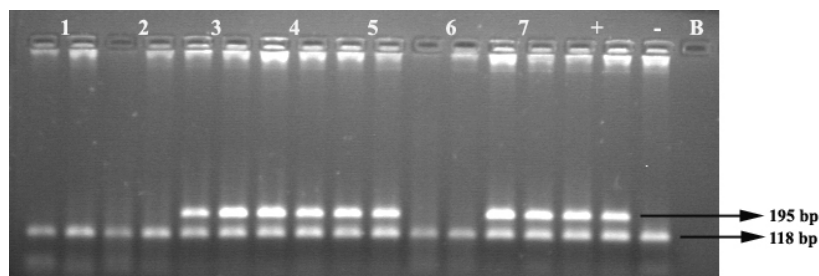


Fig. 3. Duplex PCR of soybean samples using primers for endogenous lectin gene (118bp) and 35Spromotor (195bp)

B blank test, + positive control, - negative control, 1- 7 samples in duplicate

b) Quantitative PCR

GMO quantification based on PCR can be performed after completed PCR (competitive PCR) or during PCR reaction (Real Time analysis).

Competitive PCR: is based on comparison of the final quantity of amplified two targeted DNA, one from sample and DNA competitor. Competitor DNA is added to known concentration and it co-amplifies with DNA sample. Primer is also linked to DNA competitor, but at different sites, and fragment of different size in relation to GMO sample is obtained. If the intensity of strip of PCR product is the same, then initial quantity of DNA is equal to quantity of competitor DNA.

“Real Time” PCR: Much more reliable and more frequently used method for GMO quantification is Real Time PCR. This test is based on 5'-3' nuclease activity of Taq polymerase and oligonucleotide probes marked with two fluorescence dyes (Reporter and Quencher). In PCR reaction, primers hybridize to a specific sequence on the target DNA. The target sequence is then amplified (Figure 4). In the meantime, probes also hybridize to a target sequence between the binding sites of primers. When polymerase reaches site of binding probe it has to be cleaved in order to continue amplification. In that way liberated reporter day emits fluorescence registered by the apparatus. When the probe is intact, the fluorescence is disabled by Quencher day. The level of fluorescence emission is proportional to the quantity of gene found in the sample. Each analysis on GM content in the sample includes series of standards on the basis of which the percentage of genetic modification can be determined.

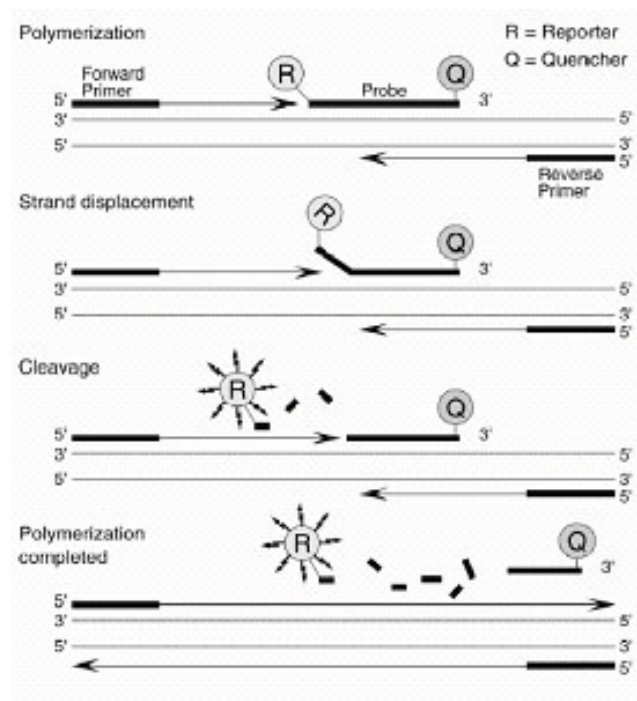


Fig. 4 Principle of Real Time analysis

In the samples where, upon detection of regulatory elements, the presence of genetic modification was determined in Laboratory for seed testing, quantification was performed using Real-Time (Taski-Ajdukovic *et al.* 2006a). Thus the monitoring of Roundup Ready soybean in meat products on the Serbian food market sample was positive for presence of soybean subjected to Real-Time quantification of the percentage of RR soya. The presence of the GMO was demonstrated in 12 cases and all contained RR soya below 0.1% (Taski-Ajdukovic *et al.* 2009). The same method was used for quantification of GM soybean in food products representing a variety of processing steps for soybean imported from different suppliers in the Serbian food market. Most analyzed samples were correctly labelled, but ten of the food products were demonstrated to contain material above the threshold level for labelling of 0.9% (Nikolic *et al.* 2009).

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METODE TRANSFORMACIJE I ODREĐIVANJA GENETIČKIH MODIFIKACIJA KOD RATARSKIH I POVRTARSKIH BILJAKA

- prethodno saopštenje -

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Rezime

Genetički modifikovani organizmi (GMO) su organizmi u koje je unesen gen ili geni iz nesrodnih vrsta metodama genetičkog inženjeringa (transformacije). Postoje različite metode transformacije. Najveći broj GM biljaka nastao je direktnim unošenjem dela DNK u genom (direktne transformacije) ili pomoću bakterije *Agrobacterium tumefaciens*, koja ima sposobnost prenosa DNK u biljnu ćeliju (transformacija putem Agrobakterijuma).

Genetski modifikovane biljke su postale sastavni deo poljoprivredne proizvodnje i sve je više GM biljnih vrsta koje su komercijalno dostupne (soja, kukuruz, krompir, paradajz, uljana repica i dr.). Kako je dokazano da neke od njih mogu uticati na ljudsko zdravlje u većini zemalja je obaveza testiranja GMO propisana zakonskom regulativom.

GMO se mogu detektovati na osnovu razlika između nemodifikovane i transgene biljke, koje mogu biti na nivou DNK, proteina ili na osnovu novostečene karakteristike. Najčešće korišćena metoda za analizu proteina transgenih biljaka je ELISA test koji se zasniva na korišćenju specifičnih antitela za protein od interesa. Metode zasnovane na analizi DNK su bazirane na reakciji lančane polimerizacije DNK (PCR) i mogu se podeliti na: kvalitativne, kojima se određuje da li su prisustvo genetske modifikacije i kvantitativne, koje pokazuju procenat GMO (kompetitivni PCR i Real Time).