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DETECTION OF GENETICALLY MODIFIED PLANTS BY USING PCR

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The PCR method for qualitative detection of GMOs was optimized. Primers, namely 35S (35S-promoter, originated from cauliflower mosaic virus), NOS (nopaline synthase-terminator, derived from *Agrobacterium tumefaciens*), were used to identify the GM maize and GM soybeans. The data further confirm that the PCR methods can be effectively used to differentiate GM soybeans and maize from non-GM products.

GMOs – PCR – maize - soybean

Գենետիկորեն վերափոխված օրգանիզմների (ԳՎՕ) իդենտիֆիկացման համար օպտիմացվել է պոլիմերազային շղթայական ռեակցիայի (ՊՇՌ) մեթոդը: Կիրառվել են 35S պրոմոտերի (35S-պրոմոտեր ծաղկակաղամբի մոզաիկայի վիրուսից) և NOS տերմինատորի (նոպալին սինթազի տերմինատոր *Agrobacterium tumefaciens*-ից) պրայմերները: Ստացված արդյունքները վերահաստատում են պոլիմերազային շղթայական ռեակցիայի մեթոդի կիրառելիությունը գենետիկորեն վերափոխված օրգանիզմների իդենտիֆիկացման համար:

ԳՎՕ – ՊՇՌ - եգիպտացորեն - սոյա

Адаптирован метод ПЦР идентификации генетически модифицированных оганизмов (ГМО). Использованы праймеры для 35S промотера (35S-промотер из вируса мозаики цветной капусты) и NOS терминатора (терминатор синтазы нопалина *Agrobacterium tumefaciens)*. Полученные результаты подтверждают эффективность применения ПЦР метода для идентификации ГМ кукурузы и сои.

ГМО – ПЦР – кукуруза - соя

According to the EU legislation, the genetically modified organisms (GMOs) are defined as “organisms, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination” [5].

Since the first experiments on tobacco in the 1980's, several plant species, including both monocots and dicots [3], have been genetically modified to improve specific characteristics (e.g. yield, quality, pest resistance), through adding one or more useful character previously not present in the non-modified counterparts. Such changes are generally obtained by the insertion of one or more genes using a range of available techniques defined as ''gene transfer technologies'' [8, 9, 20, 21]. These technologies include the following three techniques:

1) Recombinant DNA techniques using vector systems;

2) Techniques involving the direct introduction into an organism of heritable material by micro-injection, macro-injection and micro-encapsulation;

3) Cell fusion (including protoplast fusion) or hybridization techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.

Genetic modifications are carried out by the insertion of several smaller pieces of DNA from various sources, into the genome of the plant to be modified. The insert is a combination of several small pieces of DNA. In its easiest format the gene construct composes of three elements: 1) the promoter functions as an on/off switch for when and where the inserted or modified gene is active in the recipient plant; 2) the inserted/modified gene (structural gene) encodes a specifically selected trait; 3) the terminator functions as a stop signal for transcribing the inserted/modified gene. In addition, the marker genes may be present to distinguish GMOs from non-GMO during development.

Commercial cultivation of genetically modified (GM) crops has been growing since 1996, reaching from 114,3 million hectares in 2007 up to 125 million hectares in 2008. The USA (63%), Argentina (21%), Canada (6%) and Brazil (4%) are the countries with largest areas of GM crops [11]. Those countries are called mega biotech countries and accounted for 99% of the total GMO-growing area. The principal GM crops are four crop plants essentially growing since 13 years. There are almost 65,8 % of GM soybeans, followed by 30% of maiz, 12% cotton, and than some 5% are oilseed rape, and two traits in those plants including resistance to herbicides and insect damage [11]. In 2008, the stacked double and triple traits occupied a larger area 22% of global biotech crop area than insect resistant varieties at 15% [11]. But, great diversity of traits and genetically engineered organisms (plants, animals and micro-organisms) are under development. Most promising in terms of expected profits are pharmaceutical traits (enzymes, vaccines, etc.), industrial products especially these days for agro-fuels, but also other products (e.g. starch from amylopectin producing potatoes, etc.). So, it is possible to assume that GMOs play a positive role in sustainable agriculture, forestry, aquaculture, bioremediation, and environmental management, both in developed and developing countries. There are growing concerns about the impact of GM crops on the environment such as vertical or horizontal gene flow, related ecological impacts especially on non-target insects, effects on biodiversity and the impact of presence of GM material on human health, which lead to the need for risk assessment and

management (Haslberger, 2006). However, all nations have specific rules under which new biotech products are evaluated for these risks and approved before entering the market. In EU particularly, authorization to release a GM organism in the environment is regulated by Directive 2001/18/EC [5], and only approved GM cultivars are allowed to be cultivated. Furthermore, the rules for placing into the market food or feed consisting of or containing GMO are stipulated by Regulations (EC) No 1829/2003 [6] and (EC) No 1830/2003 [7]. EU legislation requires detection and monitoring of GMOs to enable safety assessment and enforce labeling, which leads to a high demand for reliable and easy to perform GMO detection and identification methods.

In the Republic of Armenia the biosafety-related activities started in 1993, when the National Assembly of RA ratified the Convention on Biological Diversity. The national and worldwide importance of biosafety in Armenia is conditioned by a number of factors. Particularly the territory of Armenia is the center of origin for different flora and fauna species, Armenia is located on the cross-road of migration routs for a number of animal and bird species, and is a habitat for some of them. In order to fulfill biosafety-related obligations under the Cartagena Protocol to the Convention on Biological Diversity, Armenia has recognized importance of pre-market safety testing and post-market testing in support of monitoring of GMOs/LMOs, such as tests for the presence of transgenic DNA or foreign proteins.

Analytical methods to detect (qualitative or yes/no answer) and quantify (percentage content) GMOs fall into two main categories: protein analysis to detect the specific protein expressed by the transgene in the GMO through the use of ELISA (enzyme-linked immunosorbent analysis) and lateral flow strip tests [17,18] or DNA analysis to detect the specific transgene in the GMO or specific elements associated with the transgene [12,16]. Significant progress has been achieved in the development of genetic analysis methods, such as high technological methods based on the use of PCR. The PCR-based methods are the most sensitive, reliable and easy to perform [2, 12]. Like all PCR techniques, GMO testing tools are designed to target and visualize the presence of specific transgene DNA fragments in plants and foods. Most of the early qualitative and semi-quantitative methodologies have been designed to target regulatory DNA sequences (promoters, terminators) common in many transgenic cultivars [14, 19]. By testing for the presence of these fragments, especially the cauliflower mosaic virus (CaMV) 35S promoter and the NOS terminator from Agrobacterium tumefaciens that are common to most of the GMOs authorized by EU, the technique is versatile in screening for many different transgenic cultivars in one step [22].

The most common procedures including GMO detection, identification and quantification begin with sampling followed by DNA extraction and GMO screening/detection (Fig. 1).

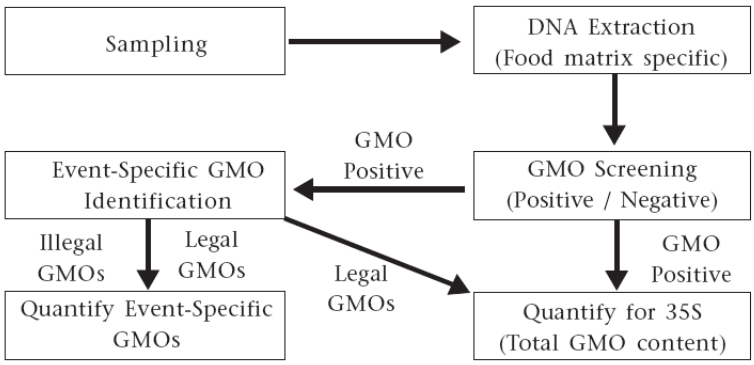

Fig.1. Scheme for DNA based GMO detection, identification and quantification

1) Screening. The purpose of screening is to determine whether a sample contains GMOs. For this objective, a screening method can be used resulting in a positive/negative statement. The screening methods are usually based on the polymerase chain reaction (PCR).

2) Event specific identification. If there is a positive detection of GMOs, further analysis is required to discover which GMO it is and thus whether the GMO is approved within the EU. The only analytical methods, which unequivocally may enable identification of each GMO variety are methods based on PCR.

3) Quantification. If a product has been shown to contain GMO(s), the next step is to assess compliance with the 1% threshold level (or the 0,3 or 0.5% level, respectively for seeds) by the determination of the exact amount of each of the GMOs present in the sample. Typically quantification is performed using semi-quantitative PCR or Real-time PCR.

The objective of the study was to optimize the procedures for the detection of genetically modified maize and soybean. At first, DNA extraction method was checked and optimized, followed by the optimization of PCR conditions.

Materials and methods. Plant material.10 samples of maize and soybean seeds where obtained from the markets in Yerevan.

DNA extraction. Cotyledon samples were excised with a 6 mm diameter cork borer. The extraction of DNA was done by CTAB method described in PN-EN ISO 21571:2006 [4]. To enhance the yield of genomic DNA from highly complex matrices the RNase 10 mg/ml and proteinase K 20 mg/ml treatments were used.

The DNA extraction was carried out under sterile conditions in flow chamber. To avoid contamination single-use equipment and decontamination solutions were used during sample preparation.

PCR amplification and product analysis. The 48 µl of PCR mix contained 1X 10x PCR Buffer, 2.5 mM MgCl, 0.025 U/µl Taq, 0.2 mM dNTPs, 0.5 µM pM each primer. PCR amplification was carried out using Apmly 25 termalcycler, Biokom. Amplification conditions for PCR 35S and NOS promoter were 95°, 3 min; then 95°, 25 sec; 62°, 30 sec; 72°, 45 sec for 50 cycles and a final extension of 72°, 7 min.

Oligonucleotide PCR primers for 35S promoter and NOS terminator were kindly received from the University of Milan. All reactions were optimised as regards primers, MgCl2, dNTP's, and polymerase concentration. Moreover, thermal profile was also optimised due to the profile of Amply 25 thermocycler

35S promoter: p35S-cf3 - 5'-CCACGTCTTCAAAGCAAGTGG-3'
p35S-cr4 - 5'-TCCTCTCCAAATGAAATGAACTTCC-3'

NOS terminator: HA-*nos* 118-f - 5'-GCATGACGTTATTTATGAGATGGG-3'
HA-*nos* 118-r- 5'-GACACCGCGCGCGATAATTTATCC-3'

As a positive control transgenic DNA was used containing no less than 100 samples per mkl of 35S promoter and NOS terminator (Syntol, Russia). As negative control DNA from reference material (maize and soybean DNA with 0% of GM, University of Milan). Negative control of the mastermix, in which water is used instead of DNA also was used .

PCR product analyses were visualized by UV transillumination on a 1.5 % agarose and TBE buffer. The gel was stained with ethidium bromide. Size markers (100 bp ladder) were electrophoresed in adjacent wells of the gel to allow accurate size determination.

Results and Discussion. Two primers, 35S and NOS as listed below were selected for PCR analysis. In 2008, 80% commercial GM crops were found to contain 35S-promoter or NOS-terminator in their inserted genes. Theoretically, using the primers specific to the above two genes for PCR analysis allows the identical PCR products to be amplified and therefore the GMOs firstly can be detected by using this method [15]. The PCR products with size 12bp (from 35S) and 118bp (from NOS) were obtained, but no PCR products are found from regular maize and soybean (negative control, 0% GMO content). In 8 of 10 samples of maize the 35S promoter was amplified, from which in 5 samples markers for NOS genes were identified also (Fig. 2). In two samples there weren't amplification products. In our study in all samples of soybeans studied, 35S promoter and NOS terminator were successfully amplified (Fig. 3). The results obtained suggest that studied samples are different lines of GM maize and GM soybeans.

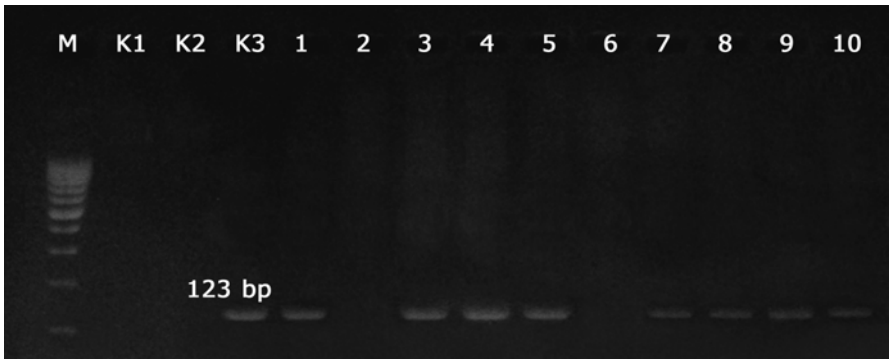

Fig.2. The amplification of the p35S-cf3/p35S-cr4 primers for CaMV35S promoter using genomic DNA of maize. M – DNA ladder (100 bp), K1 – Negative control with mastermix without DNA, K2- Negative control with reference material (o% of GMO), K3 – positive control (35S promoter), lanes 1-10 sample of maize seeds.

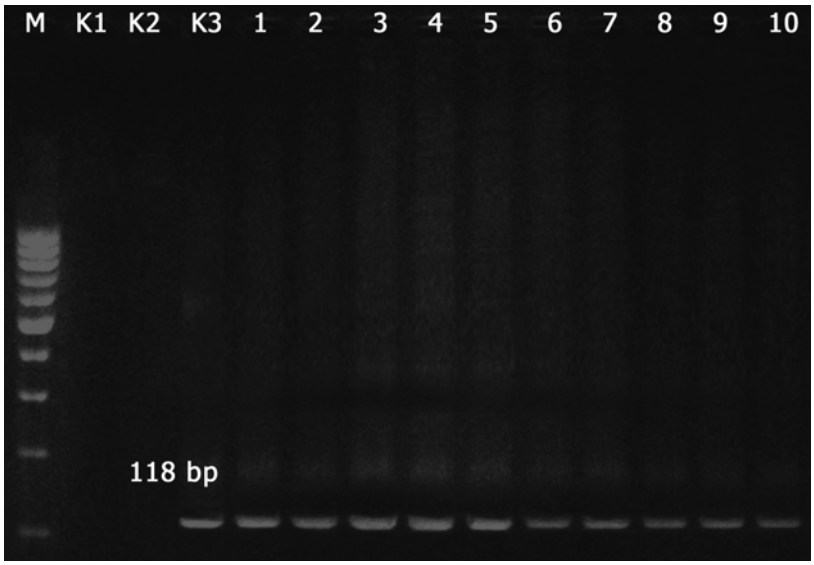

Fig. 3. The amplification of the HA-nos118-f/HA-nos118 primers for NOS terminator using genomic DNA of soybean. M – DNA ladder (100 bp), K1 – Negative control with mastermix without DNA, K2- Negative control with reference material (o% of GMO), K3 – positive control (NOS terminator), lanes 1-10 sample of soybean seeds.

From the literary data it is known that PCR analysis with 35S and NOS primers could detect as low as 0.1% GM structures in GM soybeans and maize [15]. According to the literature, the NOS primer is less sensitive in GMO detection among the studied primers [13, 15]. So, primers for 35S promoter are recommended to be used for detection of GM plants at the first stage of screening of GMOs.

So, the results obtained shows that CTAB method used and optimized for DNA extraction in this study has been reported to yield a higher quality DNA extract. PCR conditions and profiles for identification of these very important genetic structures of GM crops (35S and NOS) important for early identification of GMOs were optimized. There is a need for further event specific identification of GM inserts and their quantification in studied samples.

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