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Analysis of Genetically Modified Maize Allergens by PCR

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Detection of genetically modified (GM) maize allergens is important for food safety assessment and labeling, health protection and consumer information. This study describes novel polymerase chain reaction (PCR) methods for analysis of GM maize allergens. The investigation was focused on the insect resistant GM maize event MON 810. The set of certified reference materials consisting of 0-5% MON810 were used for the optimization of the PCR systems. New oligonucleotide primers were designed for the genes of two important food allergens, such as maize Zea m 8 (chitinase) as well as GMO-specific Cry1Ab delta-endotoxin expressed in the insect resistant GMOs. The agarose gel electrophoresis revealed that PCR method using primers Zea8m130f/ Zea8m130r enables an effective analysis of maize allergen Zea m 8 whereas PCR method with primers Cry102f/Cry102f allows accurate detection of GMO allergen Cry1Ab delta-endotoxin. Moreover, this method may be applied for screening of insect-resistant GMOs with sensitivity of 0.5%. *© 2022 Bull. Georg. Natl. Acad. Sci.*

Allergen detection, maize chitinase, Bt crops, Cry1Ab gene, polymerase chain reaction

Maize *(Zea mays* L.*)*, also called corn, has great significance for food industry and nutrition worldwide and especially in Georgia. It is widely consumed as raw material, processed product, additive and ingredient in food and feed production. Due to the widespread corn derived products, it is one of the important sources of food allergy [1]. Moreover, maize allergy can cause severe illness. In the recent years the special attention is paid to the allergenicity of genetically modified (GM) maize because it is among two major transgenic crops distributed worldwide [2]. Transgenic crops with improved agronomic characteristics and beneficial nutritional traits were produced by modern agricultural biotechnology. Cloning of new genes from other organisms into plant genome resulted in the expression of new proteins which are responsible for new traits of genetically modified organisms (GMOs) [3]. However new proteins are considered as potential allergens and therefore

allergenicity of GM foods causes consumer high interest [4]. In order to assess safety of GM foods the both plant-species specific and GMO-specific allergens should be evaluated according to the international consensus guideline outlined by the Codex Alimentarius Commission [5]. In addition, the European Union (EU) and Georgia have implemented strict legislations and mandatory rules for labeling food and feed containing GMOs or products derived thereof above a threshold of 0.9% with a requirement for the traceability of the GMO in the food and feed chains [6, 7]. The monitoring system requires reliable methods for GMO allergen detection. Moreover, currently there is no cure for food allergy, and allergic individuals must avoid foods containing their allergen. Therefore, diagnostic tests and reliable analysis of GM food allergens is in urgent need.

Allergic reactions are caused by special proteins. Characterization and reliable detection of allergenic proteins and their genes is crucial for the safety assessment of food products. The maize allergen proteins are classified into 20 different families, displaying diverse structures and functions, among them are the panallergen profilin, the Lipid Transfer Proteins (LTPs) and chitinase [1,8]. Plants contain many chitinases which participate in defense against fungi and other pathogens. This may constitute a large problem, from an allergological point of view, because they could be over expressed or used as transgenic molecules to enhance plant resistance to fungi and pathogens. The maize chitinase protein (ChiA) is involved in maize defense against ear rot. The recombinant maize chitinase, rChiA, was characterized by immunological and activity analysis, together with structural modeling [9]. Seven maize chitinase genes were identified responsible for resistance to aflatoxin and A. flavus infection and the suitable SNP and SSR markers were developed [10]. However, the available data indicate that the biochemical and molecular characterization and analyisis of food allergenic chitinases have been only partially carried out. Moreover, their presence in common allergen databases is not complete.

GM maize belongs to the Bt crops, which are resistant to insects. They are produced by the introduction of the *Cry1Ab* gene from the agrobacterium *Bacillus thuringiensis* (Bt) into the maize genome. The *Cry1Ab* gene encodes the insecticidal protein delta-endotoxin Cry1Ab. There are controversial data about safety of Bt crops [2-4]. Therefore, accurate diagnostic methods for GM maize allergens constitute a field of great interest.

Two main approaches applied for allergen detection are the protein based enzyme-linked immunosorbent assay (ELISA) and DNA based polymerase chain reaction (PCR). Each of them has advantages and limitations [11]. PCR technology allows accurate detection of ingredients in foodstuffs because DNA remains rather stable molecule than protein during food processing [12, 13]. PCR-based allergen methods target the genes encoding allergenic proteins. The expression of 5 maize allergens such as Zea m14, Zea m25, Zea m27kD, 50kD Zein and trypsin inhibitor were evaluated and compared using quantitative real time RT-PCR technique in MON 810 vs. its non-transgenic counterpart [14]. Despite the existing analytical methods, the selection of a suitable tool for allergen detection is challenging. There is a lack of techniques for accurate and fast testing of food allergens.

In this study we developed novel PCR methods for reliable detection of important maize allergen Zea m 8 (chitinase) as well as GMO-specific potential allergen Cry1Ab delta-endotoxin expressed in the insect resistant GMOs. Moreover, maize allergen Zea m 8 was investigated by PCR for the first time.

Materials and Methods

Plant material. The seeds of maize (*Zea mays var. indentata*) were purchased at local markets in

Tbilisi (Georgia). The genetically modified maize certified reference materials (CRMs) were used as GMO standards for development and optimization of PCR methods. Maize GMO Standard (ERM-BF-413) set containing 0, 0.1%, 0.5%, 1%, 2% and 5% MON 810 were purchased commercially (Fluka, Biochemika).

DNA extraction. The maize seeds were ground by electric grinder (Siemens, Munich, Germany) to obtain flour. The certified reference materials were obtained in dried powder form and used directly. DNeasy plant mini kit (Qiagen) was used for DNA extraction. Genomic DNAs were isolated and purified from 50 mg of the powdered samples according to the Qiagen protocol. The quality and amount of the extracted DNAs were evaluated by agarose gel electrophoresis. The aliquots of 5 µl from each sample were analysed on a 1% agarose gel.

Oligonucleotide Primers. The available literature, GenBank and allergen databases were screened in order to identify important allergens and corresponding genes sequences for GM maize (MON810). We selected maize common allergen Zea m 8 - Zea mays chitinase (chiA) gene and Cry1Ab delta-endotoxin (*cry1Ab*) gene, cloned in the insect resistant GMOs, such as maize MON810. The new oligonucleotide primer pairs for each gene were designed using bioinformatics tools Primer-BLAST and PrimerQuest tool (https: //eu.idtdna.com/PrimerQuest). Table 1 shows oligonucleotide primers used in this study. The oligonucleotides were synthesized and purified by Eurofins Genomics. The dried powder of the oligos were diluted to a final concentration of 5 µM with bi-distilled water and stored at -20^{0}C until use.

PCR analysis. All PCR analyses were performed with a thermal cycler Techne TC -412. The amplification reactions were carried out in a final volume of 25 µl using using 1.25 U Taq DNA polymerase with standard Taq Buffer (New England BioLabs), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Deoxynucleotide solution mix, New England BioLabs), 1 µl (60-70 ng) of genomic DNA, and 0.5 µM of each primer. The PCR cycling profile for primers Zea8m130f/Zea8m130r was as follows: 95°C initial denaturing for 3 min, followed by 40 cycles of 95°C denaturing for 30 s, 60°C annealing for 30 s, 72°C extension for 35 s; 72°C final extension for 5 min. The PCR with GMO-specific primers Cry102f/Cry102r was carried out in the following conditions: denaturing at 95°C for 3 min, 35 cycles of 30s at 95°C, 30s at 60°C, 60s at 72°C; final extension at 72°C for 5 min. The amplification products were analyzed by electro-phoresis in 2.0% agarose gels containing 1µg/ml of Ethidium Bromide (EtBr).

Table 1. Oligonucleotide primers used in PCR

Target	Primers	Sequence	Amplicon length (bp)
Zea m 8	Zea8m130f Zea8m130r	GAACGTGGCTAACGTGGTCA GAAGCCCGGGTACTTGTTGA	130
cry1Ab	Cry102f Cry102r	CCAGAAGATCGATGAGTCCAAG TTGTAGCGGATCAGGTAAATCTC	102

Results and Discussion

Maize MON 810 is an only GM event approved for direct use and processing in food and feed production in the European Union, USA, Japan and several other countries. Correspondingly, allergenicity of MON 810 is of particular interest. In this study two important allergens genes were investigated, namely maize allergen Zea m 8 - Zea

mays chitinase (chiA) gene and Cry1Ab delta-endotoxin gene (*cry1Ab*), introduced in the insect resistant GM maize MON810. The analytical procedure includes several sequential steps, such as design of PCR primers; genomic DNA extraction; development and optimization of PCR methods; analysis of genomic DNAs and PCR products by gel electrophoresis, interpreting results.

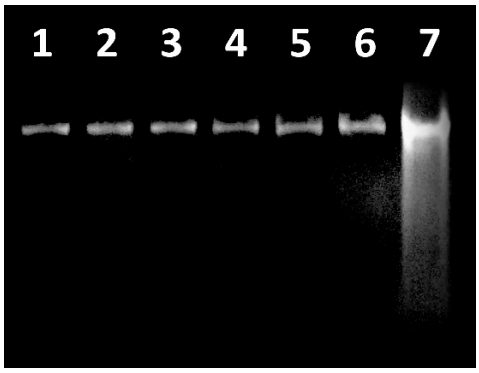

Fig. 1. Agarose gel electrophoresis of the Genomic DNAs. Samples: lanes 1-6. CRMs of maize MON 810 set: 0, 0.1, 0.5, 1, 2, 5%; lane 7. maize flour.

The assessment of the genomic DNAs from CRMs of GM maize MON 810 and the maize flour by agarose gel electrophoresis exhibited high purity and integrity of the samples (Fig. 1) and suggested that they may be applied in PCR analysis successfully. However the maize flour (Fig. 1, lane 7) gave higher amount of the genomic DNA than CRMs (Fig. 1, lanes 1-6).

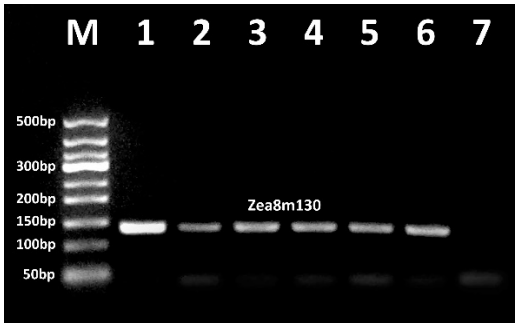

Fig. 2. PCR amplification of maize allergen Zea m 8 gene using primers Zea8m130f/ Zea8m130r (lanes 1-7). Samples: lane 1. maize flour; lanes 2-6. CRMs of maize MON 810 set: 0, 0.1, 1, 2, 5%; lane 7, water. M. GelPilot 50 bp ladder (Qiagen).

The primer pairs were designed for each allergen gene, such as chitinase (chiA) and Cry1Ab. The efficiency of primer pairs was separately tested to amplify their targets by uniplex conventional PCR.

Fig. 2 shows the gel electrophoresis of the amplification products generated by PCR primers Zea8m130f/ Zea8m130r targeted to the maize chitinase (Zea m 8) gene. The PCR produced one main amplicon of 130 bp in size for all DNA samples as was expected (Fig. 2. Lanes 1-6). Moreover maize flour produced more intensive PCR band than CRMs that corresponds to the genomic DNA amounts shown in Fig. 1. While water negative control did not give any PCR band (Fig. 2 lane 7) that confirms purity of the experimental conditions. The outcomes indicate that primers Zea8m130f/Zea8m130r are useful for accurate detection of allergen chitinase gene.

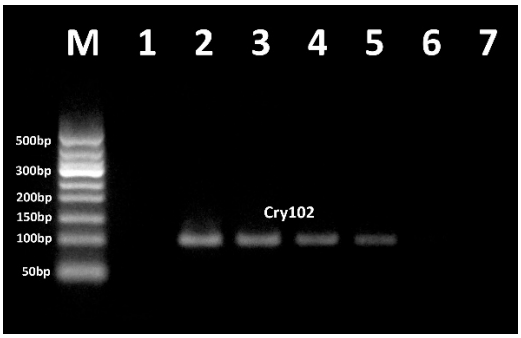

Fig. 3. PCR amplification of GM maize allergen cry1Ab gene using primers Cry102f / Cry102r. Samples: lane 1, water, lanes 2–7. CRMs of maize MON 810 set: 5, 2, 1, 0.5, 0.1, 0 %. M. GelPilot 50 bp ladder (Qiagen).

The GMO-specific primer pair Cry102f/Cry102r was used to amplify Cry1Ab delta-endotoxin (cry1Ab) gene introduced in MON810 by genetic engineering. Using of GMO standard set containing 0%, 0.1%, 0.5%, 1%, 2% and 5% MON810 enabled assessment of the reliability and the specificity of the PCR method. As shown in Fig. 3, the primer pair Cry102f/Cry102r produced single appropriate amplicon for all MON810 DNA templates except of 0% and 0.1% MON810 (Fig. 3 lanes 6, 7). In particular, primers Cry102f and Cry102r gave 102

bp PCR product for 0.5%, 1%, 2% and 5% MON810 templates as was expected. This indicates sufficient sensitivity of the PCR method for detection of 0.5% Bt maize. Moreover, the intensity of the DNA band increased correspondingly to the increased amount of transgenic material in the samples. The absence of any amplification product in water and 0% MON810 (blank) negative controls (Fig. 3, lanes 1 and 7) indicated high specificity of the method and purity of the experiment. The obtained results suggested that PCR method applied in this study may be used for analysis of GMO-specific putative allergen Cry1Ab delta-endotoxin.

Conclusion

New conventional PCR methods were developed and optimized for detection of allergens in genetically modified maize. The PCR using primers Zea8m130f/ Zea8m130r enables reliable analysis of maize allergen chitinase (Zea m 8). The outcomes of this study demonstrated that the primer pair Cry102f/Cry102r is useful for accurate detection of GMO-specific putative allergen Cry1Ab delta-endotoxin. Moreover, this method may be used for screening of insect-resistant GMOs with sensitivity of 0.5%. The results obtained suggested that the PCR methods developed in this study can be successfully applied to food allergen analysis.

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ბიოტექნოლოგია

პჯრ მეთოდების შემუშავება გენეტიკურად მოდიფიცირებული სიმინდის ალერგენების ანალიზისათვის

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(წარმოდგენილია აკადემიის წევრის დ. მიქელაძის მიერ)

გენეტიკურად მოდიფიცირებული სიმინდის ალერგენების დეტექცია მნიშვნელოვანია სურსათის უვნებლობის შეფასებისა და სწორი ეტიკეტირებისათვის, ჯანმრთელობის დაცვისა და მომხმარებელთა ინფორმირებისათვის. კვლევაში აღწერილია ახალი პოლიმერაზული ჯაჭვური რეაქციის (პჯრ) მეთოდები გმ სიმინდის ალერგენების ანალიზისათვის. კვლევა ფოკუსირებულია მწერების მიმართ რეზისტენტული გმ სიმინდის ხაზზე MON810. შესწავლილ იქნა საკვების ორი მნიშვნელოვანი ალერგენი, როგორიცაა სიმინდის ჩიტინაზა Zea m 8 და გმო-ს სპეციფიკური დელტა ენდოტოქსინი Cry1Ab, რომელიც ექსპრესირდება მწერების მიმართ რეზისტენტულ ტრანსგენურ მცენარეებში. პჯრ-სისტემების ოპტიმიზაცია მოხდა 0-5% MON810-ის სერტიფიცირებული საკონტროლო მასალების გამოყენებით. გენომური დნმ-ის ექსტრაქცია ჩატარდა ქიაგენის DNeasy მცენარის მინი კრებულით. პჯრ-ანალიზის დროს გამოყენებულ იქნა ჩიტინაზას გენის სპეციფიკური და Cry1Ab გენის სპეციფიკური ახალი პრაიმერები, რომელთა დიზაინი შესრულდა ბიოინფორმატიკის ხერხებით. პჯრ-პროდუქტების შეფასება მოხდა აგაროზას გელზე ელექტროფორეზით. კვლევის შედეგად აღმოჩნდა რომ Zea8m130f/Zea8m130r პრაიმერების გამოყენებით პჯრ-ით შესაძლებელია ალერგენი ჩიტინაზას ეფექტური ანალიზი, ხოლო პჯრ მეთოდი Cry98f/ Cry98r პრაიმერები ახდენს გმო-ს ალერგენის დელტა ენდოტოქსინის ზუსტ დეტექციას. ამასთან, ეს მეთოდი შეიძლება გამოყენებულ იქნეს მწერების მიმართ რეზისტენტული გმო-ების სკრინინგისათვის 0,5% სიზუსტით.

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