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A quantitative competitor PCR assay to detect genetically modified roundup-ready soybeans in commercially sold foods in Iran

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ABSTRACT

Due to ever-increasing global diffusion and related socio-economic implications, the detection of genetically modified organisms (GMOs) is very important. In this study, we design a plasmid containing two genes in mutated form as construct-specific (*cp4 epsp*s) and event-specific (*pd35S*). It is applied for quantitative-competitor (QC) PCR as a simple and reliable method for the detection of GM food. This plasmid is calibrated with the external standard of the IRMM (The Institute for Reference Materials and Measurements), and then used to detect the presence of *cp4 epsps* and *pd35S* sequences in five foods derived from GM round-ready (RR) soya sold commercially in Iran. The results indicate the presence of GM RR soya in these products, quantitatively. In order to detect whether they contain more or less than 1% RR soya DNA, QC-PCR with various amounts of DNA plasmids as a standard was performed. The results show that this plasmid can be used as a calibrator for 1% *cp4 epsps* and *pd35S*, and that it can also be applied instead of 1% IRMM genomics.

Keywords: genetically modified organisms, quantitative competitor PCR, roundup ready soybean, transgenic food.

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Introduction

Genetically modified foods are becoming increasingly used worldwide. Currently, more than 50 GM crops have been approved for commercial production in the US. Among them, soybean, maize, cotton and rapeseed are the most common (http://www.transgen.de). Along with the increasing market for transgenic crops, consumers increasingly demand the testing of food products to certify non-GMO food products. The labelling of food products containing more than 0.9% GM material is required by the EU. Hence, a reliable method for GMO detection is necessary (1, 2). The preferred technique for GM food detection is the polymerase chain reaction (PCR) or its derivative, real-time PCR (3, 4). The GM product analysis primers are designed based on sequences of regulatory and structural genes on transgenes (5, 6). Because of the importance of the bioethics and biosafety aspects of GM crops in relation to human health, labelling of GMOs is required in food laws (7, 8). The major brand among the GM soybeans cultivated in the US is Roundup® Ready (RR) (9). The RR soybean was introduced into agricultural production in 1996. It was genetically modified to increase its tolerance to the Roundup® herbicide (10). The structure of the gene of the inserted recombination gene in the RR soybean is *cp4 epsps* (5-enolpyruvylshikimate-3-phosphate synthase from the *Agrobacterium tumefaciens* strain CP4). This foreign gene is regulated by a constitutive cauliflower mosaic virus (CaMV) 35S promoter and the *A. tumefaciens* nopaline synthase (NOS) terminator (11). Here, we describe our attempts to identify an efficient approach to qualify and quantify the RR soybean using a QC-PCR-based protocol (12, 13). In this study, we designed a novel plasmid that has a part of *cp4 epsps* as a gene–specific and *pd35S* as an event-specific of RR soyabean. Both genes are mutated by adding a 35 bp fragment.

Materials and Methods

0%, 1%, 5% or 2% GM-soya standard were obtained from Fluka Co. (Buchs SG, Switzerland). Other commercially available soya products (soy sausage, soya flour, soya sweet and bean soy) were purchased on the open market in Iran.

DNA Extraction

DNA was extracted according to the Swiss food manual (14, 15). Approximately 100 mg of homogenized sausage samples were mixed with 500 µL CTAB buffer [50 mM CTAB, 1.4 M NaCl, 100 mM Tris-HCl (pH= 8), and 20 mM EDTA] and incubated at 65 °C for 20 min. Next, 20 µl Proteinase K (20 mg/ ml) was added, mixed and incubated at 65 °C for 90 min. Samples were deproteinized by 520 µl chloroform/isoamylic alcohol (24:1), vortexed and centrifuged for 10 min at 16.000 g. The supernatant was transferred into a new 1.5 ml tube. Nucleic acids were precipitated with one volume of isopropanol and 0.5 volume of 7.5 M ammonium acetate, and centrifuged for 10 minutes at 16.000 g. The supernatant was discarded and the pellet washed once with 500 µL 70% ethanol. The pellet was then dried at room temperature for approximately 10 minutes, and then re-suspended in 50 µl water and stored at –20 °C until use. The extracted DNA was quantitatively standardized to 100 µg/ml and monitored by O.D. 260/280 nm prior to proceeding with the necessary testing for this study.

Construction of the Standard Plasmid

Table 1 lists five sets of primers designed for

this study. For amplification of the mutated form of *cp4 epsps*, PCR was carried out in a 25 µl volume containing a 1X PCR buffer, 1.5 mM $MgCl_2$, a 2 mM dNTP mixture, a 0.5 unit *Taq* DNA polymerase, 20 ng template DNA, and 1 pmol of each primer (RRS/QRRS and RRw/QRRw), which were used individually in PCR I and II. Thermocycling conditions started with an initial denaturation step at 95 ºC for 5 min, followed by 35 additional cycles at 95 ºC for 1 min, an annealing temperature of 70 ºC for 1 min, and an extension at 72 ºC for 1 min. The PCR products were electrophoresed on a 1.5% agarose gel, and the DNA was purified using the DNA Extraction Kit (Bioneer). Two intermediate fragments of Q*cp4 epsp*s for the mutated form of *cp4 epsp*s were fused together by two-step SOE-PCR; in the first step, 56 ºC was established as the annealing temperature for 15 cycles; in step two, SOE-PCR was continued by adding 1.5 pmol of RRs/RRw primers, which annealed at 62 ºC for another 30 cycles. The reaction was set up with 0.2 mM dNTP, 1 mM $MgCl_2$, a 1X PCR buffer, and a 1 unit *Taq* DNA polymerase. For the production of mutated *pd35S*, a polymerase chain reaction with pd35S F/Qpd35S R and pd35S R /Qpd35S F primers was performed. Two intermediates of *Qpd35S* were fused by SOE-PCR using pd35S F/pd35S R, as previously mentioned. After the separation of *Qcp4 epsps* and *Qpd35S*, inserting additional fragments at 5' *cp4 epsps* and *Qpd35S*, PCR was performed using Q1RRW-RRS and p35S F/Q1pd35S R primers, respectively. Finally, two fragments were amplified by RRS/Pd35S F primers (Fig. 1).

Table 1. Information of primers used in this study

	Gene	Sequence (5' - 3')	Amplicon (bp)
Mutated *cp4 epsps* (Q*cp4 epsps*) 165 bp	*cp4 epsps*	RRs: TGGCAAAATTCACTGGCATA RRw: CATTCGAGCTTCTTCCGAAC	130
	Fragment 1 for SOEing	RRs: TGGCAAAATTCACTGGCATA QRRs:ATGGTCGGTTACGTACGGATTCATGCACTAGCTGCCGAAG AGATGAGATCGGGAG	77
	Fragment 2 for SOEing	RRw: CATTCGAGCTTCTTCCGAAC QRRW: CAGCTAGTGCATGAATCCGTACGTAACCGTCCATGCCTC AACTCTCTCCGAGG	74
Mutated *pd35S* (*Qpd35S*) 150 bp	*pd35S*	Pd35S R: TTGCTTTGAAGACGTGGTTG P35S F: AGGGAACCCAAATGGAAAAG	115
	Fragment 1 for SOEing	Pd35S F: AGGGAACCCAAATGGAAAAG Qpd35S R: GCAGCTAGTGCATGAATCCGTACGTAACCGTCCATTTC GCAAGACCCTTCCTCTA	83
	Fragment 2 for SOEing	Pd35S R: TTGCTTTGAAGACGTGGTTG Qpd35S F: ATGGTCGGTTACGTACGGATTCATGCACTAGCTGCTGGG GTTTATGGAAATTGGA	84
Mutated *cp4 epsps* + Mutated *pd35S* 355 bp	Fragment 1 for SOEing	Q1RRW: CGTACCTAATGCGATCATTCCATATCGACGATTACG CATTCGAGCTTCTTCCGAAC- RRs: TGGCAAAATTCACTGGCATA	185
	Fragment 2 for SOEing	Pd35S F: AGGGAACCCAAATGGAAAAG Q1Pd35S R: CGTAATCGTCGACGGGTATGGATCGCATTAGGTAGC TTGCTTTGAAGACGTGGTTG	201
	Q*cp4 epsps*+ Qp35S	RRS: TGGCAAAATTCACTGGCATA P35S R: TTGCTTTGAAGACGTGGTTG	335

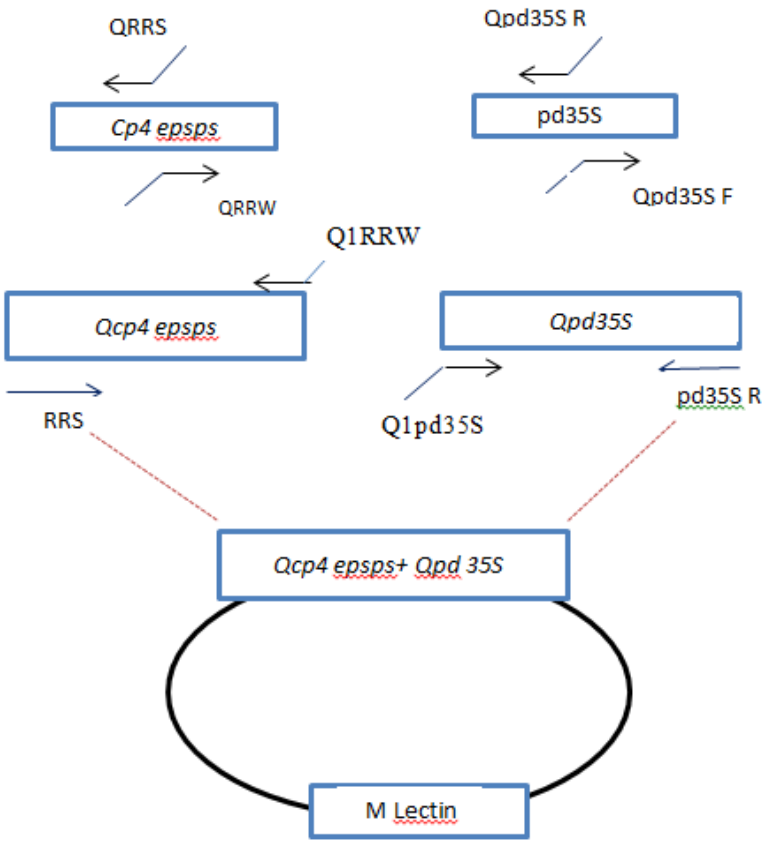

Figure 1. Schematic representation of the pGEM-*Qcp4 epsps+ Qpd 35S.* The construct contains the mutated *cp4 epsps (Qcp4 epsps)* and mutated *pd 35S* (*Qpd 35S*)

The PCR products were purified and inserted into the pGEM-T easy vector (Promega). The resulting plasmid with the two targets of RR soya was named PGEM-002, and after sequencing was used as an internal standard in QC-PCR. The copy number of the mutant plasmid was calculated based on the concentration of the plasmid using the *DS Copy Number Analyzer* online software found at www.uri.edu/research/gsc/ resources/ cndna.html and the plasmid was linearized by the *sca*I enzyme.

Calibration of QC-PCR for Two Targets (*cp4 epsps* and *pd35S*)

It is important to calibrate the sensitive and specific plasmid for the QC-PCR method. Hence, for the calibration of this plasmid for both target genes (*cp4 epsps* and *pd35S*), around 500 *ng* of the DNA template containing different proportions of GMO DNA (100, 50, 10, 2, 1, 0.5, 0.1, and 0%) was used. In QC-PCR, the amount of soybean DNA with two-targets is determined in comparison to a known amount of standard DNA. At the point where the concentration of both sequences is the same, the band intensities will be equal. Visual assessment of the band intensities or digital analysis of the gel images and the generation of a regression line can be used to determine this point (16, 17). The intensities of native and mutated

products were directly measured using an image program (http://rsb.info.nih.gov/ij) and the total lab and SAS (Statistical Analysis System, version 7.12; SAS, Cary, N.C.) software. The amplification products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

Quantification of 1% GM Content in a Food Sample for *cp4 epsps* and *pd35S*

DNA extraction was performed for the five commercial food products. Next, QC-PCR for *cp4 epsps* using a calibrated plasmid of 1% RR soybean (68 pg) was applied. To accomplish the best optimization of this plasmid around the RR soybean food at the event level (GTS 40-3-2), the commercial samples were also checked with *pd35S* primers using the QC-PCR method.

Results

Construction of the Standard Plasmid for QC-PCR

After amplification of the mutated forms of both target DNAs (*cp4 epsps* and *pd35S*), two large fragments were fused together. Standard DNA which was 355 bp in length - was made and inserted into the pGEM-T easy vector (Promega). The copy number of this standard plasmid was calculated based on the concentration of the plasmid as 5.4×10^4 copies/µl.

Standardization of the Novel Plasmid

We calibrated the plasmid for cp4 *epsps* relative to the DNA (500 *ng*) containing different proportions of GMO DNA (100, 50, 10, 2, 1, 0.5, 0.1, and 0%) as a template in 6 µl (Fig. 2). We also calibrated the plasmid for *pd35S* relative to the same samples of IRMM in 2 µl (Fig. 3). Therefore, this calibrated plasmid could be used for the analysis of any commercial foods with 1% GMO. For the analysis of five commercial foods, a tenfold amount of the standard DNA plasmid was used in the QC-PCR. The result showed that the GMO content of *cp4 epsps* for three out-of-five commercial products was lower than 1% (soy sauce, soya sausage, soya flour). One sample (soy candy) contained 1% and one sample contained more than 1% (soybean) (Fig. 4 A). Checking the samples for *pd35S* showed that soya sauce has significantly less than 1%, and that it could not be detected by this procedure, but that soya sweet, bean soy, soya sausage and soya flour have more than 1% GM material GMO (Fig. 4 B). Among these commercial foods sold in Iran, soya sauce and soya flour are the most processed foods and their DNA materials are destroyed. Therefore, the detection of these foods by PCR was difficult. Our results demonstrated that this calibrated plasmid is sensitive and specific to the detection of gene-specific (*cp4 epsps*) and event-specific (*pd35S*) levels. Soybean and soya candy - for two levels - had more than 1% RR soy. It is difficult to detect the *pd35S* level in processed foods, such as soya flour, because the DNA has been destroyed. However, the calibrated plasmid was more sensitive and specific for the detection of the *pd35S* level of soya flour. We found that it could not be used for the detection of the *pd35S* level of soya sauce because of the very high level of processing required to manufacture soya sauce. Therefore, this plasmid could be used as a sensitive detector for processed food for event-specific RR soy, while for a highly processed foods such as soya sauce it could not be trusted.

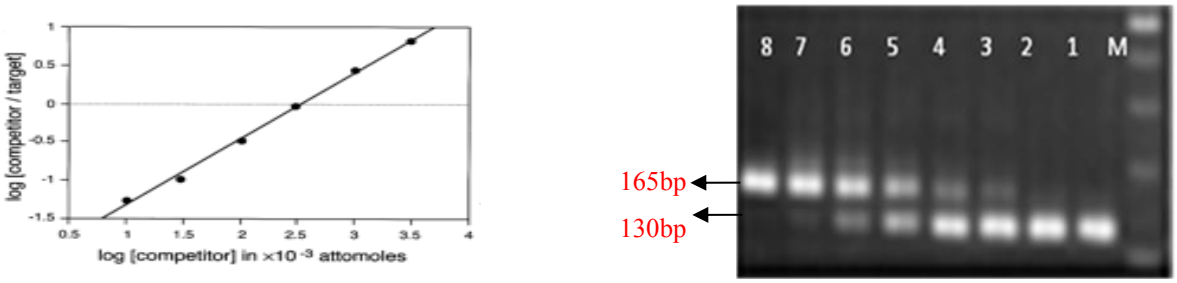

Figure 2. QC-PCR of *cp4 epsps* in target DNA in the presence of a competitor. Right: The QC-PCR products of different proportions of IRMM DNA (130 bp) and the fixed amount of the competitor (165 bp) separated on the 2% agarose gel. Lane M, 100 bp molecular ladder (Fermentas); lanes 1-7, in the same order: 100%, 50%, 25%, 5%, 5, 1%, 0.1%, and 0.5% GMO DNA; lane 8: pGEM TA easy vector as a positive control. Left: Calibration curve of *cp4 epsps* for 0.1% RR soya DNA based on the intensities of the DNA bands in the gel on the right.

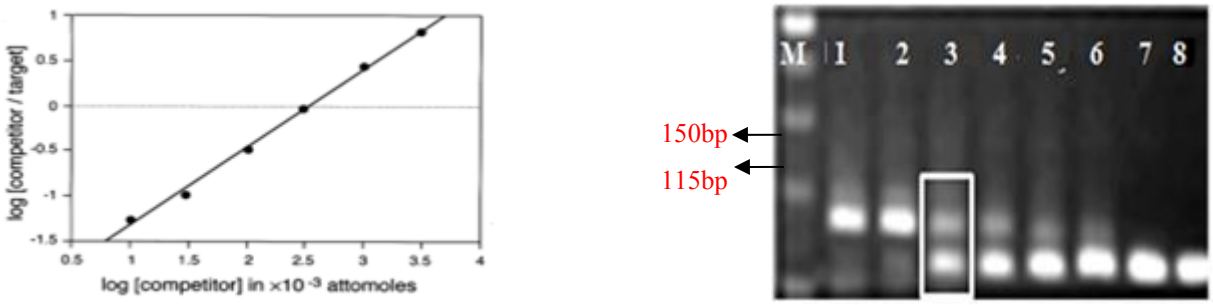

Figure 3. QC-PCR of *Pd35S* in the target DNA in the presence of a competitor. Right: The QC-PCR products of the co-amplified different proportions of IRMM DNA (115 bp) and the fixed amount of the competitor (150 bp) separated on the 2% agarose gel. Lane M, 100 bp molecular ladder (Fermentas); lanes 1-7, in the same order: 0%, 0.1%, 1%, 5%, 25%, 50%, and 100% GMO DNA; lane 8: pGEM TA easy vector as a positive control. Left: Calibration curve of *Pd35S* for 0.1% RR soya DNA based on intensities of DNA bands in the gel to the right.

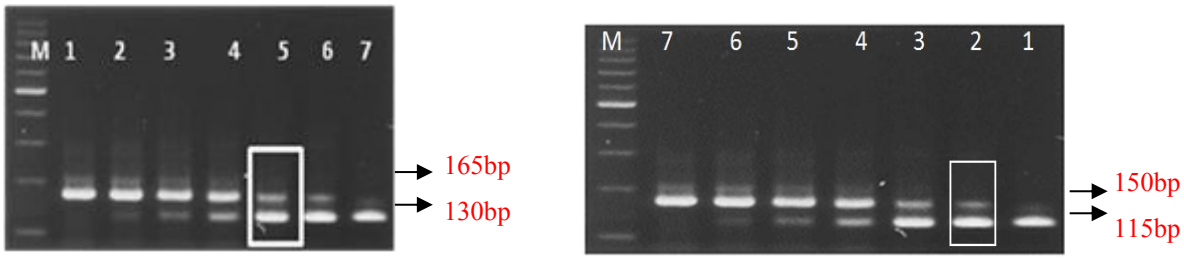

Figure 4. Five commercial food samples were tested for the detection of *cp4 epsps* (A) and *Pd35S* (B) using a plasmid calibrated to 1% RR soy. A: Lane M, 100 bp molecular ladder (Fermentas); lane 1, 68 pg of pGEM-002 as a positive control (equal to 1% RR soya DNA); lane 2, souse soya sausage; lane 3, soya sausage; lane 4, soya flour; lane 5, soya sweet; lane 6, bean soy; lane 7, 1% IRMM. B: Lane M, 100 bp ladder (Fermentas); lane 1, 1% RR soya DNA (99 fg); lane 2, 1% RR soya DNA and internal standard (99 fg); lane 3, bean soy; lane 4, soya sweet; lane 5, soya flour; lane 6, soya sausage; lane 7, positive control.

Discussion

The aim of this study was the detection of RR soybean at the construct–specific and event-level in soybean-derived samples obtained in Iran. Although Iran is not currently a member of the EU, it has adopted their biosafety and bioethics standards. We performed qualitative and quantitative testing of GMOs using detection methods with the assistance of a community reference laboratory.

The bulk of soybean foodstuffs in Iran are transgenic, so the labelling of seeds and RR foods is necessary. We performed RR soybean analysis in this study for five commercial Iranian foods. For the detection of specific GMOs, PCR methods are accepted as being reliable and the most sensitive (16). In this study, we used QC-PCR systems by designing a new plasmid for two targets of *cp4 epsps* (gene-specific) and *pd35S* (event-specific).

QC-PCR was first described in 1990 (18). Studer et al. (1998) used QC-PCR for the detection and quantification of GMOs first (19), but we constructed the internal DNA standard with different primer sequences. These primers were designed for a part of *cp4 epsps* as a gene–specific and *pd35S* as an event-specific of RR soybeans. Our protocol and plasmid can be used for labelling GMO in maize and soybean. The greatest advantage of designing this plasmid is that it can be used as a calibrator plasmid instead of as an IRMM genomic standard. Because this plasmid contains two transgenes that are most common in commercial transgenic foods, it can easily be used for the detection of multiplex transgenic foods.

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