Optimization of DNA-based screening methods for genetically modified organisms

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The worldwide distribution of genetically engineered plants has generated an urgent need for reliable and efficient methods of monitoring genetically modified organisms (GMOs) in each country. In this study, certified reference materials in the form of dried powders containing 0–5% Roundup Ready soybean and maize MON-810 were used for optimization and validation of GMO screening methods. Genomic DNAs were isolated using a Qiagen DNeasy plant minikit. The high amplification quality of extracted DNAs was revealed by a plant-specific polymerase chain reaction (PCR) suitable for the detection of chloroplast genome conserved sequences. The conventional end-point polymerase chain reactions specific to the 35S promoter from cauliflower mosaic virus and the NOS terminator from *Agrobacterium tumefaciens* confirmed the presence of transgenic material in the GMO-containing reference materials with high specificity and a sensitivity of 0.1% GMO, while negative water and non-GM samples did not generate any amplification signal. The results obtained indicate that the DNA-based analytical procedure described in this paper allows a reliable and sensitive qualitative detection of GMOs that corresponds to European regulatory requirements.

Keywords: DNA analysis, NOS terminator, qualitative detection, transgenic products, 35S promoter

1. INTRODUCTION

Recent advances in plant biotechnology have resulted in the development of a vast number of transgenic crops and substantially increased the share of genetically modified organisms (GMOs) in seeds, grains, food and feed distributed and commercialized worldwide. Correspondingly, the influence of GMOs on the environment, human and animal health has increased every year during the last decade, thus causing growing public concern over safety, especially since the long-term consequences of GMOs are unknown [1]. Due to consumer pressure, in many countries the production and distribution of GMOs are regulated by legislation. In the EU, food products containing GMO ingredients above a 0.9% threshold should be labelled accordingly [2, 3]. The regulatory system requires efficient and reliable methods of GMO detection [4, 5]. Since the monitoring of GMOs concerns international trade issues, implementation of GMO detection methods in each country and their validation and harmonization at the international level is an urgent need.

Genetically modified organisms are generated from wild-type plants by recombinant DNA techniques, in particular by introducing new genes employing vectors with commonly used regulatory elements such as 35S promoter from cauliflower mosaic virus (CMV) and the NOS terminator from *Agrobacterium tumefaciens*. These regulatory sequences do not naturally occur in the plant genomes (with the exception of plants naturally infected with the CMV), correspondingly their detection indicates the presence of transgenic material [7].

DNA-based analysis is the most widespread, reliable and specific method for the identification of genetically modified organisms [6–8]. The analytical procedure includes qualitative detection (screening), identification and quantification of GMOs. Qualitative detection is the first critical step in GMO analysis, because only positive samples revealed during the screening procedure are subjected to further identification and quantitative analysis. The qualitative detection procedure requires sample preparation and DNA extraction methods as well as different polymerase chain reactions (PCRs) specific to plant species and transgenic DNA sequences. Special interest is accorded to GMO-specific PCR for screening purposes, which determine whether a sample contains GMO material. Several PCR methods have already been developed and validated [9–12] for GMO screening, however there is a lack of regulatory and analytical coherence [4]. Published data differ in the evaluation of the efficiency and sensitivity of PCR methods [12, 7] and, therefore, new evaluations of GMO screening methods are of special interest. This study describes a simple, reliable and sensitive DNA-based analytical procedure for screening genetically modified organisms to meet EU regulatory demands.

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The DNA-based analysis described in this article includes the following main steps: sample preparation (1); genomic DNA extraction and evaluation (2); plant-specific PCR detection (3); and GMO general screening (4). Three conventional end-point qualitative PCR methods were applied. One of them is useful for the detection of plant-specific sequences and two other methods are applicable for GMO screening based on the detection of transgenic regulatory elements. The methods were optimized on samples of GM and non-GM maize and soybean varieties. A qualitative conventional end-point PCR system was developed in order to verify the presence and amplifiability of plant DNA in the DNA extracts.

In this study, detection of the 35S promoter and NOS terminator was applied to screen genetically modified organisms, because they are present in most of the authorized genetically modified plants, correspondingly use of these regulatory elements as targets allows detection of a vast number of transgenic plants, especially as CaMV 35S is a widely used promoter regulating the expression of new inserted genes in both Roundup Ready soya and MON 810 maize.

2. MATERIALS AND METHODS

Plant material. Soybean and maize certified reference materials in the form of dried powders were used as GMO standards because they are both convenient and the most frequently used for development and validation of GMO detection methods, while Roundup Ready soya and MON 810 maize were chosen as the GM varieties. Certified reference materials (ERM-BF-410) of GM soya bean powder containing 0, 0.1%, 0.5%, 1%, 2% and 5% Roundup Ready and GMO maize standard (ERM-BF-413) containing 0, 0.1%, 0.5%, 1%, 2% and 5% MON 810 were purchased commercially (Fluka, Biochemika).

Genomic DNA extraction. The certified reference materials were used directly. A Qiagen DNeasy plant minikit was chosen as suitable for DNA extraction based on the results of our previous study [13]. Genomic DNAs were isolated and purified from 100 mg of the samples. The quality and amount of the extracted DNAs were evaluated by agarose gel electrophoresis. 5 μL aliquots from each sample were analysed on a 1% agarose gel. Genomic DNAs were extracted from each sample in duplicate and analysed together with negative (pure water) controls.

Oligonucleotide primers. The available literature and GenBank databases were screened in order to identify plant-specific and GMO-specific primer sequences. The PCR primer pairs were selected based on published data [9, 12]. The primers were synthesized and purified by MWG Biotech. They were diluted to a final concentration of 5 μM with bidistilled water and stored at –20 °C until use.

PCR conditions. All PCR analyses were performed using a Techne TC -412 thermal cycler. The PCRs were carried out in a final volume of 25 μL using 0.8 U GoTaq DNA polymerase with 1x green GoTaq reaction buffer (pH 8.5) (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Promega), 0.4 μM of each primer, and 1μL (60–70 ng) of genomic DNA.

The primers plant1 and plant2 specific to conserved sequences of the chloroplast genome were used. The PCR cycling profile for primers plant1/plant2 was as follows: initial single denaturation step at 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min; final extension step at 72 °C for 5 min. Different PCR conditions were tested for optimization of GMO screening methods. Similar PCR conditions were found to be the most suitable conditions for both pairs of GMO-specific primers Cf3/Cr4 and HA-nos118f/HA-nos118r: denaturation for 3 min at 95 °C, 50 cycles of amplification for 25 s at 95 °C, for 35 s at 62 °C, for 45 s at 72 °C; final extension step for 7 min at 72 °C.

Each sample was tested in duplicate or triplicate in every PCR run. At least two independent runs were carried out for each PCR experiment.

Agarose gel electrophoresis. Both the genomic DNAs and PCR products were analysed by agarose gel electrophoresis using 1.0 and 2.0% agarose (Promega) gels for genomic and amplified DNA, respectively. Electrophoresis was in 1x TBE (tris-borate EDTA) buffer containing 1 μg/mL of ethidium bromide (EtBr). After electrophoresis the DNA bands were visualized and photographed by a Sony DSC-S600 digital still camera.

3. RESULTS

Genomic DNA extraction. Evaluation of the DNA extracts by agarose gel electrophoresis revealed high purity and integrity of the samples, and approximately equal intensities of DNA bands for duplicate samples, whereas no signal was visible in the negative control (data not shown).

Plant-specific PCR. Gel electrophoresis of the PCR products revealed as expected one amplicon approximately 450–550 bp in size for all plant DNA templates; maize samples generated a smaller amplicon than soybean samples (Fig. 1). The size was equal and the amount similar for all PCR products of the GM and non-GM soybean samples. This was true also for the size and amount of the PCR products for all of the GM and non-GM maizes, whereas no amplification signal was observed for negative (water) controls. These results

indicate a high specificity of the developed assay for plant-specific detection and high amplification quality of the DNA extracts.

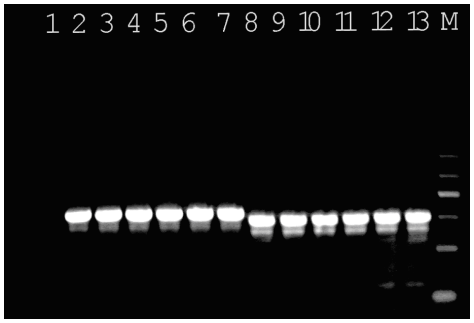

Figure 1. PCR amplification using plant-specific primer pair plant1/plant2 for GM soybean (Roundup Ready) (lanes 2–7) and GM maize (MON 810) (lanes 8–13) certified reference materials: lane 1, negative (water) control; lanes 2 and 8, blank (GMO 0%); lanes 3 and 9, GMO 0.1%; lanes 4 and 10, GMO 0.5%; lanes 5 and 11, GMO 1%; lanes 6 and 12, GMO 2%; lanes 7 and 13, GMO 5%. M, PCR markers 1.2 kb, 900 bp, 700 bp, 500 bp, 300 bp, 100 bp (Qiagen).

Detection of 35S promoter. Qualitative end-point PCR performed by the primer pair Cf3/Cr4 specific for CaMV 35S promoter gave the expected amplicon of size 123 bp when GM soybean and GM maize (including 0.1% GMO) genomic DNAs were used as templates. No PCR fragment was observed for non-GM plant samples and the negative control (Fig. 2). Moreover, the intensity of the DNA band increased corresponding to the increasing amount of transgenic material in the samples.

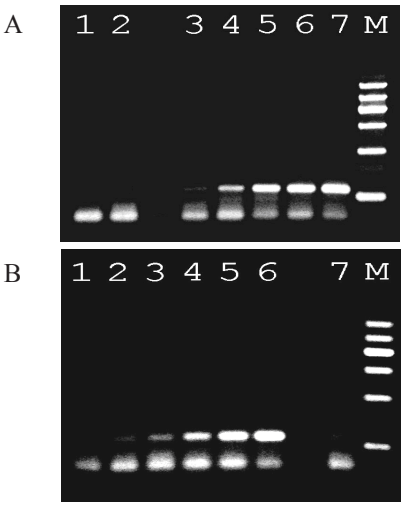

Figure 2. GMO screening by PCR using CaMV 35S promoter-specific primer pair Cf3/Cr4. PCR product is size of 123 bp.
(A), Roundup Ready soy bean: lane 1, negative (water) control; lane 2, blank (GMO 0%); lane 3, GMO 0.1%; lane 4, GMO 0.5%; lane 5, GMO 1%; lane 6, GMO 2%; lane 7, GMO 5%.
(B), MON 810 maize: lane 1, blank (GMO 0%); lane 2, GMO 0.1%; lane 3, GMO 0.5%; lane 4, GMO 1%; lane 5, GMO 2%; lane 6. GMO 5%; lane 7, negative (water) control. M, PCR markers 1.2 kb, 900 bp, 700 bp, 500 bp, 300 bp, 100 bp (Qiagen).

Detection of NOS terminator. The PCR primers HA-nos118f and HA-nos118r were used to detect the NOS terminator present in Roundup Ready soya but not in MON 810 maize. This primer pair gave the expected amplicon of size 118 bp when GM soybean (including 0.1% GMO) DNA was used as a template, however no amplification signal was detected for non-GM soybean DNA and the negative control (Fig. 3). The amount of amplicon increased corresponding to the increasing percentage of GM material. No PCR fragment was observed when maize DNA was used as the template, as expected (data not shown).

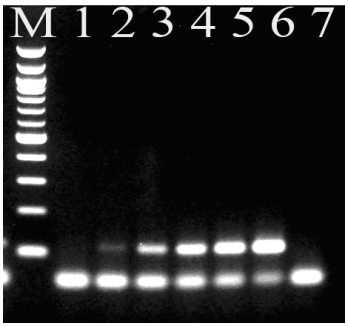

Figure 3. GMO screening by PCR using NOS terminator-specific primer pair HA-nos118f/HA-nos118R of Roundup Ready soy bean. M, 1.5 kb; 1.2 kb; 1.0 kb; 900 bp; 800 bp; 700 bp; 600 bp; 500 bp; 400 bp; 300 bp; 200 bp; 100 bp. Lane 1, blank (GMO 0%); lane 2, GMO 0.1%; lane 3, GMO 0.5%; lane 4, GMO 1%; lane 5, GMO 2%; lane 6, GMO 5%; lane 7, negative (water) control.

At least three independent experiments were carried out for each analysis. The repeated experiments provided reproducible results underscoring the reliability of the methods presented in this study.

4. DISCUSSION AND CONCLUSIONS

Qualitative PCR screening methods are the first choice for most analytical laboratories involved in the detection of GMOs in routine applications [14]. Taken together, the obtained results indicate that the DNA-based methods applied in this study are suitable for the analysis of different plant matrices such as seeds, grains and flour. Genomic DNAs from these samples extracted by the Qiagen DNeasy plant minikit produced an amount sufficient and of appropriate purity and amplifiable quality for PCR testing. The primers plant1 and plant2 specific to conserved sequences from the chloroplast genome gave positive signals for all plant DNA, whereas primer pair Cf3/Cr4 specific for CaMV 35S promoter generated an amplified product for all transgenic samples. The primers NOS1 and NOS2 specific for the NOS terminator produced amplicons only for the GM soybean samples. Reactions serving as negative and

positive controls showed high specificity and accuracy of the employed PCR methods. Therefore, the PCR systems applied in this study using primer pairs Cf3/Cr4 and HA-nos118f /HA-nos118r are suitable for the reliable and efficient detection of CaMV 35S promoter and NOS terminator, respectively. These GMO-specific methods revealed detection sensitivity of at least 0.1% of GM material. This value is higher than the GMO detection limits obtained in previous studies by Lipp et al [11, 12]. According to those studies correct identification was achievable for both soy beans and maize samples containing 2% GMOs, with the exception of 0.5% GM soybeans detected with the 35S promoter. The improvement of screening sensitivity in our study indicates an appropriate choice of DNA extraction method and PCR primer pairs, and correct PCR optimization for both soy beans and maize samples. The sensitivity of 0.1% GMOs exceeds the lowest threshold level (0.5% for unauthorized GMOs) in the labelling required for the EU. Considering that the CaMV 35S promoter and NOS terminator regulatory elements are present in almost all commercialized transgenic crops, a vast number of biotechnological plants might be detected by these methods.

In conclusion, the DNA-based analytical procedures described in this paper represent a simple, reliable and sensitive screening technology for genetically modified organisms that fulfils European regulatory requirements. Therefore, they might be well applied in international monitoring programmes for genetically modified seeds, grains, foodstuffs and feed.

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