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A multiplex nested PCR assay for the simultaneous detection of genetically modified soybean, maize and rice in highly processed products

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ABSTRACT

The use of genetically modified organisms (GMOs) as food products becomes more and more widespread. The European Union has implemented a set of very strict procedures for the approval to grow, import and/or utilize GMOs as food or food ingredients. Thus, analytical methods for detection of the GMOs are necessary in order to verify compliance with labeling requirements. There are few effective screening methods for highly processed GM (genetically modified) products. Four genes (*CP4-EPSPS*, *Cry1A(b)*, *BAR*, and, *PAT*) are common exogenous genes used in commercialized transgenic soybean, maize, and rice. In the present study, a multiplex nested polymerase chain reaction (PCR) method was developed to simultaneously detect the four exogenous genes and one endogenous gene in two runs. We tested eleven representative highly processed products samples (soya lecithin, soya protein powder, chocolate beverage, infant rice cereal, soybean refine oil, soybean salad oil, maize oil, maize protein powder, maize starch, maize jam) using the developed method, and amplicons of endogenous gene and transgenic fragments were obtained from all the processed products except for soybean refined oil, soybean salad oil and maize oil, and the sensitivity was 0.005%. These results indicate that multiplex nested PCR is appropriate for qualitative detection of transgenic soybean, maize and rice in highly processed products except for refined oil.

1. Introduction

The use of genetically modified organisms (GMOs) as food products becomes more and more widespread (Ying et al., 2005; Nikoli et al., 2009) The European Union has implemented a set of very strict procedures for the approval to grow, import and/or utilize GMOs as food or food ingredients. Thus, analytical methods for the detection of GMOs are necessary in order to verify compliance with labeling requirements (Lu, Lin, & Pan, 2010; Litao et al., 2007). The most accepted analytical methods for GMO detection are based on DNA techniques such as polymerase chain reaction (PCR), since the protein-based methods are not reliable for highly processed food analysis. PCR gel-based assay is rapid, sensitive and simple, and used as a routine GMO detection method in many countries because of the relatively inexpensive and ordinary equipment required.

Recently, several multiplex PCR assays based on the simultaneously amplification multiple sequences have been developed. Randhawa, Chhabra, and Singh (2009) developed a multiplex PCR-Based simultaneous amplification of selectable marker and reporter genes for the screening of genetically modified crops. Bahrdt, Krech, Wurz, and Wulff (2010) developed a hexaplex real-time PCR assay for screening for presence of GMOs in food, feed and seed. These studies demonstrated that multiplex PCR was a cost-effective and efficient assay for GM detection.

Multiplex nested PCR can be generated by using the product of the first as a template when high specificity is required. Multiplex nested PCR method fully combines the high sensitivity and specificity of nested PCR and the rapidness of the multiplex PCR (Anna et al., 2010; Rondini et al., 2008). Application of the multiplex nested PCR not only can greatly improve sensitivity, but also can save considerable time and effort by decreasing the number of reactions required to assess the possible presence of GMOs in a food sample.

Several methods using multiplex nested PCR for the detection of GM maize and/or soy have already been described. One multiplex nested PCR assay has been commercialized which simultaneously detects the presence of the 35S promoter as well as zein (maize) and lectin (soya) (Biosmart Allin 1.0 GMO Screening system, Promega, WI, USA). We also reported a triplex nested PCR

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assay for the simultaneous detection of lectin and transgenic construct 35S-CTP and EPSPS-NOS of soybean (Minghui et al., 2007), but these analytical methods detect only one endogenous gene and several transgenic markers of one genetically modified organism or endogenous gene of two genetically modified organism and 35S promoter. However, a positive test for this CaMV35S promoter is not always conclusive. This sequence also occurs naturally in plants and plants may be naturally infected with the Cauliflower mosaic virus, the source of the CaMV35S promoter. Therefore a positive result of CaMV35S will not be sufficient to confirm the presence of GMO, but will suggest that it is probable. In such cases further PCR tests should be run with primers designed to amplify the specific transgenic DNA. In addition, more and more GMOs lacking the 35S promoter will not be detected by such screening and an alternative screening test will be required. Here, we describe a new multiplex nested PCR assay to simultaneously detect four mainly specific transgenic sequences and a common endogenous reference gene in highly processed products of transgenic soybean, maize and rice. It would be advantageous to detect more than one sequence per genetically modified organism (one endogenous gene and several transgenic sequences) or to screen several GMOs in one analysis.

In this study, a multiplex nested PCR procedure that provides a simple and reliable identification of four main exogenous genes, *5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS)* gene, *Bacillus thuringiensis* subsp *(Cry1A(b))* gene, *ribonuclease* gene from *Bacillus amyloliquefaciens (BAR)*, *phosphinothricin acetyltransferase (PAT)* gene and a common endogenous reference *ribulose bisphosphat4e carboxylase/oxygenase large subunit (RBCL)* gene in highly processed products of genetically modified soybean (GTS40-3-2, MON89788, A2704-12, A5547-127), maize (MON810, Bt176, Bt11, T25, TC1507, CBH351, NK603, MON88017, DAS-59122) and transgenic rice has been developed. The methods are reproducible since each sample has been repeated at least three times. Host specific internal target (the gene of *RBCL*) has been tested in all assays as a control to evaluate DNA quality and PCR efficacy, reducing the risk of false negatives, thereby increasing reliability. The method we described here is simple, reliable, efficient and sensitive, which offers a cost-effective alternative for routine GMO identification in food product analysis.

2. Materials and methods

2.1. Materials

Transgenic soybean (GTS40-3-2, MON89788, A2704-12, A5547-127), transgenic maize (Bt11, Bt176, MON810, TC1507, T25, CBH351, NK603, MON88017, DAS-59122) and transgenic rice were obtained from the Academy of Agriculture Science of China, as reference materials. The non-transgenic soybean, maize and rice were gifts from the Institute of Soybean of Northeast Agricultural University (NEAU, Harbin, China).

The GM mixture samples included equal weights of each of four GMO (GM soybean containing *CP4-EPSPS* gene, GM maize T25 containing *PAT* genes, CBH351 containing *BAR* gene and GM rice containing *Cry1A(b)* gene) in non-GMO (soybean, maize and rice) were used as positive controls and the non-GM mixture samples included equal weights of non-transgenic soybean, maize and rice as negative controls.

Eleven highly processed products containing soybean, maize and rice ingredients (labeled in their trademark) including soya lecithin, soya protein powder, chocolate beverage, infant rice cereal, soybean refined oil, soybean salad oil, maize oil, maize starch, maize protein powder, oatmeal, maize paste were chosen as blind samples bought from local markets.

2.2. DNA extraction and purification

DNA extraction was performed with the Wizard® Magnetic DNA Purification System for Food Kit (Promega, Madison, WI, USA) according to the procedure outlined in the technical manual; the DNA concentrations were measured by absorbance at 260 nm and DNA purities were measured by calculating the ratio of absorbance at 260–280 nm with the spectrophotometer DU-600 (Beckman, Fullerton, CA). Three replicate extracts for per sample were measured. Also the extracted DNA was loaded onto the agarose gel (0.8%) to check its purity.

2.3. Selection of primers for the multiplex nested PCR

In order to detect the highly processed products of transgenic soybean, maize and rice, we first needed to obtain sequence information on exogenous genes to design primers. The sequences of the same exogenous gene in different breeds are much different after the gene has been transfered with different modifications. Furthermore, sometimes the sequences of the same exogenous gene such as *Cry1A(b)* gene in different lines: Bt11, Bt176 and MON810, are also different. So different primers need to be designed for one exogenous gene in different breeds or lines in routine PCR detection.

In this study, the different sequences of the same exogenous gene in different breeds or lines were compared, and the consensus sequence regions were determined, then the general primers for different breeds or lines were designed to simplify the primer design and increase the detection efficiency. Using this method, we selected and designed *Cry1A(b), PAT, BAR* and *CP4-EPSPS* gene primers by the information in the available database (GMDD, http://gmdd.shgmo.org/). The *Cry1A(b)* gene primers were from Takeshi, Hideo, and Ken (2002), who successfully used them to amplify *Cry1A(b)* gene in transgenic maize Bt11, Bt176, MON810, and Delano, Anna–Mary, Erika, Margaret, and Saad (2003) got the same result. Primer pairs CP4 WF and CP4 WR (498 bp product), Primer pairs Rbcl WF and Rbcl WR (433 bp product), Primer pairs Bar WF and Bar WR (175 bp product) were from references (Jiang et al., 2003; Quan et al., 2002; Tan et al., 2003). And the other primer pairs were from this study (listed in Table 1).

In addition, the product length was also an important factor which interfered with the PCR reaction. The DNA in processed products was destroyed and cut into little fragments, so these primers were designed to yield amplicon sizes within 100–500 bp. Each primer pair was also designed to distinguish the length of the amplified product from other amplified products.

PCR primers were designed with Primer Premier V5.0 software and listed in Table 1. The larger fragments were amplified in the first round of the multiplex nested PCR, and afterward the smaller ones were amplified in the second round. To equalize as much as possible the intensities of all the PCR products in gel, according to different amplifying efficiencies, the suitable concentration of each primer pair was accurately calibrated. The primer concentrations for multiplex PCR were optimized by determination the minimum primer concentration. The primers were premixed to minimize the differences among the primer concentrations due to pipetting variability; the primer mixture prepared at a 4× concentration was ready to be diluted during the PCR assembly. The oligonucleotides were synthesized by Shanghai Sangon Bioengineering Technological Service Ltd. (Shanghai, China).

2.4. Multiplex nested PCR conditions

The procedures of the first multiplex PCR round were performed in a final volume of 50 μl with the following reagent concentrations: genomic DNA 50 ng, PCR buffer 2×, 0.4 mmol/L of each dNTPs