Establishment of Quantitative Analysis Method for Genetically Modified Maize Using a Reference Plasmid and Novel Primers

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Abstract

For the quantitative analysis of genetically modified (GM) maize in processed foods, primer sets and probes based on the 35S promoter (p35S), nopaline synthase terminator (tNOS), p35S-hsp70 intron, and zSSIIb gene encoding starch synthase II for intrinsic control were designed. Polymerase chain reaction (PCR) products (80~101 bp) were specifically amplified and the primer sets targeting the smaller regions (80 or 81 bp) were more sensitive than those targeting the larger regions (94 or 101 bp). Particularly, the primer set 35F1-R1 for p35S targeting 81 bp of sequence was even more sensitive than that targeting 101 bp of sequence by a 3-log scale. The target DNA fragments were also specifically amplified from all GM labeled food samples except for one item we tested when 35F1-R1 primer set was applied. A reference plasmid pGMmaize (3 kb) including the smaller PCR products for p35S, tNOS, p35S-hsp70 intron, and the zSSIIb gene was constructed for real-time PCR (RT-PCR). The linearity of standard curves was confirmed by using diluents ranging from $2 \times 10^1$~$10^5$ copies of pGMmaize and the $R^2$ values ranged from 0.999~1.000. In the RT-PCR, the detection limit using the novel primer/probe sets was 5 pg of genomic DNA from MON810 line indicating that the primer sets targeting the smaller regions (80 or 81 bp) could be used for highly sensitive detection of foreign DNA fragments from GM maize in processed foods.

Key words: GM-maize, novel primer, GMO detection, real-time PCR, reference plasmid, processed food

INTRODUCTION

Genetically modified organisms (GMOs) have been broadly used in processed foods worldwide, and for that reason, most consumers are quite concerned about their bio-safety. To alleviate consumer concerns, many countries including the European Union (EU), Korea, and Japan have introduced labeling systems. The threshold levels for unintentional mixing of GMOs in food products are 0.9% in the EU, 3% in Korea, and 5% in Japan (1).

According to a report by James (2), global areas of GM crops reached 160 million hectares (395 million acres) in 2011, which exceeded 8% (12 million hectares) over 2010, indicating biotech crops have spread quickly in recent years. In 2011, GM soybeans were the principal biotech crop, which covered 47% of global biotech areas, followed by maize (32%), cotton (15%), and canola (5%). Most of these biotech crops have traits such as herbicide tolerance, insect resistance, or both. At present, many GM maize lines including MON810, GA21, NK603, MON863, DLL25, DBT418, MON88017 (above Monsanto Co., St. Louis, MO, USA), 1507, DAS-59122-7 (above Dupont Co., Wilmington, DE, USA), Bt11, Bt176, Bt10 (above Syngenta Seeds Co., Basel, Switzerland), and T25 (Byer Crop Science Co., Monheim am Rhein, Germany) have been approved for importation by the Korea Food and Drug Administration (KFDA; http://www.kfda.go.kr/gmo/index.do).

To manage the labeling system for GMOs in foods, reliable evaluation methods are essential. To date, many detection methods for GMOs as crops or food ingredients based on polymerase chain reaction (PCR), microarray, and immunoassay have been developed (3-11). Interestingly, Kalogianni et al. (12) reported a method using a dry-reagent DNA biosensor in a disposable dipstick format for the visual detection and sequence confirmation of GMO by hybridization within minutes. Among the above methods, PCR-based techniques are easily applied and very useful in detecting transgenic regions of GMOs; however, this method has some difficulties to calculate the incorporation rate of GMOs in highly processed foods due to DNA damage and food matrices. To overcome these problems, the preparation of template DNA from food samples and highly sensitive primers targeting small transgenic regions would be primarily necessary.

In this study, we designed and evaluated primer and
probe sets targeting small transgenic regions of GM maize lines harboring p35S, tNOS, and p35S-hsp70 intron specific for MON810 line. Furthermore, a reference plasmid pGMmaize (3 kb) was constructed for quantification of the target DNA fragments using real-time PCR (RT-PCR) and the sensitivity was evaluated.

**MATERIALS AND METHODS**

**Maize and food samples**

The transgenic maize events, Bt11 and MON810, were kindly provided by Dr. T. Kim from the Korean Institute of Agricultural Biotechnology (Suwon, Korea). As a negative control, non-GM maize was purchased from a local market and proved to be non-GM by PCR method using the specific primer set to detect intrinsic or transgenic regions of GM maize lines (Fig. 1) by Primer Express® software v2.0 (Applied Biosystems Co., Foster, CA, USA) and synthesized from TIB MOLBIOL Co. (Berlin, Germany). Taq-Man fluorogenic probes were employed in this study and the fluorescent reporter dye, 6-carboxy-fluorescein (FAM), was labeled on the 5'-end and the fluorescent quencher dye, 6-carboxytetramethylrhodamine (TAMRA), was labeled on the 3'-end. The oligonucleotide sequences of primers and probes are shown in Table 1.

**Qualitative PCR**

To amplify target sequences, newly synthesized primers were diluted to 50 μmol/L for use. For optimization of PCR reaction in a thermal cycler (Biometra T3 thermocycler, Biotron GmbH, Göttingen, Germany), concentrations of MgCl2 and DNA template and the annealing temperature were adjusted. The PCR cycles were as follows: 95°C for 10 min (pre-incubation); 40 cycles of 95°C for 30 s (denaturation), 55~60°C for 30 s (annealing), and 72°C for 30 s (extension); 72°C for 7 min (final extension). The PCR products were separated on 2% (w/v) agarose gels and visualized by a UV transilluminator (UVP, Upland, CA, USA) after ethidium bromide (Sigma Co., St. Louis, MO, USA) staining. For sensitivity tests of newly designed primers, the concentration of template DNA ranged from 0.005 to 100 ng.

**Construction of a novel reference plasmid**

To construct a reference plasmid for RT-PCR, the smaller target regions (80 or 101 bp) of p35S, tNOS, or hsp70 intron, and the intrinsic zSSIIb gene were amplified with the novel primers added with restriction endonuclease sites, i.e. 35F1-HindIII, 35R1-BamHI, NOSF1-BamHI, NOSR1-KpnI, MON810F1-KpnI, MON810R1-
Table 1. Oligonucleotide primers and probes used in this study

| Target | Name               | Sequence (5'→3') Primer/Probe | Product size (bp) |
|--------|--------------------|-------------------------------|-------------------|
| 35S    | 35F1               | CGTTCCAACCACGTCTTCAA forward  | 81                |
|        | 35R1               | GGAATGCGAATGGTAGGCTGCTCAGC    | reverse probe     |
|        | 35int1             | CAAGTGGATTTAATAGGTGGGACTTCC   | 101               |
|        | 35F2               | TGATGTGATATCCTCCACTGTGACGTAAG| forward probe     |
|        | 35R2               | GCATCTGAAAATATGAAATAGAATTCTC  | reverse probe     |
|        | 35int2             | CAACATCCACATTCCTCCTCGCAAGACC  |                  |
| NOS    | NOSF1              | TACTGATGACTGAAATATACTTATAACT  | forward probe     |
|        | NOSR1              | GATGGGACTTACATGCTCCTAATCAC    | reverse probe     |
|        | NOSint1            | AATGCATGCTGATATTGAGATGGGT     | 81                |
|        | NOSF2              | TTGGTTGAAATGTTAAGCAGATGT      | forward probe     |
|        | NOSR2              | TATAATGATATAATATTCCGACTTCAA   | reverse probe     |
|        | NOSint2            | AAATGCATGCTGATATTGAGATGGGT    | 101               |
| MON810 | MON810F1           | CTGCAAGGCTGACTCTGAGCAGTCTAC   | forward probe     |
|        | MON810R1           | CACTAGAGAAGTGACAGTAAACAGAAG   | reverse probe     |
|        | MON810int1         | TCTTCGGTGACGCTCTACCTCG        |                  |
|        | MON810F2           | ACCCTTCTCTATATAAGGAGAGATGTTAC| forward probe     |
|        | MON810R2           | AGTGAGCGGATACCGGAAGAGAGAG     | reverse probe     |
|        | MON810int2         | ACACGCTGCAAGCTGACTCTAGAAGATCT|                  |
| zSSIIb | zSSIIbF1           | GCTCCATGTGGCGGTACTGTG        | forward probe     |
| gene   | zSSIIbR1           | GACAGGCGAGAAATGCGGTATG        | reverse probe     |
|        | zSSIIbint1         | CTATGTTGAGCTGGCAACTTAATGTTCC |                  |
|        | zSSIIbF2           | CGTATGGAGGTCCATGTGAGATG       | forward probe     |
|        | zSSIIbR1           | GACAGGCGAGAAATGCGGTATG        | reverse probe     |
|        | zSSIIbint2         | TATGGTATGGACAACTTAATGCTAGTA  |                  |

ClaI, zSSIIbF1-ClaI, and zSSIIbR1-EcoRI. The PCR products were digested by the restriction enzymes (Takara Co., Shiga, Japan), purified, and tandem ligated. Finally the ligate was introduced into pUC19 cloning vector digested with EcoRI and HindIII enzymes and transferred to Escherichia coli JM109 strain by an electroporation method (15). The transformants were selected on an LB (Luria-Bertani) agar plate (10 g/L of tryptone, 10 g/L of NaCl, 5 g/L of yeast extract, 15 g/L of agar, pH 7.0) supplemented with 100 µg/mL of ampicillin (Sigma Co.). X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) solutions were also spread on the agar plate for color (blue/white) selection. The recombinant DNA pGMmaize was confirmed by restriction profiles and nucleotide sequencing (data not shown).

RESULTS AND DISCUSSION

Quantitative RT-PCR

Newly synthesized primer and probe sets were tested for RT-PCR. The PCR reactions were performed with a LightCycler™ system (Roche diagnostics Co., Mannheim, Germany). The TaqMan PCR reaction mixtures contained FastStart PCR master mix (Roche diagnostics Co.), 0.25 µL of primer mix (each 10 µM), 0.5 µL of probe (25 µM), and various concentrations of template DNA. The mixtures were subjected to the following thermal profile: 95°C for 10 min (pre-incubation); 45 to 50 cycles of 95°C for 30 s (denaturation), 57 to 59°C for 1 min (annealing and extension), and 40°C for 30 s (cooling). Standard curves for quantification of transgenic DNA regions were constructed with the reference plasmid pGMmaize which was serially diluted to contain 20, 200, 2,000, 20,000, and 200,000 copies.

Quantitative PCR

To confirm whether the target DNA regions are specifically amplified with novel primer sets designed in this study, qualitative PCRs were performed, resulting in the specific amplifications of the target regions (data not shown). As expected, primer sets targeting the smaller DNA regions were more sensitive than those targeting the larger ones. Particularly in the case of p35S, the primer set for the smaller region (81 bp) was even more sensitive than that for the larger one (101 bp) by 3 logscales (Fig. 2), which were reflected on real food sample tests. In the tests, 5 of 8 GM-labeled food samples including soup, cracker, frying powder, whole corn, sweet corn, and corn tea were shown to be positive with the primer set for the larger DNA region, whereas seven samples were positive with the primer set for the smaller
region; these results indicate the primer sets targeting the smaller DNA regions are more sensitive and powerful for detection of transgenic DNA fragments from GMOS or GM-foods. Additionally, in our model system, GM maize was exposed to high temperature and pressure, resulting in an increase in the rate of DNA degradation (7,16,17); the primer sets for the smaller regions were also more sensitive than those for the larger regions in detection of transgenic regions of the fragmented DNA (unpublished data). These results indicate that the newly designed primer sets for 80 or 81 bp of amplicon could be broadly used for detection of GM maize in highly processed foods.

**Construction of a reference plasmid pGMmaize**

As described in materials and methods, PCR products (80 or 81 bp) amplified by using genomic DNAs of GM maize lines were tandem ligated and cloned into the pUC19 plasmid vector and the resulting recombinant DNA was named pGMmaize. The recombinant DNA was confirmed by restriction with EcoRI and HindIII, which resulted in the liberation of 370 bp of the insert, and the nucleotide sequencing with universal primers for pUC19. The schematic representation of pGMmaize and the nucleotide sequence of the insert are shown in Fig. 3.

**Quantitative RT-PCR**

To validate the designed primers and probes targeting the smaller transgenic regions (80 or 81 bp) in RT-PCR, standard curves using 35F1-R1 for p35S, MON810F1-R1 for the MON810 line, and SSIIbF1-R1 for intrinsic zSSIIb gene were constructed with the reference plasmid pGMmaize dilutions, i.e. 20, 200, 2,000, 20,000, and 200,000 copies (Table 2). The slopes, which show direct PCR efficiency, ranged from -3.36 to -3.66 and R² values for linearity ranged from 0.999 to 1.000, which indicates that the reference plasmid is well-suited for quantitative

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Fig. 2. Sensitivity of novel primer sets for p35S. The primer set 35F1-R1 was used for the amplification of 81 bp (A) and 35F2-R2 for 101 bp (B). Lane 1, 100 ng; lane 2, 50 ng; lane 3, 5 ng; lane 4, 0.5 ng; lane 5, 0.05 ng; lane 6, 0.005 ng of genomic DNA from MON810 maize line; lane M, size marker (100 bp DNA ladder, NEB Co., Ipswich, MA, USA).

Fig. 3. Schematic representation of pGMmaize (A) and the nucleotide sequence of the insert (B). HindIII, BamHI, KpnI, ClaI, and EcoRI indicate restriction endonuclease recognition sites. Each primer and probe region is underlined and named. Arrows indicate the directions of DNA polymerization.
PCR detection of GM maize lines. The results of RT-PCR using the genomic DNA from the MON810 line as the PCR template could detect 5 pg of the genomic DNA for p35S, MON810 line-specific region, or the PCR template could detect 5 pg of the genomic DNA from the MON810 line for detection of p35S, p35S-hsp70 intron, and zSSIIb gene. Our RT-PCR method is highly sensitive but the developed quantitative method has not yet been applied to real food samples. Therefore, this method should be evaluated for its validity with all types of food samples containing transgenic DNA fragments from GM-Maize lines and compared with other developed quantitative methods in the near future.

In conclusion, we know that the use of the novel primer sets targeting the smaller transgenic DNA regions is even better than that for the larger ones in detection of transgenic DNA fragments from GM-maize lines or highly processed GM-foods, even though some practical data should be supported.

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