Prev Nutr Food Sci
Vol 17, p 274～279 (2012)
http://dx.doi.org/10.3746/pnf.2012.17.4.274

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

Gi-Seong Moon[1] and Weon-Sun Shin[2†]

[1]Department of Biotechnology, Korea National University of Transportation, Chungbuk 368-701, Korea
[2]Department of Food & Nutrition, Hanyang University, Seoul 133-791, Korea

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 2×10^1～10^5 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

†Corresponding author. E-mail: hime@hanyang.ac.kr
Phone: +82-2-2220-1204, Fax: +82-2-2292-1226

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 a GMO detection primer kit (Nippon Gene Co., Fukuyama, Japan). For feasibility test of novel primer and probe sets, processed food samples including corn and corn flour were collected from local markets.

Extraction of genomic DNA

To isolate genomic DNA from the reference GM maize, samples were homogenized by a mortar and pestle under liquid nitrogen. The homogenates were applied to DNeasy Plant Maxi Kit (Qiagen Co., Hilden, Germany) according to the manufacturer's instruction with a modification where the incubation time at 65°C was doubled after addition of the initial buffer for lysis. For the isolation of genomic DNA from food samples, a rapid-salt extraction buffer method (EasyPrep™, NEXGEN Co., Seoul, Korea), a silica resin method (DNeasy plant mini kit, Qiagen Co., Seoul, Korea), and a magnetic bead method (Wizard DNA prep kit, Promega Co., Madison, WI, USA) were combined. In cases of snacks, after homogenization, a large amount of sugar was excluded by diluting with ultrapure water and the resulting centrifugal pellets were dried at 50°C and used for DNA purification. For ham, after homogenization, 10% sodium dodecyl sulfate (SDS) or hexane was added to the homogenates to get rid of fats and the remains were dried and used for DNA purification. The quantities of isolated DNAs were determined by a UV spectrophotometer (J710, JASCO Co., Tokyo, Japan) at 260 nm.

Oligonucleotide primers and probes

Novel primers and probes based on the published sequences (13,14) and GenBank (http://www.ncbi.nlm.nih.gov/) database (GenBank accession no. V00141 and J01541 for p35S, V00087 and J01541 for tNOS, X03658 for *hsp70* exon 1, AF019297 for *zSSIIb* gene) were designed 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). TaqMan fluorescent 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.

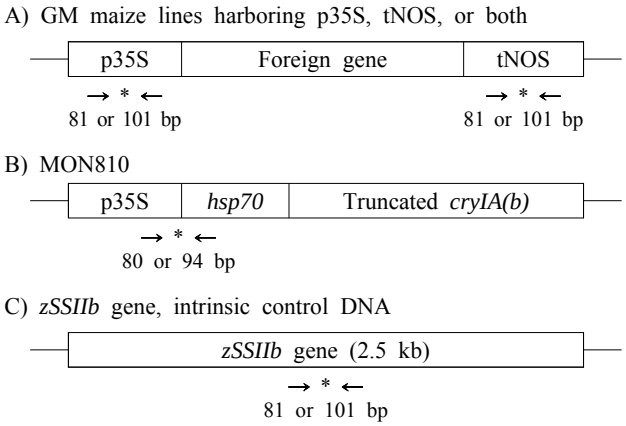


Fig. 1. Schematic representation of target regions of primers and probes designed in this study. p35S, 35S promoter region derived from cauliflower mosaic virus; tNOS, the terminator region of nopaline synthase derived from *Agrobacterium tumefaciens*; *hsp70*, DNA fragment containing intron no. 1 of *hsp70* gene, encoding a heat-shock protein, of maize; *cryIA(b)*, synthetic delta endotoxin gene derived from *Bacillus thuringiensis*; *zSSIIb* gene, starch synthase II gene. Asterisks (*) indicate probes.

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 $MgCl_2$ 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 81 bp) of p35S, tNOS, p35S-*hsp70* intron, and the intrinsic *zSSIIb* gene were amplified with the novel primers added with restriction endonuclease sites, i.e. 35F1-*Hind*III, 35R1-*Bam*HI, NOSF1-*Bam*HI, NOSR1-*Kpn*I, MON810F1-*Kpn*I, MON810R1-

Table 1. Oligonucleotide primers and probes used in this study

Target	Name	Sequence (5'→3')	Primer /Probe	Product size (bp)
35S promoter	35F1	CGTTCCAACCACGTCTTCAA	forward	81
	35R1	GGATAGTGGGATTGTGCGTCAT	reverse	
	35int1	CAAGTGGATTGATGTGATATCTCCACTGACG	probe	
	35F2	TGATGTGATATCTCCACTGACGTAAG	forward	101
	35R2	GTCCTCTCCAAATGAAATGAACTTC	reverse	
	35int2	CACAATCCCACTATCCTTCGCAAGACC	probe	
NOS terminator	NOSF1	TTACGTTAAGCATGTAATAATTAACA	forward	81
	NOSR1	AATTGCGGGACTCTAATCAT	reverse	
	NOSint1	ATGCATGACGTTATTTATGAGATGGGT	probe	
	NOSF2	TTCTGTTGAATTACGTTAAGCATGT	forward	101
	NOSR2	TTAAATGTATAATTGCGGGACTCTAA	reverse	
	NOSint2	AATGCATGACGTTATTTATGAGATGGGTTT TTATG	probe	
MON810	MON810F1	CTGACAAGCTGACTCTAGCAGATCTAC	forward	80
	MON810R1	CACTAGAGAAACGTGACAGTAACAAAG	reverse	
	MON810int1	TCTTCGGTACGCGCTCACTTCG	probe	
	MON810F2	ACCCTTCCTCTATATAAGGAAGTTCATT	forward	94
	MON810R2	AGTGAGCGCGTACCGAAGA	reverse	
	MON810int2	ACACGCTGACAAGCTGACTCTAGCAGATCTAC	probe	
zSSIIb gene (intrinsic control)	SSIIbF1	GCTCCATGTGGCGGTACTG	forward	81
	SSIIbR1	GACAGGCAGAAGTGCGGTATG	reverse	
	SSIIbint1	CTATGGTGATGGCAACTTAGTTTTCATTGCTAATGA	probe	
	SSIIbF2	CTGTTGAGGTTCCATGGTATGC	forward	101
	SSIIbR1	GACAGGCAGAAGTGCGGTATG	reverse	
	SSIIbint2	TATGGTGATGGCAACTTAGTTTTCATTGCTAATG	probe	

*Cla*I, SSIIbF1-*Cla*I, and SSIIbR1-*Eco*RI. 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 *Eco*RI and *Hind*III enzymes and transferred to *Escherichia coli* JM109 strain by an electrotransformation 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).

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.)

RESULTS AND DISCUSSION

Qualitative 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 log-scales (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

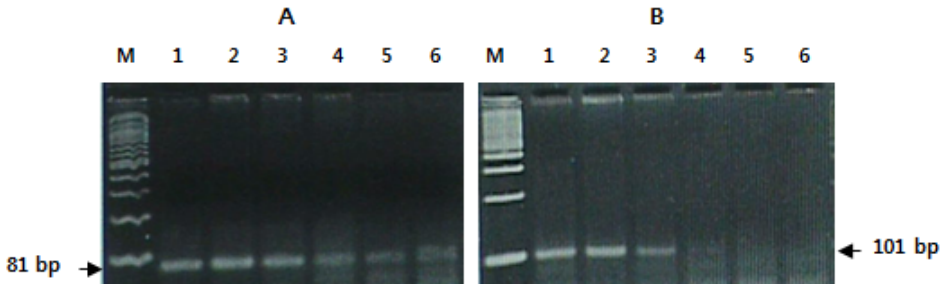


Fig. 2. Sensitivity of novel primer sets for p35S. The primer set 35F1-R1 was used for the amplification of 81 bp (A) and 35F2-R2 for 101 bp (B). Lane 1, 100 ng; lane 2, 50 ng; lane 3, 5 ng; lane 4, 0.5 ng; lane 5, 0.05 ng; lane 6, 0.005 ng of genomic DNA from MON810 maize line; lane M, size marker (100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

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.

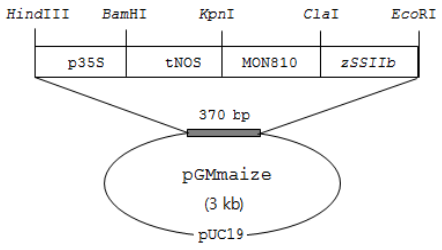
Construction of a reference plasmid pGMmaize

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 *Eco*RI and *Hind*III, 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 R^2 values for linearity ranged from 0.999 to 1.000, which indicates that the reference plasmid is well-suited for quantitative

(A)



(B)

```
CATGATTACG CCAAGCTTGG GCGTTCCAAC CACGTCTTCA AAGCAAGTGG ATTGATGTGA   60
           HindIII           35F1                      35int1

TATCTCCACT GACGTAAGGG ATGACGCACA ATCCCACTAT CCCGGGATCC CGTTACGTTA  120
                           35R1             BamHI

AGCATGTAAT AATTAACATG TAATGCATGA CGTTATTTAT GAGATGGGTT TTTATGATTA  180
  NOSF1                      NOSint1

GAGTCCCGCA ATTGGGGTAC CCCCTGACAA GCTGACTCTA GCAGATCTAC CGTCTTCGGT  240
 NOSR1          KpnI              MON810F1

ACGCGCTCAC TTCGCCCTCT GTCTTTGTTA CTGTCACGTT TCTCTAGTGC CATCGATGGG  300
 MON810int1                 MON810R1                ClaI

CTCCATGTGG CGGTACTGTC TATGGTGATG GCAACTTAGT TTTCATTGCT AATGATTGGC  360
      SSIIbF1                SSIIbint1

ATACCGCACT TCTGCCTGTC GGAATTCACT GGCCGTCGTT  400
      SSIIbR1          EcoRI
```

Fig. 3. Schematic representation of pGMmaize (A) and the nucleotide sequence of the insert (B). *Hind*III, *Bam*HI, *Kpn*I, *Cla*I, and *Eco*RI indicate restriction endonuclease recognition sites. Each primer and probe region is underlined and named. Arrows indicate the directions of DNA polymerization.

Table 2. Reliability of copy number of pGMmaize in real-time PCR

Target region	Copy No.		
	True value	Calculated mean	RSD[1)]
p35S	20	19.05	13.84
	200	225.1	23.95
	2000	2449.8	13.23
	20000	18028	24.39
	200000	201420	13.09
p35S-*hsp70* (for MON810 line)	20	22.83	11.28
	200	167.74	24.37
	2000	2023.8	30.65
	20000	18392.2	15.87
	200000	242320	21.55
zSSIIb gene (intrinsic control)	20	20.62	6.62
	200	200.54	11.73
	2000	1975	13.00
	20000	14726	10.25
	200000	258880	9.84

[1)]RSD: relative standard deviations of reactions, average across five replicates.

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 *zSSIIb* gene, which indicate that the developed system using the novel primers and probes targeting the smaller transgenic regions is sensitive and can be effectively applied for quantification of target DNA in highly processed foods.

At present, many GMOs are cultivated worldwide and the area of cultivation is growing rapidly (2). Therefore GM crops are naturally incorporated into food chains and the labeling system for giving choices to consumers is a way to eradicate their concerns. In the system, a reasonable detection method is a prerequisite and the sensitivity is a key factor to effectively detect transgenic DNA fragments in highly processed foods. For that reason, novel primers and probes targeting transgenic DNA regions such as p35S, tNOS and p35S-*hsp70* intron from GM-maize lines were designed in this study (Table 1; Fig. 1). In qualitative tests, primer sets for targeting the smaller regions (80 or 81 bp) were more sensitive than the larger ones (94 or 101 bp). In particular, the p35S primer set for the smaller region (81 bp) was even more sensitive than the larger one (101 bp) by 3 log-scales (Fig. 2). These results were correlated with real food sample tests. The primer set for the smaller region detected 87.5% of GM-labeled food samples (7/8), whereas the primer set for the larger one detected 62.5% of the samples (5/8), indicating the former primer set is more suitable for the detection of transgenic DNA fragments in highly processed food samples where intact DNA molecules are excessively digested during the processes. In quantitative RT-PCR using the primer sets targeting the smaller regions (80 or 81 bp), the detection limit was 5 pg of 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.

ACKNOWLEDGMENTS

This work was supported by the research funds of Korea Food and Drug Administration (KFDA) and Korea Food Research Institute (KFRI).

REFERENCES

1. Lee SH, Kang SH, Park YH, Min DM, Kim YM. 2006. Quantitative analysis of two genetically modified maize lines by real-time PCR. *J Microbiol Biotechnol* 16: 205-211.
2. James C. 2011. Global status of commercialized biotech/GM crops. *ISAAA Brief* 43.
3. Ahmed FE. 2002. Detection of genetically modified organisms in foods. *Trends Biotechnol* 20: 215-223.
4. Fantozzi A, Ermolli M, Marini M, Scotti D, Balla B, Querci M, Langrell SR, van den Eede G. 2007. First application of a microsphere-based immunoassay to the detection of genetically modified organisms (GMOs): quantification of Cry1Ab protein in genetically modified maize. *J Agric Food Chem* 55: 1071-1076.
5. Hernández M, Rodríguez-Lázaro D, Zhang D, Esteve T, Pla M, Prat S. 2005. Interlaboratory transfer of a PCR multiplex method for simultaneous detection of four genetically modified maize lines: Bt11, MON810, T25, and GA21. *J Agric Food Chem* 53: 3333-3337.
6. Leimanis S, Hernández M, Fernández S, Boyer F, Burns M, Bruderer S, Glouden T, Harris N, Kaeppeli O, Philipp P, Pla M, Puigdomènech P, Vaitilingom M, Bertheau Y, Remacle J. 2006. A microarray-based detection system for genetically modified (GM) food ingredients. *Plant Mol Biol* 61: 123-139.
7. Moreano F, Busch U, Engel KH. 2005. Distortion of genetically modified organism quantification in processed foods: Influence of particle size compositions and heat-induced DNA degradation. *J Agric Food Chem* 53: 9971-9979.
8. Rudi K, Rud I, Holck A. 2003. A novel multiplex quantita-

tive DNA array based PCR (MQDA-PCR) for quantification of transgenic maize in food and feed. *Nucleic Acids Res* 31: e62.
9. Toyota A, Akiyama H, Sugimura M, Watanabe T, Sakata K, Shiramasa Y, Kitta K, Hino A, Esaka M, Maitani T. 2006. Rapid quantification methods for genetically modified maize contents using genomic DNAs pretreated by sonication and restriction endonuclease digestion for a capillary-type real-time PCR system with a plasmid reference standard. *Biosci Biotechnol Biochem* 70: 2965-2973.
10. Xu W, Huang K, Zhao H, Luo Y. 2005. Application of immunoaffinity column as cleanup tool for an enzyme linked immunosorbent assay of phosphinothricin-N-acetyltransferase detection in genetically modified maize and rape. *J Agric Food Chem* 53: 4315-4321.
11. Yang L, Pan A, Zhang K, Guo J, Yin C, Chen J, Huang C, Zhang D. 2005. Identification and quantification of three genetically modified insect resistant cotton lines using conventional and TaqMan real-time polymerase chain reaction methods. *J Agric Food Chem* 53: 6222-6229.
12. Kalogianni DP, Koraki T, Christopoulos TK, Ioannou PC. 2006. Nanoparticle-based DNA biosensor for visual detection of genetically modified organisms. *Biosens Bioelectron* 21: 1069-1076.
13. Brodmann PD, Ilg EC, Berthoud H, Herrmann A. 2002. Real-time quantitative polymerase chain reaction methods for four genetically modified maize varieties and maize DNA content in food. *J AOAC Int* 85: 646-653.
14. Matsuoka T, Kuribara H, Takubo K, Akiyama H, Miura H, Goda Y, Kusakabe Y, Isshiki K, Toyoda M, Hino A. 2002. Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *J Agric Food Chem* 50: 2100-2109.
15. Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor Laboratory, New York, NY, USA.
16. Yoshimura T, Kuribara H, Matsuoka T, Kodama T, Iida M, Watanabe T, Akiyama H, Maitani T, Furui S, Hino A. 2005. Applicability of the quantification of genetically modified organisms to foods processed from maize and soy. *J Agric Food Chem* 53: 2052-2059.
17. Yoshimura T, Kuribara H, Kodama T, Yamata S, Futo S, Watanabe S, Aoki N, Iizuka T, Akiyama H, Maitani T, Naito S, Hino A. 2005. Comparative studies of the quantification of genetically modified organisms in foods processed from maize and soy using trial producing. *J Agric Food Chem* 53: 2060-2069.

(Received November 11, 2012; Accepted December 5, 2012)