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Detection of Recombinant Marker DNA in Genetically Modified Glyphosate-Tolerant Soybean and Use in Environmental Risk Assessment

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Abstract The genetically modified glyphosate-tolerant soybean contains the following introduced DNA sequences: the EPSPS (5-enol-pyruvylshikimate-3-phosphate synthase) gene from *Agrobacterium* sp. strain CP4, the 35S promoter from the cauliflower mosaic virus, and the NOS terminator from *Agrobacterium tumefaciens*. In the present study, detection of these introduced DNAs was performed by amplification using the polymerase chain reaction (PCR). A multiplex PCR method was also applied to prevent false positive results. When primers for 35S promoter, nos3', CTP(chloroplast transit peptide), and CP4 EPSPS (EPSPS from *Agrobacterium* sp. CP4) were used, positive results were obtained in PCR reactions using DNA from genetically modified glyphosate-tolerant soybeans. There were no false positive results when using DNA from non-genetically modified soybeans. The CP4 EPSPS gene was detected when less than 125 pg glyphosate-tolerant soybean DNA was amplified. Lectin *Le*1 and *psb* A were amplified from both non-genetically modified and genetically modified glyphosate-tolerant soybean DNA. Multiplex PCR was performed using different primer sets for actin *Sac*1, 35S promoter and CP4 EPSPS. The actin gene was detectable in both non-genetically modified and glyphosate-tolerant soybeans as a constant endogenous gene. Target DNAs for the 35S promoter, and CP4 EPSPS were detected in samples containing 0.01–0.1% glyphosate-tolerant soybean, although there were variations depending on primers by multiplex PCR. Soybean seeds from five plants of non-genetically modified soybean were co-cultivated for six months with those of genetically modified soybean, and they were analyzed by PCR. As a result, they were not positive for 35S promoter, nos3' or CP4 EPSPS. Therefore, these results suggest there was no natural crossing of genes between glyphosate-tolerant and non-genetically modified soybean during co-cultivation, which indicates that gene transfer between these plants is unlikely to occur in nature.

Key words: Glyphosate-tolerant soybean, multiplex PCR, risk assessment, gene transfer, natural crossing

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The development of transgenic crops with new traits has become one of the main activities of agro-industry today. Using molecular biology techniques, DNA sequences from different organisms have been combined and integrated into plants. Glyphosate-tolerant soybeans show specific tolerance towards glyphosate (*N*-phosphonomethyl-glycine), the active ingredient in the nonselective herbicide Roundup. EPSPS (5-enol-pyruvylshikimate-3-phosphate synthase) is the only physiological target of glyphosate, and expression of CP4 *epsps* from *Agrobacterium* sp. strain CP4 confers glyphosate tolerance in soybeans [6]. Glyphosate-tolerant soybeans also harbor the CAMV 35S promoter of the cauliflower mosaic virus and the NOS terminator of *Agrobacterium tumefaciens*.

According to recent regulations, new foodstuffs made from genetically modified crops must be labeled. The labeling of such foodstuffs is based on detection of foreign DNA sequences borne by genetically modified organisms. One of the analytical methods used for enforcement of these regulations is polymerase chain reaction (PCR).

The transfer of genetic information between distantly or even unrelated organisms may pose a major risk to natural environments. Genetically modified soybean may cross with non-genetically modified or wild soybean and cause unexpected spread of artificially modified genes in the environment. For this reason, a PCR method was developed for detecting introduced recombinant DNA in genetically modified glyphosate-tolerant soybeans. A risk assessment study was performed to investigate any natural gene

Table 1. Primer sequences and amplicon length used in polymerase chain reaction assays performed in the present study.

Oligonucleotide name	primer-1 (5'-3') primer-2 (5'-3')	Amplicon length (bp)	Target sequence
35S[a]	GCTCCTACAAATGCCATCA GATAGTGGGATTGTGCGTCA	195	CAMV 35S promoter
35S-2[b]	GAAAAGGAAGGTGGCACTACAAATG GTCCTCTCCAAATGAAATGAACTTC	259	CAMV 35S promoter
CTP[a]	TGATGTGATATCTCCACTGACG TGTATCCCTTGAGCCATGTTGT	172	35S promoter Petunia CTP gene
EPSPS[b]	CCATAAACCCCAAGTTCCTAAATC ATCCTGGCGCCCATGGCCTGCATG	366	35S promoter *Agrobacterium* CP4 EPSPS
NOS[a]	GAATCCTGTTGCCGGTCTTG TTATCCTAGTTTGCGCGCTA	180	nos3' of nopaline gene
LE1[b]	GGCTGATAACACACTCTATTATTGT TGATGGATCTGATAGAATTGACGTT	818	lectin *Le*1 of soybean
PSB	GATGGTATTCGTGAGCCTGT GGCCAAGCAGCTAAGAAGAA	658	*psb* A of soybean
ACT	TTATGCCCTCCCACATGCAA TGCTGGAAGGTGCTTAGAGA	560	*SAc*1 of soybean

[a]Nucleotides sequence from Hemmer [4].
[b]Nucleotides designed by Matsuoka *et al.* [5].

transfer that may have occurred during co-cultivation of non-genetically modified and genetically modified soybean in a model system.

Glyphosate-tolerant soybeans were kindly provided by Monsanto Co. (St. Louis, U.S.A.). Soybean samples were incubated in distilled water for 12 h at room temperature prior to DNA isolation. Swelled germs were separated from soybeans and ground to a fine powder in liquid nitrogen. DNA from 1 g of homogenate samples was prepared using the DNeasy Plant Maxi Kit (QIAGEN, Hilden, Germany).

The glyphosate-tolerant soybean contains the *Agrobacterium* CP4 EPSPS gene that confers resistance to glyphosate [1], the 35S promoter from cauliflower mosaic virus [3], the coding sequence for CTP [1] and the nopaline gene terminator sequence [2]. To detect glyphosate-tolerant soybeans, oligonucleotide primers that have been previously synthesized for PCR were used. Actin *SAc*1 [7], lectin *Le*1 [5] and soybean chloroplast *psb* A [8] gene coding for the photosystem II thylakoid membrane protein were used as negative controls in PCR (Table 1).

PCR was performed on an i-cycler (Bio-Rad, California, U.S.A.). About 100 ng of isolated DNA was added to 20 μl reaction mixture containing 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 1.25 μM of each primer, 250 μM dNTP and 1 unit Taq polymerase (AccuPower PCR PreMix, BIONEER, Korea). Amplification was performed under the following conditions: first denaturation for 3 min at 94°C, 40 cycles (94°C for 20 sec, 50°C for 40 sec, 72°C for 60 sec), and terminal elongation for 3 min at 72°C. The amplification products (20 μl) were separated using 2% agarose gels (SeaKem LE agarose, BMA, Rockland, U.S.A.) in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), and visualized under UV light after staining for 10 min in 1 μg/ml ethidium bromide solution.

The detection of foreign DNA depends on the primers and PCR conditions. Primer sequences from Hemmer [4] were used to detect the 35S promoter, nos 3' and Petunia CTP genes. The primer sequences for CP4 EPSPS and Lectin *Le*1 were obtained from Matsuoka *et al.* [5]. PCR amplification conditions were based on thermal cycler profiles from Promega Notes [9].

DNAs from genetically modified glyphosate-tolerant or non-genetically modified soybeans were examined in PCR experiments using different primers (Fig. 1). Primers were used to amplify gene fragments that naturally occur in the plant under investigation, i.e. actin *SAc*1, lectin *Le*1 and *psb* A and used as negative controls. In addition, DNAs

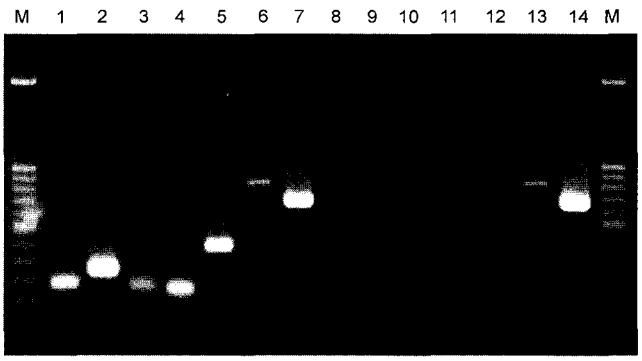

Fig. 1. PCR amplification products from glyphosate-tolerant (lanes 1-7) and non-genetically modified soybeans (lanes 8-14). M, 100 bp ladder; Lane 1, 35S; Lane 2, 35-2; Lane 3, NOS; Lane 4, CTP; Lane 5, EPSPS; Lane 6, LE1; Lane 7, PSB; Lane 8, 35S; Lane 9, 35S-2; Lane 10, NOS; Lane 11, CTP; Lane 12, EPSPS; Lane 13, LE1; Lane 14, PSB.

extracted from non-genetically modified soybeans were used as the negative controls not contaminated by modified DNA. When primers for 35S promoter, nos3', CTP and CP4 EPSPS were used, positive results were obtained from genetically modified glyphosate-tolerant soybean DNA samples. There were no false positive results in non-genetically modified soybean DNA samples. Negative controls of lectin *Le*1 and *psb* A were successfully amplified from both non-genetically modified and genetically modified glyphosate-tolerant soybean DNA.

To evaluate the detection limit of the assay, serial dilutions ranging from 10 pg to 1 ng glyphosate-tolerant soybean DNA per reaction were amplified using *Agrobacterium* CP4 EPSPS primers. Products were clearly detected when less than 125 pg DNA was amplified (data not shown).

Multiplex PCR was performed in a reaction mixture containing two or more different primer sets. Serial dilutions of glyphosate-tolerant soybean DNA were added to reaction mixtures containing 0.125 μM ACT primers, 1.25 μM 35S primers, and 0.25 μM EPSPS primers. In this multiplex PCR, individual primer concentrations varied between 0.125 and 1.25 μM. Although equimolar primer mixtures did not usually provide optimal amplification, high primer concentrations may inhibit the reaction, whereas low primer concentrations may not be sufficient. The actin gene PCR product was detected when using non-genetically modified and glyphosate-tolerant soybean DNA in multiplex PCR with primer concentrations of 0.125 μM. Positive signals for the 35S promoter and CP4 EPSPS

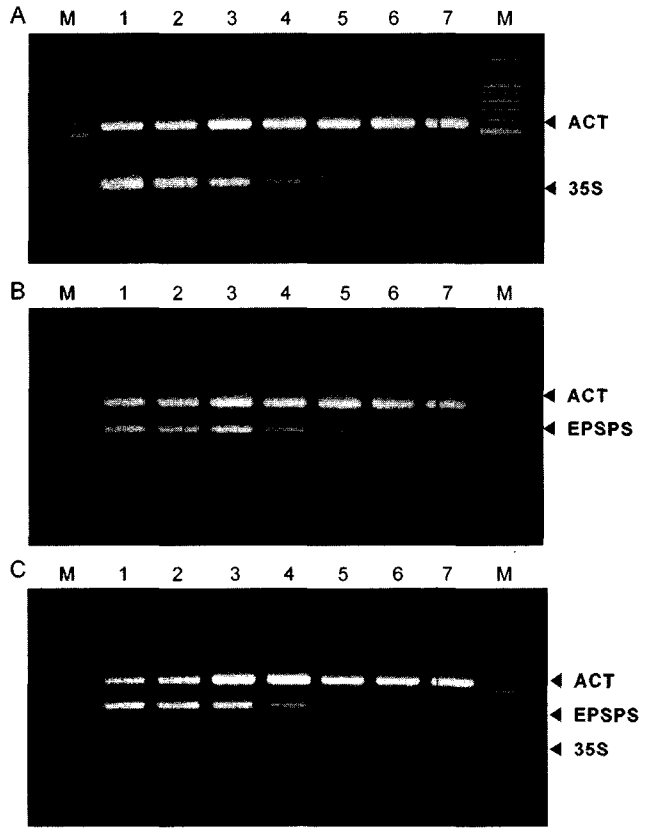

Fig. 2. PCR products amplified from 100 ng DNA mixture of glyphosate-tolerant and non-genetically modified soybean. Primers used for PCR were 0.125 μM of ACT and 1.25 μM of 35S primer (A), 0.125 μM of ACT and 0.25 μM of EPSPS primer (B), and 0.188 μM of ACT, 0.625 μM of 35S, and 0.25 μM of EPSPS primer (C).
M, 100 bp ladder; Lane 1, 5% glyphosate-tolerant soybean; Lane 2, 2% glyphosate-tolerant soybean; Lane 3, 0.5% glyphosate-tolerant soybean; Lane 4, 0.1% glyphosate-tolerant soybean; Lane 5, 0.05% glyphosate-tolerant soybean; Lane 6, 0.01% glyphosate-tolerant soybean; Lane 7, non-genetically modified soybean.

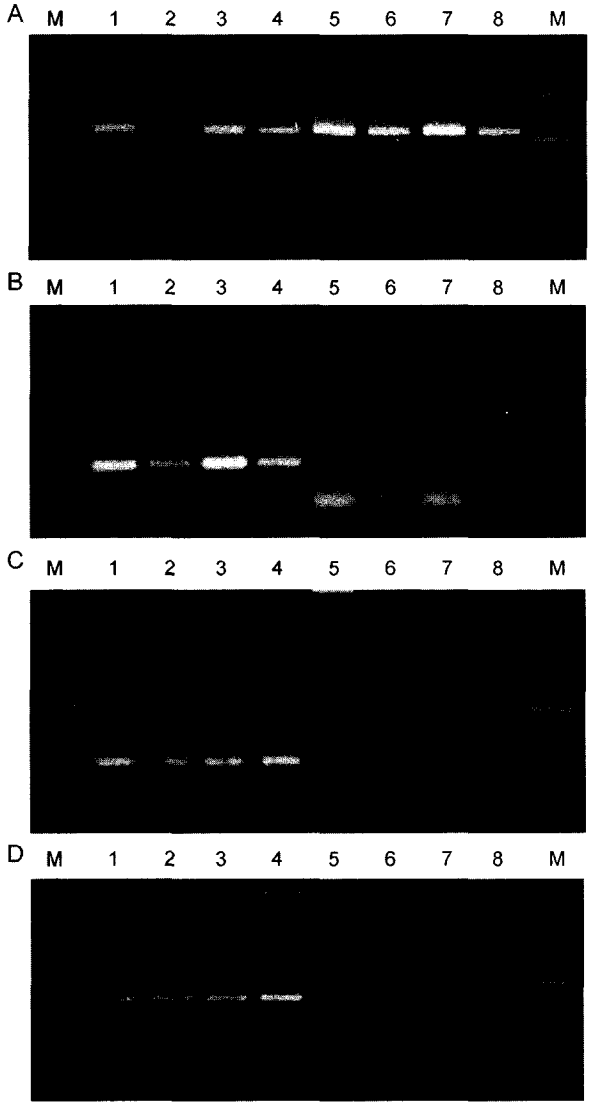

Fig. 3. Comparison of PCR products of ACT (A), 35S (B), NOS (C) and EPSPS (D) detected from LMO/non-LMO soybeans harvested after co-cultivation of LMO or non-LMO soybeans for 6 months in the same pot.
M, 100 bp ladder; Lanes 1-4, LMO soybean; Lanes 5-8, non-LMO soybean.

were obtained using 1.25 μM of 35S and 0.25 μM of EPSPS primers (Figs. 2A, 2B). In Fig. 2C, three pairs of primers were used in multiplex PCR with the concentration of ACT, 35S, and EPSPS primers being 0.188 μM, 0.625 mM, and 0.25 mM, respectively. The detection limit of the multiplex PCR ranged from 0.01% to 0.1% of the glyphosate-tolerant soybean DNA.

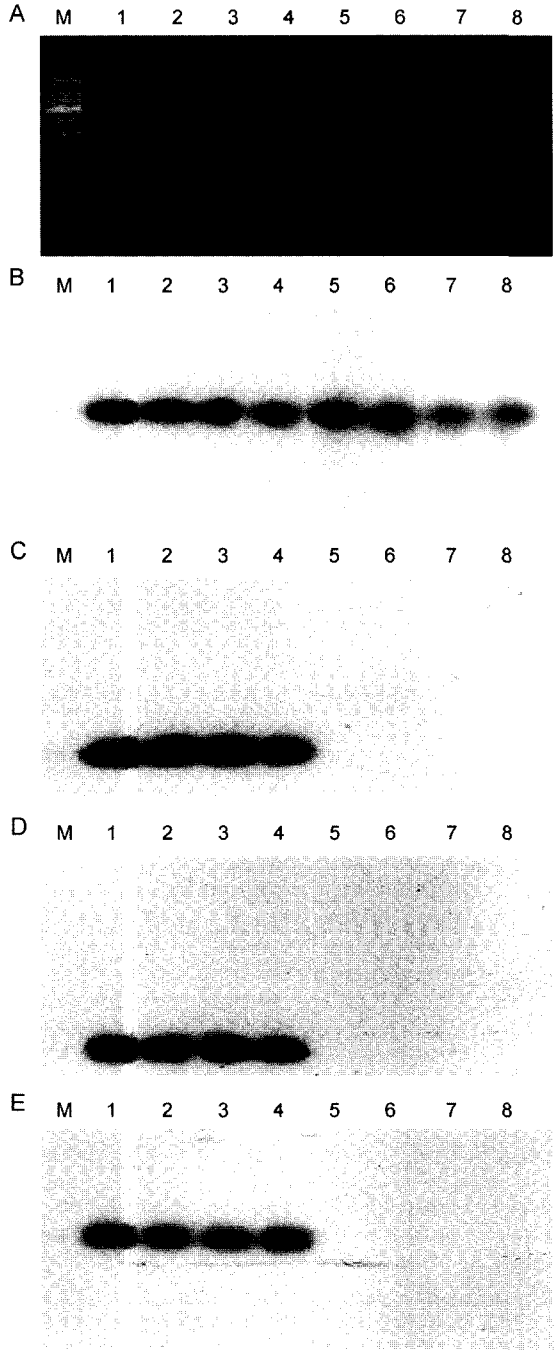

Fig. 4. *Eco*RI-digested DNA from soybean samples after co-cultivation of LMO and non-LMO soybeans for 6 months in the same pot (A), and Southern hybridization with labelled PCR products of ACT (B), 35S (C), NOS (D) and EPSPS (E).
M. 1 kb ladder; Lanes 1-4, LMO soybean; Lanes 5-8, non-LMO soybean.

Five plants of glyphosate-tolerant soybean were co-cultivated with those of non-genetically modified soybean for six months in the same pot. About 1,000 non-genetically modified soybean seeds from each pot were collected and subjected to PCR analysis. No 35S promoter, nos3', or CP4 EPSPS signals were amplified from DNA of non-genetically modified soybean co-cultivated with genetically modified soybean (Fig. 3). Southern analysis was also performed, in which genomic DNA from each soybean sample was digested with *Eco*RI, separated on 1% agarose gels, and blotted onto nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, England). PCR products of ACT, 35S, NOS, and EPSPS primers were purified using the Qiaquick gel extraction kit (QIAGEN, Hilden, Germany) and labeled with α^{32}P-dCTP using the Prime-a-gene labeling system (Promega, Wisconsin, U.S.A.). The modified church buffer (1 mM EDTA, 250 mM $Na_2HPO_4 \cdot 7H_2O$, 1% hydrolysated casein, 7% SDS, 85% H_3PO_4 adjusted to pH 7.4) was used as a hybridization solution and hybridization proceeded at 65°C for 16 h. The data presented in Fig. 4 show that 35S, nos3', and CP4 EPSPS signals were not detected when examining DNA from non-genetically modified soybean co-cultivated with genetically modified soybean.

These studies show that PCR and Southern hybridization methods are very useful for detecting modified genes. Risk assessment experiments revealed that after co-cultivation for 6 months there was no evidence of natural gene transfer of glyphosate-tolerant genes from glyphosate-tolerant soybean to non-genetically modified soybean.

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