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Investigation of Possible Gene Transfer to Soil Microorganisms for Environmental Risk Assessment of Genetically Modified Organisms

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Abstract The current study was conducted to monitor the possibility of the gene transfer among soil bacteria, including the effect of drift due to rain and surface water, in relation to the release of genetically modified organisms into the environment. Four types of bacteria, each with a distinct antibiotic marker, kanamycin-resistant *P. fluorescens*, rifampicin-resistant *P. putida*, chloramphenicol-resistant *B. subtilis*, and spectinomycin-resistant *B. subtilis*, were plated using a small-scale soil-core device designed to track drifting microorganisms. After three weeks of culture in the device, no *Pseudomonas* colonies resistant to both kanamycin and rifampicin were found. Likewise, no *Bacillus* colonies resistant to both chloramphenicol and spectinomycin were found. The gene transfer from glyphosate-tolerant soybeans to soil bacteria, including *Rhizobium* spp. as a symbiotic bacteria, was examined by hybridization using the DNA extracted from soil taken from pots, in which glyphosate-tolerant soybeans had been growing for 6 months. The results showed that 35S, T-nos, and EPSPS were observed in the positive control, but not in the DNA extracted from the soilborne microorganisms. In addition, no transgenes, such as the 35S promoter, T-nos, and EPSPS introduced into the GMO soybeans were detected in soilborne bacteria, *Rhizobium leguminosarum*, thereby strongly rejecting the possibility of gene transfer from the GMO soybeans to the bacterium.

Key words: Genetically modified organisms, gene transfer, antibiotic-resistant gene, small-scale soil-core device, soil bacteria, environmental risk assessment

The release of genetically modified microorganisms (GMOs) into the environment as a means to improve agriculture or remediate environmental hazards has raised concern over the fate of the organisms and their engineered genes [3]. The concerns related to releasing GMOs include the possibility of transferring genetic information into indigenous microflora and the conferment of unusual and potentially deleterious characters on these organisms that may perturb the natural ecosystem [1].

Although there is no clear evidence, it has been suggested that GMOs may create risks for human health and the environment such as the possibility of toxic or allergic effects, disturbance of the ecosystem, and horizontal gene transfer, all of which may emerge as long-term effects.

Despite such negative concerns, the cultivation of GMOs and the number of species have rapidly increased over the last decade. However, the techniques and methods involved in the risk assessment of GMOs have not been fully developed. Therefore, there is an urgent need for the development of a science-based methodology for the risk assessment of GMOs.

Accordingly, the current study investigated the possibility of gene transfer among soil bacteria, such as *Bacillus* and *Pseudomonas*, and from GMO soybeans to soilborne bacteria, including root nodule symbiotic bacteria, to establish basic techniques for the risk assessment of GMOs and related products.

Materials and Methods

Strains, Media, and Culture Conditions

Kanamycin-resistant *Pseudomonas fluorescens* and rifampicin-resistant *Pseudomonas putida* were incubated at 30°C for 12 h using 500 ml of LB broth containing kanamycin (50 μg/ml) and rifampicin (50 μg/ml), respectively. In addition, chloramphenicol-resistant *Bacillus subtilis* and spectinomycin-

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resistant *Bacillus subtilis* were incubated at 37°C for 12 h with the same medium containing chloramphenicol (5 μg/ml) and spectinomycin (100 μg/ml), respectively. The culture broths of the antibiotic resistant strains were then centrifuged at 6,000 ×*g* for 10 min and the pellets were suspended twice in sterilized distilled water. Thereafter, each suspension was used as an inoculum for a small-scale soil-core device.

Polymerase Chain Reaction (PCR) and Southern Hybridization

The oligonucleotide primers used to amplify the glyphosate-tolerant soybeans and *Rhizobium* genes are shown in Table 1. The genomic DNA from the glyphosate-tolerant soybeans was prepared using a DNeasy Plant Maxi Kit (QIAGEN, Hilden, Germany). About 100 ng of the isolated DNA was added to 20 μl of a 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 dNTPs, and 1 unit Taq polymerase (AccuPower PCR PreMix, Bioneer, Daejeon, Korea). Meanwhile, a cell suspension (>1×10^1 cfu/μl) of *Rhizobium leguminosarum* was added to 20 μl of a reaction mixture containing 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM of the primer, 250 μM dNTPs, and 1 unit Taq polymerase. The amplification of the 35S, NOS, and EPSPS sequences was performed under the following conditions: an initial denaturation for 3 min at 94°C; 40 cycles of PCR (94°C for 20 s, 54°C for 40 s, and 72°C for 60 s); and a final extension for 3 min at 72°C. Meanwhile, the amplification of FIXL was performed under the following conditions: an initial denaturation for 3 min at 94°C; 30 cycles of PCR (94°C for 1 min, 45°C for 1 min, and 72°C for 2 min); and a final extension for 5 min at 72°C. The PCR products of 35S, NOS, EPSPS, and FIXL were purified using a Qiaquick gel extraction kit (QIAGEN, Hilden, Germany) and labeled with α-^{32}P-dCTP using a Prime-a-gene labeling system (Promega, Madison, Wisconsin, U.S.A.). A rapid method was used for the direct extraction of DNA for the soil samples [7], then the DNA was separated on 1% agarose gels and blotted on nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, England) for Southern hybridization [5]. The *Rhizobium* spp. isolated from 10 g of homogenate root nodules of the glyphosate-tolerant soybeans was incubated on a *Rhizobium* medium (Mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4\cdot7H_2O$ 0.2 g, NaCl 0.2 g, $FeCl_3\cdot6H_2O$ 0.01 g, yeast extract 1 g, agar 20 g, D.W. 1 l, pH 7.2) using a serial dilution method. The colonies of *Rhizobium* spp. were also transferred to nylon membranes and subjected to colony hybridization [5]. A modified Church buffer (1 mM EDTA, 250 mM $Na_2HPO_4\cdot7H_2O$, 1% hydrolysated casein, 7% SDS, 85% H_3PO_4 adjusted to pH 7.4) was used as the solution for hybridization, which proceeded at 65°C for 16 h.

Risk Assessment of GMOs

To monitor the GMOs released into the environment, different antibiotic markers were used in the *Pseudomonas* and *Bacillus*. In addition, a small-scale soil-core device was constructed to monitor the GMOs, which can drift due to rain and surface water and then release GMOs into the environment (Fig. 1). Kanamycin-resistant *P. fluorescens* was inoculated into core I, while rifampicin-resistant *P. putida* was inoculated into core II. The soil cores were then sprinkled daily with 50 ml of sterilized water for 3 weeks and incubated at 25°C. The cell numbers within each soil core were examined by spreading the soil samples on LB plates containing kanamycin or rifampicin. In addition, chloramphenicol-resistant *B. subtilis* and spectinomycin-resistant *B. subtilis* were inoculated into core I and core II, respectively. The viable cell count was taken in the same way as for *Pseudomonas* except the LB plates contained chloramphenicol or spectinomycin. The flow of microorganisms in each soil-core compartment was monitored by counting the number of viable cells grown on plates containing each antibiotic. Furthermore, the possibility of gene transfer between the microorganisms in the soil core was examined by spreading the soil samples on LB plates containing two antibiotics as described above. Kanamycin-resistant *P. fluorescens* and rifampicin-resistant *P. putida* were detected in core IV after 1 week. The number of

Table 1. Primer sequences for target genes and amplicon length used in the current study.

Primer name	Primer sequence	Amplicon length (bp)	Target sequence
35S-1[a]	5'-GCTCCTACAAATGCCATCA-3'	195	CAMV
35S-2	5'-GATAGTGGGATTGTGCGTCA-3'		35S promoter
NOS-1[a]	5'-GAATCCTGTTGCCGGTCTTG-3'	180	T-nos of nopaline gene
NOS-2	5'-TTATCCTAGTTTGCGCGCTA-3'		
EPSPS-1[b]	5'-CCATAAACCCCAAGTTCCTAAATC-3'	366	35S promoter
EPSPS-2	5'-ATCCTGGCGCCCATGGCCTGCATG-3'		*Agrobacterium* CP4 EPSPS
FIXL-1[c]	5'-CCGCCCTTACTATTATCAGG-3'	609	Nitrogen fixation regulatory proteins of
FIXL-2	5'-ACAACGAGATGCGCGAAACT-3'		*Rhizobium leguminosarum* bv. *phaseoli*

[a]Spoth and Strauss [6].
[b]Matsuoka *et al.* [4].
[c]D'hooghe *et al.* [2].

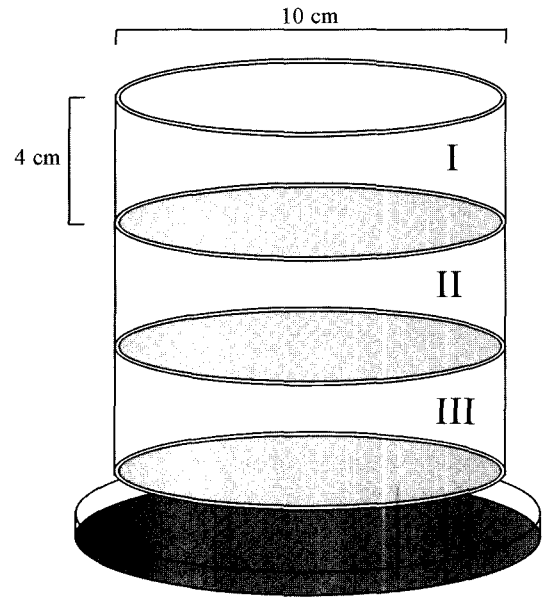

Fig. 1. Diagram of small-scale soil-core device inoculated with bacteria containing different antibiotic markers.
Fifty milliliters of sterilized water was drained from cores I to III everyday, and the drained water was collected in core IV.

viable cells differed in each soil-core compartment, and the cell density declined gradually from the top to the bottom of the soil-core device as time elapsed. No antibiotic-resistant gene transfer was detected between the *Pseudomonas* strains with different antibiotic markers in any of these experiments (Table 2).

Conversely, the number of chloramphenicol-resistant *Bacillus* after 1 week was 1.5×10^7 cfu/g in core I and 0.8×10^4 cfu/g in core IV. As such, the viable cell number of *Bacillus* in each soil-core compartment declined 10-fold as compared to the initial inoculum from the top to the bottom of the soil-core device. However, the number of chloramphenicol- or spectinomycin-resistant *Bacillus* in cores III and IV, respectively, after 3 weeks was higher than that in the initial inoculum. Therefore, the release of *Bacillus* into the environment should be carefully considered due to their high survival rate. No gene transfer was detected between the two *Bacillus* strains in double-antibiotic selection (Table 2). Although the *Bacillus* strains were incubated in an enrichment culture with both chloramphenicol and spectinomycin for 24 h, no gene transfer between the strains was observed (data not shown). Accordingly, the current results can be useful as a basis for the detection, monitoring, and risk assessment of GMOs released into the environment. In addition, the small-scale soil-core device used in the present study would seem to be effective for investigating the possibility of gene transfer from GMOs to other microorganisms before releasing GMOs directly into the environment.

Gene Transfer Between GMO Soybeans and Soilborne Bacteria

To investigate the possibility of gene transfer from GMO soybeans to soilborne bacteria including root nodule bacteria such as *Rhizobium*, total DNA was extracted from the soils taken from plastic pots, in which glyphosate-

Table 2. Number of *Pseudomonas* (A) and *Bacillus* (B) colonies recovered on antibiotic-containing LB agar plates using soil samples obtained from each soil core during 3 weeks of culture in a small-scale soil-core device.

A

Soil core	Total recoverable cfu ($\times10^4$) per 1 g of soil samples								
	1 week			2 weeks			3 weeks		
	Kam	Rif	Kam/Rif	Kam	Rif	Kam/Rif	Kam	Rif	Kam/Rif
I	1624	-	-	504	-	-	286	-	-
II	41	751	0	49	284	0	7	112	0
III	9	5	0	5	2	0	5	3	0
IV	39	16	0	0.3	1	0	0	1	0

B

Soil core	Total recoverable cfu ($\times10^4$) per 1 g of soil samples								
	1 week			2 weeks			3 weeks		
	Cam	Spc	Cam/Spc	Cam	Spc	Cam/Spc	Cam	Spc	Cam/Spc
I	1514	-	-	1026	-	-	892	-	-
II	279	914	0	516	907	0	546	826	0
III	24	18	0	248	72	0	390	83	0
IV	0.8	0.2	0	137	19	0	357	99	0

A: Kanamycin (50 μg/ml)-resistant *P. fluorescens* and rifampicin (50 μg/ml)-resistant *P. putida* were initially inoculated into core I and core II, respectively.
B: Chloramphenicol (5 μg/ml)-resistant *B. subtilis* and spectinomycin (100 μg/ml)-resistant *B. subtilis* were initially inoculated into core I and core II, respectively.

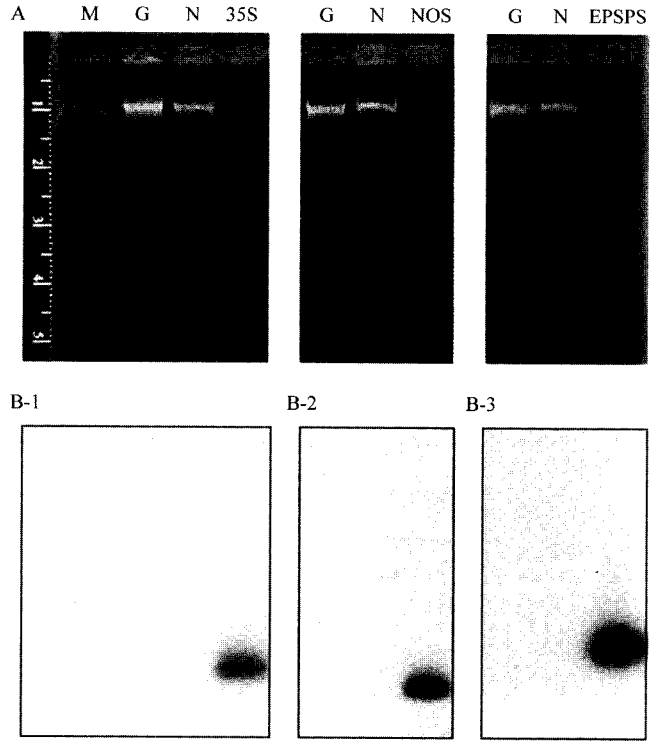

Fig. 2. Crude DNA extract from soil samples (A) and Southern hybridization with probe DNAs of 35S (B-1), NOS (B-2), and EPSPS (B-3).

M, λ *Hin*dIII; G, soil sample supplemented with glyphosate-tolerant soybeans; N, soil sample supplemented with nonglyphosate-tolerant soybeans; 35S, positive control of 35S promoter; NOS, positive control of T-nos; EPSPS, positive control of EPSPS.

tolerant soybeans had been growing for 6 months, and analyzed by Southern hybridization. As a result, the 35S promoter, T-nos, and EPSPS were all observed in the positive control, but not in the total DNA extracted from the soilborne bacteria, suggesting that no gene transfer occurred from the glyphosate-tolerant soybeans to the soilborne bacteria (Fig. 2). In addition, the possible gene transfer from glyphosate-tolerant soybeans to root nodule symbiotic bacteria such as *Rhizobium* spp. was also examined. The endogeneous gene, *fixL*, of *Rhizobium leguminosarum* was detected by colony hybridization. However, no foreign genes, such as the 35S promoter, T-nos, and EPSPS introduced into the GMO soybeans were detected in the *Rhizobium leguminosarum*. Therefore, these results strongly suggested the negative possibility of the gene transfer from the GMO soybeans to root nodule bacteria (Fig. 3).

The current study examined the possibility of gene transfer using soil microorganisms and GMO plants for an environmental risk assessment of GMOs. The results showed that the use of a soil-core device was very effective in monitoring the gene transfer among soil bacteria, including the impact of rain and surface water, in relation

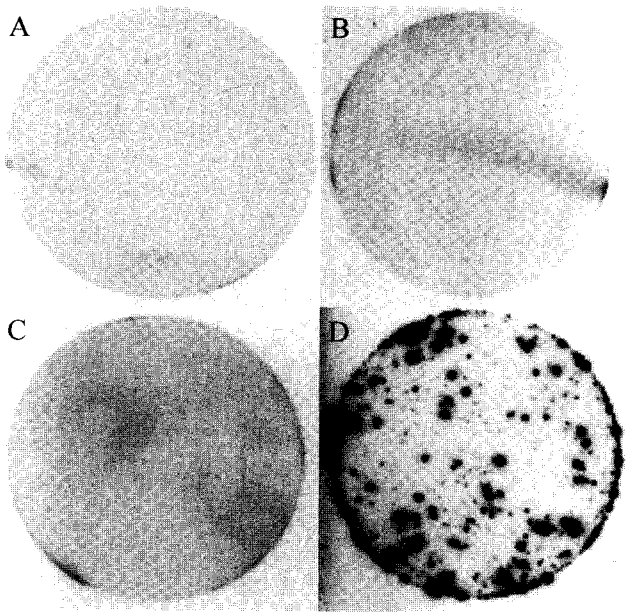

Fig. 3. Colony hybridization blot with probe DNA of 35S (A), NOS (B), EPSPS (C), and FIXL (D).

to the release of GMOs into the environment. In addition, environmental risk assessment experiments revealed that, after cultivation of 6 months, there was no evidence of gene transfer of glyphosate-tolerant genes from GMO soybeans to soilborne bacteria, including the root nodule bacteria, *Rhizobium*. Although these experiments were only performed in a small scale using soil-core devices and pots, they can still serve as an effective model system for a large-scale environmental risk assessment before releasing GMOs directly into the environmental ecosystem.

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