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Investigation of Possible Horizontal Gene Transfer from the Leaf Tissue of Transgenic Potato to Soil Bacteria

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Abstract To monitor the possibility of horizontal gene transfer between transgenic potato and bacteria in the environment, the gene flow from glufosinate-tolerant potato to bacteria in soils was investigated. The soil samples treated with the leaf tissue of either glufosinate-tolerant or glufosinate-sensitive potato were subjected to PCR and Southern hybridization to determine possible occurrence of glufosinate-resistant soil bacteria and to detect the *bar* (phosphinothricin acetyltransferase) gene, conferring tolerance to glufosinate. The *bar* gene was not detected from genomic DNAs extracted at different time intervals from the soil samples, which had been treated with the leaf tissue of either transgenic or non-transgenic potato for 2 to 8 weeks. In addition, the level of glufosinate-resistant bacteria isolated from the soil samples treated with the leaf tissue of transgenic potato was similar to that of the samples treated with non-transgenic potato after 4 months of incubation at 25°C. The *bar* gene was not detected in the genomic DNAs extracted from colonies growing on the plate containing glufosinate, indicating that the bacteria could acquire the resistant phenotype to glufosinate by another mechanism without the uptake of the *bar* gene from glufosinate-tolerant potato.

Key words: Transgenic potato, herbicide tolerance, glufosinate, horizontal gene transfer, microcosm, environmental risk assessment

Numerous field releases of genetically modified plants has been performed during the last decade. Most of them contain selectable markers such as bacterial antibiotic resistance genes. It has been suggested that the transfer of plant-harbored antibiotic resistance genes to bacteria from transgenic plants may have harmful effects on human health and the natural ecosystem in large-scale application of transgenic plants [3, 4, 7, 13]. However, it has not been shown experimentally that horizontal gene transfer of genetic materials from transgenic plants to bacteria occurs [2]. Recently, however, transformation of *Acinetobacter* sp. BD413 with DNA from transgenic plant carrying the *npt*II gene of Tn5, which encodes an aminoglycoside phosphotransferase conferring neomycin and kanamycin resistance, was demonstrated [4, 5].

The horizontal gene transfer from transgenic plants to microorganisms in soils requires the extracellular DNA available for bacterial uptake. The DNA can be released into soil from decaying plant materials [14]. If such DNA released into the environment is rapidly degraded by the DNase present in soil, it is unlikely to be taken up by bacteria. However, several groups have shown the persistence of free DNA absorbed to purified soil components and to clay-amended soils [1, 9–11, 15]. Transgenic markers, including NOS (terminator of nopaline synthase gene from *Agrobacterium tumefaciens*) and the *npt*II gene from the leaf tissue of transgenic tobacco, were found to be initially degraded at a high rate in soils, but a small fraction of these DNA fragments were still detected in soil samples even after a few months [14, 15]. Furthermore, many workers have recently demonstrated that, in soil samples collected from fields, the targeted specific sequences are detected for more than two years after the harvest of crops [6]. These results indicate that DNAs in soils may be stabilized on soil particles and persist in the environment for a considerable period of time.

Considering the fast growing ag-biotechnology and increasing cultivation area of living modified organisms

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(LMOs) for the last decade, there are urgent needs to establish basic techniques for the environmental risk assessment of LMOs [8]. Thus, we have investigated the possibility of horizontal gene transfer from herbicide-tolerant potato, which have the *bar* gene encoding phosphinothricin acetyl transferase from *Streptomyces hygroscopicus*, to soilborne bacteria.

The leaf tissues of non-glufosinate-tolerant and glufosinate-tolerant potatoes (1 g each) were mixed with 9 g of commercial horticulture nursery medium (Bio-media, Gyeongju, Korea) and incubated at 25°C for 4 months in a small-scale microcosm. The enrichment culture of soil samples, which had been treated with the leaf tissue of either transgenic or non-transgenic potato, with LB broth (Difco, Detroit, MI, U.S.A.) containing glufosinate were performed at 30°C for 4 days. The colony forming unit (CFU) was determined by the dilution plate method from each mixture of the leaf tissue of either transgenic or non-transgenic potato and soil samples. Glufosinate-resistant bacteria, *Bacillus* and *Pseudomonas*, from soil samples treated with the leaf tissue of either transgenic or non-transgenic potato were isolated using LB agar and *Pseudomonas* agar F (Difco, Detroit, MI, U.S.A.), respectively. LB and *Pseudomonas* agar F plates containing glufosinate were incubated at 30°C for 24–72 h.

Each soil DNA was extracted at different time intervals from a mixture of the leaf tissue of potato and soil samples using a modified protocol for small-scale DNA extraction from a wide variety of environmental samples [15]. One gram of samples was mixed with 7 ml of extraction buffer (250 mM NaCl, 100 mM EDTA, pH 8, 2% SDS) and vortexed for 15 sec. After the addition of 50 μl of 5 M guanidine isothiocyanate, samples were again mixed for 15 sec and stored at −20°C. For DNA extraction, samples were heated to 68°C for 10 min, sonicated for 3 min in a Branson 5510 bath sonicator (Branson, Danbury, CT, U.S.A.), and further incubated for 1 h with vigorous shaking every 15 min. Samples were centrifuged at 18,600 ×*g* for 5 min at room temperature, and an aliquot of isopropanol (7 ml) was added to precipitate the DNA, and the preparation was stored at −20°C overnight. After centrifugation for 30 min at 4°C, pellets were washed with 70% ethanol and dissolved in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The crude DNA was separated on 1% low-melting agarose (SeaPlague GTG agarose, BMA, Rockland, MA, U.S.A.) gel. The DNA was further purified from the gel slice containing the DNA band using DNA Prepmate II (Bioneer, Seoul, Korea) and used in the following experiments.

Primers used for PCR amplification of the *bar* gene were specific for the glufosinate ammonium (Basta, Aventis Korea) tolerance gene encoding phosphinothricin acetyl transferase from *Streptomyces hygroscopicus*. A 429-bp sequence from the *bar* gene was obtained by PCR amplification of the genomic DNA of transgenic potato with forward primer, 5'-CACTACATCGAGACAAGCAC-3' and reverse primer, 5'-TGAAGTCCAGCTGCCAGAAA-3'. PCR was performed on an i-cycler (Bio-Rad, Hercules, CA, U.S.A.). About 100 ng of isolated DNA was added to 20 μl of reaction mixture containing 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 1.25 μM each of primer, 250 μM dNTP, and 1 unit of Taq polymerase (AccuPower PCR PreMix, Bioneer, Seoul, Korea). Amplification was performed under the following conditions: denaturation for 3 min at 94°C, 30 cycles (94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min), and terminal elongation for 5 min at 72°C. An aliquot (20 μl) of amplification products was separated using 1% agarose gels (SeaKem LE agarose, BMA) 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 ethidium bromide solution.

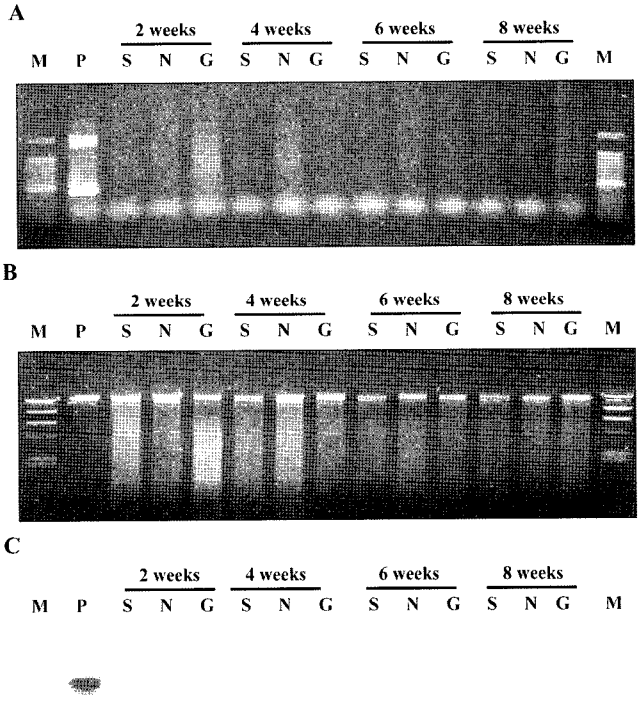

Fig. 1. PCR amplification products of the *bar* gene (A) and genomic DNAs extracted from soil samples treated with the leaf tissue of either glufosinate-tolerant or non-glufosinate-tolerant potato after 2 to 8 weeks (B), and Southern hybridization with the probe DNA of the *bar* gene (C).
M, *Hin*dIII digested lambda DNA; P, Genomic DNA from glufosinate-tolerant potato; S, DNA extracted from soil sample untreated with the leaf tissue of either glufosinate-tolerant or non-glufosinate-tolerant potato; N, DNA extracted from soil sample treated with the leaf tissue of non-glufosinate-tolerant potato; G, DNA extracted from soil sample treated with the leaf tissue of glufosinate-tolerant potato.

Table 1. Number of total colonies recovered from the enrichment culture broth of soil samples treated with the leaf tissue of non-transgenic and transgenic potato after 4 months of incubation

(Unit: $\times 10^8$ CFU/g)

Samples	Glufosinate (%)	CFU
Non-transgenic potato	0	87
	0.1	79
	0.5	78
	1.0	36
Transgenic potato	0	124
	0.1	105
	0.5	104
	1.0	37

*The number of soil bacteria was counted after the enrichment culture on LB broth for 4 days with soil samples treated with the leaf tissue of non-transgenic and transgenic potato after 4 months of incubation.

Soil DNAs extracted from each soil sample were also separated on 1% agarose gel (SeaKem LE agarose, BMA) in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and blotted to nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) for Southern hybridization [12]. A PCR amplification product of the *bar* gene was 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, Madison, WI, 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, pH 7.4) was used as a hybridization solution, and hybridization proceeded at 65°C for 16 h.

To examine the possibility of horizontal gene transfer released into the environment from transgenic potato to soil bacteria, genomic DNAs were extracted from the soils treated with the leaf tissue of either glufosinate-tolerant or non-glufosinate-tolerant potato after 2 to 8 weeks and analyzed by PCR and Southern hybridization. The result showed that the *bar* gene was observed in the positive control, but not in the genomic DNA extracted from soil samples, which had been treated with the leaf tissue of either transgenic or non-transgenic potato for 2 to 8 weeks (Fig. 1). The detection limit of the *bar* gene using genomic DNA of transgenic potato was as low as 125 pg (data not shown).

To further investigate the possibility of horizontal gene transfer from the leaf tissue of glufosinate-tolerant potato to soilborne bacteria, the soil samples were treated with the leaf tissue of either transgenic or non-transgenic potato for 4 months, and the possible occurrence of glufosinate-resistant bacteria was examined. The number of glufosinate-resistant colonies did not differ significantly after enrichment for 4 days with LB broth containing glufosinate (Table 1). The population of glufosinate-resistant bacteria, *Bacillus* and *Pseudomonas*, isolated from the soil samples treated with the leaf tissue of glufosinate-tolerant potato was similar to that of non-glufosinate-tolerant potato (Table 2). The soil samples treated with up to 50% of leaf tissue of either glufosinate-tolerant or non-glufosinate-tolerant potato gave a similar result (data not shown).

The genomic DNAs extracted from colonies growing on the glufosinate-containing LB agar plates were subjected to PCR and Southern hybridization analysis for the detection of the *bar* gene. However, no positive signal was obtained from genomic DNA of glufosinate-resistant colonies, except for the probe DNA (Fig. 2), indicating that the bacteria could acquire the phenotype of resistance to glufosinate by

Table 2. Number of colonies recovered from soil samples treated with the leaf tissue of non-transgenic and transgenic potato after 4 months of incubation.

(Unit: $\times 10^4$ CFU/g)

Samples		No glufosinate		Glufosinate (0.0025%)		Glufosinate (0.005%)		Glufosinate (0.0125%)	
		Pseudomonas spp.	*Bacillus* spp.	*Psudomonas* spp.	*Bacillus* spp.	*Pseudomonas* spp.	*Bacillus* spp.	*Pseudomonas* spp.	*Bacillus* spp.
Non-LMO	1	207	242	147	182	17	36	0	0
	2	203	280	124	256	16	42	0	0
	3	209	311	164	228	20	42	0	0
	4	219	320	148	213	16	51	0	0
	5	213	315	133	198	16	49	0	0
	Mean	210	294	143	215	17	44	0	0
LMO	1	201	260	125	199	18	51	0	0
	2	248	251	144	216	15	50	0	0
	3	234	289	163	189	15	38	0	0
	4	244	262	150	221	12	46	0	0
	5	214	299	137	221	19	53	0	0
	Mean	228	272	144	209	16	48	0	0

Non-LMO, Soil sample treated with the leaf tissue of non-glufosinate-tolerant potato; LMO, Soil sample treated with the leaf tissue of glufosinate-tolerant potato; *Bacillus* spp. and *Pseudomonas* spp. were incubated at 30°C for 24–72 h.

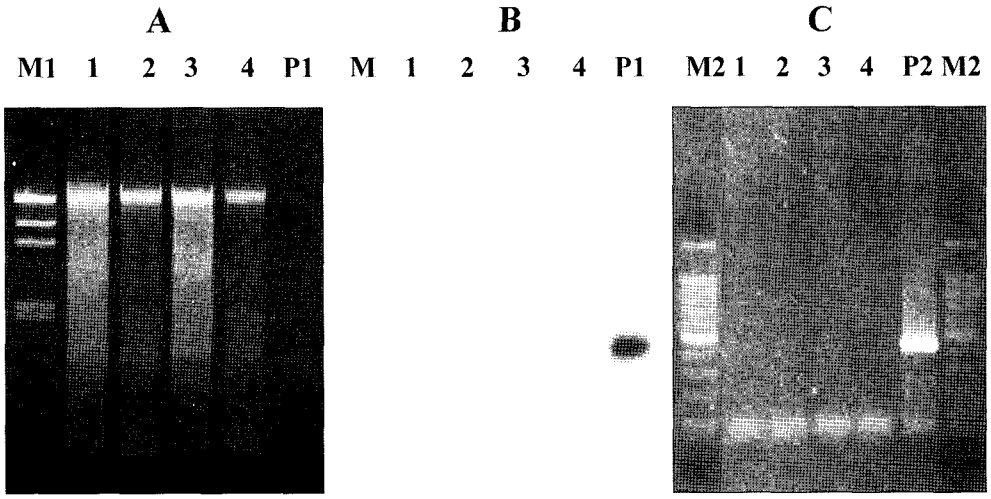

Fig. 2. Electrophoretic pattern on agarose gel of genomic DNAs extracted from soil samples treated with the leaf tissue of either glufosinate-tolerant or non-glufosinate-tolerant potato for 4 months (A), autoradiograms obtained after Southern hybridization with probe DNA of the *bar* gene (B), and PCR amplification products of the *bar* gene (C).
M1, *Hin*dIII-digested lambda DNA; M2, 100 bp DNA ladder; 1, 2, DNAs extracted from soil samples treated with the leaf tissue of either glufosinate-tolerant or non-glufosinate-tolerant potato for 4 months; 3, 4, DNAs extracted from soil samples treated with the leaf tissue of glufosinate-tolerant potato for 4 months; P1, Probe DNA of the *bar* gene as a positive control; P2, DNA extracted from the leaf tissue of glufosinate-tolerant potato as a positive control.

other mechanisms, but not by taking up the *bar* gene from glufosinate-tolerant potato.

The results of the present study showed no evidence of transfer of the glufosinate-resistant gene from the leaf tissue of glufosinate-tolerant potato to soil bacteria during 4 months of incubation. Although these experiments have been performed in a small-scale microcosm and are far short of being applied in the natural ecosystem, it can serve as a useful model system for the investigation of possible gene transfer of transgenic plants before releasing them directly into the environment. However, a final conclusion should be made after experimental data from confined field tests have been repeatedly collected.

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