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Molecular Analysis of Bacterial Community Structures in Paddy Soils for Environmental Risk Assessment with Two Varieties of Genetically Modified Rice, Iksan 483 and Milyang 204

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The impacts of planted transgenic rice varieties on bacterial communities in paddy soils were monitored using both cultivation and molecular methods. The rice field plot consisted of eighteen subplots planted with two genetically modified (GM) rice and four non-GM rice plants in three replicates. Analysis with denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes revealed that the bacterial community structures were quite similar to each other in a given month, suggesting that there were no significant differences in bacterial communities between GM and non-GM rice soils. The bacterial community structures appeared to be generally stable with the seasons, as shown by a slight variation of microbial population levels and DGGE banding patterns over the year. Comparison analysis of 16S rDNA clone libraries constructed from soil bacterial DNA showed that there were no significant differences between GM and non-GM soil libraries but revealed seasonal differences of phyla distribution between August and December. The composition profile of phospholipid fatty acids (PLFA) between GM and non-GM soils also was not significantly different to each other. When soil DNAs were analyzed with PCR by using primers for the *bar* gene, which was introduced into GM rice, positive DNA bands were found in October and December soils. However, no *bar* gene sequence was detected in PCR analysis with DNAs extracted from both cultured and uncultured soil bacterial fractions. The result of this study suggested that, in spite of seasonal variations of bacterial communities and persistence of the *bar* gene, the bacterial communities of the experimental rice field were not significantly affected by cultivation of GM rice varieties.

Keywords: 16S rRNA, transgenic plant, DGGE, bacterial community, rice field

In spite of considerable public concerns about the impact of genetically modified (GM) crops on the environment, the total agricultural area planted with GM crops has increased more than 60 fold during the 11-year period from 1996 to 2006 [15]. Among commercialized transgenic crops, in particular, those conferring tolerance to herbicides continuously increased so that they occupied 68% of the global GM crop area in 2006 [15]. Transgenic plants of rice, maize, and soybean, which are tolerating a nonselective herbicide, glufosinate ammonium, are among the dominant herbicide-tolerant crops. Glufosinate-tolerant plants contain the phosphinothricin acetyltransferase gene (*bar* or *pat* gene) derived from *Streptomyces hygroscopicus* [42] and *Streptomyces viridochromogenes* [45], respectively.

One of the major concerns regarding the release of transgenic crops is their potential for unwanted adverse effects on the soil microbial community structure. In recent studies, although significant differences in microbial communities between GM plant and non-GM plant were observed during the plant growing period [8, 38], the differences were temporary and dependent on the presence of the viable plant [9, 46]. Other studies showed that the

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microbial communities were mostly not altered in the rhizosphere of transgenic plants [13, 33, 34, 36].

A variety of experimental methods have been employed to assess the influences of transgenic plants on various aspects of soil microorganisms. Culture-dependent methods such as plating, most probable number, and Biolog GN methods, and cultivation-independent methods such as DNA fingerprint analyses of amplified rDNA genes and microbial lipid analyses, have been generally used by integrating two or three of the above methods in a risk assessment experiment [19, 23]. In addition to structural and functional changes of soil microbial community structure, potential impacts such as horizontal gene transfer of integrated gene and particular marker genes to bacteria are a great problem in risk assessment issues. Recently, it has been reported that transgenic DNA could persist in soil for a long-term period under microcosm and field conditions [6, 44]. It is suggested that transgenic DNA released from plant debris can serve as a reservoir of genetic information for indigenous soil bacteria. Experimental studies confirmed that the horizontal gene transfer (HGT) events from transgenic plant to bacteria occurred at extremely low frequencies in optimized laboratory conditions [12, 24] and have not yet been detected under field conditions. In spite of these recent safety researches with GM plants, still very limited information is available about their impacts on bacterial communities and gene flow in soil.

This study aimed to assess the impacts of transgenic rice varieties on bacterial communities and monitor the possibility of horizontal gene transfer from transgenic rice varieties to bacteria. The diversity and variation of bacterial community structure were analyzed by a culture-dependent plating method and cultivation-independent methods such as DGGE profiles, soil FAME (fatty acid methyl ester) analysis, and direct cloning of amplified 16S rDNA. In order to monitor the fate of released transgenic plant DNA, bacterial community DNAs obtained by both direct and indirect extraction methods were analyzed with PCR using specific primers that targeted the integrated *bar* gene sequence.

Materials and Methods

Soil Samples and Culture Media

Soil samples were obtained from the risk assessment experimental area at the Agricultural Research site at Yesan, Chungchungnamdo, Korea. The plot consisted of eighteen subplots, each 12.0 by 16.2 m. Six different rice varieties (two glufosinate-resistant GM rice plants, Iksan 483 and Milyang 204; four parental non-GM rice plants, Junam, Anjung, Dongjin, and Sindongjin) were planted in April and grown on the subplots in three replicates. Soil samples taken from each subplot in the middle of April, August, October, and December were sieved through a 2-mm mesh and kept at 4°C prior to use. A 10-g soil sample from each site was homogenized with 95 ml of a sterilized 0.85% saline solution by shaking at 200 rpm on a rotary shaker (Vision Co., Bucheon, Korea). Total counts of bacteria, actinomycetes, and fungi were measured with tryptic soy agar, sodium caseinate agar, and malt extract agar media, respectively [43].

PCR Amplification of Soil Bacterial 16S rDNA

Soil microbial community DNA was extracted using a FastDNA Spin Kit (Qbiogene, Carlsbad, U.S.A.). PCR was carried out using the primers 27f and 1492r, which target the 16S rRNA genes of a wide range of members of the domain Bacteria at positions 28 through 1491 (*E. coli* 16S rRNA gene sequence numbering) [20]. PCR amplification was performed in 50-μl reaction mixtures containing 10×PCR buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris/HCl, pH 8.8, 25 mM $MgCl_2$, 0.1% Tween 20], 1 μl of template DNA, 25 pmol of each primer, 200 μM of each dNTP (GeneCraft, Munster, Germany), and 2.5 U of *Taq* polymerase (GeneCraft, Munster, Germany). PCR cycles consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. After PCR amplification, 2-μl samples of the PCR products were checked by electrophoresis on horizontal 1.5% agarose gels.

Cloning and Sequencing

The 16S rDNA PCR products obtained from the soil bacterial community DNA were cloned into the pGEM-T Easy Vector as recommended by the manufacturer (Promega, Madison, U.S.A.). The preparation of randomly selected clones followed by PCR amplification of cloned inserts and purification of PCR products were carried out as described previously [14]. Sequencing was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, U.S.A.) according to the manufacturer's instructions with the sequencing primers 27f and 519r.

Denaturing Gradient Gel Electrophoresis Analysis

Soil bacterial community DNA was extracted using a FastDNA Spin Kit (Qbiogene, Carlsbad, U.S.A.). PCR amplification of the 16S rRNA genes was performed with primer pairs F352T-519r [1] and 1070f-1392r (*E. coli* 16S rRNA gene sequence numbering) [11]. The PCR product contains a GC clamp of 40 bases, added to F352T and 1392r, respectively, including the highly variable V3 and V8 regions. PCR reaction mixtures were prepared as previously described [1]. PCR cycles consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were subjected to DGGE analysis with the Dcode Universal Mutation Detection System (BIO-RAD, Hercules, U.S.A.) as described previously [17]. After electrophoresis, the gels were stained with SYBRGreen I (Cambrex BioScience, Rockland, ME, U.S.A.) for 15 min, rinsed for 25 min, and photographed with UV transillumination (302 nm). DGGE profiles were finally analyzed by the GelCompar 4.0 program (Applied Maths, Ghent, Belgium) with a slight modification of normalization settings [38].

PLFA Analysis

The analysis of phospholipid fatty acid (PLFA) was performed as previously described by Bossio and Scow [3]. Briefly, soil samples (5 g dry weight) were extracted for 2 h in 23 ml of a one-phase

extraction mixture containing chloroform:methanol:phosphate buffer (1:2:0.8 v/v/v). Supernatant from the extraction was mixed with phosphate buffer and chloroform, and the phases were allowed to separate overnight. Phospholipids were separated from other lipids on a silicic acid column. Following a mild alkaline methanolysis of the phospholipids, the resulting FAMEs were separated and quantified by a Hewlett Packard 6890A (Palo Alto., CA, U.S.A.) Gas Chromatograph with a 25-m Ultra 2 (5%-phenyl)-methylpolysiloxane column (J&W Scientific, Folson, U.S.A.). Peaks were identified using MIDI peak identification software (MIDI, Inc., Newark, U.S.A.). To compare the microbial communities, the samples were subjected to principal component using SAS (SAS Institute, Cary, U.S.A.). The principal component data were analyzed by analysis of variance (ANOVA).

Extraction of DNA from Bacterial Fractions of the Soil Matrix

Bacterial cells were separated from soil particles using high-speed centrifugation on a Nycodenz density gradient (Axis-Shield PoC, Oslo, Norway) with different pretreatment processes as described previously [5]. Soil samples (10 g) were suspended in 200 ml of 0.85% NaCl and homogenized in a 1-l Waring blender (Waring, Torrington, U.S.A.) for three 1-min intervals at maximum speed (22,000 rpm), with cooling in ice for 1 min between intervals. Twenty ml soil suspension was placed on top of a 7-ml Nycodenz cushion, followed by centrifugation at 10,000 ×*g* in a swing-out rotor (Kontron Instruments, Milano, Italy) for 60 min at 4°C. The separated bacterial cell layer was recovered with a pipette. Bacterial suspensions were washed with sterile water to remove the Nycodenz solution from the bacterial cells before centrifugation for 20 min at 10,000 ×*g* [22]. The cell pellet was finally resuspended in 1 ml of sterile water with 0.85% NaCl. As the second option, the soil pellet obtained after the first Nycodenz density gradient centrifugation was resuspended in sterile 0.85% NaCl solution by vortexing and sonication, and one additional extraction was carried out as above to improve the recovery of bacterial fraction. The second bacterial fraction recovered was added to the first bacterial fraction. As the third option, incubation of soil sample in 6% yeast extract solution for 1 h at 40°C, to induce bacterial spore germination before blending, was applied to increase the population of *Actinobacteria* [5], because the *bar* gene was originated from *Streptomyces* sp. belonging to *Actinobacteria*. Total bacterial DNA was extracted from both the bacterial fractions recovered and the soil sediments after Nycodenz density gradient centrifugation using a FastDNA Spin Kit (Qbiogene, Carlsbad, U.S.A.). To get cultivated microbial community DNA, each soil sample of six GM sites was homogenized with sterilized 0.85% saline, and after sieving with 0.5-mm mesh to exclude plant debris, the mixture was inoculated in Nutrient Both. Total soil bacterial DNA was extracted after incubating for 7 days. All DNA yields were estimated on agarose gels (0.7% w/v) by comparing the band intensity against a standard curve of Lambda DNA (Fermentas, Hanover, U.S.A.).

PCR Amplification of *bar* Gene Sequence

PCR amplification of *bar* gene fragments was carried out using the primers barF and barR (5'-TCTGCACCATCGTCAACCACTACATC-3' and 5'-CAGAAACCCACGTCATGCCAG-TTC-3'). Flanking regions of the *bar* gene were also amplified using primer pairs pbarF-pbarR (5'-TCTACACCCACCTGCTGAAGTC-3' and 5'-TAATCATCGCA-AGACCGGCAAC-3') and bbarF-bbarR (5'-AACTGGCATGACGT-GGGTTTCT-3' and 5'-ATGCTTCCGGCTCGTATGTTGT-3'), which were designed in this study. Rice genomic DNA, positive control DNA for *bar* gene PCR, was isolated from the young leaves of GM rice plants (Iksan483 and Milyang204) using a method of Rogers and Bendich [27]. For the 430-bp *bar* fragment amplification, the PCR amplification of the target gene was performed using published PCR conditions [25]. The amplification conditions were as follows; initial denaturation at 94°C for 10 min, subsequent denaturations at 94°C for 1 min, annealing at 68°C for 2 min, extension at 72°C for 10 min (36 cycles) and the final extension at 72°C for 10 min. For pbar and bbar primer pairs, annealing temperatures were 65°C and 58°C, respectively. After PCR amplification, 2 μl samples of the PCR products were checked by electrophoresis on horizontal 1.0% agarose gels.

Analysis of 16S rDNA Sequences

16S rDNA sequences were checked for chimeras using the Mallard program [2]. Sequences from this study and reference sequences were subsequently aligned using CLUSTAL X [41]. An average of at least 600 nucleotides was included in the phylogenetic analysis. Phylogenetic trees were constructed by the bootstrapped (1,000 iterations) neighbor-joining method, using PAUP 4.0 (http://paup.csit.fsu.edu). For analysis of clone libraries, statistical tools for comparing community structure were used. After aligning all sequences, the Jukes-Cantor corrected distance matrix was constructed using the DNADIST program from PHYLIP [10]. Species richness and diversity of each community were estimated by DOTUR [31], which assigns sequences to operational taxonomic units (OTUs) based on the genetic distance between sequences. To determine whether the community structures of two communities are the same, different, or subsets of one another, both the distance-based and phylogeny-based tests were performed. The ∫-LIBSHUFF program [30] was used to compare clone libraries as the distance-based test. This tool compares homologous and heterologous coverage curves by integral form of Cramér-von Mises statistic and performs multiple pair-wise comparisons among a set of libraries. For the phylogeny-based statistical analysis, clone libraries were compared using TreeClimber [32], which implements a parsimony-based test to determine whether the differences between two communities arose from random variation or whether lineages from one community had become more dominant through negative or positive selection pressures.

Nucleotide Sequence Accession Numbers

The partial 16S rRNA gene sequences from the clone libraries have been deposited in the GenBank nucleotide sequence databases under accession numbers EF613726 through EF614125.

RESULTS

Changes of Total Microbial Populations

The total viable counts of bacteria, fungi, and actinomycetes in rice soils were estimated over the year. The bacterial cell densities, which were in the range of $1.4\times10^7-4.0\times10^7$ cells/g soil over the year, were not significantly different between GM and non-GM rice soils in a given month. The fungal and actinomycetes population densities, which were in the range of $1.9\times10^4-4.3\times10^4$ cells/g soil

and $1.4\times10^5-5.0\times10^5$ cells/g soil, respectively, also showed no significant difference between GM and non-GM rice soils during this study. One-way analysis of variance (ANOVA) was used to determine statistical significance of subplot (position) and transgenic rice (GM versus non-GM) effects on the number of microbial populations. As a result of the analysis, no significant difference was observed between 18 subplots in the number of microbial populations ($P>0.05$), and the influence of transgenic rice on the number of microbial populations also was not statistically significant ($P>0.05$). The total population densities of soil microorganisms, however, tended to fluctuate slightly with season. The bacterial cell densities in April, which ranged from 1.4×10^7 to 1.8×10^7 cells/g soil, were slightly increased to $3.0\times10^7-4.0\times10^7$ cells/g soil in December. The actinomycetes population densities showed the similar

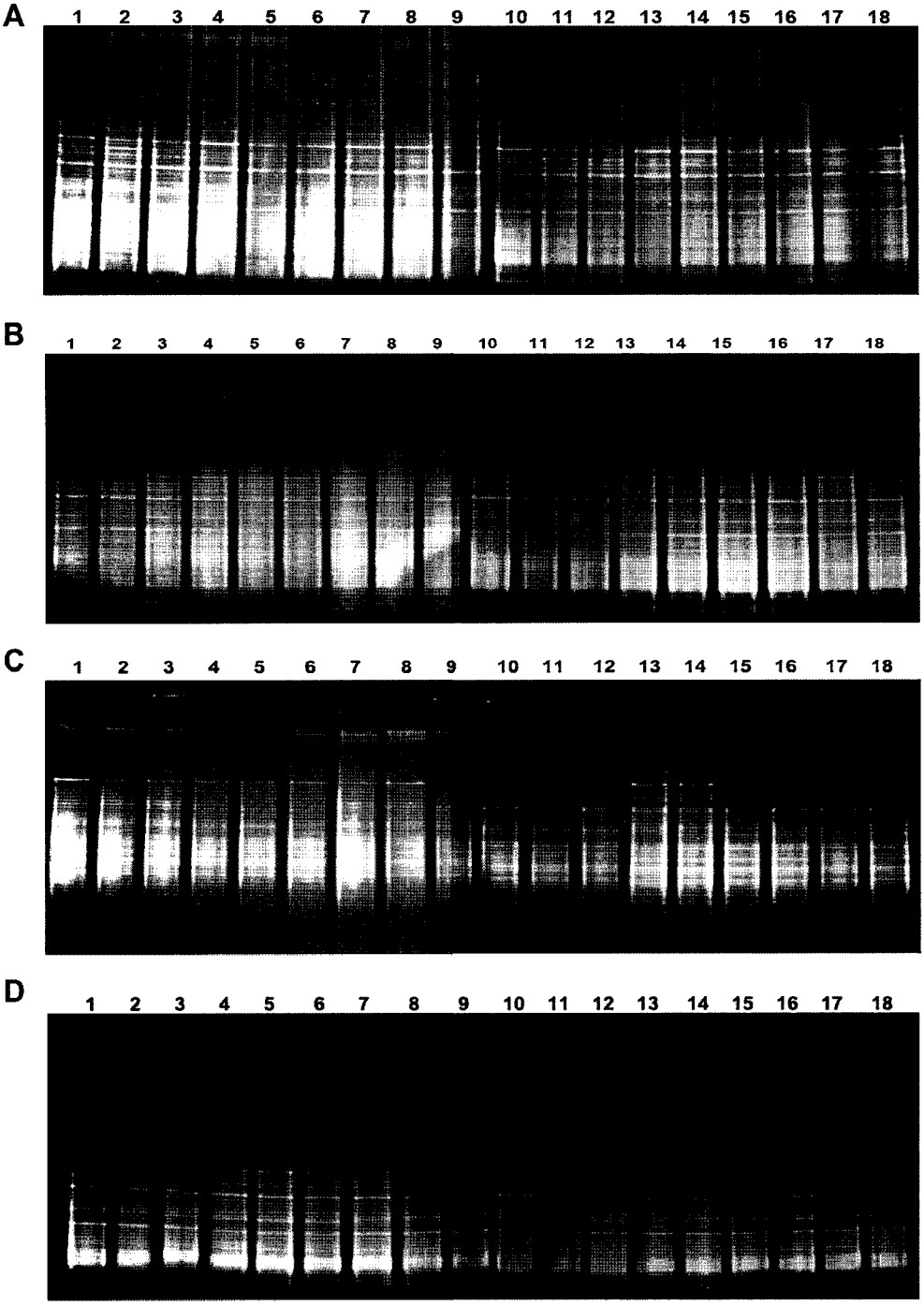

Fig. 1. DGGE analysis of 16S rDNA fragments obtained after PCR amplification with eubacterial primers 1070F and 1392R.
DGGE profiles for April (**A**), August (**B**), October (**C**), and December (**D**) paddy soils of Milyang204 (lanes 3, 11, and 13), Iksan483 (lanes 6, 9, and 17), Anjung (lanes 4, 7, and 14), Dongjin (lanes 1, 10, and 15), Junam (lanes 2, 12, and 16), and Sindongjin (lanes 5, 8, and 18).

fluctuation pattern to that of the bacterial densities over the year. Meanwhile, the fungal cell densities were slightly decreased to $1.9\times10^4-2.3\times10^4$ cells/g soil in August after flooding and then increased to $3.6\times10^4-4.3\times10^4$ cells/g soil in October.

DGGE Analysis

To investigate the impacts of GM rice on bacterial community structures, total soil microbial DNAs extracted from rice field soils were analyzed by DGGE after PCR amplification of the variable V8 region of the 16S rRNA gene. The band patterns of all samples were generally similar to each other in a given month (Fig. 1), and thus no significant differences in band patterns were observed between GM and non-GM rice subplots when checked with cluster analysis. The random fluctuation of intensities of some bands could be due to the heterogeneity of the soil, since none of them reoccurred repeatedly in three replicate subplots. To investigate the change of soil bacterial DGGE profiles over the year, soil bacterial DNA was extracted from each representative subplot of four seasons and analyzed on the same DGGE gel (Fig. 2). The DGGE profiles from the different months also looked similar to each other throughout the year (Fig. 2A). However, minor variations were detected among different seasons with computer-aided cluster analysis (Fig. 2B). The profiles of August and October grouped together with a high similarity of 95.5%, and those of April and December grouped with a similarity of 91.5%. Similar results were obtained from the analysis with the V3 region of the 16S rRNA gene (data not shown). The results suggested that the bacterial communities of the rice paddy soils were relatively stable over the year, without being significantly influenced by cultivation of GM rice plants.

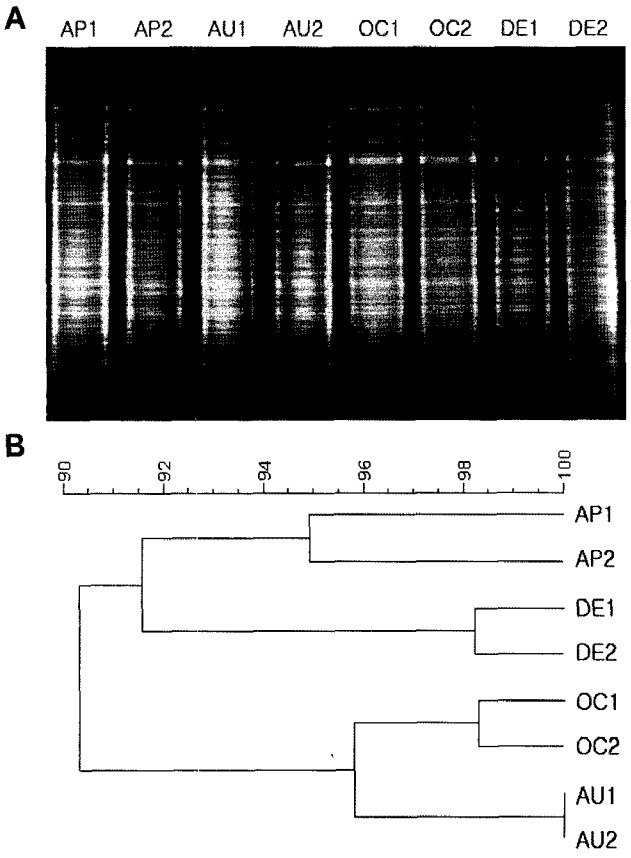

Fig. 2. DGGE analysis of 16S rDNA fragments obtained after PCR amplification with eubacterial primers 1070F and 1392R.
A. DGGE profiles of rice field soil samples taken in April (AP1, AP2), August (AU1, AU2), October (OC1, OC2), and December (DE1, DE2). No. 1 represents non-GM line, Anjung, and No. 2 represents GM line, Iksan483. **B.** The dendrogram represents the similarity between the patterns according to cluster analysis based on Dice's algorithm.

PLFA Profiles of Microbial Communities

In addition to DGGE analysis, to investigate the change of soil microbial communities, microbial fatty acid profiles of GM and non-GM rice soils were analyzed for August and December. Principal component analysis (PCA) of the fatty acid profiles of soil microbial communities revealed that there were no significant differences between GM and non-GM rice soils in both August and December (Fig. 3). In contrast, August and December soils exhibited distinct fatty acid profiles to each other ($P<0.001$), possibly due to environmental and physiological differences. The result indicated that the fatty acid compositions of the microbial communities of rice field soils changed with season, whereas the microbial community structures were not affected by GM rice varieties in a given month.

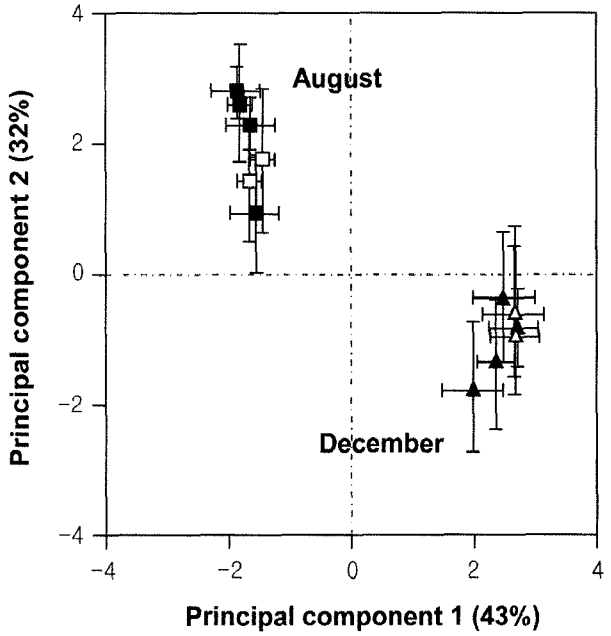

Fig. 3. Principal component analysis of PLFA profiles for rice paddy soils.
August (■) and December (▲) rice paddy soils (n=3). Open symbols represent GM soil and closed symbols non-GM, respectively. Error bars represent the standard errors of the means.

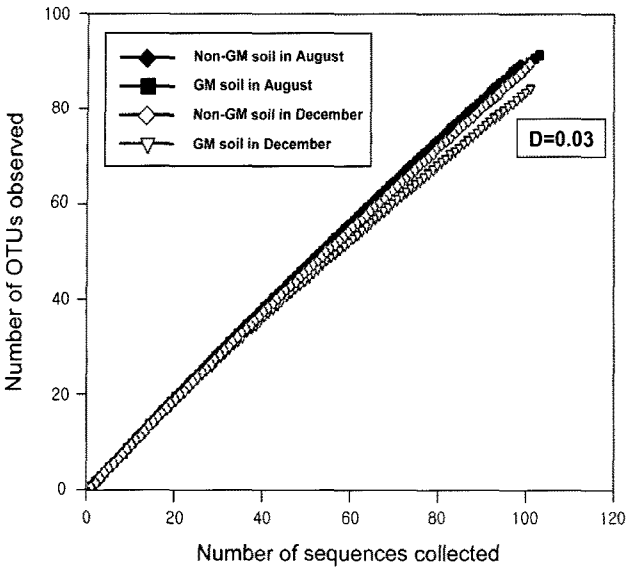

Fig. 4. Comparison of the taxonomic diversity of GM and non-GM soil clone libraries in August and December using rarefaction curves for distance of 0.03.
OTUs, operational taxonomic units.

Comparison of 16S rDNA Clone Libraries

Since the DGGE and fatty acid profiles of soil microbial communities were very similar to each other between GM and non-GM rice soils, 16S rDNA clone libraries were constructed from representative GM (Iksan 483) and non-GM (Anjung) soils for comparison of bacterial 16S rDNA sequences. A total of four clone libraries, each library having approximately 100 sequences, were constructed from GM and non-GM soils in August and December. The rarefaction curve generated from the clone libraries of GM and non-GM plots, with the operational taxonomic unit (OUT) definition at 3%, showed that the number of OTUs observed was between 84 and 91 among the four clone libraries (Fig. 4). This result indicated that there is no significant difference in species richness between GM and non-GM soils, as well as between August and December soils.

Although the four clone libraries represent the same level of richness, it is not clear whether the community structures are similar to each other. To determine the similarities of the four bacterial communities, the four libraries were compared with each other by distance-based and phylogeny-based methods (Table 1). First of all, the ∫-LIBSHUFF program [30] was used to compare the clone libraries by the distance-based method. ∫-LIBSHUFF is an integral form of tool for making statistical comparisons of the diversity of taxonomic lineages represented in 16S rRNA gene libraries. The *P* values for the comparison between GM and non-GM soils, with 10,000 randomizations, were 0.0196 in August and 0.3712 in December (margin of error for the *P* value's 95% confidence interval of 0.004). This suggested that there was not a significant difference in community structure between GM and non-GM soil libraries of each of August and December. In contrast, the *P* values for the comparison between August and December soils were very small (all *P* values were <0.001, margin of error for the *P* value's 95% confidence interval of 0.004). To determine whether these communities were significantly different, a parsimony-based approach was implemented for comparisons between 16S rRNA gene libraries. TreeClimber is a parsimony-based tool used to determine whether the differences between two communities arose from random variation or whether lineage from one community has become more dominant. The comparison between GM and non-GM soil libraries, with the neighbor-joining tree bootstrapped 1,000 times, produced the *P* values of 0.137 and 0.232 in August and December, respectively. On the other hand, the comparisons across GM and non-GM libraries in different seasons resulted in very low *P* values (100% of trees with the *P* values less than 0.001). The result suggested that although the bacterial communities between August and December showed differences in their compositions of 16S rDNA sequences, there were no meaningful differences between GM and non-GM soil libraries in a given month.

Detection of Recombinant *bar* Gene in Rice Soils

The persistence of transgenic rice DNA in soil and putative horizontal gene transfer (HGT) from transgenic rice to soil bacteria were monitored by using both cultivation and culture-independent methods. First, PCR amplification using primer set barF and barR was performed with soil DNAs directly extracted from all subplots. Any positive signals were not detected in April and August soils (data not shown). In contrast, surprisingly, five positive PCR

Table 1. Comparison of *P* values obtained using ∫-LIBSHUFF and TreeClimber for 16S rDNA clone libraries.

Library name (no. of sequences)	∫-LIBSHUFF *P* value[a]	TreeClimber *P* value at 95th percentile
		Neighbor joining
GM-Aug (100), non-GM-Aug (98)	0.0196	0.137
GM-Dec (100), non-GM-Dec (102)	0.3712	0.232
GM-Aug (100), GM-Dec (100)	0.0003	0.002
non-GM-Aug (98), non-GM-Dec (102)	0.0001	<0.001

[a]The margin of error for the *P* value's 95% confidence interval for the *P* values near 0.05 is 0.004 (10,000 randomizations).

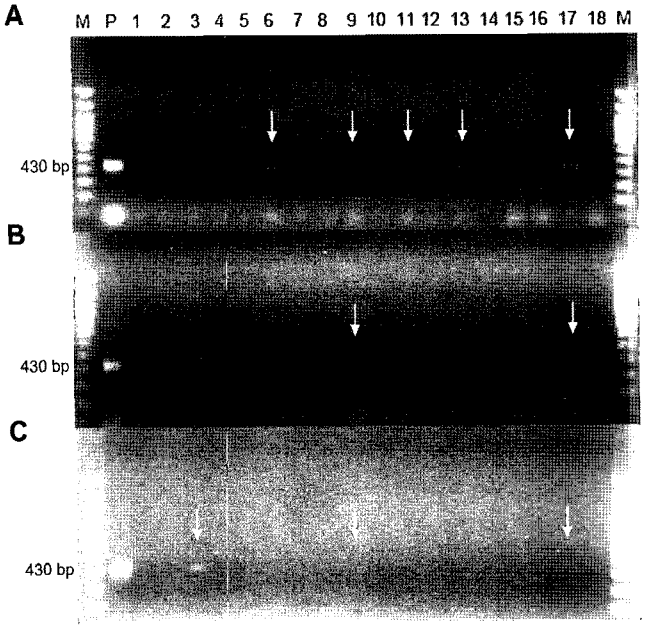

Fig. 5. Detection of the construct-specific *bar* fragment in soil. M, 100 bp DNA ladder; P, positive control; October (**A**), December (**B**) in 2005, and April (**C**) of the following year paddy soils of Milyang204 (lanes 3, 11, and 13), Iksan483 (lanes 6, 9, and 17), Anjung (lanes 4, 7, and 14), Dongjin (lanes 1, 10, and 15), Junam (lanes 2, 12, and 16), and Sindongjin (lanes 5, 8, and 18). Arrows indicate PCR-amplified DNA bands of the *bar* gene.

signals were observed among GM rice soils in October, and two positive signals were detected in December, one month later after harvesting (Figs. 5A and 5B). Even in April of the following year, 6 months later after harvesting, PCR-amplified products were still observed (Fig. 5C). When the sequences of the positive bands were analyzed and compared with the *bar* gene sequence, the sequence similarity was 100%, indicating that the positive signal, 430 bp fragment, was the *bar* gene fragment. PCR amplification of soil DNAs with primer pairs pbarF-pbarR and bbarF-bbarR, which targeted flanking regions of the *bar* gene, produced positive DNA bands, confirming that the *bar* gene sequences amplified in GM rice soil were derived from GM rice DNA (data not shown).

In order to investigate whether the detected *bar* sequence was obtained from soil fraction (*e.g.*, free DNA bound to soil particles and plant debris) or from soil bacteria, PCR analysis was performed on DNAs extracted from bacterial fractions obtained by cultivation-based and cultivation-independent methods. PCR analysis revealed that there were no *bar* gene sequences in bacterial DNAs obtained after cultivation on nutrient broth (data not shown). For the analysis of uncultured bacterial DNA, December soils that showed positive signals in PCR analysis were subjected to further experiments. To improve the recovery rate of bacterial fraction from soil, several modified procedures such as blending and sonication were implemented as pretreatment before extracting bacterial DNA. No positive

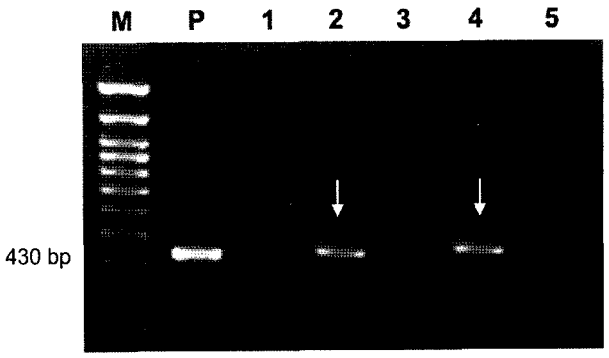

Fig. 6. Amplification of the *bar* gene sequence with DNAs extracted from bacterial fractions and soil sediments.
DNA was extracted from the bacterial fraction of two April soils of 2006 (lanes 9 and 17 in Fig. 5C) by density gradient centrifugation with different pretreatment processes. Lanes: P, positive control; 1, blending; 2, sediment after blending; 3, sonication after blending; 4, sediment after sonication; 5, incubation of soil for 1 h at 40°C before blending. Arrows indicate PCR-amplified DNA bands of the *bar* gene.

PCR signals were detected in bacterial DNAs obtained with these procedures, but positive bands were persistently observed in DNAs obtained from soil pellets remained after density gradient centrifugation (Fig. 6). An additional step (incubation in 6% yeast extract solution for 1 h at 40°C to induce bacterial spore germination before blending) was applied to increase the population of *Actinobacteria* [5], because the *bar* gene was originated from *Streptomyces* sp. belonging to *Actinobacteria*. No positive signal was detected in this case either. The result demonstrated that some *bar* gene sequences remained in rice paddy soil for a relatively long time, but potential evidence for horizontal gene transfer from transgenic rice to bacteria was not detected during this experiment.

DISCUSSION

A lot of concerns regarding potential adverse effects of transgenic crop plants on the environment have been debated. Microbial ecologists especially have tried to assess the influence of transgenic plant on soil microbial communities. With new molecular biological techniques, some previous researches showed that soil microbial communities could be changed by the introduction of transgenic plant [4, 8, 9, 35]. Likewise, in the case of glufosinate-tolerant transgenic oilseed rape, recent studies showed that soil microbial communities were slightly altered in the rhizosphere of transgenic plants [13, 35]. However, the effects were minor as compared with the nontransgenic plant and with the plant developmental stage-dependent shifts [13]. It was also reported that there were no meaningful differences in soil microbial communities between glufosinate-resistant sugar beet and its nontransgenic cultivar [33, 34].

This study aimed to analyze the effects of transgenic, glufosinate-tolerant rice on soil microbial communities. In this study, several methods, such as the plating method, PCR-DGGE analysis, FAME analysis, and comparison of 16S rDNA clone libraries, were implemented together to investigate the changes in microbial communities. First, total microbial populations in rice soils were measured by total counts of bacteria, fungi, and actinomycetes. The plating method is a useful tool to simply identify and characterize the changes of culturable microorganisms, but it usually could not detect the significant influences of transgenic plants on microbial communities [7, 16, 29]. Similarly, we also could not find anymore meaningful differences in soil microbial population levels between GM and non-GM rice soils compared by seasonal shift.

DGGE analysis, one of the DNA-based fingerprint methods, was performed by analyzing 16S rDNA fragments amplified from total soil DNA. In conclusion, even though soil heterogeneities could be detected in this study, no differences could be seen in DGGE band patterns between GM and non-GM lines. For oilseed rape (*Brassica napus*), although the effects were minor as compared with the plant developmental stage-dependent shifts, the study showed that the soil microbial communities were slightly changed in the rhizosphere of transgenic plants [13]. From the results, they estimated that an altered root exudate composition in transgenic plant may lead to changes in bacterial community structures and enzyme activities. However, for maize (*Zea mays*) and sugar beet (*Beta vulgaris*) engineered with the *pat* gene, there were no significant differences in the bacterial diversities in rhizospheres of the two transgenic cultivars and their isogenic, nontransgenic cultivars [33, 34]. In general, the DGGE fingerprinting method is not enough to detect subtle changes in banding patterns and the changes in closely related species [39]. Most likely, in our study, DGGE could not exclusively differentiate the seasonal shifts of soil bacterial populations. Therefore, it is possible that similar band patterns between GM and non-GM soils would be resulted from the masked effects of other more selective factors (plant age, soil heterogeneity) or relative low resolution of DGGE technique unable to perceive subtle changes.

To investigate the effects of GM rice on microbial communities in a different way, fatty acid profile, which is culture-independent but not a DNA-targeted method like DGGE, of soil microbial composition was analyzed in August and December. The PLFA patterns of microbial communities of GM and non-GM rice soils were not significantly different as shown by the result of PCA analysis (Fig. 3). In glyphosate-resistant canola varieties, Dunfield and Germida [8] showed that despite that the difference was depended on the presence of the viable plant, there were significant differences between the rhizosphere microbial communities associated with transgenic and nontransgenic plants in the composition of fatty acids [9]. However, in our study, any difference was not observed between GM and non-GM rice soils in the plant-growing season as well as unplanted period, which were consistent with the results of DGGE and viable count analyses.

16S rDNA sequences of dominant bacterial clones obtained from GM (Iksan483) and non-GM (Anjung) subplots were compared for determining whether the two bacterial communities consist of similar or different members of soil bacteria. Four clone libraries, two GM and non-GM lines in August and December, exhibited a similar level of high species diversity when compared by rarefaction curve (Fig. 4). Moreover, when the clone libraries were compared using the distance- and phylogeny-based methods, there were no statistically meaningful differences between GM and non-GM lines. However, the taxonomic distribution of the four libraries showed some differences between the libraries in the relative abundance of the most common phyla (Fig. 7). In particular, the number of the bacteria belonging to α-*Proteobacteria* in the GM lines was more than three times higher than those in non-GM lines. In addition, in comparison between August and December, relatively more *Firmicutes* sequences were detected in August soils as compared with December soils, and β-*Proteobacteria* were rarely discovered in August soils. Phylogenetic trees were constructed for the phyla that differed in relative abundance to determine which specific members of bacteria determine the differences. The neighbor-joining phylogenetic tree of 16S rDNA clones within the *Firmicutes* is shown in Fig. 8. In this dendrogram, there were no specific groups of bacterial clones that differentiate bacterial members between GM and non-GM soils. However, several members (shown as boldfaced clones in Fig. 8) belonging to the class *Bacilli* were detected in

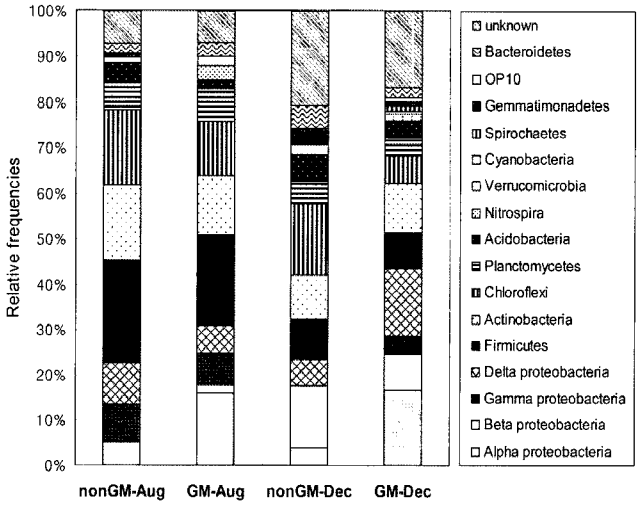

Fig. 7. Phyla distribution of four 16S rDNA clone libraries obtained from GM and non-GM soils in August and December.

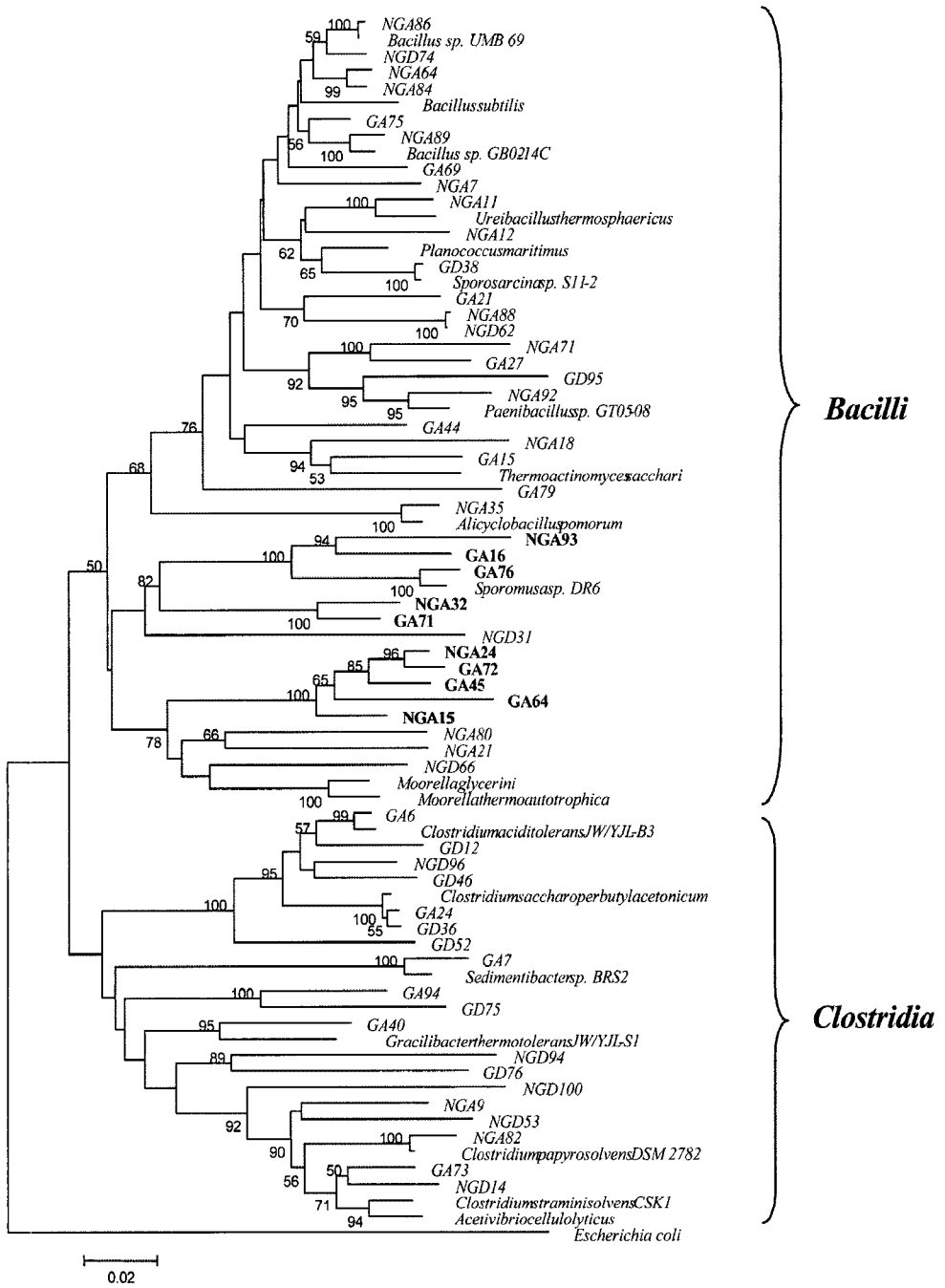

Fig. 8. Neighbor-joining tree of 16S rDNA clones within the *Firmicutes* division.
GA, GM soil clones in August; GD, GM soil clones in December; NGA, non-GM soil clones in August; NGD, non-GM soil clones in December. The scale bar indicates 0.02 estimated changes per nucleotide, and the numbers indicate bootstrap values representing percent confidence of 1,000 replicates.

August alone. Some of them were closely related to *Sporomusa* sp., which is known to degrade rice straw in anoxic paddy soils [21], and other members showed low sequence similarity (<93%) to known bacteria in the database. Their occurrence may be related to the original field condition of rice paddy soil in August, which is characterized by high temperature and anoxic environment due to flooding. For the α- and β-*Proteobacteria* (Fig. 9), unlike the different distribution of phyla, any of the sequences was not clustered as a specific group of GM or non-GM clones. Yet, more than half of the members (shown as boldfaced clones) belonging to β-*Proteobacteria* showed seasonal differences, being detected only in December. A majority of these members were also aligned to known bacteria at low sequence similarity, like the unknown *Bacilli* members as described above. It appeared that cold-tolerant, autotrophic bacteria dominated in rice paddy soil during December, considering that these sequences were clustered to the bacteria known as chemolithotrophs such as *Nitrosospira* sp. and *Gallionella* sp.

Fig. 9. Neighbor-joining tree of 16S rDNA clones within the α- and β-*Proteobacteria* divisions.
GA, GM soil clones in August; GD, GM soil clones in December; NGA, non-GM soil clones in August; NGD, non-GM soil clones in December. The scale bar indicates 0.02 estimated changes per nucleotide, and the numbers indicate bootstrap values representing percent confidence of 1,000 replicates.

The possibility of horizontal gene transfer of the recombinant DNA from GM plants to soil microorganisms is one of the important issues of GMO risk assessment. That's because the unintended changes could occur in soil ecosystem from uptake of recombinant DNA by microorganisms. It has already been reported that transgenic plant DNAs, such as plasmid and marker gene, persisted in soil for several months [28, 44]. Similarly, we also observed

that the *bar* gene introduced into transgenic rice persisted in paddy soils for more than six months after harvest, indicating that rice material was not degraded yet or released DNA was stabilized by adsorption to soil particles. However, it is extremely difficult for soil bacteria to take up extracellular DNA and integrate it into their genome [24]. To date, even though several studies showed that transformation occurred at low frequencies in microcosms experiments [37], transfer of the recombinant DNA from transgenic plants to microbes in the field soil has not been found [18]. Thus, we investigated the probability of HGT from transgenic plant to bacteria by analyzing the DNA extracted from both cultivated soil bacteria and uncultured bacterial fraction of soil. The result showed that uptake of the *bar* gene by soil bacteria was not detected in both cases. Even though a spore germination step was added before blending (for increasing the populations of *Actinobacteria*), naturally transformed bacteria were not found, indicating that the HGT event did not happen during the experimental period or we could not detect the event because of some limitation of the method. In fact, routine PCR applications generally require $>10^3$ target molecules (*e.g.*, 10 ng of template DNA) for the amplification of single-copy genes from eukaryotic genomes [26]. Moreover, it is known that substances such as humic acids in soil inhibit the binding of template DNA in PCR [40]. Hence, with respect to HGT, a more sensitive measurement is needed to increase the detection limit, rather than routine PCR amplification.

In summary, this study showed that, in spite of seasonal variations of bacterial communities and persistence of the *bar* gene, the bacterial communities of the experimental rice field were not significantly affected by cultivation of GM rice varieties. The bacterial community structure was less affected by genetically modified property than by other environmental factors such as soil heterogeneities and seasonal changes. Moreover, horizontal gene transfer from transgenic rice to soil bacteria was not observed during the experiment. However, the existence of the *bar* gene fragment in the soil for several months is a potential reservoir of natural transformation. Therefore, a continuous long-term study is required to address the genetic impacts of GM plants on soil ecosystems.

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REFERENCES

1. Ahn, J. H., M. C. Kim, H. C. Shin, M. K. Choi, S. S. Yoon, T. S. Kim, H. G. Song, G. H. Lee, and J. O. Ka. 2006. Improvement of PCR amplification bias for community structure analysis of soil bacteria by denaturing gradient gel electrophoresis. *J. Microbiol. Biotechnol.* **16:** 1561–1569.
2. Ashelford, K. E., N. Chuzhanova, J. C. Fry, A. J. Jones, and A. J. Weightman. 2006. New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl. Environ. Microbiol.* **72:** 5734–5741.
3. Bossio, D. A. and K. M. Scow. 1998. Impacts of carbon and flooding on soil microbial communities: Phospholipid fatty acid profiles and substrate utilization patterns. *Microb. Ecol.* **35:** 265–278.
4. Castaldini, M., A. Turrini, C. Sbrana, A. Benedetti, M. Marchionni, S. Mocali, A. Fabiani, S. Landi, F. Santomassimo, B. Pietrangeli, M. P. Nuti, N. Miclaus, and M. Giovannetti. 2005. Impact of Bt corn on rhizhospheric and soil eubacterial communities and on beneficial mycorrhizal symbiosis in experimental microcosms. *Appl. Envir. Microbiol.* **71:** 6719–6729.
5. Courtois, S., A. Frostegard, G. Pernilla, G. Depret, P. Jeannin, and P. Simonet. 2001. Quantification of bacterial subgroups in soil: Comparison of DNA extracted directly from soil or from cells previously released by density gradient centrifugation. *Environ. Microbiol.* **3:** 431–439.
6. de Vries, J., M. Heine, K. Harms, and W. Wackernagel. 2003. Spread of recombinant DNA by roots and pollen of transgenic potato plants, identified by highly specific biomonitoring using natural transformation of an *Acinetobacter* sp. *Appl. Envir. Microbiol.* **69:** 4455–4462.
7. Donegan, K. K., C. J. Palm, V. J. Fieland, L. A. Porteous, L. M. Ganio, D. L. Schaller, L. Q. Bucao, and R. J. Seidler. 1995. Changes in levels, species and DNA fingerprints of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var. *kurstaki* endotoxin. *Appl. Soil Ecol.* **2:** 111–124.
8. Dunfield, K. E. and J. J. Germida. 2001. Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica napus*. *FEMS Microbiol. Ecol.* **38:** 1–9.
9. Dunfield, K. E. and J. J. Germida. 2003. Seasonal changes in the rhizosphere microbial communities associated with field-grown genetically modified canola (*Brassica napus*). *Appl. Environ. Microbiol.* **69:** 7310–7318.
10. Felsenstein, J. 2004. PHYLIP (Phylogenetic Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.
11. Ferris, M. J., G. Muyzer, and D. M. Ward. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl. Environ. Microbiol.* **62:** 340–346.
12. Gebhard, F. and K. Smalla. 1998. Transformation of *Acinetobacter* sp. strain BD413 by transgenic sugar beet DNA. *Appl. Environ. Microbiol.* **64:** 1550–1554.
13. Gyamfi, S., U. Pfeifer, M. Stierschneider, and A. Sessitisch. 2002. Effects of transgenic glufosinate-tolerant oilseed rape (*Brassica napus*) and the associated herbicide application on eubacterial and *Pseudomonas* communities in the rhizosphere. *FEMS Microbiol. Ecol.* **41:** 181–190.
14. Hengstmann, U., K.-J. Chin, P. H. Janssen, and W. Liesack. 1999. Comparative phylogenetic assignment of environmental sequences of genes encoding 16S rRNA and numerically

abundant culturable bacteria from an anoxic rice paddy soil. *Appl. Environ. Microbiol.* **65:** 5050–5058.
15. James, C. 2006. Global status of commercialized biotech/GM crops: 2006. ISAAA Briefs No. 35, Ithaka, NY.
16. Kim, M. S., J. H. Ahn, M. K. Jung, J. H. Yu, D. H. Joo, M. C. Kim, H. C. Shin, T. S. Kim, T. H. Ryu, S. J. Kweon, T. S. Kim, D. H. Kim, and J. O. Ka. 2005. Molecular and cultivation-based characterization of bacterial community structure in rice field soil. *J. Microbiol. Biotechnol.* **15:** 1087–1093.
17. Kim, T. S., M. S. Kim, M. K. Jung, M. J. Joe, J. H. Ahn, K. H. Oh, M. H. Lee, M. K. Kim, and J. O. Ka. 2005. Analysis of plasmid pJP4 horizontal transfer and its impact on bacterial community structure in natural soil. *J. Microbiol. Biotechnol.* **15:** 376–383.
18. Kim, Y. T., S. E. Kim, K. D. Park, T. H. Kang, Y. M. Lee, S. H. Lee, J. S. Moon, and S. U. Kim. 2005. Investigation of possible horizontal gene transfer from the leaf tissue of transgenic potato to soil bacteria. *J. Microbiol. Biotechnol.* **15:** 1130–1134.
19. Kowalchuk, G. A., M. Bruinsma, and J. A. van Veen. 2003. Assessing responses of soil microorganisms to GM plants. *Trends Ecol. Evol.***18:** 403–410.
20. Lane, D. J. 1991. 16S/23S rRNA sequencing, pp. 115–148. *In* E. Stackebrandt and M. Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley and Sons, Chichester, England.
21. Liesack, W., S. Schnell, and N. P. Revsbech. 2000. Microbiology of flooded rice paddies. *FEMS Microbiol. Rev.* **24:** 625–645.
22. Limdahl, V. 1996. Improved soil dispersion procedures for total bacterial counts, extraction of indigenous bacteria and cell survival. *J. Microbiol. Methods* **25:** 279–286.
23. Liu, B., Q. Zeng, F. Yan, H. Xu, and C. Xu. 2005. Effects of transgenic plants on soil microorganisms. *Plant Soil* **271:** 1–13.
24. Nielsen, K. M., A. M. Bones, K. Smalla, and van J. D. Elsas. 1998. Horizontal gene transfer from transgenic plants to terrestrial bacteria - A rare event? *FEMS Micobiol. Rev.* **22:** 79–103.
25. Przetakiewicz, A., A. Karas, W. Orczyk, and A. Nadolska-Orzyk. 2004. Agrobacterium-mediated transformation of polyploidy cereals. The efficiency of selection and transgene expression in wheat. *Cell. Mol. Biol. Lett.* **9:** 903–917.
26. Roche Diagnostics GmbH. 1999. *PCR Application Manual*, 2nd Ed. Germany.
27. Rogers, S. O. and A. J. Bendich. 1985. Extraction of DNA milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol.* **5:** 69–76.
28. Romanoski, G., M. G. Lorenz, G. Sayler, and W. Wackernagel. 1992. Persistence of free plasmid DNA in soil monitored by various methods including a transformation assay. *Appl. Environ. Microbiol.* **58:** 3012–3019.
29. Saxena, D. and G. Stotzky. 2001. *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. *Soil Biol. Biochem.* **33:** 1225–1230.
30. Schloss, P. D., B. R. Larget, and J. Handelsman. 2004. Integration of microbial ecology and statistics: A test to compare gene libraries. *Appl. Environ. Microbiol.* **70:** 5485–5492.
31. Schloss, P. D. and J. Handelsman. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl. Environ. Microbiol.* **71:** 1501–1506.
32. Schloss, P. D. and J. Handelsman. 2006. Introducing TreeClimber, a test to compare microbial community structures. *Appl. Environ. Microbiol.* **72:** 2379–2384.
33. Schmalenberger, A. and C. C. Tebbe. 2002. Bacterial community composition in the rhizosphere of a transgenic, herbicide-resistant maize (*Zea mays*) and comparison to its non-transgenic cultivar Bosphore. *FEMS Microbiol. Ecol.* **40:** 29–37.
34. Schmalenberger, A. and C. C. Tebbe. 2003. Genetic profiling of non-cultivated bacteria from the rhizospheres of sugar beet (*Beta vulgaris*) reveal field and annual variability but no effect of a transgenic herbicide resistance. *Can. J. Microbiol.* **49:** 1–8.
35. Sessitsch, A., S. Gyamfi, D. Tscherko, M. H. Gerzabek, and E. Kandeler. 2004. Activity of microorganisms in the rhizosphere of herbicide treated and untreated transgenic glufosinate-tolerant and wildtype oilseed rape grown in containment. *Plant Soil* **266:** 105–116.
36. Shen, R. F., H. Cai, and W. H. Gong. 2006. Transgenic Bt cotton has no apparent effect on enzymatic activities or functional diversity of microbial communities in rhizosphere soil. *Plant Soil* **285:** 149–159.
37. Sikorski, J., S. Graupner, M. G. Lorenz, and W. Wackernagel. 1998. Natural genetic transformation of *Pseudomonas stutzeri* in a non-sterile soil. *Microbiology* **144:** 569–576.
38. Smalla, K., G. Wieland, A. Buchner, A. Xock, J. Parzy, S. Kaiser, N. Roskot, H. Heuer, and G. Berg. 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. *Appl. Environ. Microbiol.* **67:** 4742–4751.
39. Susuki, M. T. and S. J. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* **62:** 625–630.
40. Tebbe, C. C. and W. Vahjen. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and yeast. *Appl. Environ. Microbiol.* **59:** 2657–2665.
41. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25:** 4876–4882.
42. Thompson, C. J., N. R. Mowa, R. Tizard, R. Crameri, J. E. Davies, M. Lauwereys, and J. Botterman. 1987. Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. *EMBO J.* **6:** 2519–2523.
43. Weaver, R. W., J. S. Angle, and P. S. Bottomley. 1999. *Methods of Soil Analysis: Part 2 – Microbiological and Biochemical Properties*. Soil Science Society of America, Madison, U.S.A.
44. Widmer, F., R. J. Seidler, and L. S. Watrud. 1996. Sensitive detection of transgenic plant marker gene persistence in soil microcosms. *Mol. Ecol.* **5:** 603–613.
45. Wohllenben, W., R. Alijah, J. Dorendorf, D. Hillemann, B. Nussbaumer, and S. Pelzer. 1992. Identification and characterization of phosphinothricin-tripeptide biosynthetic genes in *Streptomyces viridochromogenes*. *Gene* **115:** 127–132.
46. Wu, W. X., Z. F. Ye, H. Min, X. J. Duan, and W. M. Jin. 2004. Bt-transgenic rice straw affects the culturable microbiota and dehydrogenase and phophatase activities in a flooded paddy soil. *Soil Biol. Biochem.* **36:** 289–295.