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Investigation of Possible Horizontal Gene Transfer from Transgenic Rice to Soil Microorganisms in Paddy Rice Field

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In order to monitor the possibility of horizontal gene transfer between transgenic rice and microorganisms in a paddy rice field, the gene flow from a bifunctional fusion (TPSP) rice containing trehalose-6-phosphate synthase and phosphatase to microorganisms in soils was investigated. The soil samples collected from the paddy rice field during June 2004 to March 2006 were investigated by multiplex PCR, Southern hybridization, and amplified fragment length polymorphism (AFLP). The *TPSP* gene from soil genomic DNAs was not detected by PCR. Soil genomic DNAs did not show homologies on the Southern blotting data, indicating that gene transfer did not occur during the last two years in the paddy rice field. In addition, the AFLP band patterns produced by soil genomic DNAs from both transgenic and non-transgenic rice fields appeared similar to each other when analyzed by the NTSYSpc program. Thus, these data suggest that transgenic rice does not give a significant impact on the communities of soil microorganisms, although long-term observation may be needed.

Keywords: Horizontal gene transfer, transgenic TPSP rice, soil microorganisms, AFLP, environmental risk assessment

Since the genesis of commercialization in 1996, transgenic crops containing novel traits have been rapidly adopted in several important agricultural markets during the last decade [6]. In 2008 alone, 125 million hectares of transgenic crops were grown across 25 countries, with an estimated market value of $7.5 billion dollars [7]. Although living modified organisms (LMOs) may cause harmful effects on the environment and human health [1, 3], global adoption rates of transgenic crops have grown over 10% each year since 1996 and are inferred to continue to grow at this rate in the future [7].

Horizontal gene transfer (HGT), which is the flow of genes between unrelated species, is an evolutionary phenomenon [11, 13]. HGT between bacterial species involving plasmid and transposon is particularly common [2]. The most popular technology of gene transfer to plants using *Agrobacterium tumefaciens* is based on HGT. Whereas the mechanisms of HGT from *A. tumefaciens* to plant cells is known in considerable detail, little is known of the mechanism of HGT from plants to other organisms [1]. The gene flow of plant DNA to soil bacteria was proposed to be a major evolutionary force [11]. Although DNA in decaying plant cells is rapidly degraded, DNA of the appropriate amounts can remain in some soils [12], aquatic environments [14], or the digestive tract of mice [18], long enough to be available for uptake. In order for natural transformation to occur in a soil environment, free DNA needs to be available and competent bacteria in the soil need to be in close vicinity to the DNA [20]. In a previous report, some researchers quantified marker gene persistence in the field, and found that marker genes from tobacco and potato were detectable for 77 and 137 days, respectively [22]. Similarly, other workers showed that the DNA of transgenic sugar beet plants after release was detectable up to two years in the soil under field conditions [5]. Whereas evidence for the persistence of transgenic plant DNA exists, the transformation of plant DNA to indigenous soil microorganisms in the field has not been found. Several experimental studies have been published that all failed in demonstrating HGT from transgenic plants to bacteria [5, 22]. However, with a more elaborate marker-rescue approach with large stretches of homology, the kanamycin resistance gene from transgenic maize could be retrieved in a naturally transformable bacterium, *Acinetobacter* sp. BD413, which can uptake and integrate transgenic plant DNA and does not distinguish against DNA from

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other microorganisms during uptake [4]. In addition, it is reported that transgenic Bt corn (NK4640Bt) containing an inserted Bt toxin gene, *cry1Ab*, releases the toxin protein from the root into the surrounding rhizosphere soil, together with other proteins normally present in root exudates [17]. These results indicate that HGT from transgenic plants to soil microorganisms may occur in the environment for a long-term period .

Based on the development of ag-biotechnology and rapidly increasing cultivation area of transgenic crops for the last decade, there are needs to establish various techniques for the environmental risk assessment of transgenic plants. Thus, we have investigated the possibility of horizontal gene transfer from domestically developed transgenic TPSP rice (*Oryza sativa*) [8], which has trehalose-6-phosphate synthase (TPS) and phosphatase (TPP) conferring resistance to salt and drought stress, to soil microorganisms in paddy rice fields.

The non-transgenic (*Oryzae sativa* cv. Nakdong) and transgenic TPSP rice containing ubiquitin and ABC promoter, respectively, were cultivated in paddy rice fields during the last two years. The TPSP rice containing the maize (*Zea mays*) ubiquitin (Ubi) promoter (Fig. 1A) was grown from June 2004 to May 2005 in the paddy rice field (5 m×5 m) of Kyungpook National University, which is located at Chilgok, Kyungsangbuk Province, whereas the TPSP rice with ABC promoter (Fig. 1B) was cultivated from July 2005 to March 2006 in the paddy rice field (18 m×18 m) of the Bioevaluation Research Center, Korea Research Institute of Bioscience and Biotechnology, which is located in Ochang, Chungcheongbuk Province. The transgenic TPSP rice with ubiquitin or ABC promoter was provided from Myongji University, and the transgenic TPSP rice containing the ABC promoter was constructed by replacing the ubiquitin promoter with the ABC promoter in front of the trehalose-6-phosphate synthase gene (Fig. 1).

Soil samples were collected every month from the rhizosphere of the paddy rice fields where both transgenic TPSP and non-transgenic rice had been cultivated. The roots of rice plants and residues from collected soil samples were removed by using the sieve No. 14 (aperture

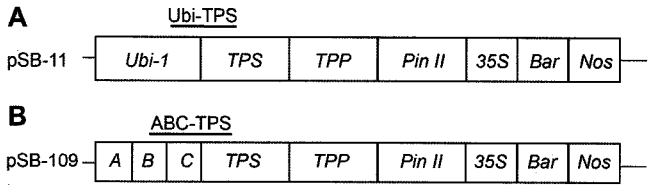

Fig. 1. Schematic diagrams of designed PCR primers to detect the inserted DNA of transgenic TPSP rice plants, Ubi-TPSP (**A**) and ABC-TPSP (**B**).
Ubi1: maize ubiquitin promoter; PinII: potato proteinase inhibitor II; TPS: trehalose-6-phosphate synthase; TPP: trehalose-6-phosphate phosphatase; Nos : nopaline synthase.

1.4 mm). Soil genomic DNAs (80–100 μg/g) were obtained by using a FastDNA spin kit for soil (MP Biomedicals, Solon, OH, U.S.A.). The extracted DNAs containing humic acid were further purified by using 0.8% agarose gel electrophoresis and a QIA quick gel extraction kit (Qiagen, Valencia, CA, U.S.A.), and the purified DNAs (80% yield) were used in the following experiments.

Fresh young leaves (1 g) of transgenic TPSP and non-transgenic rice were frozen in liquid nitrogen and grounded for the isolation of genomic DNAs from rice plants by using a DNAase Plant Maxi kit (Qiagen, Valencia, CA, U.S.A.). PCR amplifications of the transgenic TPSP rice were carried out by using specific primers to the sequences flanking the Ubi promoter, ABC promoter, and trehalose-6-phosphate synthase. 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 primers, 250 μM dNTP, and 1 unit of *Taq* polymerase (AccuPower PCR PreMix; Bioneer, Seoul, Korea). Amplification was conducted under the following conditions: denaturation for 7 min at 95°C, 40 cycle (95°C for 45 sec, 55°C for 45 sec, and 72°C for 90 sec), and terminal elongation for 7 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.

Soil DNAs extracted from each soil sample were also separated on 1.5% agarose gel (SeaKem LE agarose, BMA) in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and blotted to a nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) for Southern hybridization [16]. PCR amplification products of the Ubi and ABC promoter genes 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, 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 was carried out at 65°C for 16 h [10].

Genomic DNA samples obtained from soil samples of a paddy rice field, where both transgenic TPSP and non-transgenic rice had been cultivated, were used for amplified fragment length polymorphism (AFLP) analysis [9]. DNA samples were treated with restriction enzymes EcoR1 and Tru91 (Promega, Madison, WI, U.S.A.), and DNA fragments produced were ligated to adapters as shown in Table 1 by using a ligation kit (Promega, Madison, WI, U.S.A.). Primers (M01 5'-GAT GAG TCC TGA GTA AA-3' and E01 5'-GAC TGC GTA CCA ATT CA-3'), which were designed based on DNA sequences of the restriction sites,

Table 1. List of primers and adapters used in this study.

Oligonucleotide name	Primer-1 (5'-3') Primer- 2 (5'-3')	Amplicon length (bp)	Target sequence
Ubi-TPS	GCCCTGCCTTCATACGCTAT GACTGTGGTTTGGCTGGAGT	260	Ubi-trehalose-6-phosphate synthase gene
Act I	CAGCCACACTGTCCCCATCT ACTCCAGCCAAACCACAGTC	534	Rice actin gene
ABC-TPS	AATCTGATCATGAGCGGAGAAT TACAGGTATGCGGATCCTCTA	640	ABC-trehalose-6-phosphate synthase gene
Act II	AGAAAATCCTTGTCGGAACTATGT TGGCATGGCCAATTGAGTGGT	370	Rice actin gene

AFLP adapter			
Adapter name	Tru9I adapter (5'-3')	Adapter name	EcoRI adapter (5'-3')
M-up	GACGATGAGTCCTGAG	E-up	CTCGTAGACTGCGTACC
M-down	ATGAGTCCTGAGTA	E-down	TTAACCATGCGTCAGATG

were used to amplify the DNA fragments ligated with adapters. The other primer sets (M13411 5'-GAT GAG TCC TGA GTA AAG TAA-3' and E13411 5'-GAC TGC GTA CCA ATT CAG TAA-3') with longer DNA sequences were also used to obtain PCR amplification products, which produced more specific bands than M01 and E01 primers. The primer M13411 was used after labeling the 5'-end with fluorescein. The same reaction buffer was used for PCR reaction with the different primers. The first amplification reaction was performed by 13 cycles of denaturation at 94°C for 30 sec, annealing at 67°C for 20 sec, and extension at 72°C for 1 min, with lowering of the annealing temperature by 0.7°C at every cycle. The second amplification reaction was carried out with 23 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 20 sec, and extension at 72°C for 1 min. DNA products obtained from PCR were analyzed by using an ABI 3730XL Capillary DNA Sequencer (SolGent, Daejon, Korea). Similarity between transgenic TPSP and non-transgenic rice was analyzed by a software NTSYSpc (version 2.1) program [15].

To examine the possibility of horizontal gene transfer released into the environment from transgenic rice to soil microorganisms, genomic DNAs were extracted every month from the soils, which had been cultivated with both transgenic and non-transgenic rice in paddy rice fields for two consecutive years. Soil genomic DNAs were subjected to multiplex PCR using primers of the flanking regions specific to ubiquitin, ABC promoter, trehalose-6-phosphate synthase (TPS), and actin (Table 1, Fig. 2). An aliquot of amplified genomic DNAs was loaded into the agarose gel and examined first for whether the PCR products corresponding to specific primers were detectable, and then to confirm its homologies by Southern hybridization, which is known to detect less than 0.1 pg of DNA with radiolabeled ^{32}P probe under the best condition [16]. All samples showed similar band patterns when PCR products were separated in the agarose gel. Genomic DNAs from rice actin and TPS as positive controls were amplified to produce the expected PCR products, and showed positive bands when the TPS products were subjected to multiplex PCR and Southern blotting analysis, which was performed by using DNA probes amplified with Ubi-TPS and ABC-TPS primers (Fig. 2). However, soil genomic DNAs did not show positive homologies by Southern hybridization analysis, indicating that gene transfer from transgenic rice to soil microorganisms did not happen for the two consecutive years in the paddy rice fields (Fig. 2). Specifically, any homologous signals for the radiolabeled probes, Ubi-TPS and ABC-TPS, from both transgenic rice were not detected in the paddy rice soils collected for 7 months after harvest. These results suggest that the horizontal gene transfer, including DNA uptake and its integration into their genome, from transgenic rice to soilborne microorganisms is quite difficult in field condition. However, considering the evidence that the persistence of transgenic sugar beets plant DNA lasts up to two years [5], it is thought that a long-term monitoring for gene flow will be needed in the future.

In addition, to investigating the effect of transgenic rice on the microbial community in soil during the cultivation and after harvest, amplified fragment length polymorphism (AFLP) analysis [9, 19, 21], one of the fingerprinting techniques, was performed by using soil genomic DNAs to examine whether there are particular differences of DNA band patterns on the molecular level. Soil samples used were collected at intervals of one (Fig. 2A) and two months (Fig. 2B) from three different sites of transgenic and non-transgenic rice paddy fields. The AFLP band patterns produced by both soil DNAs collected from

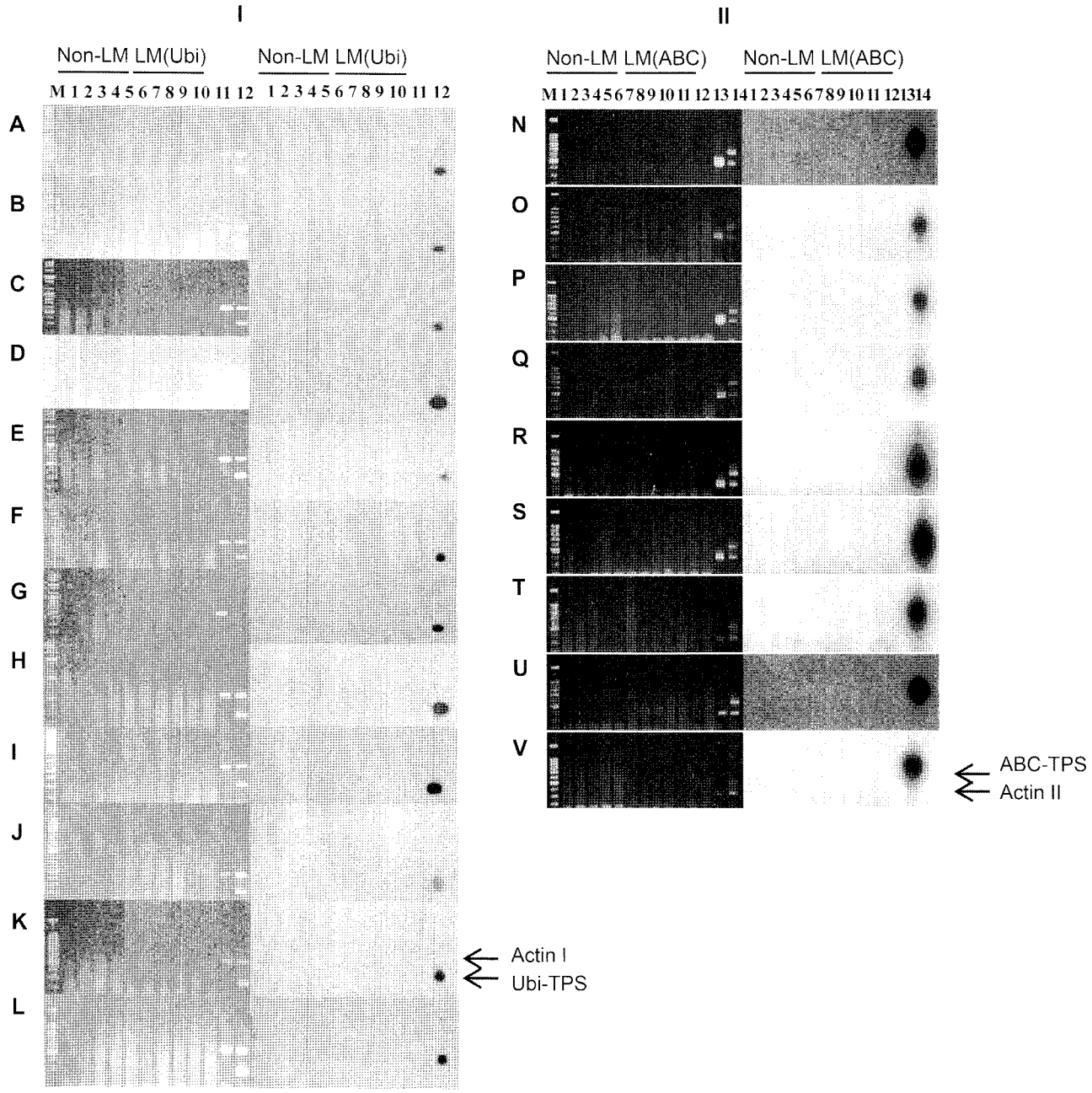

Fig. 2. Multiplex PCR products amplified from total DNA extracted from the soil samples collected monthly (**A** to **V**) at different locations by using specific primer sets for transgenic and non-transgenic rice and autoradiograms obtained after Southern hybridization with the probe DNA of the *Ubi-TPS* and *ABC-TPSP* genes.

I. PCR products (left) and autoradiograms (right) of non-transgenic and transgenic rice containing the Ubi-TPS promoter from June 2004 to May 2005. Lanes 1–5: non-transgenic rice; lanes 6–10: transgenic rice; lane 11: *act* gene; lane 12: *Ubi-TPS* gene; M: 1-kb DNA ladder. **II.** PCR products (left) and autoradiograms (right) of non-transgenic and transgenic rice containing the ABC-TPS promoter from July 2005 to March 2006. Lanes 1–6: non-transgenic rice; lanes 7–12: transgenic rice; lane 13: *act* gene; lane 14: *ABC-TPS* gene.

transgenic and non-transgenic rice fields were analyzed by the NTSYSpc program. The profiles of AFLP patterns derived from transgenic and non-transgenic rice soils showed 93–94% similarities to each other depending on the sites and time of sampling (Fig. 3A and 3B). However, the AFLP profile between Ubi-TPS and non-transgenic rice paddy soils clustered into three groups (1 and 10, 2 and 11, 9 and 12 months) showed over 96% similarities depending on sampling time. Moreover, the profile between ABC-TPS and non-transgenic rice soils was more diverse than Ubi-TPS rice, but it exhibited over 95.4% similarities, except for the transgenic and non-transgenic

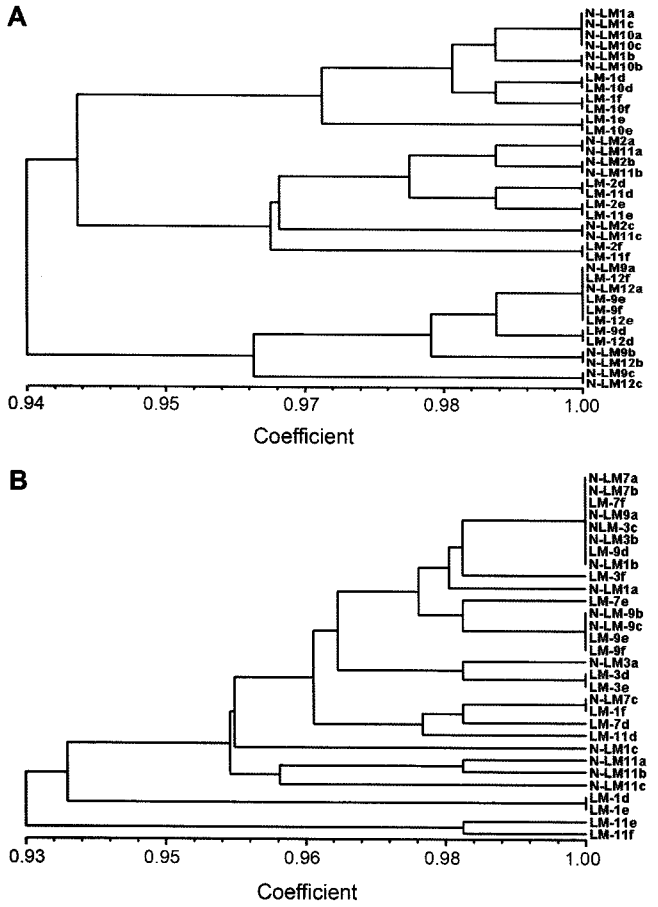

Fig. 3. Dendrogram derived from AFLP patterns.
A. Dendrogram derived from transgenic Ubi-TPSP (LM) and non-transgenic rice (N-LM) from September 2004 to February 2005. B. Dendrogram derived from transgenic ABC-TPSP (LM) and non-transgenic rice (N-LM) from July 2005 to March 2006. 1–12 and a–f: Numerical and alphabetical orders indicate the month of samples used and sites of non-transgenic (a, b, c) and transgenic (d, e, f) samples collected at different locations of paddy rice field, respectively.

soils collected in November (LM-11-d, e, f and non-LM-11-a, b, c) and January (LM-1-d, e, f and non-LM-1-a, b, c) (Fig. 3B), which seemed to be associated with the seasonal changes in winter. Thus, these data suggest that transgenic TPSP rice did not give a significant impact on the communities of soil microorganisms during the two-year experimental period, although long-term observation and continued research effort may be necessary to make a decisive conclusion.

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