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Denaturing Gradient Gel Electrophoresis Analysis of Bacterial Community Profiles in the Rhizosphere of *cry1AC*-carrying *Brassica rapa* subsp. *pekinensis*

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The effect of genetically modified (GM) *Brassica rapa* subsp. *pekinensis* (Chinese cabbage) expressing Bt toxin gene (*cry1AC*) to the rhizosphere bacterial community was examined using the denaturing gradient gel electrophoresis (DGGE) fingerprinting method. From the visual comparison of the DGGE profiles, there were no significant differences between the profiles of Bt and control rhizosphere in both Suwon and Yesan samples. From the sequence analysis of the individual bands, *Sphingomonas* sp. of *Alphaproteobacteria* and several actinobacterial members were identified as the main bacterial taxa in both Suwon and Yesan samples. In the multiple correspondence analysis, no clear separation between Bt and control rhizosphere was seen in both Suwon and Yesan datasets. The profiles of bulk soils were separated from those of rhizosphere. The DGGE fingerprinting analyses indicated that Bt crops did not significantly alter the genetic composition of rhizosphere bacterial communities.

***Keywords*:** cry1AC, rhizobacteria, *Brassica rapa* subsp. *pekinensis*, denaturing gradient gel electrophoresis

Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) is one of the most important agricultural crops in Korea, which is the main material of Kimchi. Since the crop is susceptible to insect damage, in particular that caused by diamondback moth (*Plutella xylostella*), a transgenic variety was developed earlier to reduce the use of chemical pesticides (Park *et al*., 2003). The main advantage of insect-resistant transgenic plants would be the less need for conventional insecticides. Nonetheless, cultivation of such transgenic plants has been a subject of concern to the public and scientific community due to the public perception regarding human consumption, and also to any possible effects to environment (Dunfield and Germida, 2004; Byrne, 2006).

Possible impact of GM crops expressing Bt toxins to soil biota, including bacteria, protozoa, fungi, nematodes, and earthworms, has been investigated previously (Donegan *et al*., 1995; Saxena and Stotzky, 2001; Dunfield and Germida, 2003, 2004; Blackwood and Buyer, 2004; Castaldini *et al*., 2005; Fang *et al*., 2005). Since Bt maize and cotton are the two major Bt crops cultivated worldwide, most of the studies have been focused on these crops.

Rhizobacterial communities are in nature greatly influenced by the plants, and vice versa (Park *et al*., 2005; Ahn *et al*., 2007). Enumeration of cultivable bacteria, community level physiological profiling (CLPP), phospholipid fatty acid (PLFA) analysis and cloning of environmental 16S rDNAs have been the main tools for the assessment of any changes in the physiological and genetic composition of the microbial communities associated with Bt crops (Donegan *et al*., 1995; Saxena and Stotzky, 2001; Blackwood and Buyer, 2004; Castaldini *et al*., 2005; Fang *et al*., 2005). However, the DGGE fingerprinting techniques have not been widely used in the comparison to the other methods, and only one case with experimental microcosms was reported (Castaldini *et al*., 2005).

Following the study in which the genetic composition and metabolic activities were compared using environmental 16S rDNA clones and community level physiological profiles (Jung, 2007), the aim of this study was to assess any possible effects of the transgenic *Brassica rapa* subsp. *pekinensis* carrying *cry1AC* gene to the rhizosphere bacterial communities using the DGGE fingerprinting approach.

Materials and Methods

Sampling of rhizosphere soil

Cultivar "Samjin", carrying *cry1AC*, was developed by the Rural Development Agency (RDA) of the Ministry of Agriculture and Forestry (Park *et al*., 2003). The GM variety was grown alongside with non-GM crops as control at the isolated fields in Suwon, Kyonggi Province and Yesan, Chungnam Province, managed by RDA. Rhizosphere and soil samples were collected in November 2005 and June 2006, when the crops were grown for about 70 days and ready for harvest. The samples were kept at 4°C and transported to the laboratory for the immediate treatment and subsequent analyses.

Extraction of total community DNA

DNA was extracted in triplicates from 0.5 g of rhizosphere and bulk soil with the FastDNA Spin Kit for Soil (Q-BIO Gene, USA), following the manufacturer's instructions. The

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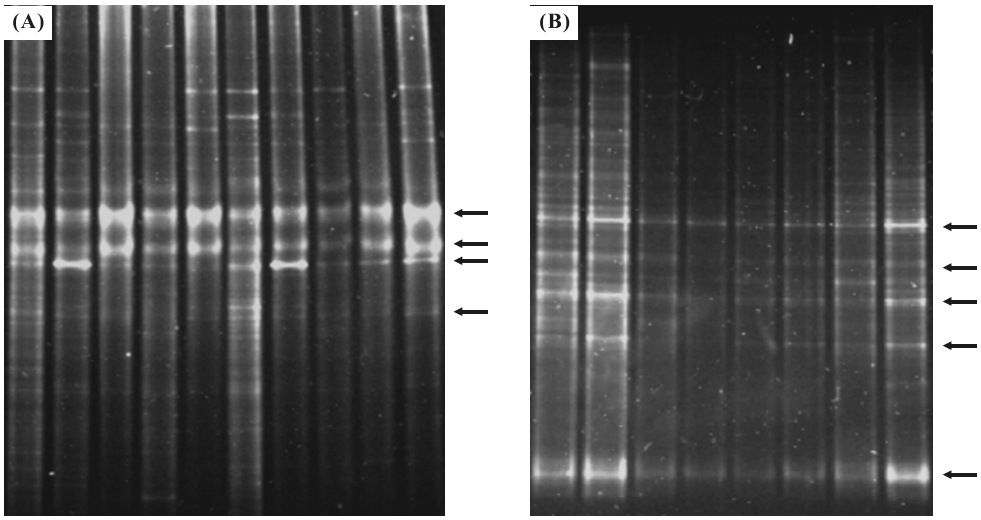

Fig. 1. Denaturing gradient gel electrophoresis profiles, (A) control (lanes 1~5 from left) and Bt (lanes 6~10) rhizosphere, Suwon (B) control (lanes 1~3 from left) rhizosphere, control bulk soil (lane 4), Bt rhizosphere (lanes 5~7) and Bt bulk soil (lane 8), Yesan.

three samples were then collected and combined for DGGE analysis.

PCR amplification of 16S rDNA V3 region

A variable region of the 16S rRNA gene was PCR-amplified using the primers 341f with GC clamp; 5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC G CC TAC GGG AGG CAG CAG-3' and 518r; 5'-ATT ACC GCG GCT GCT GG-3' (Muyzer *et al*., 1993). The expected product size for the PCR amplification with these primers was around 193 bp. Touchdown PCR was performed with an initial denaturizing step of 95°C for 5 min; followed by 20 cycles of 94°C for 1 min, 65°C~55°C for 45 sec with 0.5°C decrease at every cycle and 72°C for 1 min; followed by 10 cycles of 94°C for 1 min, 55°C for 45 sec, and 72°C for 1 min, and by a final elongation step of 72°C for 10 min. The PCR mixture contained 10 pmol of each primer, 200 μM dNTP, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 1.5 units *Taq* DNA polymerase (TaKaRa, Japan) and template DNA. Amplification products were checked on agarose gels.

Denaturing gradient gel electrophoresis (DGGE)

Parallel DGGE was carried out using the D-Code system (Bio-Rad, USA). PCR products were separated by using 10% (w/v) polyacrylamide gel containing a 35 to 65% linear gradient of denaturants (7 M urea and 40% formamide as 100% denaturant) increasing toward the direction of electrophoresis. PCR samples were mixed with 2× loading buffer (0.05% bromophenol blue and 0.05% xylene cyanol in 70% glycerol), and applied to gels in aliquots of 20 μl per lane. The running buffer was 1× TAE buffer (40 mM Tris, 20 mM acetic acid, and 50 mM EDTA, pH 8.0). Electrophoresis was performed at 60°C with 60 volts for 15 h, following an initial running with 20 volts for 30 min. After the run, gels were stained with the Nucleic acid staining kit (Bioneer, Korea) for 60 min and visualized by using the Gel Doc 2000 system (Bio-Rad, USA).

Band identification

Individual bands were excised and resuspended in distilled water overnight. PCR amplification was performed using the primer set 341f and 518r, and the PCR products were purified and sequenced. The obtained sequences were identified using the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

Statistical analysis

DGGE bands were detected using Gelcompar II (Applied Maths, Belgium). Detected band patterns were transferred to absent/present matrix, and multiple correspondence analysis was performed with SPSS release 12.0.1 (Apache Software Foundation, USA) to visualize differences among the DGGE profiles.

Results

Denaturing gradient gel electrophoresis (DGGE) fingerprinting

Ten DGGE profiles were obtained from Suwon samples, five each from Bt and control rhizosphere (Fig. 1A). Four major common bands could be recognized in all of the lanes. Around 40 individual bands could be recognized from all of the lanes, and no characteristic band was found for either Bt or control rhizosphere.

Eight DGGE profiles were obtained from Yesan samples, three each from control and Bt rhizosphere, and one each from Bt and control bulk soil, respectively (Fig. 1B). Five major common bands could be recognized in all of the lanes. Like Suwon samples, around 40 individual bands could be recognized from all of the lanes, and there was no characteristic band for either Bt or control rhizosphere. The profiles of bulk soils also apparently shared the same main bands.

Identificaiton of individual bands

The four common major bands from Suwon profiles were

identified as those of *Methylobacterium* sp., *Clostridium* sp., *Sphingomonas* sp. and an unidentified actinobacterium in the descending order at the profiles, respectively (Table 1). The five common major bands from Yesan profiles were identified as those of *Sphingomonas* sp., *Bradyrhizobium* sp., *Actinokineospora* sp., *Streptomyces* sp., and *Gemmatimonadetes* bacterium in the descending order, respectively.

Sphingomonas sp., mostly similar to the nucleotide accession number AY167840, was found to be commonly present as one of the major groups in both Suwon and Yesan, and various actinobacterial taxa were also present as other main groups. Since no characteristic band existed for either Bt or control profiles, no specific organism could be identified as Bt or non-GM specific.

Multiple correspondence analysis

In the multiple correspondence analysis to visualize qualitative differences in the DGGE profiles, separation between Bt and control rhizosphere, although not obvious, was observed in Suwon samples (Fig. 2A). However, a high level of variation existed among the individual profiles.

The rhzisophere profiles of Yesan clustered together, and those of control and Bt soils were separated from each other and also from the rhizosphere (Fig. 2B). No separation between Bt and control rhizosphere could be observed.

Table 1. Identification of individual DGGE bands

Sample*	Organism	Accession no.
Suwon	*Methylobacterium* sp.	DQ848137 (85%)
	Clostridium sp.	DQ196621 (88%)
	Sphingomonas sp.	AY167840 (92%)
	Unidentified actinobacterium	DQ054609 (92%)
Yesan	*Sphingomonas* sp.	AY167840 (99%)
	Bradyrhizobium sp.	DQ447782 (87%)
	Streptomyces sp.	AJ971910 (91%)
	Actinokineospora sp.	AY048864 (93%)
	Gemmatimonadetes bacterium	DQ829402 (89%)

* The corresponding bands are indicated as arrows sequentially from the top of the gel in Fig. 1.

Discussion

The bacterial community in an environment is dynamic, undergoing fluctuations and successions all the time. In addition, soil is not a homogeneous environment at all, and thus it is hard to observe reproducible community profiles from the samples taken at the same area (Jung, 2007). To assess any changes in bacterial composition caused by a defined factor is therefore never an easy task. DGGE fingerprinting has some advantages over other methods such as cloning of environmental 16S rDNAs or terminal restriction fragment length polymorphism (T-RFLP) analysis in that the total community composition among samples can be directly compared at a glance, and the individual components can be identified by excision and and sequencing of bands.

From the visual examination of DGGE profiles and correspondence analysis, no clear differences between the Bt and control rhizosphere could be seen. In each of the Suwon and Yesan datasets, the profiles shared virtually most major bands in common, and yet variations among the profiles were observed in the multiple correspondence analysis. These observations imply that the main bacterial constituents may remain unchaged, but the presence or absence of minor groups may be the main cause of variation. From the DGGE analysis alone, it is not clear which bacterial groups were responsible for the change.

The effects from cultivation of transgenic crops expressing Bt toxins to the genetic composition of soil microbial communities have not been clearly shown in previous studies (Donegan *et al*., 1995; Saxena and Stotzky, 2001). The phospholipid fatty acid (PLFA) or community level physiological profiles (CLPP) were not significantly different in field studies (Blackwood and Buyer, 2004; Fang *et al*., 2005), although changes in the soil respiration, bacterial composition and mycorrhizal symbiosis were reported with the Bt corn in experimental microcosms (Castaldini *et al*., 2005). The genetic

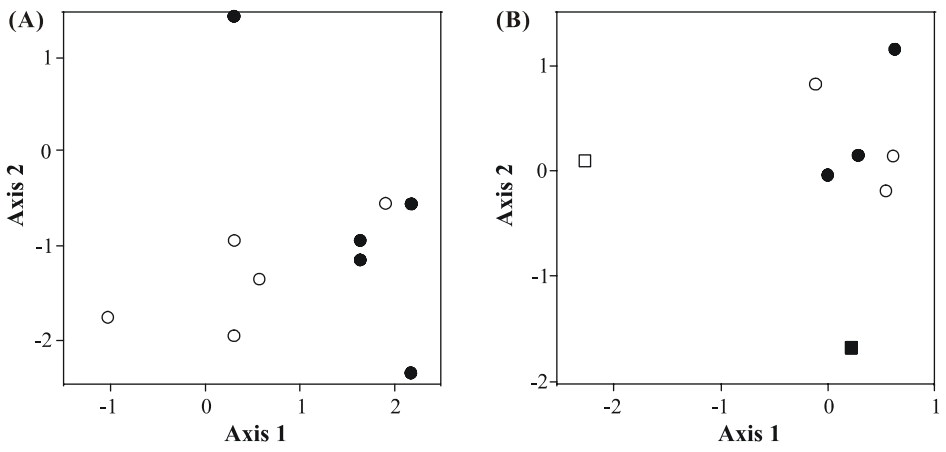

Fig. 2. Multiple correspondence analysis plots based on the DGGE profiles of (A) Suwon and (B) Yesan. Symbols indicate; (○) control rhizosphere, (●) Bt rhizosphere, (□) control bulk soil, (■) Bt bulk soil.

modification of the plants may lead to the alteration in their biochemical composition, which can affect soil organisms in turn (Donegan *et al*., 1995; Flores *et al*., 2005). In contrast, the Bt toxins produced by the transgenic plants may not have any substantial effect to the soil organisms (Saxena and Stotzky, 2001). However, GM effects are considered temporary and transient, and thus would disappear after the removal of GM crops from the fields (Dunfield and Germida, 2003, 2004).

A previous study indicates that the Bt Chinese cabbage did not seem to assert any outstanding influence to the genetic composition and metabolic activity of rhizosphere microbial community (Jung, 2007). Natural differences among samples, rather than GM effects, were suggested as the main causes of variation, which was also observed in some of the previous studies (Heuer *et al*., 2002; Blackwood and Buyer, 2004; Fang *et al*., 2005).

The question if GM crops have any substantial influence to the soil bacterial community is never an easy question to answer. Continuous and systematic monitoring of such effects using different approaches would be ideal for answering such question with confidence.

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