The Journal of Microbiology, February 2009, p. 33-39 Vol. 47, No. 1
DOI 10.1007/s12275-008-0254-4

Impact of *cry1AC*-Carrying *Brassica rapa* subsp. *pekinensis* on Leaf Bacterial Community

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(Received October 14, 2008 / Accepted November 25, 2008)

The effects of Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) carrying *cry1AC* derived from *Bacillus thuringiensis* (Bt) on leaf bacterial community were examined by analyzing the horizontal transfer of transgene fragments from plants to bacteria. The effect of plant pathogenic bacteria on the gene transfer was also examined using *Pseudomonas syringae* pathovar. *maculicola*. The frequency of hygromycin-resistant bacteria did not alter in Bt leaves, though slight increase was observed in *Pseudomonas*-infected Bt leaves with no statistical significance. The analysis of bacterial community profiles using the denaturing gradient gel electrophoresis (DGGE) fingerprinting indicated that there were slight differences between Bt and control Chinese cabbage, and also that infected tissues were dominated by *P. syringae* pv. *maculicola*. However, the cultured bacterial pools were not found to contain any transgene fragments. Thus, no direct evidence of immediate gene transfer from plant to bacteria or acquisition of hygromycin resistance could be observed. Still, long-term monitoring on the possibility of gene transfer is necessary to correctly assess the environmental effects of the Bt crop on bacteria.

***Keywords*:** *Brassica rapa* subsp. *pekinensis*, *cry1AC*, denaturing gradient gel electrophoresis, *Pseudomonas syringae* pathovar. *maculicola*, horizontal gene transfer

Bt toxin gene has been used in the development of various genetically modified crops including corn, cotton, and oilseed rape in order to avoid massive use of chemical pesticides (Estruch *et al*., 1997). However, these genetically modified crops expressing Bt toxin proteins in its tissue are likely to have possible influence to soil bacteria and other microbial communities (Donegan *et al*., 1995; Escher *et al*., 2000; Castaldini *et al*., 2005). The possibility of horizontal gene transfer (HGT) in natural habitats also represents potential risk when these genetically modified crops are cultivated in the open field. Released DNAs from genetically modified crops can spread into soil and persist for several years (Widmer *et al*., 1997; Meier and Wackernagel, 2003). It has been reported that there are naturally competent bacteria in soil (Lorenz and Wackernagel, 1994; Nielsen *et al*., 1997), and the genus *Pseudomonas* and *Acinetobacter* are able to integrate an antibiotic resistance marker gene released from genetically modified crops (Gebhard and Smalla, 1998; Nielsen *et al*., 2000; De Vries and Wackernagel, 2001).

Endophytic bacteria reside in plant hosts without causing disease symptoms and can be isolated from surface disinfected internal plant tissue (Hallmann *et al*., 1997). It has been demonstrated that the endophytic niche is a hot spot for horizontal gene transfer (HGT) (Taghavi *et al*., 2005). The endophytic *Pseudomonas* showed high transfer rates of plasmids into a range of autochthonous endophytes (Ryan *et al*., 2007). The colonization of ecological niches by endophytic bacteria is similar to that by plant pathogens (Ulrich *et al*., 2008). If plants are colonized simultaneously by endophytic bacteria and plant pathogenic bacteria, it can be speculated that HGT in the endophytic niche may be influenced or facilitated by the presence of plant pathogens. However, little is known on the roles of plant pathogens in the HGT from genetically modified plants to endophytic bacteria.

Regarding the Bt Chinese cabbage, it has been recently shown that there were no apparent differences in the rhizosphere bacterial community composition between the Bt and non-Bt crops (Jung *et al*., 2008). In the present study the impact of Bt Chinese cabbage to leaf-decomposing endophytic bacterial community composition and possible HGT of transgenes from plants to bacteria were examined. In addition, the effects of plant pathogens on the HGT were also examined using *Pseudomonas syringae* pathovar. *maculicola*, which causes bacterial leaf spot disease in Chinese cabbage. The following analyses were carried out and the results compared between Bt and control crops; a) viable counts of leaf-decomposing endophytic bacteria, b) size and frequency of hygromycin-resistant bacteria; c) bacterial community composition based on denaturing gradient gel electrophoresis (DGGE) fingerprinting. Specific primers were used to detect presence of any gene fragments derived from plants in the pools of cultured bacteria. To see if plant pathogens facilitate gene transfer from plants to bacteria, the leaves were infected by *Pseudomonas syringae* pv. *maculicola*, and the above experiments were repeated.

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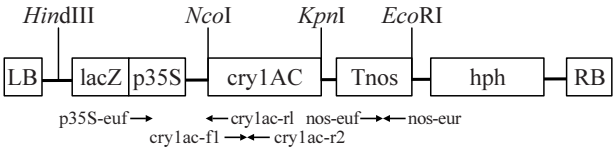

Fig. 1. The structure of pBT21 containing a synthetic *cry1AC* gene and locations of primers used for PCR (p35S, 35S promoter; Tnos, NOS terminator; hph, selective marker encoding hygromycin resistance; lacZ, β-galactosidase; LB, left border; RB, Right border).

Materials and Methods

Plant sample and treatment

The Bt Chinese cabbage (*Brassica rapa* subsp. *pekinensis*, cultivar Samjin) used in this study has 35S promoter, NOS terminator for the expression of synthetic *cry1AC* gene (GenBank accession no. AY126450) and a hygromycin-resistant selection marker (Fig. 1; Park *et al*., 2003). Control and Bt Chinese cabbage samples were grown for 10 weeks in an isolated experimental field maintained by National Institute of Agricultural Biotechnology (NIAB), Korea, collected and transported to the laboratory under refrigeration.

The leaves were washed thoroughly with tap water to remove soil particles. Subsequently the leaves were immersed in 70% aqueous ethanol for 1 min, disinfected in 1% aqueous sodium hypochlorite solution for 10 min, and finally washed twice with sterile distilled water. The washed water was inoculated onto the nutrient agar (NA) plates to check the efficiency of disinfection. Leaves from both Bt and control crops were prepared from three individual plants, and the leaf samples were prepared in triplicates from each individual, respectively.

Viable counts of total and hygromycin-resistant bacteria

The numbers of total and hygromycin-resistant bacteria were counted from the decomposing leaves of control and Bt Chinese cabbage. The viable counts were calculated from numbers of bacterial colonies per 1 g dry sample. The leaves infected by *Pseudomonas syringae* pv. *maculicola* were also used for the counting. *P. syringae* pv. *maculicola* (KACC 11637) was cultured in nutrient broth media (Difco, USA) for 1 day at 30°C. The 100 μl of the culture solution (~10^7 CFU/ml) was added onto 1 g of surface-sterilized Chinese cabbage leaf tissue slice and incubated for 1 week at 30°C. Sterile uncultured nutrient broth media was used as infection control. After the incubation, the samples were diluted and plated on nutrient agar (NA) media (Difco, USA) without or with hygromycin (NA^{hyg}, 50 μg/ml final concentration), and colony forming units (CFU) were counted after 1 day at 30°C. The occurrence rate of hygromycin-resistant colonies was calculated as the percentage of CFU counts on NA^{hyg} divided by the CFU counts on NA. Any differences in the estimates between Bt and control data were examined by t-test using SPSS version 10.0 (SPSS, USA). The *P*-values were calculated, and the significance of differences was evaluated accordingly.

Denaturing gradient gel electrophoresis (DGGE)

To extract bacterial gDNAs directly from leaves, infected or non-infected Chinese cabbage tissue slices were placed in mortar with liquid nitrogen and ground to powder with pestle. The powdered sample was mixed with resuspension solution (10 mM Tris-HCl; pH 8.0, 1 mM EDTA; pH 8.0, 0.5% SDS, 0.1 mg/ml proteinase K) and incubated at 37°C for 1 h. To this, 1/6 volume of 5 M NaCl was added and mixed thoroughly. Subsequently 1/8 volume of CTAB/NaCl solution (0.2 M Tris-HCl; pH 8.0, 0.05 M EDTA; pH 8.0, 2 M NaCl, 2% cetyltrimethyl ammonium bromide) was added and incubated at 65°C for 10 min. The mixture was then extracted with phenol/chloroform/isoamyl alcohol, and DNA was precipitated with 0.6 volume of isopropanol. The pellet was washed with 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl; pH 8.0, 1 mM EDTA, pH; 8.0). The primers GC341f and 518r were used to amplify the V3 variable region of 16S rDNA for DGGE analysis (Table 1; Temmerman *et al*., 2003). The DGGE analysis and identification of individual bands followed previously described procedures (Jung *et al*., 2008).

Table 1. Primer sequences and amplicon length used in polymerase chain reaction assays performed in the present study

Primers	Primer-1 Primer-2	Amplicon (bp)	Target	Reference
27f-1492r	5'-AGAGTTTGATCMGGCTCAG-3' 5'-GGTTACCTTGTTACGACTT-3'	~1,485	16S rDNA	Lane (1991)
GC341f-518r	5'-CGCCCGCCGCGCCCCGCGCCC GGCCCGCCGCCCCCGCCCGCCTA CGGGAGGCAGCAG-3' 5'-ATTACCGCGGCTGCTGG-3'	193	16S rDNA	Muyzer *et al*. (1993)
p35S-euf-cry1ac-r1	5'-GCTCCTACAAATGCCATCA-3' 5'-AA GAGATGTCGATGGGAGT-3'	380	35S promoter *cry1AC* gene	Pietsch *et al*. (1997) This study
cry1ac-f1-cry1ac-r2	5'-TGTCCGTGTACGTTCAAGCA-3' 5'-TAGGGTAACGTCTGGAGTCA-3'	299	*cry1AC* gene	This study
nos-euf-nos-eur	5'-GAATCCTGTTGCCGGTCTTG-3' 5'-TTATCCTAGTTTGCGCGCTA-3'	180	NOS terminator	Pietsch *et al*. (1997)

Table 2. The numbers of total and hygromycin-resistant bacteria from control Chinese cabbage and Bt Chinese cabbage

Samples[a]	CFU×10[10]/g dry sample (standard error)				Frequency of resistant bacteria		*P*-value
	Control		Bt		Control	Bt	
	NA^{hyg}	NA	NA^{hyg}	NA	% ± Standard Error		
Uninfected	0.6 (0.3)	2.4 (0.6)	3.7 (0.7)	13.0 (2.1)	33.3 (18.4)	34.9 (9.0)	0.918
Infected	12.2 (3.4)	26.6 (7.0)	24.3 (3.7)	39.6 (4.2)	46.9 (4.7)	62.2 (6.7)	0.121

[a] Each average was calculated from 7~9 independent plate counts. NA, nutrient agar media; Na^{hyg}, NA with hygromycin B (50 μg/ml).

PCR amplification of transgenes and Southern hybridization analysis

Bacterial colonies from non-infected and infected Chinese cabbage samples by dilution plate culture method on NA media were collected by adding 2 ml of sterilized distilled water. After centrifugation of bacterial cell suspension for 5 min at 12,000 rpm, DNA was isolated from the pellet by using Genomic DNA Prep Kit for bacteria (Solgent, Korea). PCR primers for detecting 35S promoter and NOS terminator were taken from a previous study (Pietsch *et al*., 1997), and those for synthetic *cry1AC* gene were designed and evaluated in this study (Table 1). The PCR amplification followed previously described procedures (Kim *et al*., 2004). The primer pair for 16S rDNA was used as a positive control in PCR. Southern blot hybridization was performed to confirm genuine amplification of target products, using purified pBT21 vector (Park *et al*., 2003) as the probe DNA. The probe was labeled using DIG High Prime DNA labeling and detection kit (Roche, Germany), and hybridization was performed at 65℃ for 16 h.

Results

Viable counts of total and hygromycin-resistant bacteria

The average dry weight of each 1 g fresh sample was 0.05 ±0.01 g, and there was no difference between control and Bt Chinese cabbage tissue. The average viable count of total bacteria in Bt Chinese cabbage was $13.0\pm2.1\times10^{10}$ CFU/g, which was about 5.4 times higher than that of control crop, and the average viable count of hygromycin-resistant bacteria was $3.7\pm0.7\times10^{10}$ CFU/g, which was about 6.2 times higher than that of control (Table 2). In contrast, there was no significant statistical difference by t-test in the frequencies of hygromycin-resistant bacteria between Bt and control crop, as the frequencies were 34.9±9.0% and 33.3±18.4%, respectively, thus indicating no specific increase in the proportion of hygromycin-resistant bacteria among the total bacterial populations.

Similar results were observed when the Chinese cabbage was infected with *P. syringae* pv. *maculicola*, as the average viable count of total bacteria in Bt Chinese cabbage was $39.6\pm4.2\times10^{10}$ CFU/g, which was about 1.5 times higher than that of control crop infected with *P. syringae* pv. *maculicola*. The average viable count of hygromycin-resistant bacteria was $24.3\pm3.7\times10^{10}$ CFU/g, which was about 2.0 times higher than that of control (Table 2). Consequently, increase in the frequency of hygromycin-resistant bacteria was observed, as the frequency in the Bt crop was 62.2± 6.7% and that in the control crop was 46.9±4.7%. However, such difference should not be considered statistically significant, as the *P*-value was far higher than 0.05 (Table 2).

Bacterial community analysis using DGGE

The Bt and control Chinese cabbage samples shared some major bands in common (Fig. 2), but the Bt samples (lanes 1~4) had characteristic bands with higher GC ratio compared to those of control (lanes 5~8). The DNA sequences of one of these bands (band M) matched to *Mesorhizobium* sp. with 98.5% similarity in the 131 bp of 16S rDNA sequences (GenBank accession no. DQ659081). These bands did not appear in control samples (lanes 5~8). The DGGE profiles of *P. syringae* pv. *maculicola* infected samples showed two common major bands (bands P1 and B3, bands P2 and B4) in both control and Bt samples (lane 9~16). The common bands to all samples (bands B1, B2, B3, and B4) were identified as those of *Brassica rapa* subsp. *pekinensis* chloroplast sequences (GenBank accession no. DQ231548). The common bands in the infected samples (bands P1 and P2) were identified as those of *P. syringae* pv. *maculicola* with 100% similarity in the 138 bp of 16S rDNA sequences (GenBank accession no. AB001444), thus showing that the population of *P. syringae* pv. *maculicola* was predominant in all samples after the infection. The differences of DGGE profiles between infected and non-infected samples clearly

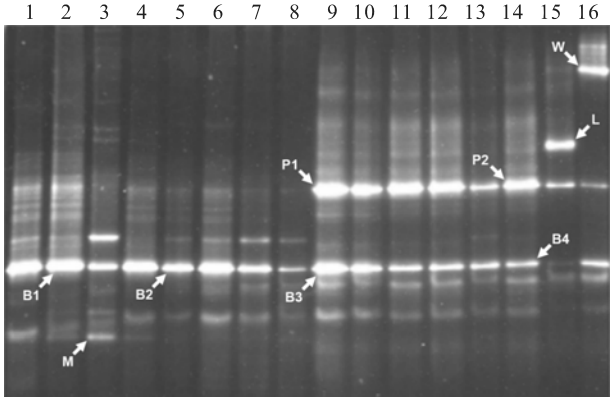

Fig. 2. DGGE analysis of V3 variable region of 16S rDNA from bacteria in non-infected and infected Chinese cabbage samples (1~4, non-infected Bt leaf; 5~8, non-infected control leaf; 9~12, Bt leaf infected with *P. syringae* pv. *maculicola*; 13~16, control leaf infected with *P. syringae* pv. *maculicola*). Arrows indicate the bands used for sequence analysis. The sequence has similarity to *Brassica rapa* subsp. *pekinensis* chloroplast (B1~B4), *Pseudomonas syringae* pv. *maculicola* (P1~P2), *Mesorhizobium* sp. (M), *Lactobacillus* sp. (L), and *Weissella* sp. (W).

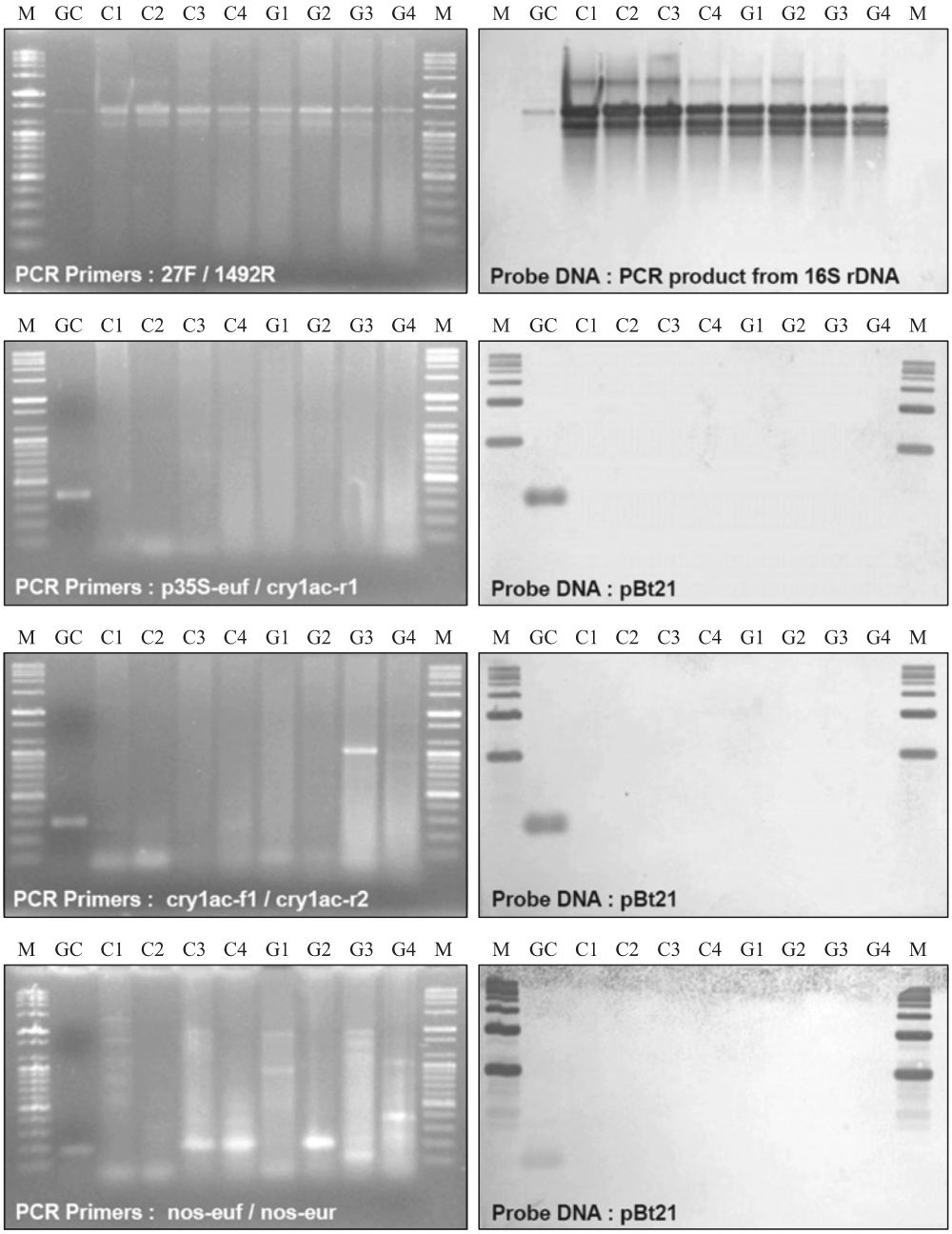

Fig. 3. PCR detection of transgenes in cultured bacterial pools from non-infected Chinese cabbage leaf tissue and Southern hybridization analysis with each probe DNA (M, 100 bp plus DNA ladder; GC, Bt Chinese cabbage gDNA; C1∼C4, bacterial gDNAs isolated from control leaf; G1∼G4, bacterial gDNAs isolated from Bt leaf).

indicated that the bacterial communities had been altered by the infection with *P. syringae* pv. *maculicola*. In addition, two of the infected control samples (Lanes 15 and 16) showed distinct strong bands (bands L and W) that were not present among all non-infected samples and other infected samples (Fig. 2). The sequence of band L was closest to *Lactobacillus* sp. (98.7% similarity from the comparison of 152 bp, GenBank accession no. AF368385), and that of band W to *Weissella* sp. (99.3% similarity from 152 bp, accession no. EU360131), respectively.

Detection of transgenes

Every primer pair successfully amplified target sequences from the genomic DNA (gDNA) of Bt Chinese cabbage. However, no obvious amplification of transgenes could be observed from most cultured bacterial pools (Fig. 3). After Southern blot hybridization with probe pBT21, there were no positively hybridizing bands on PCR products from bac-

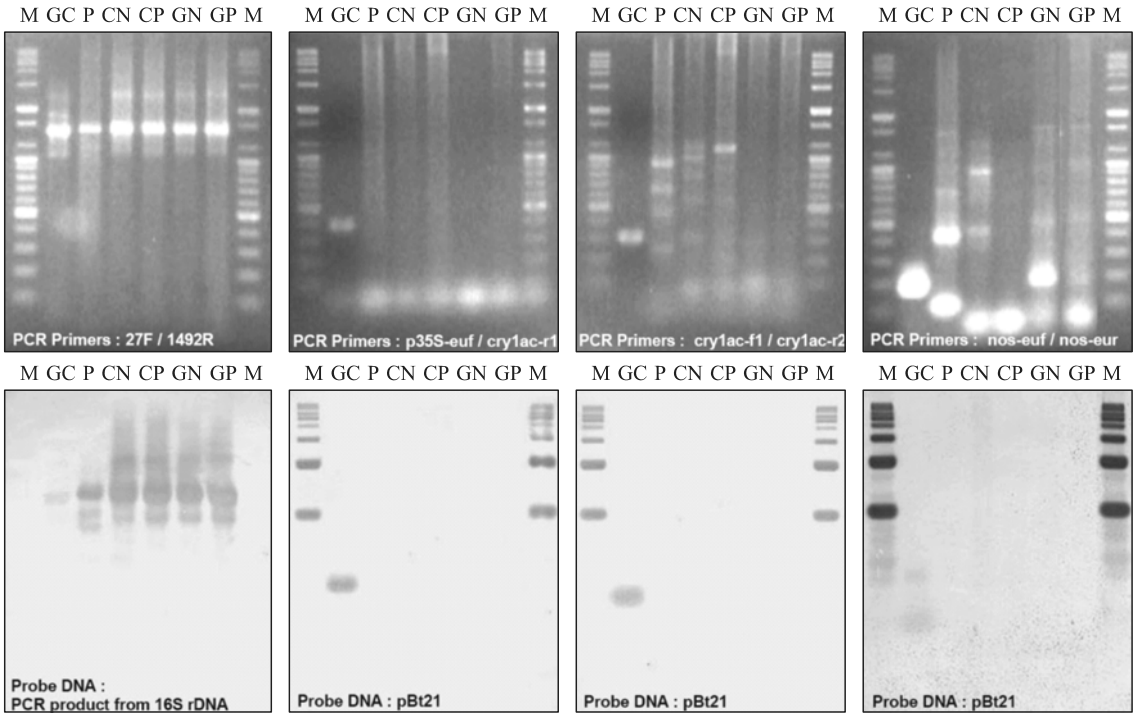

Fig. 4. PCR detection of transgenes in cultured bacterial pools from infected samples and Southern hybridization analysis with each probe DNA. Template DNA for each PCR reaction was a mixture of four replicates (M, 100 bp plus DNA ladder; GC, Bt leaf; P, *Pseudomonas syringae* pv. *maculicola*; CN, non-infected control leaf; CP, control leaf infected with *P. syringae* pv. *maculicola*; GN, non-infected Bt leaf; GP, Bt leaf infected with *P. syringae* pv. *maculicola*).

terial gDNAs except for Bt Chinese cabbage gDNA as a positive control, thus showing that the amplified products in some samples, such as G3 with primers cry1ac-f1/cry1ac-r2, did not actually contain the target sequences. Similar results were obtained with the *P. syringae* pv. *maculicola* infection, as positive amplification was seen in some samples but with no positive hybridization signal (Fig. 4).

Discussion

The decomposing Bt Chinese cabbage was found to contain higher numbers of bacteria, but there was no observable increase in the relative frequency of hygromycin-resistant bacteria in the Bt leaves. Apparent increase in the frequency of hygromycin resistant bacteria in *Pseudomonas*-infected samples also proved statistically insignificant. The subsequent analyses including the comparison of bacterial community composition and the examination of gene transfer from plant to bacteria imply that the changes in bacterial density or frequency of hygromycin-resistant bacteria, if any, may not be a consequence of the uptake of transgene fragments by bacteria.

The culture-based analysis of bacterial community, although it has its own value, for example the screening of antibiotic resistant bacteria as in this study, may not render correct views on the actual diversity, as exemplified in some of our previous studies (Park *et al*., 2005; Lee *et al*., 2006). In this study, DGGE fingerprinting method was employed for the comparative analysis of community profiles between Bt and control leaves. The resultant data suggest that the composition of bacterial communities can change after the infection of pathogenic bacteria. A similar result was also observed in potato plants infected with *Erwinia carotovora* subp. *atroseptica*, as increased endophytic bacterial diversity was seen in infected potato plants (Reiter *et al*., 2002). In the present study, differences in DGGE profiles were observed between control and Bt Chinese cabbage samples in both infected and uninfected samples. However, differences among the individual samples could also be observed, and therefore it was not clear whether such differences in the profiles could be largely attributed to the individual differences or differences between Bt and control samples. A possible explanation on the differences would be the altered biochemical contents in Bt plant tissue. For example, it has been demonstrated that Bt corn plants showed differences in fructose and other soluble carbohydrate contents compared to non-transgenic plants (Escher *et al*., 2000). Other works reported higher lignin content in the tissue of Bt corn compared with non-Bt isolines (Flores *et al*., 2005; Poerschmann *et al*., 2005). The examination of Bt Chinese cabbage also revealed that there were slight differences in nutrient composition compared to control, such as carbohydrates, proteins and inorganic nutrients (data unpublished).

It has been reported that the frequency of possible HGT from plant to bacterial community is extremely low (Schlüter *et al*., 1995), and also that it is difficult to detect rare HGT

events (Nielsen and Townsend, 2004). In the present study, a model system with a plant pathogen *P. syringae* pv. *maculicola* under an optimized condition was used to examine the possible effects in HGT event. *P. syringae* pv. *maculicola* was expected not only to release transgene DNA fragments to endophytic niche after degradation of Chinese cabbage tissue cells to facilitate HGT event within endophytic bacterial communities, but also to be a recipient itself of transgenes. The results showed that no direct evidence of immediate uptake of transgenes from Bt Chinese cabbage with infection or without infection by *P. syringae* pv. *maculicola*.

In this study, variation in the community size or composition of leaf-decomposing bacteria was observed in Bt crops compared to control. However, no direct evidence of immediate uptake of transgenes from Bt crops by bacteria could be seen. The infection of a plant pathogen *P. syringae* pv. *maculicola* also did not facilitate the uptake of transgenes. The hygromycin-resistant bacteria in Bt leaves may not be explained by the uptake of transgene fragments, since similar level of hygromycin resistance was already present among the bacteria from control leaves. Thus, the variation in the leaf-associated bacterial community size and composition is likely attributed to the alteration in biochemical composition of host plant.

However, the present study only implies the fact that immediate uptake of plant-derived transgenes is unlikely. Further works, including the examination of the fate of transgene fragments in soil, their uptake by rhizosphere and soil bacteria, and secondary transfer to any other organisms, would give more comprehensive information on the possibility of the spread of transgenes to environment.

Acknowledgements

This work was carried out with the support from the BioGreen 21 Program, sponsored by the Rural Development Administration (grant no. 20050601-034-857-007-07-00), Republic of Korea. The authors also acknowledge the support from the Brain Korea 21 program of the Ministry of Education, Science and Technology, Republic of Korea.

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