Research Article

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Soil Microbial Community Assessment for the Rhizosphere Soil of Herbicide Resistant Genetically Modified Chinese Cabbage

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Abstract

BACKGROUND: Cultivation of genetically modified(GM) crops rapidly has increased in the global agricultural area. Among those, herbicide resistant GM crops are reported to have occupied 89.3 million hectares in 2010. However, cultivation of GM crops in the field evoked the concern of the possibility of gene transfer from transgenic plant into soil microorganisms.

In our present study, we have assessed the effects of herbicide-resistant GM Chinese cabbage on the surrounding soil microbial community.

METHODS AND RESULTS: The effects of a herbicide-resistant genetically modified (GM) Chinese cabbage on the soil microbial community in its field of growth were assessed using a conventional culture technique and also culture-independent molecular methods. Three replicate field plots were planted with a single GM and four non-GM Chinese cabbages (these included a non-GM counterpart). The soils around these plants were compared using colony counting, denaturing gradient gel electrophoresis and a species diversity index assessment during the growing periods. The bacterial, fungal and actinomycetes population densities of the GM Chinese cabbage soils were found to be within the range of those of the non-GM Chinese cabbage soils. The DGGE banding patterns of the

GM and non-GM soils were also similar, suggesting that the bacterial community structures were stable within a given month and were unaffected by the presence of a GM plant. The similarities of the bacterial species diversity indices were consistent with this finding.

CONCLUSION: These results indicate that soil microbial communities are unaffected by the cultivation of herbicide-resistant GM Chinese cabbage within the experimental time frame.

Key Words: Chinese cabbage, DGGE, GM, herbicide-resistant, soil microbial community

Introduction

Chinese cabbage (*Brassica campestris* L. ssp. *Pekinensis*) is a commercially important vegetable in Korea, Japan, China and many other countries of South Asia (Cho *et al.*, 2001). The phosphinothricin acetyltransferase (*pat*) gene has been isolated from *Streptomyces viridochromogenes* and transgenic plants expressing this gene are resistant to the herbicide glufosinate (Wohlleben *et al.*, 1992). Glufosinate and glyphosate are relatively environmentally benign, non-residual, post-emergence herbicides that target both monocotyledonous and dicotyledonous weedy species (Duke, 2005; Espinoza-Ésquivel and Arrieta-Espinoza, 2007; Olofsdotter *et al.*, 2000). Through the use of these herbicides, the productivity

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of GM crops can be increased and labor costs can be reduced. Hence, herbicide-resistant GM crops offer a valuable and environmentally friendly alternative for effective weed control. In 2010, herbicide resistant GM crops are reported to have occupied 89.3 million hectares (Peng, 2011). Although the total global cultivation area of GM crops is increasing markedly, legislation to regulate their cultivation is being introduced in a number of countries.

There are a number of well established concerns regarding the commercial cultivation of GM crops including plant invasiveness, dispersal of the plant into the native ecosystem, gene flow through pollen transfer, development of resistance in target organisms and non-target effects on native flora and fauna including effects on the biodiversity of beneficial and antagonistic microorganisms. However, the potential for interaction between transgenic plants and plant residues and the soil microbial community is not well understood (Eastham and Sweet, 2002; Nielsen *et al.*, 2001; Wolfenbarger and Phifer, 2000; Riba *et al.*, 2000). Novel proteins have been shown to be released from transgenic plants into the soil ecosystem, and their presence can influence the biodiversity of the microbial community by selectively stimulating the growth of organisms that can use these factors (Dunfield and Germida, 2004). It is therefore important to identify the transgene-associated pertinent target population and investigate the possible effects of GM plants on the microbial community as part of a soil environmental risk assessment. Despite the controversy surrounding the environmental impact of GM crops, the global transgenic crop hectareage and number of countries utilizing these transgenic varieties is growing yearly. According to the International Service for the Acquisition of Agrobiotech Applications (ISAAA), the estimated global area of genetically modified crops was 160 million ha in 2010, and has increased 94-fold increase since 1996 (James, 2011; Peng, 2011). It is therefore essential to perform pre-commercialization tests of these plants for environmental safety and ensure that every GM crop undergoes post approval monitoring to guarantee that crops engineered using biotechnology continue to be safe for both consumers and the environment.

In our present study, we have assessed the effects of herbicide-resistant GM Chinese cabbage on the surrounding soil microbial community. Using

culture-dependent and -independent molecular methods, soil microbial community density, soil bacterial community changes and the composition of the soil bacterial communities during growing periods were compared for GM and non-GM Chinese cabbages.

Materials and Methods

Plant materials and field design

GM Chinese cabbage seeds, cultivar Samjin, into which the *bar* gene had been introduced to produce herbicide resistance (Cho *et al.*, 2001) were provided by Dr. Hyun-Suk Cho from RDA, Korea. Field testing was performed at the National Academy of Agricultural Science (NAAS), Korea beginning in July 2008. GM and non-GM Chinese cabbage (cv. Samjin, Jangmi, Gawulmat and Hwangseong) seeds were sown in a nursery box and transplanted into a field two weeks later (Fig. 1). Three subplots were assigned per plant type (3 x 3 meters). Soil samples were taken from each subplot in the middle of September and October, 2008. The roots were shaken

Fig. 1. Herbicide-resistant transgenic and conventional non-GM Chinese cabbages photographed after four weeks of culture in the field. HR, herbicide-resistant; SJ, Samjin; GWM, Gawulmat; HS, Hwangseong; JM, Jangmi.

vigorously to separate loosely adherent soil. These rhizosphere soil samples were then sieved through a 2-mm mesh and stored at 4 C until needed.

Growth media and culture conditions

Culture media used for bacterial growth were prepared with 20 g of R2A agar (BD Diagnostic Systems, Sparks, MD) and 0.05 g of cycloheximide per liter of distilled water. Media used for the growth of actinomycetes contained 0.2 g/L sodium caseinate, 0.5 g/L KH_2PO_4 , 0.2 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.5 g/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. Fungi were cultured in broth containing 20 g of R2A agar supplemented with 0.2 g/L chloramphenicol. To prepare solid medium, agar (BD Diagnostic Systems, Sparks, MD) was added to this liquid broth at a concentration of 1.5%. One gram of each sieved soil sample was suspended in 100 mL of 0.85% NaCl solution. This diluted soil suspension was then serially diluted tenfold (up to 10^{-7}), and 200 μL of each of the last three dilutions was used to inoculate 3 or 5 Petri plates at each dilution level to generate a counting set of 9 or 15 plates. Inocula were then spread over the surface of the agar-containing medium using sterile glass spreading rods. Plates containing bacteria, actinomycetes and fungi were incubated at 28 C for 48 h, 120 h, and 96 h, respectively. Microbial community density results were analyzed by one-way ANOVA using SPSS 15.00 (SPSS Inc., Chicago, USA). The significance level was set at $P < 0.05$.

DGGE(denaturing gradient gel electrophoresis) analysis

Soil DNA was extracted using a FastDNA Spin Kit (Qbiogene, Carlsbad, CA). PCR amplification of the 16S rRNA genes was performed with the primer pairs 1070f: 5'-ATGGCTGTCGTCAGCT-3' and 1392r: 5'-CGCCCCGCCGCGCCCCGCGCCCCGGCCCGC CGCCCCCGCCCCACGGGCGGTGT-3' (*E. coli* 16S rRNA gene sequence numbering). The 1392r primer contains a GC clamp of 40 bases with a highly variable V9 region. PCR amplification was performed in a 50 μL reaction mixture containing 10X PCR buffer [160 mM $(\text{NH}_4)_2\text{SO}_4$, 670 mM Tris/HCl, pH 8.8, 25 mM MgCl_2 , 0.1% Tween 20], 1 μL 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 step at 95 C for 5 min, followed by 30 cycles of 95 C for 1 min,

55 C for 1 min, 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, CA). A linear gradient of 40 to 70% (vol/vol) formamide plus 7M urea was used for DGGE, which was performed in 1X Tris-acetate-EDTA buffer at 60 C and a constant voltage of 60 V for 18 h. After electrophoresis, the gels were stained with SYBR Green I (Cambrex BioScience, Rockland, ME) for 15 min, rinsed for 25 min, and photographed under UV transillumination (302 nm).

PCR amplification of soil bacterial 16S rRNA gene

Soil microbial community DNA was extracted using a FastDNA Spin Kit (Qbiogen, Carlsbad, CA). PCR was carried out using the primers, 27f: 5'-AGAGTTTGATCM TGGCTCAG-3' and 1492r: 5'-GGYTACCTTGTTACGACTT-3', which target the 16S rRNA genes of a wide number of domain bacteria at positions 28 through 1492 (*E. coli* 16S rRNA gene sequence numbering). The PCR protocols used were as described for DGGE and 2 μL aliquots of the PCR products were evaluated by electrophoresis on horizontal 1.5% agarose gels.

Cloning and Sequencing

The 16S rDNA PCR products obtained from the soil bacterial community DNA samples were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and a clone library was then constructed from pooled PCR products of the soil DNA sampled in September. For bacteria, 96 clones were chosen randomly from each clone library. Plasmids were prepared using a Bioneer miniprep kit (Bioneer, Daejeon, Korea) in accordance with the manufacturer's instructions. Sequencing was performed using an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, CA) with the sequencing primers 27f: 5'-AGAGTTTGATCMTGG CTCAG-3' and 519r: 5'-ACCGCGGCTGCTGGCAC-3'.

Analysis of 16S rRNA gene sequence and bacterial species diversity indices

16S rDNA sequences were checked for chimeras using the Mallard program. Sequences were then compared with those in the GenBank, Bioinformatics Bacteria Identification and Ribosomal Database Project databases. Bacterial diversity was measured using the Shannon-Wiener's index, which determines the soil

Table 1. Number of microbes detected in the rhizosphere soils of herbicide resistant (HR) and conventional Chinese cabbages

Microbes	Month	HR	Non-transgenic Chinese cabbage			
			SJ	JM	GWM	HS
Bacteria ($\times 10^6$)	Sept	4.0 $\pm$ 0.2	3.2 $\pm$ 0.9	7.6 $\pm$ 0.7*	2.8 $\pm$ 0.2*	4.3 $\pm$ 0.4
	Oct	1.1 $\pm$ 0.7	7.1 $\pm$ 0.3*	3.1 $\pm$ 0.3*	1.5 $\pm$ 0.1	2.0 $\pm$ 0.2*
Actinomycetes ($\times 10^6$)	Sept	1.7 $\pm$ 0.4	1.1 $\pm$ 0.6*	4.0 $\pm$ 0.3*	1.2 $\pm$ 0.7	2.5 $\pm$ 0.2*
	Oct	2.1 $\pm$ 0.2	1.9 $\pm$ 0.2	1.1 $\pm$ 0.1	3.1 $\pm$ 0.4*	1.1 $\pm$ 0.1*
Fungi ($\times 10^4$)	Sept	2.9 $\pm$ 0.1	1.7 $\pm$ 0.6*	1.9 $\pm$ 0.1*	1.8 $\pm$ 0.9*	3.6 $\pm$ 0.4
	Oct	2.9 $\pm$ 0.2	2.2 $\pm$ 0.4	1.7 $\pm$ 0.4*	2.7 $\pm$ 0.3	1.8 $\pm$ 0.3

Values indicate the colony forming unit (CFU)/g fresh weight $\pm$ standard deviation from three replicates. The asterisk denotes a significant difference ($P < 0.05$). SJ, Samjin; JM, Jangmi; GWM, Gawulmat; HS, Hwangseong.

Fig. 2. DGGE analysis of the 16S rDNA V9 region obtained after PCR amplification with the eubacterial primers 1070f and 1392r. Shown are DGGE profiles of field soil samples taken in September (A&B) and October (C&D) of herbicide-resistant Chinese cabbage (HR1, 2 & 3), and non-GM Samjin (SJ1, 2 & 3), Jangmi (JM1, 2 & 3) Gawulmat (GWM1, 2 & 3) and Hwangseong (HS1, 2, & 3) Chinese cabbage cultivars. M, DGGE molecular markers.

microbial diversity index according to the following formula (Odum, 1998):

$$H' = -\sum_{i=1}^S P_i \ln P_i$$

Where H' = the Shannon-Wiener's diversity index, S = the number of genera, $P_i = n_i/N$ as the proportion of type i (n_i = the total number of individuals of microbe in type i ; N = the total number of all the individuals in total n). The criteria adopted for interpreting the Shannon-Weiner's diversity (Ferianita-Fachrul *et al.*, 2005) were as follows: $H' < 1$, low diversity; $H' = 1-3$, fair diversity, and $H' > 3$, high diversity.

Results

Culturable microbial communities

The numbers of culturable bacteria, actinomycetes, and fungi from Chinese cabbage soils were estimated during an assigned growing period (September to October 2008). The bacterial cell densities ranged from 1.1×10^6 - 7.6×10^6 cfu/g and there was little difference found between GM and non-GM Chinese cabbage soils in this regard during the period examined (Table 1). The actinomycete and fungal

population densities ranged from 1.1×10^6 - 4×10^6 and 1.7×10^4 - 3.6×10^4 , respectively, and also showed few differences between GM and non-GM Chinese cabbage soils. The actinomycete cell densities of the September and October GM soil samples and the fungi cell density of the September GM soil were also within the range of the non-GM Chinese cabbage soils. In October, the fungal cell density showed no significant difference between GM and non-GM Chinese cabbage soils apart from the Jangmi Chinese cabbage soil.

DGGE analysis

DGGE analysis was performed to investigate the impacts of GM Chinese cabbage growth on the overarching structure of the soil bacterial community. Total soil DNA samples were PCR-amplified using the V9 region of the 16S rRNA gene. The band patterns were similar between all of the soil samples tested in a given month (Fig. 2) but some minor variations were detected. These results suggested that the bacterial communities of Chinese cabbage soils are quite stable during growing period and that the cultivation of GM Chinese cabbage has no significant effect on the soil microbial community structure.

Fig. 3. Bacterial community analysis of soil samples from field-grown Chinese cabbages. Rhizospheric soils were sampled in September 2008 from the following cultivars: A, Samjin non-GM. B, Herbicide-resistant GM Chinese cabbage (Samjin background). OTU, operational taxonomic unit; H' , Shannon-Weiner's diversity index.

Taxonomic identification of bacterial isolates

Ninety six colonies were selected from 16S rRNA gene clone library of GM and non-GM Chinese cabbage soils (Samjin variety only). All the bacterial clones were closely related to the bacterial sequences in the GenBank/EMBL databases. The Blast analysis showed that the rhizospheres of the GM and non-GM Chinese cabbage comprised 17 classes and 12 classes, respectively (Fig. 3). Among these different classes, *actinobacteria*, *bacilli*, *alphaproteobacteria*, and *betaproteobacteria* comprised the largest groups contributing 18, 20, 11 and 17% of the total isolates from the GM Chinese cabbage and 20, 14, 10 and 20% from the non-GM Chinese cabbage, respectively. These results suggested that there was no significant difference between the main rhizo-bacterial groups from GM and non-GM soil libraries in a given month, although the Shannon-Wiener index (H) measured for the GM-Chinese soil (2.36) was higher than that of the control Samjin Chinese cabbage soil.

Discussion

This study assesses the impact of herbicide-resistant GM Chinese cabbage on the soil- and plant-associated microbial communities in the field. We employed a comparison of dilution plate method, PCR-DGGE analysis and 16S rDNA clone libraries to investigate any differences between GM and non-GM Chinese cabbage in this regard. Total counts for bacteria, fungi, and actinomycetes in soil samples revealed no meaningful influence of GM Chinese cabbage on the soil microbial community. Rhizosphere microorganisms play a major role in nutrient transformation and element cycling. Hence, any impact that genetically modified plants have on the dynamics of the rhizosphere and root-interior microbial community could have either positive and negative effects on plant growth and health, and in turn on ecosystem sustainability (Dunfield and Germida, 2004). In previous studies of the rhizospheres of plants that were transformed to produce opine and mannopine, the concentration of mannopine utilizers was found to be 80 times higher than non-transformed plants, whilst the number of culturable bacteria was not observed to be significantly different (Oger *et al.*, 1997; Savka and Farrand, 1997). However, these authors speculated that many metabolites overproduced by engineered

plants would specifically stimulate the growth of bacteria degrading these metabolites. Their findings thus demonstrated that the interaction between transgenic plants and their root-associated bacteria is highly specific. The incorporation of transgenic plant products into the soil could therefore alter the soil microbial biodiversity due to variable responses by microorganisms to novel proteins produced by GM plants. This is a potential cause of some concern because Tilman and Dowing (1994) have suggested that the preservation of biodiversity is essential for the maintenance of productivity in an ecosystem. Although we could not detect significant impacts on the soil microbial communities between GM and non-GM Chinese cabbage soils in our present analysis, the effects of genetically modified plants on specific groups of ecologically important soil microorganisms still remain to be fully investigated.

Bacteria are considered to be useful bio-indicators in environmental impact studies because they interact continuously with plants and most species react quickly to changes in their environments (Arias *et al.*, 2005). Denaturing gradient gel electrophoresis (DGGE) is one of the most common and comprehensive molecular approaches to assessing the status of soil microbial communities. Changes in the soil microbial community composition might occur directly through transgene products or indirectly via for example the altered composition of root exudates (Kowalchuk *et al.*, 2003). DGGE analysis of 16S rRNA gene fragments previously obtained with bacteria-specific primers has generated useful reference profiles of the most dominant populations (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998). A nested PCR approach using taxon-specific primers has further allowed the analysis of minority populations (Heuer *et al.*, 1997, 2001, 2002). Our extensive investigation of soil microbial communities associated with GM and non-GM Chinese cabbage using PCR-DGGE revealed no significant impact of the herbicide resistance transgene. We also found from our analysis that DGGE could not exclusively differentiate shifts in the soil bacterial population structures, although this remains a powerful method for assessing microbial communities. It is possible that similar band patterns between GM and non-GM soils mask the effects of other more selective factors (plant age, soil heterogeneity) or may reflect a threshold issue in terms of the ability of the DGGE technique to detect

subtle changes (Kim *et al.*, 2008).

The 16S rDNA sequences of the dominant bacterial clones we obtained from GM and non-GM Chinese cabbage subplots were compared to evaluate whether the associated soil communities comprised similar or different bacterial types. *Bacilli*, *Actinobacteria*, and *Betaproteobacteria* were the most commonly detected classes in the GM and non-GM Chinese cabbage soils, between which their distribution rates were found not to significantly differ. Similar results were also observed in paddy soils planted with GM rice expressing trehalose-6-phosphate synthase/phosphatase (TPSP) fusion gene and non-GM rice (Lee *et al.*, 2011). *Bacilli* were the most common class of bacteria found in Chinese cabbage soil and this is related to the field conditions for rice paddy soils in August, which are characterized by high temperatures and an anoxic environment due to flooding (Kim *et al.*, 2008). Some of the bacteria that can be found in rice paddy soils at this time of year have also been shown to be related to *Sporomusa* sp., which degrades rice straw in anoxic soils (Liesack *et al.*, 2000). However, the overall composition of the bacterial communities was similar between the GM and non-GM Chinese cabbage soils.

This study is to our knowledge the first attempt to assess the possible effects of growing herbicide-resistant Chinese cabbage on the soil microbial community. Our data show no measurable effects in this regard as four non-GM Chinese cabbage cultivars in fact showed a greater difference in their soil microbial community structure. Nevertheless, further studies will be needed to provide a more complete assessment of the effects on soils of cultivating herbicide resistant Chinese cabbage.

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