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ORIGINAL ARTICLE

Investigation into Effects of Transgenic Glufosinate-Resistant *Zoysia* Grasses with Herbicide Application on Bacterial Communities under Field Conditions

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Abstract *Zoysia* grass (*Zoysia japonica* Steud.) is a commercially valuable turfgrass that is popularly cultivated for use in sports and recreational environments. A field study was conducted in 2013 to evaluate the effects of transgenic herbicide-resistant *Zoysia* grasses in combination with the herbicide glufosinate on microbial communities. We investigated population changes in cultivable total bacteria and glufosinate-resistant bacteria using the traditional plate-count method. The results showed that total and glufosinate-resistant bacteria counts decreased in September. Significant differences in the abundance of cultivable bacteria were observed between *Zoysia* grass lines as well as between March and September samples in both glufosinate-treated and non-treated plots. However, there was no significant difference in the abundance of total cultivable bacteria between *Zoysia* grass lines in March or between Basta-treated and untreated plots in September. To assess the possible horizontal gene transfer (HGT) of the *bar* gene across microorganisms, total soil DNAs and genomic DNAs of glufosinate-resistant bacteria isolated from soils and water were analyzed by PCR. No positive DNA bands were found, indicating that HGT did not occur during this experimental period. We also investigated changes in taxonomic distribution of the bacterial community using 16S rRNA gene clone libraries constructed from soil samples. Although bacterial diversities increased in September, there were no significant differences in species richness between genetically modified (GM) and non-GM *Zoysia* grasses as well as between glufosinate-treated and untreated soils.

Keywords: Bacterial community, Glufosinate-resistant bacteria, Horizontal gene transfer, Transgenic herbicide-resistant *Zoysia* grasses, *Zoysia japonica* Steud.

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Introduction

The worldwide cultivation of genetically modified (GM) plants has increased rapidly due to economic and agronomic growth. The continued development and use of GM plants has raised concerns about their potential side effects on the environment. Major safety concerns related to the environmental impact of GM plants include horizontal gene transfer (HGT) and influences on the soil microbial community (Nap et al. 2003; Liu et al. 2005). The diversity and activity of soil microbial communities are essential to the terrestrial ecosystem since these could influence microorganism-mediated processes such as recycling of nutrients and maintenance of soil structure (Lupwayi et al. 2004). Consequently, it is necessary to investigate the possible impact of GM plants on indigenous bacterial populations in the field (Heinemann and Traavik 2004).

Herbicide-resistant crops are the most cultivated GM plants, and increasing cultivation of herbicide-resistant plants in combination with use of herbicides can influence the biodiversity of soil microbial communities (Dunfield and

Germida 2001; Gyamfi et al. 2002). Therefore, it is important to examine the impact of cultivating herbicide-resistant GM plants along with application of corresponding herbicides on the soil microbial population. Turfgrass is a commercially valuable species, and its cultivation is mainly related to sports and recreational environments. The transgenic *Zoysia* grasses (*Zoysia japonica* Steud.) used in this study contain the *bar* gene (Toyama et al. 2003). The *bar* gene, originally cloned from *Streptomyces hygroscopicus*, encodes phosphinothricin acetyltransferase (PAT; Thompson et al. 1987) and confers tolerance to the glufosinate-based herbicide Basta in transgenic crops. Basta (glufosinate ammonium) is a non-selective herbicide used for broad spectrum control of weeds. This herbicide acts by inhibiting glutamine synthetase, a key enzyme in the nitrogen metabolism, and was shown to exhibit antibiotic effects on several microorganisms (Dunfield and Germida 2001; Gyamfi et al. 2002).

The objective of this study was to assess the possible effects of herbicide-resistant *Zoysia* grasses as well as associated herbicide application on bacterial communities during short-term exposure under field conditions.

Results and Discussion

Effects of GM *Zoysia* Grasses and Glufosinate Treatment on Soil Bacterial Populations

Cultivation of transgenic herbicide-resistant plants in combination with herbicide application may affect indigenous soil microorganisms in the field. However, the combined effects of herbicide-resistant plants and herbicide application on the soil microbial community have rarely been addressed. To investigate the effects of GM *Zoysia* grasses and herbicide treatment on soil bacterial populations under field conditions, we first estimated total and glufosinate-resistant bacterial populations by a culture-dependent approach. The plate count is still a useful method to characterize changes in the number of cultivable bacteria (Liu et al. 2005). *Zoysia* grasses (JG21, JG21-MS, and WT) were distributed among plots according to a randomized scheme (Fig. 1A, B). Six pools, each containing soils from two different plots of the same *Zoysia* grass line (Basta-treated and untreated, respectively), were evaluated. In each pool, the numbers of

A

B

JG21-MS	JG21	WT	JG21	JG21-MS	JG21
WT	JG21-MS	JG21	JG21-MS	JG21	WT
JG21	WT	JG21-MS	WT	WT	JG21-MS

C

Fig. 1. Experimental site (A) and schematic illustration (B) of the field trial carried out with transgenic (JG21, JG21-MS) and wild-type (WT) *Zoysia* grasses. Each box corresponds to a single plot. Gray and white boxes indicate glufosinate-treated and untreated plots, respectively. (C) An artificial ecological pond built in a test field located in Nam Jeju County.

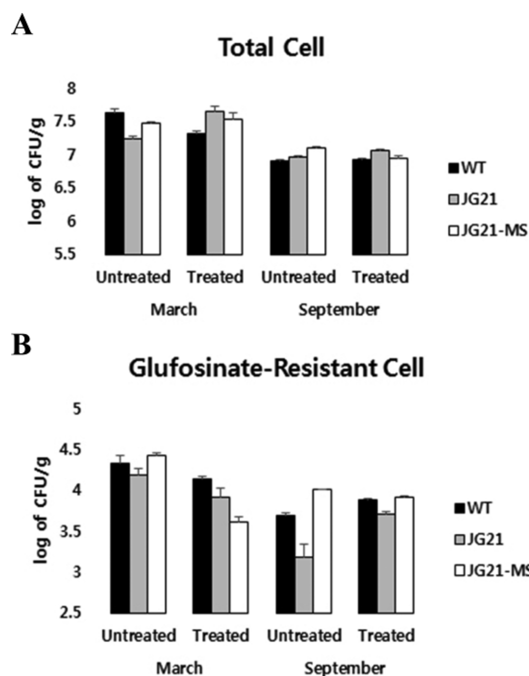

Fig. 2. Effects of GM *Zoysia* grasses and Basta application on populations of total bacteria (A) and glufosinate-resistant bacteria (B) in March and September. Error bars indicate standard error of mean of six independent experiments. CFU, colony-forming units.

total and glufosinate-resistant bacteria (evaluated as CFU per gram of dry soil) were determined by collecting soils in late March and September 2013. Mean comparison of cultivable total and glufosinate-resistant bacterial populations in 12 different soil samples is shown in Fig. 2. Cultivable bacterial numbers showed significant differences between seasons in both total and glufosinate-resistant bacterial populations, with a decrease in September. Reasons for this could be due to the increased soil surface temperature during the summer season. Among the various environmental factors, soil temperature greatly affects the microbial population (Kaur et al. 2014). Two-way analysis of variance (ANOVA) was used to determine the statistical significance of the effects of transgenic *Zoysia* grasses and Basta treatment on bacterial populations. In both Basta-treated and untreated plots, significant differences ($P < 0.05$) were observed in the total CFU of cultivable bacteria between *Zoysia* grass lines as well as between March and September samples. However, there was no significant difference in the abundance of total cultivable bacteria between *Zoysia* grass lines in March or between Basta-treated and untreated plots in September (Fig. 2A). Significant differences ($P < 0.05$) in the abundance of cultivable glufosinate-resistant bacteria were observed between *Zoysia* grass lines as well as between March and September samples in both treated and untreated plots (Fig. 2B). Glufosinate ammonium, the active ingredient of Basta, is a non-residual herbicide that is rapidly degraded from soil and has an estimated half-life ranging from 1 to 25 d depending on microbial activity and soil properties (Ismail and Ahmed 1994; Faber et al. 1997). Therefore, short-term investigation is desirable for evaluating the effects of Basta on soil microorganisms. Haney et al. (2000) pointed out that experiments conducted on a short-term basis may provide a more realistic evaluation of the effects of herbicides on soil microorganisms.

Detection of *bar* Gene in Total DNA from Soil and PCR Analysis of *bar* Gene in Glufosinate-Resistant Bacteria

Although several studies have indicated that HGT from GM plants to indigenous soil bacteria is not expected to occur at detectable frequencies under field conditions (Badosa et al. 2004), the extent to which such events takes place is still a controversial issue (Baumgarte and Tebbe 2005; Castaldini et al. 2005). To assess possible HGT of the *bar* gene from transgenic *Zoysia* grasses to soil microorganisms, total community DNAs extracted directly from the soils of field plots were subjected to PCR using the primer set (Bar443-F and Bar443-R) specific for the *bar* gene (Table 1). DNA bands corresponding to the *bar* gene were observed in the positive control but not in the genomic DNAs extracted from soil samples of the six plots (three Basta-treated and three untreated *Zoysia* grass lines) collected in March and September 2013 (data not shown). These results suggest that HGT of the *bar* gene from herbicide-resistant *Zoysia* grasses to the soil microbial community did not occur or PCR amplification failed due to the detection limit of *bar* DNA concentration in soil. To confirm the above result, 10

Table 1. Nucleotide sequences of the primers used in this study.

Primer	Sequences (5'→3')	Size (bp)	Target gene
Bar443-F	GTCTGCACCATCGTCAACCACTA	443	*bar*
Bar443-R	AAGTCCAGCTGCCAGAAACCCAC		
Ubi-Bar-F	CGGGTCATCTTTTCATGCTT	810	ubiquitin promoter
Ubi-Bar-R	GTACGGAAGTTGACCGTGCT		
BBS-F	TGCAGGTCGACTCTAGAGGATCC	598	*bar* border
BBS-R	GGGCGATGCCACGCGCACTCCGT		
968F	AACGCGAAGAACCTTAC	433	16S rRNA
1401R	CGGTGTGACAAGGCCCGGGAACG		

Table 2. Taxonomic identification of glufosinate-resistant bacteria isolated from JG21-MS plots and artificial ecological pond along with homologies with the *bar* gene introduced into transgenic *Zoysia* grasses

Basta-untreated			Basta-treated			Artificial pond		
Isolate	Genus	Homology (%)	Isolate	Genus	Homology (%)	Isolate	Genus	Homology (%)
SN1	*Cupriavidus* sp.	99	ST1	*Burkholderia* sp.	100	W1	*Klebsiella* sp.	99
SN2	*Burkholderia* sp.	99	ST2	*Burkholderia* sp.	99	W2	*Enterobacter* sp.	99
SN3	*Cupriavidus* sp.	99	ST3	*Pseudomonas* sp.	99	W3	*Enterobacter* sp.	99
SN4	*Burkholderia* sp.	99	ST4	*Burkholderia* sp.	99	W4	*Enterobacter* sp.	99
SN5	*Pantoea* sp.	99	ST5	*Pseudomonas* sp.	99	W5	*Aeromonas* sp.	99
SN6	*Burkholderia* sp.	99	ST6	*Pantoea* sp.	99	W6	*Aeromonas* sp.	99
SN7	*Cupriavidus* sp.	99	ST7	*Pantoea* sp.	99	W7	*Enterobacter* sp.	99
SN8	*Enterobacter* sp.	99	ST8	*Burkholderia* sp.	100	W8	*Enterobacter* sp.	99
SN9	*Cupriavidus* sp.	99	ST9	*Burkholderia* sp.	99	W9	*Plesiomonas* sp.	98
SN10	*Ralstonia* sp.	99	ST10	*Pantoea* sp.	99	W10	*Plesiomonas* sp.	99

bacterial isolates were randomly selected from plates that were screened for glufosinate-resistant bacteria from plots of treated and untreated JG21-MS, respectively. Genomic DNA from individual isolates was amplified by PCR with a *bar* gene-specific primer set (Table 1). Meanwhile, PCR amplification was also carried out using a set of universal bacterial 16S rRNA gene primers (968F and 1401R) to determine the taxonomic identities of the isolates. The amplified fragments were subsequently sequenced, and BLASTN analysis for homologous sequences in the GenBank database was performed. The results are summarized in Table 2. The *bar* gene amplified from these isolates showed 99 or 100% homology with the *bar* gene of *S. hygroscopicus*, which was originally introduced into transgenic *Zoysia* grasses. Isolates were predominantly classified as members of the *Burkholderia* genus, although the *Pantoea* and *Cupriavidus* genera were also represented from Basta-treated and untreated plots, respectively (Table 2). ST1 and ST8, two isolates which showed 100% homology with the *bar* gene from *S. hygroscopicus*, were recultured, and genomic DNAs from these isolates were analyzed by PCR using three primer sets (Bar443-F + Bar443-R, Ubi-Bar-F + Ubi-Bar-R, and BBS-F + BBS-R) (Table 1). Using the primer set Bar443-F and Bar443-R, all corresponding DNA bands were amplified. However, using the primer sets Ubi-Bar-F + Ubi-Bar-R and BBS-F + BBS-R, which are specific for the border region of the *bar* gene in *Zoysia* grass, only positive control DNAs were amplified (Fig. 3). These results indicate that the *bar* genes in ST1 and ST8 are not outcomes of HGT from transgenic *Zoysia* grasses.

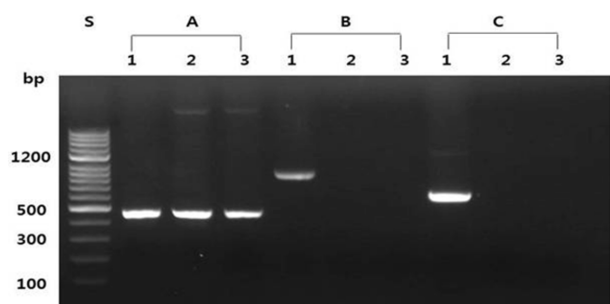

Fig. 3. PCR analysis for detecting *bar* gene in glufosinate-resistant bacteria isolated from JG21-MS plot. (A) primer set Bar443-F+Bar443-R. (B) primer set Ubi-Bar-F+Ubi-Bar-R. (C) primer set BBS-F+BBS-R; lane S, size marker (100 bp ladder); lane 1, positive control (pGPTV-HB DNA); lane 2, isolate ST1; lane 3, isolate ST8.

Monitoring of Possible HGT from GM Plants to Aquatic Microorganisms

Most studies have examined the effects of GM plants on soil microorganisms (Dunfield and Germida 2001; Gyamfi et al. 2002; Baumgarte and Tebbe 2005; Liu et al. 2005). However, HGT from GM plants to aquatic microorganisms in a water environment has not been extensively investigated. Application of herbicide to herbicide-resistant GM plants may affect microbial communities in water environments through agricultural waste water. To examine the identity of glufosinate-resistant bacteria in a water environment, water samples from the artificial environmental pond (Fig. 1C) were subjected to culture-based analysis. Total counts of cultivable bacteria and glufosinate-resistant bacteria were $1.44 \times 10^5 \pm 3.47 \times 10^4$ CFU/L and $2.20 \times 10^4 \pm 7.50 \times 10^3$ CFU/L, respectively. To reveal the nature of the cultivable glufosinate-resistant bacteria community in water, 10 glufosinate-resistant colonies were randomly selected, and genomic DNAs were extracted. The *bar* genes from genomic DNAs were amplified, sequenced, and their nucleotide sequences were compared with the *bar* gene sequence of *S. hygroscopicus*, which was introduced into GM *Zoysia* grasses. Moreover, PCR-amplified 16S rRNA gene products from the isolates were sequenced

Table 3. Phylogenetic affiliation and abundance of soil bacterial 16S rRNA gene sequences in the clone libraries at different sampling times

Phylum	Basta-untreated						Basta-treated					
	March			September			March			September		
	WT	JG21	JG21-MS	WT	JG21	JG21-MS	WT	JG21	JG21-MS	WT	JG21	JG21-MS
Chloroflexi	0	0	1	2	0	0	0	0	1	2	4	2
Proteobacteria	20	18	22	4	13	4	21	23	22	9	3	2
(*β-Proteobacteria*)	(19)	(18)	(19)	(2)	(6)	(2)	(21)	(23)	(21)	(5)	(0)	(1)
Actinobacteria	0	0	0	0	0	1	0	0	0	1	2	1
Chlamydiae	0	0	0	0	1	0	0	0	0	0	0	0
Acidibacteria	1	0	0	4	0	4	0	0	0	2	5	7
Bacteroidetes	0	0	0	0	1	1	0	0	0	1	0	0
Verrucomicrobia	2	5	0	5	1	6	4	2	0	0	3	3
Armatimonadetes	0	0	0	0	0	0	0	0	0	0	1	0
Candiadatus saccharibacteria	0	0	0	0	2	0	0	0	0	0	0	0
Unclassified	1	1	0	5	3	2	0	0	0	4	2	4
Total OTU	24	24	23	20	21	18	25	25	23	19	20	19

to uncover the taxonomic identities of these isolates. The results are shown in Table 2. The *Enterobacter* genus was predominant in the glufosinate-resistant isolates, and *bar* genes of these isolates exhibited 98-99% sequence homology with that of *S. hygroscopicus*. No *bar* gene identical to the *bar* gene introduced into GM *Zoysia* grasses was detected, indicating that HGT of the *bar* gene from transgenic *Zoysia* grasses into aquatic bacteria did not occur during the experimental period or we could not detect the event due to some limitation of the detection method.

Effects of GM *Zoysia* Grasses and Basta Treatment on Diversity of Soil Bacterial Communities

In order to assess the effects of herbicide-resistant *Zoysia* grasses and associated Basta application on the soil bacterial community, clone libraries of partial 16S rRNA genes were constructed from soils of Basta-treated and untreated *Zoysia* grasses in March and September. Although bacterial diversity analysis based on 16S rDNA sequencing has shortcomings such as biases in DNA extraction and PCR steps (Suzuki and Giovannoni 1996), useful information can still be obtained in order to understand phylogenetic diversity and microbial community structures through identification of uncultivable populations. A total of 12 clone libraries were established, and a total of 300 partial 16S rRNA gene sequences (25 sequences from each library) were determined. Thirty-nine sequences were identified as chimera sequences and subsequently eliminated. Phylogenetic affiliation of the remaining 261 16S rDNA sequences was analyzed using RDP Classifier. The results showed that these sequences could be assigned to 10 different phyla, among which *Proteobacteria*, *Verrucomicrobia*, and *Acidobacteria* dominated (Table 3). These three groups together made up 82% of all obtained OTUs. *Proteobacteria*, composed of five subdivisions, was the most abundant group, and β-*Proteobacteria* held major proportions in this phylum. Although the distribution of *Proteobacteria* from the plots was almost the same, proportions of this group between seasons were completely different. Between 75-96% of sequences in the March fractions belonged to the *Proteobacteria* phylum, whereas 15-62% of sequences from the September fractions could be ascribed to this phylum. In addition, relatively more *Acidobacteria* sequences were detected in September soils as compared with March soils (Table 3).

Diversity indices, including the Chao1 species richness estimation and Shannon diversity index, were calculated for OTUs with an evolutionary distance of 0.03 (Table 4). These indices indicated no significant difference in species richness between GM and non-GM soils as well as between Basta-treated and untreated soils. However, bacterial populations in the September fraction were more diverse than those in the March fraction (Table 4). The species richness of the September fraction estimated by Chao1 was higher than that of the March fraction. Similarly, the Shannon index estimated for the September community was 4.24, which was markedly higher than the 0.87 value estimated for the March community. Basta treatment caused minor shifts in soil bacterial community structures possibly due to the short half-life of glufosinate ammonium, which is the active ingredient of Basta (Ismail and Ahmed 1994). Statistical comparisons of bacterial community compositions of 16S rDNA libraries were performed using the LIBSHUFF computer program (Schloss et al. 2004). Pair wise comparisons of all libraries revealed statistically significant differences between March and September libraries ($P < 0.0001$) but failed to

Table 4. Summary of comparisons between 16S rRNA gene libraries for number of sequences and OTUs, richness (Chao1), diversity (Shannon index), and similarity (Libshuff)

Libraries†	Number of sequences‡	Number of OTUs	Chao1	Shannon index	Libshuff *P* value (XY, YX)§
WT vs. GM	261 (88, 173)	93 (39, 65)	235.06 (105.43, 192.50)	2.99 (2.61, 2.83)	0.5109, 0.0783
JG21 vs. JG21-MS	173 (90, 83)	65 (34, 35)	192.50 (88.17, 110.60)	2.83 (2.45, 2.49)	0.0424, 0.0902
Basta-untreated vs. treated	261 (130, 131)	93 (55, 45)	235.06 (129.54, 171.00)	2.99 (2.92, 2.48)	0.0104, 0.1523
March vs. September	261 (144, 117)	93 (15, 84)	235.06 (33.33, 291.09)	2.99 (0.87, 4.24)	<0.0001, <0.0001

†WT indicates the non-transgenic *Zoysia* grass. JG21 and JG21-MS represent the transgenic herbicide-tolerant lines. GM represents the combined clone libraries for JG21 and JG21-MS.
‡The numbers before parentheses represent the total number, and two numbers in the parenthesis represent the number of individual groups that were compared.
§XY and YX represent the *P* values obtained when the first library is compared with the second library and vice versa, respectively. If the *P* value of either of these comparisons has a $P<0.005$, the libraries are considered significantly different.

yield significant differences between libraries from WT and GM *Zoysia* grasses, JG21 and JG21-MS, and Basta-treated and untreated soils (Table 4). Similar findings were obtained by Hart et al. (2009), who found that seasonality was a stronger determinant of microbial communities in soil than crop type and herbicide.

Conclusions

Significant differences in the abundance of cultivable bacteria were observed between *Zoysia* grass lines as well as between March and September samples in both treated and untreated plots. However, there was no significant difference in the abundance of total cultivable bacteria between *Zoysia* grass lines in March or between Basta-treated and untreated plots in September. PCR and sequence analysis of the *bar* gene from total soil and genomic DNAs of glufosinate-resistant bacteria indicated absence of HGT during the experimental period. Investigation of taxonomic distribution and bacterial diversity using the 16S rRNA gene clone libraries showed no significant differences in species richness between GM and non-GM *Zoysia* grasses as well as between Basta-treated and untreated soils. Although the total cultivable and glufosinate-resistant bacteria populations decreased, bacterial diversities increased during September. We concluded that the effects of GM *Zoysia* grasses and associated Basta treatment on soil microbial communities are likely to be minor as compared to seasonal variation.

Materials and Methods

Field Site and Plant Materials

This study was conducted on a test field located in Nam Jeju County, Jeju, Korea, which was approved for environmental risk assessment of GM plants by the Rural Development Administration/Korea Ministry of Agriculture and Forestry (Bae et al. 2008). The experimental area within the test field included a total of 18 plots (1 m × 1 m each). These plots were planted with three different *Zoysia* grass lines, which were arranged in a randomized block design with three replications (Fig. 1A, B). An artificial ecological pond was built within the GM test field to assess the effects of GM plants on aquatic organisms (Fig. 1C).

Herbicide-resistant *Zoysia* grass transgenic line JG21 carries a single copy of the *bar* gene from *S. hygroscopicus* (Toyama et al. 2003). Another transgenic line, JG21-MS, is a mutant of JG21 with defects in reproduction and was generated by gamma ray irradiation-induced mutagenesis (Bae et al. 2009). Wild-type (WT) *Zoysia* grass was also cultivated as the control for the test. In order to assess the effects of herbicide on soil bacterial populations, Basta solution (0.054% glufosinate, Bayer Crop Science) was applied to the herbicide treated plots on 28 February, 8 April, 23 May and 11 July, respectively.

Sampling and DNA Extraction

Soil samples were collected from each plot in the test field in late March and September 2013. After removing the surface soil, samples were collected at a depth of 5-15 cm, transferred into plastic bags, kept in a cooler with an ice pack, and transported to the laboratory. Once in the laboratory, soils were air-dried and passed through a 2-mm sieve. Water samples (50 mL each) were collected at three randomly selected points from the artificial ecological pond on 27 September 2013.

Total microbial community DNA was directly extracted from 1 g (dry weight) of each soil sample using a GeneAll Exgene™ Soil SV Kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's protocol. Extracted DNAs from each replicate were pooled to provide representative samples from each field plot and then analyzed by agarose gel electrophoresis. Genomic DNAs from glufosinate-resistant bacteria were isolated using the GeneAll Exgene™ Cell SV Kit following the protocol provided by the manufacturer.

Culture-Dependent Microbiological Analyses

Total numbers of cultivable bacteria were enumerated by the plate counts method. Plant Count Agar (PCA; Oxoid, Basingstoke, Hampshire, UK) was used to determine the total aerobic cultivable bacteria. For soil bacteria counts, 5 g of each soil sample was suspended in 45 mL of sterile saline solution and gently shaken for 1 h. Each suspension was then serially diluted, and 0.1 mL aliquots were plated onto triplicate plates. The plates were incubated at 30ºC for 3 d. Glufosinate-resistant bacteria were isolated using PCA medium containing Basta (0.1% glufosinate, w/v) as described above. The culture media were supplemented with cycloheximide (100 µg

mL^{-1}, Sigma) to inhibit fungal growth. Plate counts were expressed as colony-forming units (CFU) per gram of dry soil. Water samples were directly plated without dilution on PCA medium and Basta-containing PCA medium for total bacteria and glufosinate-resistant bacteria counts, respectively.

PCR Detection of *bar* Gene

The GM *Zoysia* grasses contain the *bar* gene as a selection marker (Toyama et al. 2003). To test for the presence of the *bar* gene in microbial community DNA, the extracted total soil DNA samples were subjected to PCR analysis using the primer set (Bar443-F and Bar443-R) specific for the *bar* gene (Table 1). Amplifications were performed in 50-μL reactions with Taq DNA polymerase (GeneAll) as described previously (Bae et al. 2007). pGPTV-HB DNA, which contains the *bar* gene (Toyama et al. 2003), was used as a positive control. To detect potential HGT events, genomic DNAs isolated from glufosinate-resistant bacteria were subjected to PCR analysis using three primer sets specific for the *bar* gene region (Bar443-F and Bar443-R), including the ubiquitin promoter and terminator (Ubi-Bar-F and Ubi-Bar-R) and border region of the *bar* gene (BBS-F and BBS-R) (Table 1). PCR was conducted in a 50-μL reaction containing 1 μL of genomic DNA (50 ng μL^{-1}), 1×PCR buffer, 0.1 μM of each primer, 2.5 U of Taq DNA polymerase, and 0.2 mM dNTPs. The amplification program was as follows: 1 cycle at 95ºC for 5 min; 30 cycles at 95ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 50 sec, and a final cycle at 72ºC for 10 min. The PCR products were analyzed by 1% (w/v) agarose gel electrophoresis.

PCR Amplification of 16S rRNA Gene for Bacterial Community Analysis

The genomic DNAs directly extracted from soils of each plot were used as templates for PCR. 16S rDNAs were amplified using the 968F and 1401R primers set (Table 1) spanning the region roughly between nucleotides 968 and 1401 of the 16S rRNA gene of *Escherichia coli*, which includes the variable regions V6 through V8 (Heuer et al. 1997). PCR were carried out in 50-μL volumes containing 25 pmol of each primer, 0.2 mM dNTPs, 1×PCR buffer, 2.5 U of Taq polymerase, and 100 ng of the extracted DNA. Reactions were carried out in a T100™ thermal cycler (Bio-Rad, Hercules, USA) under the following conditions: initial denaturation at 95ºC for 3 min, 25 cycles of denaturation at 94ºC for 30 sec, annealing at 54ºC for 3 min, extension at 72ºC for 1 min, and a final extension step at 72ºC for 7 min. The resulting PCR products were purified using a GEL SV Gel Extraction Kit (General Biosystem, Seoul, Korea) and combined separately for each plot. The mixed populations of PCR products from each plot were separately ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into *E. coli* JM109 competent cells. Twenty-five recombinant colonies from each plot library were randomly selected, and recombinant DNAs were purified using a Plasmid SV Mini Kit (GeneAll) as recommended by the manufacturer. DNA sequencing was conducted at the Genotech DNA sequencing facility (Daejeon, Korea) by automated sequencing using the dideoxynucleotide chain termination method.

Sequence Identification and Statistical Analysis

A total of 300 clone inserts were partially sequenced and analyzed. After removing PCR primer and vector sequences, remaining sequences were checked for chimeric artifacts by the DECIPHER program (Wright et al. 2012), and all chimera sequences were eliminated. The remaining sequences were identified by performing BLASTN analysis on the GenBank database at the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990) (http:/www.ncbi.nlm.nih.gov/BLAST) and the Classifier program (Wang et al. 2007) of the Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu).

Statistical analysis of bacterial populations was performed on log-transformed CFU data using two-way analysis of variance (ANOVA) at a significance level of $p < 0.05$ using the Microsoft® EXCEL2007 statistical package. For calculations of diversity indices, clones were first clustered into Operational Taxonomic Units (OTUs) with an evolutionary distance of 0.03 (about 97% sequence similarity). Shannon and Chao1 diversity indices were calculated from the OTU data using the RDP Pipeline. LIBSHUFF analysis was performed for pair-wise comparisons in each library to determine the significance of differences between clone libraries using the LIBSHUFF function available in MOTHUR (Schloss et al. 2009).

Nucleotide Sequence Accession Numbers

The sequences of the partial 16S rRNA genes obtained in this study were deposited in the GenBank database under accession numbers KM822237-KM822577.

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Author's Contributions

LYE and LHY designed the experiments. LSH and RGD performed molecular and microbial experiments. KHG, KYI and SHJ contributed to field work and data analysis. PKW, LB and SIJ contributed to data analysis and discussion. LPO performed bioinformatic analysis. LYE drafted the initial manuscript; LPO and LHY revised the paper. All the authors agreed on the contents of the paper and post no conflicting interest.

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