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CONTENTS

Effect of vegetation of transgenic Bt rice lines and their straw amendment on soil enzymes, respiration, functional diversity and community structure of soil microorganisms under field conditions

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Abstract

With the development of transgenic crops, there is an increasing concern about the possible adverse effects of their vegetation and residues on soil environmental quality. This study was carried out to evaluate the possible effects of the vegetation of transgenic Bt rice lines Huachi B6 (HC) and TT51 (TT) followed by the return of their straw to the soil on soil enzymes (catalase, urease, neutral phosphatase and invertase), anaerobic respiration activity, microbial utilization of carbon substrates and community structure, under field conditions. The results indicated that the vegetation of the two transgenic rice lines (HC and TT) and return of their straw had few adverse effects on soil enzymes and anaerobic respiration activity compared to their parent and distant parent, although some transient differences were observed. The vegetation and subsequent straw amendment of Bt rice HC and TT did not appear to have a harmful effect on the richness, evenness and community structure of soil microorganisms. No different pattern of impact due to plant species was found between HC and TT. It could be concluded that the vegetation of transgenic Bt rice lines and the return of their straw as organic fertilizer may not alter soil microbe-mediated functions.

Key words: anaerobic respiration; enzyme activity; functional diversity; community structure; transgenic Bt rice

DOI: 10.1016/S1001-0742(11)60939-X

Introduction

Transgenic Bt rice is transformed with the crylAb gene from *Bacillus thuringiensis* to produce a kind of insecticidal toxin to kill *lepidopteran* pests. Some studies have been conducted to assess the effect of Bt rice vegetation on soil health. It was reported that CrylAb protein will not accumulate in rhizosphere soil throughout the course of transgenic Bt rice development (Wang et al., 2006). Liu et al. (2008) found that the vegetation of transgenic Bt rice will not affect the enzyme activities and microbial composition in the rhizosphere during crop development. Nevertheless, the amendment of their straw altered the population of soil microorganisms and some biological properties (dehydrogenase activity, methanogenesis, hydrogen production and anaerobic respiration) in water-flooded soil under laboratory conditions (Wu et al., 2004). To date, the patterns found often showed no effect or only minor effects by Bt crops on soil microorganisms (Flores et al., 2005; Shen et al., 2006; Sun et al., 2007; Liu et al., 2008; Lawhorn et al., 2009; Miethling-Graff

et al., 2010; Raubuch et al., 2010). However, most of the reported results were obtained under laboratory conditions. Few studies have been performed under field conditions with the combination of transgenic Bt rice and the return of their straw to the soil. Considering the fact that rice straw is usually returned to the field as organic fertilizer after harvest, it is necessary to assess the effect of the vegetation of transgenic Bt rice lines together with the return of their straw to the field on soil microorganisms and soil biological activities.

The aim of this study was to evaluate the effect of the vegetation of transgenic Bt rice lines followed by the return of their straw to the field on soil microbe-mediated functions under field conditions. Two transgenic rice lines, Huachi B6 (HC) and TT51 (TT), were used to assess the possible different impacts due to plant species. Soil biological functions were measured, including enzyme (catalase, urease, neutral phosphatase and invertase) activities, anaerobic respiration activity, and microbial utilization of carbon substrates and community structure.

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1 Materials and methods

1.1 Sites and crops

The study was conducted on the experimental farm located in Jiande, Zhejiang, China (29°21′N, 119°13′E). The farm soil is classified as heavy loam soil (pH: 5.81; total N: 0.2%; organic matter: 3.4%; CEC: 20.1 me/100 g; sand: 38.6%; clay: 61.4%).

The study was carried out with two transgenic rice lines (HC and TT) containing the crylAb gene from *Bacillus thuringiensis* under the control of maize ubiquitin promoter. Non-transgenic parental rice varieties Jiazao 935 (JZ) and Minghui 63 (MH), and non-transgenic distant parental rice varieties Zhongjiu B (ZJ) and 9311 were adopted as controls for HC and TT, respectively.

1.2 Treatments

All treatments were performed in plots (2 m × 8 m each) on the experimental farm in May, 2009. The experiment was set up as a completely randomized design with three replicates. The rice seedlings were transplanted to the plots and irrigated under normal conditions. The plants were harvested normally at the maturing stage, and the straw was air-dried at room temperature and cut into small pieces of about 2 cm length and then were returned and spread onto the plots at a normal level (3.5 kg for each).

1.3 Soil sampling

Ten rice plants from each plot were excavated (about 20 cm depth) together with rhizosphere soils, with as much of their associated roots, at the tillering, heading, filling and maturing stages of rice (July 23, August 1, 17 and 29 in 2009 for HC, JZ and ZJ; July 23, August 29, September 9 and 21 in 2009 for TT, MH and 9311). Soils and plants were placed into plastic bags and transported immediately to the laboratory and prepared within a few hours. The rice rhizosphere (root system) was shaken vigorously to remove big soil particles and then the rhizosphere soil was collected from the rice roots surface using a paint brush, and subsequently passed through a sieve of 10 mesh (2 mm).

Surface soil (0–15 cm) samples were freshly collected 97, 128, 158 and 189 days after the rice straw was added (on March 12, April 12, May 12 and June 12 in 2010), transported to laboratory and passed through a sieve of 10 mesh (2 mm).

1.4 Measurement of soil enzyme activities

The activities of catalase, urease, neutral phosphatase and invertase in soil were determined according to the procedures of Yu et al. (2006). Each measurement was performed in triplicate.

1.5 Anaerobic respiration activity

Soil anaerobic respiration activity was expressed as the evolution of CO_2. To measure the evolution of CO_2, 5 g of fresh soil and 2 mL of sterile glucose solution (0.1 mol/L) were added into a 50-mL narrow-mouthed bottle.

The bottle was flushed with oxygen-free N_2 for 3 min, and then covered with gas-tight butyl rubber to ensure an anaerobic condition, and incubated at 30°C. Following incubation for 7 and 24 hr, 500 μL of the gas in the headspace of the bottle was extracted and analyzed by a gas chromatography 9720 system equipped with a converter for the transformation of CO_2 into CH_4, a flame ionization detector and a TDX-01 column (1.5 m × 2 mm i.d.) for the detection of CH_4 (Fuli Analytic Instrument Co., Zhejiang, China). The amount of CO_2 was quantified using standard curves obtained by injecting a known concentration gradient of CO_2. The anaerobic respiration activity was expressed as mg CO_2/(g dry soil·7 hr) and mg CO_2/(g dry soil·24 hr). Each measurement was performed in triplicate.

1.6 Carbon substrate utilization

Microbial utilization of carbon substrates was assessed using Biolog ECO microplates (BIOLOG Co., Hayward, CA, USA), which contained three replicate wells of 31 carbon sources and a blank well without any carbon source. A soil microbial suspension was prepared by suspending a certain fraction of the fresh soil equivalent to 10 g of dry soil in 100 mL of sterile physiological saline solution (0.85% NaCl), and the microbial suspension was then stepwise diluted to 10^{-3} with the sterile physiological saline. The diluted microbial suspension of 150 μL was added to each well of the Biolog ECO plate using an 8-channel repeating pipettor. The plates were incubated at 25°C in the dark for 7 days and color development in the wells was measured as the absorbance at 590 nm wavelength every 24 hr with a BIO-TEK Elx808 automated microplate reader (BIO-TEK Instruments Inc., Winooski, VT, USA).

1.7 Total DNA extraction and PCR amplification of 16S rDNA V₃ region

Total soil DNA was extracted from soil samples with a FastDNA Spin Kit according to the manufacturer's protocol (Qbiogene, Carlsbad, CA, USA), and then dissolved in 50 μL of TE and used as template to amplify the V_3 region of the 16S rDNA using primers GC341F and 518R. Polymerase chain reaction (PCR) was performed with a TPersonal Thermalcycler (Biometra, Göttingen, Germany). Initial denaturation at 95°C for 5 min was followed by 30 cycles consisting of denaturation for 30 sec at 94°C, annealing for 45 sec at 55°C and extension for 45 sec at 72°C. Finally, there was an extension step for 10 min at 72°C. The reaction mixture (50 μL) contained 0.15 μL of Taq polymerase (5 U/μL, TaKaRa, Japan), 5 μL of 10× buffer, 3 μL of $MgCl_2$ (25 mmol/L), 1.5 μL of dNTPs (dATP, dCTP, dGTP, and dTTP, 10 μmol/L each), 1 μL of primer (1 μmol/L each), and double-deionized H_2O to a final volume of 50 μL. PCR products were analyzed by 1.0% agarose gel electrophoresis with TAE buffer that contained 1.5 mg/mL ethidium bromide.

1.8 Temperature gradient gel electrophoresis (TGGE)

The TGGE Mini system (Biometra, Göttingen, Germany) was used for sequence-specific separation of PCR products. Polyacrylamide gels were composed of 8% acry-

lamide/bis (37.5:1, *V*/*V*), 8 mol/L urea, 20% formamide and 2% glycerol. Runs were performed at a constant voltage of 200 V for 3 hr in 1× TAE buffer (40 mmol/L tris-acetate, pH 8.0). A temperature gradient of 42 to 50°C in parallel with the direction of electrophoresis was used. After the electrophoresis, gels were stained with AgNO₃ as described by the manufacturer. The stained gels were immediately photographed using a digital camera (DSC-F717, Sony, Tokyo, Japan). Sequencing and phylogenetic analysis of TGGE bands were conducted according to the method of Wang et al. (2009).

1.9 Statistical analysis

The absorbance values at 72 hr were used to calculate the Shannon-Wiener index, Simpson Index and McIntosh index, which are used to assess the richness, the dominant population and evenness of soil microorganisms, respectively. The differences in the effects of transgenic rice lines on soil enzymes activities, anaerobic respiration activities

and the diversity indices were analyzed by one-way analysis of variance (ANOVA) using SPSS 15.0 software (SPSS for Windows, Version 15.0, USA).

2 Results

2.1 Enzyme activities in soil

The activities of soil enzymes (catalase, urease, neutral phosphatase and invertase) during the tillering, heading, filling and maturing stages of HC, JZ, ZJ, TT, MH and 9311 rice lines as well as the corresponding straw amendment are shown in Fig. 1. There was no significant difference ($p < 0.05$) in the activities of catalase, neutral phosphatase and invertase between HC, JZ and ZJ at the tillering stage. A significant difference was, however, observed in the activity of urease between HC, JZ and ZJ. The activity of urease in the rhizosphere soil of HC was significantly higher than those of JZ and ZJ by 32.5% and
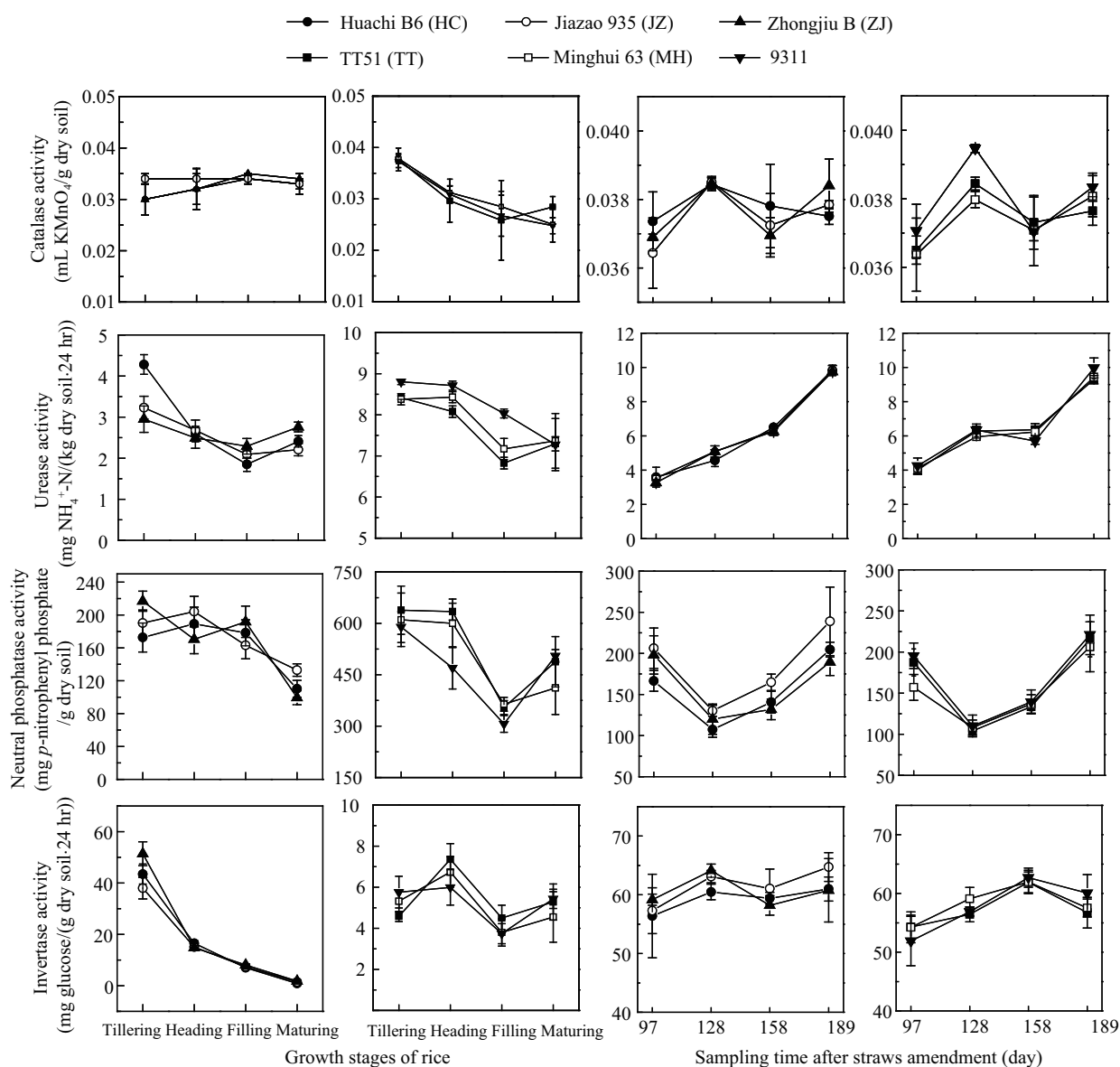

Fig. 1 Effects of different growth stages of transgenic rice lines and different sampling time after rice straw amendment on soil enzyme activities under field conditions.

45.1%, respectively. Afterwards, no significant difference was observed in the activities of the tested soil enzymes between HC, JZ and ZJ at the heading, filling and maturing stages.

The variation pattern of the soil enzyme activities in the rhizosphere soil of TT, MH and 9311 throughout their development was generally found to be similar to that of HC, JZ and ZJ. However, a significant difference was observed in the activity of urease in the rhizosphere soil of TT and 9311 at the stages of tillering, heading and filling, and in the activity of neutral phosphatase in the rhizosphere soil of TT and 9311 at the heading stage, respectively. Additionally, it should be noted that the activities of urease and neutral phosphatase in the rhizosphere soil of TT were always similar to those of its non-transgenic parent MH. This indicated that the difference observed in the activity of urease at the tillering, heading and filling stages, as well as the neutral phosphatase at the heading stage, may not be caused by the Bt-transgenic event.

As shown in Fig. 1, no significant difference was observed in the activities of enzymes in soils amended with Bt transgenic rice straw under field conditions, compared with those of non-transgenic parental and distant parental rice varieties, respectively.

2.2 Anaerobic respiration activities in rhizosphere soil

Figure 2 shows the anaerobic respiration activities of rhizosphere soils of the two transgenic rice lines (HC and TT) and their non-transgenic parental rice varieties and non-transgenic distant parental rice varieties. After 7 hr incubation, the anaerobic respiration activity of HC rhizosphere soil decreased by 22.4% and 26.9% at the heading stage and by 27.8% and 25.7% at the filling stage, respectively, compared with those of non-transgenic parental rice variety JZ and non-transgenic distant parental rice variety ZJ. This difference was not detected at the

maturing stage. It is well known that the CO_2 released during 0–7 hr results from the activity of the *in situ* microbial population (Shi et al., 2005). The present data indicated that the *in situ* microbial population might be adversely affected by the vegetation of HC at the heading and filling stages. However, this harmful effect was found to be transient and to disappear at the maturing stage.

As shown in Fig. 2, there was not any significant difference in the CO_2 released between HC and JZ during the 7–24 hr period, nor between HC and ZJ at the tillering stage. However, a significant reduction in the rhizosphere soil respiration of HC was monitored at the heading stage compared with those of JZ and ZJ. This effect was not consistent and had disappeared at the filling and maturing stages. The CO_2 released during 7–24 hr reflects the effect of microbial growth with the added substrate. This may mean that the Bt-transgenic event does not adversely affect microbial growth on the added substrate.

The CO_2 released during 0–7 hr from the rhizosphere soil of TT was significantly lower (49.7%) at the tillering stage, but 31.9% and 29.7% higher at the heading and filling stages than that of 9311, respectively. However, no significant difference in anaerobic respiration was found between TT and MH throughout rice development. It could, therefore, be concluded that the difference between TT and 9311 was not caused by the Bt-transgenic event. The released CO_2 during 7–24 hr from the rhizosphere soil of TT was similar to those of MH and 9311, suggesting that the vegetation of TT has no harmful effect on microbial growth with the substrate.

Figure 2 also shows the anaerobic respiration activity of soils amended with rice straw of HC, JZ, ZJ, TT, MH and 9311. There was no consistent significant difference in the CO_2 released during the 0–7 hr and 7–24 hr periods from straw-amended soils between Bt rice and its non-transgenic parental and distant parental rice. The CO_2
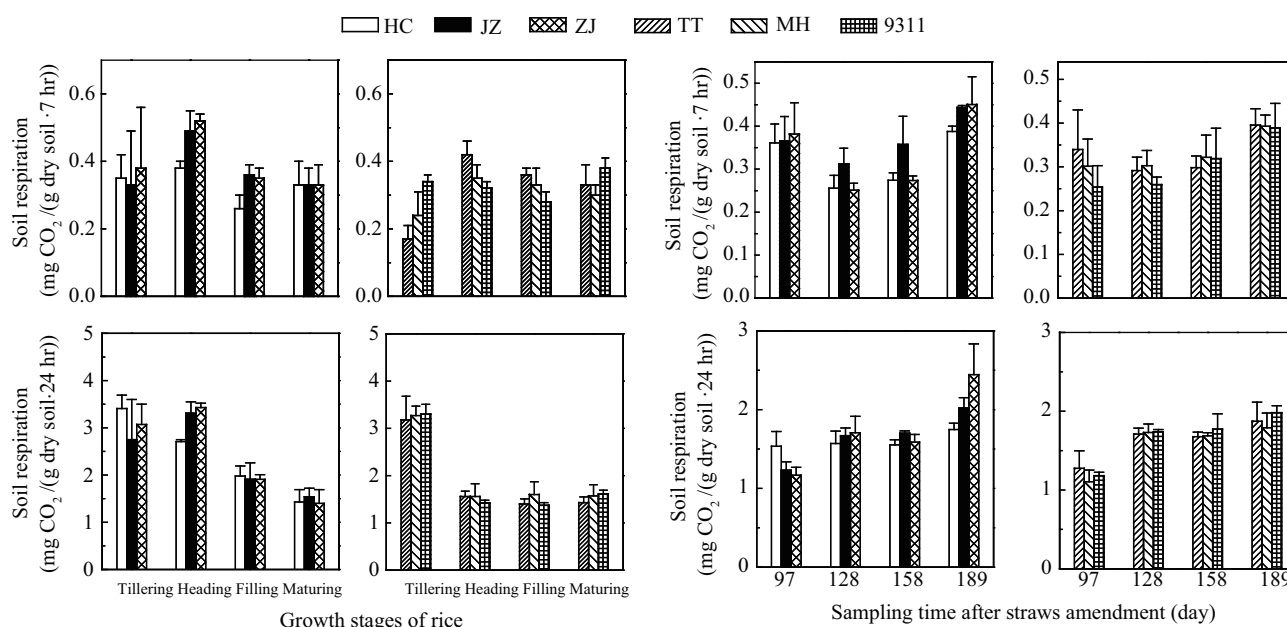

Fig. 2 Effects of different growth stages of transgenic rice lines and different sampling time after rice straw amendment on anaerobic respiration activities of rhizosphere soil.

released during 7–24 hr from soil amended with HC straw was significantly higher (31.4%) and lower (28.5%) than that of ZJ after 97 and 189 days amendment, respectively.

2.3 Functional diversity of microbial communities

The Biolog system is usually adopted as an effective approach to distinguish spatial and temporal changes in microbial communities (Sprocati et al., 2006). The community-level physiological profiles in the rhizosphere soils of the two transgenic rice lines and the corresponding non-transgenic parental rice varieties and non-transgenic distant parental rice varieties were assessed with the Biolog EcoPlate system. As shown in Table 1, the McIntosh and Shannon indexes of the rhizosphere soil of transgenic rice lines (HC and TT) were both similar to those of the corresponding non-transgenic parental rice varieties and non-transgenic distant parental rice varieties. Although the Simpson index of HC rhizosphere soil was significantly lower at the heading stage and higher at the filling stage than that of its non-transgenic distant parental rice variety ZJ, the index was similar to that of its non-transgenic parental rice variety JZ. Similarly, the Simpson index of TT rhizosphere soil was significantly higher at the heading and filling stages and lower at the maturing stage than that of its non-transgenic distant parental rice variety 9311. However, such change was only observed at the heading stage between TT and MH.

As shown in Table 1, the Shannon and McIntosh indices of the soils amended with the two transgenic rice straw were similar to soils amended with non-transgenic rice straw. The Simpson index of HC straw-amended soil was

greater than that of the soil amended with ZJ straw after 128 and 158 days, whereas such difference was not found between HC and JZ straw amendment.

2.4 Soil microbial community structure and diversity

As shown in Fig. 3, no apparent visible differences in TGGE profiles of the bacterial 16S rDNA fragments in rhizosphere soils of HC, JZ and ZJ were observed. Similar bands were detected in the TGGE profiles among HC, JZ and ZJ, aside from special band 8 in ZJ rhizosphere soil at the filling stage. However, there were some differences in the intensity of bands 1–7 and 9 among HC, JZ and ZJ. A similar pattern was observed in the TGGE profiles of TT, MH and 9311, except for special band 11 in 9311 rhizosphere soil at the heading stage and special band 13 in TT and MH rhizosphere soils. These bands (1–14) were sequenced and compared with the NCBI GenBank database using the BLAST procedure, and then phylogenetic analysis was performed. The 16S rDNA sequences of bands 1–14 were distributed throughout the bacterial phylogenetic tree (Fig. 4). As shown in Table 2, the majority (bands 1–8, 10 and 13–14) of bacterial sequences recovered from TGGE profiles were closely associated with clusters of γ- or α-Proteobacteria, and the sequences of bands 9, 11 and 12 belonged to Anaerolineae, Actinobacteria and Clostridia, respectively. Most of these showed higher than 90% similarity to clone sequences.

Figure 3 also shows the microbial community composition of the soils amended with biomass of the two transgenic rice lines and the corresponding parental and distant parental rice varieties. Although there were no

Table 1 Effect of transgenic rice vegetation and the corresponding straw amendment on the functional diversity of the soil microbial community under field conditions

| Diversity	Sampling time*	Varieties of rice			Varieties of rice		
		HC	JZ	ZJ	TT	MH	9311
Shannon (richness)	Tillering	3.09 ± 0.03 a	3.08 ± 0.04 a	3.08 ± 0.02 a	3.03 ± 0.10 a	3.02 ± 0.10 a	3.15 ± 0.08 a
	Heading	3.01 ± 0.08 a	2.96 ± 0.02 a	3.05 ± 0.02 a	3.07 ± 0.07 a	2.98 ± 0.08 a	2.90 ± 0.01 a
	Filling	3.12 ± 0.03 a	3.11 ± 0.06 a	2.88 ± 0.09 a	3.04 ± 0.07 a	3.04 ± 0.06 a	2.85 ± 0.05 a
	Maturing	2.88 ± 0.04 a	2.74 ± 0.09 a	2.88 ± 0.09 a	2.92 ± 0.06 a	2.74 ± 0.10 a	2.98 ± 0.04 a
	97 days	2.54 ± 0.31 a	2.40 ± 0.22 a	2.41 ± 0.13 a	2.10 ± 0.29 a	2.25 ± 0.15 a	1.82 ± 0.38 a
	128 days	2.15 ± 0.08 a	2.25 ± 0.17 a	2.28 ± 0.10 a	2.18 ± 0.10 a	2.29 ± 0.20 a	2.33 ± 0.22 a
	158 days	2.09 ± 0.07 a	2.18 ± 0.03 a	2.05 ± 0.41 a	2.38 ± 0.02 a	2.04 ± 0.02 a	2.18 ± 0.42 a
	189 days	2.95 ± 0.05 a	3.03 ± 0.05 a	2.90 ± 0.14 a	3.01 ± 0.02 a	3.06 ± 0.05 a	3.05 ± 0.05 a
McIntosh (evenness)	Tillering	2.99 ± 0.03 a	2.98 ± 0.04 a	3.13 ± 0.02 a	2.88 ± 0.10 a	2.96 ± 0.10 a	2.90 ± 0.08 a
	Heading	3.28 ± 0.01 a	3.27 ± 0.11 a	3.92 ± 0.32 a	3.82 ± 0.16 a	3.53 ± 0.12 a	3.27 ± 0.28 a
	Filling	3.74 ± 0.16 a	3.58 ± 0.09 a	3.50 ± 0.28 a	3.63 ± 0.08 a	3.94 ± 0.47 a	3.63 ± 0.27 a
	Maturing	2.91 ± 0.13 a	3.29 ± 0.48 a	3.50 ± 0.28 a	3.55 ± 0.21 a	3.26 ± 0.34 a	3.77 ± 0.13 a
	97 days	2.23 ± 0.82 a	1.61 ± 0.29 a	1.81 ± 0.31 a	1.65 ± 0.31 a	1.47 ± 0.25 a	1.63 ± 0.25 a
	128 days	1.47 ± 0.12 a	1.51 ± 0.10 a	1.37 ± 0.10 a	1.43 ± 0.16 a	1.48 ± 0.13 a	1.36 ± 0.09 a
	158 days	1.49 ± 0.11 a	1.65 ± 0.14 a	1.79 ± 0.21 a	1.38 ± 0.34 a	1.39 ± 0.11 a	1.68 ± 0.23 a
	189 days	3.05 ± 0.29 a	3.10 ± 0. 23 a	2.90 ± 0.21 a	2.93 ± 0.16 a	2.80 ± 0.52 a	2.94 ± 0.13 a
Simpson (dominant population)	Tillering	18.57 ± 0.49 a	18.53 ± 1.07 a	18.78 ± 0.27 a	17.55 ± 2.06 a	17.33 ± 1.62 a	18.46 ± 2.03 a
	Heading	17.68 ± 1.72 ab	16.29 ± 0.90 a	18.55 ± 0.46 b	19.37 ± 1.36 a	17.29 ± 1.59 b	15.59 ± 0.56 c
	Filling	19.76 ± 0.86 a	19.85 ± 1.28 a	15.43 ± 1.57 b	18.50 ± 1.44 a	18.46 ± 0.76 a	14.86 ± 0.56 b
	Maturing	14.43 ± 0.82 a	13.17 ± 1.08 a	15.43 ± 1.57 a	15.73 ± 0.94 a	14.72 ± 1.18 a	17.16 ± 0.86 b
	97 days	10.01 ± 2.76 a	7.10 ± 1.62 a	8.19 ± 1.68 a	5.43 ± 3.35 a	5.85 ± 0.81 a	4.09 ± 1.69 a
	128 days	13.78 ± 1.49 ab	14.56 ± 0.83 a	11.53 ± 0.51 b	12.91 ± 1.51 a	11.73 ± 1.37 a	12.23 ± 1.41 a
	158 days	9.61 ± 0.29 ab	10.43 ± 0.23 a	8.79 ± 0.64 b	10.18 ± 0.62 a	9.40 ± 0.29 a	9.49 ± 0.27 a
	189 days	16.13 ± 0.91 a	17.83 ± 0.90 a	16.20 ± 1.06 a	16.89 ± 0.20 a	18.36 ± 1.49 a	18.07 ± 1.16 a

* Sampling time after rice straw amendment: 97, 128, 158 and 189 days.
All values represent mean ± SD of triplicate samples.
Values in horizontal rows followed by the same letters are not statistically different ($p \leqslant 0.05$).

Table 2 Phylogenetic affiliations of partial 16S rDNA sequences of bands excised from TGGE gels at different growth stages of rice and different sampling times after rice straw amendment

Band No.	Fragment length (bp)	Closest relative in GenBank	Similarity	Putative phylum
Band 1	139	*Pseudomonas syringae* Cit 7	100%	γ-Proteobacteria
Band 2	132	*Pseudomonas syringae* pv. *morsprunorum* str.	94%	γ-Proteobacteria
Band 3	142	*Azotobacter vinelandii*	90%	γ-Proteobacteria
Band 4	136	*Pseudomonas syringae* pv. *aptata* str.	97%	γ-Proteobacteria
Band 5	136	*Shigella dysenteriae* 1617	98%	α-Proteobacteria
Band 6	156	*Selenomonas* sp. oral taxon 149 str.	96%	α-Proteobacteria
Band 7	138	*Escherichia coli* str.	82%	α-Proteobacteria
Band 8	145	*Escherichia coli* W strain W ctg00072	98%	γ-Proteobacteria
Band 9	152	*Clostridium bartlettii*	96%	Anaerolineae
Band 10	146	*Pseudomonas aeruginosa* PAO1	96%	γ-Proteobacteria
Band 11	141	*Ruminococcus albus*	100%	Actinobacteria
Band 12	152	*Reinekea blandensis*	82%	γ-Clostridia
Band 13	153	*Shigella dysenteriae* 1617	90%	γ-Proteobacteria
Band 14	155	*Shigella dysenteriae* 1617	92%	γ-Proteobacteria
Band 15	153	*Escherichia coli* MS 85-1	99%	γ-Proteobacteria
Band 16	151	–	–	γ-Clostridia
Band 17	155	*Escherichia coli* ED1a	90%	γ-Proteobacteria
Band 18	143	*Shigella flexneri* 2a str.	98%	γ-Proteobacteria
Band 19	148	*Escherichia coli* 2362-75	99%	γ-Proteobacteria
Band 20	149	*Zunongwangia profunda*	91%	Bacteroidetes
Band 21	151	*Escherichia coli* 1827-70	99%	γ-Proteobacteria
Band 22	145	*Bifidobacterium dentium*	99%	Anaerolineae
Band 23	152	*Methylibium petroleiphilum*	99%	β-Proteobacteria
Band 24	159	*Lactobacillus gasseri*	100%	Anaerolineae

Lanes 1–3: HC; lanes 4–6: JZ; lanes 7–9: ZJ   Lanes 1–3: TT; lanes 4–6: MH; lanes 7–9: 9311
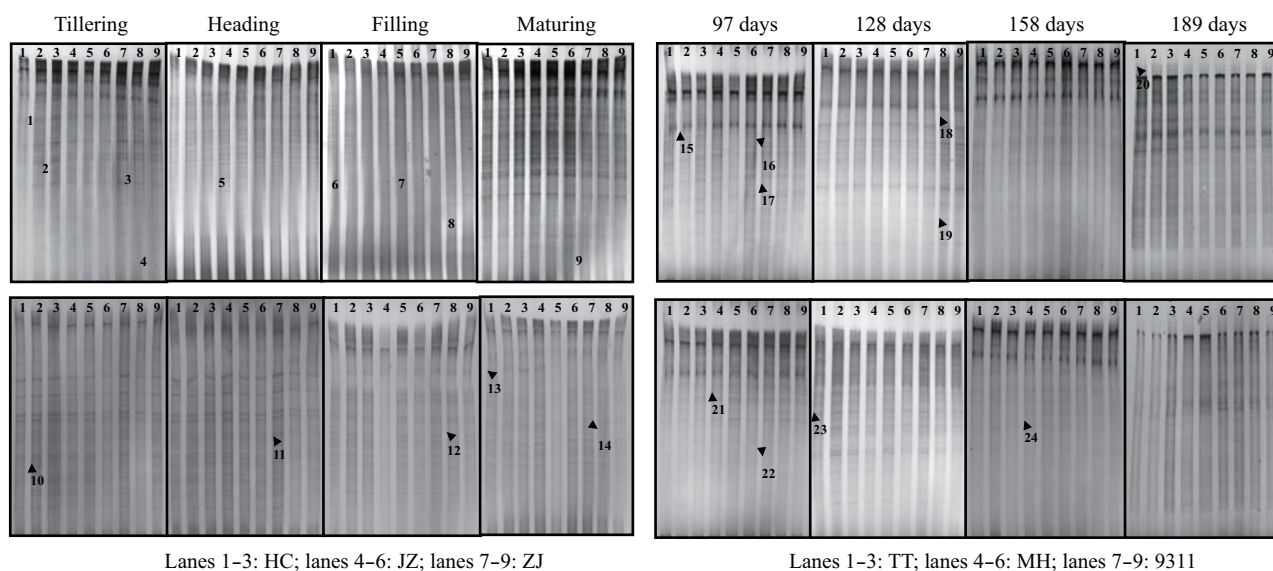
Fig. 3 TGGE analysis of 16S rDNA fragments amplified from rhizosphere soils of HC, JZ, ZJ, TT, MH and 9311 at different growth stages and from soils amended with their straw at different sampling times.

special bands between the soils amended with Bt, parental and distant parental rice straw, the intensity of bands 15–24 was different. These bands were excised from TGGE gels and then cloned and sequenced. Figure 5 shows the phylogenetic relationship of these ten sequences in combination with 16S rDNA sequences obtained from the NCBI GenBank database. As shown in Table 2, the sequences of bands 15, 17–19, 21 and 23 were closely related to clusters of γ- or β-Proteobacteria. The band 16 was associated with Clostridia, but it had low similarity to any of the bacterial 16S rDNA sequences available in the NCBI GenBank database, the band 20 belonged to Bacteroidetes, and the sequences of bands 22 and 24 were most closely related to Anaerolineae. Overall, these clone sequences showed high (⩾ 90%) similarity.

3 Discussion

Activities of soil enzymes (phosphatase, invertase, urease, catalase etc.) could be used as indicators of the impact of Bt toxins on soil microbiological activity (Liu et al., 2008). The data shown in Fig. 1 indicated that the vegetation of the two transgenic rice lines, HC and TT, and return of their straw to the field have few adverse effect on soil enzymes activities, compared to their parent and distant parent, although some transient or even significant differences were observed. These results were in agreement with previous studies, in which some transient significant differences were found in the activity of some enzymes (arylsulfatases, phosphatases, dehydrogenases, urease, invertase and proteases) between the soils with Bt and their near-
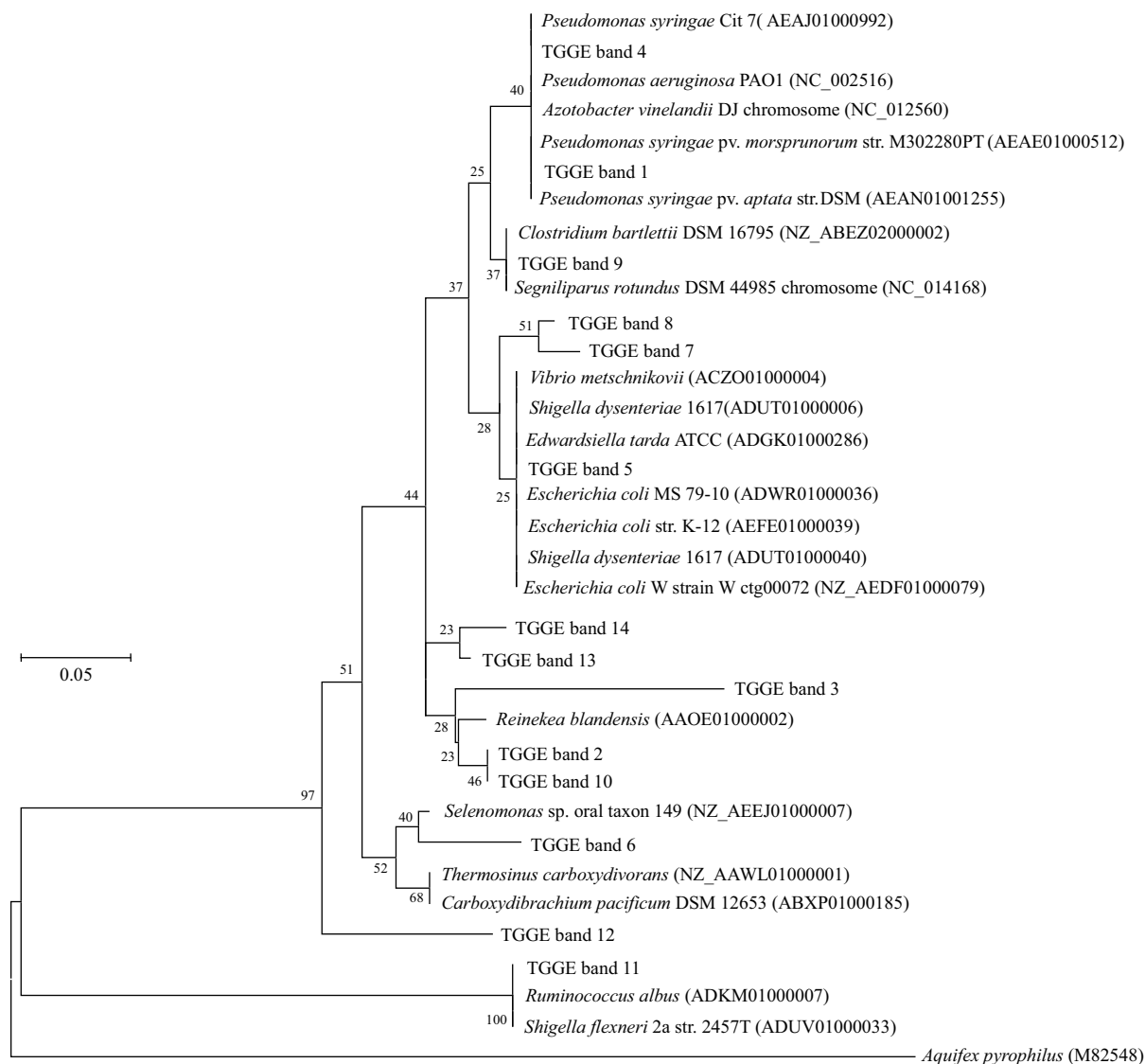
Pseudomonas syringae Cit 7(AEAJ01000992)
TGGE band 4
Pseudomonas aeruginosa PAO1 (NC_002516)
Azotobacter vinelandii DJ chromosome (NC_012560)
Pseudomonas syringae pv. *morsprunorum* str. M302280PT (AEAE01000512)
TGGE band 1
Pseudomonas syringae pv. *aptata* str. DSM (AEAN01001255)
Clostridium bartlettii DSM 16795 (NZ_ABEZ02000002)
TGGE band 9
Segniliparus rotundus DSM 44985 chromosome (NC_014168)
TGGE band 8
TGGE band 7
Vibrio metschnikovii (ACZO01000004)
Shigella dysenteriae 1617(ADUT01000006)
Edwardsiella tarda ATCC (ADGK01000286)
TGGE band 5
Escherichia coli MS 79-10 (ADWR01000036)
Escherichia coli str. K-12 (AEFE01000039)
Shigella dysenteriae 1617 (ADUT01000040)
Escherichia coli W strain W ctg00072 (NZ_AEDF01000079)
TGGE band 14
TGGE band 13
TGGE band 3
Reinekea blandensis (AAOE01000002)
TGGE band 2
TGGE band 10
Selenomonas sp. oral taxon 149 (NZ_AEEJ01000007)
TGGE band 6
Thermosinus carboxydivorans (NZ_AAWL01000001)
Carboxydibrachium pacificum DSM 12653 (ABXP01000185)
TGGE band 12
TGGE band 11
Ruminococcus albus (ADKM01000007)
Shigella flexneri 2a str. 2457T (ADUV01000033)
Aquifex pyrophilus (M82548)

0.05

Fig. 4 Neighbor-joining tree showing the phylogenetic affiliations of partial 16S rDNA sequences (V3-hypervariable region) obtained from bands excised from TGGE gels at different growth stages of rice. The tree was rooted with the partial 16S rDNA sequence of *Aquifex pyropklius* as an outgroup. Values at nodes represent the percentage of 1000 bootstrap replicates. The scale bar indicates an estimated change of 5%.

isogenic non-Bt plants (Icoz and Stotzky, 2008; Liu et al., 2008). Some studies reported that there was no significant difference in the activities of phosphatases and catalase between the soils planted with Bt and non-Bt maize (Flores et al., 2005; Lang et al., 2006; Icoz et al., 2008). Wu et al. (2004) showed no apparent differences in the activity of neutral phosphatase between the soils amended with Bt and non-Bt rice straw. There were few significant differences in urease, alkaline phosphatase, dehydrogenase, phenol oxidase and protease activities between Bt and non-Bt cottons at any of the growth stages and after harvest (Shen et al., 2006). However, Sun et al. (2007) reported that activities of soil urease, acid phosphomonoesterase, invertase and cellulase were stimulated by the addition of Bt cotton tissues, which may be due to increased microbial activity caused by straw addition.

The data shown above indicate that cultivation of Bt transgenic rice and the addition of their straw to the field have no significant effects on soil anaerobic respiration activities. Similar results were found in Bt and non-Bt maize

associated rhizospheres (Castaldini et al., 2005). Fang et al. (2007) also reported that Bt corn residues in field studies did not affect cumulative soil CO_2 efflux and rates of soil CO_2 evolution. By contrast, during the cultivation of Bt crops, the evolution of CO_2 from soils associated with Bt maize was about 30.5% lower than that from soils associated with its near isogenic non-Bt maize (Dinel et al., 2003). Sarkar et al. (2008) reported a significant reduction in soil respiration in the rhizosphere of Bt-cotton over non-Bt isoline. A 3-year comparative field assessment of Cry3Bb Bt corn vs. non-Bt corn grown also demonstrated that Bt corn had adverse effects on respiration rates (Devare et al., 2004). During the straw amendment of Bt crops, Flores et al. (2005) reported that the amounts of C evolved as CO_2 were significantly lower from soil microcosms amended with the biomass of Bt plants compared to those of non-Bt plants. Wu et al. (2004) found that differences in anaerobic respiration between soils supplemented with Bt-transgenic rice straw and non-transgenic rice straw were persistent over the course of incubation. Lower evolution
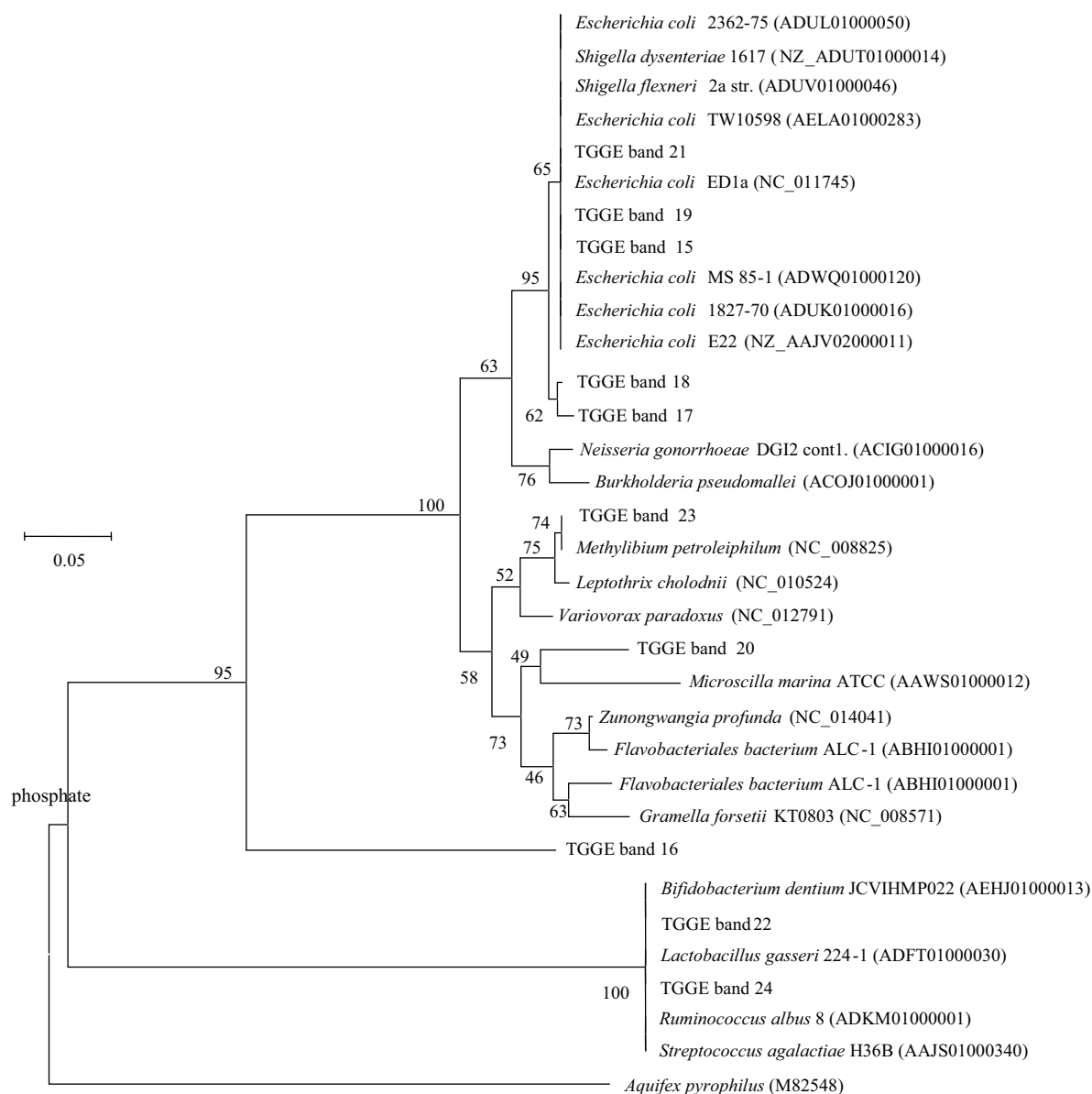
Fig. 5 Neighbor-joining tree showing the phylogenetic affiliations of partial 16S rDNA sequences (V3-hypervariable region) obtained from bands excised from TGGE gels at different sampling times after amendment with straw. The tree was rooted with the partial 16S rDNA sequence of *Aquifex pyropklius* as an outgroup. Values at nodes represent the percentage of 1000 bootstrap replicates. The scale bar indicates an estimated change of 5%.

of CO_2 from soils amended with biomass of Bt maize, potato, cotton, canola and tobacco than with biomass of near-isogenic non-Bt counterparts was also observed (Icoz and Stotzky, 2008). However, the addition of Bt-maize straw resulted in increased respiration rates within the first day of incubation, compared to that of near-isogenic non-Bt varieties straw (Raubuch et al., 2007). Raubuch et al. (2010) also reported that the addition of Bt-maize straw significantly increased CO_2 production rates and the specific respiration rates CO_2-C/microbial biomass C and CO_2-C/ATP compared with the addition of non-Bt maize straw. These contradictory results of the effects of Bt plants on microbes may be the result of differences in the type of Cry protein, plant variety and experimental method used, as well as soil type and environmental factors (Icoz and Stotzky, 2008).

The data on the functional diversity of soil microbial communities indicated that the vegetation and straw

amendment of Bt rice HC and TT did not affect the richness and evenness of soil microorganisms. The dominant population of soil microorganisms seemed to be more sensitive and was altered temporarily by the vegetation and straw amendment of Bt rice. This alteration was, however, smaller than that caused by the vegetation and straw amendment of non-transgenic distant parental rice. Similar to this result, differences in soil microbial communities were also observed during two years cultivation of Bt maize and non-Bt maize (Griffiths et al., 2005). Fang et al. (2007) also reported that Bt corn residues in field studies could alter substrate utilization patterns of soil microbial communities based on Biolog metabolic profiles. By contrast, some studies indicated that transgenic plants had less influence on soil microbial communities (Wu et al., 2004; Lee et al., 2011). Shen et al. (2006) indicated that the richness and functional diversity of microbial communities were no different when comparing

the rhizosphere soils of Bt (expressing Cry1A) and non-Bt cotton after a complete cotton growth cycle. Chun et al. (2011) reported that no differences were observed in the Shannon and Simpson diversity indices between transgenic rice and its parental non-transgenic counterpart (cultivar Dongjin) at the seedling, tillering, heading, and maturing stages over two successive years. Lawhorn et al. (2009) reported that Bt corn exuding the cry3Bb1 toxin had no adverse effects on saprophytic microbial communities of the soil and decaying roots or on decomposition. Wu et al. (2004) also showed that decomposing transgenic Bt rice straw had no significant effects on culturable bacteria, actinomycetes, and fungi in a flooded paddy soil under laboratory conditions, although some transient effects were observed on populations of anaerobic fermentative bacteria, denitrifying bacteria, hydrogen-producing bacteria and methanogenic archaea.

During all growth stages of rice and straw amendment, all special bands 1–24 with differences in band intensity were selected to excise, clone, sequence and then analyze. These clone sequences were distributed into Proteobacteria, Anaerolineae, Actinobacteria, Clostridia, and Bacteroidetes by phylogenetic analysis, which are common residents in agricultural soils. The majority of the 24 bacterial clone sequences belonged closely to clusters of γ- or α- or β-Proteobacteria. A similar result was also reported by Lee et al. (2011), who showed that most clone sequences belonged to the Proteobacteria. The data shown in Figs. 3–5 indicate that the vegetation of the two transgenic rice lines and return of their straw to the field had little effect on the soil microbial community structure compared to their parent and distant parent, but minor changes in some microbial population biomass were observed. Most studies have also indicated that Bt plants cause only minor changes in microbial community structure that are often transient in duration, if any change at all (Blackwood and Buyer, 2004; Devare et al., 2004). Fang et al. (2007) reported that Bt corn residues did not affect soil microbial community structure. Icoz et al. (2008) reported no differences in microbial community structure between soils with Bt and non-Bt maize as measured by denaturing gradient gel electrophoresis (DGGE). Liu et al. (2008) found that KMD1(Bt) rice expressing the crylAb gene had no measurable adverse effect on the key microbial processes or microbial community composition in rhizosphere soil over two years of rice cropping. Miethling-Graff et al. (2010) revealed that there were no significant differences between the rhizosphere bacterial community structure of Bt maize and the other cultivars over the course of three consecutive years of study. Lee et al. (2011) reported that GM rice and non-GM rice cropped soils did not differ significantly in soil bacterial and fungal community structures. The composition of bacterial and fungal communities within the soil also did not differ between *Myxococcus xanthus* protoporphyrin oxidase (Mx PPO) transgenic rice and non-transgenic parental rice at the seedling, tillering, heading and maturing stages of rice over two successive years (Chun et al., 2011). However, a few studies have reported significant differences in

microbial community structure between soils with Bt and non-Bt crops. Castaldini et al. (2005) found consistent significant differences in microbial community structure between soils with Bt and non-Bt maize. Similar to our results obtained for rice straw amendment treatments, Lu et al. (2010) reported that the effects of crylAb gene insertion into the Xiushui 11 rice genome on the residues' decomposition-associated bacterial and fungal community compositions were minor over two successive years.

In addition, a number of studies showed that different plant species (no matter whether transgenic or conventional) may exert different effects on soil microbial communities by exudation of organic compounds, root senescence and possibly other mechanisms (Viebahn et al., 2005). Our data also provided evidence that the differences of rhizosphere soil microbial communities between parents and distant parents might be larger than those between transgenic rice lines and the corresponding parents or distant parents. A related study was reported by Marschner et al. (2004), who reported that the rhizosphere community structure of three plant species (chickpea, canola and Sudan grass) differed from each other in the sand and the loam. Berg et al. (2006) revealed that the rhizosphere effect on the antagonistic bacterial community was influenced by the plant species. Garbeva et al. (2008) also found that four plant species (maize, oat, barley and commercial grass mix) had clear and different effects on the microbial community and diversity as well as on the abundance of the bacterial antagonistic community of the soil borne pathogen *Rhizoctonia solani* AG3.

Overall, in the present study, significant variations in soil enzymes, anaerobic respiration activities, microbial diversity indices and community structure were caused by different growth stages of rice and sampling time after rice straw amendment. This may be explained by seasonal or temperature fluctuations. Some studies showed that the expression products of the crylAb gene may have a lesser effect than the physicochemical and environmental factors (soil pH, moisture, redox potential, nitrogen concentration, temperature, precipitation etc.) (Kennedy et al., 2004). A similar phenomenon was proved by Chun et al. (2011), who reported that the changes in the activity and structure of microbial communities were influenced by seasonal variations. Liu et al. (2008) also reported that the changes in rhizosphere soil microbial community composition associated with the crop growth stage outweighed the effects of application of triazophos and the crylAb gene transformation.

4 Conclusions

The results obtained in this study indicated that the vegetation of the two transgenic rice lines (HC and TT) and subsequent straw amendment had few adverse effects on soil enzymes (catalase, urease, neutral phosphatase and invertase), anaerobic respiration activity and functional diversity of soil microorganisms when compared to the corresponding parent and distant parent, although some transient differences were observed. The community

structure of soil microorganisms was not altered by the vegetation of Bt-rice (HC and TT) and the addition of their straw, but minor changes in microbial population biomass were observed. No different pattern of impact due to plant species was found between HC and TT. However, the results of this study do not mean that the two transgenic rice lines (HC and TT) have no long-term effects on soil biology functions. Longer term studies over several consecutive growing seasons should be carried out to assess the potential effects of the cultivation of Bt rice and straw amendment on soil microbial ecology and functional microflora.

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