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REGULAR ARTICLE

Effect of soil type and soybean genotype on fungal community in soybean rhizosphere during reproductive growth stages

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Abstract Fungal communities in soybean rhizosphere from reproductive growth stages R_1 (beginning bloom) to R_8 (full maturity) were studied based on the polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) banding patterns of partial rDNA internal transcribed spacer regions (ITS1) and sequencing methods. Pot experiment subjecting three soybean genotypes grown in two soils (Mollisol and Alfisol) indicated that the soil type was the major factor in shaping the fungal communities in the soybean rhizosphere. Field experiment was conducted in an Alfisol field with three soybean genotypes, and both pot and field experiments showed that rhizosphere fungal communities shifted with growth stages, and more diversity of communities was found in early reproductive growth stages than later stages. No major difference among fungal communities of three soybean genotypes was detected at individual growth stage. BLAST search of ITS sequence data generated from excised DGGE bands showed that fungi belonging to Ascomycetes and Basidiomycetes predominantly inhabited in the soybean rhizosphere. In addition, a few bands had low similarity with database sequences inferred that unknown fungal groups existed in soybean rhizosphere.

Keywords DGGE · Fungal community · ITS region · Rhizosphere · Soil type · Soybean genotype

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Introduction

The rhizosphere is the soil region influenced by plant roots and characterized with high microbial activities (Hiltner 1904). Rhizosphere microbial communities carry out fundamental processes that contribute to nutrient cycling, healthy root growth, and plant growth promotion (Buchenauer 1998; Atkinson and Watson 2000; Sylvia and Chellemi 2001). Plant roots release a wide variety of compounds into the rhizosphere, which form unique micro-environments for soil microorganisms. It is commonly recognized that the root exudates differ according to plant species, even cultivars, and plant growth stages (Rovira 1959; Nelson 1990; Whipps 2001; Rengel 2002). As different microbes respond differently to the compounds released by roots, different composition of root exudates is believed to explain the plant specific rhizosphere microbal communities (Marschner et al. 2001, 2002; Smalla et al. 2001; Kowalchuk et al. 2002). Soil type is another important factor for the determination of the rhizosphere microbal communities (Buyer et al. 1999; Dalmastri et al. 1999; Kowalchuk et al. 2000), as different soils show

different particle size distribution, pH, aeration, and physico-chemical characteristics that can affect microbial communities either directly, *e.g.*, by providing a specific habitat for selecting specific microbes, and indirectly, *e.g.*, by affecting plant root exudation (Garbeva et al. 2004). Therefore, the interactions among soil types, plant species/genotypes and growth stages complicatedly affect microbial communities in the rhizosphere (Marschner et al. 2001). The effect of soil type on the community is greater than that of plant species in some cases (Dalmastri et al. 1999; Buyer et al. 2002; Singh et al. 2007), while in other cases plant species show greater effect on the community composition than the soil type (Grayston et al. 1998; Wieland et al. 2001). Although most of the previous studies focused on the rhizosphere bacterial communities, only a few reports were related to fungal communities in plant rhizosphere (Gomes et al. 2003; Singh et al. 2007).

Soybean (*Glycine max* (L.) Merrill) is a major crop in Northeast China. The acreage of soybean cultivation and its total yield in this region account for about 33% and 44% of nation's total, respectively (Liu and Herbert 2002). Since the first cultivar was released in 1923, more than 600 cultivars had been released in Northeast China by the end of the last century (Liu et al. 2008). Although soybean breeding and genotype improvement contributed primarily to increase soybean yield in this region, the influence of soybean genotype on microbial communities, especially on fungal communities in the rhizosphere is not clear.

Culture-dependent techniques were traditionally used for assessing fungal diversity. It has become increasingly know that only 5–10% of fungal community members can be detected by culture methods (Hawksworth and Rossman 1997; Hawksworth 2001). Recently, culture-independent techniques, such as analyses of the microbial DNA extracted directly from environments have pushed a remarkable progress in microbial ecology research (Marschner et al. 2001; Ebersberger et al. 2004; Yao et al. 2006). Our previous researches have focused on bacterial communities in soybean rhizosphere (Xu et al., unpublished data). In this work, we analyzed the potential effects of two soil types (Mollisol and Alfisol), and three soybean genotypes (Hefeng 25, Suinong 14, and D2003-1) on fungal community structures during soybean reproductive growth stages by the pot and field experiments, and the community structures were estimated by using denaturing gradient gel electrophoresis (DGGE) for community evaluation and sequencing of DGGE bands for phylogenetic determination of dominant fungal members.

Materials and methods

Pot experiment

A Black soil (Mollisol) and a Dark Brown soil (Alfisol) were collected from farming fields at Hailun Agro-Ecological Experimental Station, Chinese Academy of Sciences (47°26′N, 126°38′E) and Lanling village, Jixi City (45°17′N, 130°42′E), respectively, in March 2005 (Table 1). Both sites were located in Heilongjiang Province, which is the largest soybean cropping province in Northeast China. Fifteen kilograms of soil were transferred into each pot (25.5 cm diameter and 29.0 cm deep), and the moisture content was adjusted to 70% field capacity. Two soybean genotypes of Hefeng 25 (average yield: 2260 kg ha^{-1}) and Suinong 14 (average yield: 2550 kg ha^{-1}) that were released in 1984 and 1996, respectively, and a new line of D2003-1 with the yielding potential of 3000 kg ha^{-1} were chosen in this study. All three genotypes take about 120–125 days to maturity in Heilongjiang Province, China.

Three genotypes were sown to the Dark Brown soil, while Hefeng 25 and D2003-1 were sown in the Black soil (six seeds per pot). After the seed germination, seedlings were thinned to three plants per pot. All pots were placed in a glasshouse (daytime: 24–28°C, night time 16–20°C). Four replicate pots were prepared for each genotype, soil and sampling time combinations. At the soybean

Table 1 Some characteristics of soil used in the study

Property	Black soil	Dark brown soil
Total C (g kg^{-1})	27.6	12.1
Total N (g kg^{-1})	2.1	0.9
Total P (g kg^{-1})	0.9	0.4
pH (H_2O)	6.5	5.2
Soil texture	Clay loam	Sand loam
Clay (%)	29.4	55.3
Silt (%)	40.5	36.2
Clay (%)	28.1	8.5

reproductive growth stages of R_1 (beginning bloom), R_3 (beginning pod), R_4 (full pod), R_5 (beginning seed) and R_8 (full maturity), the shoot was cut off, and the roots were carefully separated from soils by inverting the pots. Only the soil adhering the roots was considered as rhizosphere soil (Nazih et al. 2001), and the rhizosphere soils were collected by shaking off from roots in the air. A portion of composite soil samples was placed into autoclaved microcentrifuge tubes (2 ml) immediately. The tubes were stored at −80°C until use.

Field experiment

A field experiment was conducted on a Dark Brown soil at Lanling village in 2005. Three genotypes of Hefeng 25, Suinong 14 and D2003-1 were sown with the density of 30 plants m^{-2} on 15 May. Randomized design with three replicates for each genotype was performed, and each plot contains ten rows with the 15 m long and 0.67 m wide. Fertilization was followed to the local recommendation with N 50 kg ha^{-1}, P 45 kg ha^{-1} and K 60 kg ha^{-1}. At the growth stages of R_1, R_3, R_4, R_5 and R_8, ten plants were harvested, and the rhizosphere soils were collected and treated in the same way as the pot experiment.

DNA extraction and purification

DNA was extracted from the soil samples (0.5 g wet weight) by bead-beating method based on the method of Zhou et al. (1996) and Watanabe et al. (2004). DNA extracts were dissolved in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and purified with Sephadex G-200 (Cahyani et al. 2004).

PCR-DGGE

A nested PCR amplification was conducted targeting fungal rDNA internal transcribed spacer (ITS) regions (Bastias et al. 2006). Fungal-specific primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990), GC-ITS1-F and ITS2 (Gardes and Bruns 1993) were used for the first and second round of PCR amplifications, respectively. PCR amplification was performed according to the description by Bastias et al. (2006). DGGE was performed by using 8% (w/v) acrylamide gel with a 20–60% denaturant gradient and run in 1×TAE (Tris-acetate-EDTA) buffer for 16 h under conditions at 60°C and 75 V. After the electrophoresis, the gel was stained in 1:3300 (v/v) GelRed (Biotium, USA) nucleic acid staining solution for 20 min. DGGE profiles were photographed by using Bio-Rad transilluminator (BIO-RAD Laboratories, Segrate, Italy) under UV light.

Analysis of the DGGE profile

Banding patterns of the DGGE profile were analyzed by the Quantity one software (version 4.5). The position and intensity of each band were determined automatically. The density value of each band was divided by the average band density of the lane in order to minimize the influence of loaded DNA concentrations among samples (Garland and Mills 1991; Graham and Haynes 2005). Normalized data were used for principal component analysis as described previously (Matsuyama et al. 2007).

Sequencing of DGGE bands

Several common bands and bands showing variations with soil types, growth stages and genotypes were carefully excised from the DGGE gel and subjected to sequencing. DNA extraction from DGGE bands, verification of the mobility of bands and sequencing were performed as described previously by Cahyani et al. (2004).

Phylogenetic analysis

Closest relatives and phylogenetic affiliations of the obtained sequences were determined by using the BLAST search program at the NCBI web site. All sequences determined in the present study were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers from AB438048 to AB438071.

Results

Pot experiment

DGGE profiles of fungal communities in the rhizosphere are shown in Fig. 1. In total, 56 bands with different mobility were detected. The average number of DGGE bands across growth stages from R_1 to R_8 for genotypes Hefeng 25, D2003-1 and Suinong 14 in

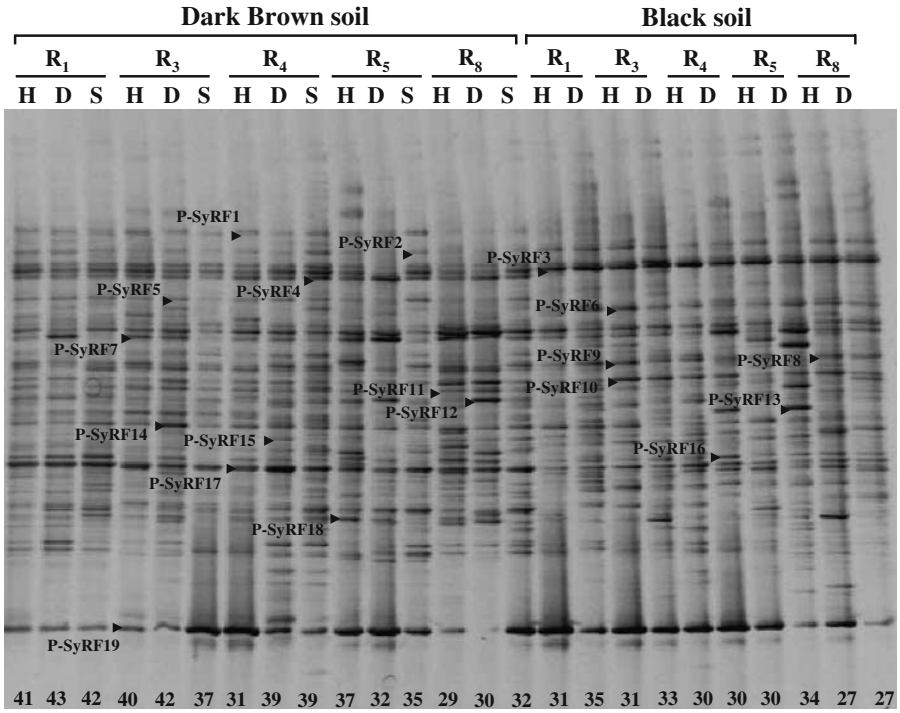

Fig. 1 DGGE profiles of fungal communities in rhizospheres of three soybean genotypes grown in the Black and the Dark Brown soils (pot experiment). R_1, R_3, R_4, R_5 and R_8 represent soybean stages at beginning bloom, beginning pod, full pod, beginning seed and full maturity, respectively. H, D and S represent soybean genotype of Heifeng 25, D2003-1 and Suinong 14, respectively. Numbers below the profiles indicate the DGGE band abundances of the line, and arrows indicate bands excised from the gel for sequencing

Dark Brown soil were 35.6±5.4, 37.2±5.9 and 37.0±3.8, respectively, and for genotypes Hefeng 25 and D2003-1 in Black soil were 29.8±1.6 and 31.8±3.3, respectively. Under both soil conditions, the DGGE band number was in the highest level at stage R_1 for every genotype, and then it decreased with soybean development to the lowest level at growth stage R_8 (Fig. 1).

DGGE banding patterns of rhizosphere fungal communities among all three genotypes were very similar to each other at the individual sampling time, but different between the two soil types (Fig. 1). For examples, bands P-SyRF4 and P-SyRF5 were specific to Dark Brown soil, band P-SyRF6 was restricted to Black soil, and more abundances of bands P-SyRF3 in Black soil, and P-SyRF7 in Dark Brown soil than those in counterpart soil.

Excluding band P-SyRF19 (ITS region of soybean), principal component analysis clearly separated fungal communities in soybean rhizospheres between Black soil and Dark Brown soil, irrespective of genotypes (Fig. 2). In addition, the fungal communi-

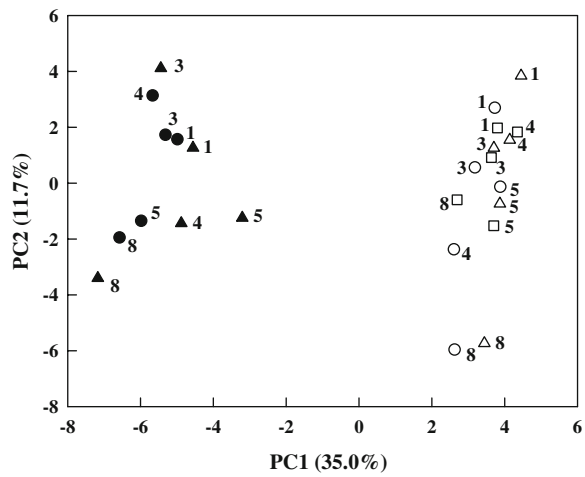

Fig. 2 Principal component analysis of DGGE profiles of fungal communities in rhizospheres of D2003-1 (Δ), Hefeng 25 (○) and Suinong 14 (▫) (pot experiment). Numbers beside the symbols indicate soybean reproductive growth stages. Solid and open symbols represent the soybean grown in the Black and the Dark Brown soils, respectively

ties in soybean early reproductive growth stages were different from those in later stages (Fig. 2), and the succession pattern of fungal communities from growth stages R_1 to R_8 was similar in Black soil and Dark Brown soil. No significant differences were found in the fungal community among the genotypes at individual growth stage.

Field experiment

As shown in Fig. 3, DGGE profiles of fungal communities in the field experiment were very similar with each other among the genotypes, and the difference was only observed for a few bands or band density. For instance, band F-SyRF1 showed the highest intensity at stage R_8, and band F-SyRF6 had relatively high intensity at stages R_5 and R_8 than preceding growth stages.

In total, 74 bands with different mobility were observed in the field experiment, and average band numbers across growth stages for Hefeng 25, D2003-1 and Suinong 14 were 49.0±6.5, 48.2±6.2 and 46.4±4.2, respectively. Similar to the pot experiment, the band number tended to be more numerous in the early reproductive growth stages than in the later stages (Fig. 3).

Excluding band F-SyRF7 (ITS region of soybean), principal component analysis divided the bacterial communities into three groups (A, B and C). Groups A, B and C contained all three soybean genotypes at stages R_1~R_4, R_5 and R_8, respectively (Fig. 4). The succession of fungal communities of all three genotypes with growth stages had a similar tendency, and the significant effect of genotypes on fungal community changes was also not detected at individual growth stage.

Sequence analysis of DGGE bands

Nineteen common, dense and soil-specific bands in Fig. 1 were excised for sequencing. BLAST search indicated that all bands have closest relatives with

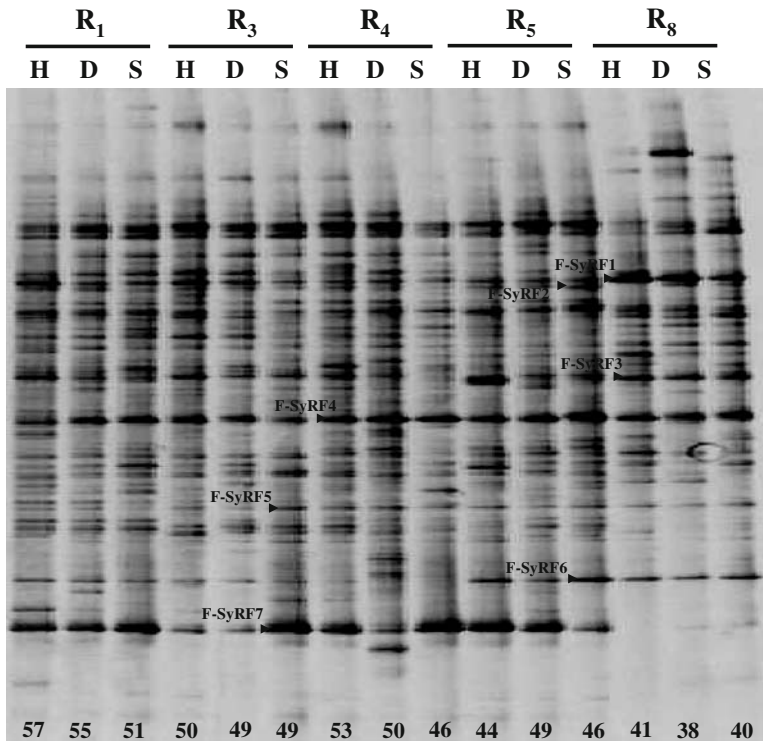

Fig. 3 DGGE profiles of fungal communities in the rhizospheres of three soybean genotypes (field experiment). R_1, R_3, R_4, R_5 and R_8 represent soybean stages at beginning bloom, beginning pod, full pod, beginning seed and full maturity, respectively. H, D and S represent soybean genotypes of Hefeng 25, D2003-1 and Suinong 14, respectively. Numbers below the profiles indicate the DGGE band abundances of the line, and arrows indicate bands excised from the gel for sequencing

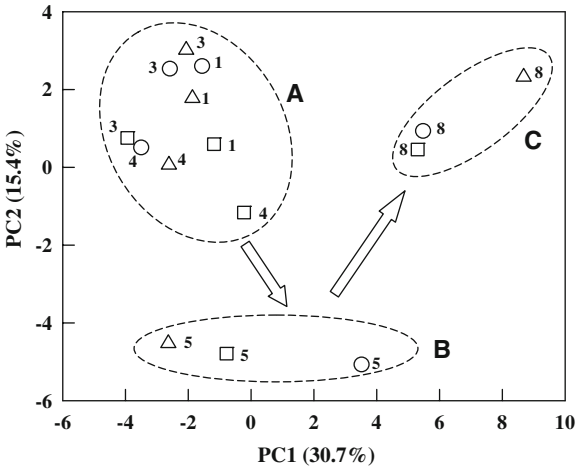

Fig. 4 Principal component analysis of DGGE profiles of fungal communities in the rhizospheres of D2003-1 (Δ), Hefeng 25 (○) and Suinong 14 (▫) (field experiment). Numbers beside the symbols indicate soybean reproductive growth stages

fungal clones or isolates (Table 2), except for band P-SyRF19 come from the ITS region of soybean. Among the 18 fungal related bands, 11 bands affiliated to Ascomycota, five bands to Basidiomycota, one band to Oomycetes, and one band to an uncultured soil fungal clone.

In the field experiment, seven bands were excised and sequenced. Results showed that the nucleotide sequence of band F-SyRF7 was identical to P-SyRF19 in pot experiment, and it was the ITS region of soybean. Other four bands belonged to Ascomycota, and one band was closely related to uncultured soil fungal clone (Table 2). Comparison of nucleotide sequences among the bands elucidated that bands P-SyRF7, P-SyRF15 and P-SyRF17 in the pot experiment were identical to bands F-SyRF2, F-SyRF3 and F-SyRF4 in the field experiment, respectively.

Table 2 Closest relatives of excised DGGE bands those were commonly present or characteristic with soil types, growth stages and genotype. Bands named as P- and F- indicated bands excised from pot and field experiments, respectively

DGGE band	Seq (bp)	Closest relatives			Similarity (%)	Alignment
		Microorganisms	Phylogenetic affiations	Accession number		
P-SyRF2	196	*Penicillium restrictum*	Ascomycota	AY373928	98%	195/197
P-SyRF4	198	*Ascomycete* sp. RS010	Ascomycota	EU082789	100%	198/198
P-SyRF6	165	*Fusarium oxysporum* f. sp. *strigae*	Ascomycota	EU264074	100%	165/165
P-SyRF7	159	*Fusarium solani* isolate FKCB-015	Ascomycota	EU314982	100%	159/159
P-SyRF8	176	*Trichocladium opacum*	Ascomycota	AY970224	100%	175/175
P-SyRF10	158	*Alternaria longissima* strain FG40	Ascomycota	EU030349	100%	156/156
P-SyRF14	182	*Phoma glomerata*	Ascomycota	AJ428532	82%	101/123
P-SyRF15	186	*Corynespora cassiicola*	Ascomycota	DQ780421	90%	172/190
P-SyRF16	171	*Podospora didyma*	Ascomycota	AY999127	81%	126/155
P-SyRF17	179	*Phoma* sp.	Ascomycota	EU530003	100%	179/179
P-SyRF18	149	*Nectriaceae* sp. LM109	Ascomycota	EF060478	99%	147/148
P-SyRF1	231	*Puccinia rupestris*	Basidiomycota	EF635898	51%	115/224
P-SyRF3	190	Uncultured *tremellomycete*	Basidiomycota	EU030400	100%	189/189
P-SyRF5	208	*Cryptococcus terreus*	Basidiomycota	AY591343	99%	207/208
P-SyRF9	206	Uncultured *basidiomycete*	Basidiomycota	AY970109	98%	205/207
P-SyRF12	197	*Ganoderma lipsiense*	Basidiomycota	EF060006	100%	197/197
P-SyRF13	184	*Pythium myriotylum*	Oomycetes	AM396958	100%	184/184
P-SyRF11	165	Uncultured soil fungus clone 9b36	Fungi	DQ421268	82%	138/168
F-SyRF1	182	*Thelebolus microsporus*	Ascomycota	DQ028268	100%	179/179
F-SyRF2	159	*Fusarium solani* isolate FKCB-015	Ascomycota	EU314982	100%	159/159
F-SyRF3	186	*Corynespora cassiicola*	Ascomycota	DQ780421	90%	172/190
F-SyRF4	179	*Phoma* sp. P45A	Ascomycota	EU530003	100%	179/179
F-SyRF5	179	*Plectosphaerella cucumerina*	Ascomycota	EU594566	100%	179/179
F-SyRF6	246	Uncultured soil fungus clone 138-40	Fungi	DQ420877	100%	244/244

Discussion

Although many primers have been designed for amplification of fungal environmental DNA, the fungal ITS region was found to take more information in taxonomy than other genomic regions (*e.g.* 18S rDNA) (Bruns et al. 1991). The primers ITS1 and ITS2 were tested suitable for DGGE analysis of fungal communities associated with the forest and the moorland ecosystems (Anderson et al. 2003), and used for a research on forest soils (Bastias et al. 2006). In the present research, we also used this primer set for analysis of soybean fungal community, and clear and sharp DGGE banding patterns were obtained (Figs. 1 and 3). In addition, four out of six bands in field experiment were sequenced with identical nucleotide sequences to those of counterpart bands in pot experiment, suggesting that the primers used in this study were suitable. However, BLAST search indicated that identical sequence of bands P-SyRF19 and F-SyRF7 at the lowest position in Figs. 1 and 3 were 100% similarity with the ITS region of soybean, inferred that those primers are not specific enough for fungal environmental DNA analysis. This finding was not discovered in previous studies, although some bands were sequenced in their researches (Anderson et al. 2003; Bastias et al. 2006).

Many researches on rhizosphere microbial community based on pot or microcosm container experiments (Wieland et al. 2001; Buyer et al. 2002; Marschner et al. 2002, 2004; Singh et al. 2007), only a few studies based on field sampling (Gomes et al. 2001, 2003; Costa et al. 2006). In this study, both pot and field experiments were used to reveal the fungal community in soybean rhizosphere. Although more bands were obtained in DGGE profiles from field experiment than pot experiment of soybean grown in Dark Brown soil, comparison of the two DGGE profiles indicated that the banding patterns of majority bands between the two experiments were similar (Figs. 1 and 3), suggesting that the pot experiment in this study can reflect the fungal community dynamics in field condition. The reasons for more bands in field experiment are not know, one might be the contamination of weeds grown nearby the soybean, in some extent, contributed to increase fungal diversity in soybean rhizosphere.

Principal component analysis in Fig. 2 clearly demonstrated that the fungal communities were separated into Black soil and Dark Brown soil groups, suggesting that the soil type is the major factor to determine the fungal community in the soybean rhizosphere. This finding was consistent with our previous research on bacterial community in soybean rhizosphere (Xu et al., unpublished data), and also consistent with other studies, indicating that the soil type was the most important factor in determining microbial community in the rhizospheres of various plants (Girvan et al. 2003; De Ridder-Duine et al. 2005; Singh et al. 2007). Marschner et al. (2004) reported that many factors contributed to change rhizosphere bacterial community, such as soil pH, nutrition and soil type. As the two soil types have difference soil properties (Table 1), we conclude that the difference of rhizosphere fungal community between two soils is related to soil characteristics.

Relative impact of soil type on microbial communities varies with soil properties such as soil texture and organic matter contents. Given that clayey soils exert a greater influence on microbial communities than sandy soils (Garbeva et al. 2004; Marschner et al. 2004), and the contents of clay is more and sand is less in Black soil than those in Dark Brown soil in this study (Table 1), higher similarity of microbial communities in soybean rhizosphere grown in Black soil than those in Dark Brown soil would be detected. However, this presumption was not observed in this study for analysis of fungal community (Fig. 2), but was detected in bacterial community (Xu et al., unpublished data). Those results inferred that the relative impact strength of soil type on fungal community in soybean rhizosphere was less than on bacterial community. In addition, more DGGE bands in Dark Brown soil than in Black soil suggested that more diversity of fungal communities in Dark Brown soil, although it had relative low organic matter content.

Gomes et al. (2001, 2003) reported that bacterial and fungal abundance in maize rhizosphere increased with growth development, and no relevant differences in TGGE/DGGE banding patterns of both bacterial and fungal communities were observed between two maize cultivars. Similar to their results, in this study, principal component analyses indicated the shifts of soybean rhizosphere fungal communities with growth stages, and the community structures among three soybean genotypes were similar (Figs. 2 and 4), suggesting that the growth stage is the second major factor in shaping fungal communities in the soybean

rhizosphere. However, in contrast to the band abundance observed by Gomes et al. (2001, 2003), a decline tendency of DGGE band number from growth stages R_1 to R_8 was observed in both pot and field experiments (Figs. 1 and 3). This finding suggested that more complex fungal community in soybean early reproductive growth stages than that in later stages. Similar result was also observed in our previous study on bacterial communities (Xu et al., unpublished data). It should be noticed that the primers used in our research were difference with those used by Gomes et al. (2001, 2003), a future examination is necessary to elucidate the diversity of rhizosphere microbial communities between soybean and maize.

The succession of microbial communities with growth stage might be related with two mechanisms. One might be the environmental changes such as temperature and soil moisture with the growth stage (Nazih et al. 2001). However, this mechanism could be minor, because the temperature and water regime were relatively uniform throughout the growth stage in the pot experiment. The other mechanism might be ascribed to the changes in quality and quantity of root exudates/rhizodepositions with the growth stage (Marschner et al. 2002). Although root exudates were not measured in the present study, there are many evidences that root exudates are strongly affected by the growth stage, which in turn can affect rhizosphere microbial communities over time (Yang and Crowley 2000; Duineveld et al. 2001; Garbeva et al. 2004). Thus, the succession of fungal communities in the soybean rhizosphere is concluded due to the change in root exudates or rhizodepositions.

Eighteen DGGE bands were excised from pot experiment and their sequences were positively assigned to fungal ITS regions (Table 2). BLAST search showed that about 61% and 28% of bands belonged to Ascomycete and Basidiomycete, respectively. The fungi belonging to Ascomycete and Basidiomycete dominated in soil environments were also observed by Anderson et al. (2003) in transect ecosystems from moorland to forest and by Bastias et al. (2006) in a prescribed burning sclerophyll forest.

Although the majority of sequenced DGGE bands had high similarity (≥90%) with database sequences, four bands (*i.e.,* P-SyRF1, P-SyRF11, P-SyRF14 and P-SyRF16) had low similarity values (50~82%) suggesting that some unknown fungal members exist in soybean rhizosphere. Gomes et al. (2003) reported that young maize plants seemed to select the Ascomycetes order Pleosporales, and senescent maize seemed to favor different members of the Ascomycetes and Basidiomycetes living in mazie rhizospheres. However, those phenomena were not detected in soybean rhizosphere, and bands P-SyRF3 and P-SyRF5 belonging to Basidiomycetes were evenly observed from soybean growth stages from R_1 to R_8 (Fig. 1). Furthermore, the sequences of bands P-SyRF2, P-SyRF6, P-SyRF7 and P-SyRF10 had 100% similarity with the fungal groups usually isolated from soybean rhizosphere, and some species, such as *Fusarium oxysporum* and *Fusarium solani* were identified as common pathogens to cause soybean root rot in Heilongjiang Province, China (Xin and Ma 1987).

In conclusion, the present study indicated that fungal communities in the soybean rhizosphere were primarily regulated by soil type, and also changed with growth stage. No significant differences of fungal communities among three genotypes were detected from both experiments. More diversity of fungal community observed in Dark Brown soil than in Black soil, and in early reproductive growth stages than in later stages. Sequence analysis of the DGGE bands revealed that fungal belonging to Ascomycetes and Basidiomycetes dominantly inhabited in the soybean rhizosphere.

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