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Changes in soil microbial community structure associated with two types of genetically engineered plants analyzing by PLFA

XUE Kai, LUO Hai-feng, QI Hong-yan*, ZHANG Hong-xun

(Department of Environmental Biotechnology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. E-mail: qihy@mail.rcees.ac.cn)

Abstract: With the rapid expansion of GEPs(genetically engineered plants), people are more and more concerned about the ecological risks brought by their release. Assessing the effect of GEPs on soil microbial ecology is indispensable to study their ecological risks. In our study, the phospholipids fatty acid(PLFA) method was used to analyze the microbial community of soil samples collected from fields with two types of GEPs-Bt transgenic corn and PVY(potato virus Y) cell protein gene transgenic potato. The principal components analysis (PCA) showed all controls were on the right of related GEPs samples along the PC1 (the first principal component) axis, which means a decrease of fungi in soils with genetically engineered crop since most of PLFAs that are strongly positively correlated with PC1 represent fungi. For samples collected from Bt transgenic cornfield, the ratios of gram-positive to gram-negative bacteria were less than those of controls. For samples of transgenic potato field, these ratios were lower than those of controls when soils were collected from deep layer (20—40 cm), but were higher when soils collected from surface layer(0—20 cm). For soils collected from 0—20 cm, the ratios of fungi to bacteria for all GEPs samples were at the same level. So were such rations for all controls. Changes of soil microbial community in two types of GEPs fields were detected in our study, but the causes and more information still needs further study.

Keywords: GEPs; microbial community; PCA; PLFA; GC-MS

Introduction

Genetically engineered plants(GEPs) have been cropped on a large scale so far in many countries, playing a great role in creating economic benefits. At the same time, an increasing attention had also been paid to the ecological risks brought by the release of GEPs(Zhang, 2002).

Plants provide the primary source of energy to life, both above- and belowground; however, most environmental risk assessment studies for GMPs (genetically modified plants) have focused on potential aboveground effects (Kowalchuk, 2003). In fact, GEPs assessment research on soil microbial community is an important aspect of studying the effect of GEPs. Microbial ecology is an indispensable part of the environment. Microorganisms dominate soil-borne communities, accounting for 80% of the total biomass (excluding roots) and largely determine ecosystem functions, such as nutrient cycling and decomposition. Their direct and indirect interactions with plants create strong feedback mechanisms, influencing primary production and vegetation dynamics (Bever, 1997; Van der Putten, 1997). A number of soil microbiological parameters have been suggested as possible indicators of soil quality. A close relationship has been reported between soil fertility and the microbial biomass (Brookes, 1995; Insam, 1991; Pankhurst, 1995). Moreover, microbial diversity(community structure) has also been recommended as a biological indicator of soil quality (Kennedy, 1995).

That the underground component is often neglected in risk assessment of GMPs stems mainly from the inherent difficulties of studying soil-borne microbes (Kowalchuk, 2003). Soil systems are complex and heterogeneous, and we are currently unable to culture most soil microorganisms (Amann, 1995). However, recent methodological advances, including phospholipids fatty acids(PLFA) method, are now helping us to understand these communities. Using PLFA profiles to characterize microbial communities can overcome the limitation of traditional techniques, because it does not require the removal and the culture of the microbes. The PLFA method can be utilized to measure the viable microbial biomass and the community structure in environmental samples, such as sediments and soils, etc. It can provide comprehensive information for detecting the microbial community(Qi, 2003).

In this study, the soils with two types of GEPs-Bt transgenic corn and PVY(potato virus Y) cell protein gene transgenic potato were assessed by PLFA, in order to find out whether changes exist and what the changes are.

1 Materials and methods

1.1 Soils and sampling site

PVY cell protein gene transgenic potato soils (Huhehaote, the Inner Mongolia Autonomous Region, in north China) were taken both from the surface layer(0—20 cm) and the deep layer(20—40 cm) in the field. Bt β toxin protein expression corn soils (Chinese Academy of Agricultural Sciences, Beijing, China) were collected from the surface layer(0—20 cm) in the field. The genetically engineered potato has already been planted for more than four years, while the genetically engineered corn has been planted for less than one year.

The "tgene20" and "tgene40" were used to represent

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PVY cell protein gene transgenic potato soil samples collected from 0—20 cm and 20—40 cm, respectively. The "cgene20" and "cgene40" represented samples collected from 0—20 cm and 20—40 cm in the potato control field, respectively. The "tbt" and "cbt" represented samples collected from 0—20 cm with Bt transgenic corn and the related control, respectively.

1.2 Phospholipid fatty acid(PLFA) analysis

The total lipid was extracted from the soil's organic matter using a single-phase extraction based on the method of Bligh and Dyer(Bligh, 1959) with modifications by White *et al*.(White, 1979). The extraction procedure was similar to the one described by Wilkinson(Wilkinson, 2001). At last, the FAMEs(fatty acid methyl esters) were suspended in 100 μl chloroform: hexane (1∶4 (v/v)) containing 33 μg/ml nonadecanoic acid methyl ester(Sigma Aldrich Co., USA) as an internal standard.

FAMEs were quantified by gas chromatography mass spectrometry. Fatty acid separation was carried out using a Hewlett Packard HP6890 gas chromatograph equipped with a Hewlett Packard hp5 capillary column(60 m × 0.32 mm × 25 (μm film thickness)). The injector was at 230℃ and the oven was held at 50℃ for 1 min after injection. The oven temperature was then increased to 180℃ at 12℃/min and held for 2 min, then increased to 220℃ at 6℃/min and held for 2 min, then increased to 240 at 15℃/min and held for 1 min. Finally, the oven temperature was increased to 260℃ at 15℃/min and held for 15 min. The transfer line was held at 280℃ throughout. Helium was used as the carrier gas(1 ml/min). FAMEs were identified by retention time and/or electron ionization mass spectrometry (Hewlett Packard HP5973 Mass Selective Detector) with reference to standards. Electron energy in electron impact was 70 eV.

1.3 Fatty acid nomenclature

Fatty acids are designated in terms of total number of carbon atoms, with the number of double bonds given after a colon. The position of the double bond is defined by the symbol ω followed by the number of carbons from the methyl end of the fatty acid molecule. *Cis* and *trans* configurations are indicated by *c* and *t*; the *i* and *a* refer to *iso* and *ante-iso* branching; *br* indicates an unknown branch position; and *cy* refers to cyclopropyl fatty acids. 10Me indicates a methyl group on the 10th carbon atom from the carboxyl end of the molecule.

1.4 Statistics

PLFA data were analyzed using principal components analysis (PCA), employing varimax rotation. This was performed on log10 transformed mole percentages of individual PLFAs. PCA scores were subsequently analyzed by analysis of variance(ANOVA) with correlation matrix using SPSS Version 10.0. Cluster analysis was performed by SPSS software too.

2 Results and discussion

2.1 PLFAs profiles

All soils contained a variety of PLFAs composed of saturated, unsaturated, methyl-branched and cyclopropane fatty acids. As shown in Table 1, 23 PLFAs with a chain length from C_{14} up to C_{19}(internal standard) were detected by GC-MS. Most of them were identified on the basis of comparison with mass spectra obtained from standards, database or previously reported in the literature. But a number of peaks, designated 18∶2ωa to 18∶2ωf, could not be completely identified but regarded as fatty acids whose total number of carbon atoms is 18 and the number of double bonds is 2 because of the presence of several ions in their mass spectra characteristic of methyl esters of fatty acids.

2.2 PCA analysis

Data for PLFA concentrations (as log10 mol percentages) were subjected to principal components analysis. The first two principal components explained 63.75% and 19.37% of the variation in PLFA patterns, respectively.

The PLFA changes were analyzed by the PCA loading plot of samples (Fig. 1). Specific identified PLFAs, including 14∶0, 18∶0, the branched a17∶0, a18∶0, and the monounsaturated 18∶1ω10, 18∶2ω6, 18∶2ω7, as well as the unidentified PLFAs 18∶2ωa to 18∶2ωf(with unknown double bond position), were found on the right in the plot. The PLFAs 16∶0, the branched a15∶0, a16∶0, i5∶0, i17∶0, the cyclopropane fatty acid cy17∶0, cy19∶0, and the monounsaturated 16∶1ω7, 16∶1ω9 were found on the left in the plot.

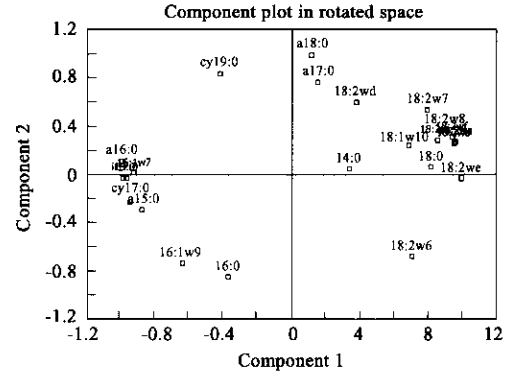

Fig. 1 PCA showing loading values for individual PLFAs

PC loadings showed that 18∶0, 18∶1ω10, 18∶2ω6, 18∶2ω7, 18∶2ωa, 18∶2ωb, 18∶2ωc, 18∶2ωe, 18∶2ωf were strongly positively correlated(i.e., loading 0.6 or greater) with PC1(the first principal component). Except 18∶0, all these PLFAs were monounsaturated, whether specific identified monounsaturated PLFAs or unidentified PLFAs with unknown double bond position. These monounsaturated PLFAs are considered to be typical for fungi other than 18∶1ω10 represents gram-negative bacteria. On the contrary,

a15:0, a16:0, i5:0, i17:0, cy17:0 and 16:1ω7 were strongly negatively correlated(i.e., loading −0.8 or less) with PC1. All these PLFAs are common in bacteria. Along PC2(the second principal component) axis, cy19:0, a18:0 and a17:0, were strongly positively correlated(i.e., loading 0.7 or greater) with PC2, while 16:0, 16:1ω9 and 18:2ω6 were strongly negatively correlated(i.e., loading −0.6 or less) with PC2. No PLFA was close to, or on, the origin in the plot.

Table 1 PLFA concentrations(mean mole percentages) in soil samples

Peak No.	PLFA	Proportion of PLFA, mol%					
		cgene20	tgene20	cgene40	tgene40	cbt20	tbt20
1	14:00	1.54(0.41)	1.86(0.23)	1.92(0.28)	2.29(0.14)	1.62(0.24)	1.85(0.14)
2	i15:0	7.69(0.81)	8.81(0.70)	4.12(0.51)	6.02(0.63)	8.84(0.64)	10.00(0.97)
3	a15:0	5.92(0.60)	6.38(0.35)	4.97(0.58)	5.80(0.48)	5.53(0.39)	6.45(0.41)
4	a16:0	2.08(0.59)	2.30(0.34)	1.42(0.21)	1.85(0.21)	2.35(0.34)	2.87(0.34)
5	16:1ω9	5.14(1.18)	5.79(0.38)	3.38(0.52)	4.06(0.27)	4.10(0.29)	4.36(0.46)
6	16:1ω7	3.20(0.87)	3.79(0.19)	2.09(0.65)	3.44(0.25)	3.76(0.35)	4.11(0.21)
7	16:00	14.19(1.72)	18.16(0.89)	11.81(1.14)	14.48(0.69)	12.88(0.54)	12.85(0.65)
8	i17:0	7.62(2.31)	7.49(0.73)	3.67(2.15)	5.65(0.97)	7.50(1.01)	9.02(2.01)
9	a17:0	3.16(0.49)	3.20(0.19)	3.55(0.60)	3.69(0.42)	3.42(0.08)	3.70(0.15)
10	cy17:0	2.27(0.69)	2.37(0.13)	1.39(0.19)	1.65(0.18)	2.13(0.14)	2.40(0.40)
11	18:2ωa	2.08(0.74)	1.66(0.40)	3.27(0.70)	2.76(0.38)	2.12(0.62)	1.70(0.37)
12	a18:0	1.95(0.21)	1.40(0.25)	2.55(0.32)	2.43(0.51)	2.64(0.27)	2.92(0.30)
13	18:2ω8	1.58(0.79)	1.10(0.11)	2.28(0.35)	1.94(0.14)	1.45(0.29)	1.30(0.08)
14	18:2ω7	2.74(1.22)	1.82(0.07)	3.36(0.63)	3.09(0.71)	2.77(0.51)	2.25(0.45)
15	18:2ω6	4.53(1.16)	4.63(0.93)	4.92(1.03)	4.17(0.95)	3.82(0.50)	3.56(0.68)
16	18:1ω10	8.66(0.37)	8.01(0.26)	13.68(2.10)	7.81(0.71)	8.93(0.47)	8.78(0.51)
17	18:2ωb	4.39(1.88)	3.04(0.50)	6.37(0.58)	4.89(0.51)	4.22(0.60)	3.09(0.83)
18	18:2ωc	3.50(0.96)	2.64(0.51)	4.11(0.42)	3.41(0.76)	3.59(0.57)	2.63(0.99)
19	18:00	7.01(0.92)	7.00(0.27)	7.90(1.51)	8.48(0.29)	7.24(0.65)	6.53(0.76)
20	18:2ωd	3.19(1.18)	2.40(0.41)	2.99(0.86)	3.64(0.78)	3.25(0.48)	2.87(0.64)
21	18:2ωe	2.92(0.25)	2.25(0.54)	4.69(1.19)	3.59(0.39)	2.39(0.57)	1.78(0.60)
22	18:2ωf	1.36(0.28)	0.94(0.21)	2.24(0.49)	1.75(0.11)	1.47(0.26)	0.96(0.22)
23	cy19:0	3.27(0.54)	2.95(0.37)	3.32(0.52)	3.14(0.84)	3.97(0.89)	4.04(0.40)

Notes: standard error ($n = 4$) shown in parenthese

In Fig. 2, the PCA of soils showed that points representing tgene40 and cgene40 were in the first quadrant of the plot, indicating the PLFAs positively correlated with PC1 were present in higher proportions for these samples. The points representing tbt and cbt were in the second quadrant, tgene20 and cgen20 were in the third quadrant. For tbt, cbt and tgene20, the PLFAs negatively correlated with PC1 were present in higher proportions. The cgene20 point was very close to the origin of PC1 axis, indicating that there was no significant difference between the PLFAs positively correlated and negatively correlated with PC1 in this sample. Meanwhile, the tbt, tgene40 and their related control samples were found to the upper in the plot, which means the PLFAs positively correlated with PC2, especially cy19:0, a18:0 and a17:0, were present in higher proportions for tbt, tgene40 and their controls. The tgene20 and cgene20 samples were found to the nether in the plot, suggesting a corresponding increase in the proportions of the PLFAs that was negative correlated with PC2, especially16:0, 16:1ω9 and 18:2ω6.

PCA analysis showed GEPs samples appeared to differ from the related control and the changes brought by GEPs to soil microbial community had the same trend to a certain extent. All control samples were on the right of the related

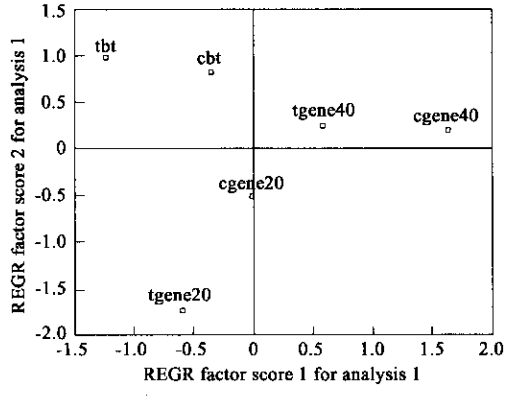

Fig. 2 PCA showing variations in PLFA pattern in different soils

GEPs samples and certainly had higher PC1 scores, indicating the proportions of PLFAs positively correlated with PC1 were higher in controls than in GEPs samples. Because most of PLFAs strongly positively correlated with PC1 were typical for fungi, there was a decrease of fungi in soils with genetically engineered crop. While the PLFAs negatively correlated with PC1, all representing bacteria, increased in GEPs samples.

The causes of such changes may be very complicated. It may result from the release of toxin protein to soils, thus affecting the microbial community. Some scientists found that

the Bt (*Bacillus thuringiensis*) toxin was bound to surface-active particles, which is probably responsible for their persistence in soil (Saxena, 1999). This indicates GEPs probably have considerable effects on non-target organisms in soil, including microbes, directly or indirectly. The HGT (horizontal gene transfer) may be another probable explanation. The potential for HGT of traits introduced into GEPs has received widespread scientific and public attention (Hails, 2000). Except out-crossing of introduced genes to related non-GEPs, there is also the possibility that genetic material can be transferred to microorganisms, although plant-microbe HGT events occur at extremely low levels. Transgenes of microbial origin could be present in the soil and the selective pressure that acts on transformed microbial populations might result in the shift of PLFA pattern. Some other factors should also be considered. Soil microbial communities exist within complex soil food webs that include other important players, such as nematodes, collembola, mites, earthworks, and so on (Kowalchuk, 2003). Soil-borne organisms also respond to the quantity and quality of plant litter(Priha, 1999). These disturbing reasons may lead to the shift too. In a word, the reasons of the increase of bacteria and the decrease of fungi brought by two types of GEPs still need further study.

Along the axis of PC2, tgene40 and tbt samples approximately had the same PC2 values as their controls. The cgene20 was on the upper of tgene20, which indicated PFLAs positively correlated with PC2 were present in higher proportions of cgene20 and PLFAs negatively correlated with PC2 increased in tgene20.

For soils with PYV cell protein gene transgenic potato and the controls, samples collected from 20—40 cm were found to the right and upper in contrast to samples collected from 0—20 cm. This meant samples with higher depth had higher proportions of PFLAs strongly positively correlated with PC1, most of which were typical for fungi, and PFLAs positively correlated with PC2, especially cy19:0, a18:0 and a17:0. However, it could not be concluded that PC1 and PC2 scores were higher in deeper soils for the samples that could be compared for different depth in our study were only two groups.

2.3 Ratios of fungi to bacteria and gram-positive to gram negative bacteria

The ratios of PLFA amount (in moles percent) between the GEPs and control samples are shown in Table 2. The result was consistent with the analysis of PCA. For example, the ratios at 18:1ω10, 18:2ω7, 18:2ω8, 18:2ωa, 18:2ωb, 18:2ωc, 18:2ωe, 18:2ωf were less than 1. All these PLFAs were strongly positively correlated with PC1.

The ratios of fungi to bacteria and the ratios of gram-positive to gram-negative were calculated according to the literatures (Frostegård, 1996; Wilkinson, 1988). The unidentified PLFAs 18:2ωa to 18:2ωf (with unknown double bond position) were regarded as lipid markers for fungi.

Table 2 Ratios of PLFA amount between GEPs and control samples

Peak No.	PLFA	Proportion of PLFA, mol%		
		tgene20/cgene20	tgene40/cgene40	tbt20/cbt20
1	14:00	1.21	1.19	1.14
2	i15:0	1.15	1.46	1.13
3	a15:0	1.07	1.17	1.17
4	a16:0	1.11	1.30	1.22
5	16:1ω9	1.13	1.20	1.06
6	16:1ω7	1.18	1.64	1.09
7	16:00	1.28	1.23	1.00
8	i17:0	0.98	1.54	1.20
9	a17:0	1.01	1.04	1.08
10	cy17:0	1.04	1.18	1.13
11	18:2ωa	0.80	0.84	0.80
12	a18:0	0.72	0.96	1.10
13	18:2ω8	0.69	0.85	0.90
14	18:2ω7	0.66	0.92	0.81
15	18:2ω6	1.02	0.85	0.93
16	18:1ω10	0.93	0.57	0.98
17	18:2ωb	0.69	0.77	0.73
18	18:2ωc	0.76	0.83	0.73
19	18:00	1.00	1.07	0.90
20	18:2ωd	0.75	1.22	0.88
21	18:2ωe	0.77	0.77	0.74
22	18:2ωf	0.69	0.78	0.65
23	cy19:0	0.90	0.94	1.02

As shown in Fig.3, the proportions of fungi in GEPs soils were less than in related controls. The result also was consistent with the analysis of PCA. The samples collected from the depth of 20—40 cm had a relative higher proportion of fungi in contrast to soils from the depth of 0—20 cm. For samples collected from 0—20 cm, tbt and tgene20 samples, which were all GEPs, had very similar ratio of fungi to bacteria, the same as cbt and cgene20, which were both control samples.

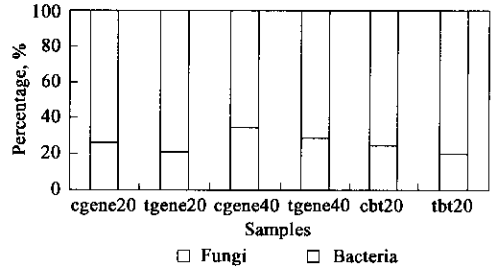

Fig.3 Ratios of fungi to bacteria in different samples

In Fig.4, the ratios of gram-positive to gram-negative in samples collected from 20—40 cm were higher than samples from 0—20 cm. There was no accordant trend of ratios' change for GEPs and related controls. For tbt and tgene40, the ratios of gram-positive to gram-negative were less than their related controls', though the difference between cbt and tbt was small. The ratio for tgene20 was greater than the ratio for cgene20.

3 Conclusions

Changes of soil microbial community brought by two types of GEPs were detected in our study. There existed an

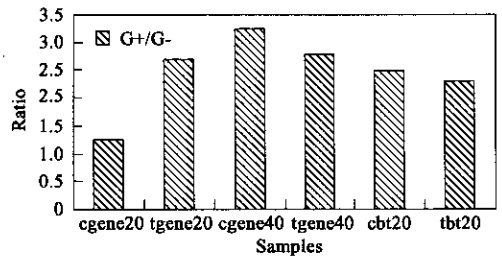

Fig.4 Ratios of gram-positive to gram-negative in different samples

increase of bacteria and a decrease of fungi in GEPs samples. For the ratio of gram-positive to gram-negative, there was no accordant trend of change in GEPs samples and related controls. The causes of these changes and more information still needs further study.

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Introduction to author's research group—The Group of Microbial Ecology

The group of microbial ecology is a part of The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. The mostly research scope of the group is microbial ecology and molecular biotechnology.

Microbial ecology is an interdisciplinary science that promotes an understanding of microbial interactions among members of microbial communities as well as the interactions of the microbial community with its environment. New concepts of microbial communications between microorganisms as well as with higher organisms have recently emerged.

The group has successfully accomplished the National Key Scientific and Technological Project "Study of new technology of ecology evaluation to soil resource of country" in 2004. Both of PCR-DGGE and PLFA, analytical techniques of microbe diversity, were established. The results showed visible differences from cultivated methods and give more information of microorganism diversity variety in soil samples.

A new research item running by The National Natural Science Foundation of China will be implemented immediately in the group to study bacteria based biosensor technology to test and assay heavy metal polluted soil environment from 2005 to 2007.

It is very necessary to develop a method to detect heavy metal pollution rapidly and accurately for pollution treatment.

In this area, the greatest constraint in China appears to be the lack of modern and advanced analytical techniques, although these techniques have already been widely applied in the world. For instance, bacteria based biosensor technology is a powerful alternative to conventional analytical techniques, harnessing the specificity and sensitivity of biological systems in small, low cost devices. However, its application in China is very limited.

In this new project, the molecular biological methods will be used, such as soil total DNA extraction, 16SrRNA gene sequencing, DGGE and DNA library, to test the bacterial community in heavy metal polluted soil and try to find some relationships between heavy metals and microbe. The research will supply a gap of concerning a specific microbial sensor to detect certain heavy metals in soils.

Address: Associate Prof. Hongyan QI, Prof. Hongxun ZHANG, The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, P.O. Box 2871, Beijing 100085, Tel. & Fax: +86-10-62849156, 62849819, E-mail: qihy@mail.rcees.ac.cn