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Technical Review

Soil microbial community analysis in the environmental risk assessment of transgenic plants

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Abstract Soil microbial community analysis is one of the most important elements in the environmental risk assessment of transgenic plants. Recent technical advances in this area now enable us to assess the impact of plant genotypes on soil microbial communities with rapid, simple and less biased molecular techniques than the previously used conventional microbiological methodologies. We review the use of these modern molecular techniques from the aspect of environmental assessments of transgenic plants.

Key words: Environmental risk assessment, plant-microbe interaction, rhizosphere, soil microbial community.

Soil microbes play important roles in various aspects of the terrestrial ecosystem, such as soil fertility during plant growth, including agronomic activities, (O'Donnell et al. 2001) and in the biogeochemistry of the cycling of organic and non organic compounds throughout entire natural ecosystems (Molin and Molin 1997; Trevors 1998; Wall and Virginia 1999). It is well known that soil microorganisms that colonize the rhizosphere assist plants in the uptake of several vital nutrients, such as phosphorous, potassium and nitrogen, from the soil (George et al. 1995; Timonen et al. 1996; Trolove et al. 2003; Cocking 2003). Soil microbes can also exert considerable influence upon the status of a plant's health (Smith and Goodman 1999). Recently, a role for root-associated microbes has been reported, whereby these organisms maintain soil composition by identifying a glycoprotein from field and forest soils (Sen 2003). Consequently, the productivity and sustainability of agricultural systems are deeply dependent upon the functional processes of the microbial communities in soil (Doran and Zeiss 2000).

It is also well known that plants have significant influence on the diversity, spatial distribution and abundance of soil microbes through the rhizospheres (Wall and Virginia 1999). Thus, the cultivation of transgenic plants could have an influence upon soil microbes through rhizospheres and plant residues, over both the short and long term. Consequently, transgenic plant technology could have the potential to significantly alter microbial community structures. However, until recently, the analysis of soil microbe communities has been considered to be one of the most difficult challenges for the risk assessment of transgenic plants. This is due to the extreme complexity of the physical, chemical and biological properties of soil, and is also due to the lack of appropriate methodologies to analyze microbial diversity in soil.

Conventional environmental risk assessments of transgenic plants upon soil microbial communities in the past have mainly been based on plate counts, using selective media for specific groups of soil microorganisms such as fungi and bacteria. These culture-dependent methods have provided useful information for evaluating microbial diversity in various environmental samples, including the risk assessment of transgenic plants. However, it also has been shown that these conventional methods are limited by strong inherent biases that are caused by medium selection and the cultivation conditions. Moreover, a significant disadvantage of these techniques is the inability to culture most of the soil microbes that are found in the natural microbial community (Prosser 2002). Torsvik et al. (1998) have in fact suggested that as little as 1% of the soil bacterial population can be cultured using standard laboratory procedures. Furthermore, this small percentage of culturable organisms is highly unlikely to be representative of the entire bacterial population in soil as new taxonomic groups have been continuously

reported via sequence analyses of non-culturable microbes (Rondon et al. 1999, 2000). Similarly, the majority of microorganisms that inhabit rhizospheres have not been successfully cultured (Kent and Triplett, 2002).

In order to address these limitations, culture-independent methods, such as molecular biological techniques that target ribosomal RNA gene regions, have been introduced for the assessment of the structural and functional diversity of soil microbial communities (Head et al. 1998; O'Donnell and Gorres 1999). Most of these DNA- and RNA-based methods consist of nucleic acid extraction from the soil matrix and the subsequent profiling of microbial communities by PCR. This is considered to be a more reliable procedure due to the high stability of the genome when compared to the relatively high fluctuations in the profiles of physiological markers such as fatty acids (Lechevaliar 1977; Minnikin et al. 1984). Moreover, nucleic acid based techniques are expected to provide more sensitive and less biased information for soil microbial communities.

In the present review, we summarize the published reports of microbial community analyses using culture-independent methods and discuss the current status and the issues associated with risk assessments of transgenic plants in soil environments.

Field management

Prior to or during the environmental assessments, particular care needs to be taken with field management practices, since differences in soil management procedures, such as the application of fertilizers and pesticides, have been shown to cause structural changes in soil microbial communities (Singh et al. 2003; Girvan et al. 2004; Saeki and Toyota 2004) (Figure 1). In the case of some transgenic plants that are being used to test for pest or disease resistance, careful consideration of the chemical controls should be taken in order not to create a bias in the microbial community analyses. In addition to plant and soil effects, infection with arbuscular mycorrhiza fungi (AMF) has also been shown to influence the rhizosphere microbial community composition (Marschner and Baumann 2003). Since the effects on microbial communities could be due to differences in the intensity of AMF infections in different sampling locations for a field, efforts should also be made to maintain the homogeneity of the test field as much as possible.

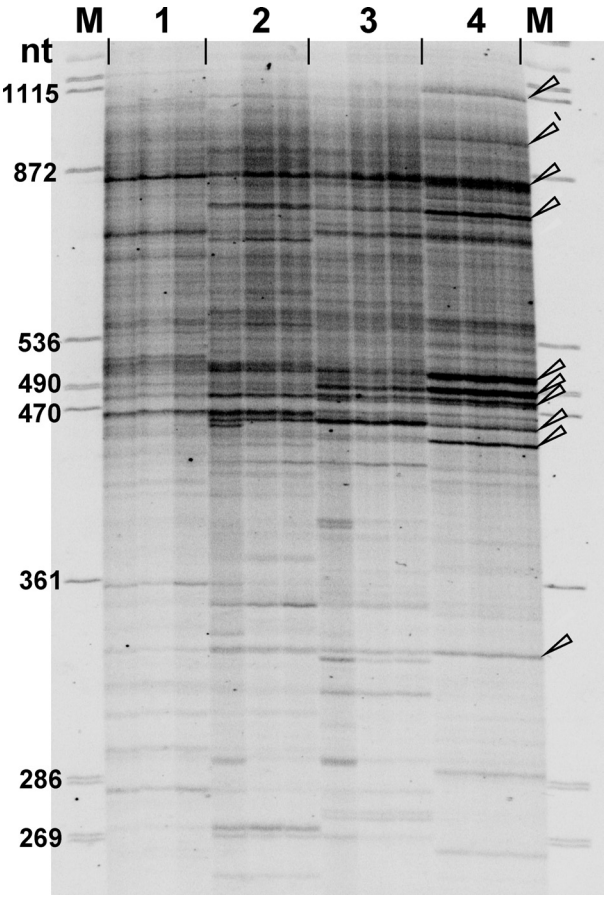

Figure 1. The effects of field management on soil microbial community structures assessed by RISA. Lanes: M, GeneScan-2500 ROX molecular size markers (Applied Biosystems); 1, no treatment with cattle slurry; 2, fertilization with cattle slurry (60 t/ha/year); 3, fertilization with cattle slurry (150 t/ha/year); 4, fertilization with cattle slurry (300 t/ha/year). Triplicate results for each fertilization treatment are shown. Arrows indicate possible DNA bands that are responsive to the increase in slurry treatments.

Sampling scale and spatial heterogeneity of bulk soils

The sampling of bulk (non-rhizospheric) soils requires a number of cautions, one of which is the sampling scale. In recently reported molecular ecological analyses of soil environments, samples of less than a few grams are often used in the evaluation of microbial diversity. Microbial communities can exist on such a small scale, but these sample sizes could conceivably bias the results and generate unrealistic conclusions. It has also been reported that there are "microbiological hot spots in soils", which are distinct microhabitats with high levels of biological activity including aggregates, rhizospheres and water flow pathways (Sextone et al. 1985; Joergensen 2000; Bundt et al. 2001; Kirk et al. 2004). In addition, considerable spatial variability of the vertical distribution of soil microorganisms exists due to the presence of a surface gradient for several environmental factors, including oxygen availability and various nutrients (Franklin and Mills 2003). In order to control for these variations in a given sampling site, it is advisable that the multiple soil samples should be collected rather than increasing an individual sampling volume (Grundmann and Gourbiere 1999). In addition,

collected soil samples should be mixed thoroughly, and debris such as plant residues must be removed by sieving (a 2 mm diameter sieve is described in most reports) and by manual inspection. In general, sample variability is greater for fungal communities than for the bacterial community (Girvan et al. 2004).

Transportation and storage conditions

Environmental factors such as repeated freezing and thawing, rapid changes in moisture, and natural daylight can cause cell lysis of microbes with fragile cell structures. Hence, the transportation and storage conditions of the soil samples could alter the microbial community structures in a few hours without the use of appropriate safeguards. Both transportation and storage conditions should therefore be carefully regulated to minimize any possible impact upon subsequent analyses. In our laboratory for example, soil samples are rapidly transported under gentle conditions (with shading at 4°C in a sealed container) and processed immediately after arrival or stored at −80°C. It is noteworthy however, that microbial community structures have also been shown to change even when soil specimens are previously frozen (Pesaro et al. 2003) or stored at ambient temperature (Anderson 1987).

Sampling of rhizospheric soils

The evaluation of the impact of transgenic plants on soil microbial community analysis requires the sampling of rhizospheric soils. The rhizosphere is defined as the soil environment directly under the influence of living roots (Kent and Triplett 2002). The effects of the rhizosphere can usually be observed by fingerprinting analyses, as indicated by profiles that contain fewer bands of greater intensity than the patterns found in bulk soils (Figure 2). Since the degree of influence of the roots upon neighboring soil environments could be affected by several factors such as plant species, plant aging and soil properties, the physical definition of rhizospheric soils is in fact extremely difficult. For practical purposes therefore, two methods are usually employed. The first of these is the recovery of adherent soil from the roots by agitation (shaking) of root systems that have been decomposed carefully from the ground in air or water. This method can minimize contamination by plant tissues which could affect the results of microbial community analysis. However, the quality and quantity of the recovered rhizospheric soils could also be greatly affected by the handling procedures and by other factors such as the properties of both the soil and root systems. These concerns should be taken into consideration during any biological evaluations of the rhizosphere. Moreover, bulk soil could be carried over into rhizospheric soil samples and thereby mask the impact of the plant genotypes in question upon the microbial communities.

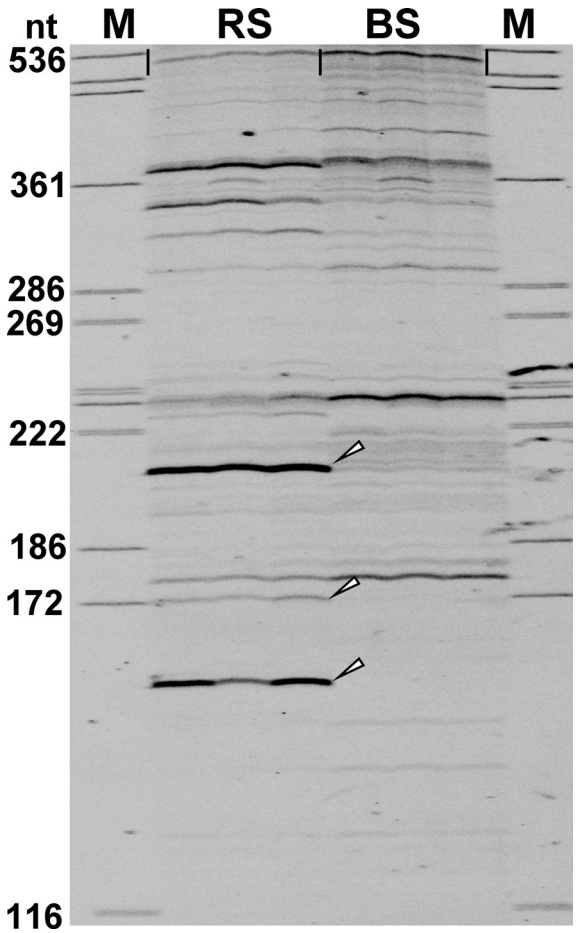

Figure 2. Comparison of soil microbial community structures between rhizospheric and bulk soils, revealed by T-RFLP. Lanes: M, GeneScan-2500 ROX molecular size markers (Applied Biosystems); RS, rhizospheric soils; BS, bulk soils. Soils were obtained from cultures of *Lotus corniculatus*. Triplicate samples for each soil are shown. Arrows indicate rhizosphere specific DNA bands.

A second established method for rhizospheric soil sampling is the direct use of root systems with tightly adherent soils for DNA extraction, without the separation of plant tissues. In this case, there are potential problems with possible contamination by excess plant DNA for analyses involving PCR. However, the appropriate choice of PCR primers and careful technique could either circumvent or minimize this potential bias. Furthermore, this sampling procedure recovers microbial DNA from the rhizoplane region and retains less bulk (non-rhizospheric) soils when compared to the first method. As a result of this, the microbial DNA composition that is sampled could be expected to provide a better reflection of the impact of the plant genotype under study. However, since it is practically impossible to extract microbial DNA directly from the entire root systems of most field crops, several root tips are usually collected for DNA extraction. A potential bias that can be introduced by this procedure, however, derives from

Table 1. Common procedures employed in soil DNA extraction

Procedures and reagents	Advantages	Disadvantages	References[6]
Cell lysis			
Bead-beating	rapid and simple	harsh condition for some microbial groups	(1)
Ultrasonic treatment	rapid and simple	harsh condition for some microbial groups	(2)
Freezing and thawing	simple and low cost	bias of cell lysis for some microbes groups	(3)
Enzymatic treatments	simple and gentle condition	bias of cell lysis for some microbes groups	(4)
Buffer			
Tris	low cost	high variability for DNA yield	(5)
SPB[1]	low cost	contamination	(6)
Tris-SPB mixture	low cost	need for mixing of two reagents, contamination	(7)
Guanidium isothiocyanate	high stability of DNA	high cost and hazardousness	(8)
CTAB	high quality of DNA	high cost and high variability for DNA yield	(9)
Additives			
SDS	low cost	deposition at low temperature	(10)
Sodium sarcosinate	no deposition at low temperature	high cost	(11)
EDTA	high stability of DNA	co-extraction of humic acids	(12)
RNA	high DNA yield	high cost, need of RNase treatment	(13)
Skim milk	low cost	contamination	(14)
Commercial kits			
FastDNA SPIN Kit for Soil[2]	rapid, simple and stable	high cost	(15)
ISOL for beads Beating[3]	low cost	high variability for DNA yield	(16)
The SoilMaster DNA Extraction Kit[4]	low cost	high variability for DNA yield and purity	(17)
Ultra Clean Soil DNA Isolation Kit[5]	low cost	high variability for DNA yield and purity	(18)

[1] Sodium Phosphate Buffer, [2] Qbiogene, [3] Nippon Gene, [4] Epicentre, [5] MoBio Inc., [6] (1) Bürgmann et al. 2001; (2) Krsek and Wellington 1999; (3) Zhou et al. 1996; (4) Krsek and Wellington 1999; (5) Berthelet et al. 1996; (6) Holben et al. 1988; (7) Miller et al. 1999; (8) Porteous et al. 1997; (9) Cho et al. 1996; (10) Ogram et al. 1987; (11) Holben et al. 1988; (12) Krsek and Wellington 1999; (13) Frostegård et al. 1999; (14) Kageyama et al. 2003; (15) Martin-Laurent et al. 2001; (16) Tsuda 2005; (17) Hügler et al. 2005; (18) Martin-Laurent et al. 2001.

the reported shift in the profile of microbial community structures from the root tip to the basal area within the same root system (Yang Crowley 2000). Another caveat of this method, with regard to the environmental assessment of transgenic plants, is a growth stage of the plants at the time of sampling. In particular, the senescence of root systems will occur extensively during the reproductive phase and this may increase the variability of the data being analyzed (Baudoin et al. 2002).

At the present time, both of these principal methods for the sampling of rhizospheric soils should be considered for full assessments of the impact of transgenic plants on the rhizospheric microbial communities. There may be significant ecological roles for the microbes loosely related to root systems. Additionally, it is also advisable to perform continuous assessments not only during the cultivation of transgenic plants, but also after their cultivation in order to evaluate the persistence of any impact of these plants over a long period (Smalla et al. 2001).

Nucleic acid extraction from soil

To date, there are several reports describing methods for nucleic acid extraction from soil. However, the recovery of DNA/RNA from soil has not yet become a routinely practiced work, and often requires refinement of the extraction and purification conditions due to the extreme diversity of the physical, chemical and biological properties of soils. It is also well recognized that the methods employed for DNA or RNA extraction can also bias the subsequent results of microbial community analyses, in terms of both qualitative and quantitative interpretation of data (Maarit-Niemi et al. 2001).

Whereas RNA based examination of microbial communities may provide a better reflection of the naturally occurring profiles, the expression of RNA transcripts may be too sensitive to environmental changes, such as those that occur during the extraction process to obtain reliable data. In addition, RNA molecules are extremely labile both *in vitro* and *in vivo* and may not be an appropriate marker for evaluating environmental impacts. Consequently, soil DNA is analyzed in most recent reports of risk assessments of transgenic plants, due to the lack of reliable methodologies for soil RNA extraction. We therefore mainly focus on soil DNA extraction procedures in the present review (Table 1).

Cell lysis

The cell lysis techniques employed in soil DNA extraction can be generally divided into physical and chemical procedures. Among these methods, treatment by bead-beating has been reported as the most reliable and efficient procedure for cell lysis (Bürgmann et al. 2001). Both the diameter and the number of beads need to be optimized for the target organisms. In general, bead diameters of 0.1 and 2 mm are used in this method to lyse bacterial and fungal cells, respectively. Zirconium beads are the most effective to use, although are more

Table 2. Reagents employed in soil DNA purification

Reagents	Advantages	Disadvantages	References
Gene Clean Spin-kit[1]	simple, rapid	high cost	(1)
Wizard total DNA cleanup system[2]	simple, rapid	high cost	(2)
Gel extraction	high purity of DNA	labor intensive	(3)
Phenol/Chloroform	simple, low cost	hazardousness	(4)
PEG600/PEG8000	high purity of DNA	high variability of DNA yield	(5)
PVP	simple, rapid	low purity of DNA	(6)
PVPP	simple, rapid	low purity of DNA	(7)
Potassium acetate	simple, rapid, low cost	low purity of DNA	(8)
Sephadex/Sepharose	simple, rapid	low purity of DNA	(9)
Microcon YM-100	simple, rapid	low purity of DNA, high cost	(10)
DEAE-cellulose	simple, low cost, high purity of DNA	labor intensive	(11)

[1] Qbiogene, [2] Promega, [3] (1) Smalla et al. 1993; (2) Schneegurt et al. 2003; (3) Zhou et al. 1996; (4) Steffan et al. 1988; (5) Cullen and Hirsch 1998; (6) Boivin-Jahns et al. 1996; (7) Holben et al. 1988; (8) Smalla et al. 1993; (9) Miller 2001; (10) Berthelet et al. 1996; (11) Schneegurt et al. 2003.

expensive than their glass alternatives. However, zirconium beads provide a better efficiency of cell lysis, under conditions that are limited by the number of beads that can be used and the processing time, because of their higher density than glass beads.

Since the lysis efficiency of microbial cells can vary considerably, both among and even within the same taxonomic groups, depending on the physiological and developmental status of these organisms (Prosser 2002), cell lysis conditions that have been optimized for some microbial groups may be too harsh for other groups which have more fragile cell structures. Hence, extraction via bead beating can cause excess shearing of nucleic acids, which has been highlighted previously as a potential problem for PCR amplification due to mis-priming (Wintzingerode et al. 1997). Therefore, in addition to bead-beating treatments, gentle cell lysis procedures such as enzymatic treatments and freeze/thaw protocols should also be undertaken during full environmental assessments. However, these milder procedures may not be as effective for DNA extractions from certain soils, such as some types of Andosol in Japan, and we have observed that such treatments can result in a poorer quality and quantity of the recovered soil DNA (Ikeda et al. unpublished data).

DNA extraction Buffers and additives

A number of different buffer systems have been employed for soil DNA extraction. As a result of extensive testing in our laboratory, however, we have concluded that a mixture of Tris and sodium phosphate gives the most stable and satisfactory results when recovering soil DNA from most arable lands in Japan (Ikeda et al. 2004b). The presence of detergents in extraction buffers is essential for recovering soil DNA, but they should be ionic in composition, such as SDS or sodium sarcocinate. Moreover, the presence of EDTA in the extraction buffer is also essential for the stable extraction of soil DNA from Japanese arable lands. In addition, other additives such as skim milk and RNA are often required for Japanese samples due to DNA adsorption by the soil matrix in some soil types (Kageyama et al. 2003; Ikeda et al. 2004b). We recommend skim milk due to the ease with which it can be removed during the purification steps.

Purification

With environmental samples, it is necessary to employ careful purification strategies, since contaminants in DNA samples can disrupt various enzymatic reactions, including PCR amplification. However, several purification steps can also lead to a decrease in the DNA yield. This may cause problems during the analysis of soil DNA, since the high degree of heterogeneity of DNA molecules in soil may result in a bias against rare sequences when amplifying from DNA preparations of lower yield. Therefore, purification of soil DNA isolates should be designed to be as effective as possible in a minimal number of steps.

Extraction with organic solvents is a common technique for the purification of nucleic acid. However, we have found that the extraction of DNA from some soil samples with either phenol or phenol-chloroform can cause deleterious effects upon both the quality and quantity of the final product. We assessed most of the previously reported procedures (Table 2) and determined that a combination of salting out and DEAE cellulose column treatments was the most effective method for obtaining the highest purity and yield of soil DNA. This protocol is in fact comparable or superior to commercially available soil DNA extraction kits (Ikeda et al. 2004b).

Commercially available kits for extracting DNA from soil

Several commercial kits for soil DNA extraction are now available, among them, the FastDNA SPIN Kit for Soil (Qbiogene, Inc., Irvine, CA) has been widely used in a

Table 3. PCR primers employed in microbial community analysis in soils

Method	Organism	Primer name (Forward/Reverse)	References[2]
DGGE	Archaea	344f-GC/915r	(1)
DGGE	Bacteria	F341-GC/R518	(2)
DGGE	Bacteria	338f/518r	(3)
DGGE	Actinomycetes	F243/R513-GC	(4)
DGGE	*Burkholderia*	Burk3/BurkR	(5)
DGGE	*Pseudomonas*	8f-GC1/PSMG	(6)
DGGE	Eukarya	1Af/516r-GC	(7)
DGGE	Fungi	EF4f/Fung5r	(8)
DGGE	Fungi	NS1/FR1-GC	(9)
DGGE	Nematode	NEMF1/S3	(10)
SSCP	Archaea	133FN6F/248R5P	(11)
SSCP	Bacteria	Com1/Com2	(12)
T-RFLP	Archaea	Ar109f/Ar912r	(13)
T-RFLP	Bacteria	8f/1406R	(14)
T-RFLP	Bacteria	8-27f/1507-1492r	(15)
T-RFLP	Eukarya	1Af/516r	(16)
T-RFLP	Fungi	nu-SSU-0817-5′/nu-SSU-1536-3′	(17)
RISA	Archaea	915f/71r	(18)
RISA	Bacteria	S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18	(19)
RISA	Bacteria	ITSF/ITSReub	(20)
RISA	Fungi	ITS 1F/ITS 4	(21)
RISA	Fungi	1406f/3126T	(22)
RISA	Ascomycetes	ITS 1F/ITS 4A	(23)
RISA	Basidiomycetes	ITS 1F/ITS 4B	(24)

[1] The addition of GC clamp to the 5′ end of the primer reported by Muyzer et al. (1993)., [2] (1) Casamayor et al. 2002; (2) Muyzer et al. 1993; (3) Øvreas et al. 1997; (4) Heuer et al. 1997; (5) Salles et al. 2002; (6) Gyamfi et al. 2002; (7) Casamayor et al. 2002; (8) Van Elsas et al. 2000; (9) Gomes et al. 2003; (10) Waite et al. 2003; (11) Sliwinski et al. 2004; (12) Schwieger and Tebbe 1998; (13) Ramakrishnan et al. 2001; (14) Tiquia et al. 2002; (15) Dunbar et al. 2000; (16) Casamayor et al. 2002; (17) Brodie et al. 2003; (18) Casamayor et al. 2002; (19) Ranjard et al. 2000; (20) Cardinale et al. 2004; (21) Gardes and Bruns 1993; (22) Hansgate et al. 2005; (23) Larena et al. 1999; (24) Gardes and Bruns 1993.

number of published studies. Other kits may also be effective for certain types of soils, but uncertainties in the quality of the yield and purity of the recovered DNA have been observed when using these commercial kits with arable soils from Japan (Ikeda et al., unpublished data). The use of commercial products does guarantee a degree of stability of results with soil DNA extractions. However, it is our experience that there are difficulties with the direct use of these commercial kits for some soil samples and these extractions require additional purification steps or modifications, such as the addition of skim milk in the extraction buffer (Ikeda et al. 2004b; Takada-Hoshino and Matsumoto 2004). Moreover, soils can be contaminated with chemicals or heavy metals, which can be the case for assessments of transgenic plants, and these compounds may not be eliminated during DNA purification steps which could affect subsequent analyses. The use of kits can also create a heavy dependency on particular commercial products which could cause complications if there were problems with supply. We contend, however, that it would be inappropriate to compare results from soil microbial community analyses, for the purposes of environmental risk assessments of transgenic organisms, when these data had been generated using different commercial kits.

PCR amplification

PCR amplification of ribosomal RNA regions has been extensively used to study microbial diversity (Pace 1997). Initially, molecular-based methods for microbial diversity studies relied on the cloning of target genes isolated from environmental samples. Although sequencing has become routinely used in this endeavor, it is both labor intensive and incurs high costs. Hence, many other methods, mainly based on DNA fingerprinting techniques, have been developed in combination with PCR amplification to assess microbial community diversity.

Primers

A great number of primers and probes of different specificities, ranging from universal to specific primer sets, are now routinely used for the molecular detection and identification of microorganisms (Table 3). In general, small subunit and large subunit rRNA genes and their intergenic spacer regions are utilized for primer design in microbial diversity studies as these genes/fragments are present in all organisms. In addition, these regions are well defined by taxonomic classifications and are not subjected to horizontal transfer. Although bacteria and fungi have been mainly

examined in studies of microbial diversity in rhizospheric soils, recent reports suggest that members of the archaeal division Crenarchaeota also colonize the rhizosphere (Simon et al. 2000; Chelius and Triplett 2001; Sliwinski and Goodman 2004). Although a series of PCR primer sets are available as "universal primers", it should be remembered that none of the presently available primers will amplify all sequences from the corresponding eukaryotic, bacterial and archaeal domains. In addition, a report by Wintzingerode et al. (1997) discusses a number of important issues regarding the successful outcome of differential PCR amplification, including the different affinities between primers and templates, the different copy numbers of target genes, hybridization efficiency and primer specificity.

PCR facilitators and cycling programs

Due to the technical limitations in the purification of environmental DNA samples, it is not feasible to expect the complete elimination of all potential contaminants from diverse soil specimens. In order to overcome the potential inhibition of PCR by such contaminants and to perform stable PCR amplification, special additives are often incorporated into the reaction mixtures. The examples of these are T4 phage 32p (Tebbe and Vahjen 1993), BSA (Kageyama et al. 2003), Betaine (Henckel et al. 1999), DMSO (Felske et al. 1996), Formamide (Van Elsas et al. 2000), GC-Melt (Smalla et al. 2001), and Glycerol (Felske et al. 1996). Among these, we recommend bovine serum albumin (BSA) in particular, since it is relatively inexpensive and has so far helped to generate stable PCR amplifications in our laboratory analysis of arable soil samples collected from diverse locations in Japan. In addition, other additives such as GC-Melt[b] (Clontech Laboratories, Inc., Mountain View, CA) may be of great assistance in PCR amplifications with soil DNA templates in which the target sequences contain a high GC content, as is the case for actinomycetes.

Whilst the microbial community in an environmental sample may contain a high level of diversity, it may also consist of similar microbial groups. In the later case, mis-priming during PCR may become a major problem and can result in the formation of chimeras during amplification. In order to minimize this possibility, two strategies are usually employed for microbial community analysis. These are the use of a "hot start program" (McVeigh et al. 1996) and a "touch down program" (Duineveld et al. 1998), and these conditions can also be used in combination.

Potential biases in PCR amplification

It is estimated that microbial community analysis such as DGGE can only detect 1–2% of the microbe population and that this represents only the dominant species that are present in an environmental sample (MacNaughton et al. 1999). Furthermore, it has been reported that PCR analysis can heavily bias the relative abundance ratios of the original samples. Hence, dominant amplicons are not necessarily derived from dominant members of the microbial community (Wintzingerode et al. 1997; Suzuki and Giovannoni 1996). However there are no molecular methods currently available that can assess universal microbial diversity without any inherent bias. Less dominant members of the microbial community may well play an essential ecological role in nature (Bothe et al. 2000), but might not be properly assessed with universal PCR primer sets due to competition effects during amplification.

In cases where certain microbes are not easily detected by standard PCR based methods, the use of taxon-specific primers has been shown to be sometimes effective (Kowalchuk et al. 2000; Boon et al. 2002). Furthermore, in the case of the rhizosphere, several microbial groups, such as *Burkhoderia* and *Pseudomonas*, have been shown to be important community members and specific primer sets have thus been developed for these species (Salles et al. 2002; Gyamfi et al. 2002). Holben et al. (2004) reported the use of DGGE in combination with G+C fractionation to assess microbial community diversity and to detect minority populations of bacteria in the digestive tracts of chickens. This approach is therefore an alternative method that can reduce the complexity of the community under analysis and allow for the detection of species that are present in low abundance.

Primer specificity for fungal community analysis

Whereas the assessment of bacterial diversity is less problematic due to the availability of universal primers for bacterial domains, effective fungal community analysis suffers from the effects of co-amplification of DNA from other eukaryotic organisms such as plants, algae and nematodes (Kowalchuk et al. 1997). Although there are several studies that have reported attempts to resolve this problem (Kowalchuk et al. 1999), the specificity of these primer sets is still not sufficient enough in our experience.

Molecular techniques for the analysis of microbial communities

There are four principal fingerprinting techniques that have been widely applied to soil microbial community analysis (Figure 3). These are denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP) and ribosomal intergenic spacer analysis (RISA).

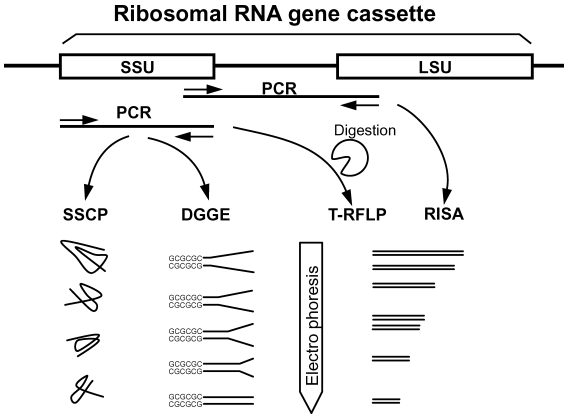

Figure 3. Principles underlying the major DNA fingerprinting methods employed for microbial community analysis. In all of these methods, the highly conserved regions in the ribosomal RNA genes are used for primer design (small arrows). The small subunit rRNA gene is used for PCR amplification in DGGE, SSCP, and T-RFLP, whereas the intergenic spacer region between the small and large subunit rRNA genes is amplified in RISA. PCR products generated using both DGGE and SSCP are resolved based on their three dimensional conformations. PCR products produced by T-RFLP and RISA are separated in a linear fashion, based on DNA fragment length. The boxed arrow indicates the direction of electrophoresis.

Denaturing gradient gel electrophoresis (DGGE)

DGGE was originally developed for the detection of point mutations in DNA sequences, but Muyzer et al. (1993) applied this technique to the study of microbial genetic diversity. DNA is extracted from soil samples and amplified using universal PCR primers that target parts of the small or large subunit rRNA gene sequences. The 5′-end of the forward primer contains a 35–40 base pair GC clamp to ensure that at least part of the DNA remains double stranded. This is necessary to separate the DNA fragments on a polyacrylamide gel along a gradient of increasing concentration of denaturants (formamide and urea), the resolution of which will occur based on melting behavior of the double-stranded DNA. Hence, DGGE can separate DNA fragments of identical sizes that have differences in their sequences. In theory, DGGE can resolve DNA bands that differ by only one base pair (Miller et al. 1999). Temperature gradient gel electrophoresis (TGGE) uses the same principle as DGGE, except that the gradient used is temperature rather than chemical denaturants.

DGGE has the capacity to detect subtle changes in microbial community structures because of its detection principle. Specific DGGE bands can also be excised from the gels, re-amplified and then either sequenced or transferred to membranes and hybridized with specific probes to provide more information regarding structural or functional diversity (Theron and Cloete 2000). By sequencing excised bands, one can obtain information about the specific microorganisms in a particular community. DGGE has also been used to assess the diversity of bacteria and fungi in the rhizosphere (Duineveld et al. 1998, 2001; Smalla et al. 2001).

The limitations of DGGE include PCR bias (Wintzingerode et al. 1997) and laborious sample handling, which could potentially influence microbial community analysis (Muyzer 1999; Theron and Cloete 2000). There are also noteworthy difficulties in DGGE primer design due to the limitation in the size of PCR products that can be analyzed, which is a maximum of a few hundred base pairs. In addition, DNA fragments of different sequences may have similar mobility characteristics in polyacrylamide gels (Ferris et al. 1996; Sekiguchi et al. 2001), and the presence of multiple melting domains in a PCR product was also reported as a cause of fuzzy bands (Kisand and Wikner 2003), which may also pose serious problems for the re-amplification and cloning of DNA bands of interest. Moreover, Nikolausz et al. (2005) have shown not only that different sequences with similar melting behavior can migrate together, but also that dominant amplicons are generally dispersed in a gel. Another possible factor influencing re-amplification is the formation of heteroduplexes. Satokari et al. (2001) and Speksnijder et al. (2001) reported that heteroduplexes during DGGE analysis causes an increase in band numbers. Hence, sequence analysis of DGGE bands should be performed carefully, and it must be remembered that a single band may not necessarily represent one species (Gelsomino et al. 1999).

Single strand conformation polymorphism (SSCP)

Another technique that relies on electrophoretic separation that is based on differences in DNA sequence is single strand conformation polymorphism (SSCP). Similar to DGGE, this technique was originally developed to detect polymorphisms or point mutations in DNA sequences (Orita et al. 1989). Single-strand DNA is separated on a polyacrylamide gel based on differences in mobility that are caused by their folded secondary structures (Lee et al. 1996). SSCP has all the same advantages and limitations of DGGE but does not require a GC clamp or the construction of gradient gels. Thus, technical requirements such as the design of primers and handling facilities are less problematic than DGGE. Furthermore, SSCP has been used to analyze microbial communities in rhizospheres (Schwieger and Tebbe 1998; Schmalenberger et al. 2001).

Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) is a technique that can address some of the limitations of both DGGE and SSCP (Tiedje et al. 1999). It follows the same basic principle as PCR-RFLP except that one of the PCR primers is labeled with a fluorescent

dye. This allows for the detection of only the labeled terminal restriction fragment (Liu et al. 1997). This also simplifies the banding patterns, thus allowing for the analysis of complex communities as well as providing information on diversity as each visible band represents a single operational taxonomic unit or ribotype (Tiedje et al. 1999). The banding pattern can therefore be used to measure species richness and evenness as well as similarities between samples (Liu et al. 1997). Moreover, T-RFLP can be automated to enable the sampling and analysis of a large number of soil specimens (Osborn et al. 2000). Osborn et al. (2000) tested the reproducibility of this method and found that the banding patterns both within a sample and between samples were highly reproducible. Significantly, T-RFLP has been used to assess the diversity of AMF in rhizospheres of metal-contaminated soil (Tonin et al. 2001).

One of the major differences between T-RFLP and DGGE/SSCP is that different community fingerprints can be obtained from same PCR products by digestion with different restriction enzymes (Dunbar et al. 2000). It is therefore advisable to use at least two to four different restriction enzymes (Tiedje et al. 1999). Incomplete digestion by restriction enzymes, however, could also lead to an overestimation of diversity (Osborn et al. 2000). T-RFLP also has the disadvantage of having both high facility and running costs, which could prevent its routine use in ecological assessments due to their requirement for a large set of samples. In addition, although the species corresponding to each profile in T-RFLP may be postulated, based on the consensus sites of the restriction enzymes and from fragment size information, there is no method to directly clone DNA bands of interest with this technique. Despite these limitations, T-RFLP can be a very useful tool for the study of microbial diversity in the environment (Liu et al. 1997; Tiedje et al. 1999; Osborn et al. 2000). There is however some current controversy regarding the reliability of T-RFLP, due to the variability in data generated from the use of different restriction enzymes. Dunbar et al. (2000) have reported statistics that detect inconsistencies in T-RFLP DNA banding patterns, depending on the restriction enzyme used. In contrast, Tiedje et al. (1999) have reported the superiority of T-RFLP over DGGE due to the presence of multiple domains for variable regions using this method.

Ribosomal intergenic spacer analysis (RISA)

RISA generates ribosomal-based fingerprinting of the microbial community. With this method, the intergenic spacer (IGS) region that is located between the small and large rRNA subunit genes is amplified by PCR, denatured and resolved by polyacrylamide gel electrophoresis under denaturing conditions. This region may encode tRNA genes in the case of prokaryote or 5.8S rRNA gene in the case of eukaryote and is useful for differentiating closely related species because of inherent heterogeneities in IGS length and sequence (Fisher and Triplett 1999). In RISA, sequence polymorphisms are detected by silver staining, whereas the forward primer is fluorescently labeled and is automatically detected using the automated RISA method (ARISA). Both methods provide highly reproducible bacterial community profiles but ARISA increases the sensitivity of the method and reduces the operation time. Similar to DGGE and SSCP, a DNA band of interest that has been obtained from a RISA profile can be cloned for sequence analysis. Because of the use of sequencing gels, however, RISA provides the high levels of resolution that can be obtained by T-RFLP (Ikeda et al. 2004a). Furthermore, RISA has also been used to compare microbial diversity in the rhizospheres of plants (Borneman and Triplett 1997; Ikeda et al. 2005).

As one of the potential biases in RISA, Milyutina et al. (2004) have demonstrated in their previous report that intragenomic differences in the IGS in some cases exceed the differences in these sequences among strains. This could bias the interpretation of both the quantitative and qualitative interpretation of the data obtained by RISA. However, the high variability of the target regions may also allow for subtle discrimination within a particular species, which is in contrast to the difficulties in distinguishing species, or even genera, using 16S rRNA sequences. Since RISA targets different regions of rRNA genes and can also provide sequence data, it would also be a useful complement for polyphasic analyses, in combination with other methods that target the small subunit of the rRNA gene.

The limitations and potential biases in DNA fingerprinting analysis of microbial communities

A major limitation of the established DNA fingerprinting techniques for community analysis for soil microbes is that the complexity of rDNA fragments can exceed the resolving power of the current electrophoresis techniques, due to the extreme complexity of soil ecosystems (Nakatsu et al. 2000). In addition, it is known that some bacterial groups have variations in length and sequence between intragenomic copies of ribosomal operons (intercistronic heterogeneity), for both the RNA gene coding and intergenic regions (Maarit-Niemi et al. 2001). Furthermore, different species will have different gene copy numbers and this can also bias the interpretation of fingerprinting results (Liu et al. 1997).

Comparison of the advantages and disadvantages of the current molecular techniques for microbial community analysis

Each of the molecular techniques described in the

Table 4. Comparison of DNA fingerprinting methods for microbial community analysis

Elements	RISA[1]	DGGE[2]	SSCP[3]	T-RFLP[4]	Sequencing
Facility cost	low	medium	medium	high	high
Running cost	low	medium	medium	high	high
Processing time	short	medium	medium	medium	long
Technical requirements	low	medium	medium	medium	high
Automation	possible	not possible	possible	possible	possible

[1] Ribosomal Intergenic Spacer Analysis, [2] Denaturing Gradient Gel Electrophoresis, [3] Single Strand Conformation Polymorphism, [4] Terminal Restriction Fragment Length Polymorphism.

Table 5. Microbial community analysis in the rhizospheres of transgenic plants

Plant species	*Transgene*	Phenotype	Method	Target microbe	References[2]
Solanum tuberosum	*barnase*/*barstar*	disease resistance with cell death	T-RFLP	Bacteria	(1)
Brassica napus	*pat*	herbicide tolerance	DGGE	Bacteria	(2)
Solanum tuberosum	*gpe*	T4 lysozyme producing	DGGE	Bacteria	(3)
Zea mays	*pat*	herbicide tolerance	SSCP	Bacteria	(4)
Beta vulgaris	*pat*	herbicide tolerance	SSCP	Bacteria	(5)
Zea mays	*Cry1Ab*	insect resistance	RISA	Bacteria	(6)
Nicotiana tabacum	*YenI*	quorum-sensing signal producing	DGGE	Bacteria	(7)
Solanum tuberosum	*gbss*	altered starch composition	DGGE	Bacteria/*Pseudomonas*/Fungi	(8)
Chrysanthemum[1]	*Cry1Ab*	insect resistance	RISA	Bacteria/Fungi	(9)

[1] Chrysanthemum [*Dendranthema*×*grandiflorum* (Ramat.) Kitamura], [2] (1) Lukow et al. 2000; (2) Gyamfi et al. 2002; (3) Heuer et al. 2002; (4) Schmalenberger and Tebbe 2002; (5) Schmalenberger and Tebbe 2003; (6) Brusetti et al. 2004; (7) d'Angelo-Picard et al. 2004; (8) Milling et al. 2004; (9) Ikeda et al. 2005.

previous sections has been widely used for microbial community analysis. However, there are some considerations that are vital from the view point of environmental risk assessments (Table 4). Both DGGE and SSCP have been evaluated as highly sensitive methods as they have the ability to detect point mutations. However, compared to T-RFLP and RISA, they also require more sensitive experimental conditions for reproducibility. In addition, the mobility of the DNA profiles in DGGE and SSCP is not simply linear. Thus, the data sets provided by DGGE and SSCP may not be appropriate for comparisons between the findings of different laboratories or institutes. This is a critical issue for the performance of effective environmental risk assessments, in which objectivity of interpretation is required for the full complement of data sets. Moreover, T-RFLP and RISA can be much more easily automated because of their technical simplicity, and their underlying principles of amplicon separations, which are based on the linear resolution of amplicon size. However, DGGE and SSCP may well be more sensitive and effective for some samples where the detection of subtle sequence differences is required. In addition, several taxon specific primers are now available for DGGE analysis.

Current status of environmental assessments of the impact of transgenic plants upon soil microbial diversity

By employing a number of the fingerprinting techniques described in the previous sections, several transgenic plants have been evaluated for their impacts on the soil microbial communities in a number of reports (Table 5). Currently, disease and insect resistance are the major traits that are conferred by the genetic modifications of transgenic plants. In such cases, however, some of the transgenes are predicted to be directly toxic to the microbial communities in the rhizosphere. Since some rhizospheric microbes are known to be beneficial for plant growth and disease resistance (Bashn and Holguin 1998), there is a degree of concern about the impact of antimicrobial substances derived from transgenic plants on important soil microbes. Heuer et al. (2002) concluded, however, that the T4 lysozyme producing potato has negligible effects on the bacterial community in its rhizospheric soil. For insect resistance, several *Bt* transgenic crops have now been widely commercialized (James 1997). Recently, Brusetti et al. (2004) have reported that the introduction of Cry protein genes could disrupt microbial communities in the rhizosphere of *Bt* corns. The authors speculated that these changes could be caused by the unintentional effects of altered root exudate patterns, which were observed in all of the *Bt* corns examined by them (Saxena et al. 2002). Rhizosphere effects are well known to increase microbial density and activity, compared to bulk soil, and to provide selective pressure for specific microorganisms. As a consequence, any variation in root exudation could induce variations in the structures of microbial communities.

Herbicide resistance also has been shown to be an important target trait for transgenic plants. Schmalenberger and Tebbe (2002, 2003) examined the

impacts of transgenic herbicide resistant corn and sugar beet on their corresponding soil microbial communities, and reported no effects. In contrast, Gyamfi et al. (2002) reported moderate effects of herbicide resistant transgenic oilseed rape on *Pseudomonas* communities in its rhizosphere, and concluded that unintentional modifications by the transformation events most likely caused these effects. Noticeably, they detected no effects of transgenic plants when they conducted whole bacterial community analysis, indicating the usefulness of taxon specific community analysis for sensitive detection.

In all of the studies which have observed some impact of transgenic plants on soil microbial communities, it was reported that the effects upon community structures were negligible in comparison to the changes caused by other environmental factors. Moreover, there was no observation of any beneficial effects against harmful microbes in cloning experiments involving differential profiles between transgenic and non-transgenic plants. In addition, Milling et al. (2004) have described their results in the context of natural genetic variance by introduction of a "second non-transgenic line", which has a different genetic background to the parental line of transgenic plants. They demonstrated that the differences in the microbial community profiles between transgenic and non-transgenic plants could be considered to be within the normal range of natural variance. We have recently reported similar results for the *Bt* transgenic chrysanthemum (Ikeda et al. 2005).

In contrast to number of environmental assessments of upland transgenic crops on soil microbial communities, no transgenic rice has been investigated for possible effects upon soil microbes. Environmental risk assessments of soil microbial communities may be more important for transgenic rice, however, since it has been shown that methane-oxidizing bacteria play an important role in the reduction of methane emissions from rice fields (Eller et al. 2005). It is possible that transgenic rice could have an impact on either the increase or decrease of the global CH_4 emissions from rice fields, which is now of great concern in relation to global warming (Lelieveld et al. 1998; Neue 1997).

In summary, we conclude that environmental assessments of the impact of transgenic plants on soil microbial communities will be made possible by employing the molecular techniques described in the present review with appropriate cautions. These methods allow for the handling of multiple samples, facilitate efficient processing times, and provide less biased data sets for the overall evaluation of soil microbes. Furthermore, in addition to evaluating the impact of transgenic plants in environmental risk assessments, these molecular methodologies may also provide alternative strategies for plant molecular biologists to investigate plant-microbe interactions in their natural environments.

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