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Review

Effects of transgenic plants on soil microorganisms

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Abstract

The rapid development of agricultural biotechnology and release of new transgenic plants for agriculture has provided many economic benefits, but has also raised concern over the potential impact of transgenic plants on the environment. Considerable research has now been conducted on the effects of transgenic plants on soil microorganisms. These effects include unintentional changes in the chemical compositions of root exudates, and the direct effects of transgenic proteins on nontarget species of soil microorganisms. Most studies to date suggest that transgenic plants that have been released cause minor changes in microbial community structures that are often transient in duration. However, due to our limited knowledge of the linkage between microbial community structure and function, more work needs to be done on a case-by-case basis to further evaluate the effects of transgenic plants on soil microorganisms and soil ecosystem functions. This review summarizes the results of a variety of experiments that have been conducted to specifically test the effects of transgenic plants on soil microorganisms, and particularly examines the types of methods that are being used to study microbial interactions with transgenic plants.

Introduction

The advent of agricultural biotechnology and genetic engineering of plants has created new transgenic crop plants with superior yields and improved traits for resistance to insect pests and pathogens, tolerance to herbicides, and improved ability to endure environmental stress. In countries where this technology has been embraced, the land area that has been planted for commercial production of transgenic plants has increased dramatically from 1.7 million ha in 1996 to 67.7 million ha in 2003 (James, 2003) and is predicted to increase even more in the future. Along with modification of agronomic traits, genetically modified plants are now also being developed to produce industrial and pharmaceutical compounds. This new technology has the potential to provide enormous economic and agronomic benefits in the future and can also provide environmental benefits such as reducing the need for pesticide usage (Huang et al., 2003). Nevertheless, the release of transgenic plants is still highly controversial in many countries due to concern over the potential detrimental effects of genetically modified plants on human health and the environment. Among the major concerns are the possibility of creating invasive plant species, the unintended consequences of transgene flow to indigenous plants and microorganisms, development of super pests, and the effects of transgenic plants on non-target organisms including soil microbial communities (Wolfenbarger and Phifer, 2000). These are all serious issues and require that the effects of transgenic plants on the

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environment be carefully evaluated. This is especially true when considering the impact of transgenic plants on our soil resources and ecosystem function since our understanding of the complexity of soil microbial communities is still rudimentary and we are only now developing the tools to understand the relationship between microbial community structure and ecosystem function. As new transgenic plants are developed, it is essential that soil microbiologists participate in developing criteria for evaluating the safety of transgenic plants that includes the study of their effects on soil organisms (Jepson and Pratt, 1994).

In this article, we summarize recent published studies that have examined the effects of transgenic plants on soil microorganisms, with a particular emphasis on the methods that have been used for this purpose. As shown in these studies, there are many different methods that are currently being used and the results of these experiments are often difficult to interpret. In many cases, there are consistent effects of transgenic plants on soil microorganisms that can be observed, but the ecological relevance of these data is difficult to interpret. In other situations, there are no observable effects, but it is still uncertain whether the methods that have been used are sufficiently sensitive to detect changes or whether the results with a particular experimental system can be broadly applied to all soils. However, the studies that have been conducted to date do provide valuable insight into the possible mechanisms by which transgenic plants might affect soil microorganisms, and have led toward the evaluation of appropriate methods that can be used to study the fate of transgene products when they are released into the soil environment.

Mechanisms by which transgenic plants affect soil microorganisms

There are a variety of considerations as to how transgenic plants may affect soil microorganisms that include both direct and indirect effects of the plant that has been modified. Direct effects will depend on the spectrum of activity of the transgene proteins (Oger et al., 1997) and the quantities of the protein that accumulate in the environment. In contrast, indirect effects are mediated by changes in plant protein and root exudate composition that arise as a result of modifying the metabolic pathways in the plant tissues. The direct effects are of the most concern since the introduction of transgene proteins for pest and disease resistance can involve the production of chemical substances that are potentially toxic to non-target soil organisms, including mycorrhizal fungi and soil microfauna that are involved in organic matter decomposition. Indirect effects caused by changes in root exudates are also possible consequences of transgenic plants, but are much more difficult to evaluate since so many factors may affect root exudates composition and microbial community structures in soil.

Accumulation of transgene proteins in soil

Many factors may potentially influence the accumulation of transgene proteins in soil, including the amount that is contained in the plant tissues, the resistance of the proteins to degradation, and soil chemical, physical, and environmental factors that influence bioavailability and persistence of proteins in the soil. The amount of protein that enters the soil will depend both on the location of the protein in the plant tissues and the amount of that tissue that enters the soil. The use of constitutive promoters results in transgene expression in all organs and at all growth stages of the transgenic plant (Odell et al., 1985). In this manner, the production of large quantities of a transgene protein increases its potential for adverse effects on non-target organisms. Since the expression of transgenes in transgenic plants is frequently controlled by constitutive promoters such as the Cauliflower Mosaic Virus (CaMV) 35S promoter, plant litter is potentially one of the largest sources for introduction of transgene proteins. Moreover, transgene proteins can also enter soil via other sources. One of the most common transgene proteins that has been released into the environment in large quantities is the crystal toxin protein produced by transgenic plants expressing the gene of *Bacillus thuringiensis* (Bt), which can enter the soil through deposition of plant litter, death and turnover of sloughed root cells and in root exudates. Although the Bt toxin is used to prevent insect damage to the above ground plant parts, experimental studies have shown that Bt toxin as well as another common transgene protein, T4 lysozyme, are not only present in root exudates but that they maintain biological activity after entering the soil (Ahrenholtz et al., 2000; de Vries et al., 1999; Saxena and Stotzky, 2000; Saxena et al., 1999, 2002). On the other hand, if expression of transgenes in transgenic plants can be controlled temporally or spatially by organ/tissue specific promoters or inducible promoters, the potential environmental risks caused by transgenic plants may be reduced (Maizel and Weigel, 2004; Wilkinson et al., 1997).

During plant litter decomposition, most transgene protein(s) appear to be rapidly degraded, but some proteins can bind to surface-active particles and reduce their availability to microbes. In the case of transgenic cotton which produces the CryIIA protein, the plant litter contains 34 μg of CryIIA protein/g of fresh weight of tissue prior to harvest. If the transgenic cotton plant matrix is uniformly incorporated into the top 7.6 cm of soil, then the estimated maximum content of CryIIA protein is 1.6 μg/g dry soil (Sims and Ream, 1997). Although this is a relatively small quantity of protein, the potential impacts on soil organisms will depend on the persistence of the transgene protein(s) in plant residues after they undergo degradation. For example, when purified Bt toxin was added to non-sterile soils, activity against the larvae of the tobacco hornworm (*Manduca sexta*) was still detected after 234 days (Saxena and Stotzky, 2000). A high soil clay content and low soil pH increased the persistence of Bt toxin in soil. In another study examining this question, no degradation of the Cry1Ab toxin in transgenic Bt corn leaves was observed during the first month, after which the Cry1Ab toxin concentrations decreased to 20% of their initial values during the second month. There was no further degradation during winter and the toxin continued to degrade slowly when temperatures again increased in spring (Zwahlen et al., 2003). These results suggest that the transgene protein(s) can remain in soil for a long time. At this time, it is still not known whether continuous planting of transgenic plants over many years will lead to increasing accumulation of transgenic proteins, or what impact this might have on soil microorganisms or the soil fauna.

Unintentional changes in chemical compositions in transgenic plants

During genetic manipulations, the practice of cell and tissue culturing (especially when antibiotics are used as the selection agents) can provoke unintended alterations in plant characteristics. The position effects from the sites of the inserted gene(s) and the expression of the inserted genes disturb the normal expression of genes of the parental plants and can cause unexpected changes in plant characteristics (Stelly et al., 1989; Zhang et al., 2001). For example, the expression of an antisense GIGANTEA gene fragment in transgenic radish causes delayed bolting and flowering (Curtis et al, 2002). Unintentional characteristics may also occur in plant tissues and root exudates of transgenic plants, e.g. significant differences have been observed in the contents of fructose and soluble carbohydrates between transgenic plants and the parental plants (Escher et al., 2000). Transgenic alfalfa that was engineered to over-express the nodule-enhanced malate dehydrogenase (neMDH) gene also significantly increased the exudation of citrate, oxalate, malate, succinate and acetate as compared to the non-transgenic plant (Tesfaye et al., 2001). Transgenic plants may also have altered metabolism of pesticide residues. As an example of this phenomenon, metabolites of herbicides were shown to be exuded from the roots of transgenic rice plants containing the P450 CYP1A1 gene that was inserted into the genome to confer tolerance to herbicides (Hiroyuki et al., 2003). Because the composition and diversity of soil microbial communities that are associated with plant roots are influenced by root exudation in a selective manner, changes in the composition of root exudates between transgenic plants and non-transgenic plants may produce different effects on microbial communities. Currently our understanding of the relationship between rhizosphere community structure and plant growth and health is limited, but in the future this will become an important consideration as plant breeders and microbiologists focus on optimization of plant beneficial microbial populations in the rhizosphere.

Case studies

Transgenic plants with pest-resistant traits

Transgenic plants producing Bt toxins

The insecticidal proteins produced by *B. thuringiensis*, a naturally occurring soil bacterium, have proved to be effective tools for the control of a wide variety of insect pests. Preparations of Bt have been used as insecticides in agriculture for over 40 years and are considered relatively safe due to their specificity for only a narrow range of insects (Schnepf, 1995). Transgenic plants containing Bt genes such as the CryIA(c) and CryIA(b) first appeared on the market on a large scale in 1996, and at least 26 different transgenic Bt crop and tree species have been developed and proved effective for control of specific insect pests (Cannon, 2000).

Experiments have been conducted to evaluate the non-target effects of transgenic Bt plants on soil microorganisms. When Bt toxins such as CryIA(c) and CryIA(b) were incorporated into soil at the concentration of 0.05 μg toxin g^{-1} soil, or the activated insecticidal toxins from *B. thuringiensis* subsp. *kurstaki*

(B*tk*), *morrisoni* strain *tenebrionis* (B*tt*), and *israelensis* (B*ti*) were dissolved into liquid mediums for dilution and disk-diffusion assays, no significant effects on soil microbial communities or on specific microorganisms were observed (Donegan et al., 1995; Koskella and Stotzky, 2002). In an experiment lasting 56 days, leaves of three transgenic cotton lines producing Bt toxins were added to soil. Numbers and species of indigenous soil bacteria and fungi were monitored using culture based methods (i.e. colony-forming units (CFUs) in culture media and metabolic fingerprinting using Biolog Gram negative microtiter plates) and a DNA fingerprinting method (restriction endonuclease digested fragment patterns of amplified rDNAs from soil samples). Experimental results indicated that two of the three transgenic cotton lines containing the CryIA(c) gene caused a significant but transient stimulation of cultural, aerobic bacterial and fungal populations. In contrast, neither the third transgenic cotton line containing a CryIA(b) gene, nor the purified CryIA(c) toxin or CryIA(b) toxin had any detectable effects on the total numbers of bacteria and fungi (Donegan et al., 1995). According to the results of experiments performed in a plant-growth room, there were no significant differences in the CFUs of culturable bacteria and fungi after 40 days between rhizosphere soils of transgenic corn containing the Cry1Ab gene and the non-transgenic corn or after 45 days between soils amended with biomass of the two corn lines (Saxena and Stotzky, 2001).

In another study on Bt proteins, an experiment was carried out under laboratory conditions to investigate the differences in the population of culturable microorganisms and the enzymatic activities between soils amended with the straw of transgenic rice (*Oryza sativa*) containing the Cry1Ab gene and the isogenic non-transgenic rice. No significant differences were observed in the CFUs of culturable bacteria, actinomycetes and fungi between the two soils. Some apparent but transient differences were observed in the populations of ammonifying bacteria, nitrogen-fixing bacteria and cellulose-decomposing bacteria in the middle of the incubation. No significant differences in the activities of some soil enzymes (protease, neutral phosphatase, urease) and respiration activity were found between the two soils. Soil dehydrogenase activity in the soil amended with transgenic rice straw was significantly higher compared with the soil amended with non-transgenic rice straw, but the difference disappeared after incubation for 63 days (Wu et al., 2004).

Transgenic plants producing lectins and proteinase inhibitors (PIs)

Lectins are a class of proteins capable of controlling both root-feeding nematodes through intervention in chemotactic host-finding course and phloem-feeding insect pests through binding to carbohydrate receptors in their alimentary tracts (Griffiths et al., 2000). Proteinase inhibitors are proteins which inhibit the digestive proteinases of many plant-parasitic nematodes (Cowgill et al., 2002). The genes coding for these two proteins have been isolated and introduced into plants for enhanced pest resistance.

In one study examining the effects of these proteins on soil microorganisms, litterbags containing leaves of transgenic tobacco (*Nicotiana tabacum*) constitutively producing the tomato proteinase inhibitor I and the parental tobacco plant were buried in field plots. Results of this study showed that the nematode populations in the soil surrounding the transgenic plant litterbag were greater than those in the soil surrounding the parental plant litterbag and had a different trophic group composition, including a significantly higher ratio of fungal feeding nematodes to bacterial feeding nematodes on sample day 57 (Donegan et al., 1997). Whether this might have long term consequences on soil community structure was not determined and would require a multiyear study to examine the possible consequences with respect to plant health, productivity, and soil function. It would also be relevant to include different plant cultivars to determine whether the observed changes are specific to transgenic plants or might arise simply through the planting of different crop cultivars.

The transgenic potato (*Solanum tuberosum* L.) expressing the cysteine proteinase inhibitors (cystatins) has been developed to control the potato-cyst nematode (PCN), an important plant-parasitic pest. The microbial community structure and litter decomposition of the transgenic potato were recently studied in field tests that were conducted over two growing seasons. In the first year, the transgenic lines had no effect on the abundance, evenness or metabolic activity of the soil microbial community as determined by ester-linked phospholipid fatty acid analysis (PLFA). However, one transgenic line favored fungal growth relative to bacterial growth during the latter parts of the growing season, and a second transgenic suppressed the fungal growth in late season. In the second year, the microbial abundance was reduced by 23% in the transgenic treatment relative to the control. The transgenic plants suppressed both the bacterial and

fungal community components. The observed changes in soil microbial community structure did not result in changes in the rate of leaf litter decomposition (Cowgill et al., 2002).

A tiered experiment, initially involving laboratory studies with purified lectins, and then with microcosm and pot studies before moving into studies with field plots, was performed to determine whether transgenic potatoes producing the concanavalin A (Con A) and *Galanthus nivalis* agglutinin (GNA) had effects on non-target soil microorganisms and processes. In the laboratory studies and pot trials, both the two lectins and transgenic potatoes producing the two lectins had no detectable effects on soil bacterial communities. Controlled field-release experiments demonstrated that although GNA-producing potato lines consistently altered the Biolog GN metabolic fingerprinting of the rhizosphere microbial community at harvest, the effect did not persist from one season to the next over a trial period of two field seasons (Griffiths et al., 2000).

Transgenic plants with pathogen-resistance traits

Plant diseases caused by phytopathogenic microorganisms lead to high losses of crop yields worldwide. To obtain durable and broad-spectrum resistance against pathogens, plants are being transformed with genes coding for antimicrobial proteins such as chitinases, glucanases, lysozymes, thionins, defensins, and systemic acquired resistance (SAR) gene products (Salmeron and Vernooij, 1998).

Transgenic plant producing T4 lysozyme

Lysozymes are enzymes capable of degrading the murein layer of bacterial cell walls. While the cell wall of gram-positive cells is directly accessible to externally added lysome, the action on gram-negative cell requires the enzyme to overcome the barrier of outer lipopolysaccharide membrane. Experimental results showed that most of the gram-negative and all the gram-positive bacteria tested were sensitive to T4 lysozyme (de Vries, 1999). A chimeric gene coding T4 lysozyme was inserted into the genome of potato plant with the aim of increasing resistance to a phytopathogenic strain of *E. carotovora* (During et al., 1993). Roots from potato lines expressing the T4 lysozyme gene always showed significantly higher killing activity (1.5- to 3.5-fold) on root-adsorbed *Bacillus subtilis* cells than control lines (Ahrenholtz et al., 2000).

To further evaluate the transgenic potato line, a field release of the transgenic potato producing T4 lysozyme and non-transgenic potato was performed to study their effects on plant-associated bacteria. No significant differences in total soil bacterial counts, the percentage and function of potentially soil beneficial bacteria were found between the two lines over a period of two years at three stages of potato development and at two different locations, but seven isolates of the 28 potato-associated species with antagonistic effects on phytopathogens were isolated only from non-transgenic potato (Lottmann et al., 1999). The two potatoes varieties were planted at two field sites in three consecutive years and rhizosphere samples were taken from young, flowering, and senescent plants. The bacterial structure and dynamics of the two soil samples were analyzed by three different approaches including, the cultivation and characterization of isolates by fatty acid analysis, catabolic profiling of bacterial community using Biolog GN microplates, and the analysis of 16S rRNA gene fragments amplified from total rhizosphere DNA by denaturing gradient gel electrophoresis (DGGE). The experimental results indicated that environmental factors related to season, field site, or year but not to the T4 lysozyme expression of the transgenic plants influenced the rhizosphere bacterial communities (Heuer et al., 2002). According to the result of another field study with the same two potato varieties, the phenotypic and genotypic features of the isolated antagonistic bacteria did not reveal correlations between bacterial isolates and the transgenic character of the plants (Lottmann and Berg, 2001).

Transgenic plant producing cecropin B

Cecropins are a family of small, highly basic peptides that exhibit lytic and antibacterial activity against a number of gram-negative and gram-positive bacteria *in vitro*. Cecropin B has been proved to be highly toxic to several plant pathogenic bacteria. Cecropin B gene has been introduced into a variety of plants in order to enhance resistance to phytopathogenic bacteria (Nordeen et al., 1992). A greenhouse experiment was carried out with transgenic cecropin B-producing potato in order to determine whether such plants affect non-target *Bacillus* communities in the rhizosphere. Six hundred and twenty-one *Bacillus* isolates were obtained from the potato rhizospheres at the flowering and the tuber production stage, and strains were analysed by polymerase chain reaction (PCR) – restriction fragment length polymorphism

(RFLP) analysis of the 16S rRNA gene and the 16S-23S rDNA intergenic spacer. Representative isolates were further analyzed by partial 16S rDNA sequence analysis. At the flowering stage, the transgenic potato caused a transient, but significant effect on the diversity and community structure of *Bacillus* spp compared to the parental line. However, rhizospheres of transgenic and parental potatoes had comparable *Bacillus* community structures and diversities at the tuber production stage (Sessitsch et al., 2003).

Transgenic plants expressing pathogenesis-related proteins (PRs)

The expression of some PRs such as chitinases and β-1, 3-glucanases can make plants exhibit enhanced resistance to pathogenic fungi. In a greenhouse experiment, tobacco lines genetically modified to express various PRs constitutively were grown in a steam-sterilized mixture (sand and loam) and their effects on arbuscular mycorrhiza (AM) fungi were examined. Constitutive expression of some acidic isoforms of tobacco PRs such as chitinases PR-1a and PR-4 did not affect the time course or the final level of colonization by the VAM fungus *Glomus mosseae* (Vierheilig et al., 1993). Although β-1, 3-glucanase had very little antifungal potential when assayed with various fungi on artificial growth media (Sela-Buurlage et al., 1993), a delay of colonization by *G. mosseae* was observed in tobacco plants expressing the acidic isoform of tobacco PR-2, a protein with β-1,3-glucanase activity (Vierheilig et al., 1995). The authors indicated that beneficial symbiotic fungi might be affected adversely by the antimicrobial proteins expressed in the transgenic plant.

Chitinase genes from different sources have been isolated and inserted into plant genomes for control of fungal pathogens (Punja, 2001). To examine the effects of these genes on soil microorganisms, field studies were conducted in which roots of transgenic rice varieties producing rice-chitinase and the parental rice plants in tasseling stage were sampled to analyze their rhizosphere microbial communities. The results showed that the microbial communities of the root and root surface in two transgenic varieties were different from the non-transgenic variety. Transgenic plants had less endo-fungi, but their numbers of endobacteria were about 10 times more than the control. The root-ratios with endo-fungus in two transgenic varieties were 55.2% and 81.1%; whereas the control was 100%. The species of bacteria and fungi in the root systems were also different from the control (Yang et al., 2002).

Transgenic plants expressing the defense mechanisms

Systemic acquired resistance (SAR) is a constitutive and inducible defense mechanism that is widely used by plants to respond to infectious agents. The SAR induction is strongly correlated with the coordinate expression of a set of PR genes and requires the signal molecule salicylic acid (SA), which accumulates in plants prior to the onset of SAR (Metraux et al., 1990). Transgenic tobacco plants expressing the bacterial nahG gene (NahG tobacco), which results in the reduction of SA level, and the transgenic tobacco expressing two bacterial genes (CSA tobacco) coding for enzymes that enhance the SA levels have been shown as a valuable tool to study plant defense responses against pathogens. Since colonization of roots by mycorrhizae may be influenced by SAR, it is relevant to test how changes in SAR expression may affect mycorrhization. To examine this question, an experiment was conducted with wild type tobacco, transgenic NahG tobacco and transgenic CSA tobacco, which were inoculated with the AMF *G. mosseae*. During early plant development, NahG plants had enhanced AM root colonization levels, whereas in CSA plants, mycorrhization was reduced. However, at the end of the experiment with *G. mosseae* root colonization was similar in both the transgenic and wild type plants, indicating that enhanced SA levels in plants might sometimes delay AMF root colonization, but in this case, did not affect the level of final root colonization (Medina et al., 2003).

Antimicrobial proteins are rarely specific and exhibit lytic activity against a wide range of microorganisms. Consequently, transgenic plants producing such substances may not only affect the target pathogens, but also the soil or rhizosphere microflora, especially beneficial microorganisms such as mycorrhizas and rhizobia (Glandorf et al., 1997). Transgenic pathogen-resistant plants have been found to have effects on microbial community structure and specific microorganisms such as *B. subtilis* and *G. mosseae* in rhizosphere soils (Ahrenholtz et al., 2000; Lottmann et al., 1999; Vierheilig et al., 1995; Yang et al., 2002), which coincides with the theoretical prediction (Glandorf et al., 1997). However, there is still not evidence that the effects are the results of antimicrobial protein expressed in transgenic plants. Furthermore, some transgenic pathogen-resistant plants have no effects on the microbial communities and specific non-target

microorganisms in rhizosphere soils (Heuer et al., 2002; Lottmann and Berg, 2001; Medina et al., 2003; Sessitsch et al., 2003). There are several possible reasons for this. First, the rhizosphere communities may be stable enough to resist the effects of antimicrobial proteins. Second, the amount of transgene proteins exuded into soil may be too limited to have significant effects on soil microbial communities (Heuer et al., 2002). And third, the released proteins may be rapidly inactivated in soil by factors such as chemical conditions and rapid microbial transformation into less active compounds or by adsorption to solid soil surfaces (de Vries, 1999).

Transgenic plants with herbicide-resistant traits

Among transgenic crops that have been commercially planted in the world, transgenic crops (soybean, maize, canola and cotton) tolerating herbicides have consistently been predominant during the eight-year period between 1996 to 2003 (James, 2003). Herbicides are often sprayed on the green parts of certain plants, but the herbicide- resistant genes are expressed not only in the green parts, but also in the roots.

To study the effect of herbicide resistance genes on soil microorganisms, glyphosate-resistant oilseed rape (*Brassica napus*) varieties were genetically transformed with the 5-enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium sp*. strain CP4 and glyphosate oxidoreductase (Siciliano and Germida 1999). Approximately 2300 bacteria were isolated from roots of the transgenic oilseed rape and the non-transgenic oilseed rape. According to the results, fewer *Bacillus, Micrococcus, Variovarax* isolates, and more *Flavobacterium*, *Pseudomonas* isolates were found on the roots of the transgenic cultivar compared with the non-transgenic cultivar. The bacterial root-endophytic community of the transgenic cultivar exhibited a lower diversity compared with the non-transgenic cultivar (Siciliano and Germida, 1999). This result coincided with other two experimental results which similarly indicated there were significant differences in the composition and functional diversity of the microbial community between the same two varieties of oilseed rape (Dunfield and Germida, 2001; Siciliano et al., 1998). In an more recent study by these researchers, a 2-year experiment with the two oilseed rape lines was conducted at different field sites and three different methods including community-level physiological profiles (CLPP), fatty acid methyl ester profile (FAME) and terminal amplified ribosomal DNA restriction analysis profiles (T-ARDRA) were used to assess the rhizosphere microbial communities. The results again showed that there were significant differences between the rhizosphere microbial communities associated with the two lines that were observed at several times throughout the growing season; however, there were no differences between microbial communities from field plots containing harvested transgenic line and field plots containing no oilseed rape during the field season after winter. This means that differences between the rhizosphere microbial communities associated with the transgenic plants and the parental non-transgenic plants were temporary and depended on the presence of the viable plant (Dunfield and Germida, 2003).

The phosphinothricin acetyltransferase (*pat*) gene has been isolated from *Streptomyces viridochromogenes* and transgenic plants containing the *pat* gene show tolerance against another herbicide glufosinate (Wohlleben et al., 1992). A greenhouse experiment was conducted in order to evaluate possible shifts in eubacterial and *Pseudomonas* rhizosphere community structures due to the environmental release of transgenic oilseed rape containing the *pat* gene. Rhizosphere soil was sampled from early and late flowering plants as well as from senescent plants. A culture-independent approach was chosen to characterize microbial communities based on denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments amplified from rhizosphere DNA. Results showed that the microbial communities were slightly altered in the rhizosphere of transgenic plants; however, the effects were minor as compared to the non-transgenic line and to the plant developmental stage-dependent shifts (Gyamfi et al., 2002). For maize (*Zea mays*) and sugar beet (*Beta vulgaris*) engineered with the same *pat* gene, there were no significant differences in the bacterial diversities in rhizospheres of the two transgenic cultivars and their isogenic, non-transgenic cultivars either (Schmalenberger and Tebbe, 2002, 2003).

Interestingly, while the transgenic plants (oilseed rape) tolerating the herbicide glyphosate had significant effects on soil microbial communities, the transgenic plants (oilseed rape, maize and sugar beet) tolerating another herbicide glufosinate did not cause any significant effects. Besides factors such as different herbicide treatment and soil types that may account for the different effects on microbial communities, the difference may also result from the effects of different transgene (5-enolpyruvylshikimate-3-phosphate synthase gene and *pat* gene) products, or may be related

to the indirect effects of different root exudates compositions altered by insertion of different transgenes in transgenic plants. The basis for the differences between transgenic cultivars and the parental lines is yet to be investigated.

Transgenic plants with other new traits

Transgenic alfalfa producing industrial compounds
The gene from *Bacillus licheniformis* coding for alpha-amylase and that from *Phanerochaete chrysosporium* coding for lignin peroxidase have been introduced into alfalfa (*Medicago sativa* L.) lines to produce the two industrial enzymes (Austin et al., 1995). The two genes were expressed in all parts of the plants with units of soluble protein highest in the root. During a 2-year field study, multiple experimental methods, including soil enzyme activities, Biolog metabolic fingerprinting, DNA fingerprinting of soil bacterial communities by enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR), soil microbial respiration, population counts of indigenous soil microorganisms, were employed to monitor the effects of the two transgenic alfalfas on the rhizosphere bacterial communities. The study results revealed that consistent differences existed in the types of rhizosphere bacteria of the two transgenic alfalfas and the parental alfalfa, particularly of the lignin peroxidase producing alfalfa. Significantly higher levels of culturable, aerobic spore-forming and cellulose-utilizing bacteria, higher soil pH levels, and lower activity of the soil dehydrogenase and alkaline phosphatase were also associated with the lignin peroxidase transgenic alfalfa (Donegan et al., 1999; Giovanni et al., 1999).

Transgenic alfalfa over-expressing the neMDH gene
The transgenic alfalfa plant over-expressing the neMDH gene displays a 1.6-fold increase in the activity of malate dehydrogenase compared with the untransformed alfalfa cultivar. Differences also exist between the alfalfa lines in the amount and composition of root organic acids produced and exuded into the rhizosphere (Tesfayea et al., 2001). The two alfalfa plants were grown at the same field site and rhizosphere soils were collected after 53 weeks of plant growth in order to assess the activities of bacterial communities in the rhizospheres. Nucleotide sequencing of PCR-based 16S rDNA clone libraries and Biolog GN microtiter plates were employed to assess the activity of naturally occurring rhizobacteria in the two alfalfa rhizospheres. There were qualitative changes in the abundance of bacterial phylogenetic groups between rhizosphere soils of transgenic and untransformed alfalfa. Comparisons of the mean average well color development (AWCD), catabolic richness and evenness showed that bacteria in the rhizosphere of transgenic alfalfa utilized significantly more substrates and indicated significantly greater functional diversity than that of untransformed alfalfa. However, there were no significant differences in the populations of total culturable bacteria between the rhizosphere soils of transgenic and untransformed alfalfa (Tesfayea et al., 2003).

Conclusions of studies on the effects of transgenic plants on soil microorganisms

Transgenic plants have been found to have significant effects on soil populations of non-target bacteria and fungi (Ahrenholtz et al., 2000; Donegan et al., 1995, 1999; Giovanni et al., 1999; Lottmann et al., 1999; Lottmann and Berg, 2001; Tesfayea et al., 2003; Wu et al., 2004), soil enzyme activities (Donegan et al., 1999; Giovanni et al., 1999), root colonization of *G. mosseae* (Medina et al., 2003; Vierheilig et al., 1995) and the structure of microbial community (Cowgill et al., 2002; Dunfield and Germida, 2001, 2003; Griffiths et al., 2000; Sessitsch et al., 2003; Siciliano et al., 1998; Siciliano and Germida 1999; Yang et al., 2002). Some of the effects caused by transgenic plants on soil microorganisms are transient and temporary (Donegan et al., 1995, 1997; Wu et al., 2004), occur only at certain growth stages of transgenic plants (Cowgill et al., 2002; Griffiths et al., 2000; Medina et al., 2003; Sessitsch et al., 2003; Yang et al., 2002) or occur only when viable plants are present in the soil (Dunfield and Germida, 2003). In other cases, no effects were found on either specific microorganisms or on microbial community structure (Gyamfi et al., 2002; Heuer et al., 2002; Saxena and Stotzky, 2001; Schmalenberger and Tebbe, 2002, 2003).

Experimental results with transgenic plants producing opines suggest that interactions between a transgenic plant and its root-associated microorganisms are transgene specific and therefore, that any assessment studies relative to the introduction of a transgenic plant are probably valid only for the given transgene (Oger et al., 1997, 2000). For transgenic plants producing antimicrobial proteins such as the T4 lysozyme and PR-2, the significant effects on specific soil microorganisms could be related to the transgene

products. However, because the biological effects of many transgene proteins, such as malate dehydrogenase, PIs and proteins coded by herbicide-resistant genes, on soil microorganisms are unclear, it is impossible to determine whether the observed effects were due to the transgenic nature of the plants.

The unintentional characteristic changes in transgenic plants (especially the changes in the composition of the root exudates), other than the transgene proteins, were speculated to be the causes for some significant effects on soil microorganisms of the transgenic plants (Dunfield and Germida 2001; Gyamfi et al. 2002; Sessitsch 2003; Siciliano et al., 1998; Siciliano and Germida, 1999). However, there is no direct and powerful evidence for this perspective and this hypothesis awaits for further experimental verification.

Evaluation of methods used for assessing the effects of transgenic plants on soil microorganisms

Great strides have been made in the development of new methods to analyze soil microbial communities, but limitations of these methods still exist and prevent the understanding of soil microorganisms comprehensively and extensively (Ogram, 2000). Four types of experimental methods have been employed to assess the effects of transgenic plants on different aspects of soil microorganisms.

The plating method offers a simple but useful tool to identify and characterize the changes of specific strains, but it usually could not detect significant effects of transgenic plants on microbial communities (Donegan et al., 1995; Wu et al., 2004; Saxena and Stotzky, 2001). The reason might be that the great majority of microorganisms in the environment remain unidentified because they are not culturable with standard methods (Ward et al., 1990). The plating method and the most probable number method (MPN) can be used to detect the effects of transgenic plants on specific soil microorganisms such as symbiotic nitrogen-fixing bacteria, degraders of recalcitrant organic matter, nitrifying bacteria recommended by Bruinsma et al. (2003) (Lu, 1999). However, when measuring the effects of transgenic plants on microbial communities, it will be better to combine the plating method with other methods mentioned below.

The substrate utilization assay with Biolog GN microtiter plates is one of the methods for metabolic fingerprinting. This is also a cultural technique and cannot detect the activity of all microorganisms in soil samples. It is impossible to determine the microorganisms responsible for the observed metabolic fingerprints with this method (Haack et al., 1995; Giovanni et al., 1999). However, the method is often used and has proved effective in revealing the effects of transgenic plants on soil microbial communities (Donegan et al., 1995, 1999; Dunfield and Germida, 2003; Giovanni et al., 1999; Griffiths et al., 2000; Heuer et al., 2002; Tesfayea et al., 2003).

DNA fingerprint analysis based on PCR-amplified rRNA genes from soil-extracted DNA enables cultivation-independent analysis of microbial community through the detection and characterization of microbial nucleic acid sequences within samples (Amann et al., 1995). Though more comprehensive for determining differences in microbial community structure than the plating method, these methods still have low resolution and it is difficult to detect subtle changes in banding patterns and the changes in closely related species (Susuki and Giovannoni, 1996). Nonetheless, several DNA fingerprint methods such as DGGE, RFLP, ARDRA and SSCP have been employed to evaluate the effects on soil microorganisms of transgenic plants (Donegan et al., 1995, 1999; Dunfield and Germida, 2003; Giovanni et al., 1999; Heuer et al., 2002; Lottmann and Berg, 2001; Sessitsch et al., 2003).

Similarly to DNA based methods, microbial lipid analysis such as FAME and PLFA are cultivation-independent and are capable of providing a quantitative measure of the viable or potentially viable biomass. Effects of transgenic plants on soil microbial communities often can be detected with the analysis (Cowgill et al., 2002; Dunfield and Germida, 2003). As with DNA fingerprinting the main drawback is the low resolution. Many microorganisms share common fatty acids and may change their fatty acid composition depending on the availability of nutrients and other environmental factors (Drijber et al., 2000; Kozdroj and van Elsas, 2001).

Activity-based assays such as the substrate-induced respiration and the use of soil enzymes have been used to monitor the microbial community changes caused by transgenic plants (Donegan et al., 1999; Giovanni et al., 1999; Wu et al., 2004). These methods can provide information on general microbial activities, but not on specific microbial groups that actually contribute to different types of soil enzymatic activities. Moreover, the activities of soil enzymes and respiration may come from not only microbial

communities but also other organisms such as the protozoa in soil, and are influenced by the microbial community as well as other conditions such as the temperature and water content in soil (Lu, 1999).

Based on the advantages and disadvantages of all of these different methods, it is apparent that the application of multiple methods will provide different information and allow a more accurate and extensive assessment than with the application of any one method of the effects of transgenic plants on microbial communities and processes. In the studies reviewed here, two or three of the above-mentioned methods were generally integrated in a single experiment to evaluate the effects of transgenic plants on soil microorganisms. However, the employment of multiple methods often makes it difficult to interpret the experimental results. For example, significant differences were detected among transgenic alfalfa lines and the parent line in metabolic fingerprints in Biolog GN plates, population levels of culturable bacteria and certain soil enzyme activities, but no differences in DNA fingerprints and rates of microbial substrate-induced respiration (Donegan et al., 1999). The authors suggested from the results that transgenic plants had some effects on soil ecosystem. However, it is difficult to resolve the relationship between the microbial community structure and the various activities that were monitored. Furthermore, the employment of multiple methods also hampers efforts at comparison between studies unless a full set of standardized procedures are used in all studies.

Scales of the studies of the effects on soil microorganisms of transgenic plants

Among the studies reviewed here, some were conducted in contained conditions, i.e. in laboratories (Donegan et al., 1995; Wu et al., 2004) or in greenhouses (Medina et al., 2003; Saxena and Stotzky, 2001; Sessitsch et al., 2003; Vierheilig et al., 1995), and the other studies were field tests (Cowgill et al., 2002; Donegan et al., 1997; 1999; Dunfield and Germida, 2001, 2003; Giovanni et al., 1999; Gyamfi et al., 2002; Heuer et al., 2002; Lottmann et al., 1999; Lottmann and Berg, 2001; Schmalenberger and Tebbe, 2002, 2003; Siciliano et al., 1998; Tesfayea et al., 2003; Yang et al., 2002). One complete experiment consisted of three sequential tiers: laboratory study, then microcosm and pot studies in the greenhouse followed by field tests (Griffiths et al., 2000). These three-tier experiments differ in the research objectives, conditions and results.

For studies in contained conditions, the objectives studied were transgene proteins (Donegan et al., 1995), organs of transgenic plants (Donegan et al., 1995; Wu et al., 2004) or living transgenic plants (Medina et al., 2003; Saxena and Stotzky, 2001; Sessitsch et al., 2003; Vierheilig et al., 1995). The durations of these studies were generally several weeks or months. Because experimental conditions such as the soils, humidity and temperature could be controlled artificially in the contained studies, it was easier to conduct the contained studies, and the results were more repeatable and interpretable compared to that of field experiments (Donegan et al., 1995). However, these studies were highly artificial and are not representative of the natural situation. In studies involving field tests, the durations of these ranged from one to several years, but were normally conducted only for one year. Although field experiments are more realistic than contained experiments, the results were highly variable and difficult to interpret partly due to the fluctuating weather conditions and the complexity of natural soil ecosystem (Cowgill et al., 2002; Dunfield and Germida, 2003; Heuer et al., 2002; Lottmann et al., 1999). Furthermore, field experiments are often costly, time consuming and labor intensive.

Based on the advantages and disadvantages of the contained studies and field experiments, we propose that assessments of the effects of transgenic plants on soil microorganisms begin with experiments in contained conditions which mimic natural conditions. Field trials can then be designed and conducted according to the results of the contained studies.

Prospects

Transgenic plants are considered by many scientists to be critically important to the future of agriculture, and the second generation of transgenic plants containing economically useful and health-related genes is under development in many commercial and academic laboratories (Dunwell, 2002). Although studies reviewed here have improved our understanding of the effects of transgenic plants on soil microorganisms, our knowledge is far from complete. According to the above discussions, we do not know for the time being the relationship between the effects of transgenic plants on soil microorganisms and the amounts of transgenic products in specific soils, organs and root exudates of

transgenic plants. Furthermore, soil microbial communities are very plastic in their species composition and structure and change constantly in different root zones, agricultural practices, and with respect to various other environmental variables (Buckley and Schmidt, 2003; Lipson and Schmidt, 2004.

The present studies have revealed effects of transgenic plants on certain aspects of soil microorganisms, and such studies are important for determining the potential risks associated with the release of transgenic plants. However, what ecological consequences will result from these effects, especially whether or not the microbial functions in maintaining soil quality and improving plant growth will be impacted adversely, remains unknown. Because some ecological consequences, such as the effects on soil fertility and quality, are a function of the temporal scale of the introduction, limited field experiments can not always sufficiently mimic future reality prior to widespread planting of transgenic plants. In addition, there are several fundamental knowledge gaps that need to be bridged for the future efforts in this field. The main knowledge gaps are the very limited understanding of the relationship between the microbial community species composition and its function; the poor knowledge of the structural and functional responses of the microbial community to "normal" variation in soil systems (such as season, weather, crop rotation, pesticide use, etc) (Bruinsma et al., 2003), the conceptual limitation on the relationship of effects and risks, i.e. what effects are detrimental and should be studied further? A better understanding of these issues is essential for designing universal experimental protocols that can be recommended to provide guidance to research scientists and government regulatory agencies for assuring the safety of transgenic plants on soil ecosystems.

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