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Influence of the production of antibacterial and antifungal proteins by transgenic plants on the saprophytic soil microflora

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SUMMARY

To obtain durable and broad-spectrum resistance against plant pathogens, plants are transformed with genes coding for antimicrobial proteins from plant, animal or microbial origin. An obvious concern is that increased levels of these antimicrobial compounds affect not only the target pathogen, but also beneficial micro-organisms such as mycorrhizae, rhizobia and other micro-organisms involved in plant health, litter decomposition and nutrient cycling. This literature study focuses on effects of these transgenic plants on the non-target saprophytic soil microflora.

Transgenic plants that constitutively express proteins with potential antifungal and/or antibacterial activity, can reduce activities of specific soil-borne plant pathogens in the rhizosphere. Reports on non-target effects on the saprophytic soil microflora are scarce and incomplete, and mainly focused on mycorrhizal symbiosis. Constitutive expression of antifungal pathogenesis-related proteins in tobacco in most cases did not affect root colonization by the mycorrhizal fungus *Glomus mosseae*. However, increased levels of a class II tobacco β-1,3-glucanase reduced the colonization potential, indicating that non-target effects can occur. Concerning other members of the plant-beneficial rhizosphere microflora, it can be assumed that they will come into contact with the transgenic product. By natural wounding, senescence and sloughing-off of root cells, at least some of the antimicrobial protein(s) will be released in the rhizosphere. Despite proteolytic activity of the rhizosphere microflora, part of the protein can remain active due to protective adsorption to clay minerals or humic components.

Key-words: pathogenesis-related proteins, antimicrobial proteins, transgenic plants, soil microflora, vesicular arbuscular mycorrhizae, review.

INTRODUCTION

Plants exhibit natural resistance to most potential pathogens, and disease is the exception rather than the rule. Yet, specific pathogens are adapted to specific plant species and are able to circumvent or suppress host resistance mechanisms. Pathogens

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of cultivated crops are a major threat and many examples of devastating epidemics in agriculture exist (Agrios 1988). In order to increase crop production, plant breeding programmes have focused on selection of high-yielding cultivars and the basis for genetic traits other than agricultural performance has been narrowed. Although major resistance genes against specific diseases have been retained or bred in, the level of general resistance is likely to be decreased. Moreover, continuous cropping practices on large surface areas favour rapid spread of diseases for which resistance genes are not available or have become ineffective. Classical breeding of disease resistant cultivars has focused on vertical resistance, thereby conferring plant resistance to only specific races of the pathogen. This classical approach is costly and often too time-consuming to react adequately to the evolution of new virulent races of pathogens (Cornelissen & Melchers 1993).

In the last decade genetic engineering technology for plants has become available. Currently it is being applied to obtain non-conventional types of disease resistance. To acquire resistance to specific races of fungal or bacterial pathogens, recombinant DNA technology is used to identify and isolate major resistance genes that, upon transfer, can render susceptible plant cultivars or species resistant (Cornelissen & Melchers 1993; Lamb *et al.* 1992). Other approaches involve, for example, expression in transgenic plants of microbial enzymes that reduce virulence by inactivating toxins produced by the pathogen (De la Fuente-Martínez *et al.* 1992), of fungal toxins that kill related, sensitive strains of the pathogen (Kinal *et al.* 1995), or of antibodies directed against enzymes produced by the pathogen (Van Engelen *et al.* 1994). To confer resistance to viral pathogens, constitutive expression in transgenic plants of the gene coding for the coat protein of the virus has proven highly successful (Van den Elzen *et al.* 1993). A limitation of both cultivar-specific vertical resistance and introduction of single resistance genes in certain cultivars is that these plants obtain resistance only to specific (races of the) pathogens and remain sensitive to other pathogens. Moreover, selective pressure will favour the evolution of new virulent races of the pathogen when newly bred transgenic crops are grown on a large scale (Cornelissen & Melchers 1993; Rissler & Mellon 1993).

To obtain both broad-spectrum and durable resistance against fungal and bacterial pathogens, various strategies are being employed (for review see Cornelissen & Melchers 1993; Lamb *et al.* 1992). Most research is focused on constitutive expression of activities that become manifest only when a plant is induced to defend itself effectively against pathogenic attack, such as during a hypersensitive reaction. Although the induction of these defence responses is pathogen-specific, the responses themselves are not. Commonly, an integrated set of responses is coordinately induced, with various activities acting synergistically to reduce pathogen penetration, multiplication, spread and reproduction. These inducible responses include reinforcement of cell walls by deposition of lignin-like polymers and structural proteins, formation of low-molecular-weight antimicrobial phytoalexins and accumulation of high-molecular-weight 'pathogenesis related' (PR) proteins with potential antimicrobial activity. Since activities such as phytoalexin biosynthesis or lignin deposition in the cell wall require the action of several genes (Lamb *et al.* 1992), these activities are not easy to manipulate. On the other hand, PR-proteins are individually coded by single genes and, therefore, directly amenable to manipulation by gene transfer. Transgenic plants which constitutively express one or more PR-proteins are available and have been demonstrated to be more resistant to selected plant pathogens (Broglie *et al.* 1991; Alexander *et al.* 1993;

Vierheilig *et al.* 1993; Van den Elzen *et al.* 1993; Lawton *et al.* 1993; Yoshikawa *et al.* 1993; Liu *et al.* 1994; Zhu *et al.* 1994; Jach *et al.* 1995; Jongedijk *et al.* 1995; Lin *et al.* 1995).

Other groups of broad-spectrum antimicrobial plant proteins that can enhance disease resistance in transgenic plants are plant defensins (specific low-molecular weight cysteine-rich seed proteins) (Terras *et al.* 1995), ribosome inhibiting proteins (RIPs) (Logemann *et al.* 1992; Jach *et al.* 1995) and thionins (Carmona *et al.* 1993; Florack 1994). The search for effective antimicrobial genes has not been restricted to higher plants. Plants have also been modified with genes coding for chitinase or chitosanase from bacteria (Howie *et al.* 1994; Elquakfaoui *et al.* 1995), antimicrobial peptides of animal origin (e.g. cecropins) (Jaynes *et al.* 1993; Allefs *et al.* 1995) and lysozyme from bacteriophage T4 (Düring *et al.* 1993) or hen egg white (Trudel *et al.* 1995).

Introduction of transgenic crops into the environment may impose several non-target effects on the ecosystem (Rissler & Mellon 1993; Morra 1994). For example, effects may be caused by dispersal of transgenic plants in wild populations (Van Raamsdonk & Schouten 1997, this issue). Other non-target effects may be caused by the presence of selection markers and reporter genes used during selection of the transgenic plant (Metz & Nap 1997, this issue). For transgenic crops that express antimicrobial proteins, an obvious possibility is that increased production of broad-spectrum, antimicrobial components suppresses not only target pathogens but also influences plant symbionts such as mycorrhizae or rhizobia. Other non-target groups that could be affected are plant beneficial rhizosphere-inhabiting micro-organisms, including those involved in the decomposition of decaying plant material and nutrient cycling. This study addresses possible effects of transgenic plants, with increased levels of antimicrobial proteins, on the saprophytic soil microflora.

PRODUCTION OF ANTIMICROBIAL PROTEINS BY TRANSGENIC PLANTS

Levels of antimicrobial proteins produced in transgenic plants

Commonly, genes of interest are placed under the control of the cauliflower mosaic virus (CaMV) 35S promotor, ensuring constitutive expression in all plant organs, including roots. In some cases, wound-inducible promoters such as from the potato *wun* I (Logemann *et al.* 1992) or proteinase inhibitor II genes (Jaynes *et al.* 1993) are used for expression of the foreign gene. Expression then occurs only during wounding of the plant, which usually accompanies pathogenic attack. So far, no reports have been published on the use of tissue–specific promoters that would prevent gene expression in tissues that are not affected by the target pathogen.

In transgenic plants, levels of antimicrobial proteins may reach levels found in untransformed plant organs as a result of, for example, pathogenic attack. In some further examples, chitinase concentrations in roots of transgenic plants were seven times higher than in roots of ethylene-induced, untransformed plants (Neuhaus *et al.* 1991). Expression of bean chitinase under the control of the 35S promoter in transgenic tobacco resulted in a similar chitinase activity in all plant tissues. Compared to non-induced control plants, chitinase activity in roots of transgenic plants was only 2–4 times higher, whereas in leaves the activity was increased 23–44 times (Broglie *et al.* 1991).

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Several groups of antimicrobial proteins such as the basic PR-proteins of tobacco, thionins or RIPs are localized intracellularly. When targeted to the intercellular space, these proteins are assumed to be effective towards the pathogen at an earlier stage of infection (Neuhaus *et al.* 1991; Vierheilig *et al.* 1993; Howie *et al.* 1994; Jach *et al.* 1995). There is no clear evidence to support this hypothesis, since extracellularly targeted chitinases or RIPs were not more effective in the control of *Rhizoctonia solani* than their vacuolar forms (Vierheilig *et al.* 1993; Howie *et al.* 1994; Jach *et al.* 1995). However, it can be assumed that vacuolar proteins also reach the intercellular space and rhizosphere of plants as a result of natural phenomena such as wounding and senescence of roots.

The actual concentration of the transgenic compounds in the rhizosphere can be reduced by proteolysis or binding of the product *in planta*. Examples are proteolysis of cecropin B by plant proteases (Florack *et al.* 1995) or the binding of transgenic lysozyme to tobacco component(s) (Trudel *et al.* 1995). Although it has not been explicitly investigated for class I chitinases, glucanases and other vacuolar antimicrobial PR-proteins, these are likely to be highly resistant towards proteolytic degradation by endogenous, intracellular, plant proteases (Van Loon 1985; Van Loon & Gerritsen 1989). Only specific aspartyl proteinases, active exclusively at low pH values, have been found to be able to degrade PR-proteins from tomato (Rodrigo *et al.* 1989) and tobacco (Rodrigo *et al.* 1991, Linthorst 1991).

Release of antimicrobial proteins into the soil

There are several ways in which transgenic plant products can be introduced into the soil. First, through rhizodeposition of living plants (Lynch & Whipps 1991). Plant roots can actively secrete extracellularly targeted antimicrobial proteins into the rhizosphere, or the product can be passively lost from roots during tissue senescence, sloughing-off of root cap cells, natural wound openings occurring during formation of lateral roots, or injury. Passive loss probably applies to both vacuolar and extracellularly localized gene products. For example, approximately 5000 root cap cells are lost from the roots of a single maize plant each day (Moore & McClelen 1983). Some plants, especially grasses, contain most of their biomass in the form of roots and most crop species have root systems of several hundred metres per plant. Since rhizodeposition by plant roots is a major source of organic nutrients, proliferation of micro-organisms is strongly stimulated around plant roots. Thus, in the rhizosphere many micro-organisms are likely to come into contact with transgenic plant products.

Another way of introduction of transgenic plant products into soil is the return of plant residues after crop harvest. In no-tillage procedures, plant residues are not mixed into the soil but come in contact with the soil top layer only. In conventional-tilling, however, residues are incorporated into and mixed with deeper soil layers. Furthermore, in both cultivation systems transgenic plant products will be released by the decaying root system, that is not harvested.

No information is available on levels of transgenic or even naturally produced plant enzymes in the rhizosphere, because of difficulties in extracting enzymes from soil (Dick 1992). It is proposed that extracellular plant enzymes are secreted by living cells in the aqueous phase of the soil, whereafter they are degraded or adsorbed to humic substances or clay minerals (Burns 1982). Indeed, growing roots of transgenic tobacco seedlings that constitutively express the gene for lysozyme (from hen egg white) were found to release active lysozyme, as evidenced by a lytic zone around the root during growth on agar plates containing test micro-organisms (Trudel *et al.* 1995). However,

β-1,3-glucanase and chitinase produced by sterile barley were found to remain firmly bound to barley root surfaces and were not released into the cultivation medium (water) (Sotolová *et al.* 1989; Hanzlíková *et al.* 1989). Enzymes that are produced intracellularly, and are released from lysing cells, will probably leak passively into the rhizosphere, where degradation or adsorption will occur.

Stability of antimicrobial proteins in the soil

Information on the stability of plant-encoded (antimicrobial) proteins in soil is generally lacking. However, it is known that the soil microflora exhibits proteolytic activity, and protease activity is found to be significantly higher in the microbially active rhizosphere than in bulk soil (Jandera *et al.* 1989). For example, attacin and cecropins are readily degraded by a protease from *Bacillus thuringiensis in vitro* (Dalhammer & Steiner 1984). Besides enzymatic degradation, non-biological denaturation and inactivation can occur through pH shifts and changes in temperature or moisture (Burns 1982). On the other hand, enzymes adsorbed to inorganic clay minerals or organic humic colloids (Sarkar *et al.* 1989; Dick 1992) can show enhanced resistance to proteolysis in comparison to enzymes in the aqueous phase of the soil (Sarkar *et al.* 1989; Burns 1982). A single example addressing this question concerns the proteinaceous *Bacillus thuringiensis* delta toxin. Adsorption of this protein to clay was essentially complete within 30 min (Tapp *et al.* 1994). Toxin bound to clay was immunologically detectable in non-sterile soil after 40 days (Tapp & Stotzky 1995a) being more resistant to degradation by micro-organisms than free toxin (Tapp & Stotzky 1995b). Moreover, the insecticidal activity of the toxin was retained and sometimes enhanced by adsorption (Tapp & Stotzky 1995b). However, the amount of enzyme immobilized and the stabilizing action on enzyme activity will be highly dependent on soil type (Burns 1982; Tapp & Stotzky 1995b).

One study has addressed the stability of microbial and plant derived β-1,3-glucanase in a silty loam soil (Lethbridge *et al.* 1978). Laminarin degrading activity did not decrease during 28 days of storage at 4, 25 or 50°C. In these experiments a bacteriostatic agent was included, so no active bacterial microflora was present. In the presence of an active rhizospheric microflora, chitinase and β-1,3-glucanase activity were observed in the rhizosphere of germinating barley and they were higher than in bulk soil (Hanzlíková *et al.* 1989; Sotolová *et al.* 1989). Glucanase and chitinase that were tightly bound to the root surface accounted for the major part of the enzyme activity. Based on the findings that under sterile conditions plant-derived chitinases and glucanases were closely associated with the root surface, root surface-bound enzymes can be assumed to be of plant origin. However, microbial enzymes and other proteins have been demonstrated to be absorbed by or adsorbed to plant roots (McLaren *et al.* 1960); thus, part of root-bound enzyme activity can be of microbial origin. Although far from conclusive, these studies suggest that at least part of the plant enzymes produced in the rhizosphere can be active there and be protected from immediate degradation.

EFFECTS OF ANTIMICROBIAL PROTEINS ON THE MICROFLORA

Plant-encoded antimicrobial enzymes will be present mainly in root tissues or in close vicinity of roots as part of the rhizodeposition. The rhizodeposition of plant roots provides the main source of organic substrate for micro-organisms in soil and microbial growth is especially stimulated in and around roots. When an antimicrobial protein is

introduced into the rhizosphere, it may affect specific plant-beneficial components of the saprophytic rhizosphere microflora and thereby affect plant functioning. Obvious non-target micro-organisms include symbionts such as vesicular arbuscular (VA) mycorrhizal fungi and symbiotic nitrogen-fixing bacteria, such as rhizobia. Other bacteria and fungi that interfere with plant pathogens or are involved in decomposition and in nutrient cycling can similarly be affected. These groups of micro-organisms and possible effects of transgenic plants on these groups are described in the next section.

Mycorrhizae

Under low phosphate conditions, most plant roots form a symbiotic association with mycorrhizae. Two major groups of mycorrhizae are recognized: the ectomycorrhizae, common to gymnosperms and many wood angiosperms, and the vesicular–arbuscular mycorrhizae (VAM) (Morton 1988). Most agronomically important crops, except for the *Cruciferae*, *Chenopodiaceae*, *Cyperaceae* and *Caryophyllaceae*, are colonized by VAM fungi (Millner 1991). These fungi, for which about 150 species have been described (Morton 1988; Morton & Bentivenga 1994) have the potential to enhance plant growth through increased uptake of water and nutrients (Marschner & Dell 1994), resistance to drought and defence against pathogens (Toth *et al.* 1990; Hwang *et al.* 1992; Linderman 1994). In addition, they play a role in soil aggregation (Abbott & Gazey 1994). However, their contribution to crop growth under field conditions is far from clear (Abbott & Gazey 1994; Morton & Bentivenga 1994). Plants are commonly colonized by more than one VAM strain (Read 1991) and no real evidence for host–fungal species specificity exists (Millner 1991).

Infection of plant roots by a VAM fungus takes place when resting chlamydospores germinate and form hyphae that penetrate host cell walls. The fungal hyphae proliferate both outside and inside the root, with the internal hyphae differentiating into arbuscules and vesicles that function in nutrient exchange and food storage. Induction of some defence reactions of the host during the establishment of endomycorrhizal symbiosis has been suggested (Volpin *et al.* 1994; Lambais & Mehdy 1995; Liu *et al.* 1995; Blee & Anderson 1996), including increases in chitinases and/or β-1,3-glucanases (Spanu *et al.* 1989; Volpin *et al.* 1994; Dumas-Gaudot *et al.* 1994; Vierheilig *et al.* 1994). Chitinase activity was increased in roots of leek during the early stages of symbiosis with *Glomus versiforme*. However, mycorrhizal roots contained much less chitinase than uninfected roots when symbiosis was fully established (Spanu *et al.* 1989). A similar phenomenon was observed for the VAM fungus *Glomus intraradix* in its association with alfalfa roots (Volpin *et al.* 1994). Dumas-Gaudot *et al.* (1994) and Dassi *et al.* (1996) found evidence for the induction of a special isoform of acidic chitinase upon *Glomus mosseae* infection of pea plants. This specific chitinase was not detected in uninfected roots, nor in roots of mutated pea plants that are resistant to mycorrhizal infection. In general, defence-related components, such as chitinase, are only slightly or transiently induced and limited to the very early stages of infection (Spanu *et al.* 1989; Lamblais & Mehdy 1993; Volpin *et al.* 1995). These observations indicate that the plant defence response is somehow controlled or suppressed during symbiosis establishment (Gianinazzi-Pearson *et al.* 1994).

VA mycorrhizae contain up to 27% of chitin in their cell walls. Extracellular hyphae are usually very thick and embedded in a non-chitinous matrix, the so-called polysaccharide sheath. This sheath is probably less developed in penetrating hyphae and during intercellular growth (Miller 1993). Nevertheless, Spanu *et al.* (1989) observed that in

leek roots mycelium of *G. versiforme* does not bind chitinase. It has been proposed that the chitin layer of intercellular hyphae is covered with proteins or alkali-soluble polysaccharides (Bonfante-Fasolo & Grippiolo 1982) which render them inaccessible to plant enzymes such as chitinases. Based on this study alone, however, it cannot be excluded that transgenic plants producing high, constitutive levels of chitinase could lyse intercellularly growing hyphae and affect mycorrhizal symbiosis (Miller 1993).

Three studies have addressed this question. Tahiri-Alaoui *et al.* (1994) focused on the effect of constitutive expression of either a basic bean endochitinase or an endochitinase from the hyperparasitic fungus *Aphanocladium album* on the symbiosis of transgenic tobacco with arbuscular mycorrhizae. By using enzyme markers, the metabolic state of the fungal tissue during development of the mycorrhizal association was monitored. No effects of constitutive chitinase expression were evident on the metabolic activity of intraradical hyphae, nor on ultrastructural aspects of the symbiosis. Surprisingly, transgenic plants expressing bean chitinase were more receptive to mycorrhizal infection than untransformed control plants, despite their higher chitinase activity.

Vierheilig *et al.* (1993) reported that constitutive expression of tobacco vacuolar chitinase in transgenic *Nicotiana sylvestris*, resulted in 14 times higher chitinase levels in roots, but did not significantly affect the colonization potential of the VAM fungus *G. mosseae* when measured after 8 weeks. In contrast, colonization by *Rhizoctonia solani* was significantly reduced in these same plants after 5 weeks of growth. No differences in fungal structures were seen when mycorrhizal roots of control and transgenic plants were observed under a light microscope. Recently, effects of increased levels of several PR-proteins, including chitinases, on colonization by *G. mosseae* were studied in transgenic tobacco (Vierheilig *et al.* 1995). Constitutive expression of either acidic tobacco PR-1a, chitinase (PR-3), PR-4, PR-5 or class III β-1,3-glucanase did not affect mycorrhizal symbiosis. Neither was symbiosis reduced by expression of tobacco basic chitinase or glucanase, a combination of the latter two, or by an acidic cucumber chitinase. Targeting the basic tobacco chitinase to the intercellular space had no additional effect. Interestingly, a two-fold delay in colonization by *G. mosseae* was demonstrated in tobacco plants constitutively expressing an acidic tobacco β-1,3-glucanase (class II). In these plants, light microscopy showed distinct differences in fungal structures compared to control plants. It is not clear whether this glucanase acts directly on the mycorrhizal fungus or has an indirect effect, for example by releasing elicitors from the fungal cell wall that stimulate defence responses in the host. The presence of β-1,3-glucans has recently been established in selected *Glomus* species (Lemoine *et al.* 1995). The lack of effect on the mycorrhizal symbiosis in the transgenic plants that express high levels of the other glucanases and chitinases, alone or in combination, may be explained by the proposed protective layer around the fungal hyphae (Bonfante-Fasolo & Grippiolo 1982). Why such a layer would not protect against class II acidic tobacco β-1,3-glucanase, remains an open question.

These studies indicate that high levels of homologous or heterologous antifungal PR-proteins in transgenic *Nicotiana* species can, but do not necessarily, affect mycorrhizal symbiosis. However, more generalized conclusions can only be drawn when also other host-VAM combinations are studied. VAM fungi could well be affected by transgenic plants coding for RIPs or thionins, since these components exhibit antifungal properties *in vitro*. However, no literature is available on effects of such plants on VAM fungi.

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Rhizobia

Under conditions of nitrogen limitation, *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, collectively referred to as rhizobia, elicit on their leguminous hosts the formation of specialized organs, nodules. In these root or stem structures, the bacteria are able to convert atmospheric nitrogen into ammonia, which is used by the plant as a nitrogen source (Van Rijn & Vanderleyden 1995). The host range of individual bacterial species and strains can be either broad or narrow (Fisher & Long 1992).

Nodule initiation can be characterized as a two-way molecular conversation between bacterium and plant. The host legume releases host-specific flavonoid signals, that stimulate the coordinate expression of bacterial genes that are required for nodulation (*Nod* genes). These *Nod* genes, in turn, encode enzymes necessary for the synthesis of *Nod* factors, that cause morphological changes in the plant root. The *Nod* factors are oligomers of N-acetylglucosamine, with different host-specific modifications, that apparently determine host specificity (Vijn *et al.* 1993). The bacteria invade the plant by means of infection threads which are initiated from curled root hairs and grow towards the root cortex (Fisher & Long 1992). Simultaneously, inner cortical cells dedifferentiate and start dividing. In the nodule primordium the rhizobia differentiate into bacteroids, that are the actively nitrogen-fixing organelles.

There is no clear evidence for the elicitation of a plant defence response during a compatible interaction in which effective, nitrogen-fixing nodules are formed. One of the rhizobial strategies to evade plant defence during infection seems to be the presence of specific extracellular polysaccharides (EPS) (Parniske *et al.* 1994) or lipopolysaccharides (LPS) (Perotto *et al.* 1994). Autoregulation of nodule formation by *Rhizobium meliloti* on its host alfalfa seems, however, dependent on induction of a plant defence response (Vasse *et al.* 1993). After the first nodule primordia have been induced, an increasing proportion of infection threads abort before a nodule is initiated. This abortion takes place in a single or a few root cortical cells in which both symbionts simultaneously under go necrosis, similar to a hypersensitive response (HR) (Vasse *et al.* 1993). The authors propose that the elicitation of the HR is part of the mechanism by which the plant controls infection and, thereby, regulates nodulation.

Of particular interest is the observation that plant chitinases can inactivate *Nod* factors produced by *Rhizobium* and *Bradyrhizobium* strains (Roche *et al.* 1991; Staehelin *et al.* 1994a). Cytochemical and immunological characterization of abortive infection during the *R. meliloti*-alfalfa symbiotic interaction has led to the idea that acidic chitinases that accumulate in cells undergoing a hypersensitive reaction, can specifically hydrolyse the oligomeric N-acetylglucosamine backbone of *Nod* factors (Vasse *et al.* 1993). The finding that structural modifications in *Rhizobium meliloti* nodulation factors influence their stability against root chitinases has supported the notion that chitinases, at least in part, may regulate activity and specificity of rhizobial nodulation factors (Staehelin *et al.* 1994a,b). Thus, chitinases from pea, bean and soybean are able to lyse *Nod* factors differentially (Collinge *et al.* 1993). Pea and bean chitinase did not influence *Nod* factors that cause nodulation of pea and bean roots, but soybean chitinase was active on pea and bean, as well as on soybean *Nod* factors. It has been postulated that rhizobial *Nod* factors resemble comparable glycolipid structures of plant origin (Fisher & Long 1992). These compounds may be endogenous substrates for chitinases, and may give rise to plant signal molecules. Indeed, molecules resembling bacterial *Nod* factors have been observed following chitinase treatment of butanol extracts from *Lathyrus*

flowers (Collinge *et al.* 1993). Conversely, bacterial *Nod* factors can substitute for a specific chitinase in the requirement to rescue a carrot mutant blocked in somatic embryogenesis (Schmidt *et al.* 1994). These results suggest that chitinases may also be involved in rhizobium-plant signalling. Although transformation and regeneration of leguminous plants is far from routine, it can be assumed that in these plants expression of high constitutive levels of heterologous chitinases could interfere with rhizobial symbiosis.

It is not clear to what extent chitinases of microbial origin can influence symbiosis. *R. meliloti*, transformed with a chitinase gene from *Serratia marcescens*, demonstrated no decrease in nitrogenase activity on alfalfa compared to its wild type (Sitrit *et al.* 1993). Antibacterial compounds, such as thionins, cecropins or lysozymes, could interfere with the symbiosis by affecting the bacterium itself. However, no information is available on this subject.

Other antagonistic and plant growth-promoting micro-organisms

Many other species of rhizosphere-inhabiting fungi and bacteria have been demonstrated to stimulate plant growth in many crops (i) directly, by increasing nutrient availability or producing plant growth regulators or (ii) indirectly, by affecting plant pathogens or deleterious rhizobacteria through parasitism, competition, antibiosis or induction of resistance in the plant. Many genera of fungi and bacteria exhibit antagonistic and plant growth-promoting activities, as demonstrated in different plant-pathogen combinations and growing conditions (Weller 1988; Gerhardson & Larsson 1991). The importance of the antagonistic microflora in disease suppression is best illustrated by the suppresssiveness of certain soils to plant pathogens (Cook & Baker 1983). Many genera of fungi and bacteria have been implicated in this phenomenon, among them *Trichoderma*, non-pathogenic *Fusarium* and fluorescent *Pseudomonas* spp. (Chet & Baker 1981; Alabouvette 1990; Schippers 1992).

So far, only one study has been directed at studying non-target effects of transgenic plants coding for PR-proteins on specific groups of the saprophytic soil microflora (DeGraeve 1994). The non-mycorrhizal oil seed rape, constitutively expressing bean endochitinase, was tested for its effect on root-associated total bacteria, total fungi, *Bacillus* spp., fluorescent pseudomonads and *Pseudomonas corrugata* populations in three soil types. *Bacillus* spp. and pseudomonads were included because bean endochitinase has weak lysozyme activity. After 45 days of plant growth in a rhizotron, plate countings revealed no consistent differences in numbers of the various members of the microflora obtained from roots of transgenic and control plants. Since *P. corrugata* proved to be the dominant culturable species in the rhizosphere of oil seed rape, genotypic and phenotypic differences in this population were studied using several biochemical and genetic methods. Among others, a Biolog system was used to determine differences in utilization of several carbon substrates. To compare strains at the genetic level, specific DNA fragments were amplified by PCR-RFLP. In addition, certain regions of the 16S ribosomal RNA genes of strains were compared. No differences were demonstrated between strains present on transgenic and control plants. Thus, no direct or indirect effects of increased levels of chitinase on the culturable, saprophytic microflora were evident. However, except for the *P. corrugata* population, no information was sought on potential qualitative changes within the microflora.

Micro-organisms involved in organic matter decomposition and mineralization

All soil heterotrophic micro-organisms are saprophytes that require organic carbon and nitrogen for growth. The organic matter content of the soil, consisting mainly of plant litter and root-derived carbon compounds, represents the dominant food source. Decomposition of plant residues not only supplies carbon, but also releases other essential elements, particularly nitrogen, phosphorus and sulphur (mineralization). Although soil animals are intimately involved (Lee & Pankhurst 1992), it is the microbial activity that is the driving force in nutrient cycling in soil (Van Veen *et al.* 1989). Microbes involved in nutrient cycling are particularly abundant in the rhizosphere, where root exudates and dead root tissue may comprise up to 30–40% of the total input of organic matter in soils.

No information is available on possible effects of transgenic plants on micro-organisms involved in nutrient cycling. DeGraeve (1994) looked into total numbers by plate counting, but did not address soil microbial activities. From studies in which non-target effects of pesticides on micro-organisms were investigated, it has become clear that microbial activities are only transiently influenced, even when various pesticides are added in succession (Atlas *et al.* 1978). If non-target effects of pesticides on soil fertility occur, they predominantly affect the nitrogen and the carbon cycle (Bollen 1979). This is understandable because only a few genera of specialized microbes are involved in these cycles. Non-target effects on less specialized micro-organisms will be less evident, since those can be replaced by other, more tolerant, species (Bollen 1979).

It can be concluded that the data provided by DeGraeve (1994) are a valuable start but are insufficient to conclude that transgenic plants do not affect the soil microflora. The study of DeGraeve particularly lacks in-depth information on possible effects on the fungal population, a primary target group for transgenic chitinases. Moreover, only total numbers of culturable members of the microflora were studied. Since it is estimated that only 1–10% of the soil microflora is culturable (Ward *et al.* 1990), plate counts give information on a minor part of the microflora. Nothing has been reported so far on effects on the activities involved in, for example, nutrient cycling. Information is fully lacking on non-target effects of transgenic plants producing combinations of PR-proteins, increased levels of RIPs, etc. on the saprophytic fungal population. Further data also need to be obtained on non-target effects of various antibacterial proteins on the bacterial microflora.

METHODS FOR STUDYING EFFECTS ON THE SOIL MICROFLORA

It is evident that current knowledge about non-target effects of transgenic plants on functional groups of micro-organisms in the rhizosphere is insufficient to draw any conclusions. Yet, broad-spectrum antimicrobial compounds will be produced by transgenic plants at possibly high levels in the rhizosphere throughout one or repeated cropping seasons. Changes in the rhizosphere microflora are likely to occur and may be long lasting.

For symbionts such as rhizobia and VA mycorrhizae, relatively straightforward methods are available to measure the impact of transgenic plants on their activities. In the case of rhizobia, the numbers of active nodules on transgenic and control plants can

be counted. The presence of specific rhizobial strains in nodules can be demonstrated by antibiotic resistance, genetic markers or specific antisera (Schmidt 1991). Effects on VA mycorrhizal colonization can be assessed by studying the percentage of root length infected by the fungus (Giovannetti & Mosse 1980; Green *et al.* 1994), or measuring specific enzymatic activities (Bothe *et al.* 1994, Tahiri-Alaoui *et al.* 1994) under defined conditions (Wyss *et al.* 1992). Until recently, no reliable methods were available to define species of VA mycorrhizae, especially *Glomus* spp., when growing symbiotically, distinction between strains being based exclusively on spore morphology (Millner 1991; Morton & Bentivenga 1994). However, recently a molecular method was reported to identify specific isolates of *Glomus mosseae*, also during root colonization, by using specific primers (Lanfranco *et al.* 1995). This method may be used in the near future to determine qualitative changes in the mycorrhizal symbiosis under field conditions.

Difficulties arise for measuring the impact on other functional groups of rhizosphere micro-organisms, due to our lack of knowledge on the complex interactions that occur in the soil and rhizosphere. However, a variety of methods has been used to assess changes in the soil and rhizosphere microflora as a result of cultural management procedures, including pesticide application. Basically, two approaches have been taken. Within the first approach the microbial community is considered as a single entity, the so-called biomass (Powlson 1994). Measurements to study the size and activity of the biomass include total cell numbers by microscopy (Atlas *et al.* 1978; Brendecke *et al.* 1993), soil respiration (Wardle *et al.* 1993; Powlson 1994; Heilman *et al.* 1995), total community enzyme activity (Bolton *et al.* 1985; Beese *et al.* 1994; Kennedy & Smith 1995), or physiological characteristics (Winding 1994; Garland & Mills 1994). Although these methods provide valuable information on overall microbial activity and nutrient cycling, measurements are averaged over the entire community, with little attention given to a mechanistic understanding of the individual components and their variation under different conditions.

In contrast, the second approach is focused on changes in the composition of the soil community. So far, the most commonly used method is quantification of groups of culturable soil micro-organisms (Bolton *et al.* 1985; Harris *et al.* 1995). Thereafter individual micro-organisms, or groups of micro-organisms, can be tested for a variety of genetic, biochemical and biological properties (Atlas *et al.* 1991; Beare *et al.* 1993; DeGraeve 1994; Lemanceau *et al.* 1994; Kennedy & Smith 1995). Although in this way valuable information on soil communities is obtained, it must be stressed that only a very limited part of the microbial community is considered. Only 1–10% of the soil bacteria are culturable, and the major part is defined as 'viable but non-culturable' (Ward *et al.* 1990). Although it is assumed that the overall diversity of the soil community is reflected in the culturable part, Torsvik *et al.* (1990) demonstrated the complexity of soil-extracted microbial DNA to be about 200 times higher than the diversity of microbial DNA after culturing of soil samples.

More recent techniques therefore omit the culturing step and, instead, make use of direct soil extraction of total cell mass and/or DNA, followed by biochemical or molecular methods to analyse community structure. Differences in community structures have been demonstrated by analysis of fatty acids (Cavigelli *et al.* 1995), lipopolysaccharides and phospholipids (Zelles *et al.* 1995) in soil samples. Another promising approach is analysing 16S (bacterial) and/or 18S (fungal) ribosomal RNA (rRNA) sequences as fingerprints of microbial communities (Lee 1994). Initially, this method made use of 16S/18S rRNA sequences that were directly extracted from soil.

Nowadays, rRNA sequences are isolated and amplified either from complementary DNA (cDNA) generated from extracted RNA, or more directly from rRNA genes present in extracts of total genomic DNA. Because of the presence of both conserved and hypervariable regions in rRNA molecules, micro-organisms can be identified at the taxon and species level. A disadvantage of these latter methods (e.g. fatty acid analysis and 16S/18S rRNA analysis) is that there is not necessarily a direct link between the number of species and their activity in the soil.

It will be clear that all methods described above have their limitations. Preferentially, several approaches should be integrated in order to gain an understanding of the links between community structure and function, and their alterations due to perturbation.

DISCUSSION

Disturbances of crop ecosystems are by no means uncommon in agriculture; alterations in the biological component of the soil have been demonstrated as resulting from organic amendments, crop rotation, or other crop management regimes (Baker & Cook 1974; Dick 1992; Scott Angle 1994; Pankhurst *et al.* 1995). Although the mechanisms involved are often unclear, such regimes can increase crop yield through suppression of soil-borne plant pathogens (Baker & Cook 1974; Cook & Baker 1983). In general, organic amendments or tillage of crop residues reduces the activity of pathogens through a relatively non-specific stimulation of biological activity (Dick 1992) resulting in increased soil mycostasis (Cook & Baker 1983). Crop rotation suppresses activity and survival of soil-borne plant pathogens in the absence of the appropriate host, and can at the same time increase activity of the microbial biomass (McGill *et al.* 1986; Dick 1992). It is assumed that the saprophytic microflora changes upon cultivation of a different crop, by changes in root exudates and physical conditions (Weller 1988). However, changes in the microbial community have only been clearly demonstrated for symbiotic micro-organisms such as mycorrhizae and rhizobia (Miller 1993; Ryan *et al.* 1994).

To reduce crop losses due to plant pathogens, mainly physical and chemical methods of plant protection are being employed. These methods are directed towards eliminating harmful micro-organisms, with beneficial micro-organisms receiving scant attention. Physical soil treatments, such as steaming at high temperatures, have a rather drastic impact on the soil microflora. Steam disinfection can eliminate all or most part of the (antagonistic) microflora, thereby creating a biological vacuum that can foster growth of accidently introduced pathogens (Baker & Cook 1974). However, steaming at lower temperatures and milder methods like solarization have demonstrated that the biological balance can be shifted in favour of surviving or recolonizing antagonists and can even create disease-suppressive soils (Baker and Cook 1974; Stapleton & DeVay 1984; Greenberger *et al.* 1987). This indicates that a shift in the soil microflora is not necessarily unfavourable for plant health. The outcome will depend on how the antagonistic potential of the microflora is affected.

Control of plant diseases with chemical pesticides is generally effective but may be hazardous to the environment. Various unexpected side-effects were observed after large-scale application of chemical pesticides for the control of plant diseases, due to non-target effects on the soil microflora. (Bollen 1979; Jalali 1979; Rodriguez-Kabana & Curl 1980; Beare *et al.* 1993). For this reason, one of the prerequisites for admission of pesticides for plant pathogen control in several countries is a study on effects of the

non-target saprophytic microflora (Atlas *et al.* 1978). Most studies published so far indicate that effects of these pesticides are transient and reversible, as far as total cell numbers and/or activity of the microflora are concerned (Atlas *et al.* 1978; Lewis *et al.* 1978; Bollen 1979; Rodriguez-Kabana & Curl 1980; Banerjee & Dey 1992). However, for some broad-spectrum pesticides unexpected, long lasting, non-target effects have been observed, especially after repeated applications over long periods (Jenskinson & Powlson 1970; Bollen 1979; Jalali 1979; Rodriquez-Kabana & Curl 1980). Although these broad-spectrum pesticides may be more toxic and persistent in the soil than antimicrobial proteins produced by transgenic plants, the latter will also be present in the rhizosphere during one or several cropping seasons.

Reports on non-target effects of transgenic plants, expressing antimicrobial proteins, on the saprophytic soil microflora are scarce. Most studies indicate that there are no obvious effects, but methods employed do not justify generalization of conclusions. However, in one study it has been demonstrated that mycorrhizal symbiosis can be affected (Vierheilig *et al.* 1995), indicating that non-target effects on beneficial fungi can occur. Also the plant-beneficial rhizosphere microflora will come into contact with the transgenic plant product. By natural wounding, senescence and sloughing-off of root cells, antimicrobial proteins will be released from plant roots. In spite of the proteolytic activity in the rhizosphere, part of the protein may remain active due to protective adsorption to clay minerals or humic components.

Chances that the saprophytic microflora will be affected by the antimicrobial compounds, will depend on many factors. *In vitro* sensitivity does not predict sensitivity in the rhizosphere of the transgenic plant. Thus, various plant pathogens are sensitive when grown on nutrient media, but their activity is not reduced upon natural infection of transgenic plants (Neuhaus *et al.* 1991; Nielsen *et al.* 1993; Hironaka *et al.* 1993; Florack 1994; Allefs *et al.* 1995). Levels of the transgenic plant product in the rhizosphere or phyllosphere can be too low, or micro-organisms can be insensitive because of protective structures *in situ.*

Plant-derived antimicrobial proteins (chitinases, glucanases, RIPs, thionins, etc.) are also produced by untransformed plants during their normal life cycle. In addition, several rhizosphere micro-organisms themselves are able to produce a range of antimicrobial compounds, including chitinases and glucanases. Many antimicrobial proteins are, therefore, natural compounds in the soil ecosystem and one may doubt whether increased levels produced by transgenic plants can significantly affect the rhizosphere microflora. However, these plants are designed to withstand plant pathogens and results so far do indicate effects on at least specific members of the microflora. It is not clear to what extent these effects can be due to expression of heterologous plant enzymes or to the increased levels of homologous components in the transgenic plant. Lawton *et al.* (1993) demonstrated that constitutive expression of either tobacco chitinase, the SAR 8·2 gene or tobacco PR1a in homologous tobacco plants, resulted in resistance against several pathogens. Lin *et al.* (1995) obtained similar results with chitinase-expressing transgenic rice. Moreover, expression of a homologous tobacco glucanase in transgenic tobacco reduced root colonization by the mycorrhizal fungus *Glomus mosseae*. This strongly indicates that increased levels of these proteins *per se* can be responsible for enhanced resistance. Moreover, combinations of antimicrobial proteins can have synergistic effects (Zhu *et al.* 1994; Jach *et al.* 1995; Jongedijk *et al.* 1995). Since salicylic acid (SA) enhances plant resistance to fungi, bacteria and viruses, and at the same induces several types of PR-proteins (Malamy & Klessig 1992; Raskin

1992), research in different laboratories is aimed at constructing transgenic plants that constitutively produce SA. Such plants could have a significant effect on the microflora, the more so because the spectrum of antimicrobial activities induced by SA is only partly characterized.

So far, it appears that transgenic plants expressing heterologous antimicrobial plant proteins are not better protected from diseases than those constitutively expressing homologous ones, and their non-target effects can be expected to be similar. Antimicrobial proteins from non-plant origin such as cecropins, lysozymes and other non-plant components that are, or will be, cloned in plants are less common for the rhizosphere microflora and therefore may have a different impact.

Possible non-target groups to be affected by the transgenic plants are symbionts like VAM fungi and rhizobia. On the other hand, these symbionts are adapted to overcome plant defence responses (Gianinazzi-Pearson *et al.* 1994). Indeed, results by Vierheilig *et al.* (1993, 1995) indicate that constitutive expression of a range of antifungal PR-proteins in transgenic tobacco did not affect mycorrhizal colonization. However, not all PR-proteins are induced to the same extent during the symbiosis and mycorrhizae may be less adapted to PR-proteins that are not, or only slightly induced. One would expect the fungus to be more sensitive to these less-induced proteins. This may be the reason that constitutive expression of a class II β-1,3-glucanase affects the tobacco–*G. mosseae* symbiosis, whereas other PR-proteins do not.

Compared to the use of chemical pesticides, a distinct advantage of transgenic plants coding for antimicrobial proteins is the restriction of the transgenic product to the vulnerable plant itself. Moreover, proteinaceous transgenic compounds are probably less persistent in soil than most chemicals. On the other hand, continuous exposure of the pathogen to the transgenic compound may lead to populations that become insensitive to the specific compound. It should also be noted that, although the transgenic plants are designed for broad-spectrum disease resistance, pathogens may have differential sensitivity towards antimicrobial protein(s), as is often observed *in vitro*. In this situation the control of a sensitive pathogen could result in an increase of a tolerant one, that initially was of minor importance. This phenomenon, called 'dominance change of pathogens', has been observed more than once as a result of fungicide applications (Bollen 1979). Dominance change of pathogens can also occur due to differential sensitivity of pathogens and their microbial antagonists towards antimicrobial protein(s).

An important issue is the nature, magnitude and reversibility of the effect, as well as how to evaluate data in view of common fluctuations in the microflora by environmental impact (e.g. temperature, moisture) or man-induced effects (e.g. crop rotation, organic amendments, fertilizers). Host-dependent micro-organisms, such as VAM fungi and rhizobia, can be greatly influenced by crop rotation (Miller 1993) or type of farming (Ryan *et al.* 1994). For example, mycorrhizal spore density on linseed was 25 per gram of soil when sunflower was grown as a preceding crop, whereas hardly any spores were detected when linseed was preceded by fallow (Thompson 1991). Under low N-input, indigenous *Bradyrhizobium* spp. populations can be stimulated 10–100-fold when the homologous host legume is cropped after non-host cropping of fallow (Thies *et al.* 1995). Domsch *et al.* (1983) proposed a model to evaluate side-effects of agrochemicals on populations and functions of soil micro-organisms as compared with reduction of microbial populations caused by natural stress factors. Reductions of more than 50% in numbers and activities of culturable micro-organisms were observed as a result of

natural fluctuations in temperature, water availability and other abiotic processes (Domsch *et al.* 1983). In general, microbial populations recovered within 60 days. In studies on side-effects of pesticides, therefore, this recovery period of 60 days is considered ecologically tolerable. Persistence of the effect after this period may be critical, depending on the magnitude of the effect. Based on this model, a possible impact of the transgenic plant on the microflora may be critical because antimicrobial compounds will be produced during the whole cropping season. This risk-assessment model, however, only considers the activity and numbers of (culturable) micro-organisms and not the diversity of the microflora, and does not take into account the possible replacement of sensitive species by less sensitive ones. The study of differential sensitivity of micro-organisms to antimicrobial proteins in the rhizosphere of transgenic plants is a major challenge for the future.

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