Influence of the expression of antibacterial and antifungal genes in transgenic plants on the saprophytic soil microflora

Introduction

Plants exhibit natural resistance to most potential pathogens, and disease is the exception rather than the rule. In order to increase crop production, plant breeding programs have been focussed on selection of high-yielding cultivars and less on characteristics conferring general disease resistance. Continuous cropping of monocultures of agriculturally important crops on large surface areas has favoured occurrence to a high incidence of diseases as compared to wild plant communities. Classical breeding of disease resistant cultivars has been concentrated on monospecific resistance genes, effective only against races of the pathogen. This classical approach is costly and often too time-consuming to react adequately to the emergence of new virulent races of pathogens (Cornelissen and Melchers, 1993).

Antimicrobial activities

To obtain more durable and broad-spectrum disease resistance, research in plant genetic engineering is now directed towards constitutive expression of activities that are normally induced only when a plant is able to defend itself effectively against pathogenic attack, such as during a hypersensitive reaction. Although induction of these defense 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 and structural proteins, formation of low-molecular-weight antimicrobial phytoalexins and accumulation of high-molecular-weight "pathogenesis related" (PR) proteins with potential antimicrobial activity. This latter group is attractive for genetic modification of plants, because PR-proteins are individually coded by single genes and therefore easily amenable to manipulation by gene transfer. Expression of PR proteins in a heterologous host is thought to be most effective on the assumption that co-evolution of host and pathogen has evolved in pathogens that exhibit a diminished sensitivity towards the set of PR proteins expressed by their own hosts (Lamb *et al.*, 1992).

Other groups of proteins with antimicrobal activity that are used to transform plants are ribosome-inactivating proteins (RIPs) and thionins, low-molecular-weight plant proteins. The

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search for active antimicrobial genes has not been restricted to higher plants. Plants have also been modified with genes coding for a bacterial chitinase, antimicrobial peptides of animal origin (cecropins) and lysozyme from bacteriophage T4.

Introduction of such transgenic crops into the environment may impose nontarget effects on the ecosystem (Morra, 1994). An obvious possibility is that increased production of broad-spectrum, antimicrobial components suppresses not only target pathogens but also directly or indirectly influences plant-beneficial symbionts like mycorrhiza or rhizobia. Other nontarget groups that could be affected are rhizosphere-inhabiting microorganisms with plant growth-promoting and disease-suppressing activities, like fluorescent *Pseudomonas spp.* (Bakker *et al.*, 1991) and non-pathogenic *Fusarium oxysporum* (Lemanceau *et al.*, 1992), and soil microbes involved in the decomposition of decaying plant material and nutrient cycles. Nothing is known about possible effects of antimicrobial genes on the latter two groups. However, symbiosis of transgenic plants with mycorrhiza has been the subject of recent studies (Vierheilig *et al.*, 1993; Tahiri-Alaoui *et al.*, 1994).

Chitinases

By now, 11 families of PR-proteins have been recognized, of which PR-3, 8 and 11 exhibit chitinase activity (Van Loon, 1994). Plant chitinases (Collinge *et al.*, 1993; Graham and Sticklen, 1994) exhibit mainly endochitinase activity. Since chitin is a major component of the cell wall of the majority plant pathogenic fungi and the plant itself seems to lack substrates for chitinases, these enzymes are thought to act in plant defense. Various chitinases inhibit the growth of many fungi *in vitro* by causing lysis of hyphal tips, especially in combination with ß-1,3-glucanase (PR-2) (Mauch *et al.*, 1988). In addition to being induced in response to pathogenic attack, many chitinases seem to be developmentally regulated, with specific isoforms appearing in specific organs during certain stages of the plant's life. The relevance of this occurrence of chitinases during development is not clear, but protection against fungal attack has been advocated.

The fact that chitinases may accumulate to high levels during normal plant development, implies that transgenic plants constitutively expressing chitinase only increase enzyme levels that are already occurring naturally in the plant. Nevertheless, constitutive expression of increased levels of chitinase has been reported to significantly affect activities of at least one soil-borne, chitin-containing plant pathogen in the plant's rhizosphere. In tobacco, constitutive expression of chitinase from bean, tobacco, cucumber or the bacterium *Serratia marcescens* caused a significant reduction of symptoms caused by the root rot fungus *Rhizoctonia solani*, but not of *Pythium aphanidermatum*, a fungus that does not contain chitin (Table 1; Broglie *et al.*, 1991; Howie *et al.*, 1994; Lawton *et al.*, 1993; Vierheilig *et al.*,1993).

On the other hand, enhanced chitinase levels did not result in protection against the wilt pathogen *Fusarium oxysporum* (Logeman *et al.*, 1994) and in no or slight protection against the leaf blight pathogen *Cercospora nicotianae* (Neuhaus *et al.*, 1991; Nielsen *et al.*, 1993; Zhu *et al.*, 1994) or the leaf pathogen *Alternaria solani* (Hironaka *et al.*, 1993), despite the fact that the intracellular chitinases were targetted extracellularly and thereby could act on the leaf pathogens in an early stage of the infection process. However, symptoms caused by *C. nicotianae* and *F. oxysporum* were significantly reduced when in addition to chitinase also ß-1,3-glucanase was expressed constitutively (Logeman *et al.*, 1994; Zhu *et al.*, 1994). The

Table 1. Disease reduction by constitutive chitinase expression in transgenic tobacco and tomato plants

Source chitinase	Pathogen	Disease reduction	Reference
Bean	*R. solani*	+	Broglie *et al.*, 1991;
Tobacco	*R. solani*	+	Lawton *et al.*, 1993 Vierheilig *et al.*, 1993
Cucumber	*R. solani*	+	Lawton *et al.*, 1993
Tobacco	*F. oxysporum*	-	Logeman *et al.*, 1994
Bacterial	*R. solani* *P. ultimum*	+ -	Howie *et al.*, 1994

results on the effects of constitutive expression of various chitinases in transgenic tobacco and tomato are summarized in Table 1.

Mycorrhiza

At low phosphate availability, roots of most herbaceous plants are colonized by mycorrhiza. These symbiotic fungi play an important role in water and nutrient uptake by the plant and are also involved in the defense of plants against pathogens. Vesicular-arbuscular (VA) mycorrhiza contain up to 27% of chitin in their cell walls. The extracellular hyphae are usually rather thick and are embedded in a non-chitinous matrix, the so-called polysaccharide sheath, that may offer protection of the hyphae to e.g. chitinase. However, this sheath is probably not well-developed in penetrating- and intercellularly-growing hyphae (Miller, 1993). The cell wall thins when the fungus penetrates the cortical host cells and when intercellular hyphae run through the roots, such that they may be highly sensitive to constitutively expressed chitinase. Interestingly, VA fungi themselves induce PR-proteins, such as chitinase, upon penetration of the host, but chitinase levels decline dramatically when colonization becomes fully established. An obvious concern is that constitutive chitinase production by the plant diminishes its colonization by mycorrhiza, leading to reduced plant growth (Miller, 1993). Two studies have adressed this question.

Vierheilig *et al.* (1993) reported that constitutive expression of tobacco vacuolar chitinase in transgenic tobacco, resulted in 14 times higher specific chitinase levels in roots, but did not affect the colonization potential of the VA fungus *Glomus spp.* as measured after 8 weeks. In contrast, colonization by *R. solani* was significantly reduced in these same plants. In earlier

work it was observed that living intercellular mycelium of VA mycorrhizal fungi did not bind chitinase (Spanu *et al.*, 1989). Therefore the authors hypothesize that the intruding hyphae of the VA fungi are protected from the chitinase by the polysaccharide sheath.

A study by Tahiri-Alaoui *et al.* (1994) focussed on the effect of constitutive expression of either a basic bean endochitinase gene or an endochitinase gene from the hyperparasitic fungus *Aphanocladium album* on the symbiosis of transgenic tobacco with arbuscular mycorrhiza. 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.

Conclusions

Studies published to date indicate that increased chitinase levels in transgenic plants, even when expressed in a heterologous host, do not significantly affect mycorrhizal symbiosis. However, more research on species other than tobacco and on more strains of mycorrhizal fungi will be necessary before generalized conclusions can be drawn. Moreover, many plant pathogens are not sensitive to chitinases, but can be inhibited by combinations of different enzymes, such as chitinase and ß-1,3-glucanase. Thus, expression of combinations of enzymes may have an effect on mycorrhizal fungi. So far, no results are available on the possible effects of the expression of antimicrobial genes in transgenic plants on the saprophytic soil microflora that is ecologically important in maintaining plant health, soil fertility and nutrient cycling.

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Unanswered Safety Questions when employing GMO's

Workshop Proceedings
Leeuwenhorst Congress Centre Noordwijkerhout
the Netherlands
May 2 - 4, 1995

Organised by:

Coordination Commission Risk Assessment Research (CCRO)
with finances from the Ministery of Economic Affairs.