# Colonization of Transgenic *Nicotiana sylvestris* Plants, Expressing Different Forms of *Nicotiana tabacum* Chitinase, by the Root Pathogen *Rhizoctonia solani* and by the Mycorrhizal Symbiont *Glomus mosseae*

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Transgenic plants constitutively expressing bean chitinase have been shown to display enhanced resistance to the root pathogen *Rhizoctonia solani* (K. Broglie, I. Chet, M. Holliday, R. Cressman, P. Biddle, S. Knowlton, C. J. Mauvis, and R. Broglie, Science 254:1194-1197, 1991). We used transgenic *Nicotiana sylvestris* plants, expressing different forms of tobacco chitinase under the control of the expression signals of the 35S transcript of cauliflower mosaic virus, to compare the colonization of the root system by *R. solani* and by the mycorrhizal symbiont *Glomus mosseae*. Plants of *N. sylvestris* expressing the vacuolar tobacco chitinase A or the same chitinase without the N-terminal chitin binding domain were less colonized by *R. solani* than control plants and showed less reduction of the root fresh weight when exposed to an attack of this fungus. Plants of *N. sylvestris* expressing chitinase A without the C-terminal signal for vacuolar targeting showed no enhanced resistance since they were colonized by *R. solani* like control plants. All transgenic *N. sylvestris* investigated were equally well colonized by *G. mosseae*, indicating that the enhanced resistance conferred by constitutive expression of chitinase in transgenic plants does not interfere with the vesicular-arbuscular mycorrhizal symbiosis.

Chitinases (E.C. 3.2.1.14) are involved in the natural defense of plants against fungal infection (Boller 1987, 1988; Meins *et al.* 1992). Plant chitinases degrade chitin in fungal cell walls and can inhibit fungal growth (Schlumbaum *et al.* 1986; Broglie *et al.* 1991), particularly in combination with $\beta$-1,3 glucanase (Mauch *et al.* 1988). In many plant tissues, chitinases are strongly induced by pathogen infection or by the plant stress hormone, ethylene (Boller 1988; Meins *et al.* 1992).

Since chitinase has a low constitutive activity in many tissues and is often induced only relatively late upon pathogen attack (Boller 1988), it has been speculated that constitutive expression of chitinase may increase the resistance of plants against chitin-containing fungal pathogens. *Nicotiana sylvestris* plants constitutively expressing chitinase A of *Nicotiana tabacum* (tobacco) did not show a significantly increased resistance to the leaf pathogen, *Cercospora nicotiane* (Neuhaus *et al.* 1991a). However, constitutive expression of a bean chitinase gene has been found to increase the resistance of transgenic tobacco and canola plants to the root pathogen, *Rhizoctonia solani* (Broglie *et al.* 1991).

Plant roots are colonized not only by pathogens but also by beneficial symbiotic fungi. Most herbaceous plants are hosts for vesicular-arbuscular (VA) mycorrhizal fungi that enhance the uptake of mineral nutrients in exchange for assimilates provided by the plant (Smith and Gianinazzi-Pearson 1988). The fungi involved in this symbiosis have chitin in their cell walls and may therefore be influenced by the chitinases of their hosts (see Bonfante-Fasolo 1984; Spanu *et al.* 1989).

To test if chitinase constitutively expressed in plant roots might influence their colonization by VA mycorrhizal fungi, we examined transgenic *N. sylvestris* plants, expressing tobacco chitinase A and two derivatives of this gene product, with regard to their colonization by *R. solani* and *G. mosseae*. Our results demonstrate that constitutive expression of vacuolar chitinases increases the resistance to *R. solani* but does not reduce the symbiotic potential of the plants.

**Plant material.**

Progeny of four different primary transformants of *N. sylvestris*, selected to express a high level of the transgene, were used in this work. They were obtained by *Agrobacterium*-mediated leaf disk transformation using derivatives of the binary vector pCIB200 containing a plant-selectable chimeric *NOS/NPTII* gene (Neuhaus *et al.* 1991a). Control plants were transformed with the vector pCIB200 without insert (Neuhaus *et al.* 1991a). Chimeric genes encoding the following derivatives of tobacco chitinase A under the control of the 35S promoter of cauliflower mosaic virus were introduced into *N. sylvestris*: wild-type tobacco

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chitinase A (Tob) (Neuhaus *et al.* 1991a); tobacco chitinase A from which the entire cysteine-rich, chitin-binding domain, the so-called hevein domain, had been deleted (TobΔH) (Sticher *et al.* 1992); and tobacco chitinase without its C-terminal extension of seven amino acids (TobΔT) (Neuhaus *et al.* 1991b). The C-terminal extension functions as a targeting signal for the plant vacuole since the tobacco chitinase is located in the vacuole in transgenic *N. sylvestris* plants expressing the wild-type sequence (Tob), while it is present in the intercellular space in plants expressing sequence TobΔT with the C-terminal deletion (Neuhaus *et al.* 1991b).

Seed from heterozygous primary transformants were surface-sterilized for 5 min in 0.75% (w/v) NaOCl, washed extensively with sterile distilled water, and grown axenically in petri dishes containing 15 ml of LS medium (Linsmaier and Skoog 1965) supplemented with 400 mg/L of kanamycin sulfate under continuous light at 25° C. Kanamycin-resistant plants are a mixture of heterozygous and homozygous plants.

## Plant inoculation.

Six-week-old kanamycin-resistant plantlets were transferred to a low phosphorus steam-sterilized mixture of 50% loam and 50% sand (v/v). For inoculation with the mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe, the soil was mixed with 5% (v/v) soil from a stock plant culture (*Tagetes tenuifolia* grown on a sand/expanded clay mixture, containing sporocarps, spores, hyphae, and infected root pieces). For inoculation with the pathogenic fungus *R. solani* Kühn (*Thanatephorus cucumeris* (Frank) Donk), strain 126 of collection Dahmen (CIBA-GEIGY, Basel), the soil was mixed with 2% (v/v) agar pieces of a fungal stock culture (*R. solani* grown for 7 days on 1.5% malt extract agar). The corresponding controls received corresponding stocks of soil or agar without the respective fungi.

Plants were grown in a greenhouse under a day/night cycle of 14hr/10hr, with temperatures of 27° C during the day and 20° C during the night. They were watered daily with tap water and once a week with a Hoagland-type nutrient solution lacking phosphorus (Finck 1979).

## Measurements.

Plants inoculated with *R. solani* and corresponding controls were harvested 5 wk later, and plants inoculated with *G. mosseae* inoculated plants 8 wk later. Infection by *G. mosseae* and *R. solani* was estimated by visually scanning the root samples in the stereo microscope for fungal structures according to the gridline intersection method (Giovannetti and Mosse 1980). Data are given as percentage of root length infected. The root fresh weight of mock-inoculated and *R. solani*-inoculated plants was also measured. For chitinase assays, plant roots were extracted with a 50 mM Tris buffer adjusted with HCl to pH 8.0, containing 500 mM NaCl and 0.2% Triton X-100. Chitinase was assayed as described (Boller *et al.* 1983), and protein was determined according to Bradford (1976), using bovine serum albumin as a standard. All experiments were repeated twice using a minimum of 10 replicates per

transformant per treatment. Means and standard errors of means of a typical experiment are shown.

## Specific activity of chitinase in the roots of transgenic plants.

Plants constitutively expressing different forms of chitinase showed an increased chitinase activity in their roots. As shown in Table 1, the specific chitinase activity was determined in root systems of the various transgenic plants after infection with *R. solani* or *G. mosseae* in order to relate the data directly to the colonization ability by fungi (see below). Compared to the plants transformed with a vector without chitinase sequences, the plants constitutively expressing wild-type tobacco chitinase (Tob) or the same chitinase lacking the hevein domain (TobΔH) showed an activity about 14 times higher than the control (Table 1). Activity in the roots of plants with the tobacco chitinase lacking the C-terminal vacuolar targeting sequence (TobΔC) was about six times higher than the control (Table 1).

## Colonization of the roots by the pathogen *R. solani.*

*R. solani* colonized about 30% of the root system of control *N. sylvestris* plants within 5 wk under the conditions employed. There were no discernible symptoms in shoot growth or appearance. Colonization of the root system of the different transgenic *N. sylvestris* plants by *R. solani* was examined (Fig. 1A). In transformed control plants, about 30% of the root length was colonized by *R. solani*. In plants constitutively expressing tobacco chitinase A (Tob), only about 15% of the total root length was infected. The results were similar for the plants expressing a chitinase lacking the hevein domain (TobΔH), which is also targeted to the vacuole (Sticher *et al.* 1992). Plants expressing a chitinase without the C-terminal vacuolar targeting sequence (TobΔT) and consequently secreting it into the intercellular space (Neuhaus *et al.* 1991b), were colonized by *R. solani* to the same extent as the control transformants.

**Table 1.** Chitinase activity in roots of *Nicotiana sylvestris* transformed with a vector lacking chitinase sequences or containing sequences encoding various forms of chitinase under the control of a CaMV 35S promoter

| Line[a] | Specific chitinase activity in roots[b] (nkat/mg protein ± SE) | |
| | Infected with *R. solani* | Infected with *G. mosseae* |
| --- | --- | --- |
| Control | 18 ± 5 | 13 ± 2 |
| Tob | 233 ± 28 | 200 ± 64 |
| TobΔH | 254 ± 39 | 204 ± 32 |
| TobΔT | 97 ± 22 | 83 ± 28 |

[a] *N. sylvestris* lines were progeny from plants transformed with a vector containing no insert (control) or chimeric genes encoding the vacuolar tobacco chitinase A (Tob), the same vacuolar tobacco chitinase A without its N-terminal chitin-binding domain (TobΔH) or the same chitinase A without its C-terminal vacuolar targeting peptide, causing secretion of the chitinase into the intercellular space (TobΔT).

[b] Specific activity of chitinase was measured 5 wk after infection of plants with *R. solani* and 8 wk after inoculation with *G. mosseae*, respectively.

As in previous work of others (Broglie *et al.* 1991), the pathogenic potential of *R. solani* was further examined by comparing the fresh weight of the root systems of uninoculated and *R. solani*-infected plants (Fig. 2). Taking the root fresh weight of uninfected control plants as 100%, the fresh weight of the infected controls was reduced to 35%. The transformants expressing wild-type tobacco chiti-

nase (Tob) or the same chitinase without the hevein domain (TobΔH) showed much less or no reduction of the root fresh weight: The fresh weight was 77% and 101% of the corresponding uninfected controls, respectively. In transformants expressing tobacco chitinase without the C-terminal extension (TobΔT), on the other hand, the fresh weight of infected roots was reduced to a similar extent (to 31% of the value of uninfected plants) as in the control plants.

The results presented in Figures 1A and 2 corroborate and extend the observation of Broglie *et al.* (1991) that constitutive expression of plant chitinase enhances resistance of roots to *R. solani*. Both in the previous study (Broglie *et al.* 1991) and in the present work, the constitutively expressed chitinases providing enhanced resistance are expected to be located in the vacuole (Boller 1987, 1988). In our study, we compared plants accumulating the same chitinase either in the vacuole (Tob) or in the intercellular space (TobΔT), as determined by extraction of intercellular fluid and vacuole isolation from leaf tissue (Neuhaus *et al.* 1991b). Surprisingly, plants expressing the intact vacuolar chitinase showed enhanced resistance to *R. solani*, while plants expressing the same chitinase without the vacuolar targeting signal were as susceptible as control plants (Figs. 1A and 2). A priori, assuming the same localization in roots as in leaves, one might have expected a better success with the extracellular chitinase (TobΔT) since *R. solani* is growing in the intercellular spaces. However, our unexpected result might be explained at least in part by the lower chitinase activity in the transformants containing the extracellular form, TobΔT, compared to those with the vacuolar form, Tob (Table 1). In addition, chitinase might be present in the extracellular
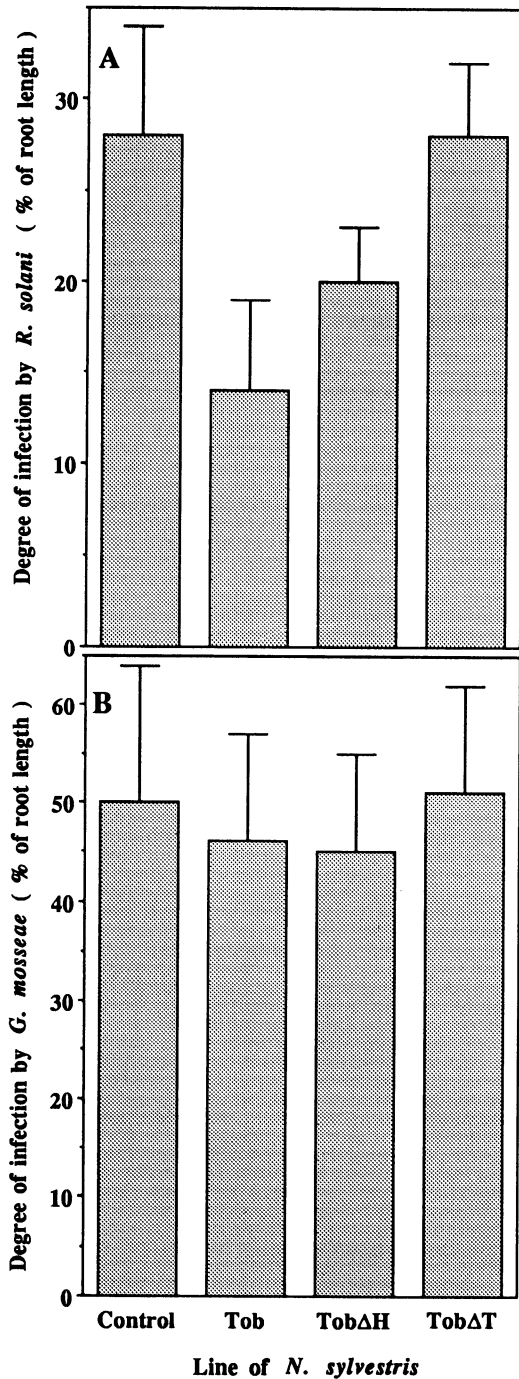


Fig. 1. Percentage of root length infected by A, *Rhizoctonia solani*, or B, *Glomus mosseae* in transformed *Nicotiana sylvestris* plants. Samples of the root systems were examined 5 wk after infection with *R. solani* or 8 wk after infection with *G. mosseae*, using the gridline intersection method. The transgenic *N. sylvestris* lines are designated as in Table 1.
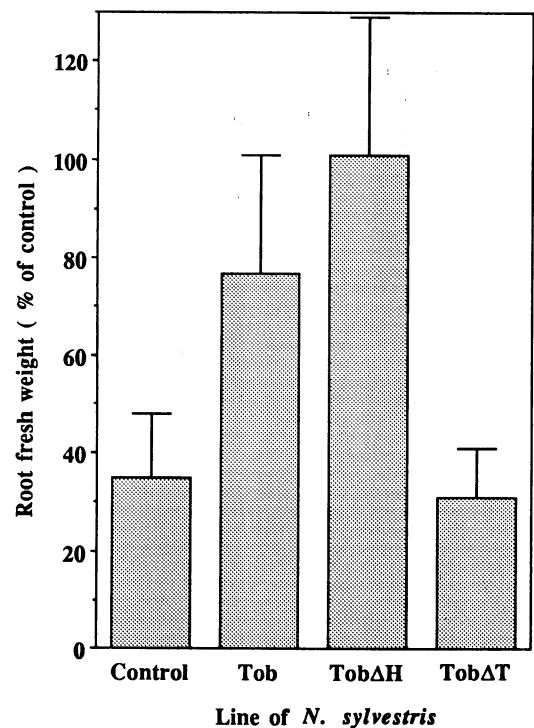


Fig. 2. Root fresh weight of transgenic *Nicotiana sylvestris* plants 5 wk after infection with *Rhizoctonia solani*, expressed as percentage of the root fresh weights of mock-inoculated plants of the same age. The transgenic *N. sylvestris* lines are designated as in Table 1.

space even in roots containing the vacuolar form of chitinase, because of release from the decaying root cap and rhizodermis. Furthermore, vacuolar localization may also be advantageous in defense, for example if the vacuolar contents are released suddenly upon approach of a fungus, curtailing its chances of adaptation (Boller 1987).

## Colonization of the roots by the symbiotic VA mycorrhizal fungus G. mosseae.

Under the conditions of low phosphorus nutrition employed here, roots of *N. sylvestris* plants are extensively colonized by *G. mosseae*. The colonization ability of *G. mosseae* on the root system of different transgenic *N. sylvestris* plants was studied (Fig. 1B). In control plants, transformed with a vector without chitinase insert, about 50% of the root length was colonized by *G. mosseae* within 8 wk. This high degree of colonization was not significantly affected by expression of any of the chitinases studied here (Fig. 1B). Light microscopy did not reveal any conspicuous difference in the structure of hyphae, vesicles, and arbuscules in any of the transgenic plant lines tested.

It is known that arbuscules of VA fungi are surrounded by a specific plant membrane, the periarbuscular membrane, and do not come in direct contact with the vacuolar content and the intercellular space (Bonfante-Fasolo 1984; Bonfante-Fasolo et al. 1990). Therefore, the failure of the constitutively expressed chitinases to affect arbuscules may not seem surprising. However, it is still possible that the chitinases entering the secretory pathway are also secreted through the periarbuscular membrane, and that the arbuscule may need protection against these enzymes. In contrast, intercellular hyphae should readily come into contact with extracellular chitinase, expressed constitutively in plants carrying a gene for chitinase lacking the C-terminal extension (Neuhaus et al. 1991b). Nevertheless, the intercellular hyphae of *G. mosseae* showed no obvious alteration in these transgenic plants. In earlier work, it was observed that living intercellular mycelium of VA mycorrhizal fungi did not bind chitinase (Spanu et al. 1989), and it was proposed that the chitin layer of the cell wall may be protected by a cover of proteins or alkali-soluble polysaccharides (see Bonfante-Fasolo and Grippiolo 1982).

## Conclusion.

Transgenic *N. sylvestris* plants constitutively expressing vacuolar chitinase of *N. tabacum*, independent of the presence or absence of the hevein domain, show reduced susceptibility to colonization by the root pathogen *R. solani* but are still colonized normally by the root symbiont *G. mosseae*. Thus, it appears possible to make use of the antifungal properties of chitinase in transgenic plants without reducing their symbiotic potential. However, many pathogens are not susceptible to chitinase alone, but can be inhibited by chitinase in combination with other enzymes like β-1,3-glucanase (Mauch et al. 1988). Constitutive expression of combinations of chitinase and β-1,3-glucanase may provide better protection against a wider range of pathogens. It remains to be seen whether or not this has an effect on the ability of the plants to enter symbiosis with VA mycorrhizal fungi.

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