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Research Article

Chitinase activities, scab resistance, mycorrhization rates and biomass of own-rooted and grafted transgenic apple

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Abstract

This study investigated the impact of constitutively expressed *Trichoderma atroviride* genes encoding exochitinase *nag70* or endochitinase *ech42* in transgenic lines of the apple cultivar Pinova on the symbiosis with arbuscular mycorrhizal fungi (AMF). We compared the exo- and endochitinase activities of leaves and roots from non-transgenic Pinova and the transgenic lines T386 and T389. Local and systemic effects were examined using own-rooted trees and trees grafted onto rootstock M9. Scab susceptibility was also assessed in own-rooted and grafted trees. AMF root colonization was assessed microscopically in the roots of apple trees cultivated in pots with artificial substrate and inoculated with the AMF *Glomus intraradices* and *Glomus mosseae*. Own-rooted transgenic lines had significantly higher chitinase activities in their leaves and roots compared to non-transgenic Pinova. Both of the own-rooted transgenic lines showed significantly fewer symptoms of scab infection as well as significantly lower root colonization by AMF. Biomass production was significantly reduced in both own-rooted transgenic lines. Rootstock M9 influenced chitinase activities in the leaves of grafted scions. When grafted onto M9, the leaf chitinase activities of non-transgenic Pinova (M9/Pinova) and transgenic lines (M9/T386 and M9/T389) were not as different as when grown on their own roots. M9/T386 and M9/T389 were only temporarily less infected by scab than M9/Pinova. M9/T386 and M9/T389 did not differ significantly from M9/Pinova in their root chitinase activities, AMF root colonization and biomass.

Key words: apple, chitinase, mycorrhiza, transgenic.

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Introduction

Diseases caused by fungal, bacterial and viral agents are a major concern for fruit tree producers. In a society demanding a reduction in pesticide usage, fruit tree resistance to important diseases is a priority in breeding (Cummins and Aldwinckle, 1995). The fungal pathogen *Venturia inaequalis* is a major threat to apple (*Malus* x *domestica*) production and is one of the most widespread and yield-damaging plant pathogens. To prevent damage to fruit yield, fungicides need to be applied on a regular basis. Some apple cultivars such as Pinova are less susceptible to fungal attack than others, but up to 20 fungicidal treatments per year are still necessary in Western Europe to produce fruits of marketable quality (Roßberg, 2009). Alternative methods to control fungal plant pathogens, such as the use of mycoparasitic *Trichoderma* sp., have been investigated (Harman *et al.*, 2004). In particular, chitinase-encoding genes from *Trichoderma* have received considerable attention as they have much higher antifungal capacities than the corresponding plant genes (Lorito *et al.*, 1998). The insertion of *Trichoderma* genes encoding for chitinases into various plants increases plant resistance to a wide range of foliar and soil-borne pathogenic ascomycetes and basidiomycetes (Stefani and Hamelin, 2010).

In the present study, apple cultivar Pinova was transformed with the binary plasmid vector pBIN (Nag+Endo) described by Bolar *et al.* (2001). Two transgenic lines (T386 and T389) were selected from eight transgenic lines originating from three independent transformation experiments. Both lines expressed the genes *nag70* and *ech42*

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from the mycoparasitic fungus *Trichoderma atroviride* that were driven by the constitutive double CaMV 35S promoter. Lines T386 and T389 were selected based on their stable level of transgene expression and high level of scab resistance (as confirmed in the present study).

Like most fruit trees, apple forms symbiotic associations with arbuscular mycorrhizal fungi (AMF) in its roots. These symbiotic partners belong to the phylum Glomeromycota (Schüßler *et al.*, 2001). In exchange for plant-derived carbon these fungi provide their hosts with various benefits, such as a more efficient uptake of soil nutrients, especially phosphorus, enhanced tolerance to biotic and abiotic stress and resistance to pests (Smith and Read, 2008). As in most higher fungi, chitin is an important structural element in AMF cell walls (Bonfante-Fasolo *et al.*, 1990). Consequently, the susceptibility of AMF to chitinases from *Trichoderma* sp. may be similar to that of fungal pathogens.

As in many other fruit tree species, the grafting of desirable cultivars is a common practice. The art of grafting superior fruit varieties onto rootstocks has been practiced in numerous fruit species for thousands of years (Cummins and Aldwinckle, 1995). In modern apple cultivation, desirable scions are grafted onto a wide range of rootstocks that confer different attributes such as reduced tree size, increased disease resistance or improved fruit quality (Jensen *et al.*, 2003). Limited tree height facilitates management in operations such as pesticide spraying, pruning, thinning and harvesting (Webster, 2004).

In this work, we used own-rooted and grafted trees to examine how transformation of the apple cultivar Pinova with the genes *nag70* and *ech42* from *T. atroviride* affected (i) the exo- and endochitinase activities in leaves and roots, (ii) resistance to *V. inaequalis*, (iii) mycorrhization rates in an artificial substrate upon inoculation with *Glomus intraradices* and *Glomus mosseae* and (iv) biomass production.

Materials and Methods

Plant material

Shoots of transgenic lines T386 and T389 of the apple (*Malus* x *domestica*) cultivar Pinova grown *in vitro* and containing the T-DNA of plasmid vector pBIN (carrying the *Trichoderma* chitinase genes *ech42* and *nag70*) as described by Bolar *et al.* (2001) were used. Prior to the experiments, the transgenic status of the two lines was confirmed by PCR using the primers ech42_F 5'-GCCAGTGGATAC GCAAACGC-3' and ech42_R 5'-AATTCCTCTAGTTG AGACCGCTTC-3' for the *ech42* gene, nag70_F 5'-GCTCTAGACCGCCTCGGTCGTCCATCAT-3' and nag70_R 5'-CGGGATCCTTATGCGAACAAGGTGCA AGCCGTAGC-3' for the *nag70* gene and nptII_F 5'-ACAAGATGGATTGCACGCAGG-3' and nptII_R 5'-AACTCGTCAAGAAGGCGATAG-3' for the *nptII* gene (data not shown). Transgene integration was tested by Southern hybridization, as described by Flachowsky *et al.* (2010) (data not shown). Probes were amplified using the primers described before. Transgene expression was assessed by reverse transcriptase (RT)-PCR using cDNA and the primers described above.

Rooting and acclimatization

Shoots of transgenic lines and the non-transgenic control plants were propagated *in vitro* and transferred to rooting medium containing half strength of the salts and vitamins used by Murashige and Skoog (1962), 3 mg of indolebutyric acid (IBA)/L, 30 g of sucrose/L and 0.8% agar for one week in the dark and three weeks in light on the same medium without IBA. Rooted plantlets were transferred to peat pots (Jiffy, Stange, Norway) and acclimatized in a greenhouse as described by Bolar *et al.* (1998). Shoots of previously propagated transgenic lines and the non-transgenic control were grafted onto rootstock M9.

Evaluation of resistance to apple scab

Screening for resistance to *V. inaequalis* was done using transgenic and non-transgenic greenhouse plants that were own-rooted or grafted onto rootstock M9. Four to five weeks before inoculation all shoots were pruned at a height of 10 cm above soil level. The vigorously growing shoot regrowth was inoculated at the 3-5 leaf stage with a mixed local inoculum of conidia from *V. inaequalis*. The inoculum was prepared from scab-infected leaves that were harvested from *Malus floribunda 821* grown in the experimental orchard of the Julius Kühn-Institut in Dresden-Pillnitz and frozen at -20 °C. Immediately before inoculation the leaves were dipped in water and spores were wiped off. The density of secondary spores (conidia) was adjusted to 2 x 10^5/mL of inoculum. Transgenic and control plants were inoculated by spraying the inoculum on the tip and the two youngest leaves of a shoot followed by wrapping the tip in wet paper and covering it with a plastic bag. After two days, the bag and paper were removed. Infection with scab was evaluated 17 and 28 days post-inoculation (dpi). During this time, the plants were kept at >80% humidity. The incidence of scab was scored with a six-stage scale (1, 2, 3, 5, 7 and 9) that is also used in conventional apple breeding (1: no symptoms, 2: pin points, 3: sporulation, 5: deformation of leaves and sporulation, 7: sporulation and necrotic tissue, 9: necrotic tissue). Twenty to 29 plants of each transgenic line and non-transgenic control were tested.

Mycorrhization experiments

In 2007, a greenhouse experiment with 63 sample trees (nine replicates per line or variety) was set up and included non-transgenic Pinova and transgenic lines T386 and T389; the trees were either own-rooted or grafted onto rootstock M9. In addition, ungrafted rootstock M9 (*i.e.*, "complete tree") was also included. Pinova is a cultivar that

produces so-called “refreshing” or “table” apples whereas M9 is a dwarfing rootstock that is commonly used in apple production. The sample trees were grown in 5 L of artificial substrate (a 2:1 ratio of sand:vermiculite and 1 g of Osmocote long-term fertilizer) containing 75 g of vermiculite inoculated with *G. intraradices* BEG 140 and *G. mosseae* BEG 25 until September 2008. The greenhouse temperature was 18-22 °C and no artificial light was supplied. The plants were watered individually two or three times a week based on their weight and estimated water holding capacity of 60%. At the end of this period, root samples were thoroughly washed to remove soil. The youngest leaves and fine root material for measuring chitinase activity were frozen in liquid nitrogen immediately after sampling and stored at -80 °C. Fine roots for microscopical analysis were fixed in formaldehyde-acetic acid (FAA): 6.0% formaldehyde, 2.3% glacial acetic acid, 45.8% ethanol, 45.9% H_2O (v/v).

Biomass determination

During the following winter dormancy, 31 plants (4-5 individuals each from Pinova, T386, T389, M9, M9/Pinova, M9/T386 and M9/T389) were randomly selected and harvested. The roots were thoroughly washed to remove soil and stem and root material was dried for 24 h at 105 °C before weighing.

Chitinase activity

The assays used were based on the methods described by Bolar *et al.* (2000, 2001), Marx *et al.* (2001) and Pritsch *et al.* (2004) and measured both native and transgenic chitinase activities. Buffers and substrates were prepared as described by Bolar *et al.* (2000, 2001). Calibration solutions C_1-C_6 containing known concentrations of 4-methylumbelliferone and dissolved substrates were stored at 4 °C and protected from light. Stopping buffer (SB) was 2.5 M Tris (pH 11). Plant tissue (~30 mg) was ground to a fine powder in liquid nitrogen. 500 µL of assay buffer (AB) was added to each mg of plant tissue followed by mixing. The mixture was poured into 50 mL tubes and cooled on ice prior to sonication at a burst pulse of 6/10 for 2 min (Sonopuls HD2200 sonicator, Bandelin, Berlin, Germany); the samples were kept on ice throughout the sonication. Four 1.8 mL aliquots of each sample were pipetted into centrifuge tubes and centrifuged (13,000 *g*, 15 min, 4 °C) and the supernatant then transferred to fresh tubes and stored on ice. Enzymatic activity of the plant extracts was assayed immediately. Black, medium binding, 96-well microplates (Greiner, Frickenhausen, Germany) were used for the assays. The microplate setup included eight incubation wells per sample and substrate (100 µL plant tissue extract + 50 µL substrate), six calibration wells (100 µL AB + 50 µL C_1-C_6), six control wells per sample for assessing autofluorescence and fluorescence quenching by the sample (100 µL plant tissue extract + 50 µL C_1-C_6) and one control well per substrate for measuring substrate autofluorescence (100 µL AB + 50 µL substrate). The assays were done at 21 °C in the dark with shaking (500 rpm) on a microplate shaker (Eppendorf, Hamburg, Germany). The enzyme activity of the samples was measured in duplicate and the reactions were stopped with 100 µL of SB at 15 min intervals (after 15, 30, 45 and 60 min; two wells for each interval). Before measuring, 100 µL of SB was added to the calibration wells and control wells after which the microplate was briefly shaken; air bubbles in the wells were removed by piercing with a sharp needle. The plates were read in a Cary Eclipse fluorescence microplate spectrophotometer (Varian, Sydney, Australia) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Protein content

The total protein content of the plant tissue extracts was measured by the Bradford assay (Bio-Rad, Munich, Germany) according to the manufacturer’s instructions. Samples were assayed in triplicate.

Root colonization by AMF

Fungal structures were visualized by treating the roots as described by Phillips and Hayman (1970) with modifications by Schmitz *et al.* (1991). After incubation in 10% KOH (two 15 min incubations at 95 °C) the root material was rinsed with tap water, acidified with 3.7% HCl for 10 min and stained in lactophenol blue solution (Merck, Darmstadt, Germany) for 90 min. For destaining and storage the roots were washed several times with 50% lactic acid and stored in this same solution. The percentage of root colonization was determined with a Zeiss Axioplan light microscope using the magnified intersection method (McGonigle *et al.*, 1990). Three hundred segments of each root sample were checked for the presence of intraradical AMF hyphae, arbuscules and vesicles.

Statistical analysis

The results were expressed as the mean ± SD. For chitinase activities, mycorrhization rates and biomass, the data were tested for normality prior to analysis and log transformed if necessary. Statistical comparisons were done using analysis of variance (ANOVA) followed by Tukey’s Honestly Significant Difference *post-hoc* test. For scab resistance, Dunnett’s *t*-test was used for *post-hoc* comparisons. All data analyses were done using SAS JMP Statistical software version 8.

Results

Transgenic lines T386 and T389 expressed transgenes *nptII, nag70* and *ech42* (Figures 1-3, respectively). Transformation of the apple cultivar Pinova with *nag70* and *ech42* significantly increased the endochitinase activities of leaves and significantly increased the exo- and

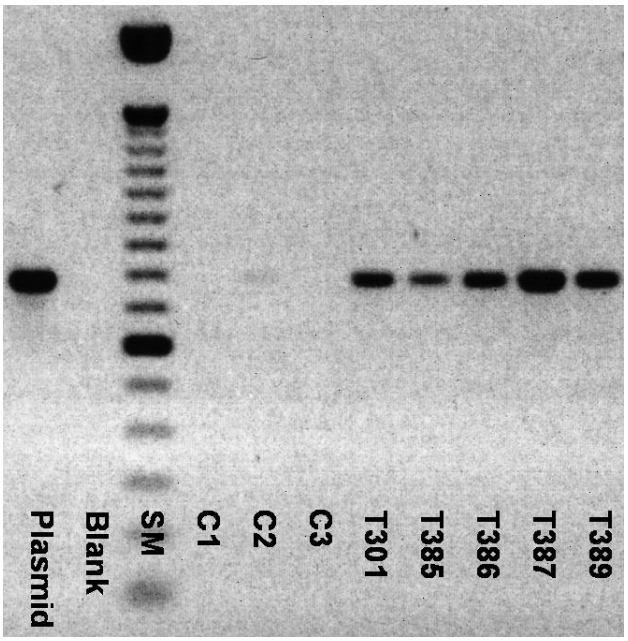

Figure 1 - Detection of *nptII* mRNA transcripts by RT-PCR. SM (size marker) – 100 bp DNA ladder, Blank – H_2O sample, C1 – non-transgenic Pinova, C2 and C3 – non-transgenic plants of two other apple cultivars, T301-T389 – Nag+Endo transgenic lines of the apple cultivar Pinova.

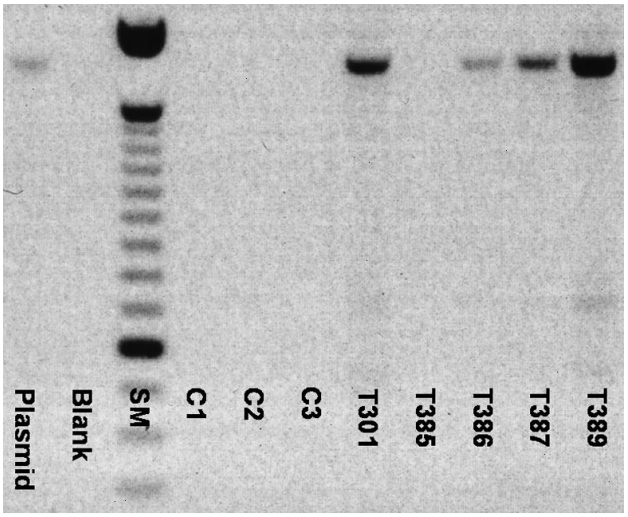

Figure 2 - Detection of *Nag70* mRNA transcripts by RT-PCR. SM (size marker) – 100 bp DNA ladder, Blank – H_2O sample, C1 – non-transgenic Pinova, C2 and C3 – non-transgenic plants of two other apple cultivars, T301-T389 – Nag+Endo transgenic lines of the apple cultivar Pinova.

endochitinase activities in the roots of own-rooted transgenic lines T386 and T389 compared to own-rooted non-transgenic Pinova (Figure 4). However, whereas the exo- and endochitinase activities in leaves were similar for both transgenic lines, T389 clones had greater chitinase activities in the roots. The Pinova and M9 varieties differed in the level of exo- and endochitinase in their leaves but not in their roots. Rootstock M9 had a variable but non-significant influence on chitinase activities of the grafted scions. In non-transformed Pinova, the leaf exo- and endochitinase

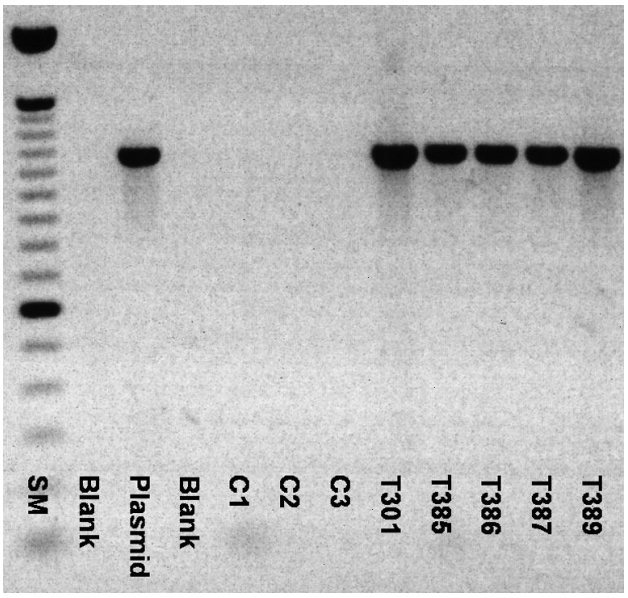

Figure 3 - Detection of *ech42* mRNA transcripts by RT-PCR. SM (size marker) – 100 bp DNA ladder, Blank – H_2O sample, C1 – non-transgenic Pinova, C2 and C3 – non-transgenic plants of two other apple cultivars, T301-T389 – Nag+Endo transgenic lines of the apple cultivar Pinova.

activities tended to increase when grafted onto M9. In transgenic line T386, grafting did not influence the level of leaf exochitinase activity whereas leaf endochitinase activity decreased slightly when grafted onto M9. In transgenic line T389, exochitinase activity tended to increase after grafting while endochitinase activity decreased slightly. In summary, grafting resulted in very similar leaf exochitinase activities in M9/Pinova, M9/T386 and M9/T389 while the differences in leaf endochitinase activities were less pronounced between M9/Pinova, M9/T386 and M9/T389 than between own-rooted Pinova and transgenic lines. Grafting of transgenic scions onto rootstock M9 (M9/T386 and M9/T389) did not significantly influence root exo- and endochitinase activities compared to the non-transgenic control M9/Pinova.

In the susceptibility test against *V. inaequalis*, own-rooted transgenic lines showed significantly fewer symptoms of scab infection than own-rooted non-transgenic Pinova at 17 dpi and 28 dpi (Figure 5). In contrast, the performance of grafted scions was different. At 17 dpi, M9/T386 and M9/T389 had significantly fewer symptoms of scab infection than M9/Pinova while there was no difference among M9/Pinova, M9/T386 and M9/T389 at 28 dpi.

After four months of cultivation in artificial substrate with an inoculum of *G. intraradices* BEG 140 and *G. mosseae* BEG 25 (from Symbio-M, Landskroun, Czech Republic), the mean root colonization (RC) rates of the transgenic lines T386 and T389 were significantly reduced (0.2% and 0.1%, respectively) compared to the RC rate of 29.8% in Pinova control plants (Schäfer *et al.*, 2009). However, as rootstock M9 and the grafted trees also exhibited very low mycorrhization rates (M9: 2.0%, M9/Pinova:

0.7%, M9/T386: 0.8% and M9/T389: 0.8%) the experiment was prolonged for another year. At the end of the second growth period, the RC of own-rooted and grafted trees grown in artificial substrate with an inoculum of *G. intraradices* and *G. mosseae* was again assessed microscopically. In own-rooted transgenic lines, the RC rates

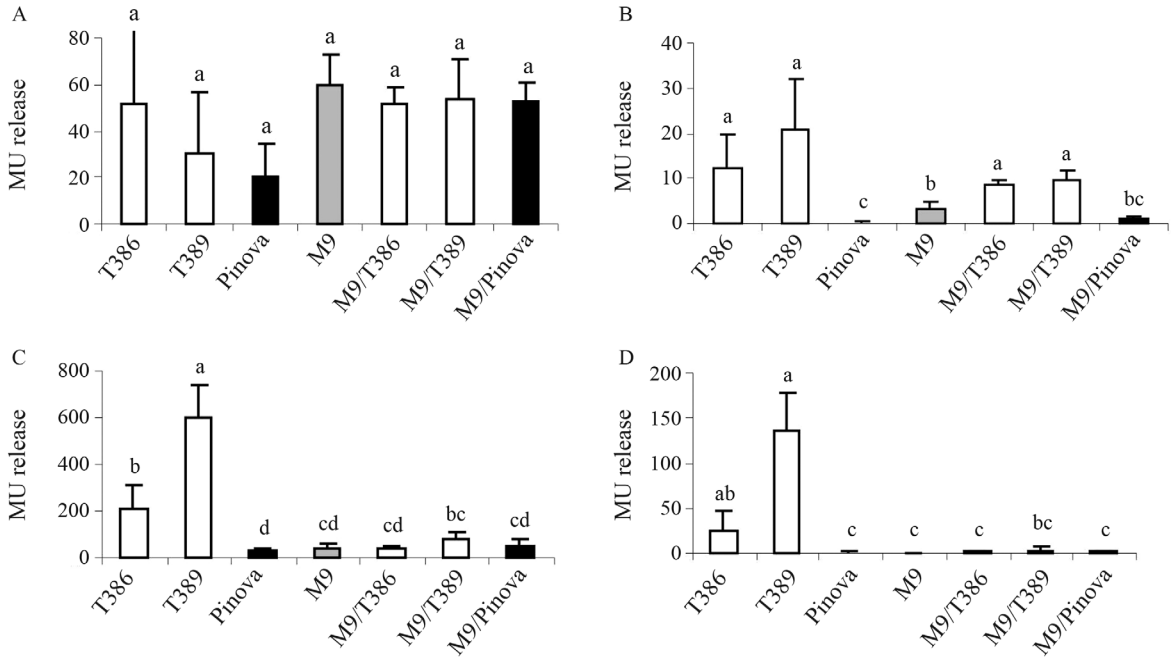

Figure 4 - Chitinase activity of tissues from greenhouse-grown trees. (A) Exochitinase and (B) endochitinase activities in the youngest leaves, and (C) exochitinase and (D) endochitinase activities in fine roots. Enzyme activities (MU release) were expressed in pmol/mg protein/min and the columns represent the mean ± SD of four independent determinations. ANOVA of log transformed data revealed significant differences in the leaf endochitinase (F: 41.4145, $p < 0.0001$), root exochitinase (F: 25.6303, $p < 0.0001$) and root endochitinase (F: 18.6769, $p < 0.0001$) activities among the varieties/lines. Different letters above bars indicate significant differences ($p < 0.05$) based on Tukey's Honestly Significant Difference test.

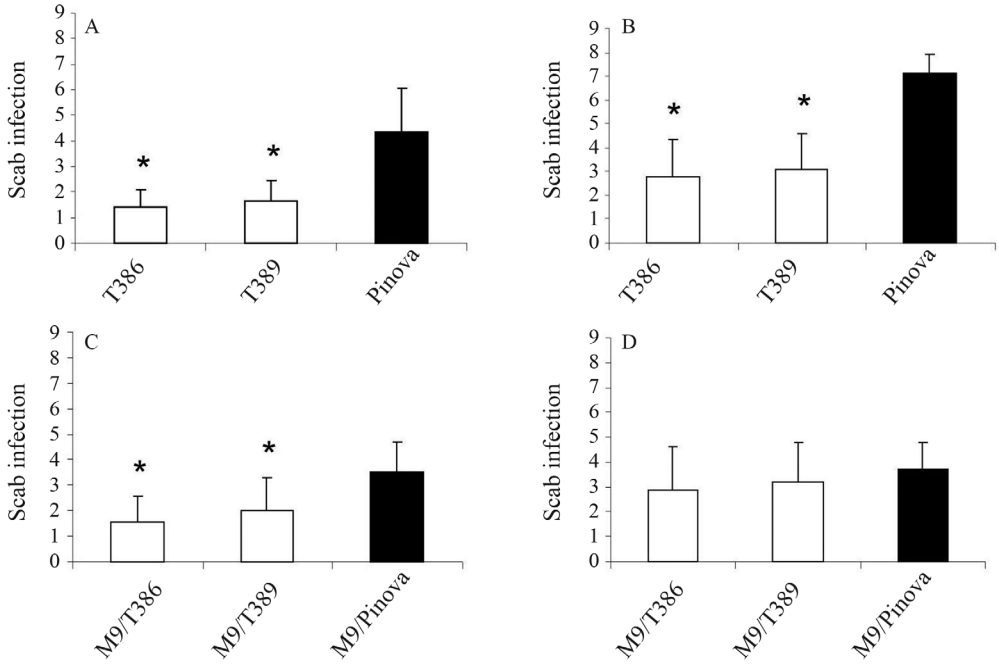

Figure 5 - Scab susceptibility tests against *V. inaequalis* in non-transgenic Pinova and transgenic lines T386 and T389 on own roots (non-grafted) 17 (A) and 28 (B) days post-inoculation (dpi) and scions of non-transgenic control Pinova and transgenic apple clones T386 and T389 grafted onto the non-transgenic rootstock M9 at 17 (C) and 28 (D) dpi. Shoot regrowth was inoculated with a local mixture of scab conidia (10^5 conidia/mL). The degree of scab infection on infected leaves was scored on a scale of 1 to 9 after 17 (A) and 28 days (B). The results of different shoots from each clone were averaged. The columns represent the mean ± SD of 20-29 determinations. Significance (*$p < 0.05$) was detected using Dunnett's *t*-test.

were still significantly reduced compared to non-transgenic Pinova (Figure 6). In contrast to transgenic line T389 which established hardly any mycorrhizal structures in its roots (RC: 0.5%), transgenic line T386 had an RC rate of 9.1%. Rootstock M9 showed the highest colonization rates. Grafted trees tended to have lower mycorrhization rates than M9. Among grafted trees, M9/T386 and M9/T389 tended to have higher mycorrhization rates than M9/Pinova.

When grown on their own roots, transgenic lines T386 and T389 produced significantly less biomass than the non-transgenic Pinova (Figure 7). In addition, grafted trees produced less biomass than M9 as a complete tree. Among grafted trees, there was no difference in the biomass of non-transgenic M9/Pinova compared to transgenic M9/T386 and M9/T389.

Discussion

Previous studies have described the transformation of apple with the *T. atroviride* genes *nag70* and *ech42* to enhance resistance to *V. inaequalis* the pathogenic agent of apple scab (Bolar *et al.*, 2001; Faize *et al.*, 2003). In accordance with these findings, the results of the present study show that own-rooted transgenic lines T386 and T389 were significantly less susceptible to *V. inaequalis* than the non-transgenic cultivar Pinova. However, in addition to this desired effect, the significantly increased chitinase activities of T386 and T389 were accompanied by a significant reduction in biomass production. Indeed, endochitinase activity is known to be negatively correlated with the growth of transgenic apple trees (Bolar *et al.*, 2000; Faize *et al.*, 2003).

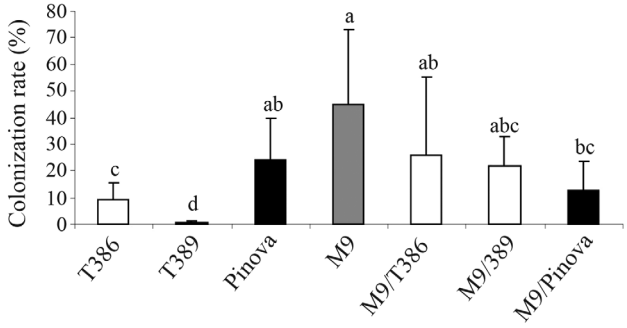

Figure 6 - AMF colonization rates of greenhouse-grown transgenic lines (T386 and T389) and non-transgenic Pinova (on own roots and grafted on rootstock M9) in the second year of cultivation in sand-vermiculite with *G. intraradices* and *G. mosseae.* The columns represent the mean ± SD of nine determinations. ANOVA of log transformed data revealed significant differences among the varieties/lines (F: 15.3322, $p < 0.0001$). Different letters above bars indicate significant differences ($p < 0.05$) based on Tukey's Honestly Significant Difference test.

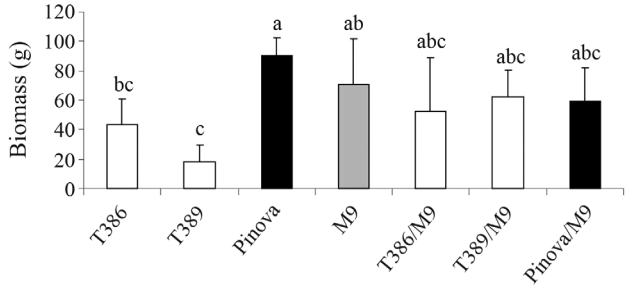

Figure 7 - Biomass of two-year-old greenhouse-grown transgenic lines (T386 and T389) and non-transgenic Pinova (on own roots and grafted on rootstock M9). The columns represent the mean ± SD of 4-5 determinations. ANOVA revealed significant differences among the varieties/lines (F: 5.0944, $p = 0.0017$). Different letters above bars indicate significant differences ($p < 0.05$) based on Tukey's Honestly Significant Difference test.

As shown here, the line T389 had the highest endochitinase activity and produced the least biomass. When own-rooted, both transgenic lines showed a significant reduction in the RC rates by AMF compared to the non-transgenic Pinova. Apple trees reportedly benefit from the symbiosis with AMF through enhanced drought tolerance (Runjin, 1989), improved uptake of nutrient elements from the soil (An *et al.*, 1993) and improved tree growth, especially in soils with low phosphorus (P) levels (Plenchette *et al.*, 1981; Gnekow and Marschner, 1989) and in soils affected by apple replant disease (Taubebaab and Baltruschat, 1993; Catska, 1994). Consequently, the suppression of AMF symbiosis in the roots of transgenic apple trees could decrease plant vitality and productivity.

The grafting of transgenic lines T386 and T389 onto rootstock M9 neither significantly increased root chitinase activities nor reduced RC rates compared to non-transgenic M9/Pinova. The grafting of transgenic scions also did not result in reduced biomass. However, grafting influenced leaf chitinase activities of the scions. Differences among the leaf chitinase activities of M9/Pinova, M9/T386 and M9/T389 were less marked than those among own-rooted Pinova, T386 and T389. This observation agreed with the results of the resistance test against *V. inaequalis*. Grafted scions of transgenic lines (M9/T386, M9/T389) were only temporarily less susceptible to *V. inaequalis* than the non-transgenic M9/Pinova. Apparently, grafting altered the characteristics of scions and, in contrast to the transgenic scions, non-transgenic Pinova scions benefited from being grafted onto rootstock M9 (cf. the scab susceptibility of own-rooted sample trees). In a recent study of rootstock-regulated gene expression patterns in apple tree scions, Jensen *et al.* (2010) reported that different rootstocks trigger distinct, reproducible scion gene expression patterns. While relatively few scion genes were affected by the rootstock at the transcript accumulation level, "transcripts predicted to have a function in responses to abiotic and biotic stimuli and response to stress were disproportionately represented among the candidate rootstock-regulated genes" (Jensen *et al.*, 2010). Further research should investigate whether the combination of transgenic scions expressing *nag70* and *ech42* with rootstocks other than M9 results in better resistance to *V. inaequalis*.

In the present study, sample trees were cultivated in artificial substrate and inoculated with *G. intraradices* and *G. mosseae*. The RC rates of own-rooted transgenic lines were significantly reduced compared to non-transgenic Pinova (local effect) while the RC of grafted transgenic scions were not reduced (no systemic effect).

Previous reports have shown that AMF root colonization rates were not adversely influenced by overexpressing plant chitinase genes in tobacco (Vierheilig *et al.*, 1993, 1995) and tomato (Girlanda *et al.*, 2008). The insertion of a single *Trichoderma* endochitinase had no adverse effect on ectendomycorrhizal fungi in spruce (Stefani *et al.*, 2010) nor on AMF in barley (Kogel *et al.*, 2010). However, multiple chitinases have been reported to exhibit synergistic and complementary antifungal effects (Bolar *et al.*, 2001; Duo-Chuan, 2006). Whether the adverse effect on AMF seen in own-rooted transgenic lines expressing the *T. atroviride* genes *ech42* and *nag70* was specific to apple (in a manner similar to the apple-specific reduction in vigor seen after interaction with endochitinase) remains to be determined, although this effect has not been reported for any other crop (Bolar *et al.*, 2001; Gentile *et al.*, 2007; Stefani and Hamelin, 2010).

Symbioses between plants and AMF are complex and are influenced by various environmental factors (Smith and Read, 2008). In a further study, we will address the impact of transforming apple with the *T. atroviride* genes *nag70* and *ech42* on AMF symbionts in an experimental set-up that simulates natural conditions more closely by cultivating trees in pots with field soil harbouring a diverse AMF community.

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