Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride (T. harzianum)* against the pathogenic fungus (*Venturia inaequalis*) in transgenic apple plants

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Abstract

Genes from the biocontrol fungus *Trichoderma atroviride* encoding the antifungal proteins endochitinase or exochitinase (N-acetyl-β-D-hexosaminidase) were inserted into 'Marshall McIntosh' apple singly and in combination. The genes were driven by a modified CaMV35S promoter. The resulting plants were screened for resistance to *Venturia inaequalis*, the causal agent of apple scab, and for effects of enzyme expression on growth. Disease resistance was correlated with the level of expression of either enzyme when expressed alone but exochitinase was less effective than endochitinase. The level of expression of endochitinase was negatively correlated with plant growth while exochitinase had no consistent effect on this character. Plants expressing both enzymes simultaneously were more resistant than plants expressing either single enzyme at the same level; analyses indicated that the two enzymes acted synergistically to reduce disease. Selected lines, especially one expressing low levels of endochitinase activity and moderate levels of exochitinase activity, were highly resistant in growth chamber trials and had negligible reduction in vigor relative to control plants. We believe that this is the first report of resistance in plants induced by expression of an N-acetylhexosaminidase and is the first report of in planta synergy between an exochitinase and an endochitinase.

Introduction

Genes from biocontrol fungi in the genus *Trichoderma* have been demonstrated to encode proteins with high antifungal activity against a wide range of plant pathogenic fungi (reviewed by Lorito, 1998a). One of these genes, encoding an endochitinase (ech42, EC 3.2.14) was inserted into tobacco, potato (Lorito et al., 1998b) and apple (Bolar et al., 2000). This gene conferred high levels of resistance to all pathogens tested, including the leaf and fruit rotting Deuteromycetes *Alternaria alternata*, *A. solani*, and *Botrytis* *cinerea* (Lorito et al., 1998b), the Ascomycete *Venturia inaequalis* (Bolar et al., 2000) and the stem and root-attacking Basidiomycete *Rhizoctonia solani* (Lorito et al., 1998b). However, in apple, the expression of even moderate levels of the enzyme caused a reduction of plant vigor (Bolar et al., 2000) even though no such effects were seen in tobacco or potato (Lorito et al., 1998b).

Further, the use of a single gene could have several potential drawbacks. First, any single disease-control gene that is used widely is likely to encourage the development of resistance to the effective molecule in the pathogen population as a consequence of selection (Wenzel, 1985; Jach et al., 1995; Fuchs, 1998). Second, the use of a single gene may require higher

levels of heterologous protein expression for adequate levels of resistance than the use of two or more genes that encode synergistic proteins. Lower production of total heterologous proteins would reduce the diversion of plant resources into production of these proteins. Further, in apple it would be advantageous to produce disease resistant lines that express a minimal level of the vigor-reducing endochitinase (Bolar et al., 2000). All of these goals could be met with additional genes for disease resistance, especially if the proteins produced by them had strong synergy in their antifungal activity.

An exochitinase, N-acetyl-β-D-hexosaminidase (nag70, EC 3.2.1.52), and the endochitinase from the biocontrol fungus *Trichoderma atroviride* are each strongly antifungal (ED$_{50}$ values of about $50\,\mu$g/ml for inhibition of conidial germination of *B. cinerea*) and strongly synergistic (ED$_{50}$ values of $9\,\mu$g/ml of total protein for a 1:1 mixture of the two proteins) (Lorito et al., 1994a, b). Wong et al. (1999) demonstrated *in vitro* synergism of *T. atroviride* exochitinase and endochitinase against *V. inaequalis*, the causal agent of apple scab and *Gymnosporangium juniperi-virginianae*, the causal agent of cedar apple rust. They are therefore good candidates for conjoint transfer into apple. The genes encoding both enzymes have been cloned from *T. atroviride* strain P1 (Hayes et al., 1994; Draborg et al., 1995; Peterbauer et al., 1996) and both contain a signal peptide at the 5′ end that probably is responsible for extracellular secretion of the enzyme (Hayes et al., 1994; Draborg et al., 1995). The endochitinase is known to be secreted from transgenic plant cells (Lorito et al., 1998b). Similar genes have been cloned from other strains of *T. atroviride* (summarized by Lorito et al., 1998a). We have reported results on scab disease resistance with the expression of endochitinase in apple (Bolar et al., 2000) and Lorito et al. (1998b) reported resistance to several pathogens in potato and tobacco conferred by expression of the same gene. However, as far as we are aware, there is no report of insertion of an N-acetylhexosaminidase gene from any source for the purpose of increasing resistance to plant disease.

Apple scab is the most destructive disease of apples worldwide (Biggs, 1990). The disease is routinely controlled in commercial apple orchards by multiple applications of chemical fungicides. Although great improvements have been made in the efficacy and safety of modern fungicides, they remain an economic burden to growers, and there is continuing concern for their non-target effects (Gadoury et al., 1989). 'McIn-

tosh' is by far the most widely-planted apple cultivar in the northeastern United States (New York Agricultural Statistics Service, 1998), and like most commercial cultivars, is very susceptible to scab (Merwin et al., 1994). Conventionally bred scab resistant cultivars, such as 'Liberty' (Lamb et al., 1979) have inadequate quality for national and international markets.

This research was conducted to evaluate the level of expression, enzymatic activity, apple scab resistance and growth effects of exochitinase alone, and of endochitinase and exochitinase together in transgenic 'McIntosh' apple lines.

Materials and methods

Plasmid binary vectors

Based on the sequence of *nag70* (Peterbauer et al., 1996), a forward primer (JNF) with *Xba*I (5′-GC-TCTAGACCGCCTCGGTCGTCCATCAT-3′) and a reverse primer (JNR, 5′-CGGGATCCTTATGCGAA-CAAGGTGCAAGCCGTAGC-3′) with *Bam*HI restriction sites (restriction sequence underscored) were designed. A PCR-based cloning system was used to amplify the entire genomic and cDNA sequence of the gene. For the genomic sequence, DNA extracted from hyphae of *T. atroviride* strain P1 (ATCC 74058), formerly classified as *T. harzianum* (Mach et al., 1999), and for the cDNA sequence, DNA from a cDNA library of strain P1 (constructed by Stratagene, La Jolla, CA), were used as templates. These amplified genomic (1.9 kb) and cDNA (1.7 kb) fragments were cloned into pBluescript phagemid (Stratagene) and sequenced. The *nag70* genes were cloned behind the enhanced 35S promoter and AMV leader sequence of pBI525 (obtained from William Crosby, Plant Biotechnology Institute, Saskatoon, Saskatchewan, Canada), and the entire *Hin*dIII/*Eco*RI cassette in pBI525 was later cloned into the binary vector pBI121 (Clontech Laboratories Inc., Palo Alto, CA). The resulting plasmids were designated as pBI121-cNag and pBI121-gNag (cDNA and genomic, respectively; Figure 1A and 1B).

Plasmid pBIN19ESR contains the cDNA sequence of *ech42* (Bolar et al., 2000; Figure 1C). pBI121-cNag (Figure 1A) was restriction digested with *Hin*dIII and ligated to synthesized oligomers containing recognition sites for *Eco*RI+*Not*I+*Hin*dIII. This fragment was digested with *Eco*RI, and the cassette of interest was ligated into pBIN19ESR that had been cut by

A. pBI121-cNag (12.6 kb)

B. pBI121-gNag (12.8 kb)

C. pBIN19ESR (12.2 kb)
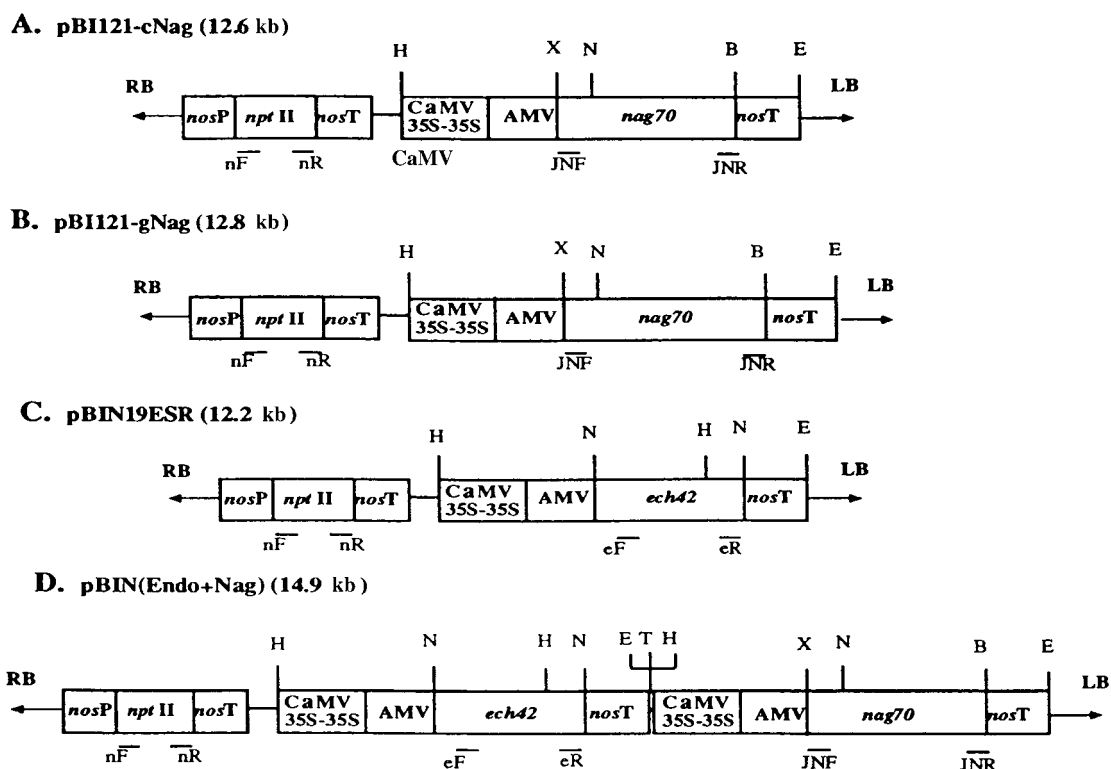

D. pBIN(Endo+Nag) (14.9 kb)

Figure 1. T-DNA of: (A) plasmid pBI121-cNag containing the cDNA of *nag70*. (B) plasmid pBI121-gNag containing the genomic DNA of *nag70*. (C) plasmid pBIN19ESR containing the cDNA of *ech42* (Bolar et al., 2000). (D) pBIN(Endo + Nag) containing the cDNA of both *ech42* and *nag70*. *nos*P: nos promoter; *nos*T: nos terminator; *npt*II, neomycin phosphotransferase II encoding gene (aminoglycoside selectable marker); CaMV-35-35S: enhanced cauliflower mosaic virus 35S promoter (Odell et al., 1985); and AMV: alfalfa mosaic virus leader sequence of RNA 4 (Jobling & Gehrke, 1987). Approximate positions of the primers used for PCR analysis: for *npt*II gene a forward primer nF and reverse primer nR (Bolar et al., 2000), for the *nag70* gene a forward primer JNF and reverse primer JNR, and for the *ech42* gene a forward primer eF and reverse primer eR (Bolar et al., 2000) were used. Restriction endonuclease sites: H: *Hind*III; X: *Xba*I; B: *Bam*HI; E: *Eco*RI; N: *Nco*I; and T: *Not*I.

the same enzyme, resulting in the double construct pBIN(Endo + Nag) (Figure 1D).

Plasmids pBI121-cNag, pBI121-gNag, and pBIN-(Endo + Nag) were transferred to *Agrobacterium tumefaciens* strains EHA105 (obtained from S. Gelvin, Purdue University, IN) and EHA105(pCH32) (Hamilton, 1997) using the procedure described by Nagel et al. (1990). The orientation of the genes within the T-DNA in all the binary vectors was confirmed by PCR and by restriction enzyme analysis. The plasmids were sequenced to confirm proper reading frames for the genes and their structure is given in Figure 1.

Plant transformation

Leaf segments of 'Marshall McIntosh' were inoculated with *A. tumefaciens* strain EHA105 or EHA105-pCH32 containing the plasmid pBI121-cNag, pBI121-gNag, or pBIN(Endo + Nag), and independent trans-

genic lines were recovered as described previously (Bolar et al., 1999; Bolar et al., 2000; Norelli et al., 1996).

PCR analysis

DNA was isolated from the youngest leaf of putative transgenics and non-transformed control plants and analyzed by the PCR procedure as described by Bolar et al. (1999), using primers specific for *nag70*, *nptII* (neomycin phosphotransferase gene that confers resistance to the aminoglycoside antibiotics) or *ech42* gene (Figure 1). Methods for Southern analysis are described in Figure 2.

ELISA for NPTII protein

The amount of NPTII protein in leaf tissue samples from the putative transgenic lines was determined

using a standard sandwich ELISA according to manufacturer's recommended procedure (5Prime→3Prime Inc., Boulder, CO).

Western analysis

Individual leaves from transgenic and control lines were processed as described by Gegenheimer (1990). Purified 42 kDa-endochitinase from *T. atroviride* (Harman et al., 1993) was used as a standard, and a Bench-Mark prestained protein ladder (Life Technologies, Grand Island, NY) was used as a molecular weight marker. SDS-PAGE was performed as described previously (Bolar et al., 2000) and the protein was detected using a polyclonal antibody specific to exochitinase or endochitinase. The western results presented are from a composite of different membranes which had similar intensities.

Activity assay for chitinases

Exochitinase and endochitinase activities of leaf tissue were detected and quantified using the procedures described by Bolar et al. (2000). The activity of exochitinase and of endochitinase was determined using the substrates 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma, St. Louis, MO) and 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside (Sigma), respectively. Activity of the detected enzyme was defined as nM of methylumbelliferone released per min per mg fresh weight of leaf tissue.

Evaluation of disease resistance and plant growth

Tissue culture plants were rooted, acclimated, established as own-rooted plants in pots, and maintained in a growth chamber or greenhouse (Bolar et al., 1998). Plants were inoculated with a conidial suspension of *V. inaequalis* and evaluated for scab development as described previously (Bolar et al., 2000; Yepes & Aldwinckle, 1993). The youngest expanded leaf was tagged and the plants were inoculated with a conidial suspension (10^6 conidia per ml) that included a mixture of races 1–5 of *V. inaequalis* (isolates 1805-2, 1770-8, 1771-2, 1778-6, and 1810-1), using an atomizer connected to compressed air supply. The isolates were originally obtained from E. B. Williams (Purdue University, IN). The plants were incubated in a mist chamber (16-h photoperiod of 40 μmol per m² per second, 18±1°C and 100% relative humidity) for 48 h and later moved to a growth chamber or greenhouse. About 2 weeks after inoculation, the severity of the disease was recorded from four leaves (the tagged leaf, one leaf above and two leaves below the tagged leaf). Data were recorded as numbers of sporulating lesions, the percentage of leaf area infected, and conidia from the four leaves. To evaluate vigor of the inoculated and uninoculated plants, data were collected on the height of the plants from soil line to the terminal leaf, number of nodes/leaves, and a qualitative estimate of the degree of chlorosis. All the experiments were repeated at least twice. Data were analyzed using SAS (SAS Institute, Cary, NC) general linear model and parametric model, and locally weighted regression.

Synergism is defined as the combined action of two or more agents that is greater than the sum of the action of each of the agents used alone, that is it implies an effect over and above the purely additive, or aggregate effect (Richer, 1987). There are various methods to analyze data for the presence of synergy. For comparisons of individual agents, Limpel's formula as modified by Richer (1987) was used. It states $E_e = (X + Y) - (XY/100)$ where E_e is the expected combined effect; X is the expected percent control when compound A is used alone and Y is the expected percent control when compound B is used alone. If the observed effect (E_o) is greater than the expected effect (E_e), then synergism has been demonstrated. In addition, entire sets of data were evaluated for synergy by comparing the three-dimensional surface generated from locally weighted regressions (Cleveland & Devlin, 1988) over the entire data set.

Results

Recovery of transgenic lines

Individual kanamycin-resistant plants were tested for the presence of transfer DNA (T-DNA) using PCR with primers specific for *nag70*, *nptII* or *ech42* coding regions (Figure 1). DNA of the appropriate size was amplified from 33 lines transformed with *nag70* cDNA (pBI121-cNag), 44 lines transformed with *nag70* genomic DNA (pBI121-gNag), 13 lines transformed with *ech42* cDNA in this, and a previous study (Bolar et al., 2000), and 27 lines transformed with a vector carrying both *ech42* and *nag70* [pBIN(Endo + Nag)]. No bands were detected in non-transformed 'Marshall McIntosh'. With primers specific to *virG* (Chen et al., 1991) a band was detected only for the plasmid control, pAL4404, indicating that
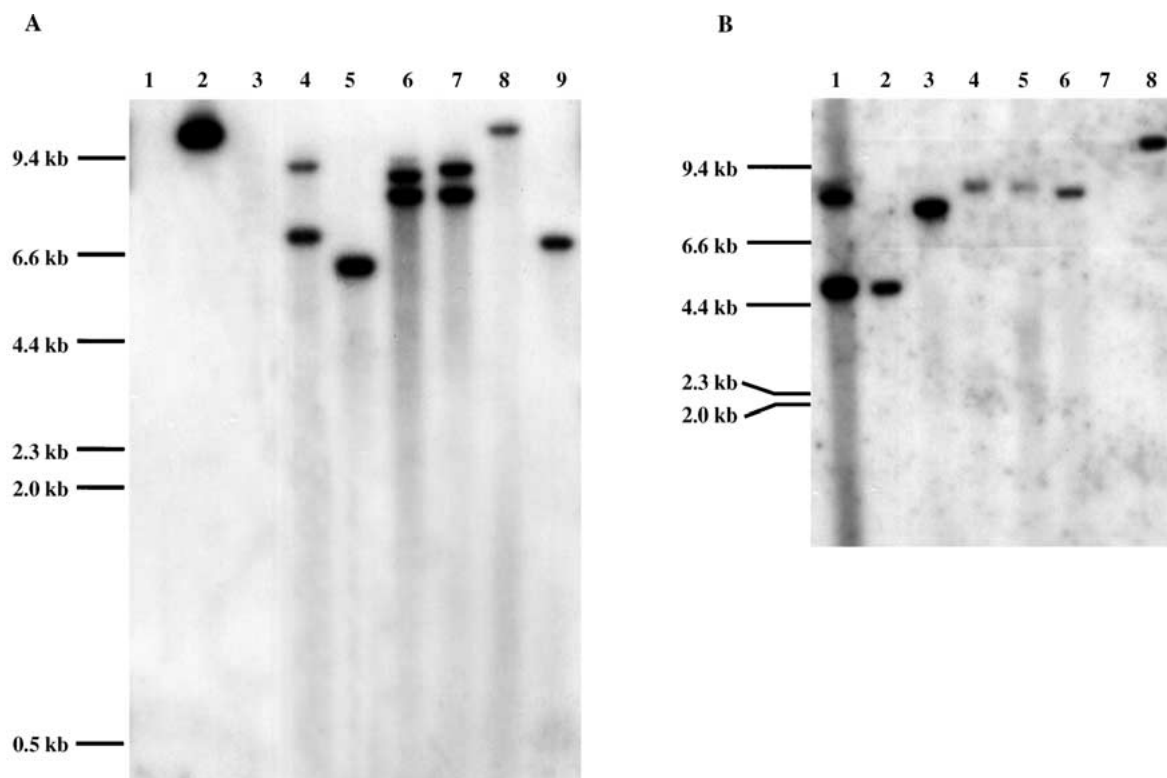
Figure 2. Southern analysis of 'Marshall McIntosh' transgenic lines. DNA was extracted from the leaf tissue of non-transformed and putative transgenic plants (Fulton et al., 1995), and Southern analyses were performed using standard procedures from directions in Genescreen Plus (NEN Research Products, Boston, MA). Genomic DNA was digested with *Hin*dIII (Refer to Figure 1 for restriction sites) and electrophoretically separated on 1.0% agarose gel. Southern blot was probed with the *nag70* (1700 bp) coding region labeled with ^{32}P using random primers (Feinberg & Vogelstein, 1983). Since the *nag70* gene is flanked by a T-DNA border and a *Hin*dIII site, each inserted T-DNA should result in an unique *Hin*dIII restricted DNA fragment which would hybridize with a *nag70* probe. Number of bands corresponds to number of inserts. Lanes as follows: (A) Gel evaluating the DNA inserted from the pBIN (Endo + Nag) 1-DNA blank, 2-pBIN(Endo + Nag), 3-'Marshall McIntosh', 4-T960, 5-T928, 6-T940, 7-T936, 8-T929, 9-T958. (B) Gel evaluating the DNA inserted from the pBI121-cNag 1-T837, 2-T950, 3-T891, 4-T832, 5-T879, 6-T870, 7-'Marshall McIntosh', 8-pBI121-cNag.

transgenic plants were not contaminated with *A. tumefaciens*. ELISA results indicated that all lines tested produced higher levels of NPT II protein than the controls. Southern blots of lines containing both genes were analyzed with *nag70* as a probe and all were found to contain the gene; the analyses from six lines are shown (Figure 2A). Similarly, 33 lines expressing *nag70* analyzed by Southern analysis demonstrated the presence of *nag70*; Southern analyses of six lines are presented (Figure 2B). Integration of the T-DNA into the genome of all the endochitinase transgenic lines has been reported previously (Bolar et al., 2000).

Characterization of chitinases in transgenic lines

There was greater Nag70 activity (0.4–33.7 nM MU/min/mg fresh weight) in lines transformed with the cDNA version (a total of 33 lines) of the gene than in lines transformed with the genomic version (a total of 44 lines) of the gene (0.2–6.54 nM MU/min/mg fresh weight). All the selected *ech42* (Bolar et al., 2000) and *ech42+nag70* lines had greater endochitinase activity than the controls (Table 1). Western analysis indicated the presence of a 42-kDa endochitinase band corresponding to Ech42 and an additional smaller band (31 kDa) that may be a degradation product of *Ech42* (Bolar et al., 2000, Figure 3A) and *ech42+nag70* lines (Figure 3B). Western blot analysis of the six nag70+ech42 lines indicated the presence of a 72 kDa Nag70 band in the transgenic lines but not in non-transformed 'Marshall McIntosh' (Figure 3C). The 72 kDa Nag70 band also was detected in the six *nag70* transgenic lines in Table 1 (data not presented). There was a correlation between enzymatic activity

Table 1. Transgenic 'Marshall McIntosh' lines: chitinase activity, scab reaction and plant height

Transgenic line	Chitinase activity[1]		Percent leaf area infected[2,3]	Plant height (cm)[3]
	ech42	*nag70*		
M. McIntosh[4]	0.02	0.5	30.1 a–b	24.1 c–e
T286 (vector)[5]	0.05	0.7	31.9 a–b	26.1 b–d
Endochitinase lines				
T776	1.2	nt	12.2 d	16.9 h–k
T777	2.4	nt	14.7 d	17.6 g–j
T568	3.4	nt	1.2 e	10.2 m
T564	5.3	nt	0.2 e	10.2 m
T778	26.2	nt	nt	ng
T775	38.1	nt	nt	ng
Exochitinase lines[6]				
T950	nt	1.1	34.1 a	29.2 a–b
T870	nt	1.6	15.6 c–d	23.5 d–f
T879	nt	2.5	28.3 a–b	29.0 a–b
T832	nt	4.8	24.2 b–c	30.4 a
T837	nt	15.9	16.6 c–d	27.7 a–c
T891	nt	20.4	12.7 d	18.0 g–j
Endochitinase + Exochitinase lines				
T940	1.9	1.7	12.2 d	19.1 g–i
T960	2.1	1.6	1.6 e	19.9 f–h
T936	3.4	2.4	2.8 e	16.1 i–l
T929	5.8	11.9	1.3 e	12.6 l–m
T958	16.3	19.9	nt	ng
T928	40.8	14.5	nt	ng
T961	0.6	14.9	2.2 e	20.7 e–g
Liberty[7]	0.04	0.8	0.0 e	nt

Using a simple parametric model for analysis of variance $y = b_0 + b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2$ where y is percent of leaf area infected, x_1 is log endochitinase activity, and x_2 is log exochitinase activity, and fitting terms sequentially, the following analysis of variance (ANOVA) was obtained:

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
x1	1	1396.897	1396.897	44.00935	0.00001628
x2	1	278.543	278.543	8.77552	0.01100748
x1:x2	1	155.184	155.184	4.88908	0.0455565
Residuals	13	412.632	31.74		

The ANOVA is evidence for the non-additivity of activity of the two enzymes ($P < 0.05$).

[1] Chitinase activity in nM MU/min/mg fresh weight.
[2] Plants were inoculated by spraying with a conidial suspension of *Venturia inaequalis*. Data presented are the mean of four leaves (five to 15 plants/line) 14 days after infection.
[3] Values followed by the same letter (for the entire column) are not significantly different ($p = 0.05$), as determined by Waller–Duncan K′-ratio *t* test.
[4] Non-transformed 'Marshall McIntosh'.
[5] Transgenic 'Marshall McIntosh' line transformed with pBI121 that does not contain the chitinase gene.
[6] Transgenic line T950 was transformed with genomic version of *nag70* and lines T870, T879, T832, T837 and T891 were transformed with the cDNA version of *nag70*.
[7] 'Liberty', scab resistant check that has the *Vf* gene from *Malus floribunda* 821 for resistance to *V. inaequalis* (Lamb et al., 1979).
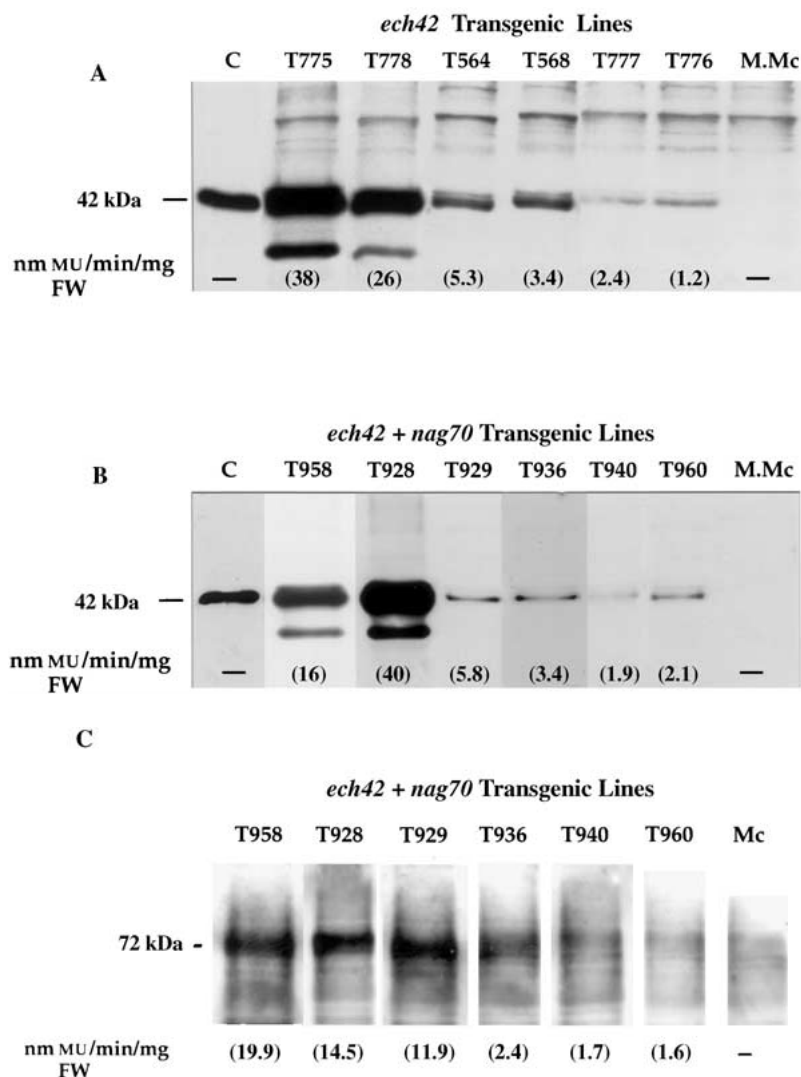ng: no growth as potted plants; nt: not tested.

Figure 3. Western blot analysis of leaf tissue extracts of endochitinase (A) and endochitinase + exochitinase (B and C) transgenic lines: Processed extracts of young leaf tissue were electrophoresed on SDS-PAGE and transferred to Immobilon-Psq membrane. The endochitinase or exochitinase protein was detected using the polyclonal antibody specific to endochitinase (A and B) or exochitinase (C). Estimated size of endochitinase (42 kDa) or exochitinase (72 kDa) is indicated. Lane C, purified endochitinase protein from *T. atroviride* MMc, 'Marshall McIntosh' control; endochitinase or exochitinase activity in nM MU per min per mg fresh weight of leaf tissue: Activity was determined by a microtiter-activity assay using 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotrioside (A and B) or 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (C) as substrate.

and western analysis for the chitinases (Figure 3 and Table 1).

Evaluation of disease resistance of plants expressing single enzymes

Two weeks after inoculation, sporulating lesions were observed on leaves of non-transformed 'Marshall McIntosh', T286 control and some of the transgenic lines. Some lines expressing *nag70*, T870, T837, and T891 (Table 1), and nine additional lines in Figure 4 had significantly less disease than the non-transformed 'Marshall McIntosh' or the empty-vector control line (T286) as measured by the number of sporulating lesions, percentage of leaf area infected, and the number of conidia recovered. There was a very weak negative correlation between Nag70 activity and number of lesions and percent of leaf area infected (Figure 4).
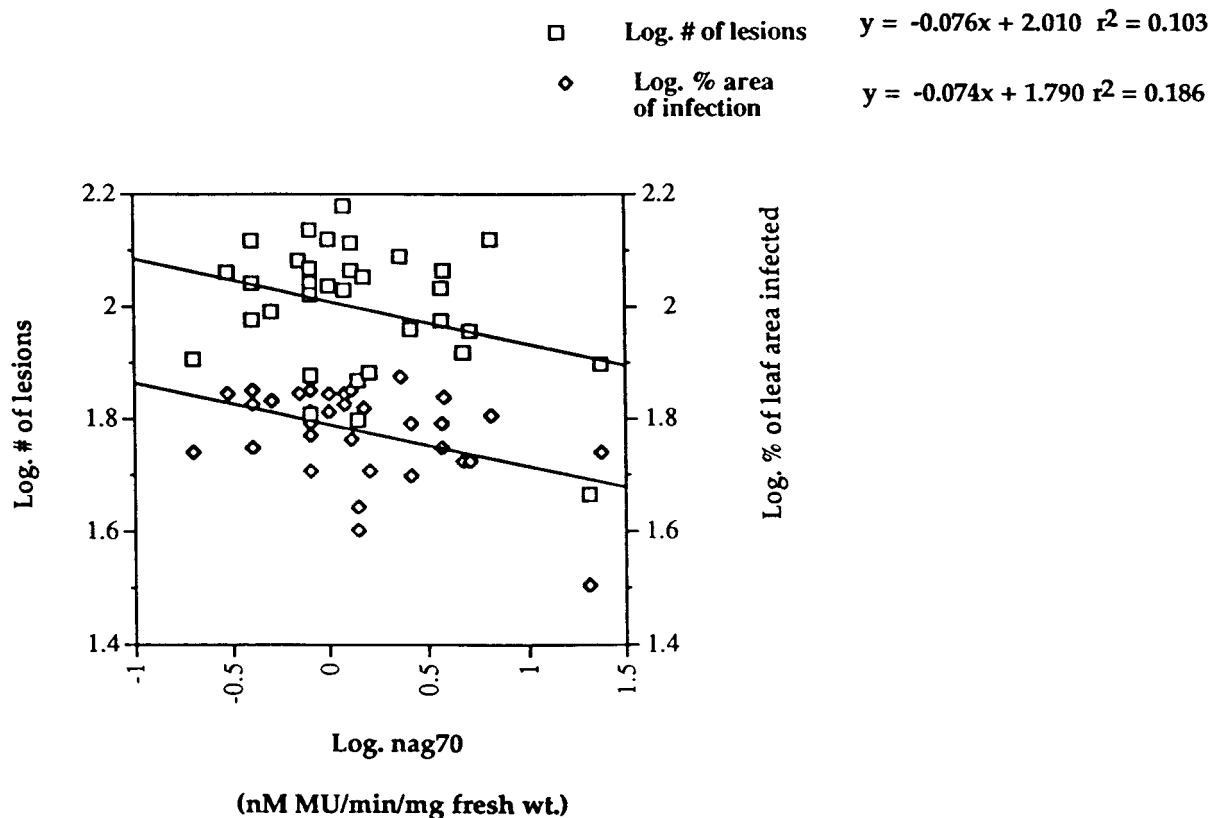
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$$y = -0.076x + 2.010 \quad r^2 = 0.103$$

$$y = -0.074x + 1.790 \quad r^2 = 0.186$$

□ Log. # of lesions

◊ Log. % area of infection

Figure 4. Effect of level of N-acetyl-β-D-hexosaminidase (*nag70*) expression in apple on scab disease caused by *Venturia inaequalis*. Plants were inoculated by spraying with a suspension of conidia of races 1–5 of *V. inaequalis*. A paper tag was hung from the youngest opened leaf at inoculation. Data presented are means of four leaves (leaf above tag, tagged leaf and two leaves below) from several replicates, 14 days after inoculation. Base 10 logarithm (Log) of number of sporulating lesions and percentage of leaf area infected were plotted against the Log of Nag70 activity (in nM MU per min per mg fresh weight of leaf tissue).

There was no significant correlation between Nag70 activity and the number of conidia recovered (data not presented). Expression of *ech42* significantly reduced levels of disease in apple lines as reported earlier (Bolar et al., 2000) and with additional lines evaluated in this study (Table 1; *ech42*-transgenic lines T564 and T568 were also analyzed in an earlier study, Bolar et al., 2000).

A comparison of disease resistance of plants expressing either enzyme singly or both enzymes together

For these studies, six lines expressing a range of levels of each individual enzyme, or both enzymes together, were selected, for a total of 19 lines (Figure 5 and Table 1). All the endochitinase transgenic lines had significantly less disease than the controls (Table 1) and there was a significant negative correl-

ation between enzyme level and disease ($R^2 = 89\%$, $P = 0.005$; data from Table 1 were used to perform a correlation analysis). Two lines, T568 and T564, had similar levels of disease as the resistant variety 'Liberty'. Of the six exochitinase transgenic lines, three had significantly less disease than the controls (Table 1) and there was a negative correlation between exochitinase activity and percent area of leaf infected ($R^2 = 62\%$, $P = 0.02$) (Table 1) but none was as resistant as 'Liberty'. All lines coexpressing the two enzymes had significantly less disease than the controls (Table 1). Lines T960, T936, T929, and T961 had similar levels of disease resistance to that of 'Liberty' (Table 1). One of the highly resistant lines, T960, expressed only modest levels of the two enzymes, while another, T961, expressed quite low levels of Ech42 and moderate levels of Nag70.

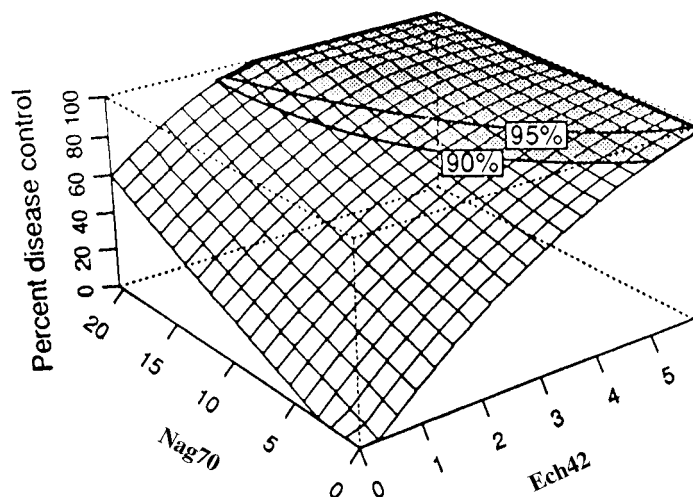The occurrence of synergy between the two enzymes was measured both in individual lines and over

Figure 5. A three-dimensional plot of local regression surface for percent disease control and chitinase activity (data from Table 1). Shaded regions indicates control $\geq$ 90% and $\geq$ 95%. Nag70 or Ech42 activity in nM MU per min per mg fresh weight of leaf tissue.

the set of 19 different lines. For individual lines, Limpel's formula (Richer, 1987) was used. *X* and *Y* values were calculated for each enzyme used alone at a specific concentration from a regression of the percent reduction in disease versus measured enzyme concentration. Based on these considerations, transgenic line T960 (E_o 95% > E_e 77%) and T961 (E_o 93% > E_e 74%) had strong synergy between the two enzymes. Transgenic line T936 (E_o 91% > E_e 85%) and T929 (E_o 96% > E_e 95%) had lower levels of synergy, and there was no synergy between the two enzymes in line T940 (E_o 60% < E_e 75%).

Synergy was also examined over the entire set using locally weighted regression analysis (Cleveland & Devlin, 1988). Synergy was clearly evident (Figure 5). A high level of disease control (95%) was never obtained with exochitinase alone and only with the very highest levels of expression with endochitinase alone. However, there is an extensive area at the 95% level of control with the combination of the two enzymes and with endochitinase at lower levels of expression (Figure 5). A parametric model of the same data demonstrates non-additivity for the two chitinase enzymes ($p < 0.05$, data from Table 1).

Chitinase activity and plant growth

To evaluate the influence of chitinolytic enzyme activity on plant vigor, growth parameters were recorded on both uninoculated and inoculated plants in the growth chamber when disease data were collected. All lines expressing exochitinase had vigor similar to that of

the controls (the analysis from six of these lines is presented in Table 1). Even though one line, T891, grew less than the controls, the other five exochitinase lines (T950, T870, T879, T832 and T837) were of similar height to the controls. There was no significant correlation between the level of exochitinase and plant height ($R^2 = 8.5\%$, $P = 0.5$) (Table 1).

As reported earlier (Bolar et al., 2000), expression of endochitinase has a negative effect on apple growth (Table 1). Lines T778 and T775 had the greatest endochitinase activity (Table 1), and did not grow when transplanted to soil in pots (Table 1). There was a significant negative correlation between endochitinase and plant growth ($R^2 = 77\%$, $P = 0.01$) (Table 1). In lines expressing both chitinases (T940, T960, T936, T929, T958 and T928), endochitinase had a negative effect on growth irrespective of the level of exochitinase (Table 1). Lines T958 and T928 had the greatest endochitinase activity, and did not grow. There was a significant negative correlation between the level of endochitinase and plant growth in these lines ($R^2 = 81\%$, $P = 0.01$) (Table 1) just as in lines expressing endochitinase alone.

Transgenic line T961 produced a very low level of endochitinase (0.6 nM MU/min/mg fresh weight) and a high level of exochitinase (14.9 nM MU/min/mg fresh weight), and had resistance that was not significantly different from that of the scab-resistant cultivar 'Liberty' (Lamb et al., 1979) (Table 1). The growth of T961 plants in the growth chamber was not significantly different from that of 'Marshall McIntosh' control plants (Table 1).

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Discussion

In this study we have successfully transferred two genes for chitinolytic enzymes from *T. atroviride*, either singly or in combination, into apples. Transgenics produced from the cDNA version of exochitinase had a higher level of enzyme expression than lines produced from the genomic version. These results suggest that the plants may not be efficient at splicing the heterologous fungal introns of exochitinase.

Constitutive expression of fungal chitinases in apple lines was correlated with increased resistance to *V. inaequalis*. There was a significant positive correlation between the level of endochitinase expression and scab resistance (Table 1), consistent with previous reports (Bolar et al., 2000; Wong et al., 1999). Some transgenic apple lines expressing exochitinase showed increased resistance to *V. inaequalis*, but the level of resistance was less than in the endochitinase lines (Table 1 and Figure 4). However, we believe this is the first report of anti-fungal activity of a eukaroytic exochitinase expressed in plants. By combining exochitinase with endochitinase, a higher level of resistance was observed at comparable levels of enzyme production. Strong synergism between the two enzymes was observed in lines T960 and T961. This is the first report to demonstrate in planta synergism by two chitinases of fungal origin.

There was no significant correlation between the level of exochitinase enzyme and plant growth. However, there was a negative correlation between the level of endochitinase and plant growth in lines expressing endochitinase alone (as reported by Bolar et al., 2000), and in lines expressing both endochitinase and exochitinase (Table 1). This reduction of vigor from the *T. atroviride* endochitinase interaction in apple has not been reported in any other crop and the mechanism of growth inhibition in apple is as yet unknown (Bolar et al., 2000).

Low expression of endochitinase with low expression of exochitinase resulted in high levels of resistance with minimal effects on plant growth, as observed in line T960 in the growth chamber. Lines with very low levels of endochitinase in combination with high levels of exochitinase (T961) had adequate plant vigor and high levels of disease resistance. Presently, some of the transgenic lines are being propagated to further evaluate disease resistance, growth effects and other horticultural qualities in the orchard. Scab-resistant lines would significantly reduce the use for chemical fungicides with beneficial economic and environmental impact.

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