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Enhanced fungal resistance in transgenic cotton expressing an endochitinase gene from *Trichoderma virens*

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Summary

Mycoparasitic fungi are proving to be rich sources of antifungal genes that can be utilized to genetically engineer important crops for resistance against fungal pathogens. We have transformed cotton and tobacco plants with a cDNA clone encoding a 42 kDa endochitinase from the mycoparasitic fungus, *Trichoderma virens*. Plants from 82 independently transformed callus lines of cotton were regenerated and analysed for transgene expression. Several primary transformants were identified with endochitinase activities that were significantly higher than the control values. Transgene integration and expression was confirmed by Southern and Northern blot analyses, respectively. The transgenic endochitinase activities were examined in the leaves of transgenic tobacco as well as in the leaves, roots, hypocotyls and seeds of transgenic cotton. Transgenic plants with elevated endochitinase activities also showed the expected 42 kDa endochitinase band in fluorescence, gel-based assays performed with the leaf extracts in both species. Homozygous T_2 plants of the high endochitinase-expressing cotton lines were tested for disease resistance against a soil-borne pathogen, *Rhizoctonia solani* and a foliar pathogen, *Alternaria alternata*. Transgenic cotton plants showed significant resistance to both pathogens.

Introduction

Microbes, including mycoparasitic fungi, represent rich sources of genes that can be utilized to genetically engineer important crops for resistance against plant pathogens and insect pests (Lorito and Scala, 1999; Punja, 2001). The use of genes encoding insecticidal proteins from *Bacillus thuringiensis* to confer resistance to various insect pests has been very successful in a number of crops (Moellenbeck *et al*., 2001; Perlak *et al*., 2001; Tu *et al*., 2000). More recently, chitinase-encoding transgenes from certain fungi have been shown to improve plant defence against a broad range of fungal pathogens. The enzyme chitinase is capable of degrading chitin, a linear homopolymer of β-1,4-N-acetyl-D-glucosamine residues, which constitutes 3–60% of the cell wall of most fungi (Collinge *et al*., 1993). *In planta* expression of endochitinase genes from diverse fungi, such as *Trichoderma harzianum* (Lorito *et al*., 1998) and *Rhizopus oligosporus* (Terekawa *et al*., 1997), has proven to be more effective both in terms of the level and in the spectrum of fungal pathogen resistance compared to the plant-derived chitinases. In addition to the direct action of chitinases on the cell wall of an invading pathogen, it has been suggested that the fungal cell wall fragments released as a result of extracellular chitinase activity may in turn activate a number of defensive responses in plants (Grison *et al*., 1996; Lorito *et al*., 1998).

Trichoderma harzianum, a mycoparasitic fungus that produces a variety of chitinolytic enzymes (Harman *et al*., 1993), is used as a biocontrol agent against plant diseases (Harman,

2000). Chitinase genes from this fungus have been used to engineer a variety of crops, such as tobacco and potato (Lorito *et al*., 1998), apple (Bolar *et al*., 2000; Bolar *et al*., 2001; Wong *et al*., 1999), petunia (Esposito *et al*., 2000), grape (Kikkert *et al*., 2000), and broccoli (Mora and Earle, 2001). Transgenic tobacco and potato plants expressing a 42 kDa endochitinase gene from *T. harzianum* were shown to be more resistant to *Alternaria alternata*, *A. solani*, *Botrytis cinerea* and *Rhizoctonia solani* (Lorito *et al*., 1998). In apple, high level expression of the same endochitinase had an adverse effect on plant development; however, in one line with a low level of expression of the endochitinase and with a moderate level expression of an exochitinase gene, a high degree of resistance to apple scab was observed with normal plant growth (Bolar *et al*., 2001).

More recently, genes encoding hydrolytic enzymes from *T. virens* have been isolated to examine their role in mycoparasitism and antifungal activities (Baek *et al*., 1999; Kim *et al*., 2002). A diverse set of chitinase and glucanase genes was isolated from *T. virens* and their structural features and expression patterns were characterized (Kim *et al*., 2002). Genetic evidence has been obtained suggesting that a 42 kDa extracellular endochitinase, common to both *T. harzianum* and *T. virens*, plays a key role in biological control of phytopathogenic fungi such as *Botrytis cinerea* and *Rhizoctonia solani* (Baek *et al*., 1999; Woo *et al*., 1999). Over-expression or disruption of a gene encoding a 42 kDa endochitinase was shown to enhance or reduce, respectively, the biocontrol activity of the mycoparasite. Results from a study by Baek *et al*. (1999) suggested that the transgenic expression of *T. virens* genes, encoding a 42 kDa class of endochitinase enzymes, might be utilized to enhance resistance of plants to various fungal pathogens.

Cotton is the most important fibre crop, grown worldwide in over 80 countries. It is the fourth largest crop in terms of economic value in the USA. Cotton seedling diseases, caused by the fungal pathogens *R. solani, Pythium* spp., and *Fusarium* spp. resulted in an estimated loss of 590 000 bales in the USA in the year 2000. In addition, other fungal pathogens (*Phymatotrichum omnivorum*, *Fusarium oxysporum* and *Verticillium dahliae*) inflict a significant financial loss for cotton producers and are difficult to control by traditional means. The present study was undertaken to examine the effectiveness of the 42 kDa endochitinase genes from *T. virens* in protecting cotton from fungal diseases. In addition, tobacco plants were initially transformed to test the expression of various endochitinase clones, and then these were evaluated for their resistance to *A. alternata*. Cotton plants transformed with one of the 42 kDa endochitinase genes from *T. virens* showed a high level of resistance to infection by *R. solani* and *A. alternata*.

Results

Regeneration of transformants and endochitinase activity in T_0 plants

Plants regenerated from transformed callus lines were grown to maturity in a greenhouse. Following establishment in the soil, both tobacco and cotton plants were examined for transgenic endochitinase activity in the leaf-protein extracts (Table 1). Transgenic endochitinase activity was only detected in the plants that were transformed with the *Tv-ech1* cDNA construct. Plants transformed with the other two cDNA constructs (*Tv-ech2*, *Tv-ech3*) and the genomic clone construct (*Tv-ech1*g) showed endochitinase activity levels that were similar to control values. The remainder of the studies were therefore conducted with plants containing the transgene *Tv-ech1*. In the case of tobacco, 21 of 47 kanamycin-resistant lines transformed with *Tv-ech1* showed endochitinase activities that were higher than the untransformed control plants (Figure 1a). The endochitinase activities ranged from 2.3 to 202 pmole 4-MU/h/μg total protein. When leaves from the higher expressing lines were assayed for endochitinase activity prior to the flowering stage, a reduction in activity levels was observed (Table 2). Ten transgenic lines with endochitinase levels ranging between 10 and 29-fold greater than the average control values were maintained in the greenhouse until maturity. All these lines were fertile and set seed.

In cotton, 71 of the 82 kanamycin-resistant lines transformed with the *Tv-ech1* cDNA construct showed elevated endochitinase activities (Figure 1b). The endochitinase values ranged from 0.1 to 56 pmole 4-MU/h/μg total protein. From

Table 1 Range of endochitinase activities* in plants transformed with four different endochitinase genes† from *T. virens*

	Control	Tv-ech1	Tv-ech1g	Tv-ech2	Tv-ech3
Cotton	0.1–0.9 (*n*‡ = 10)	0.1–55.7 (*n* = 82)	0.1–0.7 (*n* = 17)	0.1–0.9 (*n* = 17)	0.2–1.6 (*n* = 8)
Tobacco	1.6–15.1 (*n* = 10)	2.3–202.4 (*n* = 47)	0.6–9.3 (*n* = 14)	1.4–6.8 (*n* = 26)	2.2–19.4 (*n* = 25)

*Endochitinase activities are presented as pmole of 4-methylumbelliferone (4-MU) released from 4-methylumbelliferyl-β-D-*N,N′,N″*-triacetylchitotrioside/h/μg total protein.
†*Tv-ech1*, *Tv-ech2* and *Tv-ech3* are cDNA clones each encoding a different 42 kDa endochitinase and *Tv-ech1g* is the genomic clone of *Tv-ech1*.
‡*n* = the number of plants assayed in each category.

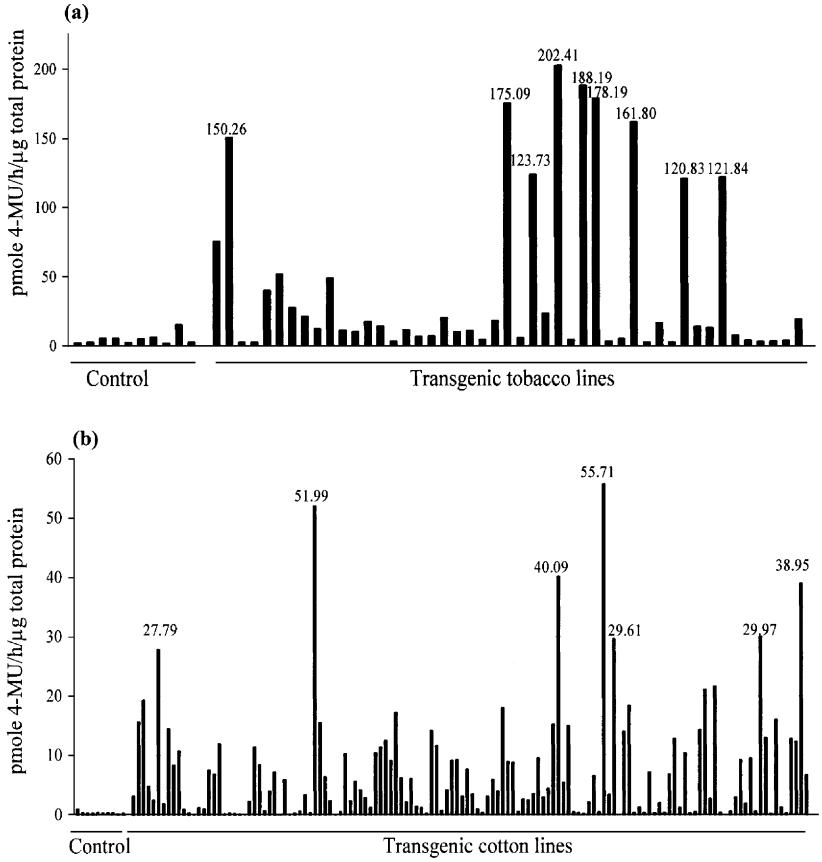


Figure 1 Endochitinase activities in young, growing leaves of T_0 plants of tobacco and cotton transformed with *Trichoderma virens* endochitinase gene (*Tv-ech1* cDNA construct). Individual bars represent endochitinase activities expressed as pmole 4-MU released/h/µg total protein. (a) Endochitinase activities in leaf tissues from 10 untransformed control plants and 47 independent transgenic tobacco lines. (b) Endochitinase activities in leaf-tissues from 10 untransformed control plants and 82 independent transgenic cotton lines. Endochitinase activity values for the high-expressing lines are shown above the respective bars.

Table 2 Endochitinase activities* in transgenic tobacco

Line	T_0 leaves	T_0 leaves (flowering)	T_1 leaves	T_2 leaves†
T4/#9	150.26	105.71	124.12	115.52 ± 11.96
T35/#5	202.41	84.82	187.75	176.50 ± 22.59
T37/#10	188.19	117.81	232.67	133.92 ± 17.91
Control	6.99	nd	nd	nd

*pmole 4-MU/h/µg total protein.
†Mean (± SE) of activities in leaf samples from 12 T_2 plants.
#: number = The number assigned to an individual T_1 plant; nd = not determined.

these, 44 lines with endochitinase activities between 20 and 180-fold greater than average control values were maintained in the greenhouse until maturity. Twenty-nine lines were fertile and set seeds. Similar to transgenic tobacco, leaves from the high expressing cotton lines assayed at the flowering stage were found to have reductions in endochitinase activities (Table 3).

In order to confirm that the elevated endochitinase activity was a result of the fungal transgene expression, a gel-based fluorescence assay was conducted to detect the 42 kDa endochitinase band in the protein extracts from the transgenic lines. A 42 kDa band illustrating the presence of a transgenic endochitinase was detected in seven tobacco (Figure 2a) and five cotton (Figure 2b) lines which was absent in the untransformed controls. Because it is not possible to apply the substrate evenly to the gel, this assay only provides qualitative information regarding the size of the endochitinase protein and either the presence or absence of the enzyme activity. The results shown in Figure 2 suggest that the endochitinase activities detected using the quantitative, fluorometric method in the leaf extracts in the transgenic plants (Figure 1) were due to the expression of the *T. virens* endochitinase gene.

Molecular characterizations of transgenic plants

Southern blot analysis was performed with genomic DNA extracted from nine different T_0 cotton plants. Of these, six transgenic lines showed a single-copy integration of the endochitinase transgene, while the others had two copies each (Figure 3a). In tobacco, Southern blot analysis was conducted with genomic DNA from T_1 plants. Transgene integration of one to as many as three copies of the endochitinase

Table 3 Endochitinase activities* in transgenic cotton

Line	T_0 leaves	T_0 leaves (flowering)	T_1 leaves	T_2 seeds†	T_2 roots‡	T_2 root-hypocotyl segment§	T_2 basal hypocotyl segment¶
H121B/#11	40.09	14.78	74.80	40.34 ± 2.37	9.17 ± 0.66	31.81 ± 2.28	36.99 ± 2.48
H32/#4	27.79	17.87	45.85	26.97 ± 1.15	15.37 ± 1.17	23.31 ± 2.16	36.10 ± 2.36
H135/#4	29.61	18.63	18.88	25.76 ± 2.72	8.35 ± 0.94	26.20 ± 2.44	33.56 ± 2.98
H135/#11			0.22	0.30 ± 0.03	nd	nd	nd
H17/#3	38.95	18.04	51.43	28.04 ± 4.21	nd	nd	nd
H17/#7			37.99	17.08 ± 1.17	nd	nd	nd
Control	0.31	0.22	nd	nd	1.31 ± 0.05	1.58 ± 0.04	1.47 ± 0.06

*pmole 4-MU/h/μg total protein.

†Mean (± SE) of activities in 12 seeds collected from a T_1 plant and individually assayed.

‡Mean (± SE) of activities in 10 roots from 10 T_2 seedlings, individually assayed.

§Mean (± SE) of activities in 1 cm-long segments from the root-hypocotyl junction in T_2 seedlings, individually assayed. Seven, 10, 7 and 12 segments were used for H121B/#11, H32/#4 H135/#4 and control, respectively.

¶Mean (± SE) of activities in 1 cm-long segments from the basal hypocotyl segment in T_2 seedlings, individually assayed. Twelve, 14, 13 and 12 segments were used for H121B/#11, H32/#4, H135/#4 and control, respectively.

#: number = The number assigned to an individual T_1 plant; nd = not determined.

gene was observed in tobacco (results not shown). Northern blot analyses on transgenic cotton revealed the presence of *T. virens* endochitinase transcript in both leaf and root tissues (Figure 3b).

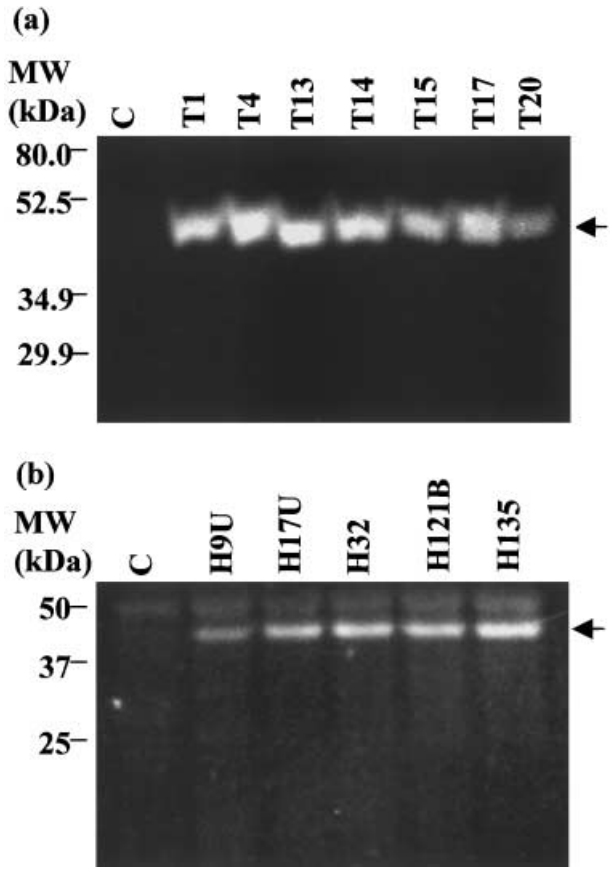


Figure 2 Fluorometric gel-based detection of the 42 kDa *T. virens* endochitinase in transgenic tobacco and cotton. The assay was conducted with total proteins extracted from leaf tissues. (a) Tobacco. Lane C: untransformed control; Lanes T1, T4, T13, T14, T15, T17 and T20: seven independent transgenic tobacco lines. (b) Cotton. Lane C: untransformed control; Lanes H9U, H17U, H32, H121B and H135: five independent transgenic cotton lines. Arrows indicate the transgenic, 42 kDa endochitinase band.

Endochitinase activities in T_1 and T_2 plants

Seeds obtained from three of the highest expressing lines in tobacco (lines T4, T35 and T37) were germinated, homozygous T_1 progeny were identified and grown to maturity to obtain T_2 seeds. Endochitinase activity was monitored in the leaf tissues over the T_1 and T_2 generations. As shown in Table 2, all three lines maintained their endochitinase activity over these generations. Leaves from T_2 plants from each of the three lines were assayed for resistance to *A. alternata*.

In the case of cotton, 15 T_1 seeds from each of the seven highest expressing T_0 lines (Figure 1b) were germinated and the leaves from the healthy plants were examined for endochitinase activities. From each set of T_1 plants, there were two or more that showed endochitinase activities similar to the control values and these were apparently the null segregants (Figure 4a, b and d). In three high expressing lines (lines H9A, H9UA and H134), there was a significant drop in endochitinase activities in the T_1 progeny compared to the T_0 parent plants (results shown only for H134, Figure 4a). These three lines were not used for further analysis and were discarded. The remaining four lines (H121B, H32, H135 and H17) were used for more extensive analyses, as their T_1 progeny

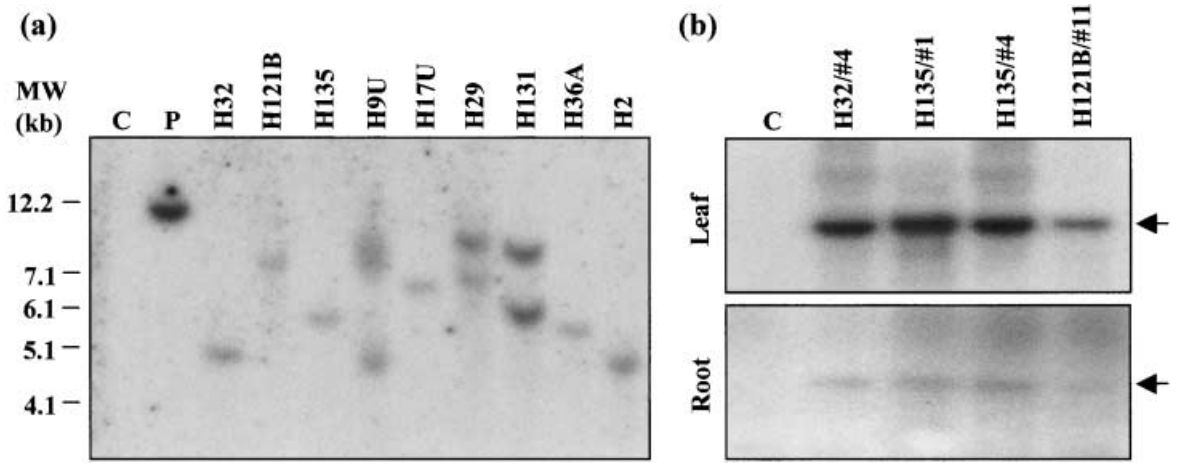


Figure 3 Molecular analyses on transgenic cotton plants. (a) Southern blot analysis of genomic DNA from leaves of a control and nine independent kanamycin-resistant cotton lines. Ten micrograms of genomic DNA was digested with *Hin*dIII. There is a single *Hin*dIII site in the T-DNA region outside the endochitinase expression cassette. The *Tv-ech1* cDNA fragment was used as the probe. Lane C: genomic DNA from a control plant; Lane P: *Hin*dIII-linearized binary vector used for transformation; Lanes H32, H121B, H135, H9U, H17U, H29, H131, H36A and H2: genomic DNA from nine independent transgenic cotton lines. (b) Northern blot analysis of RNA isolated from leaf and root tissues from control and transgenic cotton plants. Five micrograms of leaf RNA and two micrograms of root RNA were used for the analyses. Lane C: RNA from control; Lanes H32/#4, H135/#1, H135/#4 and H121B/#11: RNA from four T_2-generation cotton plants from three independent lines. Arrows indicate the endochitinase transcript.

maintained either the same or higher endochitinase activities compared to their respective T_0 parents. The results from the analyses on T_0, T_1 and T_2 progeny from line H32 are shown in Figure 4b, c, and from line H135 are shown in Figure 4d, e. Null segregants in the T_1 progeny are obvious in both lines. In line H32, some of the T_1 endochitinase values were similar to those of the T_0 parent, while others were higher than the T_0 parent. These T_1 plants with higher endochitinase levels were assumed to be homozygotes. All the T_2 seeds tested from these plants showed endochitinase activity (Figure 4c), thus confirming their homozygous status. In line H135, out of 14 T_1 plants tested, two were null segregants, and eight plants showed endochitinase activities approximately half the level of the T_0 parent. Four plants showed activity levels similar to the T_0 parent (Figure 4d). These were assumed to be homozygous and were confirmed as such based on the absence of segregation in the T_2 generation. Endochitinase activity values from one such homozygous T_1 parent and its T_2 seeds are shown in Figure 4e. Unlike line H32, where homozygous T_1 progeny showed higher levels of endochitinase compared to the T_0 parent, in line H135 the endochitinase activity levels in the homozygous T_1 progeny were similar to T_0 values. Such differences among different independent transgenic lines are not unusual and have been observed in various other transformation experiments in our laboratory. Results from the four lines H121B, H32, H135 and H17 are summarized in Table 3. Note that the T_1 plant H135/#11 was a null segregant and showed endochitinase activity levels in the leaves and seeds that were similar to control values.

Since the roots and lower parts of the hypocotyls are the potential targets for infection by soil-borne fungi, we were interested in determining the transgenic endochitinase activity levels in these tissues. Roots, hypocotyl-root junction segments and basal hypocotyl segments (1 cm-long segment excised from just above the root portion) from 3-day-old, dark-grown seedlings were examined and their endochitinase activities are presented in Table 3. The activities in T_2 roots were found to be lower in all cases compared with the activity in the respective seeds. However, the endochitinase activity in the hypocotyl-root junction tissues and basal hypocotyl tissues was higher compared with the levels detected in the roots. These differences possibly reflect the relative promoter activities in various tissues. Endochitinase activities were also examined in T_3 seeds and these were positive for transgene expression (results not shown).

Disease resistance assays with *R. solani*

Rhizoctonia solani is a soil-borne pathogen which causes seed decay and pre-emergence damping off as well as post-emergence damping off in cotton (Hillocks, 1992a). This fungus also is very susceptible to chitinase activity and therefore served as an appropriate test pathogen for our investigation. Disease resistance assays were performed with T_2 generation seeds obtained from a confirmed homozygous T_1 parent in three independent transgenic lines (H121B, H32 and H135). Thirty delinted seeds from each of the transgenic lines and 60 untransformed control seeds were sown in flats divided into

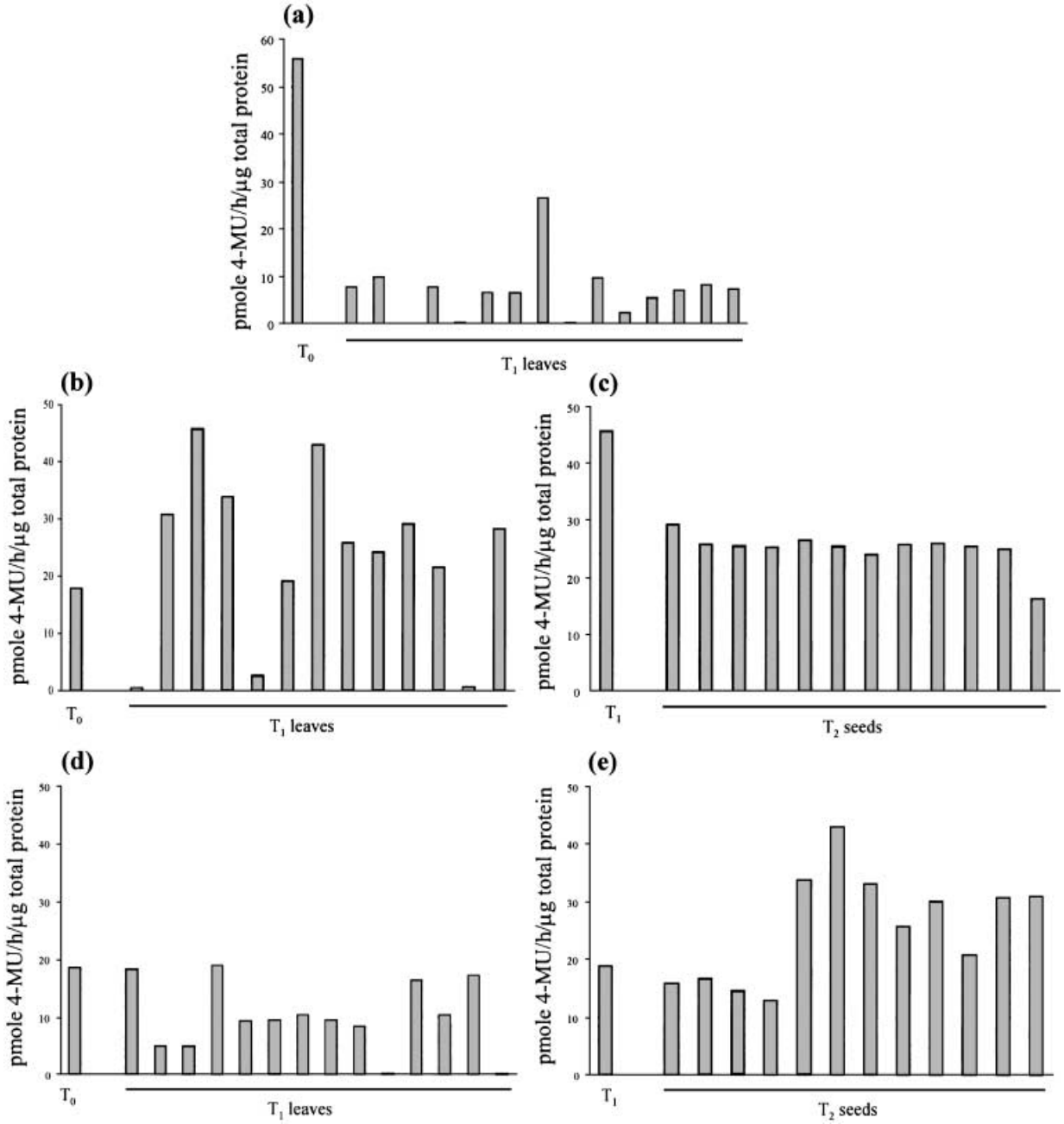


Figure 4 Endochitinase activities in transgenic cotton over T_0, T_1 and T_2 generations. Individual bars represent endochitinase activities in the leaves from T_0 and T_1 plants or in T_2 seeds. (a) Line H134 and its T_1 progeny. (b) Line H32 and its T_1 progeny. (c) Leaf from the homozygous T_1 plant (H32/#4) and its T_2 seeds. (d) Line H135 and its T_1 progeny. (e) Leaf from the homozygous T_1 plant (H135/#4) and its T_2 seeds.

six lanes with approximately 1800 mL of Metromix #366, fungus-infested soil per lane. Before distributing the soil in the flats, *R. solani*-wheat bran mix was added to the moist soil at a concentration of 0.28 g/L and mixed thoroughly. Ten seeds were sown in each lane, and every lane was watered with 500 mL of water every other day. The fungal invasion of cotton seedlings at the soil level produces a brownish, sunken lesion on the hypocotyls due to cortical decay, eventually causing the seedlings to collapse (Hillocks, 1992a). One week after the seeds were sown, seedlings were removed from the soil and assigned disease severity scores as described in the Experimental procedures. Disease symptoms appeared as brown-coloured lesions at the hypocotyl–root junction. In this experiment, only seven of the 60 untransformed seedlings were completely healthy, whereas 22 seedlings died and 21 failed to germinate. Importantly, a significant number of

the transgenic seedlings showed no disease symptoms (Figure 5a). Thirty seeds from each of the transgenic lines H135/#4, H32/#4 and H121B/#11 were sown in pathogen infested soil, and 26, 22 and 19 seedlings, respectively, germinated without obvious disease symptoms. Results presented in Figure 6a indicate that T_2 seedlings from these three transgenic cotton lines showed significant resistance to *R. solani*. A second, similar experiment was conducted except that the seedlings were allowed to grow in the infested soil for 2 weeks. In this experiment, disease symptoms were more severe in the case of the untransformed control, where 59 seeds either failed to germinate or died following germination. The only single surviving seedling showed a disease score of 4. Thirty seeds from each of the transgenic lines H135/#4, H32/#4 and H121B/#11 were sown in pathogen infested soil, and 20, 23 and 23 seedlings, respectively, germinated without obvious disease symptoms after 2 weeks. Results from this experiment, presented in Figure 6b, show that the transgenic lines were significantly resistant to fungal infection. A third experiment was conducted where the inoculum pressure was doubled by increasing *R. solani*-wheat bran concentration to 0.56 g/L of soil. Thirty control seeds and 30 T_2 seeds from transgenic line H135/#4 were used in this experiment. After 9 days, all the 21 of the germinated control seedlings died as a result of severe disease and nine seeds failed to germinate (Figure 5b). In the transgenic line, 15 of 28 germinated seedlings were completely healthy with no observable disease symptoms, 10 seedlings showed varying degrees of disease symptoms, three seedlings died, and two seeds failed to germinate (Figure 5b). The disease index value for the control was 88.33 ± 0.96 (mean ± SE). There was a significant ($P < 0.0001$) reduction in the disease index value at 28.88 ± 3.64 for the transgenic line. Results obtained from these three experiments clearly demonstrate that the expression of endochitinase, *Tv-ech1*, provides significant protection for cotton seedlings against damping-off due to *R. solani*.

Disease resistance assays with *A. alternata*

Alternaria alternata either alone or in combination with *A. macrospora* is responsible for leaf spot/leaf blight of cotton (Bashan, 1994; Hillocks, 1992b). These two pathogens are distributed world-wide (Hillocks, 1992b). Under certain conditions, such as injury created by pest attack, adverse weather conditions, moisture, or nutrient stress, these and other leaf pathogens can cause significant yield loss in cotton (Hillocks, 1992b). In tobacco, *A. alternata* causes brown spot disease where disease symptoms are characterized by the appearance of necrotic lesions often surrounded by yellow halos (Spurr, 1991). In order to assess the disease resistance of cotton and tobacco plants to foliar diseases, *A. alternata* was used as a test pathogen. Another reason for using *A. alternata* for resistance assays in cotton is that, of the two *Alternaria* species, it is the major pathogen on *G. hirsutum*, the most widely grown of the four cotton species (Bashan *et al.*, 1991). Disease resistance assays were carried out with detached leaves from the control and transgenic lines of both tobacco and cotton placed individually on wet paper towels in Petri dishes. Although the detached leaf technique is routinely used in disease resistance assays, we were interested in determining whether the transgenic endochitinase activity was maintained in the isolated leaves. The endochitinase activity as determined from isolated, individual leaves daily for 6 days was found to remain stable over the entire period (Table 4).

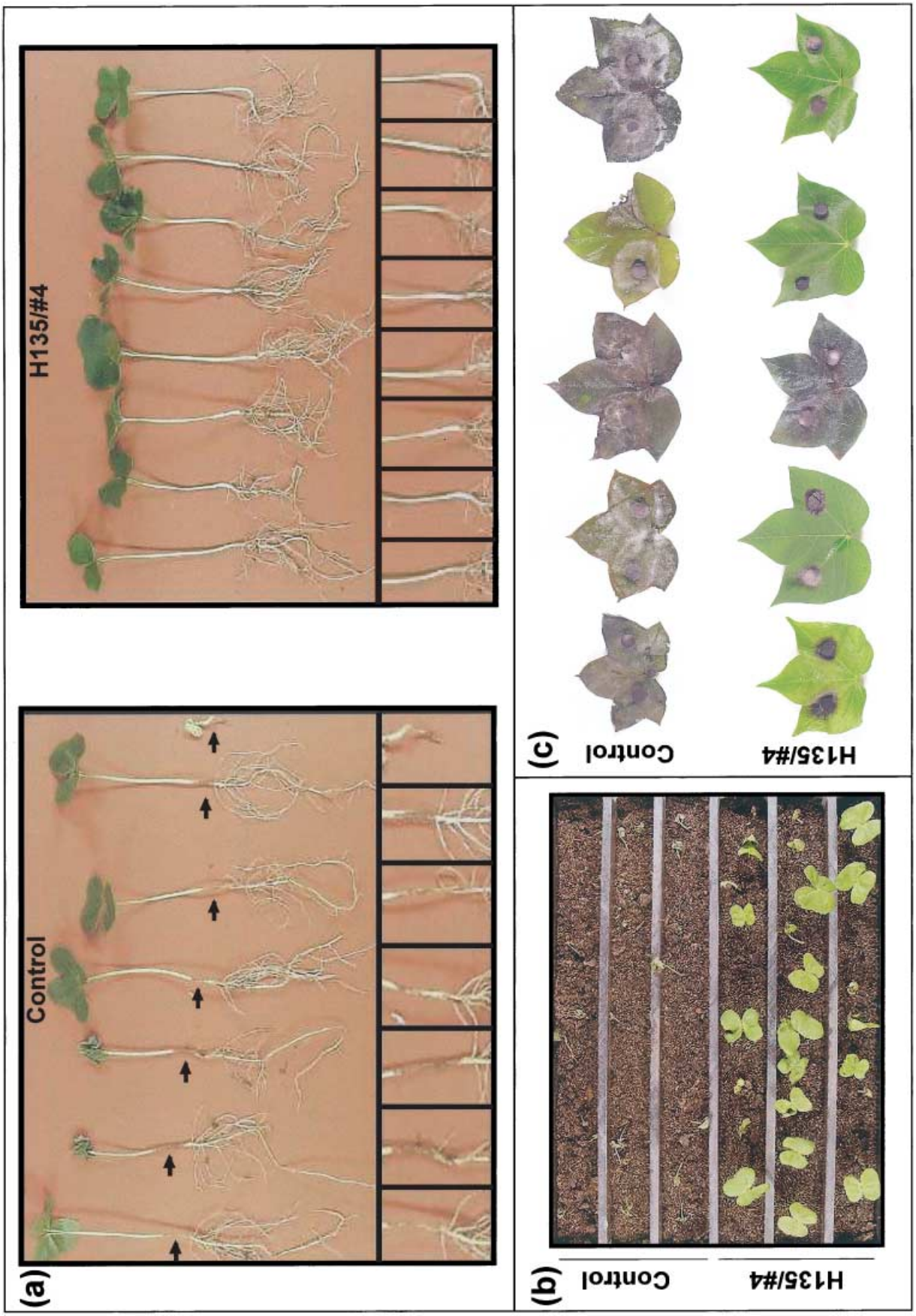
Table 4 Endochitinase activities* in the leaves of control and transgenic cotton line H135/#1 used for *A. alternata* assay

Day	Control	H135/#1
1	0.08	4.32
2	0.19	5.32
3	0.19	5.86
4	0.11	4.64
5	0.17	5.03
6	0.16	4.96

*pmole 4-MU/h/μg total protein.

In tobacco, leaves inoculated with *A. alternata* began showing symptoms, in the form of lesions surrounded by yellow halos within a week. The lesion area was measured 2 weeks following inoculations. Figures 7a, b show that the transgenic lines had significantly less damage on the leaf tissue compared to the control.

As described in the Experimental procedures, two experiments were carried out with cotton leaves. In the first experiment, where two agar plugs containing the pathogen were used, disease symptoms were generally observed over the majority of the leaf in the case of controls (average damaged area was 87%, Figures 5c and 8a). However, in the leaves from transgenic lines, the mean area exhibiting symptoms was significantly less than the control (Figures 5c and 8a). In the second experiment with a larger number of leaf samples and only one plug of inoculum, lesion development was still extensive on the control leaves. Again, leaves from transgenic cotton lines showed a significant degree of protection compared to the controls (Figure 8b). These results suggest that the expression of endochitinase *Tv-ech1* from *T. virens* also confers resistance to an important foliar pathogen in tobacco and cotton.


(a)
Control
H135/#4
(b)
Control
H135/#4
(c)
Control
H135/#4

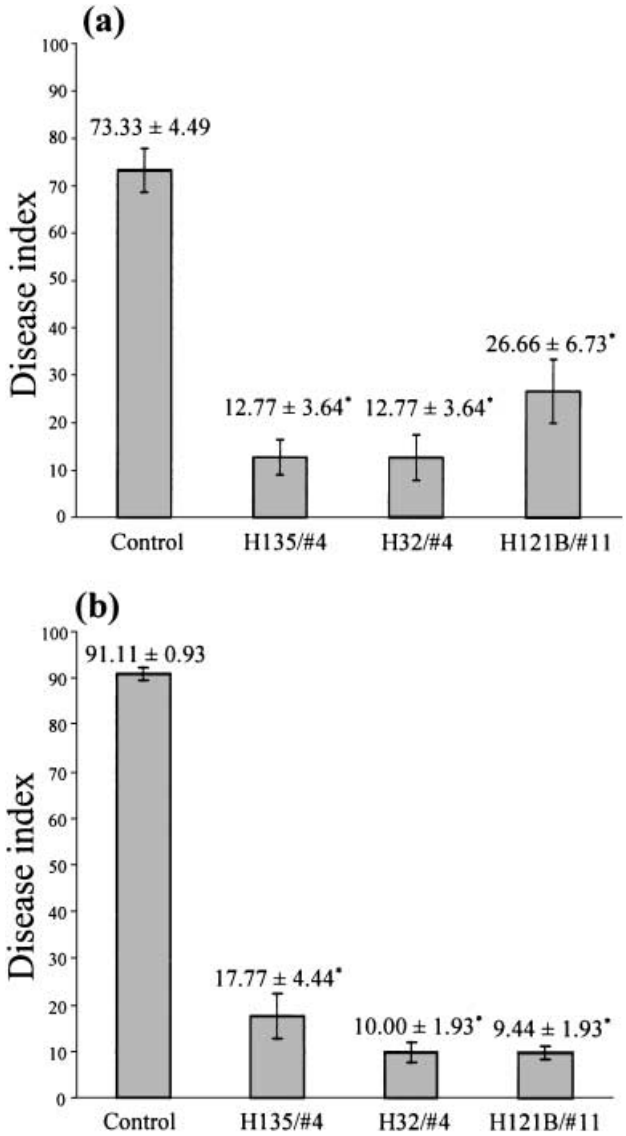


Figure 6 Resistance of transgenic cotton to *Rhizoctonia solani.* Disease indices for untransformed control and transgenic cotton plants infected with *R. solani* at an inoculum pressure of 0.28 g fungus-wheat bran culture per litre of soil (a) after 1 week (photograph shown in Figure 5a) or (b) 2 weeks. Data represent mean disease index ± standard error (*the value for the transgenic line is significantly different from the control value at $P < 0.0001$).

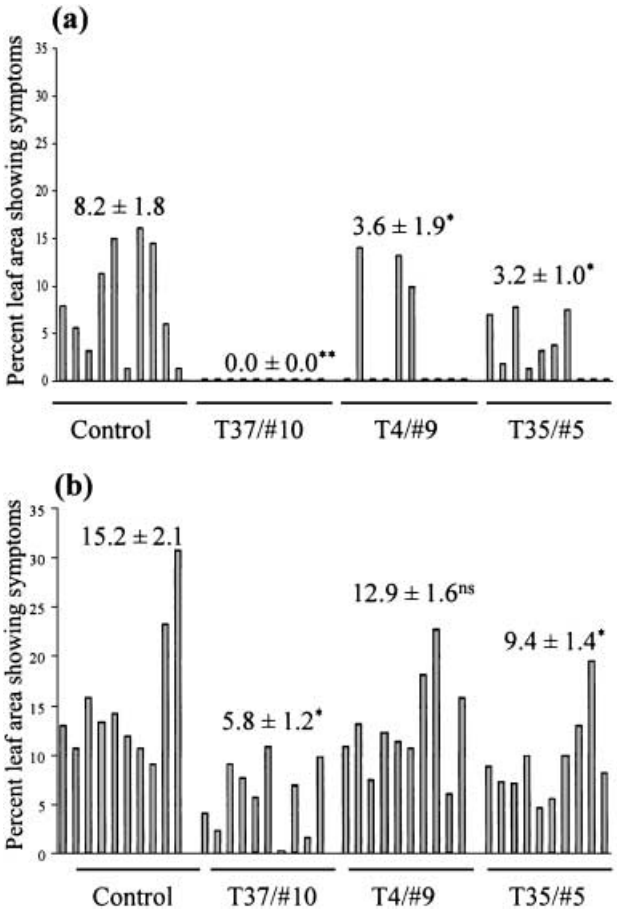


Figure 7 Resistance of transgenic tobacco to *Alternaria alternata.* Two different experiments (a, b) were conducted, each using leaves from 10 control plants and 10 T_2 plants from each line. Each bar represents percent leaf area showing infection on a single leaf. The numbers above each set of bars represent mean of percent lesion area ± SE. The value for the transgenic line is significantly different from the control value at $P < 0.05$ (*) or $P < 0.0001$(**); ns: not significant.

Discussion

To our knowledge, this is the first successful report of engineering cotton with a gene encoding a chitinase from a mycoparasite to enhance the resistance of this important crop plant against two fungal pathogens. The only other report in the literature on transgenic disease resistance in cotton involved the expression of a tobacco chitinase gene

Figure 5 Fungal disease protection in transgenic cotton plants expressing the *T. virens* endochitinase gene. (a) *Rhizoctonia solani* disease resistance assay performed with untransformed control and T_2 seeds from the transgenic line H135/#4. The image shows 1-week-old seedlings that were removed from *R. solani*-infested soil (0.28 g fungus-wheat bran culture per litre of soil) for photography. Arrows and magnified images indicate the typical disease symptoms seen as brown-coloured lesions at the hypocotyl-root junction in the untransformed control seedlings. Magnified images of the same, hypocotyl-root regions in the transgenic seedlings do not show any disease symptoms. (b) *R. solani* disease resistance assay performed by challenging 30 untransformed control and 30 T_2 seeds from transgenic line H135/#4 with an inoculum pressure of 0.56 g fungus-wheat bran culture per litre of soil. The photograph was taken 9 days after seeding. (c) *Alternaria alternata* disease resistance assay performed on detached leaves of untransformed control and transgenic line H135/#4. Two agar plugs containing the pathogen were placed on the adaxial surface of the leaves. The image shows progression of the disease 2 weeks following infection.

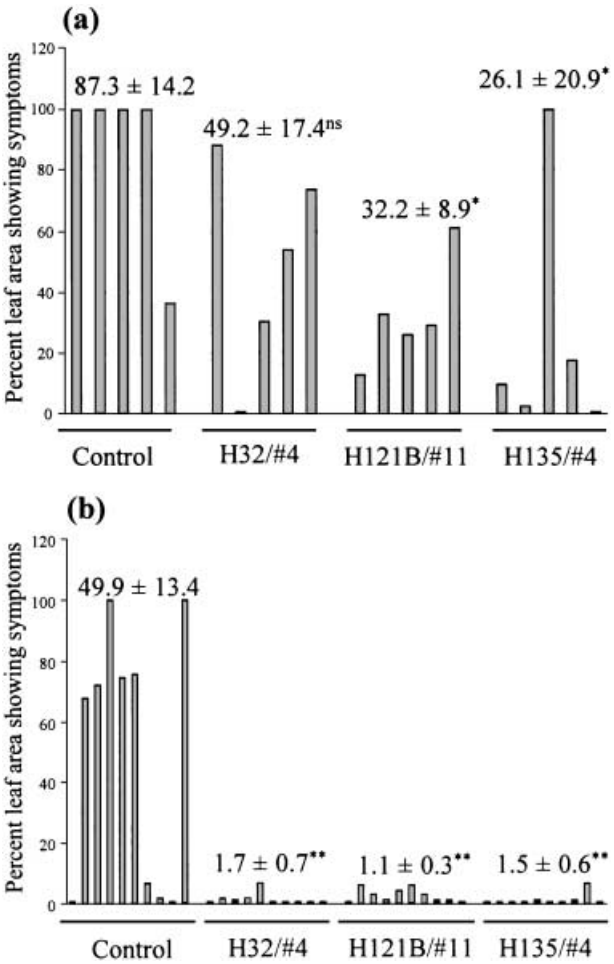


Figure 8 Resistance of transgenic cotton to *Alternaria alternata*. (a) Leaves from five control plants and five T_2 plants from each line were each infected with two plugs of inoculum. (b) Leaves from 10 control plants and 10 T_2 plants for each line were each infected with one plug of inoculum. Each bar represents percentage leaf area showing infection on a single leaf. The numbers above each set of bars represent the mean of percent lesion area ± SE. The value for the transgenic line is significantly different from the control value at $P < 0.05$ (*) or $P < 0.0001$(**); ns: not significant.

(McFadden *et al*., 2000). In that study, the expression of the plant chitinase resulted in a slight reduction in disease symptoms following *Verticillium* infection under greenhouse conditions.

We conducted a comprehensive study in which cotton and tobacco plants were transformed with three different cDNA clones (encoding three different 42 kDa endochitinases) and one genomic clone (encoding the only endochitinase whose cDNA did in fact express in transgenic plants). Both cotton and tobacco expressed only one (*Tv-ech1*) of the three *Trichoderma virens* endochitinase cDNA clones tested as determined by the enzyme activity assays on leaf extracts. This is the same *cht42* clone that was isolated and characterized by Baek *et al*. (1999) and found to play an important role in the biocontrol activity of *T. virens*. Without conducting an extensive analysis on the transformants, it is not possible to know why the other clones did not confer chitinase activity in plants.

Molecular analyses of transformed cotton plants expressing the *Tv-ech1* endochitinase demonstrated single copy integration of the transgene in six of nine plants tested, while the remaining plants contained two copies each. All of the high expressing lines used in the subsequent disease resistance assays showed single copy integrations. Northern blot analyses conducted on transgenic cotton plants using RNA isolated from both leaf and root tissues of the homozygous T_2 plants revealed the presence of the *T. virens* endochitinase transcript.

The gel-based fluorescence assay clearly showed the presence of a band (42 kDa) with endochitinase activity only in transgenic plants and not in the controls. Chitinases characterized from tobacco have molecular weights of approximately 32 and 34 kDa (Shinshi *et al*., 1987). In cotton, a sequenced chitinase clone was found to encode a 28.8 kDa protein (Hudspeth *et al*., 1996) and a purified class I chitinase had a molecular value of 31.5 kDa (Chlan and Bourgeois, 2001). Thus, the elevated chitinase levels detected in both tobacco and cotton were due to the expression of the fungal endochitinase transgene.

There is a report of a *T. harzianum* endochitinase gene expression resulting in an abnormal phenotype (reduced growth) in apple (Bolar *et al*., 2000). In our study, none of the transgenic plants expressing high levels of endochitinase showed obvious morphological abnormalities. This result was consistent with previous studies on tobacco, potato (Lorito *et al*., 1998) and broccoli (Mora and Earle, 2001) where endochitinase expression did not adversely affect the plants. There was some reduction in endochitinase enzyme activity when the plants reached the flowering stage of growth. A similar reduction was observed in tomato, tobacco (Brants, 1999) and broccoli (Mora and Earle, 2001). The promoter used in these studies, as well as in our investigation, was the CaMV 35S promoter. In our laboratory, we have examined the expression profile of the 35S promoter using the GFP gene as a reporter system in cotton (Sunilkumar *et al*., 2002). We found that the expression of the reporter gene was reduced in the leaves from older plants, suggesting that reduced promoter activity may account for the lower endochitinase activity in older tissues.

This is the first published investigation on cotton reporting the production of a large number (82) of transgenic lines that were subjected to systematic analysis to study the transgene expression levels in different lines and then monitor the

stability of the expression in selected lines over four generations. Results shown in Figure 4 highlight the importance of monitoring the expression levels over several generations to ensure the stability of transgene expression. As stated earlier, not all the high expressing lines identified in T_0 generation maintained chitinase activity in T_1 generation. Lines H9A, H9UA, and H134 showed a significant drop in chitinase activity in T_1 generation. In the other four higher endochitinase-expressing lines, H121B, H32, H135 and H17, the expression was either at the same level or showed an increase in the homozygous progeny. The differences in expression levels over generations amongst different lines reported in this study are not unusual, and have been reported by others in several plant species (Caligari *et al*., 1993; De Neve *et al*., 1999; James *et al*., 2002; Vain *et al*., 2002). Our results add to the growing literature indicating the importance of screening a large number of independent transformation events to identify lines with desired levels of transgene expression and then monitoring the stability of expression over several generations (Vain *et al*., 2002).

Rhizoctonia solani causes seed rot, lesions on the hypocotyls, and root rot in cotton seedlings. This pathogen can cause disease under a range of environmental conditions, but it can be devastating in cool, wet soil where it results in pre- and post-emergence damping-off. T_2 seeds from several high endochitinase-expressing lines were subjected to infection by planting them in soil infested with *R. solani*. At moderate inoculum pressure (0.28 g culture/L of mix), a majority of the untransformed seedlings (98%) in infested soil died due to post-emergence infections or the seeds failing to germinate; however, > 67% of the transgenic seedlings remained healthy even after 2 weeks in the infested mix. The results from these infection assays clearly demonstrate that the transgene provided significant protection to cotton seedlings against the damping-off caused by *R. solani*. Even when the inoculum pressure was doubled (0.56 g/L), 15 of the 28 transgenic seedlings examined 9 days after planting were free of disease symptoms, while none of the control plants survived (Figure 5b).

Infection due to soil-borne pathogens can occur immediately after planting, resulting in seed decay (Hillocks, 1992a). Since high endochitinase activity was observed in the seeds, protection against this pathogen may have existed in the seed prior to germination. The slight variations in the level of disease resistance observed among different transgenic lines could not be correlated with the levels of endochitinase activity in the respective lines. Lorito *et al*. (1998) showed that the expression of a *T. harzianum* endochitinase gene in tobacco and potato rendered the plants highly resistant to *R. solani*. Several studies have indicated that the biological control activity of *Trichoderma* sp. is due to the presence of hydrolytic enzymes, including chitinases (Carsolio *et al*., 1999; De La Cruz *et al*., 1995; Flores *et al*., 1997; Peterbauer *et al*., 1996; Thrane *et al*., 1997). Hadar *et al*. (1979) showed that *T. harzianum*, under greenhouse conditions, when applied in the form of wheat bran culture to *R. solani*-infested soil, effectively controlled damping-off in bean, tomato and eggplant seedlings. They attributed this protection to the production of extracellular β-(1-3) glucanases and chitinases in the mycoparasite which caused lysis of the fungal cell walls. Benhamou *et al*. (1993) studied the ultrastructural changes and cytochemical aspects of the chitin breakdown in *R. solani* using bean endochitinase and found that the chitin present in the cell wall of the fungus was rapidly hydrolysed upon exposure to endochitinase, leading to cell lysis. They also suggested that the chitin present in the cell wall was accessible to endochitinase, especially in the apical zone, making *R. solani* more vulnerable to chitinase action alone. Microscope observations in their study provided evidence that the eventual death of *R. solani* cells was a result of extensive cell wall breakdown and leakage of cytoplasm. Transgenic *Tv-ech1*-endochitinase, may, in part, be providing direct protection to cotton against *R. solani* in a similar manner.

Enhanced disease resistance was also evident against the foliar pathogen *A. alternata* in both cotton and tobacco. The significantly lower percentage of leaf area showing necrotic symptoms in transgenic leaves indicated the effective protection offered by the endochitinase transgene in cotton. Similar improved disease resistance was also seen in the three transgenic tobacco lines. An endochitinase transgene from *T. harzianum* has previously been shown to provide a high degree of resistance to *A. alternata* in tobacco (Lorito *et al*., 1998). Taken together, these results confirm that endochitinase from *Trichoderma* spp. also are effective against the foliar pathogen *A. alternata*.

In addition to coding for the chitinolytic activity, a second, indirect mechanism of protection may be afforded by the chitinase transgene. The degradation of fungal cell wall releases glycosidic components that in turn may elicit the induction of plant defence mechanisms (Benhamou, 1996; Boller, 1987; Graham and Sticklen, 1994; Lamb *et al*., 1989; Mauch and Staehelin, 1989; Roby *et al*., 1988; Shibuya and Minami, 2001). Transgenic expression of *T. virens* endochitinase gene with a constitutive promoter ensures that chitinase activity, already present at the time of fungal invasion, will hydrolyse the chitin present in the cell wall of the invading pathogen. Chitin fragments have been shown to act as potent elicitors

of a variety of defence related activities, e.g. lignification in wheat leaves (Barber *et al*., 1989), chitinase activity in melon (Roby *et al*., 1987), and β-1,3 glucanase gene expression in cultured barley cells (Kaku *et al*., 1997). In cultured rice cells, chitin oligosaccharides induced phytoalexin synthesis (Ren and West, 1992; Yamada *et al*., 1993), generation of reactive oxygen species (Kuchitsu *et al*., 1995), biosynthesis of jasmonic acid (Nojiri *et al*., 1996), and expression of defence related genes (Minami *et al*., 1996; Nishizawa *et al*., 1999; Takai *et al*., 2001). A possible explanation for the high levels of protection observed in our study may therefore be that transgenic *Trichoderma* endochitinase activity in plant tissues may release compounds from the cell wall of the invading fungi, that in turn elicit in the plant a faster and more comprehensive defensive response. Experiments with purified chitinolytic enzyme-treated *R. solani* and *A. alternata* which produced a resistance response in wild-type tobacco (Lorito *et al*., 1998) provide support for this possibility. Thus, it is possible that the effectiveness of transgenic endochitinase is due both to its direct action on the cell wall chitin of the invading fungus and to indirect action through the release of oligosaccharide elicitors from the pathogen, resulting in the induction of the plant's natural defence response. These responses may include reinforcement of the cell walls, production of phytoalexins, enzyme inhibitors, hydroxyproline-rich glycoproteins, and various lytic enzymes (Lamb *et al*., 1989). A series of pathogenesis-related proteins along with chitinases and β-glucanases have been shown to be induced in cotton as a result of exposure to invading pathogens (Cui *et al*., 2000; Dubery and Slater, 1997; Hill *et al*., 1999; Liu *et al*., 1995; McFadden *et al*., 2001). Cotton plants are also known to produce high levels of ethylene in response to attack by *Alternaria* spp. (Bashan, 1994). Ethylene has been shown to induce high levels of chitinase activity in cotton (Boller *et al*., 1983; Chlan and Bourgeois, 2001; Hudspeth *et al*., 1996). Thus in a manner similar to other species, the cotton plant will also be expected to launch a combination of defensive activities in response to fungal elicitors. Future studies may reveal that the transgenic endochitinase-mediated protection mechanism in plants is quite complex.

We also tested the chitinase transgenic cotton against *Verticillium dahliae, Fusarium oxysporum* and *Thielaviopsis basicola*; however, we did not observe any improvement in resistance against these pathogens (data not shown). This may be due to the fact that the chitinase gene alone may not provide effective protection against the pathogens where chitin is either not a major constituent of the fungal cell wall or is not easily accessible to enzyme action. It may be possible to confer resistance to these pathogens by combining chitinase genes with other antifungal genes from *Trichoderma* spp. or some other source. Regardless, our results with *R. solani* and *A. alternata* provide the first convincing demonstration of the usefulness of a mycoparasite-derived endochitinase in conferring an effective resistance against a soil-borne and a foliar pathogen in cotton.

Experimental procedures

Endochitinase constructs

Three cDNA clones (*Tv-ech1*, *Tv-ech2* and *Tv-ech3*) and one genomic clone (*Tv-ech1*g), all encoding different 42 kDa endochitinases used in this study, were isolated from *T. virens* as described by Kim *et al*. (2002). Transformation vectors were constructed in the following manner. The *Hin*dIII-*Eco*RI fragment consisting of the CaMV 35S promoter with the duplicated enhancer element, *gus*A gene and CaMV 35S polyadenylation signal from plasmid pFF19G (Timmermans *et al*., 1990) was inserted into the multiple cloning site of the binary vector, pCAMBIA2300. The *gus*A gene was removed by *Pst*I digestion and the vector was re-ligated. Each endochitinase clone was inserted as a *Kpn*I-*Bam*HI fragment between the 35S promoter and 35S polyA signal to obtain the plant transformation construct. The binary vectors were mobilized into the *Agrobacterium tumefaciens*-disarmed helper strain LBA4404 (Ooms *et al*., 1982) by the heat shock method (An *et al*., 1988).

Plant material and transformation procedures

Cotton (*Gossypium hirsutum* L.) cv. Coker 312 seeds were obtained from Seedco, Lubbock, TX. Tobacco (*Nicotiana tabacum* L. cv. Xanthi.nc) plants were kindly provided by Dr R. Smith (Texas A&M University) and were maintained through serial propagation in culture. Hypocotyl segments of cotton were used as explants for transformation and the protocols for selection of transformants and regeneration were as described by Sunilkumar and Rathore (2001). Tobacco was transformed by using the leaf disc method of Horsch *et al*. (1985). Transgenic tobacco plants were regenerated from transformed leaf discs following selection on 100 mg/L kanamycin.

Endochitinase assays

A modified method based on the fluorescence assay described by Kuranda and Robbins (1991) was used to quantify transgenic endochitinase activity in tissue extracts. In this

assay, endochitinase activity results in the hydrolysis of 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside (Sigma) to produce 4-methylumbelliferone (4-MU) which is then quantified fluorometrically to provide a measure of enzyme activity. Initial screening for transformants expressing the endochitinase transgene was performed by analysing the enzyme activities in extracts from young, growing leaves of T_0 and T_1 plants. Total protein from tobacco plants was extracted by grinding leaves in 0.1 M citrate buffer, pH 3.0. Protein extraction from cotton tissues required the use of 0.2 M boric acid-borax buffer, pH 7.6 with 0.1% (v/v) β-mercaptoethanol, 0.57 mM PMSF (phenylmethanesulphonyl fluoride) and 1% (w/v) PVPP (polyvinylpolypyrrolidone). Leaf extracts from both of the species were further diluted (16-fold) with 0.1 M citrate buffer, pH 3.0, prior to the enzyme assay. One hundred microlitres of diluted protein extract were mixed with 25 μL of substrate (250 μM) and incubated at 30 °C for 1 h. The reaction was terminated with 1 mL of 0.2 M sodium carbonate and fluorescence was measured using a DyNA Quant™ 200 fluorometer (Hoefer). Fluorometric readings were normalized to the total protein content of the samples (Bio-Rad protein assay). The endochitinase specific activity is presented as pmole 4-MU/h/μg total protein. In the case of transgenic cotton, endochitinase assays were performed with T_0, T_1 and T_2 leaves, individual T_2 seeds, roots, root-hypocotyl junction segments and basal hypocotyl segments from individual T_2 seedlings, and T_3 seeds.

Fluorometric gel-based endochitinase assay

SDS-PAGE (Laemmli, 1970) was conducted with protein samples from both tobacco (20 μg) and cotton (25 μg) leaves. Detection of the endochitinase band on the gel by fluorescence method was according to Ren *et al*. (2000).

Molecular analyses

Southern blot analyses were performed on genomic DNA from transformed cotton and tobacco plants to confirm the presence and integration of the endochitinase transgene (Sambrook *et al*., 1989). A 10 μg sample of genomic DNA digested with *Hin*dIII was used for the analyses. For transgene expression analysis, total RNA was isolated from leaves and roots of homozygous T_2 cotton plants according to Chomczynski and Sacchi (1987) with minor modifications. Five micrograms of RNA from the leaves and 2 μg from the roots were used for the Northern blot analyses. Radioactively labelled *Tv-ech1*-cDNA fragment was used as the probe for both analyses.

Rhizoctonia solani resistance assays on cotton seedlings

Cultures of *R. solani* (Strain J-1, obtained from Dr C. Howell, USDA-ARS) were maintained on Potato Dextrose Agar (PDA, Difco) in a 90 mm Petri dish. For the inoculum preparation, a quarter of the agar/culture was removed from a 3-day-old culture and placed on to 25 g of sterile wheat bran mixed with 20 mL water and incubated for 5 days. The colonized wheat bran was dried overnight and then blended in a Wiley mill. Two different levels of inoculum were tested, i.e. 0.28 g or 0.56 g fungus-wheat bran culture/L of soil (Metromix no. 366). For cotton seedling disease trials, seeds from untransformed Coker 312 and T_2 seeds from a homozygous parent were directly seeded into flats containing the infested mix. A growth chamber at 25 °C and 14 h:10 h photoperiod was used for germination and seedling growth. Disease symptoms and severity were recorded after removing the seedlings from soil. Disease severity scores for individual seedlings were assigned as follows: 0 = no symptoms, 1 = minor lesion or spot, 2 = enlarged lesion (< 1 inch, 2.5 cm), 3 = necrotic lesion (1 inch or greater), 4 = collar and tissue collapse, 5 = dead seedling and 6 = failed germination. Disease severity scores were used to calculate disease index as described by Powell *et al*. (1971). Data were analysed by ANOVA.

Alternaria alternata resistance assays on detached leaves

Cultures of *A. alternata* were grown for 2 weeks on carrot agar [5% (v/v) carrot juice + 20 g/L agar] at room temperature under light. When the fungal mycelia reached the edge of the plate, a 0.5 cm diameter agar plug with mycelium was removed from a region close to the edge using a cork borer and used for inoculations. Healthy and young leaves were collected from 3 to 4-week-old T2 cotton and tobacco plants and placed on wet paper towels in Petri dishes. Agar plugs containing *A. alternata* cultures were placed directly on the adaxial side of the leaves and incubated in the dark. In the first experiment conducted on cotton, two agar plugs were placed on each leaf, whereas in the second experiment with cotton and in both the experiments with tobacco, only one agar plug was placed on each leaf. Leaves were photographed with a digital camera after 2 weeks and the percentage of leaf area with necrosis was determined using ALPHA EASE™ software (version 5.5, Alpha Innotech Corp.). In order to confirm that the isolated leaves used in such assays maintained transgenic endochitinase activity, a set of isolated uninfected leaves were kept under the conditions described above. Endochitinase activity was monitored in a single leaf each day for 6 consecutive days.

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