Journal of Nematology 32(3):289–296. 2000.

Trichoderma harzianum Endochitinase Does Not Provide Resistance to *Meloidogyne hapla* in Transgenic Tobacco[1]

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Abstract: Eggs of *Meloidogyne hapla* contain chitin, a substrate for chitinase. Our goal was to determine if endochitinase from the biocontrol fungus *T. harzianum* expressed in transgenic tobacco increases resistance to this nematode. Endochitinase-transgenic T_1 tobacco seedlings expressing increased endochitinase activity in leaves (11 to 125 times over control) and roots (2 to 15 times over control) were transferred to quartz sand:loam soil mix (4:1 ratio) and inoculated with 5,000 *M. hapla* eggs/pot. Tomato (cv. Rutgers), pepper (cv. California Dream), and non-transformed tobacco plants were used as susceptible controls. Two experiments were performed in the greenhouse with nine and ten transgenic tobacco lines, respectively. Roots were harvested 55 days after inoculation, and number of eggs, second-stage juveniles (J2), reproductive factor (Rf), and (eggs + nematodes [J2])/g of fresh root weight were determined. The reproduction factor for tobacco plants ranged from 1.06 to 3.40. Significant differences in number of J2 and egg counts were found between some transgenic lines and control tobacco; however, they were not consistent for lines tested in both experiments. No statistical differences were detected for (eggs + nematodes [J2])/g of fresh root weight in either experiment. We conclude that the elevated endochitinase activity did not provide protection against root-knot nematodes.

Key words: biocontrol, chitinase, endochitinase, *Meloidogyne hapla, Nicotiana tabacum,* northern root-knot nematode, resistance, tobacco, transgenic, *Trichoderma harzianum.*

Northern root-knot nematode (*Meloidogyne hapla*) is the most widespread of all root-knot nematodes in the United States. It occurs in all states except Alaska (Walters and Barker, 1994). Many weeds are hosts, often limiting the usefulness of crop rotation as an effective control method (Belair and Benoit, 1995, 1996).

The nematode eggshell is the only structure in which the presence of chitin has been conclusively demonstrated (Bird and Bird, 1991). In *Meloidogyne* spp., the eggshell consists of three layers. The outer vitelline layer has a thickened membrane-like structure. The middle chitinous layer provides structural strength to the eggshell and is the thickest (approx. 0.4 µm) and most obvious layer (Bird and Bird, 1991). The innermost layer consists of a mixture of lipids and proteins, conferring a high degree of impermeability (Bird and McClure, 1976).

Higher chitinase activity and early induction of specific chitinase isozymes is associated with resistance to root-knot nematode in soybean (Qiu et al., 1997). Chitin amendments to soil can suppress plant nematode populations and lead to increased crop yields (Ehteshamul Haque et al., 1997; Godoy et al., 1984; Mian et al., 1982). It is suspected that some of this suppressive effect is due to release of chitinase by soil microorganisms taking advantage of the chitin as a nitrogen source (Burrows and De Waele, 1997). However, Spiegel et al. (1987) showed that the degradation of chitin releases nematicidal ammonia that can be at least partially responsible for reduction of nematode populations in the soil.

Considerable evidence indicates that chitinases from various sources affect hatching of nematode eggs. Chitinases from bean (*Phaseolus vulgaris*) and the bacteria *Serratia marcescens* and *Streptomyces griseus* increased hatch rates when extracted eggs of *M. hapla* were incubated with enzymes in vitro. Many of the bacterial chitinase-treated eggs became spherical, and the number of dead juveniles increased over controls. Purified bean chitinase at 17 µg/ml and higher af-

Received for publication 8 October 1999.

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The authors thank Christopher Hayes and Gary Harman for providing construct pStudI, Alan Blowers for help with the endochitinase activity assay, and Francoise M. Vermeylen for help with statistical problems. Thanks to George S. Abawi and Bill Brodie for reviewing this manuscript. This work was supported in part by a grant from the Cornell Center for Advanced Technology in Biotechnology, which is sponsored by the New York State Science and Technology Foundation and industrial partners and by TGT Inc, Geneva, NY.

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This paper was edited by L. D. Young.

fected egg mortality but not egg shape (Mercer et al., 1992). In other experiments, commercial chitinase inhibited egg hatching of the potato cyst nematode, *Globodera rostochiensis* Woll., in vitro by up to 70% (Cronin et al., 1997). Similarly, the chitinase-producing bacteria *Stenotrophomonas maltophilia* and *Chromobacterium* sp. reduced egg hatch by up to 90%. This inhibition was directly correlated with the initial bacterial concentration and incubation time; at least 10^6 CFU/ml or greater and incubation time of 2 weeks were needed to observe this effect. The bacteria also affected nematodes in soil planted with nematode-susceptible potato, although levels of inhibition were lower than in vitro (Cronin et al., 1997). The medium on which the actinomycete *Streptomyces saraceticus* was cultured produced high levels of chitinase and significantly reduced egg-hatching ratios of *Meloidogyne incognita* (Kofoid & White) Chitwood (Lee et al., 1996).

Like nematode eggs, many fungi contain chitin embedded in their cell walls. In recent years, transgenic plants expressing endochitinase genes from various sources (fungi, plants, bacteria, and insects) have been produced; some of these were effective in controlling various fungal pathogens and chitin-containing insects (Broglie et al., 1991; Lorito et al., 1998). Endochitinase from the biocontrol fungus *Trichoderma harzianum* has shown the most promising results, in several cases providing complete resistance to various fungi tested (Lorito et al., 1998).

Over-expression of chitinase in transgenic plants may be sufficient to damage nematode eggs. Due to the importance of chitin in the structural integrity of the egg, it is possible that relatively minor effects could destabilize egg development and durability. Changes in the permeability of the egg membrane could lead to the death of the embryo, inhibit hatching, or cause mistimed hatch. Nematodes that lay eggs within host tissues would be most at risk (Burrows and De Waele, 1997).

We have produced transgenic tobacco plants expressing an endochitinase cDNA from *T. harzianum*. The goal of the experiments described here was to determine whether progeny of these plants have increased resistance to *M. hapla*.

Materials and Methods

Plasmid: The plasmid pStudI contained the cDNA of the *ThEn-42* endochitinase gene from *T. harzianum* (Hayes et al., 1994) under control of the double 35S CaMV promoter and had the leader sequences of the alfalfa mosaic virus (AMV) for enhanced gene expression and a *nos* terminator (Fig. 1). The *nptII* gene for kanamycin-resistance served as the selectable marker.

Plant material: Young leaves of greenhouse-grown *N. tabacum* cv. Samsun NN were transformed with pStudI, using standard *Agrobacterium*-mediated transformation methods (Brants, 1999). Transformation was confirmed using PCR with *nptII*- and endochitinase-specific primers and Southern blotting using a pStudI/*HindIII* fragment as a probe.

Endochitinase activity tests were done with a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA). 4-methylumbelliferyl-β-D-N,N′,N″-

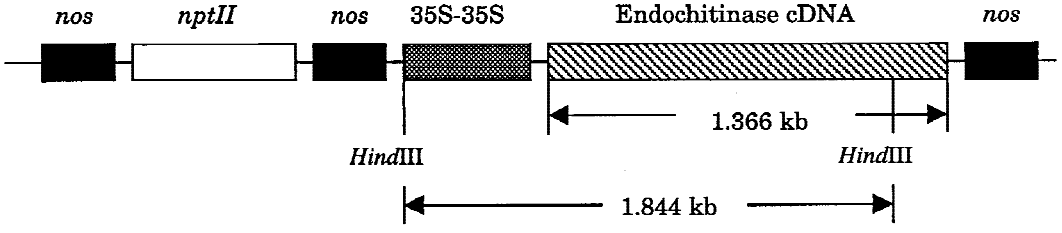

Fig. 1. Schematic diagram of pStudI construct. The *nptII* gene used as a selectable marker was controlled by and terminated by *nos* promoter and terminator. The endochitinase gene was controlled by a double 35S promoter and also contained alfalfa mosaic virus enhancer sequence. It was terminated with the *nos* terminator.

triacetylchitotriose (Sigma, St. Louis, MO) was diluted to the concentration of 25 µg/ml in 0.1 M sodium acetate (pH 5.3) and used as a substrate. Five samples of untransformed plant leaf and root tissues of the same age, grown in similar conditions as transformed plants, were used as negative controls in each experiment. Endochitinase concentrate (48 g/liter) from a *Trichoderma reesei* culture, provided by Gary Harman, was used as a positive control. Twenty-five microliters of the cell-free extract were added to 200 µl of substrate and incubated at room temperature for up to 1.5 hours. Aliquots (30-µl) were removed from the reaction mixture into 1.9 ml of 0.2 M Na_2CO_3 stopping buffer after 10 minutes, and fluorescence of the solution was determined as amount of 4-methylumbelliferyl (MU) released per minute per milligram of total protein.

Transformed plants were grown in the greenhouse until maturity, and fully developed seedpods were collected. Seeds from 11 different lines were germinated on Murashige-Skoog (1962) medium with 1% sucrose (pH 5.8), supplemented with 200 mg/l kanamycin. Kanamycin-resistant T_1 plants were transferred to Cornell soil-less mix (Sheldrake and Boodley, 1973) and grown at room temperature. Two weeks later they were moved to the greenhouse.

Control plants used: Tomato (*Lycopersicon esculentum* L.) cv. Rutgers and pepper (*Capsicum annuum* L.) cv. California Dream plants were used as susceptible controls. Stephens wheat (*Triticum aestivum* L.), a non-host species, was used to ensure that the inoculum contained no other *Meloidogyne* spp. and, in particular, was not contaminated by *M. chitwoodi* Golden et al., which can infect wheat.

Pepper seed was planted in Cornell soil-less mix 2 weeks before tobacco seed planting in vitro because of its slow germination. Tomato and Stephens wheat seed were planted in Cornell mix when tobacco seeds were planted in vitro. All controls were germinated at room temperature and a 16-hour-light/8-hour-dark photoperiod, and transferred to the greenhouse simultaneously with the tobacco plants.

Handling of plants in the greenhouse: Plants were transplanted in 15.2-cm clay pots containing autoclaved quartz sand and field soil (Cortland loam) in a 4:1 ratio in order to improve soil drainage and to allow easy movement of nematodes within the soil. Ten plants from each of nine transgenic lines (14-27, 14-29, 14-44, 14-48, 14-54, 14-56, 16-21, 16-22, and 16-29) were planted in experiment 1 (May through July 1997). Ten plants from each of 10 transgenic lines (14-6, 14-27, 14-29, 14-44, 14-48, 14-54, 14-56, 16-13, 16-21, and 16-29) were planted in experiment 2 (June through August 1997). Ten untransformed tobacco plants and five to ten plants from each control species were included in each experiment.

Potted plants were randomized on greenhouse benches and grown in the greenhouse for about 2–3 weeks until they were approximately 15 cm tall.

Inoculation of plants: Meloidogyne hapla eggs collected from Rutgers tomato plants were provided by Hassan Mojtahedi, Washington State University IAREC, Prosser, Washington. Inoculum was calibrated to provide 5,000 eggs/pot. Inoculum was agitated during the application to avoid the sedimentation of the eggs. Five 4-cm-deep holes were made with a pencil in each pot, and 1 ml of inoculum was pipeted into each hole. The holes were gently covered with soil, and plants were grown in the greenhouse for 55 days. Plants were watered once or twice daily, depending on air temperature, but were always allowed to dry between watering.

Extraction of eggs and nematodes: Plant roots were rinsed gently with water to remove the soil and air-dried until only slightly moist. Each root system was weighed, placed in a clean plastic bag, and stored at 4 °C. Roots were cut into 3 to 4-cm pieces, covered with 15% commercial bleach (i.e., 0.7875% sodium hypochlorite), and manually shaken for 10 minutes. Eggs and second-stage juveniles (J2) were collected on a 45-µm-pore sieve and washed into 50-ml plastic tubes with tap water. The eggs and J2 in 1 ml of sample diluted 5 to 20 times (depending on

egg and J2 numbers) were counted in a counting chamber at ×160 magnification.

Calculations: The following characteristics were determined and recorded for each plant: number of eggs and number of J2 extracted, sum of eggs and J2 in the extract, reproductive factor (Rf = [eggs + J2]/initial inoculum), and total eggs and J2 per gram of fresh root. Logarithmic transformation, $\log_{10}[x + 1]$ was used to transform data for all measurements except Rf. Differences between lines were determined with ANOVA and Tukey's pairwise comparisons at $\alpha = 0.05$ using Minitab v.12.0 (Minitab Inc., State College, PA). Differentials were not included in statistical analyses.

Results

Selection of T_1 progeny: Kanamycin-susceptible T_1 seedlings bleached within 2 weeks after seed planting on the selection medium, while kanamycin-resistant T_1 seedlings stayed green and developed true leaves and normal roots. Segregation ratios of 3:1 or 15:1 were observed, indicating one or two independent loci for the *nptII* gene (Table 1). The T_1 plants and control plants germinated on medium without antibiotics were at the same stage of development when they were transferred to soil.

Endochitinase activity in transformants: Endochitinase activity was determined in all tobacco primary transformants obtained and in some progenies. All tested transgenic lines, except 14-44 had significantly higher endochitinase activity than untransformed control plants. Endochitinase activity, measured in T_1 and control plant leaf and root tissues, is summarized in Table 1. All transgenic lines used had increased endochitinase activity in leaves (11 to 125 times over non-transgenic controls) and in roots (2 to 15 times over non-transgenic controls). Roots of non-transgenic plants had higher endochitinase activity than their leaves. Furthermore, endochitinase is secreted from transgenic cells in transgenic tissues according to studies of transgenic calli and cell suspensions (data not shown; for details see Brants, 1999).

Plant development: All plants grew normally in the 4:1 quartz sand:loam soil mix, and their growth rate was comparable to the plants left in Cornell soil-less mix. No signs of disease were observed on any control plants. In experiment 1, some plants from line 14-44 were stunted in comparison with other transgenic lines and untransformed tobacco after inoculation with nematodes. Other tobacco lines grew and developed normally in both experiments.

Experiment 1: Many eggs were present in the samples from the susceptible tomato and pepper plants, and none were present in the samples from Stephens wheat and uninoculated tobacco plants. In tobacco, only egg counts from 14-44 plants were significantly different from untransformed to-

Table 1. Characteristics of pStudI T_1 tobacco lines used in nematode resistance experiments.

Line	Endochitinase activity (nM MU/min/mg of total protein)				Segregation ratio on medium containing kanamycin
	In leaves	x control	In roots	x control	
Control	0.10 ± 0.02	1	2.23 ± 0.58	1	NA
14-27	9.23 ± 1.15	92	29.21 ± 4.18	13	3:1
14-29	9.14 ± 0.93	91	18.61 ± 1.73	8	15:1
14-44	1.09 ± 0.36	11	5.41 ± 1.47	2	3:1
14-48	9.67 ± 0.87	97	33.86 ± 5.81	15	3:1
14-54	10.13 ± 2.27	101	22.24 ± 3.58	10	3:1
14-56	12.51 ± 1.06	125	17.17 ± 0.78	8	15:1
14-6	8.11 ± 1.68	81	18.19 ± 1.12	8	3:1
16-13	5.74 ± 1.34	57	18.91 ± 2.05	8	3:1
16-21	11.29 ± 2.61	113	20.39 ± 1.88	9	3:1
16-22	9.41 ± 1.34	94	25.88 ± 3.23	12	3:1
16-29	6.67 ± 0.69	67	24.58 ± 3.12	11	3:1

bacco controls (Table 2). The correlation coefficient between endochitinase activity and egg counts was only 0.06, indicating that the increased endochitinase activity did not affect egg production in inoculated plants.

An unexpectedly high number of hatched J2 was observed in all samples but tomato and Stephens wheat. Three transgenic lines (14-27, 16-21, and 16-22) had significantly more J2 than tobacco controls.

Line 14-44 had significantly fewer eggs plus nematodes than lines 16-21, 14-56, 16-29, and 14-29, but no statistically significant differences were observed between transgenic and control tobacco.

After 55 days, the nematode populations increased an average of 20.8 times on tomato and 13.1 times on pepper. A low Rf (0.05) was obtained for Stephens wheat, indicating that it was not a suitable host for *M. hapla* and that on this plant the nematode population was strongly reduced in a short period of time.

Relatively low Rf values were observed in the tobacco plants, ranging from 1.03 for line 14-44 to 3.40 for line 16-21. The RF for untransformed tobacco plants was 2.0.

The highest number of nematodes and eggs per gram of fresh weight of roots was extracted from tomato and pepper plants. Very little variation was observed in tobacco lines with logarithmic values from 2.56 for tobacco line 16-21 to 2.14 for control tobacco and line 14-44. No statistical differences were detected among tobacco lines tested. The lowest value (0.29) was obtained for Stephens wheat.

Experiment 2: Line 14-44 was the only selection that behaved differently in the two experiments, providing the lowest egg counts in experiment 1 and the highest in experiment 2 (Table 3). No statistically significant differences in egg counts were observed among the tobacco plants tested. Lower nematode counts were observed in all tobacco lines in experiment 2 than in experiment 1. Only plants from transgenic line 14-6 had significantly fewer J2 than untransformed tobacco plants.

The highest Rf for tobacco (2.23) was observed in untransformed control plants. Transgenic tobacco line Rf ranged from 1.21 to 2.15. Little variation in number of nematodes and eggs per gram of fresh weight of roots was observed in tobacco lines (2.04 to 2.40). No statistical differences between any tobacco lines for these two parameters were detected in this experiment.

Discussion

A significant reduction in egg number due to the degradation of the chitin layer in the nematode eggshell was expected in transgenic plants expressing high levels of endochitinase if this enzyme interfered with the nematode parasitism or reproduction. In such a case, a reduction in nematode hatching from generation to generation should lead to reduced invasion of tobacco

Table 2. Reproduction of *Meloidogyne hapla* in endochitinase-transgenic tobacco plants (Experiment 1).[a]

Line	No. of plants	Log_{10} (eggs + 1)	Log_{10} (J2 + 1)	Log_{10} (J2 + eggs + 1)	Reproductive factor (Rf)[b]	Log_{10} ([J2 + eggs]/g root + 1)
Control	9	3.87 ± 0.09 a	2.92 ± 0.14 b	3.94 ± 0.08 ab	2.00 ± 0.35 ab	2.14 ± 0.09 a
14-27	10	2.61 ± 0.46 ab	3.64 ± 0.19 a	3.97 ± 0.05 ab	1.98 ± 0.20 ab	2.34 ± 0.12 a
14-29	9	3.16 ± 0.44 ab	3.48 ± 0.19 ab	4.05 ± 0.06 a	2.42 ± 0.34 ab	2.36 ± 0.13 a
14-44	10	2.29 ± 0.53 b	3.43 ± 0.05 ab	3.64 ± 0.08 b	1.03 ± 0.20 b	2.14 ± 0.15 a
14-48	10	3.43 ± 0.15 ab	3.35 ± 0.60 ab	3.84 ± 0.11 ab	1.92 ± 0.58 ab	2.15 ± 0.14 a
14-54	10	3.48 ± 0.11 ab	3.49 ± 0.14 ab	3.83 ± 0.10 ab	1.64 ± 0.26 ab	2.15 ± 0.12 a
14-56	9	3.96 ± 0.08 a	3.43 ± 0.13 ab	4.13 ± 0.05 a	2.78 ± 0.25 ab	2.29 ± 0.06 a
16-21	8	2.97 ± 0.68 ab	3.62 ± 0.09 a	4.14 ± 0.11 a	3.40 ± 0.89 a	2.56 ± 0.08 a
16-22	10	3.51 ± 0.22 ab	3.60 ± 0.10 a	4.03 ± 0.07 a	2.38 ± 0.35 ab	2.36 ± 0.09 a
16-29	10	3.93 ± 0.09 a	3.24 ± 0.13 ab	4.06 ± 0.06 a	2.52 ± 0.35 ab	2.35 ± 0.06 a

[a] Numbers are means ± standard error. Means followed by a common letter are not significantly different according to ANOVA analysis ($P \leq 0.05$).

[b] Rf = [eggs + J2]/initial inoculum.

TABLE 3. Reproduction of *Meloidogyne hapla* in endochitinase-transgenic tobacco plants (Experiment 2).[a]

Line	No. of plants	Log_{10} (eggs + 1)	Log_{10} (J2 + 1)	Log_{10} (J2 + eggs + 1)	Reproductive factor (Rf)[b]	Log_{10} ([J2 + eggs]/g root + 1)
Control	10	3.88 ± 0.17 a	2.24 ± 0.38 ab	3.95 ± 0.13 a	2.23 ± 0.33 a	2.37 ± 0.12 a
14-27	10	3.65 ± 0.12 a	1.31 ± 0.44 ab	3.67 ± 0.12 a	1.21 ± 0.24 a	2.26 ± 0.13 a
14-29	10	3.37 ± 0.38 a	2.28 ± 0.39 ab	3.69 ± 0.13 a	1.26 ± 0.24 a	2.04 ± 0.13 a
14-44	10	3.95 ± 0.08 a	1.63 ± 0.45 ab	3.97 ± 0.08 a	2.11 ± 0.33 a	2.40 ± 0.09 a
14-48	10	3.88 ± 0.07 a	1.65 ± 0.46 ab	3.91 ± 0.06 a	1.79 ± 0.25 a	2.34 ± 0.06 a
14-54	10	3.92 ± 0.10 a	1.97 ± 0.44 ab	3.96 ± 0.09 a	2.15 ± 0.40 a	2.40 ± 0.08 a
14-56	10	3.67 ± 0.13 a	2.60 ± 0.08 ab	3.73 ± 0.12 a	1.47 ± 0.39 a	2.27 ± 0.11 a
14-6	10	3.52 ± 0.40 a	1.16 ± 0.48 b	3.54 ± 0.40 a	1.68 ± 0.30 a	2.10 ± 0.24 a
16-13	10	3.60 ± 0.20 a	2.09 ± 0.35 ab	3.67 ± 0.18 a	1.59 ± 0.39 a	2.10 ± 0.17 a
16-21	10	3.81 ± 0.07 a	2.82 ± 0.09 a	3.87 ± 0.06 a	1.61 ± 0.20 a	2.32 ± 0.07 a
16-29	10	3.87 ± 0.09 a	2.48 ± 0.28 ab	3.91 ± 0.08 a	1.85 ± 0.27 a	2.37 ± 0.07 a

[a] Numbers are means ± standard error. Means followed by a common letter are not significantly different according to ANOVA analysis ($P \leq 0.05$).
[b] Rf = [eggs + J2]/initial inoculum.

roots and to an overall reduction of nematode population. The development of eggs to first- and second-stage juveniles might be affected, thus reducing numbers of hatched J2. Alternatively, an increase in prematurely hatched nematodes in transgenic tobacco would be expected if endochitinase had an effect on the chitin layer in nematode eggs sufficient to promote hatching, but not strong enough to suppress the nematode population. This effect would lead to a significant increase in hatched J2 in transgenic plants over the controls and, possibly, to reduced numbers of eggs due to increased hatching rate.

A total of 11 different transgenic tobacco lines were tested for resistance to *M. hapla*. All lines but 14-44 and control tobacco expressed high levels of endochitinase activity in leaves and in roots. Control tobacco roots had higher endochitinase activity in comparison with the leaves; similar findings have been reported previously (Shinshi et al., 1987). However, only 14-44 showed a statistically significant reduction in egg counts in experiment 1. Since line 14-44 did not display elevated endochitinase levels, this difference cannot be attributed to endochitinase in the transgenic plants. Experiment 2 showed no significant differences in egg counts between any transgenic line and control plants; line 14-44 had the highest egg count in this experiment. In experiment 1, the plants of line 14-44 were stunted and had a smaller and weaker root system than other tobacco plants in experiment 1 (data not shown). These factors likely led to fewer infection sites for nematodes and lower final egg counts.

Experiment 1 had higher numbers of J2 in all tobacco lines tested than experiment 2. Three lines expressing high levels of endochitinase activity (14-27, 16-21, 16-22) had significantly more hatched J2 than control plants in experiment 1. However, 14-27 and 16-21 were not different from controls in experiment 2, and line 16-22 was not tested. A positive correlation between the hatching of nematodes and temperature has been reported previously (Inserra et al., 1983; Mishra and Gaur, 1987; Wang et al., 1997), and differences in soil temperature may have affected the hatching rate in the two experiments. However, egg masses on roots contain eggs at different stages of development, and eggs at the J2 stage may hatch during the extraction procedure, as well.

Relatively low Rfs of 1.03 to 3.40 were observed in both experiments for the tobacco lines tested. However, the Rf values obtained indicated that tobacco is a suitable host for *M. hapla*.

To our knowledge, this is the first report in which transgenic plants expressing elevated levels of endochitinase were used in resistance tests against nematodes. However, our results did not support the findings of Mercer et al. (1992) on increased hatching

levels of nematodes as a result of treatment with chitinases, or those of Cronin et al. (1997) that chitinases can decrease nematode egg hatching. Instead, our results strongly suggest that elevated endochitinase levels in tobacco plants do not have an effect on *M. hapla*. The discrepancies between our results and those reported earlier may be explained by the different testing systems used. Mercer et al. (1992) used extracted eggs, increasing the possibility of interaction between chitinase and eggshell by removing the gelatinous chitin-free matrix that normally surrounds the eggs in *Meloidogyne* sp. (Bird and Self, 1995). Cronin et al. (1997) used cysts (no gelatinous matrix surrounds eggs in *G. rostochiensis*) and purified commercially available chitinase and were still able to observe decreased levels of egg hatching. In both reports the authors noted that differences in the amount or type of chitinase produced can affect egg hatch. We did not determine the overall chitinase concentration in our transgenic plants; however, it is reasonable to assume that the chitinase levels were lower than those used in the in vitro studies described above.

If nematode eggs are laid outside the root tissue, the probability of an endochitinase effect is further diminished. Our transgenic tobacco secretes endochitinase from the cells (Brants, 1999), probably resulting in a narrow layer around plant roots with significantly increased levels of chitinase activity. However, plant watering and the good drainage of the soil mixture used likely decreased endochitinase concentration in the soil by washing the enzyme out.

Although chitin amendment to the soil in order to increase the natural chitinolytic bacterial population to control nematodes can be effective (Godoy et al., 1984; Mian et al., 1982), other factors like the release of ammonia as a breakdown product of the added chitin might be responsible for the effect on nematode populations (Spiegel et al., 1987). All these results indicate that nematode control is complicated and probably cannot be attributed to a single factor. Although our experiments did not demonstrate the usefulness of chitinase in nematode control in transgenic plants, a combination of chitinase with other lytic enzymes in the same plant or use of root-specific promoters to increase enzyme concentration in roots may prove useful in the future. Alternatively, transgenic plants expressing high endochitinase activity might need lower dosages of pesticide treatment for effective control of nematode populations through synergistic interaction of chitinase with nematicides.

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