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Insect resistance of transgenic tobacco expressing an insect chitinase gene

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Chitinase expression in the insect gut normally occurs only during moulting, where the chitin of the peritrophic membrane is presumably degraded. Thus, insects feeding on plants that constitutively express an insect chitinase gene might be adversely affected, owing to an inappropriately timed exposure to chitinase. This hypothesis was tested by introducing a cDNA encoding a tobacco hornworm (*Manduca sexta*) chitinase (EC 3.2.1.14) into tobacco via *Agrobacterium tumefaciens*-mediated transformation. A truncated but enzymatically active chitinase was present in plants expressing the gene. Segregating progeny of high-expressing plants were compared for their ability to support growth of tobacco budworm (*Heliothis virescens*) larvae and for feeding damage. Both parameters were significantly reduced when budworms fed on transgenic tobacco plants expressing high levels of the chitinase gene. In contrast, hornworm larvae showed no significant growth reduction when fed on the chitinase-expressing transgenics. However, both budworm and hornworm larvae, when fed on chitinase-expressing transgenic plants coated with sublethal concentrations of a *Bacillus thuringiensis* toxin, were significantly stunted relative to larvae fed on toxin-treated non-transgenic controls. Foliar damage was also reduced. Plants expressing an insect chitinase gene may have agronomic potential for insect control.

Keywords: *Bacillus thuringiensis* toxin; *Heliothis virescens*; *Manduca sexta*; *Nicotiana tabacum*; tobacco budworm; tobacco hornworm; chitinase

Introduction

The ability to introduce foreign genes into plants has allowed the development of a variety of strategies for improving resistance to insect predation and diseases of crop plants. One such strategy has been to deploy a gene that was cloned from a pathogen or pest for biocontrol. Expression of this gene in the plant at the appropriate time and level then disrupts the development of the same or a related pathogen or pest. Examples include the expression of viral coat protein genes that confer resistance to viral infection (Abel *et al*., 1986) and the use of insect protease inhibitor genes for insect control (Thomas *et al*., 1995a,b). We describe here the use of an insect chitinase (EC 3.2.1.14) gene to enhance insect resistance in transgenic tobacco.

Chitin is a linear homopolymer of β(1 → 4) linked 2-deoxy-2-acetamido-D-glucopyranosyl residues and is a major component of the exoskeleton and alimentary canal of insects (Kramer *et al*., 1985; Peters, 1992). Together with proteinases in the moulting fluid, endo-splitting chitinases and exo-splitting β-*N*-acetylglucosaminidases break down the unsclerotized layers of the old cuticle prior to shedding of the sclerotized portion or exuvium.

A cDNA encoding the major moulting fluid chitinase of *Manduca sexta*, the tobacco hornworm, was used in our studies. Transcripts of this gene are only briefly

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detectable in epidermal and gut tissues of fifth instar larvae between 5 and 7 days after moulting and just prior to pupal transformation (Kramer *et al*., 1993). This tight developmental regulation suggested that this chitinase could be detrimental to insect growth if presented in the diet at an inappropriate time. We describe here a novel strategy to inhibit insect damage and development in transgenic tobacco by constitutively expressing an insect chitinase gene whose developmental expression in the insect gut and elsewhere normally occurs only during the moulting process. We know of no other examples of insect control in plants with an introduced chitinase gene from any source. We also report the interaction of this plant-expressed *Manduca* chitinase with *B. thuringiensis* toxin in the enhancement of insect control.

Materials and methods

Plasmid construction and plant transformation

Chimaeric gene constructs were prepared by inserting a 1.8 kb *Eco* RI fragment from a cDNA clone containing the entire coding region of the *Manduca* chitinase (Kramer *et al*., 1993) into binary vectors between single or double CaMV 35S promoters and the *nos* or *pinII* polyadenylation signal sequences, respectively (Rogers *et al*., 1987). For single CaMV 35S control, the binary pMON410 vector (Rogers *et al*., 1987) was digested with *Sma* I in order to remove the hygromycin phosphotransferase gene, filled in with the Klenow fragment of DNA polymerase I and then ligated to generate the altered vector pMON410A. The *Manduca* chitinase cDNA clone (Kramer *et al*., 1993) was then inserted into the *Eco* RI site of pMON410A to produce pMON410A:chitinase.

In order to obtain double CaMV 35S control, the binary pMON505 vector (Rogers *et al*., 1987) was digested with *Eco* RI, filled in with Klenow and ligated to destroy the *Eco* RI restriction site and generate pMON505A. The plasmid pPHI227 was digested with *Eco* RI, filled in with Klenow, mixed with *Bgl* II linker, and ligated to generate PHI227A. PHI227A was digested with *Bam* HI, filled in, and *Eco* RI linker was added and ligated to generate PHI227B. PHI227B was digested with *Bgl* II to generate a 1.2 kb fragment containing the double CaMV 35S promoter and poly A termination signal, and inserted into pMON505A at the *Bgl* II site to generate pMON505C. The *Manduca* chitinase cDNA clone (Kramer *et al*., 1993) was then inserted into the *Eco* RI site of pMON505C to produce pMON505:Chitinase.

Binary plasmid constructs were mobilized to *A. tumefaciens* EHA101 by triparental mating (Masoud *et al*., 1993). Genes were introduced into tobacco (*Nicotiana tabacum* var. Xanthi) leaf disks by *A. tumefaciens*-mediated transfer, followed by kanamycin selection and plant regeneration (Masoud *et al*., 1993).

Protein analyses

For protein immunoblot (western) analysis, leaves or leaf disks were ground in liquid nitrogen with a mortar and pestle and extracted with 100 mM sodium phosphate buffer, pH 6.7, at 25 °C. Following centrifugation in a microfuge, supernatants were subjected to SDS-polyacrylamide gel electrophoresis (Sambrook *et al*., 1989). Immunoblot analysis utilized chitinase polyclonal antibody raised against chitinase purified from *M. sexta* moulting fluid (Koga *et al*., 1983) and an alkaline phosphatase-based staining kit (Life Technologies, Gaithersburg, MD, USA). Proteins for ELISA were extracted from T_1 leaves by grinding in 100 mM sodium phosphate buffer, pH 6.7, at 25 °C. Total protein from each sample was quantified by using a protein assay kit (Sigma Chemical Co., St Louis, MO, USA) according to the manufacturer's instructions and adjusted to equal concentrations. Total protein (200 µg) was added to 200 µl of coating buffer in individual wells of a microtitre plate. Procedures followed those of Ausubel *et al*. (1989) but utilized *Manduca* chitinase polyclonal antibody diluted 1:5000 with blocking buffer. Absorbance was measured at 405 nm.

Bioassays for insect toxicity

T_0 or T_1 shoots, plants or excised leaves were compared to controls, either seed-initiated, non-transformed plants or non-expressing T_1 segregants, for budworm resistance. Neonate larvae were used in all experiments. Larvae were transferred with a camel's hair brush to excised leaves or to young terminal leaves of excised shoots or plants. Excised leaves were maintained in moist Petri plates. Larvae were restrained on shoots or plants with plastic bags in which holes had been made to reduce humidity. Plants were maintained under conditions described for each experiment. Total larval biomass and number of surviving larvae were determined for each experiment. As noted by Boulter *et al*. (1990), considerable variation in the growth and survival of larvae in budworm bioassays was observed. Many of our neonates died even when reared on control plants, and a few larvae reared on the chitinase-positive plants attained a size equal to those on chitinase-negative plants. However, significant differences in growth of the larval populations fed chitinase-positive and -negative plants were observed in all of these experiments.

Hornworm bioassays were performed by applying five neonate hornworm larvae per shoot. Shoots were covered with screen cages for ten days. Environmental conditions were as described for the budworm.

Bacillus thuringiensis *(*Bt*) toxin treatment*

Young shoots of similar size of both transgenic and control tobacco plants with 7–8 layers of leaves (16–20 leaves) were soaked in *Bt* toxin solution for 5 min,

removed from the solution and air-dried. The toxin (Attack Pesticides, Division of Ringer Corporation, Minneapolis, MN, USA) is a commercial pesticide derived from *B. t. Berliner* var. *kurstaki*. Actual *Bt* toxin concentrations, based on toxin solution consumed, were 180 and 360 ng per g fresh wt of plant tissue, for 1 and 2 μg/ml solutions. Intermediate concentrations were determined by extrapolation. The excised shoots were placed in flasks containing dilute Murashige and Skoog salts in water.

Statistical methods

Variance analyses of insect biomass and insect survival were conducted with the general linear module (GLM) procedure or the associated LSD (SAS Institute, 1991).

Results

Chimaeric gene constructs were prepared by inserting a 1.8 kb *Eco* RI fragment from a cDNA clone containing the entire coding region of the *Manduca* chitinase (Kramer *et al*., 1993) into binary vectors between single or double CaMV 35S promoters and either the *nos* or *pinII* polyadenylation signal sequences. The gene was introduced into tobacco plants (*Nicotiana tabacum* var. Xanthi) by *A. tumefaciens*-mediated transfer (Masoud *et al*., 1993). Fourteen of 44 primary transformants (T_0 plants) with the single 35S promoter were shown by immunoblot analysis to express the insect chitinase (data not shown).

Two T_0 plants, 4C16 and 4C49, transformed with the pMON410A construct and driven by the single 35S promoter, were selected. These two expressed the highest levels of chitinase based on immunoblot staining intensity. The plants were self-fertilized, and leaves from the progeny (T_1 plants) were analysed for the presence of the *Manduca* chitinase by both immunoblot analysis (Fig. 1) and activity assay using glycolchitin-containing overlay gels (Koga *et al*., 1992) (Fig. 2). The molecular mass of 46.1 kDa for the enzymatically active protein from transgenic tobacco previously was determined by mass spectrometry (Wang *et al*., 1996). This size was lower than the expected molecular mass of 60 kDa (after removal of a 2 kDa leader peptide) predicted from the cDNA sequence (Kramer *et al*., 1993). N-terminal sequencing revealed that the truncated tobacco protein and the mature moulting fluid enzyme have the same N-terminal amino acid sequence, indicating that the former protein, although enzymatically active (Fig. 2), is truncated from the C-terminal end (Wang *et al*., 1996). No residual peptide of approximately 14 kDa was observed. It may lack or differ in glycosylation and thus be unrecognized by our antibody, it may never occur, or it may be too rapidly degraded to detect.

Progeny from plants 4C16 and 4C49 each segregated 3:1 for expression of the *Manduca* chitinase with ratios

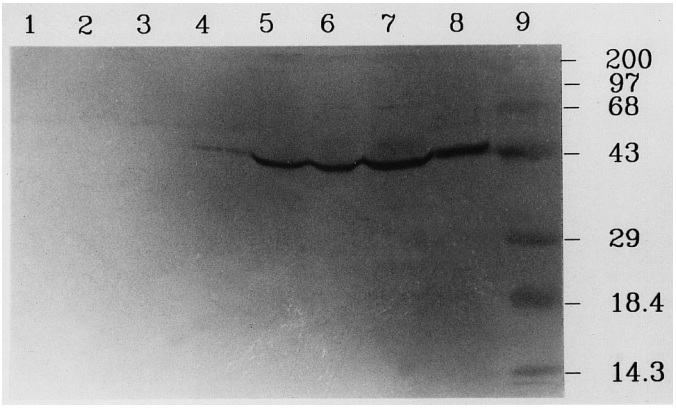

Fig. 1. Western blot showing expression of the *Manduca* chitinase transgene in progeny of transgenic tobacco. Buffer-soluble proteins were extracted from leaves of T_1 plants resulting from self-fertilization of transgenic plant 4C16. Aliquots containing 200 μg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis (Sambrook *et al*., 1989) using a polyclonal antibody raised against the insect chitinase (Koga *et al*., 1983). Lanes 1, 2, 3 and 4 contain extracts from representative progeny not expressing the chitinase transgene in 4C16. Lanes 5, 6, 7 and 8 are from different *Manduca* chitinase-positive progeny. Molecular weight markers are shown in kDa.

of 82:28 ($\chi^2 = 0.012$, $p = 0.9128$) and 56:17 ($\chi^2 = 0.014$, $p = 0.9058$), respectively, as expected for a dominant gene at a single heterozygous locus. Eight plants expressing the *Manduca* chitinase gene (chitinase-positive) and eight plants not expressing the gene (chitinase-negative) were selected on the basis of western blot activity from the segregating 4C49 T_1 population. Leaves were excised and fed to *H. virescens* (tobacco budworm) first instar larvae in moist Petri dishes. Nine and ten of the original 32 larvae that were used in this preliminary experiment survived the three-week period on leaves from chitinase-positive and chitinase-negative plants, respectively. The total mass of larvae surviving on chitinase-positive leaves was 177 mg (mean 19.7 mg per larva), whereas that on chitinase-negative leaves was 966 mg (mean 96.6 mg per larva).

Feeding bioassays on excised shoots or intact plants were also performed. Bioassays 1 and 2 each involved excised shoots from four chitinase-positive and three chitinase-negative T_1 progeny. Bioassay 1 plants were from 4C16 (Fig. 3, experiment 1) and bioassay 2 plants were from 4C49 (Fig. 3, experiment 2) T_0 plants. Only the highest-expressing plants, based on western blot intensity, were selected. Lines 4C16 and 4C49 resulted from different transformation events and presumably carry unique insertions. Shoots were inoculated with 50 neonate *H. virescens* larvae and covered with ventilated plastic bags. After 10 days, all surviving insects from each plant were counted and weighed. Larval biomass

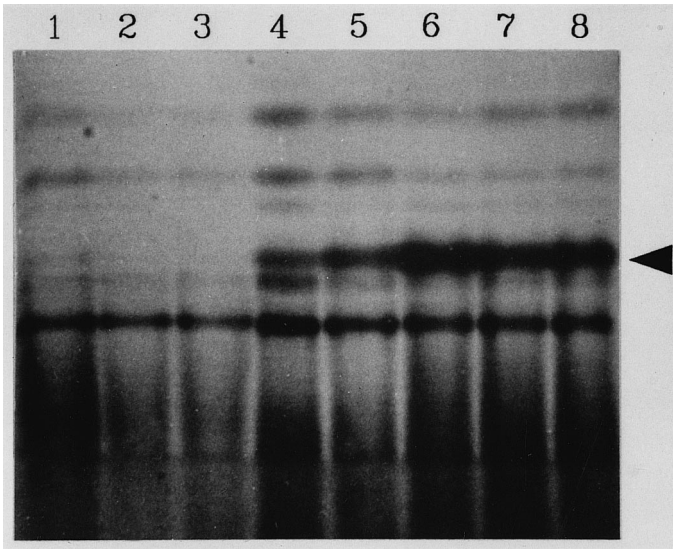

Fig. 2. Chitinase activity gel. Proteins from 4C16 progeny were extracted from leaves by grinding in 100 mM sodium phosphate buffer. An aliquot containing 40 μg protein was first subjected to 10% polyacrylamide gel electrophoresis at 4 °C under nondenaturing conditions. The gel was then overlaid with a 10% polyacrylamide gel containing 0.01% ethylene glycol chitin as a substrate, incubated at room temperature for 4 h, stained with a fluorescent brightener, and photographed (Koga *et al*., 1992). Lanes 1, 2 and 3 contain extracts from T_1 plants not expressing the *Manduca* chitinase as determined by western blot analysis. Lanes 4 and 5 contain extracts from T_1 plants expressing low levels of the insect chitinase, whereas extracts in lanes 6, 7 and 8 are from plants expressing high levels of the enzyme. Arrowhead indicates activity from chitinase transgene expression.

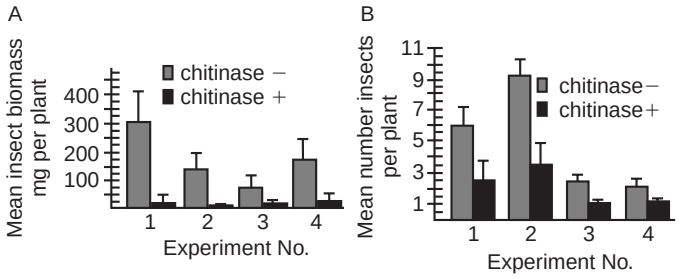

Fig. 3. Mean *Heliothis virescens* larval biomass (A) and mean number of living larvae (B) per T_1 plant expressing or not expressing the *Manduca* chitinase transgene. Determinations were made after larvae were allowed to feed for 7–11 days on shoots from T_1 progeny of 4C16 (experiment 1), 4C49 (experiment 2), D29 (experiment 3), and intact plants from D102 (experiment 4). Excised secondary shoots were placed in flasks containing 10% Murashige and Skoog salts in water. Individual shoots were infested with 20–50 neonate larvae, covered with a ventilated plastic bag, and placed in a growth chamber with a 16 h light and an 8 h dark photoperiod at 28 °C. The individual intact plants were infested with 20 neonate larvae, covered with a ventilated plastic bag, and placed in a greenhouse at 28 °C (experiment 4). F test analyses showed that mean biomass of surviving insects per plant for experiments 1–4 and number of surviving insects for experiments 1–3 were significantly different between chitinase-expressing and non-expressing plants ($p < 0.05$). Hatched bars represent values for insects feeding on plants without the *Manduca* chitinase transgene; solid bars are values for insects feeding on plants with the transgene. Values are means ± SE for each experiment.

(experiment 1, $F = 11.5$, $df = 1, 5$ and $p = 0.0209$; experiment 2, $F = 12.43$, $df = 1, 5$ and $p = 0.0168$) and the number of surviving larvae (experiment 1, $F = 11.21$, $df = 1, 5$ and $p = 0.0204$; experiment 2, $F = 8.36$, $df = 1, 5$ and $p = 0.0341$) were significantly reduced on plants expressing *Manduca* chitinase (Fig. 3, A and B, experiments 1 and 2). The reduced feeding damage on chitinase-positive plants compared to chitinase-negative plants was visually apparent (Fig. 4A). Surviving larvae from chitinase-positive plants were stunted in comparison to those on chitinase-negative plants (Fig. 4B).

In experiments 3 and 4 (Fig. 3), two other T_1 genotypes (D29 and D102) from different transformation events were used. Both genotypes were derived by transformation with the pMON505:chitinase construct that has a double 35S promoter. Expressing progeny of D29 and D102 averaged 213 ng and 265 ng of *Manduca* chitinase per mg total protein (0.02–0.03% of total protein), respectively, in leaf disks taken from the leaves being fed, when quantified by ELISA. In experiment 3, budworms feeding on the excised shoots from eight chitinase-positive D29 progeny and six wild-type *N. tabacum* cv. Xanthi plants were compared. Again, the mass ($F = 8.25$, $df = 1, 29$, $p = 0.0076$) and number ($F = 9.92$, $df = 1, 29$, $p = 0.0038$) of surviving larvae from the chitinase-positive plants were significantly lower than those from control plants (Fig. 3A and B, experiment 3). Visually assessed feeding damage was also significantly reduced in the chitinase-expressing transgenics. When plants were assigned a visual rating between 1 and 5, with 1 representing little or no damage and 5 the most extensive damage observed, 21 of the 22 chitinase-expressing plants received ratings of 2 or less, while four out of nine non-expressing plants had ratings of 4 or 5. The distribution of damaged plants was significantly different ($\chi^2 = 11.5$, $p = 0.022$) between positive and negative progeny. In a greenhouse bioassay, the insects were allowed to feed on intact plants. Again, larval biomass ($F = 4.87$, $df = 1, 12$ and $p = 0.0476$) was reduced on chitinase-positive plants when compared with the chitinase-negative plants (Fig. 3A Experiment 4), although insect number ($F = 4.6$, $df = 1, 12$ and $p = 0.0531$) was not (Fig. 3B, experiment 4). Larvae on plants lacking the *Manduca* chitinase consumed much more leaf tissue than did larvae feeding on plants with it.

In contrast to studies with the tobacco budworm, studies with the tobacco hornworm revealed no consistent

Fig. 4. Resistance to feeding damage by insects fed on T_1 tobacco plants obtained from selfed primary transformant 4C16 and effects on larval growth. Photographs are of representative plants and insects in experiment 1 (Fig. 3). (A) Feeding damage due to larvae of *Heliothis virescens* on T_1 plants expressing (left) or not expressing (right) the *Manduca* chitinase. (B) Representative surviving *H. virescens* larvae from the above shoots expressing (left) or not expressing (right) the *Manduca* chitinase transgene. Larvae were immobilized by refrigeration prior to photography.

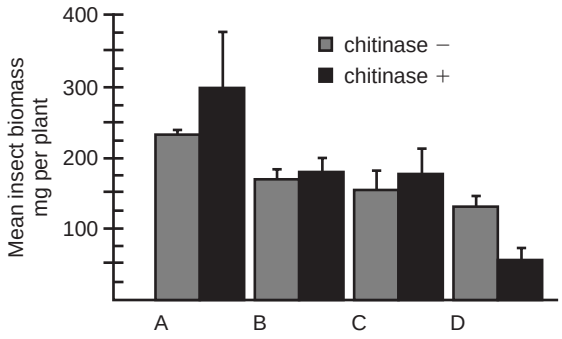

Fig. 5. Mean *Manduca sexta* larval biomass per T_1 plant shoot expressing or not expressing the *Manduca* chitinase transgene, as influenced by foliar treatment with *Bacillus thuringiensis* toxin. The D56 T_0 plant selfed to obtain the selected T_1 progeny analysed contained three or more independent insertions of the transgene Weights were taken 10 days after infestation with neonate larvae. (A) Mean insect biomass from plants without a toxin coating (water dipped). (B) Mean biomass from plants coated with 216 ng toxin per g fresh weight. (C) Mean biomass from plants coated with 234 ng toxin per g fresh weight. (D) Mean biomass from plants coated with 252 ng toxin per g fresh weight. Bars are means ± SE for each treatment.

differences in larval growth ($p = 0.9071$) or foliar damage on T_1 transgenic plants as compared to chitinase-negative controls. Fig. 5A shows representative results when the mean hornworm biomass of insects fed on transgenic and non-transgenic plants was compared. The possibility that some marginal detrimental effect on *Manduca* was occurring but not at levels significantly influencing growth rate was tested by measuring changes in growth of insects also receiving sublethal doses of *Bt* toxin. Others (Morris *et al*., 1976; Smirnoff, 1971, 1974; Sneh *et al*., 1983; Regev *et al*., 1996) have reported a potentiating interaction of chitinases and *Bt* toxin on insects. A narrow range of *Bt* toxin concentrations (234 to 252 ng/g fresh weight) was chosen for studying synergism (Fig. 5). Preliminary experiments had shown that below this level, the *Bt* toxin caused no growth inhibition interaction with chitinase-expressing plants, while significantly higher levels caused excessive inhibition of larval growth even on chitinase-negative plants. No significant interaction was detected between the presence of the chitinase transgene and the *Bt* toxin ($p = 0.1186$), although results with the highest concentration suggested that we were close (Fig. 5, B–D). When chitinase-expressing plants received a foliar coating of 288 ng of *Bt* toxin per gram fresh weight, both foliar damage and larval biomass production were significantly reduced in plants expressing the transgene (Fig. 6).

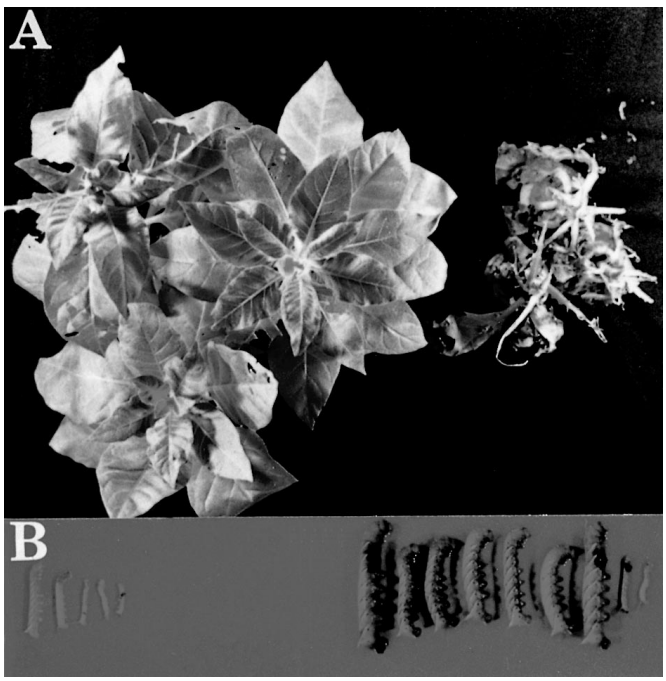

Fig. 6. Influence of the *Manduca* chitinase transgene in T_1 tobacco plants receiving a foliar application of 288 ng of *Bacillus thuringiensis* toxin per g fresh weight on feeding and growth of the tobacco hornworm, *M. sexta*. The toxin concentration was sublethal to the larvae. (A) Feeding damage ten days after infestation with neonate hornworm larvae on plants expressing (left) or not expressing (right) the chitinase gene. (B) Hornworms from plants expressing (left) or not expressing (right) the *Manduca* chitinase.

A similar *Bt* toxin-chitinase transgene interaction was observed with T_1 segregants with the tobacco budworm. Chitinase-expressing plants coated with 360 ng of toxin per gram fresh weight exhibited a 56% reduction in their mean foliar damage index relative to non-transgenic controls, and the biomass of surviving larvae was reduced by 89%.

Discussion

Enhanced resistance to fungal pathogens has been obtained following the introduction of a chitinase transgene (Broglie *et al*., 1991; Vierheilig *et al*., 1993; Zhu *et al*., 1994; Jach *et al*., 1995; Lin *et al*., 1995). Hen egg white lysozyme, an enzyme with chitinolytic activity, was isolated from transgenic tobacco expressing it and shown to inhibit the growth of some bacterial and fungal plant pathogens (Trudel *et al*., 1995). However, the enhancement of insect resistance in plants by these strategies has not been reported. Our results illustrate that an insect chitinase transgene can enhance resistance to budworm neonates feeding on tobacco and can potentiate the effect of sublethal doses of *Bt* toxin on both the budworm and the hornworm. If the expression level of the insect chitinase is properly managed, it appears that the enzyme could be utilized to promote increased protection of transgenic plants toward insect and microbial pests (Kramer *et al*., 1996).

The mechanism(s) of the *Manduca* chitinase-mediated resistance is unknown. This chitinase may be directly toxic to larvae, enhance sensitivity to existing microbes or dietary components, or elevate or induce other defensive mechanisms in the plant, which are, in turn, toxic to the larvae. The truncated chitinase is toxic to the merchant grain beetle, *Oryzaephilus mercator*, in an artificial diet (Wang *et al*., 1996). A baculovirus engineered to express the *Manduca* chitinase accelerated the mortality of the fall armyworm (*Spodoptera frugiperda*) compared to the wild-type virus (Gopalakrishnan *et al*., 1995). Microbial chitinases can also kill or retard growth of certain insects when combined with a baculovirus (Shapiro *et al*., 1987), *B. thuringiensis* (Smirnoff, 1971, 1974; Morris, 1976; Wiwat, 1996), or *Bt* spore crystals (Sneh *et al*., 1983). A synergistic toxic effect also results when both a low concentration of a truncated, recombinant CryIC protein and a bacterial chitinase are incorporated into a synthetic insect diet (Regev *et al*., 1996).

A potential site of action of chitinase is the peritrophic membrane, which is a thin, membranous sac that encloses the food in the midgut and hindgut of most insects. The chitin-containing membrane originates from the anterior portion of the midgut in *H. virescens* (Ryerse *et al*., 1992), and may function in anchoring or compartmentalization of digestive enzymes in the gut, protecting the midgut epithelium from abrasion by hard food particles, prevention of pathogen invasion, and ultrafiltration (Ryerse *et al*., 1992). The chitinase gene is not expressed by insects during feeding periods (Kramer *et al*., 1993), and the protein is detected only during a narrow period just prior to moulting (Zen *et al*., 1996). If the insect is continually exposed to chitinase, chitin-containing structures such as the peritrophic membrane might malfunction due to chitin degradation and loss of structural integrity. Chitinases purified from some bacteria can disrupt the peritrophic membrane of the insect gut (Peters, 1992; Regev *et al*., 1996). Penetration of the mosquito peritrophic membrane by the malarial parasite apparently involves chitinase (Huber *et al*., 1991), and access of the microfilarial parasite, *Brugia malayi*, to the gut cells of the mosquito host may require elaboration of a chitinase (Fuhrman *et al*., 1992). A compromised peritrophic membrane barrier may also facilitate the *Bt* toxin synergism with chitinases by enhancing contact of toxin molecules with their epithelial membrane receptors (Regev *et al*., 1996).

Why hornworm larvae were not significantly affected by feeding on transgenic plants expressing the *Manduca* chitinase is unclear. Possibly, the concentration was too low, given that the specific activity of the truncated form is about one-fourth that of the full length enzyme (Wang *et al*., 1996), to negate inactivating mechanisms likely present in this homologous system.

Relatively few genes have proven to be sources of resistance to insects. Modified forms of the δ-endotoxin gene from *B. thuringiensis* confer resistance to certain insects when expressed in plants, and commercial releases of plants expressing *Bt* toxins have occurred (Koziel *et al*., 1993; Kaiser, 1996). However, some insects acquire resistance to the *Bt* toxin after continuous exposure to it (Regev *et al*., 1996; McGaughey and Whalon, 1992). Proteinase inhibitors have also been suggested as alternate insecticidal proteins. However, their insect specificity is narrow and protection against insect predation mediated by these inhibitors in transgenic plants has often been unsatisfactory (Gatehouse *et al*., 1993).

The *Manduca* or other insect chitinase transgenes, alone or in combination with other genes for insecticidal proteins, should receive further consideration for insect control. Because the *Manduca* chitinase enhances the effectiveness of the *Bt* toxin, it may have utility as a companion gene in potentiating the effectiveness or increasing the spectrum of the *Bt* gene for insect control with transgenics. A cultivar expressing a chitinase transgene might even be used to potentiate the action of a foliar *Bt* application, if concerns are warranted regarding the effects of full-season exposure to transgene-encoded *Bt* toxin on the development of insect resistance. Further exploration of the potential of the *Manduca* or

other chitinases in the enhancement of insect control on plants is warranted.

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References

Abel, P.P., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T. and Beachy, R.N. (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738–43.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1989) *Current Protocols in Molecular Biology.* New York: Greene Publishing Associates/Wiley Interscience.

Boulter, D., Edwards, G.A., Gatehouse, A.M.R., Gatehouse, J.A. and Hilder, V.A. (1990) Additive protective effects of different plant-derived insect resistance genes in transgenic tobacco plants. *Crop Protection* **9**, 351–4.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, J. and Broglie, R. (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani. Science* **254**, 1194–7.

Fuhrman, J.A., Lane, W.S., Smith, R.F., Piessens, W.F. and Perler, F.B. (1992) Transmission-blocking antibodies recognize microfilarial chitinase in brugian lymphatic filariasis. *Proc. Natl Acad. Sci. USA* **89**, 1548–52.

Gatehouse, A.M.R., Shi, Y., Powell, K.S., Brough, C., Hilder, V.A., Hamilton, W.D.O., Newell, C.A., Merryweather, A., Boulter, D. and Gatehouse, J.A. (1993) Approaches to insect resistance using transgenic plants. *Phil. Trans. R. Soc. Lond. B* **342**, 279–86.

Gopalakrishnan, B., Muthukrishnan, S. and Kramer, K.J. (1995) Baculovirus-mediated expression of a *Manduca sexta* chitinase gene: properties of the recombinant protein. *Insect Biochem. Mol. Biol*. **25**, 255–65.

Huber, M., Cabib, E. and Miller, L.H. (1991) Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. *Proc. Natl Acad. Sci. USA* **88**, 2807–10.

Jach, G., Görnhardt, B., Mundy, J., Logemann, J., Pinsdorf, E., Leah, R., Schell, J. and Maas, C. (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *Plant J.* **8**, 97–109.

Kaiser, J. (1996) Pests overwhelm *Bt* cotton crop. *Science* **273**, 423.

Koga, D., Funakoshi, T., Mizuki, K., Ide, A., Kramer, K.J., Zen, K.C., Choi, H. and Muthukrishnan, S. (1992) Immunoblot analysis of chitinolytic enzymes in integument and molting fluid of the silkworm, *Bombyx mori*, and the tobacco hornworm, *Manduca sexta. Insect Biochem. Mol. Biol*. **22**, 305–11.

Koga, D., Jilka, J., Speirs, R.D. and Kramer, K.J. (1983) Immunological relationships between β-*N*-acetylglucosaminidases from the tobacco hornworm, *Manduca sexta* (L.). *Insect Biochem*. **13**, 407–10.

Koziel, M.G., Beland, G.L., Bowman, C., Carozzi, N.B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwell, S., Launis, K., Lewis, K., Maddox, D., McPherson, K., Meghji, M.R., Merlin, E., Rhodes, R., Warren, G.W., Wright, M. and Evola, S.V. (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis. Bio/Technology* **11**, 194–200.

Kramer, K.J., Dziadik-Turner, C. and Koga, D. (1985) Chitin metabolism in insects. In Kerkut, G.A. and Gilbert, L.I. eds., *Comprehensive Insect Physiology, Biochemistry and Pharmacology Vol. 3*, pp. 75–115. New York, USA: Pergamon Press.

Kramer, K.J., Corpuz, L., Choi, H.K. and Muthukrishnan, S. (1993) Sequence of a cDNA and expression of the gene encoding epidermal and gut chitinases of *Manduca sexta. Insect Biochem. Mol. Biol*. **23**, 691–701.

Kramer, K.J., Muthukrishnan, S., Johnson, L. and White, F. (1996) Chitinases for insect control. In Carozzi, N. and Koziel, M. eds., *Advances in Insect Control: Transgenic Plants for the Control of Insect Pests*, pp. 185–93. Washington DC, USA: Taylor and Francis Publishers.

Lin, W., Anuratha, C.S., Datta, K., Potrykus, I., Muthukrishnan, S. and Datta, S.K. (1995) Genetic engineering of rice for resistance to sheath blight. *Bio/Technology* **13**, 686–91.

Masoud, S.A., Johnson, L.B., White, F.F. and Reeck, G.R. (1993) Expression of cysteine proteinase inhibitor (oryzacystatin-I) in transgenic tobacco plants. *Plant Mol. Biol*. **21**, 655–63.

McGaughey, W.H. and Whalon, M.E. (1992) Managing insect resistance to *Bacillus thuringiensis* toxins. *Science* **258**, 1451–5.

Morris, O.N. (1976) A 2-year study of the efficacy of *Bacillus thuringiensis*-chitinase combinations in spruce budworm (*Choristoneura fumiferana*) control. *Can. Entomol*. **108**, 225–33.

Peters, W. (1992) Peritrophic Membranes, *Zoophysiology* **30**. Berlin, Germany: Springer-Verlag.

Regev, A., Keller, M., Strizhov, N., Sneh, B., Prudovsky, E., Chet, I., Ginzberg, I., Koncz-Kalman, Z., Koncz, C., Schell, J. and Zilberstein, A. (1996) Synergistic activity of a *Bacillus thuringiensis* δ-endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. *Applied Environ. Microbiology* **62**, 3581–6.

Rogers, S.G., Klee, H.J., Horsch, R.B. and Fraley, R.T. (1987) Improved vectors for plant transformation: expression cassette vectors and new selectable markers. *Meth. Enzymol*. **153**, 253–77.

Ryerse, J.S., Purcell, J.P., Sammons, R.D. and Lavrik, P.B. (1992) Peritrophic membrane structure and formation in the larvae of a moth, *Heliothis. Tiss. Cell*. **24**, 751–71.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

SAS Institute. (1991) *SAS/STAT User's Guide, release 6.03*. Cary, NC, USA: SAS Institute.

Shapiro, M., Preisler, H.K. and Robertson, J.L. (1987) Enhancement of baculovirus activity on gypsy moth (Lepidoptera: Lymantriidae) by chitinase. *J. Econ. Entomol*. **80**, 1113–6.

Smirnoff, W.A. (1971) Effect of chitinase on the action of *Bacillus thuringiensis. Can. Entomol*. **103**, 1829–31.

Smirnoff, W.A. (1974) Three years of aerial field experiments with *Bacillus thuringiensis* plus chitinase formulation against the spruce budworm. *J. Invert. Pathol*. **24**, 344–8.

Sneh, B., Schuster, S. and Gross, S. (1983) Improvement of the insecticidal activity of *Bacillus thuringiensis* var. *entomocidus* on larvae of *Spodoptera littoralis* (Lepidoptera, Noctuidae) by addition of chitinolytic bacteria, a phagostimulant and a UV-protectant. *Z. Angew. Entomol*. **96**, 77–83.

Thomas, J.C., Adams, D.G., Keppenne, V.D., Wasmann, C.C., Brown, J.K., Kanost, M.R. and Bohnert, H.J. (1995a) *Manduca sexta* encoded protease inhibitors expressed in *Nicotiana tabacum* provide protection against insects. *Plant Physiol. Biochem*. **33**, 611–4.

Thomas, J.C., Adams, D.G., Keppenne, V.D., Wasmann, C.C., Brown, J.K., Kanost, M.R. and Bohnert, H.J. (1995b) Protease inhibitors of *Manduca sexta* expressed in transgenic cotton. *Plant Cell Rep*. **14**, 758–62.

Trudel, J., Potvin, C. and Asselin, A. (1995) Secreted hen lysozyme in transgenic tobacco: recovery of bound enzyme and *in vitro* growth inhibition of plant pathogens. *Plant Sci*. **106**, 55–62.

Vierheilig, H., Alt, M., Neuhaus, J.M., Boller, T. and Wiemken, A. (1993) Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase, by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae. Mol. Plant-Microbe Interact*. **6**, 261–4.

Wang, X., Ding, X., Gopalakrishnan, B., Morgan, T., Johnson, L., White, F.F., Muthukrishnan, S. and Kramer, K.J. (1996) Characterization of a 46 kDa insect chitinase from transgenic tobacco. *Insect Biochem. Mol. Biol*. **26**, 1055–64.

Wiwat, C., Lertcanawanichakul, M., Siwayapram, P., Pantuwatana, S. and Bhumiratana, A. (1996) Expression of chitinase-encoding genes from *Pseudomonas maltophila* in *Bacillus thuringiensis* subsp. *israelensis. Gene* **179**, 119–26.

Zen, K.C., Choi, H.K., Krishnamachari, N., Muthukrishnan, M. and Kramer, K. (1996) Cloning, expression, and hormonal regulation of an insect β-*N*-acetylglucosaminidase gene. *Insect Biochem. Mol. Biol*. **26**, 435–44.

Zhu, Q., Maher, E.A., Masoud, S., Dixon, R.A. and Lamb, C.J. (1994) Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. *Bio/Technology* **12**, 807–12.