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Protease inhibitors of *Manduca sexta* expressed in transgenic cotton

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Summary. To explore the effectiveness of insect derived protease inhibitors in protecting plants against insect feeding, anti-trypsin, anti-chymotrypsin and anti-elastase protease inhibitor (PI) genes from *Manduca sexta* L. were expressed in transgenic cotton (*Gossypium hirsutum* L.). From 198 independent transformants, 35 elite lines were further analyzed. Under the control of the 35S promoter of CaMV, PI accumulated to approximately 0.1% of total protein, depending on the tissue analyzed. Using cell-flow cytometry, DNA content/ nuclei of transgenic and non-transformed cotton were identical. On cotton plants expressing PIs, fecundity of *Bemisia tabaci* (Genn.), the sweetpotato whitefly, was reduced compared to controls. Expression of these protease inhibitors may reduce the developmental rate of *B. tabaci* and other insects, and provide a strategy for cotton protection.

Key Words: Protease inhibitor - Plant transformation

Introduction

Insects such as pink boll worm, cotton boll worm, and cotton boll weevil can cause up to a 50% cotton crop loss (Schwartz 1983) even when using chemical pesticides, pheromones, insect parasites, and modified cropping practices. Feeding damage, fiber contamination with honeydew and whitefly transmitted viral diseases have significantly contributed to the loss of cotton (Brown and Bird 1992), requiring new methods of control without increasing the use of pesticides. One approach to insect control is to express the anti-insect compound within transgenic cotton. Using this strategy, expression of *Bacillus thuringiensis* (Bt) toxins have protected cotton against certain lepidoptera (Perlack et al. 1990).

In most plants, natural mechanisms to control insect attack include protease inhibitor (PI) accumulation (Ryan 1973). Addition of PIs to insect diets have been shown to limit insect development (Gatehouse et al. 1980; Broadway and Duffy 1986a). Thus, the over-expression of PI's in transgenic plants may decrease insect damage (Hilder et al. 1987; Johnson et al. 1989).

Protease inhibitor genes isolated from *M. sexta* have been expressed in transgenic alfalfa and tobacco to test their use in insect control (Thomas et al. 1995; unpublished). A cDNA encoding an anti-elastase protease inhibitor was described previously (Kanost et al. 1989). PCR-based *in vitro* mutagenesis was used to alter the active site to generate chymotrypsin-specific or trypsin-specific inhibitors. The three genes were placed under the control of two promoters, the 35S promoter of CaMV or the phospho*enol*pyruvate carboxylase (*Ppc-1*) of *Mesembryanthemum crystallinum*, and then transferred into cotton. Here we report the expression of these protease inhibitors and decreased pupae emergence of *Bemisia tabaci* (strain B) on the transgenic plants compared to controls.

Materials and methods

Cloning and manipulations. A full length cDNA encoding the anti-elastase protease inhibitor was N-terminally extended to include a second initiation ATG and 5 subsequent amino acids from the transit peptide of the RUBISCO small subunit resulting in a protein of an approximate M_r of 47 kD (Kanost et al. 1989; Thomas et al. 1995). Anti-chymotrypsin and anti-trypsin enzyme activity was predicted when the ALA at position amino acid number 343 was changed to PHE or LYS respectively (see Kanost et al. 1989). The P1 site from anti-elastase (GGTATCGTACCGGCGAGTTTGATACTA) was converted to anti-chymotrypsin (GGTATCGTACCGTTTAGTTTGATACTA) and anti-trypsin (GGTATCGTACCGAAGAGTTTGATACTA) using PCR-based oligonucleotide-directed mutagenesis according to Hemsley (et al. 1989). Promoters used were the 35S (Benfey et al. 1990) or double enhancer 35S promoter from CaMV (Guerineau et al. 1988) and the phospho*enol* pyruvate carboxylase promoter from *M. crystallinum* (Cushman et al. 1989) with the 3' 19S polyA site from CaMV. Using standard molecular methods, DNA constructions were placed into a plant transformation vector (Bevan 1984), subsequently mated into *Agrobacterium tumefaciens* LBA4404 and verified using Southern hybridization.

Plant transformation/regeneration. A. tumefaciens-mediated gene transfer into *Gossypium hirsutum* L. var. Coker 312 used adaptations

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of existing methods (Umbeck et al. 1987; Firoozabady et al. 1987; Bayley et al. 1992). Seeds were surface sterilized in 70% (v/v) ethanol for 30 s, soaked for 15 min in 10% (v/v) Clorox solution containing 1 drop of Tween 20 per 250 ml, washed three times, soaked in sterile water 30-60 min and germinated on G1 medium (hormone-free MS medium containing 3% (w/v) glucose) at 25°C, 16 h light, 30-50 µ moles PPFD $m^{-2}s^{-1}$. Cotyledons from 3-14 day old plants were sliced into 5 X 2 mm segments and co-cultivated in approximately 10^8/ml *A. tumefaciens* on G1 medium. After 3 days, explants were placed on G2 medium (MS medium with 100 mg/L inositol, 1 µM thiamine HCl, 25 µM 6-(γ-γ-dimethylallylamino)-purine (2iP), 0.5 µM naphthaleneacetic acid (NAA), 3% w/v glucose, pH 5.8 in 0.2% Gelrite (Kelko) with 15-50 mg/L kanamycin and 400 mg/L carbenicillin at 30°C, 16h light and 100 µ moles PPFD $m^{-2}s^{-1}$. Two to four week old calli were subcultured to identical media, then subcultured one month later to MSOB medium (MS salts, Gamborg's B-5 vitamins, additional 500 mM KNO_3, 2mM $MgSO_4$ and 0.16% Gelrite supplemented with 200 mg/L carbenicillin and 50 mg/L kanamycin). Arising embryos were individually placed on MSBO medium, the shoots propagated on MSBO medium without antibiotics, rooted on MS3 medium (MS salts, 0.4 µM thiamine HCL, 0.5 µM pyridoxine HCL and 0.8 µM nicotinic acid, 1% glucose, 0.8 g/L Gelrite and 4 g/L Difco Bacto agar), transferred to pots, grown and selfed in a greenhouse. Segregation of the T-1 generation was screened using cotyledon-derived callus formation on G2 medium containing 50 µg/ml kanamycin as a criteria for transformation.

DNA pg/nuclei. Analysis of the nuclear DNA with cell-flow cytometry was done according to Galbraith et al. (1983).

Western analysis. Transformed and non-transformed leaves, cotyledons and other tissues were extracted in 100 mM Tris HCl, pH 8, 100 mM NaCl, 20 mM EDTA, 10 mM DTT. Total protein was quanitated (Ghosh et al 1988), separated on 12.5% SDS-PAGE, blotted to nitrocellulose (0.45 µm), reacted with rabbit antiserum made to purified anti-elastase of *M. sexta*, and the PI band located with a secondary goat-anti-rabbit antibody conjugated to peroxidase (Sigma). Estimates of PI accumulation were based on western blot comparisons of transgenic plant extracts to several dilutions of semi-purified PI from *M. sexta* hemolymph (Kanost et al 1989; Thomas et al 1995).

Whitefly protease assay. To examine whether *B. tabaci* contained detectable protease activity, three individual lots of 125 adults were extracted in 10 mM Tris pH 7.5 and 50 mM NaCl and reacted at 25°C with N-α-benzoyl-DL-arginine p-nitroaniline (Sigma). Crude whitefly extracts contained 1.61 +/- 0.54 units of trypsin activity mg^{-1} h^{-1} total protein in comparison to 2.40 +/- 0.32 units mg^{-1} h^{-1} total protein found in *Drosophilia melanogaster* adults. Dissection and subsequent characterization of the small whitefly digestive system was not attempted.

Whitefly toxicity/aversion bioassay. To test insecticidal activity of the PIs from *M. sexta*, whitefly bioassays were performed on plants expressing or not expressing different PI proteins. Cotton plants were approximately 4 weeks olds when testing began. Growth conditions were similar to those described above, 28°C, 16h light and 100 µ moles PPFD $m^{-2}s^{-1}$. Fourty adults (20 of each sex) were surrounded by a plexiglass cylinder with 250 µm netting on T-1 selfed cotton plants (each plant was tested in triplicate). Adult insects were allowed to mate, deposit eggs, and were removed after 3 days. Following an incubation period of 25-30 days, pupae cases were counted.

Results and Discussion.

Transgenic plants

Cotton transformation/regeneration procedures were largely as described (Firoozabady et al. 1987). Selection for transformed cotton calli on 50 µg/ml kanamycin produced low rates of non-transformed (escape) plants compared to 15-25 µg/ml kanamycin, where 95% of the plants recovered were not transformed. Transformed tissues grew at a rate comparable to non-transformed cells on G2 medium without antibiotics. Once embryogenic calli emerged, 500 mm^2 pieces were subcultured repeatedly until somatic embryos were obtained. Embryos were subcultured to MSBO medium with 50 µg/ml kanamycin and shoots developed, which were subsequently rooted and hardened off in soil. Thirty five elite lines were further analyzed from 198 independent transformants, their selection based on PI expression and the ability to develop vigorous roots, thrive in soil and reproduce (self) easily. T-1 segregation indicated 80% (28) of the selected transgenic cotton segregated as a single PI-kanamycin resistance locus, while others (7) contained greated than 1 loci. Transformed and regenerated cotton plants had DNA contents indistinguishable from those found in non-transformed cotton; 4.39 +/- 0.17 pg/nuclei (N=15) versus 4.30 +/- 0.07 pg/nuclei (N=3) respectively.

Protease inhibitor expression

A large variation in the expression of transgenic genes has been observed when gene expression was under the control of the 35S promoter (Williamson et al 1989). Most (74%) of the plants containing the 35S promoter with the PI constructions were kanamycin resistant and also accumulated PI, as detected on western blot analysis. Generally, anti-trypsin and anti-chymotrypsin PI accumulated to higher amounts than anti-elastase protein as shown by immunological detection (Figure 1A, 1B). Apart from a protein migrating at the expected size of the fusion protein, approximately 48 kDa, a slightly faster migrating band was observed, perhaps the result of proteolysis or initiation at the ATG codon of the native *M. sexta* PI gene (see Material and Methods). In addition, bands corresponding to higher apparent molecular mass were observed in plants that expressed large amounts of PI which may represent PI aggregates (Figure 1B, anti-trypsin lane 4).

Gene expression also varied depending on the tissue source. An often observed, or "typical" tissue-specific expression pattern for the PI proteins expressed under control of the 35S promoter is shown in Figure 2. Mature leaves and bracts contained the greatest amount of PI, with concentrations up to 0.1% of total protein (Figure 2).

We tested two plant promoters, 35S of CaMV (both the single and double enhancer version) and *Ppc-1* from *M. crystallinum* for expression of the PIs. Both 35S promoter types led to similar levels of PI accumulation as determined by antibody detecting PI protein on a western blot (see Figure 3). *Ppc-1* in *M. crystallinum* was induced by environmental stress (Cushman et al. 1989) and in this species was able to

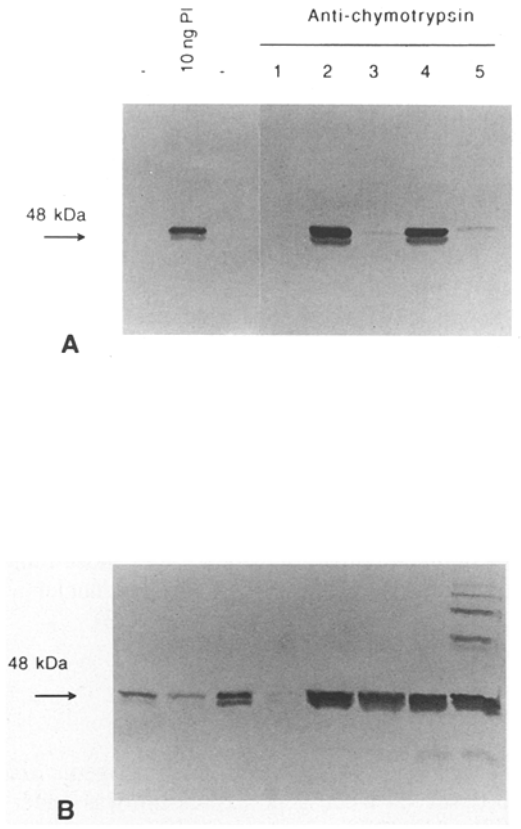

Fig. 1. Immunological detection of anti-elastase, anti-chymotrypsin and anti-trypsin in cotton plants. A. Ten nanograms of semi-purified anti-elastase from *M. sexta* serum were separated with SDS-PAGE, blotted to nitrocellulose and reacted with the rabbit anti-PI antibody (10 ng PI). Thirty micrograms of total leaf protein from different 35S-anti-chymotrypsin plants (lanes 1-5) were separated, reacted with anti-PI antibody and compared to the intensity of the antibody reaction for 10 ng PI. Detection was with goat anti-rabbit 2° antibody coupled to peroxidase. Arrow indicates a protein of molecular mass 48 kDa. B. Lanes 1-4 are from different 35S-anti-elastase plants and lanes 5-8 are 35S-anti-trypsin plants (1-4). Antibody and gel conditions were as in A.

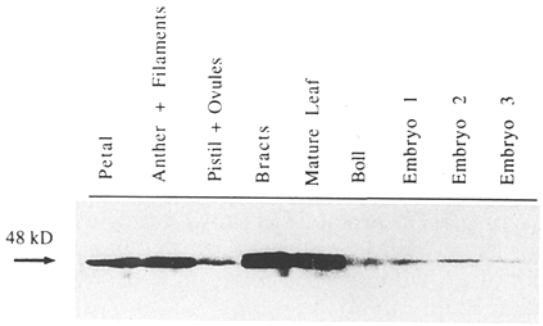

Fig. 2. Tissue specific accumulation of anti-elastase under the control of the 35S promoter of CaMV. 25 µg of total protein from each tissue source was separated on SDS-PAGE, blotted and reacted with anti-PI antibody as in Figure 1. This "typical" transgenic plant was a 35S-anti-elastase plant. Similar patterns of expression were observed in 4 other plants.

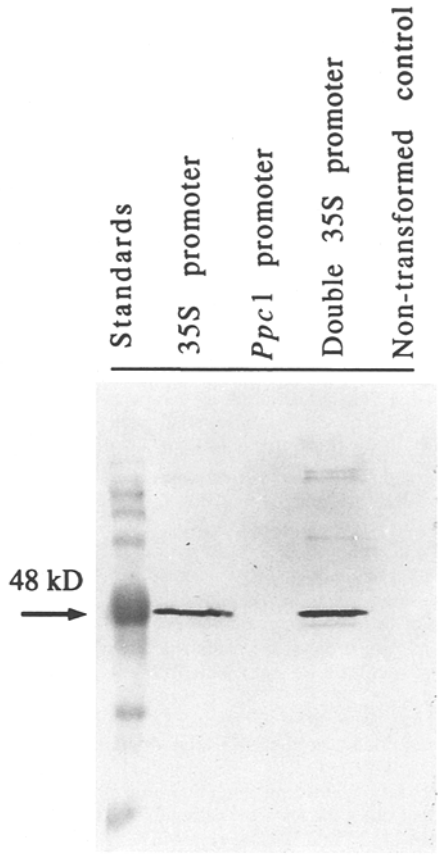

Fig. 3. Effect of promoter on PI accumulation in transgenic cotton. Leaf total protein (20 µg) of several transgenic plants was separated and reacted to the anti-PI antibody as in Figure 1 and 2.

stimulate expression of a reporter gene (Schaeffer and Cushman 1994). However, when used in transgenic cotton, the *Ppc-1* promoter did not lead to PI accumulation either when plants were kept at optimal conditions, or under stress conditions (Figure 3; data not shown). This finding was similar to results obtained in *Medicago sativa* L. (alfalfa) and *Nicotiana tabacum* (tobacco) (Thomas et al. 1995; unpublished). No further studies were conducted with these *Ppc-1* gene constructions. It is known that promoters which function well in one species may be less effective in another in inducing transcription of foreign proteins (Narvaéz-Vásquez et al. 1992).

Whitefly bioassay activity

From two separate experiments non-transformed cotton contained 224 +/- 30 pupae cases, while an anti-trypsin (#114) and an expressing anti-elastase transformant (#37) had reduced numbers of emerged whiteflies (Figure 4). An anti-elastase non-expressing transformant (#125) allowed emergence of pupae similar to controls while an anti-chymotrypsin plant #34 inhibited whitefly development compared to controls (Figure 4). Four T-1 plants from each transgenic line were tested in each experiment.

Protease inhibitors arrest digestive enzymes in the insect gut (Ryan 1973). When insects feed, plant-

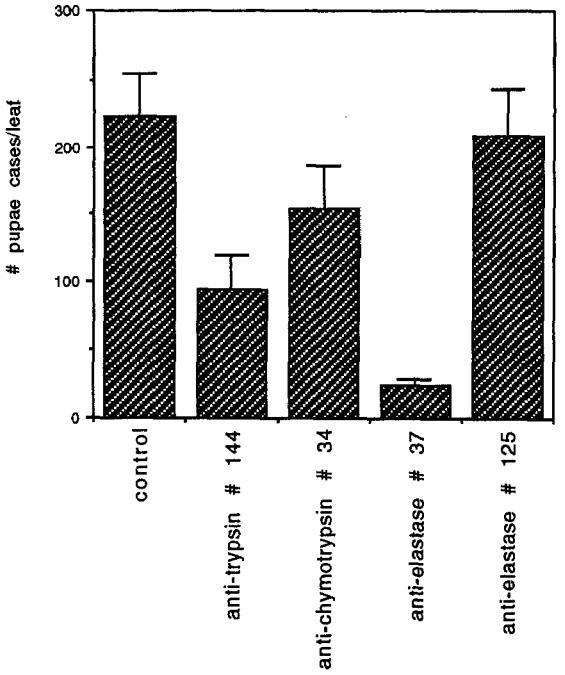

Fig. 4. Adult whitefly *B. tabaci* type B emergence from PI expressing cotton. Fourty adults (20 of each sex) were placed on leaves of T-1 selfed cotton plants and adults allowed to mate and deposit eggs. After 30 days, emerged pupae were counted on the plants. Plant number indicates an individual and unique transformation event. Controls were non-transformed plants.

encoded PI's are accumulated in non-damaged areas of the plant near the feeding site (Green and Ryan 1972), resulting in a decrease in value as a food source (Broadway et al. 1986a; 1986b). Over-expression of introduced PIs has been shown to protect plants against damage caused by some insects (Hilder et al. 1987; Johnson et al. 1989). Some PI's are more effective when presented to insects in a low protein diet, while other inhibitors act to retard insect growth in either high or low protein-containing artificial diets (Burgess et al. 1991). Diets containing trypsin inhibitors reduced growth rate of *Heliothis zea*, but measurable trypsin activity in the insect gut increased (Broadway and Duffy 1986b; Burgess et al. 1991). PI mediated hyperproduction of digestive enzymes could result in decreased concentrations of sulfur containing amino acids, thus indirectly inhibiting insect growth (Ryan 1990).

Expression of *Manduca sexta* PIs in transgenic alfalfa, tobacco and cotton (Thomas et al. 1995; unpublished) was associated with decreased whitefly adult emergence when compared to control plants. PI expression varied from tissue to tissue, suggesting that tissue specific expression of PI's will be a goal to increase effectiveness against insect feeding. Such an approach would be particularly effective if boll-specific and/or epidermis specific promoters were employed. In addition, protease inhibitors could be expressed in transgenic plants together with other anti-insect substances with different modes of action within the same transgenic crop, such as *B. thuringensis* toxin (MacIntosh et al. 1990). This combinatorial approach would lower the incidence of insect resistance to individual insecticides.

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