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Accumulation of a chymotrypsin inhibitor in transgenic tobacco can affect the growth of insect pests

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A member of the potato proteinase inhibitor II (*PPI* II) gene family that encodes for a chymotrypsin iso-inhibitor has been introduced into tobacco (*Nicotiana tabacum*) using *Agrobacterium tumefaciens*-mediated T-DNA transfer. Analysis of the primary transgenic plants (designated R_0) confirmed that the introduced gene is being expressed and the inhibitor accumulates as an intact and fully functional protein. For insect feeding trials, progeny from the self-fertilization of R_0 plants (designated R_1) were used. Leaf tissue, either from transgenic or from control (non-transgenic) plants, was fed to larvae of *Chrysodeixis eriosoma* (Lepidoptera: Noctuidae, green looper), *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) and *Thysanoplusia orichalcea* (F.) (Lepidoptera: Noctuidae) and insect weight gain (increase in fresh weight) measured. Consistently, *C. eriosoma* larvae fed leaf tissue from transgenic plants expressing the *PPI* II gene grew slower than insects fed leaf tissue from non-transgenic plants or transgenic plants with no detectable *PPI* II protein accumulation. However, larvae of both *S. litura* and *T. orichalcea* consistently demonstrated similar or faster growth when fed leaf tissue from transgenic plants compared with those fed non-transgenic plants. In agreement with the feeding trials, the chymotrypsin iso-inhibitor extracted from transgenic tobacco effectively retarded chymotrypsin-like activity measured in *C. eriosoma* digestive tract extracts, but not in extracts from *S. litura*. We conclude, therefore, that for certain insects the use of chymotrypsin inhibitors should now be evaluated as an effective strategy to provide field resistance against insect pests in transgenic plants, but further, that a single proteinase inhibitor gene may not be universally effective against a range of insect pests. The significance of these observations is discussed with respect to the inclusion of chymotrypsin inhibitors in the composite of insect pest resistance factors that have been proposed for introduction into crop plants.

Keywords: chymotrypsin inhibitor; transgenic tobacco; insect pest resistance; potato proteinase inhibitor II

Introduction

The potential use of proteinase inhibitors to protect crop plants against insect damage has been recognized since the earliest demonstration of the potency of these proteins at repressing the activity of proteinases from the insect digestive tract (Gatehouse and Boulter, 1983; Broadway and Duffy, 1986; Wolfson and Murdock, 1987). With the advent of techniques to introduce foreign genes into plants (Hernalsteens *et al.*, 1980; Herrera-Estrella *et al.*, 1983), the use of these proteins to confer resistance against insect pests to the transformants was one of the very earliest applications. Hilder *et al.* (1987) introduced a cowpea trypsin inhibitor gene into tobacco and observed that higher levels of inhibitor accumulation correlated with a decreased survival percentage in feeding *Heliothis virescens* (Lepidoptera: Noctuidae) larvae. In a more recent study (Johnson *et al.*, 1990), transgenic tobacco expressing genes that encode for either potato or tomato proteinase inhibitor II (dual trypsin and chymotrypsin inhibitors), depressed growth rates of feeding *Manduca sexta* (Lepidoptera: Sphingidae) larvae, when compared with larvae fed on control (non-transgenic) tissue. Conversely, no depression of growth rate was observed for larvae fed on transgenic tobacco expressing a member of the tomato inhibitor I gene family (predominantly a chymotrypsin inhibitor). The authors concluded that inhibitory activity against trypsin, but not chymotrypsin,

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was mainly responsible for the depressed larval growth rates they observed.

To our knowledge, these are the only two published reports that demonstrate the use of proteinase inhibitor genes solely to confer resistance against insect pests to transgenic plants. However, both studies report that trypsin inhibitors are effective, and for *M. sexta* at least, chymotrypsin inhibitors are not.

Nevertheless, as part of a longer-term strategy to produce plants with enhanced resistance to specific insect pests, we have sought to evaluate exclusively chymotrypsin inhibitors as potential resistance factors. To achieve this, we have introduced a member of the potato proteinase inhibitor II gene family (Keil *et al.*, 1986) into *Nicotiana tabacum* L., as a transcriptional fusion with the 35S promoter from cauliflower mosaic virus. This gene encodes for an iso-inhibitor with predominantly chymotrypsin, rather than trypsin, inhibitory activity (McManus *et al.*, 1990). Although *H. virescens* and *M. sexta* have not been recorded in New Zealand, one important pest of solanaceous species, *Chrysodeixis eriosoma*, is prevalent (Roberts, 1979). This paper reports results from feeding trials to evaluate the use of a chymotrypsin inhibitor as an effective defensive agent against *C. eriosoma*, and also against two closely related plusiine noctuids, *Spodoptera litura* and *Thysanoplusia orichalcea* (Hill *et al.*, 1987; Hill, 1989).

Materials and methods

Vector construction and plant transformation

A transcriptional fusion between the 35S promoter from cauliflower mosaic virus, and the coding region and the 3′ end of a potato proteinase inhibitor II gene (Keil *et al.*, 1986) cloned into vector pmpk 110 was kindly supplied by Professor J. Schell, Max-Planck-Institut, Germany. To construct pDW1 (Fig. 1), a 2.7 kb *Eco* RI restriction fragment containing the 35S:*PPI* II gene fusion was cloned into the unique *Eco* RI site of the plant transformation binary vector, pBin 19 (Bevan, 1984). pDW1 was mobilized from *Escherichia coli* strain JM 83 into the disarmed *Agrobacterium tumefaciens* strain, LBA 4404, using a triparental mating procedure (Ditta *et al.*, 1980) utilizing the helper-plasmid, pRK 2013.

Tobacco plants were transformed using a leaf disc method (Horsch *et al.*, 1985). Once regenerated plantlets were about 5 cm high, they were removed from tissue culture and transplanted into compost. When plants reached a height of about 25 to 40 cm, with vigorous vegetative growth (and before flowering), they were used for molecular analysis and designated primary (R_0) transgenic plants. These plants were self-fertilized and the progeny plants (designated R_1) used for insect feeding trials.

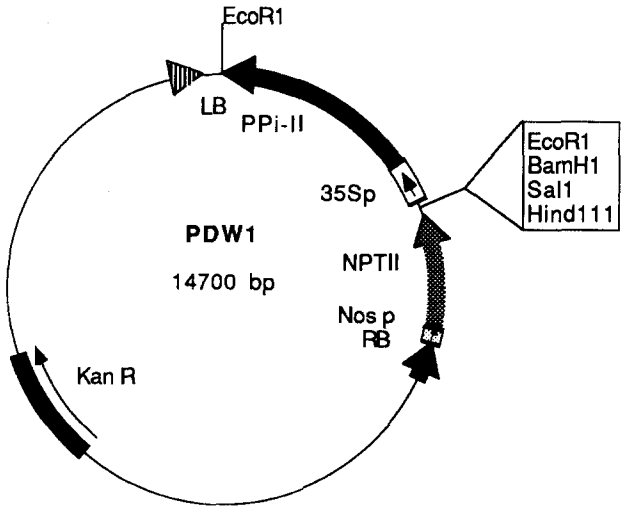

Fig. 1. The plant binary transformation vector, pDW1. LB = Left border; PPI II = potato proteinase inhibitor II coding region; 35Sp = cauliflower mosaic virus 35S promoter; NPTII = neomycin phosphotransferase II; Nos p = nopaline synthase promoter; RB = right border; Kan R = kanamycin resistance.

Nucleic acid extractions

To isolate DNA, tobacco leaf tissue was frozen in liquid nitrogen, powdered and extracted in 100 mM Tris-HCl, pH 8.0, containing 50 mM EDTA and 1.0% (w/v) SDS (1.0 g fresh weight: 3.0 ml extraction buffer). The resultant slurry was extracted twice with phenol, once with ether and the DNA precipitated with 10% (w/v) PEG (MW = 6000) after the addition of NaCl to a final concentration of 500 mM. The precipitated DNA was resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0 (TE buffer), treated with RNase (50 μg ml) at 37°C for 30 min, extracted with phenol/chloroform and finally precipitated with two volumes of 95% (v/v) ethanol, at −20°C, overnight. After washing the pellet with 70% (v/v) ethanol and then 95% (v/v) ethanol, the DNA was resuspended in sterile water and the yield quantified using standard procedures (Maniatis *et al.*, 1982).

To isolate RNA, all glassware used in the procedure was baked at 180°C, and all solutions made up with sterile water before autoclaving. Wherever possible, each step was performed on ice, using ice-cold solutions, and centrifugation carried out at 4°C. RNA was isolated by extracting frozen and powdered leaf tissue with 4 M guanidine isothiocynate in 25 mM sodium citrate, pH 7.0, containing 0.5% (w/v) sodium lauryl sarcosine and 0.7% (v/v) 2-mercaptoethanol. The slurry was centrifuged at 10000 **g** for 15 min, the supernatant acidified with 0.0025 volumes of glacial acetic acid, and the nucleic acid precipitated with 0.5 volumes of ethanol at −20°C. The precipitate was resuspended in 7 M urea in 100 mM Tris-HCl, pH 8.5, containing 0.1 mM EDTA and 0.1% (w/v)

SDS, extracted twice with phenol/chloroform and after ethanol precipitation, RNA was selectively precipitated by the addition of 2 volumes of 4.5 M sodium acetate, pH 5.0. This procedure was repeated and the salt removed by two cycles of ethanol precipitation. The RNA was resuspended in sterile water before use, and the yield quantified using standard procedures (Maniatis *et al.*, 1982).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Potato proteinase inhibitor II was partially purified from transgenic tobacco leaves by extracting leaf tissue with 50 mM Tris-HCl, pH 7.5, containing 25 mM 2-mercaptoethanol, and adjusting the pH of the supernatant after centrifugation to pH 3.0. After centrifugation of the acidified extract, the supernatant was adjusted to pH 7.5, the eluate incubated at 80°C for 10 min, centrifuged and the supernatant used for SDS-PAGE. Non-transgenic tissue was subjected to the same extraction protocol.

Purified extracts were reduced in Laemmli buffer (Laemmli, 1970) and then separated by electrophoresis through a 15% polyacrylamide gel (c = 2.7%), containing 0.1% (w/v) SDS, using 25 mA constant current at room temperature.

Western blotting was performed essentially as described by Towbin *et al.* (1979), and after transfer, the nitrocellulose sheet was blocked for 2 h at room temperature with a solution of 13.5% (w/v) low fat milk powder (Anchor Products, NZ Ltd, New Zealand), in 50 mM sodium phosphate buffer, pH 7.4, containing 250 mM NaCl (PBSalt), incubated with a polyclonal antibody raised in rabbits against purified *PPI* II and diluted 1:1000 in PBSalt, and then with PBSalt containing 2.5 μCi of ^{125}I-labelled F(ab')$_2$ fragment of donkey-anti-rabbit Ig (affinity purified, Amersham, UK). After exhaustive washing, the sheet was air dried and antibody binding detected by autoradiography. Pre-immune IgG was used as a control. It did not bind to any polypeptides which transferred onto the nitrocellulose.

Enzyme-linked-immunosorbent assay (ELISA)

To quantify the accumulation of *PPI* II in transgenic plants, the primary ELISA method (Johnstone and Thorpe, 1982) was used. Horseradish peroxidase was first coupled to purified anti-*PPI* II IgG using *N*-succinimidyl-3-(2-pyridyldithic) propionate (SPDP, Pharmacia) using the protocol provided with the product.

For ELISA, leaf tissue was extracted with 50 mM Tris-HCl, pH 7.5, containing 25 mM 2-mercaptoethanol, the slurry centrifuged at 10000 g for 5 min and the supernatant assayed. Anti-*PPI* II IgG was diluted to a final concentration of 10 μg ml^{-1} and 1.0 μg of this dilution coated onto flat-bottom 96-well plastic plates (Nunc, Gibco-BRL), the coated wells were blocked with a 0.5% solution of low-fat milk powder in PBSalt, and then coated with serial dilutions of tobacco leaf extracts diluted in PBSalt. To quantify antigen binding, peroxidase-linked-anti-*PPI* II IgG was coated, the unbound antibody washed off and bound enzyme detected using 0.05% (w/v) *o*-phenylenediamine, 0.03% (v/v) H_2O_2 in 20 mM sodium acetate, pH 5.0, as substrate. The reaction was stopped with the addition of 3 M H_2O_4 and the absorbance of each well read at 492 nm and 620 nm. The amount of *PPI* II in each extract was quantified from an internal standard curve using purified *PPI* II protein.

Northern and Southern blotting

Total RNA was reduced in the presence of 2.2 M formaldehyde, separated through a 1% (w/v) agarose gel in 50 mM MOPS, pH 7.0/1.0 mM EDTA, pH 7.5, using the gel buffer as running buffer. For Southern blotting, predigested DNA was separated through a 0.8% (w/v) agarose gel in 40 mM Tris-acetate, 2 mM EDTA, pH 8.0, using the gel buffer as running buffer. Transfer onto nitrocellulose, and subsequent treatments were performed as standard procedures (Maniatis *et al.*, 1982). Specific details of hybridization are included in the appropriate figure legends.

Insect feeding trials

Progeny plants, from self-fertilized primary transgenic plants that were accumulating the *PPI* II protein, were used in insect feeding trials. Progeny were selected by first confirming the accumulation of *PPI* II protein in each progeny plant using ELISA, and by chymotrypsin inhibition assay using *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTpNA) as substrate (Christeller *et al.*, 1989). The levels determined for each plant assayed are displayed in Fig. 4. Larvae of *C. eriosoma* and *T. orichalcea* were reared from eggs laid by light-trapped adults, and provided with artificial diet (Singh, 1983) until the second stadium. *S. litura* larvae from an insectary culture were reared on a lima bean-based diet (P. Wigley, personal communication). Insects were reared and feeding experiments conducted at 20°C ± 2°C and 16 h light. For each feeding trial, second instar larvae were used with each insect receiving a 4–6 cm^2 section of tobacco leaf, dissected from the appropriate leaf and avoiding the main veins. Larvae were examined daily, weighed every two days and then provided with fresh leaf sections.

Plant effects on live weight gain were analysed by ANCOVA with initial weight as a covariate. Differences between means were evaluated using Tukey's HSD, modified when necessary for unequal sample sizes (Spjotvoll and Stoline, 1973). When the assumptions of the analysis were violated and could not be corrected by an appropriate transformation, the data were analysed by Kruskal-Wallis non-parametric ANOVA. In these cases, means were compared using Mann-Whitney U-tests with

the overall (experiment-wise) error rate maintained at $\alpha = 0.05$ by the Bonferroni procedure.

Proteinase activity and inhibition assays

To assay proteinase activity, insect guts were isolated from second instar larvae after they had been fed control tobacco leaf tissue for seven days. Pooled guts were homogenized in 100 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, and the slurry centrifuged for 5 mins at 12000 g. To detect enzyme activity, aliquots of extract were diluted in 100 mM Tris-HCl, pH 8.0 (assay buffer), and *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (for elastase/chymotrypsin), N-benzoyl-L-arginine *p*-nitroanilide (BApNA, for trypsin), and *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTpNA, for chymotrypsin) were used as substrates at a final concentration of 4 mM. The total assay volume was 250 µl, and the rate of reaction monitored at 415 nm using a BioRad (Model 3550) Microplate reader.

For enzyme inhibition, partially purified *PPI* II (prepared as described in the SDS-PAGE and western blotting protocol) was chromatographed through a Superdex G-75 (Pharmacia, Australia, Pty Ltd, Australia) column, equilibrated with 100 mM Tris-HCL, pH 8.0. Fractions that demonstrated both chymotrypsin inhibition and binding by anti-*PPI* II IgG were pooled. Similarly treated extracts from control (non-transgenic) plants did not show any chymotrypsin inhibition or recognition by the *PPI* II antibody. To assay for chymotrypsin and trypsin inhibition, increasing amounts of the partially purified inhibitor were directly substituted in place of assay buffer in the proteinase activity assays with the final reaction volume maintained at 250 µl.

Results

Potato proteinase inhibitor II gene integration and expression in transgenic tobacco

In the Southern blot, the *PPI* II gene coding sequence hybridized to a 2.7 kb fragment (the predicted size of the combined coding region and the 35S promoter) in *Eco* RI-digested genomic DNA isolated from primary (R_0) transgenic tobacco plants (Fig. 2A). There were apparent differences between plants in the intensity of hybridization by the probe, despite equal DNA loadings being applied to each lane. However, to determine if this observed variation is a consequence of differences in the copy number or arrangement of the introduced gene, further Southern analysis would need to be undertaken using additional T-DNA probes and restriction endonuclease digestions of genomic DNA. No hybridization of the *PPI* II gene probe was observed to *Eco* RI-digested DNA extracted from control (non-transgenic) plants.

Northern analysis detected a single mRNA species of about 800 bp which was the predicted size of the fully processed *PPI* II gene transcript (Fig. 2B). Although

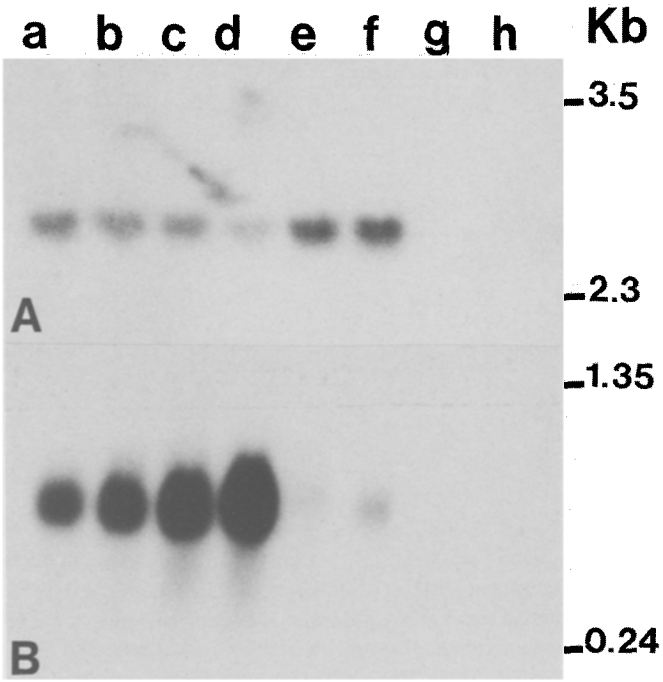

Fig. 2. Southern and Northern analysis of leaf tissue from primary (R_0) transgenic or control (non-transgenic) tobacco. A. Extracted DNA was digested with *Eco* RI, 10 µg separated through a 0.8% (v/v) agarose gel and then transferred onto nitrocellulose. The separated DNA was probed with the ^{32}P-labelled isolated *PPI* II gene coding sequence, the membrane washed with 2 × SSC and then with 0.2 × SSC, at 42°C, and hybridization detected by autoradiography. B. Extracted RNA was reduced with 2.2 M formaldehyde, 30 µg separated through a 1.0% (v/v) agarose gel and transferred onto nitrocellulose. The separated RNA was probed with the ^{32}P-labelled isolated *PPI* II gene coding *KpnI/Eco* RI digested sequence, the membrane washed with 2 × SSC and then with 0.2 × SSC, at 42°C, and hybridization detected by autoradiography. a = plant 1, b = plant 6, c = plant 12, d = plant 13, e = plant 4, f = plant 7, g,h = non-transgenic tobacco.

equal RNA loadings were applied to each lane, there were again differences between plants in the intensity of hybridization suggesting variation in the abundance of *PPI* II mRNA. No transcripts were detected by the probe in RNA extracted from control (non-transgenic) plants.

Western analysis and quantification of potato proteinase inhibitor II expression

A polyclonal antibody raised against purified *PPI* II identified, by western blotting, a single polypeptide of about 15 kDa in separated partially purified leaf extracts from primary transgenic plants (Fig. 3). This polypeptide co-migrated with purified *PPI* II, confirming that the inhibitor did not undergo unscheduled post-translational modification when expressed as a foreign protein in tobacco. The molecular weight of the protein (about

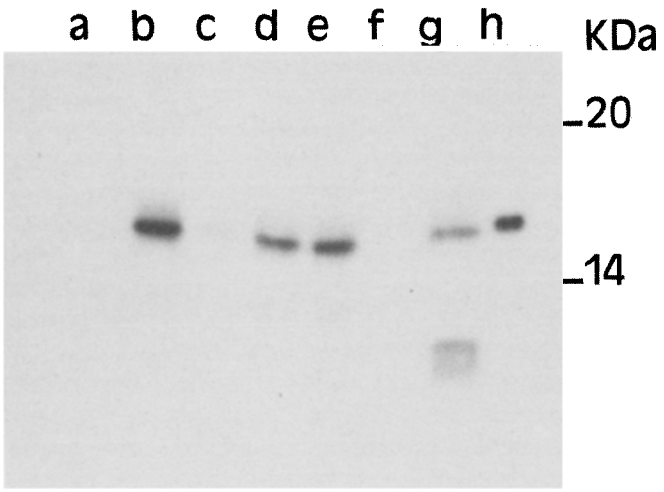

Fig. 3. SDS-PAGE and western blotting of protein extracts of leaf tissue from primary (R_0) transgenic or control (non-transgenic) tobacco. Potato proteinase inhibitor II (*PPI* II) was partially purified from transformed (and control) tobacco leaf tissue, and separated through a 15% polyacrylamide gel, containing 0.1% (w/v) SDS. The separated polypeptides were transferred onto nitrocellulose, probed with anti-*PPI* II IgG and then ^{125}I-labelled anti-rabbit Ig and recognition detected using autoradiography. a = non-transgenic tobacco, b = plant 1, c = plant 7, d = plant 12, e = plant 13, f = plant 4, g = plant 6, h = purified *PPI* II protein.

15 kDa) was higher than the reported subunit molecular weight of 10.5 kDa (Bryant *et al.*, 1976), although the lesser value was determined using phosphocellulose chromatography in the presence of 8 M urea. It is probable, therefore, that this polypeptide will behave differently when separated using SDS-PAGE. In some plants, a smaller peptide was also recognized by the antibody (for example plant 6, Fig. 3). The *N*-terminal of this peptide is homologous to a 5.4 kDa chymotrypsin inhibitor (McManus *et al.*, 1993) that has been identified in potato and designated as PCI-1 (Pearce *et al.*, 1982). The amino acid sequence of PCI-1 is entirely homologous to an internal region within the mature *PPI* II protein (Garcia-Olmedo *et al.*, 1987).

There are apparent anomalies between the level of mRNA expression of *PPI* II obtained in plants 1, 6, 12 and 13 (Fig. 2B) and the intensity of antibody recognition detected by western blotting (Fig. 3). However, neither northern analysis or western blotting is truly quantitative. To establish a correlation accurately, RNA dot blotting (with the appropriate internal standards) would need to be used and compared directly with protein levels obtained using ELISA on extracts from identical plant material.

The accumulation of *PPI* II protein in primary transgenic plants was quantified using an indirect ELISA procedure, with purified potato proteinase inhibitor II as an internal standard. For most plants assayed, *PPI* II protein expression levels were between 0.010–0.125% of total leaf soluble protein (data not shown), while progeny (R_1) plants selected for insect trials routinely accumulated *PPI* II in the range of 0.1% to 1.0% of total soluble protein. These values are within the range reported for cowpea trypsin inhibitor (CPTI) accumulation in transgenic tobacco (Hilder *et al.*, 1987) where the proteinase inhibitor gene is also expressed from the 35S promoter.

The use of ELISA in this way provided consistent results in terms of accurately identifying plants as accumulating high, moderate or low levels of *PPI* II protein. In our experience however, the precise value in terms of μg *PPI* II per mg soluble protein varied, mainly due to changes in the amount of soluble leaf protein in glasshouse-grown plants caused through environmental influences.

Insect feeding trials

C. eriosoma larvae, fed on leaf tissue from progeny from a self-fertilized primary transgenic plant that demonstrated *PPI* II protein accumulation, grew consistently slower than those fed on control (non-transgenic) tissue (data from one trial plotted as Fig. 4A). This trend was consistent over three independent trials where, in each case, larvae fed on leaf tissue that accumulated *PPI* II grew significantly slower than those fed control tissue. Moreover, growth rate was not suppressed for larvae fed on leaf tissue with no detectable *PPI* II protein accumulation when compared with control (non-transgenic) tissue (data not shown). While not statistically significant, the plant that accumulated the higher level of *PPI* II (Fig. 4A, plant 1.7) was more effective at retarding larval growth than plant 1.2. It is difficult to compare the level of *PPI* II accumulation in these plants with values for CPTI reported by Hilder *et al.* (1987) since different methods of quantification were used. Nevertheless, the level of *PPI* II accumulation required to affect the growth rate of *C. eriosoma* larvae (2.2 to 6.5 ng per μg soluble leaf protein) is in good agreement with those values for CPTI reported to be effective against *H. virescens* (2.5 ng per μg soluble leaf protein caused 62.5% insect survival, and 6.2 ng per μg soluble leaf protein caused 50%; Hilder *et al.*, 1987).

In contrast, *T. orichalcea* and *S. litura* larvae fed tissue from the progeny of selfed first generation transgenic plants that accumulated *PPI* II protein displayed more inconsistent growth trends. For *T. orichalcea*, there was essentially no difference in growth rates between larvae fed on control or transgenic plant tissue that accumulated *PPI* II protein. One trial is presented as Fig. 4B, and while plant 1.4 does have a stimulatory effect on larval growth, such a trend was not observed consistently over three independent trials.

For *S. litura*, as with *T. orichalcea*, there were no consistent differences in growth between larvae fed control or transgenic leaf tissue. In the example presented

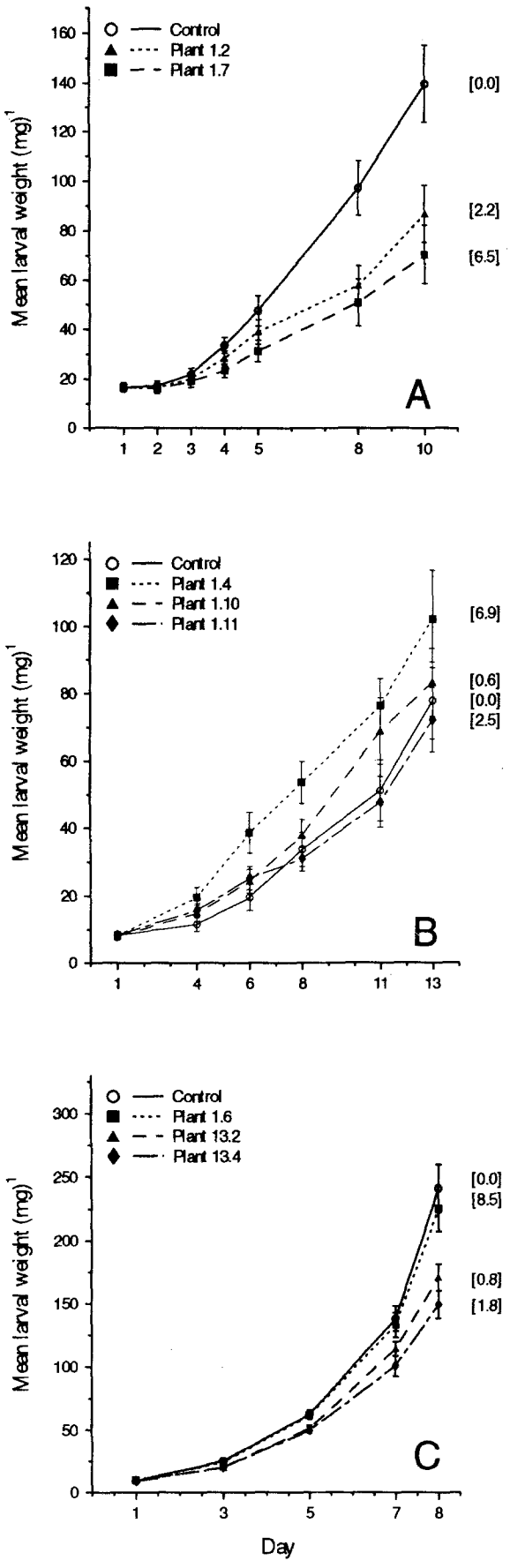

Fig. 4. Insect feeding experiments. A: Mean weights of larvae of *Chrysodeixis eriosoma* (n = 20) fed leaf tissue, either from progeny of the self-fertilized primary transgenic plant 1 (designated 1.2, 1.7), or from a control (non-transgenic) plant. B: Mean weights of larvae of *Thysanoplusia orichalcea* (n = 20) fed leaf tissue, either from the progeny of the self-fertilized primary transgenic plant 1 (designated 1.4, 1.10, 1.11), or from a control (non-transgenic) plant. C: Mean weights of larvae of *Spodoptera litura* (n = 40) fed leaf tissue, either from progeny of the self-fertilized primary transgenic plant 1 (designated 1.6) or plant 13 (designated 13.2, 13.4), or from a control (non-transgenic) plant. The levels of *PPI* II expression in each plant as determined by direct ELISA, are given in brackets as ng *PPI* II per μg leaf protein. Vertical bars = approximate 95% confidence limits.

(Fig. 4C), the growth of larvae fed on tissue from plants 13.2 and 13.4 had, by day 8, been repressed, but larvae fed plant tissue that contained a significantly higher level of *PPI* II (plant 1.6) did not display any reduction in growth.

Proteinase activity and inhibition assays

The insect feeding trials provided evidence that growth of *C. eriosoma* larvae was affected by ingestion of *PPI* II, while that of *S. litura* and *T. orichalcea* larvae was not. To ascertain whether the inhibitor could interact directly with its target digestive enzyme, extracts were prepared from digestive tracts isolated from larvae of *C. eriosoma* and *S. litura*. In these extracts, trypsin-like and chymotrypsin-like enzymes were the predominant serine proteinases (Table 1), although in *C. eriosoma* more trypsin-like activity was detected when compared with chymotrypsin-

Table 1. Serine proteinase activity in the digestive tract of *C. eriosoma* and *S. litura* larvae

Enzyme	*Activity (μmol/per min/per ml extract)*
A.	
C. eriosoma	
Trypsin	3.30 ± 0.020[1]
Chymotrypsin[2]	Neg.[3]
Chymotrypsin/elastase[4]	1.05 ± 0.012
B.	
S. litura	
Trypsin	3.75 ± 0.015
Chymotrypsin	Neg.
Chymotrypsin/elastase	3.6 ± 0.012

[1]Values are ± range of replicates.
[2]Using *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTpNA) as substrate.
[3]Neg. = negligible activity.
[4]Using *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (SApNA) as substrate.

like activity. To determine the effect of *PPI* II on these serine proteinases, the inhibitor was partially purified from a transgenic plant. For *C. eriosoma*, as the concentration of the inhibitor increased the measurable chymotrypsin-like activity decreased significantly. However, this trend was not as marked in assays where chymotrypsin-like activity from *S. litura* was measured (Fig. 5). No inhibition by the *PPI* II preparation of trypsin-like activity in extracts from either *S. litura* or *C. eriosoma* was observed (data not shown).

Discussion

This paper seeks to extend the notion that proteinase inhibitors are effective plant defensive factors against insect pests when introduced into transgenic plants. We have tested the efficiency of a chymotrypsin inhibitor (a member of the potato proteinase inhibitor II gene family) incorporated into tobacco against three closely related noctuids. As expected, *C. eriosoma* larvae fed on transgenic tissue with detectable levels of *PPI* II protein grew slower than larvae fed on control plant tissue (both non-transgenic and transgenic plants but with no detectable *PPI* II protein were used as controls). In contrast, growth of *T. orichalcea* and *S. litura* larvae was affected in a more inconsistent manner by the presence of the proteinase inhibitor in their diet. Overall though, and unlike that observed for *C. eriosoma*, larvae of these latter two insect species did not demonstrate a consistent repression of growth when fed leaf tissue that accumulated *PPI* II.

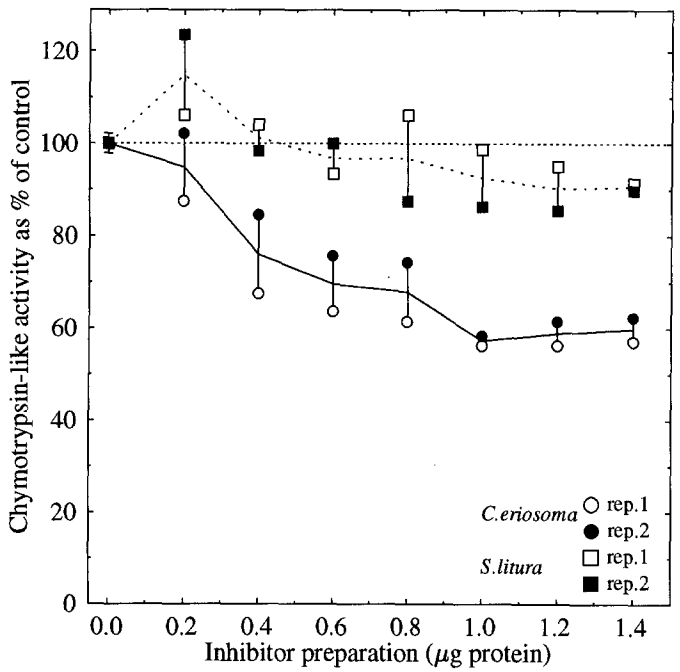

Fig. 5. Inhibition of chymotrypsin-like activity. Increasing amounts of *PPI* II inhibitor preparation (expressed as total protein) were incubated for 10 min, at 26°C, with aliquots of gut extract from either *S. litura* or *C. eriosoma* diluted in 100 mM Tris-HCl, pH 8.0 (assay buffer). To measure chymotrypsin-like activity, *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide was added to a final concentration of 4 mM and the reaction measured at 415 nm. Activity is expressed as the percentage remaining of the control value (the rate with no added inhibitor) for each inhibitor concentration. Vertical bars = range of replicates.

These data are the first evidence that growth of three closely related insect species is affected quite differently by the same proteinase inhibitor in transgenic plants. As yet, we do not know how this particular inhibitor acts, but it is known that while *PPI* II is a 'double-headed' serine proteinase inhibitor (with two proteinase binding sites), it belongs to a multi-gene family comprising several iso-inhibitors with varying affinities for trypsin and chymotrypsin (Bryant *et al.*, 1976). We have purified the iso-inhibitor encoded for by the gene used to transform tobacco in this study and have discovered that it exhibits kinetics more consistent with chymotrypsin (rather than trypsin) inhibition (McManus *et al.*, 1990). Indeed, comparison of the amino acid sequence confirms this. The amino acid pair at the putative trypsin reactive site in the inhibitor used by Johnson and co-workers is Arg-Glu, with Leu-Asn at the chymotrypsin site (Thornburg *et al.*, 1987). However, the gene used here has Leu-Glu instead of Arg-Glu (Keil *et al.*, 1986). The second reactive site has Leu-Asn, in common with the gene cloned by Thornburg and co-workers, indicating a second chymotrypsin reactive site.

Based on these observations, we have characterised the serine proteinases present in the digestive tracts, both from *C. eriosoma* (an insect whose growth rate is affected by ingestion of the inhibitor), and *S. litura* (an example of an insect that is unaffected) and determined that chymotrypsin-like and trypsin-like enzymes were the major species present. As predicted, *PPI* II inhibited chymotrypsin-like activity from *C. eriosoma* but not from *S. litura*, although the degree of inhibition was not particularly pronounced. Nevertheless, for *C. eriosoma* at least, the other digestive proteinases (including the trypsin-like activity) could not accommodate the reduction of chymotrypsin-like activity caused through ingestion of the inhibitor, and the growth rate of the feeding larvae was consequently retarded.

Overall, the data presented here indicate that inhibitors of chymotrypsin-like enzymes can be effective against certain insect pests when expressed in transgenic plants and can now be evaluated in whole plants. Moreover, these inhibitors are quite specific in their action suggesting that particular examples must be selected to provide resistance against certain insect pests. While tomato inhibitor I was ineffective against *M. sexta*, for example, it may be effective against *C. eriosoma*, whereas potent inhibition of trypsin-like activity may be necessary to retard larval development of *T. orichalcea* and *S. litura*.

This specificity is also observed in another widely used plant protective agent, *Bacillus thuringiensis* (*Bt*) toxin (Vaeck *et al.*, 1987). Here, there are many thousand isolates of the species, but each is toxic to a narrow range of insect pests (Whiteley and Schnepf, 1986).

Clearly then, the exclusive reliance on a single proteinase inhibitor (or any single resistance factor) to provide enhanced protection to plants is a questionable strategy. Instead, the use, in tandem, of different pest resistance factors that act through distinct mechanisms may be more desirable, particularly to enhance the efficacy of protection to the plant. Such dual approaches have been used successfully in transgenic plants. MacIntosh *et al.* (1990) demonstrated in feeding trials with *H. virescens* that a fusion protein comprising the *B. thuringiensis* var. *kurstaki* HD-1 protein (*Cry*IA(b)) fused to the squash trypsin inhibitor (CMTI) potentiated sublethal concentrations of the *Bt* protein alone. Boulter *et al.* (1990) used progeny whose transgenic parents expressed either the CPTI gene or a pea lectin gene. Progeny that inherited and expressed both genes demonstrated an enhanced resistance to *H. virescens*, when compared with plants that expressed either one of the two genes. This multifunctional approach, as these latter authors observe, more closely mimics the situation in nature. Here, wild plants have an array of defence mechanisms, any of which alone is not completely effective in protecting the plant from herbivores (an observation made by Kogan, 1986). Moreover, approaches that employ resistance factors with dissimilar mechanisms of action will also prolong the durability of conferred insect resistance to transgenic plants (MacIntosh *et al.*, 1990). The evaluation of multifunctional approaches both to increase the effectiveness of protection to the plant and to extend the persistence of this resistance is currently under investigation in this laboratory.

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