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Multiple insect resistance in transgenic tomato plants over-expressing two families of plant proteinase inhibitors

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Abstract

Protease inhibitors have been proposed as potential defense molecules for increased insect resistance in crop plants. Compensatory over-production of insensitive proteases in the insect, however, has limited suitability of these proteins in plant protection, with very high levels of inhibitor required for increased plant resistance. In this study we have examined whether combined used of two inhibitors is effective to prevent this compensatory response. We show that leaf-specific over-expression of the potato PI-II and carboxypeptidase inhibitors (PCI) results in increased resistance to *Heliothis obsoleta* and *Liriomyza trifolii* larvae in homozygote tomato lines expressing high levels (>1% the total soluble proteins) of the transgenes. Leaf damage in hemizygous lines for these transformants was, however, more severe than in the controls, thus evidencing a compensation response of the larvae to the lower PI concentrations in these plants. Development of comparable adaptive responses in both insects suggests that insect adaptation does not entail specific recognition of the transgene, but rather represents a general adaptive mechanism triggered in response to the nutritional stress imposed by sub-lethal concentrations of the inhibitors. Combined expression of defense genes with different mechanisms of action rather than combinations of inhibitors may then offer a better strategy in pest management as it should be more effective in overcoming this general adaptive response in the insect.

Introduction

In response to insect attack, plants accumulate a set of defense proteins including proteinase inhibitors and a range of secondary metabolites with a harmful activity to the insects. These inherent defense mechanisms confer a certain degree of natural resistance to the plant, with only a limited number of herbivores being able to feed on each individual plant species. Transfer of extra copies of these defense genes under control of constitutive promoters, together with expression of resistance genes of non-plant origin, has been favored as strategy to increase levels of plant resistance, providing an alternative to conventional insecticides and the ecological damage they cause. Insect-resistance genes transferred to plants include the *Bt* toxin from *Bacillus thuringiensis*, a range of defense genes derived from plants, and also some resistance genes derived from animals and other microorganisms (Jouanin *et al.*, 1998; Schuler *et al.*, 1998).

Codon-optimized *cry* genes encoding *Bt* toxins from different *Bacillus* strains have been transferred into several crops, including maize, rice, cotton, potato, tomato, soybean and *Brassica* species (Schuler *et al*., 1998). *Bt* crops have been commercialized in several countries, but there is concern that constitutive expression of the *cry* genes will lead to selection of resistance among insect populations. Insects resistant to *Bt* have indeed been isolated, pointing to the need of refuge islands to delay selection of resistant individuals or the alternate use of resistant plants with a different mechanism of action.

Two major groups of plant-derived genes, i.e. proteinase inhibitors and carbohydrate-binding lectins, have been also used to increase insect resistance in crops. Levels of insect control obtained with these genes are smaller than those observed for the *Bt* toxin, with much lower percentages of insect mortality than for *cry* plants. Hence, this approach has been regarded as a suitable alternative to *Bt* in pest control, as milder toxicity of these genes is expected to exert a lower selection pressure to the insect.

Plant proteinase inhibitors are part of the plant natural defense mechanism against herbivores. In response to mechanical wounding or insect attack, plants accumulate multiple inhibitory proteins specific to serine, cysteine, aspartic and metallo proteinases from insects (Ryan, 1990). The defense pathway that mediates accumulation of these proteins has been extensively studied in tomato and Arabidopsis, with jasmonates identified as the signaling compounds that trigger systemic expression of these genes (Ryan, 2000; Turner *et al*., 2002).

Insect proteases are essential digestive enzymes that catalyze the release of amino acids from dietary protein to provide the nutrients required for larval growth and development. They are found most abundantly in the midgut region of the insect digestive tract, with different proteinases found to predominate in different insects. Hence, whereas serine proteinases are predominant in lepidopteran larvae, midguts of coleopteran species are rich in cysteine and aspartic proteases. In agreement with this preferential distribution, transgenic expression of ser-proteinase inhibitors, such as tomato and potato PI-II or cowpea trypsin inhibitor, was effective to inhibit growth and development of lepidopteran larvae (Hilder *et al*., 1987; Johnson *et al*., 1989; McManus *et al*., 1994; Duan *et al*., 1996), whereas expression of cys-proteinase inhibitors, such as potato multicystatin, conferred protection against coleopteran species (Orr *et al*., 1994; Leplé *et al*., 1995). Growth inhibition effects of these defense proteins were reported to be due to direct inhibition of the respective digestive enzymes, but also to massive over-production of these enzymes, the latter leading to a depletion of essential amino acids in detriment of other proteins (Broadway and Duffey, 1986; Gatehouse *et al*., 1992). In addition to these growth retardation effects, several reports have established that insect larvae are able to adapt to the presence of inhibitors by replacing the inhibited enzymes by other PI-insensitive proteases, these larvae then exhibiting increased ingestion rates and developing faster than larvae fed on controls (Jongsma *et al*., 1995; Girard *et al*., 1998; Cloutier *et al*., 1999; Lecardonnel *et al*., 1999). This observation brought to suggest that the use of combinations of PIs, active against different classes of proteases, might be a more effective alternative to increase plant resistance, as it should be more difficult to the insect to raise expression of insensitive proteases to several families of inhibitors.

In this study, we have over-expressed in tomato plants two different classes of potato protease inhibitors, i.e. the serin-proteinase inhibitor PI-II and the carboxypeptidase inhibitor PCI, to investigate whether combined expression of two transgenes is useful to prevent insect compensatory responses to the inhibitor proteins. We have examined resistance of these plants against two common greenhouse pests: the tomato fruit worm *Heliothis (Helicoverpa) obsoleta* (Lepidoptera: Noctuidae) and the serpentine leafminer *Liriomyza trifolii* (Diptera: Agromyzidae). *Heliothis* is a highly polyphagous worm that attacks several plant species, including tomato, and causes serious economical losses by feeding on the leaves and developing fruits. Serin-proteases (trypsin- and chymotrypsin-like) are the main midgut proteases of the related species *Helicoverpa armigera* and *Heliothis virescenes*, though carboxypeptidases were also identified in these insects (Katherine, 1995; Bown *et al*., 1998). The leafminer *Liriomyza* is a major pest affecting a wide range of greenhouse *Solanaceous* and cucurbit crops, in addition to some ornamental plants. Adult flies puncture the leaf for oviposition and lay their eggs singly on

the upper surface. Larvae hatching from these eggs feed on spongy mesophyll and palisade tissues forming characteristic tunnels or mines that may vary in form depending on the host. When they reach a mature state larvae cut a characteristic slit in the leaf surface, leaving the leaf to drop and pupate on the ground. Infected leaves are significantly impaired in photosynthesis, even if they are lightly mined, thus resulting in strong reductions in fruit yield. There exist no reports on the characterization of *Liriomyza* digestive enzymes, although proteolytic enzymes of the carboxypeptidase family appear to be abundant in the midguts of these insects (Castañera *et al*., personal communication). Here we show that co-delivery of two distinct proteinase inhibitors leads to increased resistance to *Heliothis* and *Liriomyza* larvae in plants accumulating high levels of the potato inhibitors. This approach, however, did not prevent development of a feeding compensatory response in larvae reared on plants accumulating lower levels of the transgenes (0.6% of the total soluble protein), suggesting that digestive adaptation in these insects does not rely on a specific mechanism of recognition but rather respond to a general nutritional stress imposed by the inhibitors.

Materials and methods

Plant material and insects

Tomato plants (*Lycopersicon esculentum* cv. Moneymaker) were used in all experiments. Plants were grown in the greenhouse with supplementary high-pressure sodium light under 16 h light/8 h dark light regime and 26 °C of temperature. *Heliothis obsoleta* chrysalises were obtained from the Department of Entomology at the CIRAD in Montpellier (France). *Lyriomiza trifolii* pupae were obtained from the Entomology Unit at the IRTA in Cabrils (Spain). Larvae were reared on a semi-artificial diet for propagation.

PI constructs

A double plant transformation cassette expressing the PI-II coding region under control of the potato *StLS1* promoter (Stockhaus *et al*., 1989) and the potato PCI coding region under control of the Arabidopsis *rbcs1A* promoter (Donald and Cashmore, 1990) was obtained by blunt-end cloning the PI-II *Sca*I-*Mbo*II fragment into the *Sma*I site of pBluescript and further insertion as a *Bam*HI-*Sal*I fragment into the polylinker of the pBinA6 *StLS1* expression cassette, to create *StLS1*::PI-II. The PCI coding region was inserted as a *Bam*HI-*Xba*I fragment in between the *rbcs1A* promoter and the 35S terminator in the *rbcs1A*-pBin19 cassette vector. The *StLS1*::PI-II fusion was then excised by *Eco*RI/*Hind*III digestion, blunted, and inserted into the unique *Cla*I site between the *rbcs1A*::PCI fusion and the *nptII* selection gene in the *rbcs1A*-pBin19 vector, to obtain a T-DNA with the two inserted constructs as shown in Figure 1.

Plant transformation and regeneration

The double *StLS1*::PI-II/*rbcs1A*::PCI pBin19 construct was introduced into the *Agrobacterium*

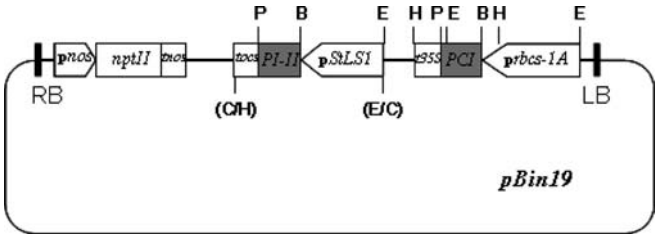

Figure 1. pBin19 derivative Ti-plasmid construct *StLS1*::PI-II / *rbcs-1A*::PCI used for tomato transformation. The PI-II coding region was inserted as a *Bam*HI-*Sal*I fragment into the polylinker of the pBinA6 *StLS1* expression cassette to create *StLS1*::PI-II. PCI was cloned as a *Bam*HI-*Xba*I fragment between the *rbcs1A* promoter and the 35S terminator in the *rbcs1A*-pBin19 vector. A double T-DNA expression construct was obtained by insertion of the *StLS1*::PI-II fusion into the unique *Cla*I site of this plasmid. The *nptII* gene conferring resistance to kanamycin was used as selectable marker for tomato transformation. B, *Bam*HI, C, *Cla*I, E, *Eco*RI, H, *Hind*III, P, *Pst*I.

tumefaciens strain LBA 4404 and used for tomato (*Lycopersicon esculentum* cv Moneymaker) leaf disc transformation as described by Koornneef *et al.* (1987). Transformants were selected on kanamycin-containing medium (50 mg/l) and propagated in soil for subsequent analysis.

Analysis of gene expression

Plants were analyzed by Southern blot and northern to determine the transgene copy number and PI expression levels. Total RNA was isolated from transformed leaves as described (Logemann *et al.*, 1987). RNA isolated from plants treated with 50 μM MeJA was used as positive control. 30 μg of total RNA were separated on agarose/formaldehyde gels, transferred onto Hybond N^+ nylon membranes and hybridized overnight at 65 °C in Church buffer [125 mM Na_2HPO_4, pH 7.2, 7% SDS, 1 mM EDTA]. Filters were washed three times for 20 min in 20 mM Na_2HPO_4 (pH 7.2), 1% SDS, 1 mM EDTA at 65 °C.

Genomic DNA was isolated from kanamycin resistant plants according to the method of Dellaporta *et al.* (1983). Southern analyses were done with 10 μg of genomic DNA digested with *Eco*RI or *Hin*dIII, and hybridized as before (data not shown). The *PI-II* and *PCI* coding sequences were radioactively labelled with a random primed DNA kit (Roche) and used as probes. Plants showing high levels of expression of both *PI-II* and *PCI* transcripts were selected to be used for further assays.

Western blot analyses

Accumulation of the PI-II protein in the transgenic lines was analyzed by western blot. Total soluble protein extracts were obtained by homogenization of leaf material in extraction buffer (100 mM phosphate buffer pH 7.0, 0.1% 2-mercaptoethanol, 0.5 mM PMSF) and centrifugation for 5 min at 10,000 rpm. Protein content in the extracts was measured by Bradford, and 25 μg of the soluble proteins loaded in a SDS/PAGE gel. Proteins were transferred to nitrocellulose membranes using a semidry transfer system and immunodetected using a 1:10,000 dilution of a rabbit antiserum raised against the PI-II protein (kindly provided by Dr. Sánchez-Serrano). Membranes were incubated with a goat anti-rabbit IgG horseradish peroxidase conjugate as a secondary antibody and ECL detected according to the manufacturer instructions (Amersham).

Estimation of the levels of PCI in transgenic tomato leaves

Levels of PCI accumulating in the transgenic tomato leaves were estimated by comparing the inhibitory effects on bovine carboxypeptidase-A activity (Sigma) of the soluble leaf extracts, with a standard inhibition curve obtained with known concentrations of the purified inhibitor. Carboxypeptidase assays were performed according to Villanueva *et al.* (1998). Reactions were carried out in 200 μl of buffer 20 mM Tris–HCl pH 7.5, 0.5 M NaCl, in the presence of 100 μM of the anisyl azo formyl phenol (AAFP) substrate and 20 μM of the enzyme. Substrate degradation was monitored spectrophotometrically at 300 nm. Inhibitory standard curves were obtained using concentrations of 0, 50, 150, 300, and 500 nM the purified PCI inhibitor. Concentrations of PCI in the transgenic plants were estimated by extrapolation of the percentage of carboxypeptidase A inhibition obtained with 100 μg of the soluble protein leaf extracts, with those obtained with known amounts of the PCI inhibitor.

Enzymatic assays

Mid-guts from fourth-instar larvae were dissected and freshly used or stored at −80 °C. Digestive protease extracts were obtained by homogenizing four mid-guts in 200 μl of buffer 50 mM Tris–HCl pH 8.0, 0.15 M NaCl, followed by 5 min centrifugation at 13,000 rpm at 4 °C. Protein content in the supernatants was determined by Bradford and adjusted to 2 mg/ml by the addition of extraction buffer. Total protease activity in the extracts was quantitatively determined using resorufin-labelled casein as a protein substrate. 100 μg of mid-gut protein extract were used per reaction in a final volume of 200 μl. Reactions were incubated for 30 min at 37 °C, stopped by the addition of 480 μl of 5% ice-cold trichloroacetic acid and centrifuged at 13,000 rpm for 5 min according to the manufacturer instructions (Universal Protease Substrate, Roche). Digested resorufin-labelled peptides were monitored in the supernatant by colour measuring at 574 nm. Two measurements

were made for each homogenate, and activities measured three independent times. Contribution of serine proteases to mid-gut protease activity was determined by pre-incubating the extracts with increasing concentrations of soybean Bowman–Birk inhibitor (BBI, Sigma). The effect of pH on the activity of the mid-gut proteases was determined using a discontinuous buffer system based on phosphate and Tris–HCl buffers, covering a pH range between 7.0 and 11.0.

Carboxypeptidase A activity in *Liriomyza* extracts was determined using the AAFP specific substrate. 100 μg of total pupae extract was used per reaction in a final volume of 200 μl, containing 100 μM of the AAFP substrate. Substrate degradation was monitored as the decrease in AAFP absorbance at 300 nm. Contribution of carboxipeptidase A to pupal protease activity was assayed by pre-incubation of the extracts with increasing concentrations of potato PCI, as indicated. Two measurements were made for each homogenate, and activities were measured three independent times.

Insect feeding assays

Heliothis obsoleta feeding assays were carried out by placing 1st-instar larvae on detached tomato leaves. Leaf petioles were placed in tubes with wet cotton and leaves maintained in closed boxes with damp absorbing paper to provide sufficient humidity. Bioassays were conducted under controlled photoperiod conditions of 16 h light/8 h dark, 22 °C, 70% relative humidity. Leaves were daily replaced and surviving larvae were monitored every second day throughout the assay. At the end of the bioassay (10 d after placing the larvae into the leaves), the developmental stage of the surviving larvae was determined by measuring their cephalic skeleton lengths, insects were individually weighed and mid-guts were dissected for digestive protease assay.

Consumed leaf area was measured at day 6th or 7th of the assay, by placing one larva per leaf and let it feed for 24 h. Leaves were photographed before and after feeding, and the consumed area calculated using the Quantity One (Bio-Rad) densitometric software. Analysis of variance (ANOVA) was used to compare growth rates, larval fresh weights and leaf consumption among lines. Differences were considered to be significant for $P < 0.05$.

Lyriomiza *bioassays*

Efficacy of PI-II and PCI expression in conferring resistance against *Liriomyza* larvae was assayed in whole plants. Six plants of each line: control and transgenic hemi- and homozygotes, were used to monitor larval growth and pupae formation. Plants were randomly distributed in the growth chamber, covered with nylon bags, and 10 pupae were laid in the soil. Plants were kept covered for 1 week to allow adult emergence and female oviposition, and bags were removed and substituted by cellophane sheets fixed around the lower leaves in order to collect emerging pupae. After two additional weeks, cellophane sheets were carefully removed and pupae counted. Bioassays were repeated three times and ANOVA was used for statistical analysis of the data. Differences among lines were considered to be significant for $P < 0.05$.

Results

Leaf-specific PI-II and PCI over-expression

Tomato plants were transformed with a double construct directing expression of the potato carboxypeptidase inhibitor (PCI) coding region under control of the Arabidopsis *rbcs-1A* promoter and expression of the potato serin-proteinase inhibitor PI-II under control of the potato *StLS1* promoter (Figure 1). Using these promoters we assured high levels of expression of the transgenes in leaf and green fruit, but a lack of expression in the mature fruit used for market consumption. The pBin19 derivative plasmid containing both constructs was introduced into *Agrobacterium tumefaciens* and used to transform tomato plants by leaf-disc infection. Insertion of both constructs was assessed by Southern blot hybridization (data not shown) and the levels of expression of the transgenes analyzed by RNA blot detection (Figure 2). No visible pleiotropic effects due to the recombinant PI-II and PCI proteins were observed in the recombinant lines. Two lines which expressed intermediate (line 8) or high levels (line 9) of both *PI-II* and *PCI* transcripts were selected (Figure 2b) and used for further characterization.

Homozygous plants were obtained for these lines, and the relative amounts of recombinant PI-II inhibitor determined by immunoblot detection. As

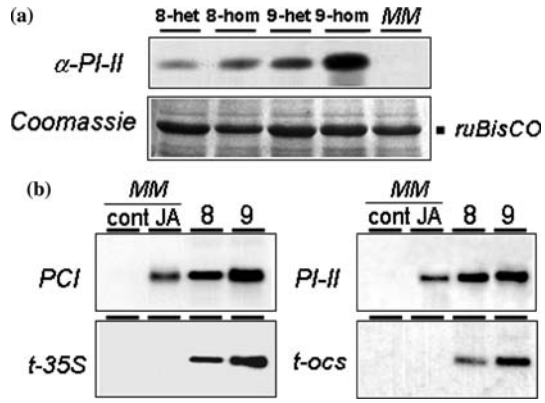

Figure 2. Western and RNA blot analyses of T1 transgenic tomato plants. (a) Detection of PI-II in leaf extracts of hemi- and homozygote plants. 50 μg of protein extracts from the untransformed controls (MM) and hemi- and homozygote plants for transformants 8 and 9 (8-het, 8-homo, 9-het, 9-homo) were separated by SDS/PAGE, transferred onto nitrocellulose membranes and immunodetected using an antibody raised against the PI-II protein. Coomassie staining of the gel is included as control for loading. (b) Northern blot detection of transcripts *PCI* and *PI-II* in transformants lines 8 and 9 (8, 9). 30 μg of total RNA were loaded per line. RNA extracted from untransformed *MM* plants treated for 12 h with 50 μM methyl-jasmonate (JA) was included for comparison. RNA blots were hybridized with probes corresponding to the *PCI* and *PI-II* coding regions or to the transgene specific *35S* and *ocs* terminators as indicated. Levels of both *PCI* and *PI-II* transcripts were higher in the transgenic lines than in the JA-treated untransformed controls.

shown in Figure 2a, levels of the PI-II protein correlated well with the levels of expression of the transgene, with a stronger signal obtained for homozygote as compared to hemizygote plants. Levels of PI-II inhibitor in high-expresser hemizygotes (9-het) were similar to those of the intermediate-expresser homozygotes (8-homo), homo- and hemizygous clones for line 9 (9-homo and 9-het) thus being used for further studies. These plants were used in the insect bioassays as a source of foliage with "high" and "intermediate" levels of accumulation of these inhibitors, to minimize uneven effects due to spatial/temporal differences in the pattern of expression of the transgenes.

Relative concentration of PCI activity in the foliage of the transgenic lines

Relative amounts of PCI in the transgenic tomato lines were determined by comparing the rates of inhibition of bovine carboxypeptidase A obtained with extracts of these plants with those of serial dilutions of the purified potato PCI inhibitor (see Materials and methods). These assays allowed estimating a relative concentration of PCI of about 1.4% of the total soluble proteins in the homozygous lines (9-homo) and 0.6% of the total soluble proteins in the hemizygotes (9-het). As shown in Figure 3, near identical rates of degradation of the AAFP substrate were obtained for extracts of the non-transformed controls (MM) as for the carboxypeptidase enzyme alone (CP-A), indicating that these extracts contained very low amounts of PCI. Similar results were obtained in western blot assays aimed to detect the PI-II protein (Figure 2a); both inhibitors accumulating to very low levels in unwounded control leaves. JA-application induced high levels of expression of both *PCI* and *PI-II* endogenous transcripts in the control plants, to levels similar to those observed in the transgenic lines (Figure 2b). These results thus are consistent with a defense-associated function of these inhibitors, they being expressed to relatively high levels in tomato flowers and fruit, and strongly induced in the leaves in response to

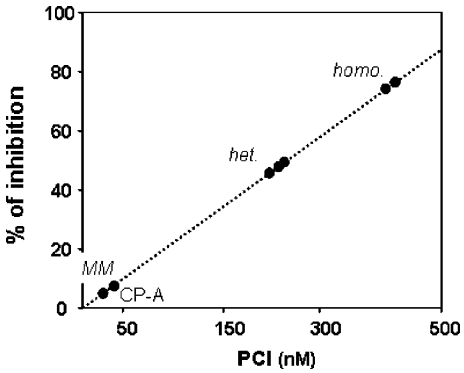

Figure 3. Relative concentration of PCI activity in the leaves of hemi- and homozygote plants for transformant 9. Levels of PCI in the transgenic leaves were estimated by measuring the inhibitory effects of 100 μg of soluble leaf protein extracts on bovine carboxypeptidase-A. Rates of inhibition were compared to a standard inhibition curve obtained with known concentrations of the purified inhibitor (50–500 nM). PCI was estimated to be about 1.4% of the total soluble proteins in the homozygous lines (homo) and 0.6% of the total soluble proteins in the hemizygous plants (het). Pre-incubation with leaf extracts of untransformed controls (*MM*) resulted in negligible inhibition of activity, with rates of substrate degradation comparable to those obtained after pre-incubation with the protein extraction buffer alone (CP-A).

wounding or insect attack (Ryan, 2000; Villanueva *et al.*, 1998).

Bioassays on Heliothis obsoleta *larvae*

Heliothis biological assays were performed by feeding first-instar larvae on detached transgenic and non-transgenic leaves. After hatching, larvae were directly placed on the transgenic or control leaves (4 larvae per leaf) and let feed for 10 days during which leaves were daily replaced by fresh ones. Leaf consumption was measured by day 8th of the assay. Leaves were photographed before and after feeding, and consumed area was determined by densitometric quantification. At the end of the assay (day 10th) surviving larvae were counted, weighted, and their developmental stage was determined by measuring their cephalic skeleton lengths. Larvae were continued to be fed until they pupate and emerging adults were separated in couples to analyze female oviposition.

As shown in Table 1, incidence of larval mortality was higher in larvae fed on the homozygous transgenic lines, with 30.4% of the larvae dead by day 10th compared to 13.8% in the controls. Foliage consumption was as well reduced in these plants (Table 1), the leaf area consumed per day being nearly 40% less in these plants than in the untransformed controls.

A slightly reduced mortality was, in opposite, observed for hemizygote plants, where only 11.2% of the larvae had died by day 10th of the assay. Leaf consumption in these plants was, in turn, higher than in the controls, with daily consumed leaf area being close to 180% that of the controls (see Table 1).

Feeding on PCI/PI-II leaves resulted in delayed larval growth, with only 17% of the larvae fed on the homozygous transgenic leaves being at the 4th-instar stage by the end of the evaluation period, compared to 60% in the controls (Figure 4). Mean weights for larvae fed on control and homozygous tomato leaves were 97.3 and 56.8 mg, respectively (Table 1). Oviposition by females emerging from chrysalis derived from larvae fed on homozygote lines was also reduced by more than 3-fold with respect to the controls, indicating a deleterious effect of the PIs over all insect developmental stages.

In contrast to the effects seen for homozygous plants, larvae fed on hemizygote leaves gained weight more rapidly, and reached L4 instar earlier than the controls (see Figure 4). At the end of the assay 84% of the larvae had reached the fourth developmental stage, with an average weight of 144.2 mg, which is near to 1.5-fold that observed for larvae fed on control plants. Adult females laid also more eggs than those reared on controls, suggesting a compensatory response in these insects to avoid the anti-metabolic effects produced by low concentrations of the PI-II/PCI inhibitors. Characteristic leaf consumption areas and larval growth of insects fed on controls, homozygous and hemizygous plants are shown in Figure 5.

Heliothis obsoleta *digestive proteinase activities*

To assess potential effects of the potato inhibitors on the digestive proteases of *Heliothis* larvae, we monitored digestive activities of dissected midguts of fourth instar larvae fed on either control, homozygous or hemizygous leaves. Proteolytic activities were measured using the casein resorufin substrate, a series of discontinuous pH buffers from pH 7.0 to 11.0 used to determine the

Table 1. Ratio of mortality, larval growth, leaf consumption and oviposition of *Heliothis* larvae reared on control or the transgenic lines.

	Mortality	Larval weight (mg/larva)	Consumed leaf area (cm^2)/day	Eggs/couple
Control	13.8%	97.3 ± 2.6	8.1 ± 0.9	53
9-homo	30.4%	56.8 ± 4.5	5 ± 0.8	16
9-hetero	11.2%	144.2 ± 6.1	14.3 ± 0.9	74

First instar larvae were placed on detached leaves which were maintained under high humidity in closed boxes. Leaves were replaced daily and consumed leaf area was determined at day 8th of the bioassay. Leaves were photographed before and after larval feeding and consumed area calculated by densitometry (Quantity One, Bio-Rad). Ten days after starting the assay, surviving larvae were counted and weighed. Larvae were fed to pupation, emerging adults separated in couples, and number of eggs scored after female oviposition. Bioassays were repeated three times.

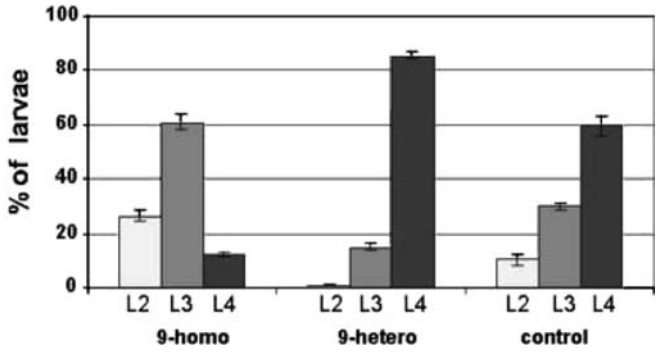

Figure 4. Developmental stage of *Heliothis* larvae fed on control, hemi- and homozygote PI-II/PCI expressing transgenic tomato plants. Larval developmental stage was scored by day 10th of the bioassay. Developmental instar of the larvae was determined by measuring their cephalic skeleton lengths. Only 17% of the larvae reared on the transgenic homozygous leaves had reached the 4th-instar stage by the end of the evaluation period. An opposite effect was observed for larvae fed on leaves of the hemizygote plants, where 84% of the larvae had reached the 4th-developmental stage compared to 60% in the controls. Results are the average of two experiments.

optimal pH for activity. Activity profiles showed a peak of activity at pH 10.5 which is in agreement with previous reports showing that extreme alkaline conditions (pH 10–12) are characteristic of lepidopteran insect midguts (Dow, 1992).

Inhibitory assays using the trypsin/chymotrypsin soybean–BBI showed a 75% inhibition of the overall activity in extracts of larvae fed on controls, thus indicating that serine-proteinases comprise a main fraction of digestive enzymes in these insects (Figure 6). Biochemical studies had indeed shown that trypsin and chymotrypsin are the major proteinase activities of lepidopteran pests (Christeller *et al*., 1992; Johnston *et al*., 1995), although elastase-like activity was as well detected and likely contributes to part of the remaining activity.

Trypsin/chymotrypsin proteinases were also abundant in gut extracts of larvae fed on the transgenic homozygous lines, BBI showing inhibitory activity against 80% of the digestive enzymes in these extracts (Figure 6). Sensitivity to BBI, however, declined by near to 2-fold in extracts of larvae fed on hemizygote plants. A BBI-inhibitory activity of only 40% was in fact observed for these extracts (see Figure 6), indicating an increased level of proteinases with a lower affinity for BBI or the presence of other classes of proteolytic enzymes. This demonstrates that larvae fed on hemizygote leaves have adapted to the presence of

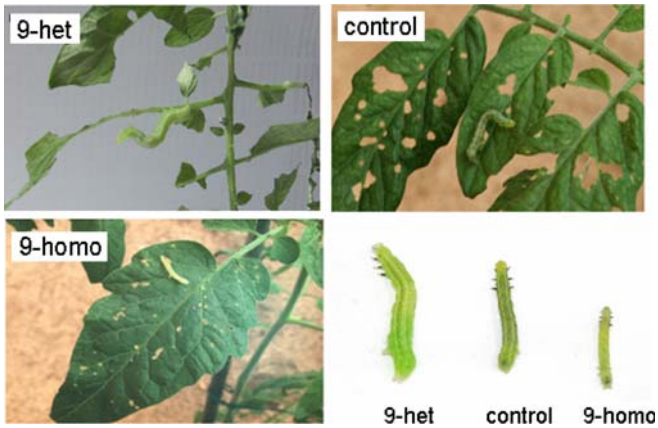

Figure 5. Plant damage and larval size of *Heliothis* larvae fed on controls or the PI-II/PCI-expressing transgenic leaves. Decreased larval weight and reduced leaf damage was observed for larvae fed on the homozygote lines. By contrast, insect larvae fed on the hemizygote plants accumulating lower levels of the PI-II/PCI transgenes were larger than larvae fed on the untransformed controls and produced more severe damage to the leaves.

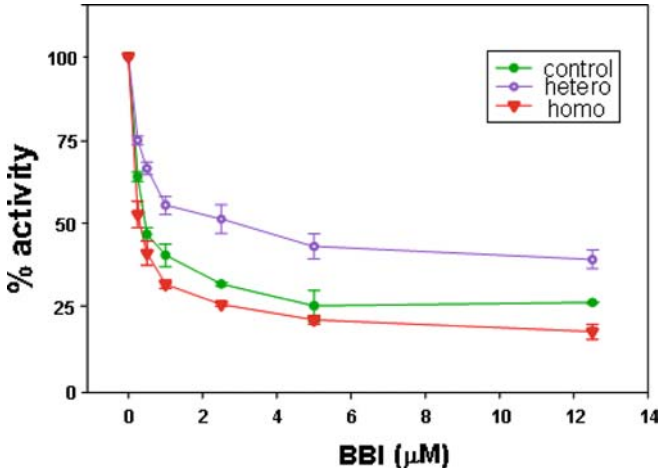

Figure 6. Inhibitory activity of soybean Bowman-Birk inhibitor (BBI) towards digestive activity of *Heliothis* larvae fed on tomato controls (non-transformed) or the PI-II/PCI-expressing transgenic leaves. Midgut digestive activity was measured using the casein resorufin substrate. Serine protease activity in the digestive extracts was determined by pre-incubation of the extracts with increasing concentrations of the BBI inhibitor. Serine proteases are relatively abundant in *Heliothis* midguts, 75% of the digestive activity in larvae fed on controls being inhibited by BBI. Addition of BBI caused 80% inhibition of the overall digestive activity of larvae fed on the transgenic homozygote lines, but 40% inhibition of the digestive activity of larvae fed on the hemizygous plants evidencing a digestive compensatory response in these larvae. Each measurement was done in triplicate.

low concentrations of the PI-II/PCI inhibitors by overproducing a new class of resistant proteases aimed to avoid the anti-metabolic effects of these inhibitors. Hyper-production of these digestive enzymes may explain the increased larval growth observed in the bioassays, larvae reared on hemizygote leaves growing faster and reaching a higher weight than larvae fed on the homozygous lines or the controls.

Parallel assays using the FAPP synthetic carboxypeptidase A substrate failed to detect any digestive activity in *H. obsoleta* midguts. Although carboxypeptidase activity was found in midguts of several lepidopteran species (Christeller *et al*., 1992) and cDNAs for carboxypeptidase A were cloned from the midgut of *Helicoverpa armigera* (Bown *et al*., 1998), we were unable to detect this activity in our population of *H. obsoleta*. Addition of the potato carboxypeptidase inhibitor PCI, in turn, did not have any significant effect on the proteolytic activity of these extracts (result not shown), pointing to a substantial amount of genetic diversity within the genera *Helicoverpa*.

Liriomyza *protease activity*

Whereas studies on *Heliothis* digestive proteases are abundant, there are no reports on the digestive activity of *Liriomyza* larvae. Therefore, we first determined whether serine proteases or carboxypeptidases are important components of the digestive system of this insect. Total protein extracts were obtained from fresh pupae and protease activity was analyzed by incubation with the protein substrate casein resorufin or the AAFP synthetic carboxypeptidase substrate. As shown in Figure 7, overall casein-resorufin protease activity was highly sensitive to the BBI trypsin/chymotrypsin inhibitor, with close to 90% of activity inhibited by pre-incubation with this inhibitor (Figure 7a).

Degradation of the carboxypeptidase substrate AAFP was as well found to be inhibited by pre-incubation with purified PCI (Figure 7b), indicating that carboxypeptidase A is also a component of the pupae digestive proteolytic system. These results indicate that overall digestive activity of this insect relies mainly on ser-proteases, carboxypeptidases accounting for an important part of the non-trypsin-like activity.

Effects of recombinant PI-II and PCI on Liriomyza *larvae*

Bioassays with the leafminer insects were carried out *in planta*. Ten pupae were inoculated into each

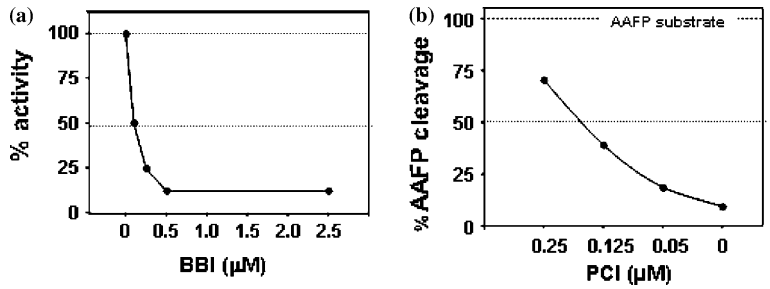

Figure 7. Proteolytic activity in total extracts of *Liriomyza* pupae. Total extracts were obtained from insects directly after pupating. Serine protease activity was measured by BBI inhibition of the casein resorufin protease activity detected in these extracts. Carboxypeptidase A activity was measured by PCI inhibition of the AAFP proteolytic activity. (a) Inhibitory activity of BBI towards *Liriomyza* extracts. (b) Inhibitory activity of PCI. Proteolytic activity is measured as a decrease in AAFP coloration at 300 nm. Each measurement was done in triplicate.

plant, and number of mines and pupating larvae monitored after 15 days of infestation. As shown in Figure 8, significant resistance to larval infestation could be observed in assays with the homozygous plants. Number of recovered pupae from these plants was reduced by 45% with respect to the controls. Larval mortality, calculated as the difference between the number of tunnels in the leaves and pupae recovered per plant was also higher in these transgenic lines, with 38% mortality in the homozygous lines versus 17.8% in the controls.

As observed in the bioassays with *Heliothis*, a higher number of pupae were recovered from the hemizygous lines, with a 40% increase in the number of pupae collected from these plants

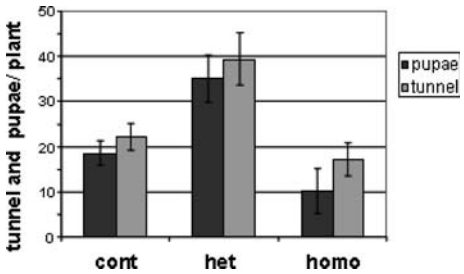

Figure 8. Number of tunnels and *Liriomyza* pupae collected from controls or the PI-II/PCI-expressing transgenic tomato lines. Ten pupae were laid per plant. Plants were covered for 1 week to let adult emergence and oviposition and bags were changed by cellophane sheets fixed around the lower leaves to collect emerging pupae. After 2 weeks, number of tunnels in the leaves was counted, as well as the number of recovered pupae. Percentage of mortality was calculated from the differences between numbers of tunnel and pupae. The assay was repeated three times, significant differences being observed among lines ($P < 0.05$).

(Figure 8). Larval mortality was also reduced, with an incidence of mortality of 11.4% compared to 17.8% in the controls. This would indicate that *Liriomyza* larvae are also able to compensate for low levels of protease inhibition by switching on to alternative proteolytic activities, this switch being correlated with an increase in the number of mines and insect pupae.

Good levels of protection against infestation by *Heliothis* and *Liriomyza* larvae are then observed in transgenic tomato lines expressing the potato PI-II and PCI inhibitors to levels higher than 1% the total soluble proteins, but not in plants accumulating lower concentrations of the inhibitors. In these plants, insects compensated for the antinutritional effect of the transgenes by expressing new digestive proteases insensitive to the expressed inhibitors. Insect adaptation correlated with a hypertrophic response and faster larval development, with bigger damage to the plant. Noteworthy, co-expression of two inhibitors appeared not to have a noticeable effect in preventing this insect compensatory response, high levels of transgene accumulation still required for insect resistance.

Discussion

Inhibition of insect digestive activity by introduction into the host plant of specific protease inhibitors has been a main strategy used in insect control (Johnson *et al*., 1989; Hilder *et al*., 1992). Nevertheless, whereas *Bt* cultivars of corn, potato, cotton and soybean were introduced worldwide with a significant impact in production, the

protective effects observed with PI gene constructs were not considered to be sufficiently convincing to lead to a serious attempt at commercializing these transgenic crops.

Reports showing that expression of protease inhibitors confer resistance to the intended target insects are numerous. Transgenic cotton lines expressing the cowpea trypsin inhibitor (CpTI), for example, were reported to be highly resistant to cotton bollworm (Li *et al*., 1998), whereas expression of potato PI-II in rice conferred resistance to *Sesamia inferens* (Duan *et al*., 1996). Transgenic rice accumulating soybean Kunitz trypsin inhibitor (SKTI) or CpTI were as well resistant to the brown planthopper *Nilaparvata lugens* (Lee *et al*., 1999) and to *Chilo suppresalis* and *Sesamia inferens* (Xu *et al*., 1996), respectively. Expression of a cysteine proteinase inhibitor in poplar trees, in turn, resulted in increased toxicity to *Chrysomela tremulae* (Leplé *et al*., 1995), whereas transgenic potato plants expressing an engineered *oryzacystatin-I* gene showed effective nematode resistance in field trials (Urwin *et al*., 2001), thus proving usefulness of these inhibitors at integrated pest control.

The ability of some insect species to compensate for protease inhibition by switching on to alternative proteolytic activities, however, has questioned practical application of these genes in plant protection (Broadway, 1996; Jongsma and Bolter, 1997). Feeding of *Spodoptera exigua* larvae with *PI-II* transgenic tobacco leaves, for example, did not affect larval growth, these larvae being able to compensate for the loss of tryptic activity by induction of new protease activities insensitive to PI-II inhibition (Jongsma *et al*., 1995). Opposite effects were also observed by feeding *Spodoptera littoralis* larvae on tobacco and Arabidopsis transgenic plants expressing the trypsin inhibitor MTI-2 from mustard (De Leo *et al*., 1998). Whereas a deleterious effect was observed for plants expressing high levels of MTI-2, both increased larval weight and higher leaf damage were observed in plants expressing lower levels of this inhibitor. Potato lines expressing the *oryzacystatin I* (*OCI*) gene from rice or the *cathepsin D inhibitor* (*CDI*) gene from tomato, showed also limited effect on Colorado beetle, with over-production of inhibitor-insensitive proteases and a hypertrophic behaviour observed in larvae fed on these transgenic lines (Cloutier *et al*., 2000; Brunelle *et al*., 2004). These results stressed on the need of new strategies based on the combined use of two or more inhibitors or hybrid defense proteins to achieve higher insecticidal activity and a broader protective spectra, at the time that minimize development of resistance in insect populations.

In this study, we have used combined expression into transgenic tomato plants of two potato inhibitors with inhibitory activity against trypsin/chymotrypsin-type serin-proteases (PI-II) and carboxypeptidase A metallo-proteases (PCI). Transgenic lines were assayed for increased resistance to the fruit worm *Heliothis obsoleta* and the serpentine leafminer *Liriomyza trifolii*, two common pests causing important economical losses in greenhouse cultivated tomatoes. Plants accumulating high levels of the PI-II and PCI transgenes were selected by RNA blot analysis and self-pollinated to homozygosis. Levels of these inhibitors were quantified by western blot detection and titration of carboxypeptidase A activity and estimated to be 1.4% of the total soluble protein in homozygous lines and 0.6% in the hemizygotes.

Effects of these transgenes in plant protection were assessed both in homo- and hemizygote lines, with significant levels of protection observed for the homozygote lines but not for the hemizygotes, which showed heavier infestation and increased plant damage with respect to the controls. Feeding bioassays carried out with *Heliothis* larvae showed that high-level transgene expression in the homozygote lines causes a strong reduction in larval weight and delayed larval growth, over-expression of these inhibitors thus providing effective protection to fruit worm attack. Feeding studies were carried out on detached leaves which were daily substituted by fresh ones. Therefore, even leaf damage produced by larval feeding is likely to induce endogenous defense gene expression; larvae should be exposed to these endogenous proteins only during the later part of the day (8–12 h following wounding), when fresh leaves are provided. Effects of endogenous defenses on larvae reared on control leaves should therefore be minimal, in opposite to larvae reared on the transgenic leaves which are permanently exposed to the expressed inhibitors. Exposure to sub-toxic inhibitor concentrations in the hemizygotes, induced a compensatory response in the insect that led to increased feeding and faster larval growth, a higher percentage of larvae reared on these plants

reaching the L4 developmental stage than in controls. Analysis of digestive activity in these larvae showed a decrease in proteases sensitive to soybean–BBI, with a reduction in BBI-inhibition from 75% in the guts of insects fed on the untransformed controls to 40% in the insects reared on these plants. This is indicative of a 1.6-fold increase in BBI-insensitive digestive proteases, these proteases then compensating for the partial loss of activity caused by the presence of the inhibitors. Interestingly, very similar results were also obtained in bioassays with *Liriomyza* larvae, with good levels of protection against insect infestation observed in homozygote lines, but negative effects observed in hemizygote lines expressing lower-level of the transgenes. Larval mortality was reduced in these plants and the number of mines was higher than in the controls, number of recovered pupae per plant being also higher than in untransformed plants. These bioassays, unlike *Heliothis* studies, were carried out *in planta*. Therefore, endogenous defense genes induced by larval feeding would be expected in these assays to affect larval growth in control leaves and to add to the effects of the recombinant proteins in the transgenic plants. However, comparable results were observed in both insect bioassays, indicating that mechanical damage produced by *Liriomyza* larval feeding does not induce a strong natural defense response in the plant.

Opposite effects similar to the ones we have observed, were also reported in bioassay studies with other insects (Bolter and Jongsma, 1995; Jongsma and Bolter, 1997; Broadway 2000). A difficulty in engineering plant resistance by means of nutritional reducer molecules in fact derives from their relatively slow mode of action. Minimal equimolar concentrations of PIs needed to achieve full inhibition of gut protease activity were estimated to be in the range of 10–30 μM, which corresponds to about 0.5–1.5% of total soluble proteins in leaves (Jongsma and Bolter, 1997). Only in the presence of such large amounts of inhibitor, toxicity of these proteins exceeds the adaptive response in the insects, with the consequent detrimental effect on larval growth.

In our bioassays, levels of the inhibitors as high as 1% of the total soluble leaf proteins (in the homozygotes) were also required for a negative effect on *Heliothis* and *Liriomyza* larvae. Although transgenic lines expressing each inhibitor alone were not obtained, the observation that high-levels of transgene expression are still required in the PI-II/PCI-expressing plants suggests that co-expression of these two inhibitors does not lead to a substantial change in toxicity towards the insects. These results would indicate that combined expression of two inhibitors does not result in a synergistic effect on insect deterrence, elevated concentrations of each transgene still required for effective pest control. Comparable adaptive responses were, on the other hand, observed for both tested insects, although *Liriomyza* digestive proteases were better targets to the expressed inhibitors than proteases present in *Heliothis* midguts. This would suggest that the mechanism of insect adaptation is relatively independent to the array of proteases present in the insect midgut and that similar compensation mechanisms are induced in the insects regardless of their protease arrays and feeding habits, i.e. leafminer and chewing insects.

Analysis of the digestive activity of *Heliothis* larvae reared on hemizygotes, showed that insect adaptation relies on the production of additional proteases that are not affected by the expressed inhibitors. The mechanisms underlying such increase in production of insensitive proteases and how this is linked to an increase in leaf consumption is still poorly understood. Also, it is unclear whether such compensatory response represents a general, non-specific response to the accumulation of anti-digestive compounds in the diet, or if specific proteases are induced depending on the type of defense genes induced in the host plant. Colorado potato beetles reared on methyl jasmonate- or arachidonic-acid induced potato plants accumulated different complements of protease activities, which suggests that digestive compensation is determined, at least in part, by the repertoire of defense-related compounds present in the plant (Rivard *et al*., 2004). However, studies in *Helicoverpa armigera* reared on artificial diets containing either a trypsin-specific, the chymotrypsin-specific chymostatin inhibitor, or a non-specific serine-protease inhibitor, showed that larvae of these insects respond to the presence of these different inhibitors by similar down-regulation of genes of the trypsin gene group and up-regulation of genes of the chymotripsin/elastase group (Gatehouse *et al*., 2002). This observation agrees with our results and indicates that such compensatory response occurs irrespectively

of the particular PI expressed in the plant, being more related to long-term changes in digestive protease expression induced in response to the total dietary protein intake or midgut proteolytic activity, than to a specific mechanism of regulation similar to that found to be involved in regulation of serine protease expression in mammals, via a monitor peptide feedback loop (Fushiki *et al.*, 1989; Spannagel *et al.*, 1996). Reductions in protein uptake were indeed reported to mimic the effects of PI addition to the diet, thus supporting a non-specific mechanism of digestive protease regulation in insects (Gatehouse, *et al.*, 2002).

An important outcome from these results is that combined use of two PIs appears not to be very effective in avoiding digestive compensatory responses in the insect. In our bioassays, insects were still able to cope with sub-lethal levels of the PI proteins by inducing the accumulation of additional sets of digestive enzymes. Therefore, it might be anticipated that combined expression of PI proteins together with other toxic proteins of completely different modes of action, such as the *Bt* endotoxin, the carbohydrate-binding lectins or other insecticidal proteins, will be more effective at enhanced insect control and stable resistance than use of different proteinase inhibitor proteins. In this respect, expression of the snowdrop lectin gene (*gna*) from *Galanthus nivalis* in transgenic rice has been shown to confer substantial protection against sap-sucking insects such as small brown planthopper and green leafhopper (Wu *et al.*, 2002; Nagadhara *et al.*, 2003). Gene pyramiding by expression of proteinase inhibitor fusions to these insecticidal proteins might then be an attractive option for integrated pest management since a recombinant fusion of the soybean cysteine protease inhibitor to the rGSII lectin from *Griffonia simplicifolia* was recently shown to synergistically enhance anti-insect activity of the soybean cystatin inhibitor by tethering it to the insect gut epithelium, thus increasing the inhibitor midgut perduration (Zhu-Salzman *et al.*, 2003).

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