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Successive Use of Non-Host Plant Proteinase Inhibitors Required for Effective Inhibition of *Helicoverpa armigera* Gut Proteinases and Larval Growth[1]

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We report on the efficacy of proteinase inhibitors (PIs) from three host plants (chickpea [*Cicer arietinum*], pigeonpea [*Cajanus cajan*], and cotton [*Gossypium arboreum*]) and three non-host (groundnut [*Arachis hypogea*], winged bean [*Psophocarpus tetragonolobus*], and potato [*Solanum tuberosum*]) in retarding the growth of *Helicoverpa armigera* larvae, a devastating pest of important crop plants. Enzyme assays and electrophoretic analysis of interaction of *H. armigera* gut proteinases (HGPs) with PIs revealed that non-host PIs inhibited HGP activity efficiently whereas host PIs were ineffective. In the electrophoretic assay, trypsin inhibitor activity bands were detected in all of the host and non-host plants, but HGP inhibitor activity bands were present only in non-host plants (except cotton in the host plant group). *H. armigera* larvae reared on a diet containing non-host PIs showed growth retardation, a reduction in total and trypsin-like proteinase activity, and the production of inhibitor-insensitive proteinases. Electrophoretic analysis of PI-induced HGP showed differential regulation of proteinase isoforms. Interestingly, HGP activity induced in response to dietary potato PI-II was inhibited by winged bean PIs. The optimized combination of potato PI-II and winged bean PIs identified in the present study and their proposed successive use has potential in developing *H. armigera*-resistant transgenic plants.

Helicoverpa armigera (Hübner) of the Lepidoptera family is a serious pest of many important crops and claims a major share in crop losses every year. It is a polyphagous pest of 181 plant species, including chickpea (*Cicer arietinum*), pigeonpea (*Cajanus cajan*), tomato (*Lycopersicon esculentum*), okra (*Abelmoschus esculentus*), and cotton (*Gossypium* species), and is expected to become an important pest in other crops such as sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*), maize (*Zea mays*), tobacco (*Nicotiana tabacum*), and groundnut (*Arachis hypogea*) (Manjunath et al., 1989). Larvae of *H. armigera* are voracious foliar feeders as early instars and later shift to the developing seeds, fruits, or bolls, leading to drastic reductions in yield (Reed and Pawar, 1982).

Exogenous chemical means to counteract *H. armigera* attack have become less feasible, mainly due to the development of pesticide resistance in insects and inherent possible environmental hazards (Armes et al., 1996). The use of genetic engineering technology for the transformation of crop plants for insect resistance has created access to genes that were otherwise beyond the scope of conventional breeding. The *Bacillus thuringiensis* (Bt) endotoxin gene has been successfully expressed in several crops to impart resistance against herbivorous insects (for review, see Jouanin et al., 1998; Schuler et al., 1998). However, insects have developed resistance to Bt endotoxin by producing a proteinase(s) that inactivates the toxin (Oppert et al., 1996; Michaud, 1997) or by lacking the proteinase allele required for activation of Bt protoxin (Oppert et al., 1997).

The development of pest-resistant transgenic plants expressing genes of PIs, amylase inhibitors, and lectins of plant origin is another approach that needs further exploration (Ryan, 1990; Boulter, 1993; DeLeo et al., 1998; Jouanin et al., 1998; Schuler et al., 1998). The use of PIs in developing insect resistance in transgenic plants is of dual benefit, as they inhibit insect mid-gut proteinases, thereby protecting other defense proteins from proteolytic degradation (Michaud, 1997). PIs block digestive proteinases in insect guts and starve them of essential amino acids (Broadway and Duffey, 1986; Ryan, 1990). They also affect a number of vital processes, including proteolytic activation of enzymes and molting (Hilder et al., 1993). Although plant PIs inhibit growth of insects, they do not lead to high selection pressure compared with the "wipeout" approach executed by other pest control measures (including Bt toxin). This minimizes the possibility of developing resistance in the insect population against PIs. Another merit of this approach lies in the fact that PIs are a plant's own natural defense response against phytophagous insects. PIs are present in the leaves and storage tissues, and are shown to be induced upon wounding, thereby significantly reducing the insect attack (Green and Ryan, 1972; Howe et al., 1996).

During our initial efforts to study the biochemical basis of chickpea-*H. armigera* interactions, we showed that the insect proteinases degrade the trypsin inhibitors (TIs) of chickpea, making it completely defenseless (Giri et al., 1998). We also demonstrated the presence of six isoprotein-

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ases in the *H. armigera* gut possessing diverse specificity (Harsulkar et al., 1998). Furthermore, we analyzed several cultivars and wild relatives of chickpea for potent inhibitors of HGP and observed that not more than a 35% inhibition of HGP was obtained (Patankar et al., 1999). These results established the biochemical basis for the susceptibility of chickpea and provided clues to explain the polyphagous nature of *H. armigera*.

In an effort to identify potential inhibitors of HGP, we screened several non-host plants and found that winged bean, groundnut, and potato PIs (PI-I, PI-II, and PI-III) are promising candidates. The present work was aimed at evaluating in vitro and in vivo effects of host and non-host plant PIs on *H. armigera*. The host group of plants selected for this study included chickpea, a wild relative of chickpea (*Cicer echinospermum*), pigeonpea, a wild relative of pigeonpea (*Cajanus scaraboides*), and cotton (*Gossypium arboreum*). The activity and in vitro stability studies of host and non-host plant PIs on the basis of their inhibition potential toward HGP were carried out. Feeding assays were performed to ascertain the potency of the inhibitors in inhibiting the growth of *H. armigera* larvae. The results provide the basis for the selection of a few non-host PIs and present an optimized combination for developing *H. armigera*-resistant transgenic plants.

MATERIALS AND METHODS

HGP Preparation

Helicoverpa armigera larvae were reared on a basal or supplemental diet or collected from chickpea fields of the Pulse Research Station (Mahatma Phule Krishi Vidyapeeth, Rahuri, India). Mid-guts isolated by dissecting the larvae were stored at −20°C till further use. For extraction, the gut tissue was mixed with 3 volumes of 0.1 M Gly-NaOH buffer, pH 10.0, and allowed to stand for 15 min. The gut luminal contents were removed by centrifugation at 10,000*g* for 10 min at 4°C. The resulting supernatant was analyzed for proteinase activity in assays and on gels.

Extraction of PIs

Seeds of chickpea (*Cicer arietinum* cv Vijay), pigeonpea (*Cajanus cajan* cv BDN-2), groundnut (*Arachis hypogea*), and cotton (*Gossypium arboreum* cv K-32) were obtained from the Pulse Research Station and Oilseed Research Station (Mahatma Phule Krishi Vidyapeeth, Rahuri, India). Seeds of a wild relative of chickpea (*Cicer echinospermum*) and a wild relative of pigeonpea (*Cajanus scaraboides*) were obtained from International Crops Research Institute for Semi Arid Tropics (Patancheru, India), while seeds of winged bean (*Psophocarpus tetragonolobus* cv iiHp Sel 21) were obtained from National Bureau of Plant Genetic Resources, (Akola, India). Purified potato PI-I, PI-II (Bowman-Birk type), and PI-III (Kunitz type) were provided by Prof. C.A. Ryan (Washington State University, Pullman). Dry seeds were ground to a fine powder, defatted, and depigmented with several washes of hexane and acetone. The solvents were filtered off and the seed powders were obtained upon air-drying. Proteins in the seed powder was extracted overnight in 6 volumes of distilled water at 4°C. The suspension was centrifuged at 10,000*g* for 20 min at 4°C. Supernatant was collected and stored frozen in aliquots. Protein in the aliquot was determined by Bradford's method (Bradford, 1976).

Proteinase and PI Assay

Total gut proteinase activity was measured by caseinolytic (Belew and Porath, 1970) and azo-caseinolytic assays (Brock et al., 1982). Trypsin and chymotrypsin-like activities were estimated using the chromogenic substrates benzoyl-arginyl *p*-nitroanilide (BA*p*NA) (Erlanger et al., 1964) and *n*-glutaryl 1-Phe *p*-nitroanilide (GLUPHEPA) (Mueller and Weder, 1989). Caseinolytic and BA*p*NAase assays were similar assays described previously (Giri et al., 1998). For azo-caseinolytic assay, 60 μL of diluted enzyme was added to 200 μL of 1% azo-casein (in 0.1 M Gly-NaOH buffer, pH 10.0) and incubated at 37°C for 30 min. The reaction was terminated by the addition of 300 μL of 5% TCA. After incubation at room temperature for 30 min, tubes were centrifuged at 10,000*g* for 10 min. An equal volume of 1 N NaOH was added to the supernatant and activity was estimated by measuring the OD at 450 nm.

Bovine chymotrypsin (25 μg) or HGP extract was added to the different tubes, and the volume was made up to 700 μL with 0.2 M Gly-NaOH buffer, pH 10.0. Twenty-five microliters of GLUPHEPA (10 mg/mL in dimethyl formamide) was added to each tube and the reaction mixture was incubated at 37°C for 1 h. The reaction was terminated by the addition of 200 μL of 30% acetic acid and the OD was measured at 405 nm. For the inhibitor assays, a suitable volume of seed extract was added to the HGP extract or to the respective proteinase and incubated at room temperature (27°C) for 15 min. The residual proteinase activity was estimated as described above. One proteinase unit was defined as the amount of enzyme that increases absorbance by 1 OD/min, and one PI unit was defined as the amount of inhibitor that causes inhibition of 1 unit of proteinase activity under the assay conditions.

Visualization of Isoforms of Proteinases and PIs

Visualization of proteinase isoforms after native and denatured electrophoretic gels (SDS-PAGE) was carried out as described in detail earlier (Harsulkar et al., 1998). TI bands were visualized by the gel x-ray film contact print technique (Pichare and Kachole, 1994; Giri et al., 1998). Gels co-polymerized with 1% gelatin were used for the detection of TI and HGP inhibitor (HGPI) bands (Felicioli et al., 1997). After electrophoresis, the gels were equilibrated in 0.1 M Tris-HCl buffer, pH 7.8, for TI activity, and in 0.2 M Gly-NaOH buffer, pH 10.0, for HGPI activity. The respective gels were transferred to solutions containing 0.1% trypsin or HGP of equivalent activity, and incubated for 1 to 2 h with constant shaking. The gels were then washed with warm water, fixed in 10% TCA, stained with Coomassie Brilliant Blue R-250, and destained. Dark blue bands of

unhydrolyzed gelatin appeared at the site of PI activity against the faint blue background.

Treatment of PIs with HGP

To confirm the stability of PIs, equal amounts of inhibitor from host or non-host plants were treated with HGP at 37°C for 30 min and 3 h, and PI activity was estimated as described above in solution assays. HGP-treated seed extracts were also analyzed on the gel as described above to detect the trypsin iso-inhibitors stable to gut proteinases.

Feeding Assay

Bioassays were conducted by feeding *H. armigera* larvae on host or non-host PIs incorporated into an artificial diet (Giri and Kachole, 1998). Composition of the diet was as follows (for 1 L): 140 g of chickpea seed meal, 14 g of yeast extract, 0.4 g of Bavistin (BASF, Mumbai, India), 0.2 mL of formalin, 4.3 g of ascorbic acid, 1.3 g of sorbic acid, 2.6 g of methyl benzoate, 0.5 g of tetracycline, one tablet of vitamin-B complex, and two drops of vitamin E were added to 450 mL of distilled water. To this mixture 17 g of agar dissolved in 500 mL of water (50°C–60°C) was added, mixed thoroughly, and poured into trays. Cubes of feed (2 g) were cut and used for the feeding experiments. The basic diet was supplemented with the seed extracts of host or non-host plants in appropriate quantities to give equal TI units (3 units/g of feed). Forty early second instar larvae were reared on this diet and any gain in weight was meticulously recorded on every 2nd d until pupation. The experiment was repeated at least three times.

RESULTS

Inhibition of HGP by Host and Non-Host Plant PIs

Several host and non-host plants were analyzed for inhibition of HGP activity, and only a few non-host plant PIs showed complete inhibition. Inhibition of HGP was studied at pH 7.8 and 10 because two groups of proteinases showing activity at specific pHs were identified earlier in the HGP complement (Harsulkar et al., 1998). Table I gives an account of the efficiency of inhibition of HGP activity by various plant PIs. A close examination of the data shown in Table I revealed that PIs from the host group of plants comprising chickpea, pigeon pea, and cotton showed 45%, 55%, and 38% inhibition of HGP activity at pH 7.8, and 33%, 48%, and 40% inhibition at pH 10.0, respectively. *C. echinospermum* PIs showed 38% inhibition at pH 7.8 and 33% at pH 10.0, while *C. scaraboides* PIs did not inhibit HGP activity. On the other hand, PIs from the non-host plants of *H. armigera* (groundnut and winged bean), along with potato PIs (PI-I, PI-II, and PI-III), showed total inhibition of HGP activity at both pHs except groundnut PIs, which inhibited HGP activity up to 84% at pH 10.

Another approach of electrophoretic visualization of inhibition of HGP isoforms by host and non-host plant PIs indicated that major HGPs were insensitive to chickpea and pigeonpea PIs (Fig. 1, lanes 2 and 3). Among the non-host PIs, those from winged bean effectively inhibited all of the HGP isoforms (Fig. 1, lane 5), whereas groundnut PIs and potato PI-II inhibited all isoforms and partially inhibited HGP-1 (Fig. 1, lanes 4 and 6). Based on the data shown in Table I and Figure 1, it can be concluded that non-host PIs are able to inhibit total proteinase activity and almost all of the isoforms of HGP effectively compared with the host plant PIs, which are poor inhibitors of HGP.

In Vitro Stability of PIs to HGP

The in vitro stabilities of host and non-host plant PIs against HGP were evaluated by enzyme assays after incubation with 0.02 BA*p*NAase units of HGP for 30 min and 3 h (Table II). In vitro stability of the host and non-host PIs against HGP was reflected by the extent of inhibition after HGP treatment for 30 min or 3 h. Interestingly, after proteolysis by HGP for 3 h, chickpea and pigeonpea PIs showed a modest increase in the inhibition of HGP. The non-host PIs, on the other hand, showed total inhibition of HGP even after incubation for 3 h.

Figure 2 reveals the stability profiles of TIs of host and non-host plants after treatment with HGP. Host and non-host seed extracts were treated with HGP for 30 min and 3 h, and TIs were visualized using the gel-x-ray film contact print technique. Stability profiles indicated that chickpea TIs were degraded by HGP, leading to the generation of active TI fragment(s) after 30 min. Incubation for 3 h led to the generation of one more TI fragment (Fig. 2A). Like chickpea TIs, *C. echinospermum* TIs were proteolyzed by HGP. Among the fast-moving four TI bands in pigeonpea, the first two bands were not stable upon 3 h of incubation with HGP, while the other two remained stable even after 3 h of incubation with HGP (Fig. 2B). *C. scaraboides* TIs were degraded after 30 min of incubation with HGP, leading to

Table I. *Inhibition potential of host and non-host plant PIs against gut proteinase activity of H. armigera*

Activity assays were performed at pH 7.8 and pH 10.0. A double concentration of gut extract was required to obtain equivalent units of BA*p*NAase activity at pH 7.8 than at pH 10.0. Five different concentrations of inhibitor extract were used to assess the potential of each inhibitor for inhibiting HGP activity. The maximum possible inhibition of HGP due to respective PIs is given in the table. The assays were performed as described in "Materials and Methods."

Plant PI	Maximum Inhibition of HGP Activity	
	pH 7.8	pH 10.0
	%	
Host plants		
Chickpea	45	33
C. echinospermum	38	33
Pigeonpea	55	48
C. scaraboides	00	00
Cotton	38	40
Non-host plants		
Groundnut	100	84
Winged bean	100	100
Potato PI-I	100	100
Potato PI-II	100	100
Potato PI-III	100	100

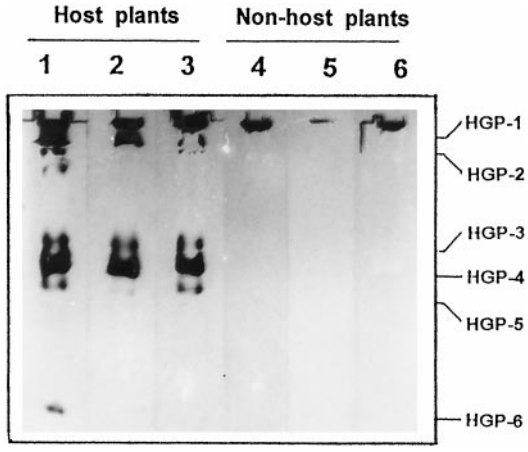

Figure 1. Inhibition of HGP isoforms by inhibitors of host and non-host plants. HGP isoforms after electrophoresis were incubated with host and non-host PIs and then visualized by the gel-x-ray film contact print technique as described in "Materials and Methods." A total of 0.02 BA*p*NAase unit HGP extract was loaded in each well. Lane 1, Control; lanes 2 to 6, HGP-resolved strips incubated in PIs of chickpea, pigeonpea, groundnut, winged bean, and potato PI-II, respectively.

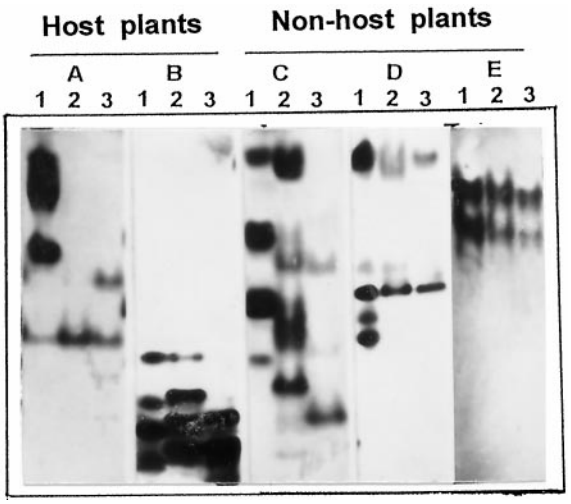

Figure 2. Stability of host and non-host plant TIs toward HGP. Seed extracts were incubated with 0.02 BA*p*NAase units of HGP for 30 min and 3 h at 37°C. TI bands were visualized as described in "Materials and Methods." Lanes 1, Control (without preincubation with HGP); lanes 2, incubated with HGP for 30 min; lanes 3, incubated with HGP for 3 h. A, Chickpea; B, pigeonpea; C, groundnut; D, winged bean; and E, potato PI-II.

the formation of four stable TI activity fragments (results not shown).

In the non-host group of plants, upon 30 min of incubation with HGP, TI isoforms of groundnut showed six fragments having TI activity, of which only three remained stable with increasing time (Fig. 2C). In winged bean, one TI was resistant to proteolysis by HGP; however, slow-moving TIs showed partial degradation and fast-moving bands disappeared after 3 h of incubation (Fig. 2D). The native form of potato PI-II was stable to proteolysis by HGP; however, there was partial proteolysis, as evident by the decreased intensity of the bands on incubation for 30 min and 3 h (Fig. 2E).

The above results suggest that most of the native TI isoforms were susceptible to proteolysis by HGP except potato PI-II and a winged bean TI to a certain extent. On limited proteolysis, some TIs generated products possessing equivalent or stronger inhibitor activities. Increased inhibition of HGP in enzyme assays in chickpea and pigeonpea might be the result of the formation of modified inhibitor fragments exhibiting higher affinity toward HGP. During incubation of purified chickpea TI with HGP, a transitory increase in HGP inhibition from 33% to 47% was observed, but with longer exposure it decreased significantly (results not shown). Although native forms of a few TIs of non-host plants were susceptible to partial proteolysis by HGP, the inhibition potential was not altered, as they showed total inhibition of HGP in the enzyme assays (Table II). This suggests that the remaining concentration of

Table II. *In vitro stability of host and non-host plant PIs against HGP*

Inhibitors were preincubated with HGP for 30 min and 3 h at 37°C and then assayed for their inhibitory activity toward HGP as described in "Materials and Methods." Each value is an average of three replicates ± SE.

Plant PI	Inhibition of HGP Activity	
	30 min	3 h
	%	
Host plants		
Chickpea	33 ± 1.5	47 ± 1.4
Pigeonpea	48 ± 1.0	55 ± 1.3
Non-host plants		
Groundnut	84 ± 2.0	100 ± 0.0
Winged bean	100 ± 0.0	100 ± 0.0
Potato PI-II	100 ± 0.0	100 ± 0.0

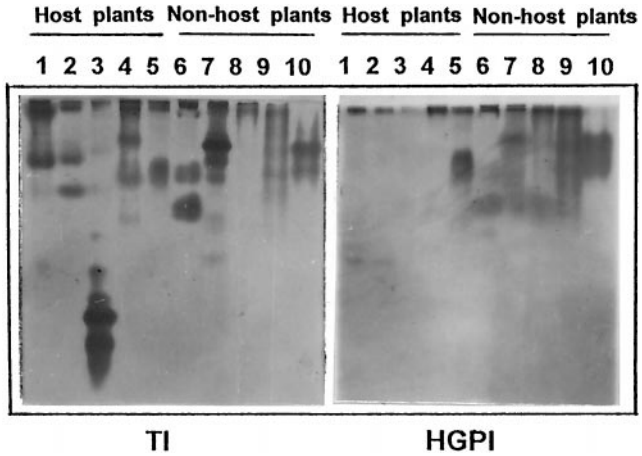

Figure 3. TI and HGPI profiles of host and non-host plants. TI and HGPI bands were visualized as described in "Materials and Methods." Equal TI units were loaded on both gels. Lanes 1, Chickpea; lanes 2, *C. echinospermum*; lanes 3, pigeonpea; lanes 4, *C. scarabioides*; lanes 5, cotton; lanes 6, groundnut; lanes 7, winged bean; lanes 8, potato PI-I; lanes 9, PI-II; and lanes 10, PI-III.

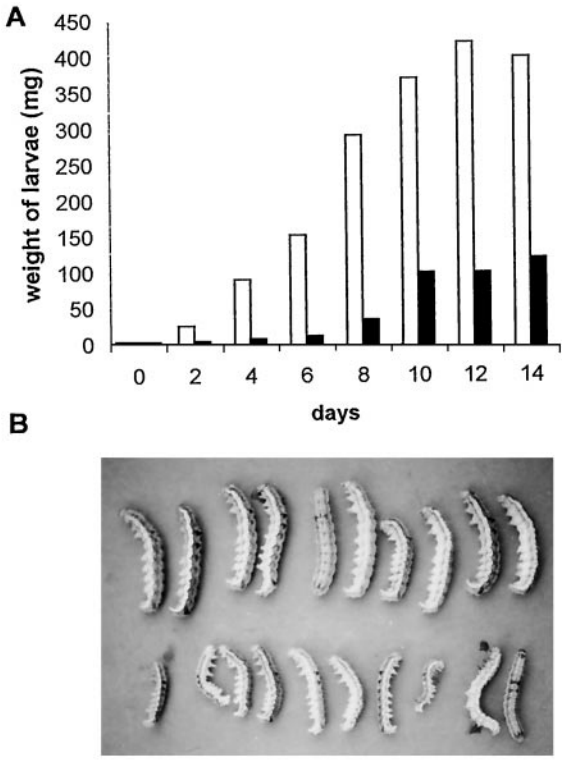

Figure 4. Development of *H. armigera* reared on artificial diet containing host (chickpea) and non-host (groundnut) plant PIs. A, Weight of larvae grown on diet containing chickpea PIs (□) and groundnut PIs (■). Weights of larvae were critically measured on every alternate day. B, Photograph of larvae grown on a diet containing chickpea PIs showing normal growth (upper row) and on a diet containing groundnut PIs showing stunted growth (lower row).

partially proteolyzed TIs of non-host plants is enough for total inhibition of HGP in the enzyme assay.

Trypsin and HGP Isoinhibitors in Host and Non-Host Plants

Figure 3 shows the electrophoretic profiles of TIs and HGPIs in seed extracts of host and non-host plants. Several bands of TI activity were present in both the host and non-host plants. Chickpea and *C. echinospermum* exhibited three TI bands (Fig. 3, lanes 1 and 2). In pigeonpea, five fast-migrating TIs were detected (Fig. 3, lane 3), while in *C. scaraboides*, four TI bands were observed (Fig. 3, lane 4). HGPI bands were absent in the host group comprising chickpea, *C. echinospermum*, pigeonpea, and *C. scaraboides* (Fig. 3, lanes 1–4), while cotton TI appeared as a HGPI band (Fig. 3, lanes 5). In the non-host group, winged bean showed six TI bands, out of which three possessed HGPI activity (Fig. 3, lanes 6). The fast-moving TI bands of winged bean did not have inhibitory activity against HGP. Groundnut seed extract revealed four bands having both TI and HGPI activity (Fig. 3, lanes 7).

The potato inhibitors PI-I, PI-II, and PI-III resolved into several TI and HGPI activity bands. Potato PI-I showed a slow-moving band active against trypsin and HGP, while PI-II showed a smear with two more bands active against trypsin and HGP (Fig. 3, lanes 8 and 9). PI-III exhibited two activity bands against trypsin, which showed higher intensity against HGP (Fig. 3, lanes 10). The results indicate that no potential inhibitor(s) of HGP were detected in chickpea, pigeonpea, and their wild relatives. In cotton the observed 40% inhibition of HGP in the enzyme assay corresponded to a single inhibitor on the activity gel. However, in chickpea and pigeonpea, HGP inhibitory activity might be distributed among the several isoinhibitors. Individual HGPI band(s), therefore, could not be detected on activity gels. To our knowledge, this is the first study in which specific insect PIs have been detected using an in-gel assay.

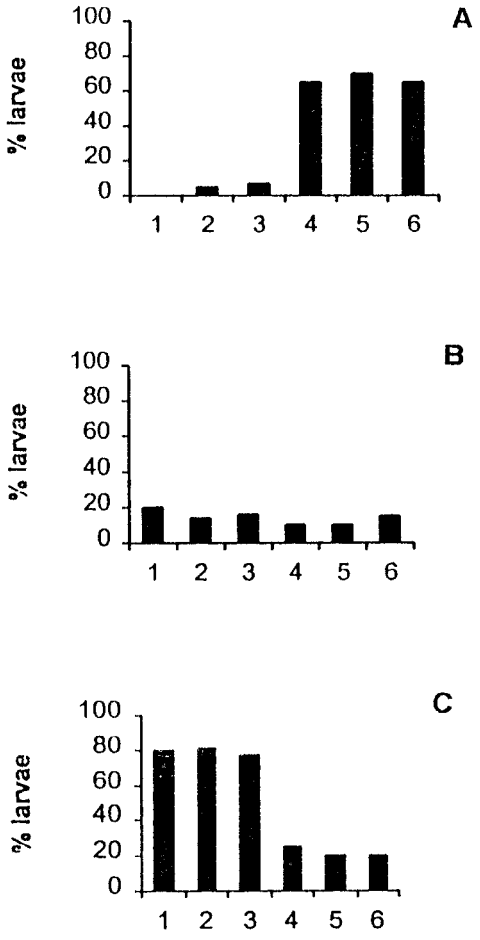

Figure 5. In vivo effects of host and non-host plant PIs on the development of larval *H. armigera*. Early second instar larvae were reared on an artificial diet supplemented with equal TI units of host and non-host seed extracts, as described in "Materials and Methods." Weights of larvae were recorded every alternate day. The larvae were classified into three groups based on their weights. A, Stunted (0.02–0.2 g); B, intermediate (0.21–0.4 g); and C, normal (0.41 g and above) growth of larvae fed on control diet (1) or a diet containing PIs of chickpea (2), pigeonpea (3), groundnut (4), winged bean (5), or potato PI-II (6).

Table III. *Gut proteinase activity of H. armigera larvae reared on host and non-host plant PIs*

Larvae fed on control diet and PI-containing diet were dissected after 8 d, and proteinase activity was estimated using different substrates as described in "Materials and Methods." Values in parentheses are the percentages of the proteinase activity of control.

HGP	Estimable Proteinase Activity per 10 Guts			
	Caseinase	Azocaseinase	BA*p*NAase	GLUPHEPHAase
Control	4.65 (100)	4.82 (100)	14.14 (100)	0.013 (100)
Host plants				
Chickpea	4.23 (90.9)	3.48 (72.2)	12.61 (89.2)	0.013 (100)
Non-host plants				
Groundnut	1.66 (35.7)	1.47 (30.5)	3.12 (22.1)	0.000 (0.00)
Winged bean	1.74 (37.4)	1.42 (29.5)	2.70 (19.1)	0.003 (23.1)
Potato PI-II	1.72 (37.0)	1.45 (30.1)	6.01 (42.5)	0.005 (38.5)

Effect of Host and Non-Host Plant PIs on Growth and Development of *H. armigera* Larvae

To estimate the in vivo effects of host and non-host plant PIs on the development of *H. armigera* larvae, feeding trials were conducted with the appropriate controls. Typical development of larvae reared on a diet containing host PIs (chickpea PIs as representative of the host group) and on a diet containing non-host PIs (groundnut PIs as representative of the non-host group) is shown in Figure 4. There was a 3- to 4-fold reduction in weight gain in the larvae fed with non-host PIs. Food intake was drastically reduced in the larvae showing growth retardation. Furthermore, pupation was also delayed for more than 10 d in larvae showing stunted growth.

On the basis of weight gain of larvae on the 10th d, the larval population was distributed into three groups: (a) stunted growth (0.02–0.2 g); (b) intermediate growth (0.21–0.4 g); and (c) normal growth (0.41–0.6 g). More than 65% of the larvae fed on potato PI-II or PIs of winged bean or groundnut showed stunted growth (Fig. 5A). A small percentage of larvae showed intermediate growth when fed host or non-host PIs (Fig. 5B). As seen in Figure 5C, 81% and 77% of larvae showed normal growth when grown on a chickpea and pigeonpea PI-containing diet, respectively. We also observed that the instar stage of *H. armigera* larvae was critical for assessing the potential of dietary inhibitors. From the above results, it can be suggested that the inhibitor concentration of non-host PIs used in the diet was sufficient to inhibit growth of early second instar larvae.

Alteration and in Vivo Inhibition of Gut Proteinases in *H. armigera* Larvae Reared on PIs

To understand the in vivo effectiveness of non-host PIs, *H. armigera* larvae fed on a control diet or on PIs of chickpea, groundnut, winged bean, or potato PI-II were dissected after 8 d and the midgut proteinase activity was estimated (Table III). Proteinase activity of control larvae was considered as 100% and the proteinase activity of PI-fed larvae was calculated accordingly. The larvae fed on chickpea PIs showed 91% caseinolytic and 72% azocaseinolytic activities, whereas in non-host PI-fed larvae the caseinolytic and azocaseinolytic activities were in the range of 35% to 37% and 29% to 30%, respectively. BA*p*NAase activity, which measures trypsin-like proteinases, was found to be lowest (19%) in winged bean PI-fed larvae, 22% in groundnut PI-fed larvae, 42% in potato PI-II fed larvae, and 89% in chickpea PI-fed larvae. Very low GLUPHEPAase activity was observed in the guts of control and PI-fed larvae. Larvae reared on non-host PIs showed a significant decrease in estimable proteinase activity, suggesting that native inhibitors or their fragments were active in the larval gut.

To assess the induction of inhibitor-insensitive proteinase activity, the inhibition potential of maximum amounts (concentration greater than that required to inhibit total proteinase activity of control HGP) of winged bean PIs and potato PI-II required to inhibit the gut extracts of *H. armigera* larvae fed the same PIs and vice versa were determined (Table IV). The larvae fed winged bean showed 27% inhibitor-insensitive activity, while those fed potato PI-II showed only 5% inhibitor-insensitive activity. Interestingly, winged bean PIs were able to inhibit as much as 96% of the HGP activity induced by potato PI-II, while potato PI-II was able to inhibit only 47% of the HGP activity induced by winged bean PIs.

To determine if the complement of gut proteinases of *H. armigera* changed following PI ingestion, gut extracts were separated on non-reducing SDS-polyacrylamide gels. The gut proteinases of larvae fed a control diet and those who were fed a diet containing plant PIs showed significant differences in the expression of individual proteinases (Fig.

Table IV. *Inhibition of gut proteinase activity of H. armigera larvae reared on winged bean PIs or potato PI-II*

Inhibition potential of winged bean PIs and potato PI-II was assessed against gut proteinases of *H. armigera* reared on the winged bean PIs or on the potato PI-II. Inhibitory activity was estimated by taking various concentrations of inhibitor to obtain maximum inhibition of proteinase activity. Activities were estimated using azocasein as a substrate, as described in "Materials and Methods."

HGP from Larvae Reared on	Inhibition of HGP Activity by	
	Winged bean PIs	Potato PI-II
	%	
Control	100	100
Winged bean PIs	73	47
Potato PI-II	96	95

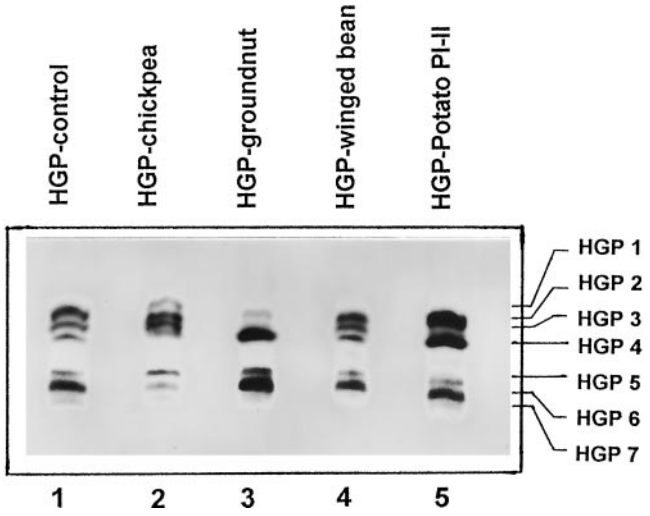

Figure 6. Isoproteinase profiles of *H. armigera* larvae fed on a diet containing chickpea or non-host PIs. Equal amounts of HGP extract of insects fed on control (lane 1), or on PIs of chickpea (lane 2), groundnut (lane 3), winged bean (lane 4), or potato PI-II (lane 5) were loaded on SDS-polyacrylamide gels and visualized as described in "Materials and Methods."

6). Gut proteinases of larvae reared on chickpea PIs showed overexpression of HGP-1 and decreased expression of HGP-2 and HGP-6. Four isoproteinases, HGP-2, HGP-3, HGP-5, and HGP-6, could be detected in the guts of larvae reared on groundnut PIs; HGP-3 and HGP-6 were highly expressed. Interestingly, isoproteinases of larvae fed winged bean PIs showed a profile similar to that of the control. In larvae fed potato PI-II, HGP-2 and HGP-3 were overexpressed; however, HGP-5 and HGP-6 showed a trend similar to that of larvae fed a control diet. HGP-7 was detected only in the control and not in the gut extracts of larvae fed either host or non-host plant PIs. The above results indicate that there are significant quantitative and qualitative changes in the gut proteinases in response to dietary PIs.

DISCUSSION

It is an apparent paradox that insects feed on plants in spite of the fact that PIs are ubiquitous, especially in case of legumes. Insect pests adapt to host plant PIs by synthesizing proteinases that are either insensitive to inhibitors (Broadway, 1995, 1996, 1997; Jongsma et al., 1995) or have the capacity to degrade them (Michaud, 1997; Girard et al., 1998a; Giri et al., 1998). In a stabilized host-pest complex, insects have evolved and adapted to overcome the effect of PIs of their host plants (Bolter and Jongsma, 1995; Broadway, 1995, 1996, 1997; Jongsma et al., 1996). It is therefore necessary to study non-host plant PIs as potential sources to overcome the host inhibitor-insensitive proteinases of insect pests. The present work evaluates non-host PIs to establish their potential against HGP through a series of in vitro and in vivo experiments. For the first time to our knowledge, specific inhibitors of insect gut proteinases have been identified, and it has been unequivocally demonstrated that all TIs do not necessarily possess HGPI activity. HGPIs fed to *H. armigera* larvae induced insensitive proteinases and resulted in antibiosis. Another important finding of our study was that HGPs induced in response to one inhibitor were different from those induced by the other PI(s). A combination of potato PI-II and winged bean PIs is thus proposed for effective control of *H. armigera*.

Molecular Evolution in Pest Proteinases and Plant PIs

Proteolytic activity of insect guts comprises many isoforms having diverse properties and specificities (Johnston et al., 1991; Jongsma et al., 1996; Bown et al., 1997; Zhu et al., 1997; Harsulkar et al., 1998). The presence of isoproteinases of different specificities in the midgut has great significance for the survival and adaptation of phytophagous insects on several host plants. The adaptation of pests to host plant PIs probably results from the selection pressure acting on an entire insect population when they encounter PIs of their host plants. Laskowski et al. (1988) have proposed that structural compatibility between the plant PIs and the insect proteinases determines the level of inhibitory activity against specific proteinases. Structural variation occurring in gut proteinases followed by selection against host plant PIs may modify insect proteinases that, although of the same class, are insensitive to host plant PIs.

An alteration in an insect proteinase isozyme may result in less inhibitor binding, leading to successful predation. In order to survive, plants also must evolve their inhibitor proteins to effectively inhibit insect proteinases. A struggle at the molecular level appears to be a course of evolution in which the proteinases and their inhibitors are variable hotspots of evolutionary changes (Laskowski et al., 1988). Both pests and plants have therefore been evolving new forms of enzymes and inhibitors to counteract each other's defense mechanisms (Ehrlich and Raven, 1964; Janzen, 1980; Ishimoto and Chrispeels, 1996; Bown et al., 1997). In fact, a few studies have demonstrated that plant inhibitor genes are prone to mutations (Laskowski et al., 1988; Ryan, 1990; Kothekar et al., 1996). Plants also seem to economize the process by producing multi-domain inhibitors (Jongsma and Bolter, 1997).

Non-Host Plant PIs Are Potent Inhibitors of HGP and Retard Growth of *H. armigera* Larvae

The present study has demonstrated the efficacy of three non-host plant PIs in inhibiting the isoproteinases and larval growth of *H. armigera*. The non-host plant PIs inhibited total proteinase activity in enzyme assays, and all of the HGP isoforms in electrophoretic assays (Table I; Fig. 1). The electrophoretic profiles of PIs in host and non-host plants revealed a number of isoforms differing in intensity and mobility. Interestingly, only the non-host plants exhibited the presence of PI bands with inhibitory activity against HGP, which were absent in most of the host plants (Fig. 3). Earlier and current reports on PIs have dealt with the identification and characterization of specific PIs such as inhibitors of trypsin, chymotrypsin, or subtilisin (Ser

proteinases) and papain (Cys proteinases). However, it is necessary to identify and evaluate PIs having specific inhibitory activity against insect gut proteinases. Our data reveal the dichotomy between the TIs and HGPIs, as not all of the trypsin PIs, even those of the non-host plants, could inhibit HGP, although most of the HGP activity is trypsin like (Johnston et al., 1991; Harsulkar et al., 1998). The TIs that did not appear in the HGPI profiles were probably either ineffective against HGP or were degraded by HGP.

Plant defense proteins can be potentially recognized as substrates by insect gut proteinases. The following reports have demonstrated that insect gut proteinases neutralize the effect of PIs by degrading them: multicystatin of potato tubers by *Diabrotica* larval proteinases (Orr et al., 1994); oryzacystatin by black vine weevil proteinases (Michaud et al., 1995, 1996); TIs of chickpea by gut proteinases of podborer (Giri et al., 1998); and oryzacystatin and soybean Bowman-Birk TI by beetle larvae (Girard et al., 1998a). Insects derive dual benefit by the digestion of PIs: the restoration of gut proteinase activity and the availability of valuable, sulfur-rich amino acids. The stability of the PIs in the proteolytic environment of the gut is thus an important criterion for selecting candidate PIs. In the present study, upon limited proteolysis by HGP, TIs generated active fragment(s), some of which remained stable up to 3 h (food retention time in the larvae) (Fig. 2). Christeller and Shaw (1989) have reported that TIs incubated with purified grass grub trypsin retain their activity after limited proteolysis. Thus, persistence of activity rather than integrity of PI proteins is a major factor in assessing their potential utility in insect resistance. Total inhibition of HGP by non-host plant PIs in the solution assay, even after incubation for 3 h, indicates their stability against the gut proteinases (Table II). It would be of further interest to identify a specific fragment(s) possessing HGPI activity from host and non-host plant PIs.

Feeding studies show that PIs of winged bean, groundnut, and potato inhibit the growth of nearly all of the early second instar larvae. A decrease in the estimable proteinase activity in these larvae was evident from the results shown in Table III. Wu et al. (1997) reported a 13% decrease in the total proteinase activity in *H. armigera* larvae fed transgenically expressed PI from giant taro. Bown et al. (1997) have reported a decrease in the overall levels of proteinases and in the levels of mRNAs encoding trypsin-like proteinases of *H. armigera* fed soybean TI, suggesting that the decrease in this activity is at the transcriptional level. The decreased mRNA levels may, however, reflect down-regulation of a particular proteinase that may be compensated for by up-regulation of the other proteinases. Furthermore, Bown et al. (1997) demonstrated the presence of at least 28 genes in *H. armigera* encoding trypsin/chymotrypsin-like proteinases having 95% homology and exhibiting certain specific changes in cDNA sequences around the active site of proteinases. That study also observed minor differences in the migration of isoproteinases.

In the present study, changes found in the sensitivity of the proteinases toward specific PIs may have been due to alterations around the active site that could not be differentiated by SDS-PAGE. Although up- and down-regulation of gut proteinases was observed, the decrease in estimable activity was not reflected in the electrophoretic profiles (Table III; Fig. 6). This observation strongly suggests the presence of active inhibitors complexed with gut proteinases, which undergo dissociation during SDS-PAGE. More intense bands actually indicate overexpression of certain proteinases. However, as evident from significant growth retardation, the larvae were suffering from the loss of proteinase activity because of the dietary non-host PIs (Table III; Fig. 5). Recently, Broadway (1997) speculated that insects might possess specific mechanisms for the regulation of individual proteinases controlled by a monitor peptide. The latter may be responsible for the induction of inhibitor-sensitive and -insensitive proteinases depending upon the nature of the ingested PI.

Winged Bean PIs in Combination with Potato PI-II Are Ideal for *H. armigera* Resistance

It has been emphasized that selection of proper PI genes out of a large variety of inhibitor genes having different specificities is a crucial step, as any one PI may not universally confer complete tolerance to a particular insect species (Hilder et al., 1993; Jongsma et al., 1996; Michaud, 1997). In this study, we observed significant PI-insensitive activity in the larvae fed winged bean PIs, which was 27% insensitive to winged bean PIs and 53% insensitive to potato PI-II (Table IV). It is known that potato PI-II is active against a wide range of Ser proteinases (Whitworth et al., 1998); however, it was not able to inhibit 53% of the HGP activity of the larvae reared on winged bean PIs (Table IV). On the contrary, winged bean PIs inhibit nearly all of the proteinase activity of the larvae reared on potato PI-II. This can be attributed to differences in the winged bean PIs and potato PI-II with respect to inhibition of HGP or to the synthesis of alternative proteinases in response to two different PIs.

The current research on PIs is mainly focused on the expression of a single PI gene in the target plant under the universal promoter (for review, see Jouanin et al., 1998; Schuler et al., 1998). However, several recent studies have proposed the use of multiple PIs to inhibit a full spectrum of gut proteinases (Jongsma and Bolter, 1997; Michaud 1997; Girard et al., 1998a, 1998b). Combinations of PIs targeted to different proteinases have been known to act synergistically (Jongsma and Bolter, 1997). The combination of PIs increases their stability in the gut due to prevention of their degradation by proteinases, and at the same time impairs digestion of dietary proteins. Based on our results, we propose a strategy using a combination of successive expression of potato PI-II and winged bean PIs in a transgenic crop to counteract *H. armigera* infestation. This involves expression of potato PI-II under the control of a universal promoter and winged bean PI under a seed-specific promoter.

H. armigera larvae of the first and second instar feed on leaves and flowers and later shift to developing seeds in chickpea. The rationale in the proposed strategy is to express potato PI-II in vegetative parts so that the growth of early instar larvae will be delayed. When these larvae

eventually shift to developing seeds, they would encounter the expressed winged bean PI that inhibits potato PI-II induced proteinases, thus forcing them to alter gut proteinase composition at least twice. Our data suggest that adaptation of *H. armigera* to one group of PIs does not mean insensitivity to other PIs, indicating different responses of *H. armigera* to different PIs. Such an inhibitor combination would significantly delay the growth and generation advance of *H. armigera* in the field.

Chickpea seed development takes around 2 months to form mature seeds. If larval development is delayed for 10 to 20 d, it will reduce at least one life cycle, with a consequent drastic decrease in the larval population, which grows exponentially with each advancing generation. This would result in a significant reduction in yield losses. In feeding studies, the larval growth remains stunted for a long time without any resultant mortality. Recently, pest management strategies have advocated containment of insect pests rather than their total elimination (Lewis et al., 1997). This can be best achieved by bolstering the system's inherent defenses. PIs of the kind reported in this paper would serve the above objective of not targeting the elimination of insect pests but merely inhibiting larval growth, thereby reducing the crop damage. Thus, tandem use of potato PI-II and winged bean PIs to develop transgenic crop plants will lead to sustainable resistance against *H. armigera*.

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