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Higher accumulation of proteinase inhibitors in flowers than leaves and fruits as a possible basis for differential feeding preference of *Helicoverpa armigera* on tomato (*Lycopersicon esculentum* Mill, Cv. Dhanashree)

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Abstract

Tomato (*Lycopersicon esculentum*, Mill; cultivar- Dhanashree) proteinase inhibitors (PIs) were tested for their trypsin inhibitory (TI) and *Helicoverpa armigera* gut proteinases inhibitory (HGPI) activity in different organs of the tomato plants. Analysis of TI and HGPI distribution in various parts of the plant showed that flowers accumulated about 300 and 1000 times higher levels of TI while 700 and 400 times higher levels of HGPI as compared to those in leaves and fruits, respectively. Field observation that *H. armigera* larvae infest leaves and fruits but not the flowers could be at least partially attributed to the protective role-played by the higher levels of PIs in the flower tissue. Tomato PIs inhibited about 50–80% HGP activity of *H. armigera* larvae feeding on various host plants including tomato, of larvae exposed to non-host plant PIs and of various larval instars. Tomato PIs were found to be highly stable to insect proteinases wherein incubation of inhibitor with HGP even for 3 h at optimum conditions did not affect inhibitory activity. Bioassay using *H. armigera* larvae fed on artificial diet containing tomato PIs revealed adverse effect on larval growth, pupae development, adult formation and fecundity.

Keywords: *Lycopersicon esculentum*; *Helicoverpa armigera*; Proteinase inhibitors; Midgut proteinases; Plant defense; Accumulation of PI

1. Introduction

Defensive role of proteinase inhibitors (PIs) against insect was demonstrated by a classical study on tomato and potato plants by Green and Ryan (1972). Their breakthrough investigations revealed that PIs are induced and accumulated in aerial tissues of tomato and potato plants as direct consequence of insect damage or mechanical wounding of leaves, making the plant tissue less palatable to invading pest. This study also showed that the effectiveness of inhibitors, as deterrents to insect, depends upon their ability to inhibit the proteinases in the insect digestive tract. During the last three decades, tomato and potato PIs were extensively characterized (Plunkett et al., 1982; Ryan, 1990; Schaller and Ryan, 1995). Two PI proteins from tomato leaves were identified and characterized of which, inhibitor I (8100 Da) was a potent inhibitor of bovine chymotrypsin while inhibitor II (12,300 Da) was strong inhibitor of bovine trypsin and chymotrypsin (Plunkett et al., 1982). Molecular mechanism of wound-inducible nature of PI genes from tomato plant was also explained by identifying a plant hormone, systemin, as polypeptide defense signal (Schaller and Ryan, 1995). Later on, PIs from numerous other plant species were studied and efforts towards application of these molecules to manipulate the insect tolerance in target plant were reported (Ryan, 1990; Boulter, 1993; Murdock and Shade, 2002). Currently, the main emphasis of plant-PI studies is on identifying

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potential inhibitors of digestive proteinases of target insect and on understanding the dynamic nature of insect midgut proteinases at molecular level (Bown et al., 1997; Lopes et al., 2004; Giri et al., 1998, 2003; Srinivasan et al., 2005; Tamhane et al., 2005).

Lepidopteron insects have serine proteinases as a major component of their digestive complement and among them, typically trypsin-and/or chymotrypsin-like are the most commonly found proteinases (Purcell et al., 1992). The PIs enter in the insect digestive tract along with the food and block the protein digestion, hence starving the insect for amino acids and energy, resulting in retardation of growth and development (Ryan, 1990). Insects on the other hand exhibit mechanisms to produce inhibitor-insensitive or inhibitor-degrading proteinases in the midgut to overcome the effect of PIs (Jongsma et al., 1995; Michaud, 1997; Giri et al., 1998; De Leo et al., 1998; Volpicella et al., 2003). Insects capable of adapting to such plant defenses have a chance to survive and to emerge as potential pests of agricultural crops.

Tomato is an agro-economically valuable vegetable crop, however, *Helicoverpa armigera*; a major insect pest of tomato heavily infests leaves and fruits. *H. armigera* moth lays eggs on the abaxial surface of tomato leaves. Neonates feed on leaves up to the first instar, thereafter travel to developing fruits and damage them. Use of pesticides to control *H. armigera* infestation on tomato fruit has little effect because insects bore inside the fruit and damage it where effective amount of pesticides is difficult to reach. Use of pesticides is also not advisable due to associated health hazards. Development of genotypic tolerance to *H. armigera* through proteinase inhibitor gene-based strategy would be appropriate approach (Giri et al., 2004). This could be achieved by expressing strong exogenous PIs or over expressing endogenous PIs in the vegetative organs of tomato plant e.g., leaves or stem, by which the growth of the larvae can be restricted at the vegetative organs only.

Our laboratory is engaged in analyzing the interaction of host and non-host plant PIs with HGPs (Chougule et al., 2003, 2004; Giri et al., 1998, 2003; Harsulkar et al., 1999; Patankar et al., 2001; Srinivasan et al., 2005; Telang et al., 2003; Tamhane et al., 2005) and has shown that the host plants like chickpea, pigeon pea and cotton do not have effective inhibition of gut proteinase activity. Our field observation of tomato plant infestation indicated that *H. armigera* larvae attack leaves and fruits but not the flowers. Secondly, tomato PIs although are well characterized, their interaction with *Helicoverpa* gut proteinases (HGPs) has not been reported so far. Both these observations prompted us to check the distribution of PIs in different organs of tomato plant and to study the inhibitory potential of tomato PIs against HGP.

In the present communication, we have analyzed the distribution of PIs in various organs of field grown tomato plants such as leaves, flowers, two stages of developing fruits (DFI and DFII) and seeds. We have also evaluated inhibition of HGPs of larvae fed on different host plants, larvae exposed to non-host plant PIs and different larval stages of *H. armigera* by tomato PIs. Finally, in vivo efficacy of the tomato PIs has been demonstrated by feeding the larvae on the diet containing different doses of tomato PIs.

2. Results

2.1. Comparative analysis of PIs from the various organs of tomato plant

Water-soluble protein content, trypsin inhibitor units (TIUs), and *Helicoverpa* gut proteinase units (HGPIUs) were determined from various organs of tomato plant viz., leaves, flowers, developing fruits (2 stages) and seeds. These results are summarized in Table 1. Statistical analysis revealed significant differences at $p < 0.05$ and < 0.01 in TI and HGPI content of various organs of tomato plant. Tomato flower showed the highest inhibitory activity against trypsin (0.6 TIUs/g fresh wt.) and HGP (0.58 HGPIUs/g fresh wt.) whereas the leaf extract showed the lowest inhibitory activity against trypsin (2×10^{-3} TIUs/g fresh wt.) and HGP (8.6×10^{-4} HGPIUs/g fresh wt.) among

Table 1
Protein, trypsin inhibitor (TI) and *Helicoverpa* gut proteinase inhibitor (HGPI) content of various organs of tomato plant: Distribution of PI activity against bovine trypsin and HGP in leaves, flowers, developing fruits and seeds

Plant tissue	Protein (μg/g fresh wt.)	TIU[c]/g fresh wt.	Specific TI activity (TIUs/mg protein)	HGPIU/g fresh wt.	Specific HGPI activity (HGPIUs[d]/mg protein)
Leaves	$5.0 \times 10^{-3} \pm 0.0003$	$2.0 \times 10^{-3} \pm 0.0002$	00.40	$8.6 \times 10^{-4} \pm 0.000008$	0.0172
Flowers	$1.3 \times 10^{-2} \pm 0.005$	$6.0 \times 10^{-1} \pm 0.01$	46.15	$5.8 \times 10^{-1} \pm 0.02$	44.62
DF-I[a]	$2.0 \times 10^{-3} \pm 0.001$	$2.3 \times 10^{-3} \pm 0.0001$	01.15	$6.2 \times 10^{-3} \pm 0.0002$	3.10
DF-II[b]	$9.0 \times 10^{-4} \pm 0.0002$	$5.0 \times 10^{-4} \pm 0.0001$	00.55	$1.2 \times 10^{-3} \pm 0.0001$	1.33
Seed	$1.7 \times 10^{-1} \pm 0.01$	ND[e]	ND[e]	ND[e]	ND[e]

Equalized units (0.4 U) of each type of proteolytic activity were used in the assays. The experiment was repeated thrice starting from extraction. Each value is shown as mean ± SE ($n = 3$).

[a] DF-I: Developing fruits stage 1.
[b] DF-II: Developing fruits stage 2.
[c] TIU: Trypsin inhibitory unit.
[d] HGPIU: *Helicoverpa* gut protease inhibitory unit.
[e] ND: Not detectable under assay conditions.

all the organs that were analyzed. In flower, the TI activity was approximately 300 and 1000 times higher while the HGPI activity was 700 and 400 times higher than leaves and developing fruit stage II (DFII), respectively. Developing fruit stage I (DFI) showed significantly, higher TI (2.3×10^{-3} TIUs/g fresh wt.) and HGPI (6.2×10^{-3} HGPIUs/g fresh wt.) content than that in leaf (2.0×10^{-3} TIUs/g fresh wt and 8.6×10^{-4} HGPIUs/g fresh wt.) and DFII (5.0×10^{-4} TIUs/g fresh wt. and 1.2×10^{-3} HGPIUs/g fresh wt.) ($p < 0.01$). The TI and HGPI activities were not detected in tomato seeds as expected in solanaceous plants except *Capsicum annum* (Antcheva et al., 2001), even though very high amount of protein (2 mg seed protein) was used for the analysis under our assay conditions.

These PI extracts of different organs of tomato plant were further analyzed on native PAGE using equal TIUs (Fig. 1). In all the organs except seeds, three major isoforms of TIs were detected upon activity visualization by X-ray film contact print technique.

2.2. Characterization of proteinase inhibitors from tomato flower

The floral extract, exhibiting similar pattern of PI isoforms as other organs and having the highest specific activity of PIs, was used as PI source in further studies. Inhibitory activity of tomato PIs against bovine trypsin and bovine chymotrypsin was found to be 100% and 90%, respectively (Fig. 2(a)). Furthermore, the inhibition potential of tomato PIs was analyzed against gut proteolytic activity of chickpea fed HGP (Cp-HGP) and tomato fed HGP (Tom-HGP) as representative HGP samples. For this purpose, fourth instar larvae, which are known to express maximum isoforms of gut proteinases (Patankar et al., 2001), were collected from chickpea and tomato fields and used for HGP extraction. One microgram per microlitre of Cp-HGP and Tom-HGP extracts were used for analyzing HGP inhibitory potential of tomato PIs. Both of these HGP activities (0.4 U) were inhibited to 50–60% at 0.032 HGPIUs (0.35 μg protein) and to ~90% at 0.124 HGPIUs (3.5 μg protein) of tomato PIs (Fig. 2(b)). These two HGPI units (0.032 HGPIUs and 0.124 HGPIUs) were further used to check their potential against host and non-host HGP activities since *H. armigera* exhibits diverse gut complement of digestive proteinases when fed on different host and non-host plants (Bown et al., 1997; Patankar et al., 2001).

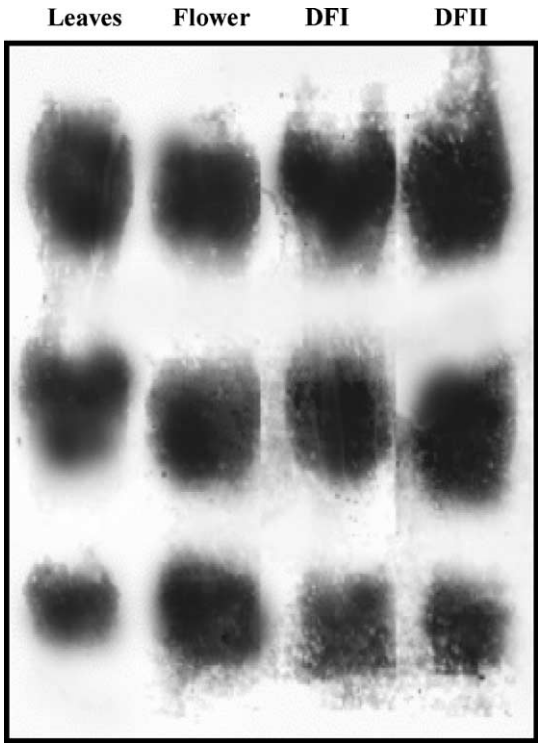

Fig. 1. Distribution of TIs in various tissues of tomato plant. Protein amount equivalent to 1 TIU trypsin inhibitor activity from leaf, flower and developing stages of fruit (DFI and DFII) were resolved on 15% native PAGE. The inhibitor bands were visualized by gel X-ray film contact print technique as described in Section 4.4. Lane 1: leaf extract; Lane 2: flower extract; Lane 3: developing fruit stage I (DFI); Lane 4: developing fruit stage II (DFII).

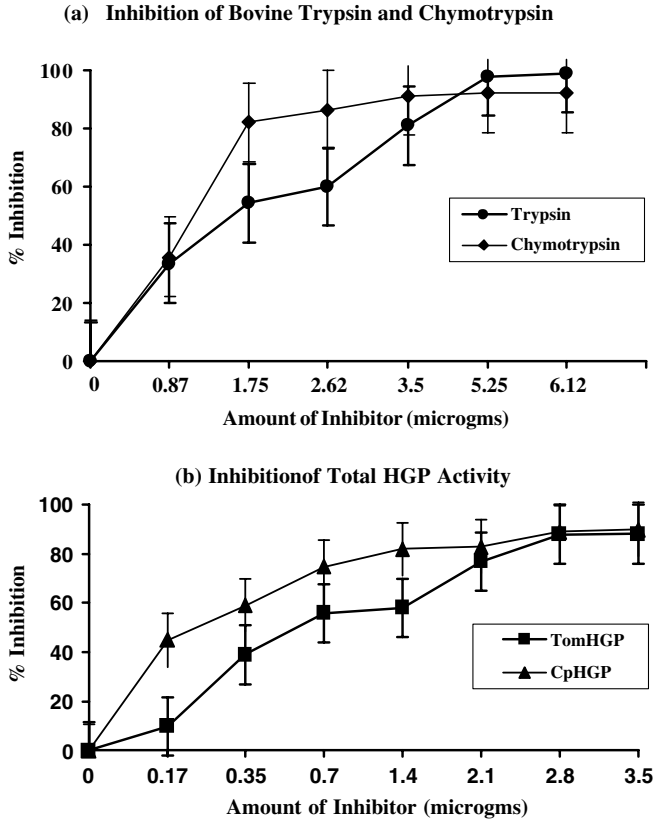

Fig. 2. Inhibition of bovine trypsin, bovine chymotrypsin and total proteolytic activity of HGP from chickpea (Cp-HGP) and tomato (Tom-HGP) fed larvae by tomato PIs: The assays were conducted using BA*p*NA, SAA*p*LNA and azocasein as substrates. The experiments were done in triplicates. (a) Inhibition of bovine trypsin and bovine chymotrypsin; (b) inhibition of total proteolytic activity of Cp-HGP and Tom-HGP.

Visualization of tomato PIs using gel X-ray film contact print technique after native PAGE exhibited similar pattern of isoforms against trypsin (TI) or chymotrypsin (CI) or HGP (HGPI) (Fig. 3), although % inhibition maxima differed against these proteases by tomato PIs.

2.3. Inhibitory potential of tomato PIs against host-, non-host- and instar-specific HGP activity

Inhibitory potential of tomato PIs was analyzed against HGP of larvae collected from infested fields of several host (tomato, chickpea, sweet pea, pigeon pea and okra) plants and HGP from larvae reared on artificial diet containing PIs from non-host (winged bean and potato) plant sources (Fig. 4(a) and (b)). Among the host plant group, at 0.124 HGPI concentration, pigeon pea HGP exhibited the lowest i.e., 72% inhibition while Tom-HGP showed the highest i.e., 85% inhibition (Fig. 4(a)). In non-host plant group winged bean fed HGP showed the lowest, i.e., 70% inhibition while winged bean + pinII fed HGP exhibited the highest, i.e., 75% inhibition by tomato PIs (Fig. 4(b)).

H. armigera is known to exhibit differential gut proteinase activity at different larval instar stages hence, inhibitory activity of tomato PIs against HGPs of various instars fed on artificial diet was estimated (Table 2). The gut proteinase activities (0.4 U), of all the instar stages except 4th instar were inhibited in the range of 62–67% and 74–83% at 0.032 HGPIU and 0.124 HGPIU tomato PI concentrations, respectively (Table 2) and found to be significant at

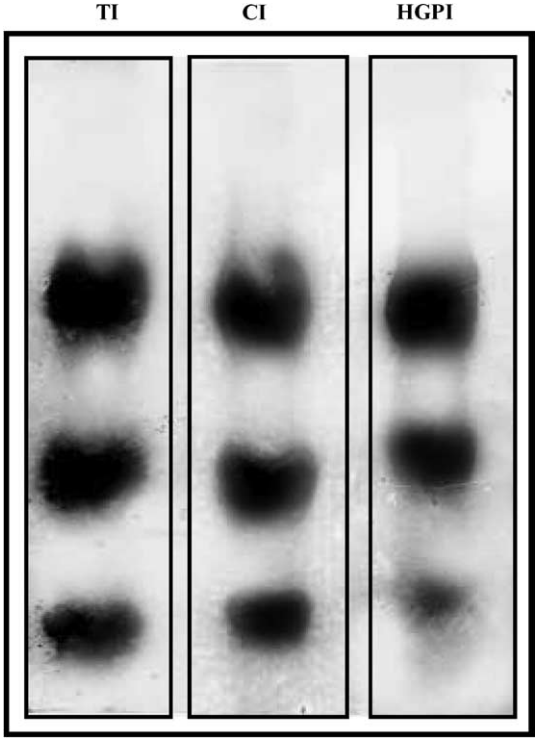

Fig. 3. Activity profiles of tomato PIs against various proteinases: Equal trypsin inhibitor units (1 TIU) of tomato flower extract were loaded on 15% native PAGE. After electrophoresis the gel strips were cut and incubated in trypsin, chymotrypsin, and HGP respectively. Lane 1: TI; Lane 2: CI and Lane 3: HGPI activities were visualized by X-ray film contact print technique as described in Section 4.4.

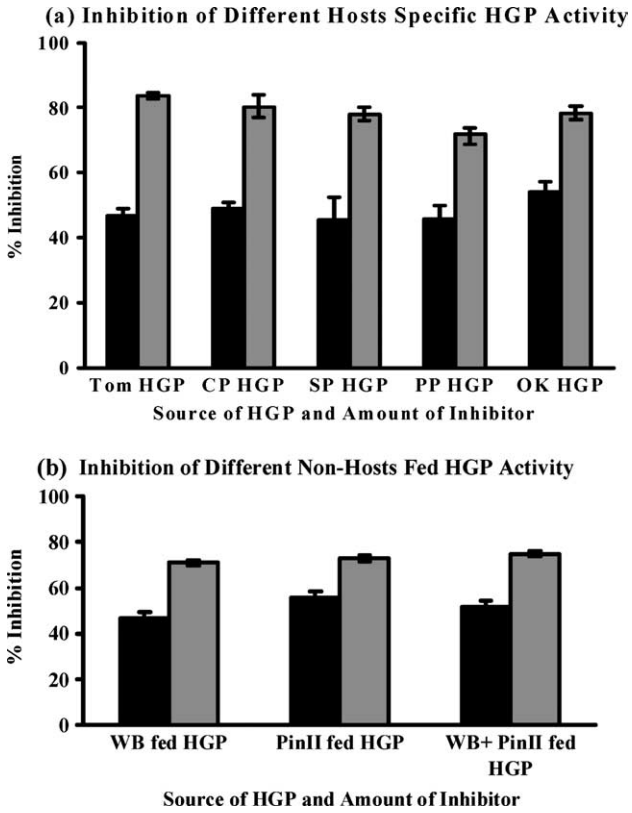

Fig. 4. Comparative inhibitions of the host and non-host PI fed HGP by tomato PIs. Two concentrations of tomato PIs (0.032 HGPIU and 0.124 HGPIU) were titrated against HGP isolated from several host and non-host plant PI fed larvae. Host plant sources: Tomato fed HGP (Tom-HGP), chickpea fed HGP (CP-HGP), sweat pea fed HGP (SP-HGP), pigeon pea fed HGP (PP-HGP) and Okra fed HGP (OK-HGP). Non-host plant PI sources: Winged bean fed HGP (WB fed HGP), potato inhibitor II fed HGP (Pin II fed HGP) and combination of winged bean and potato inhibitor II fed HGP (WB + Pin II fed HGP). The amounts of inhibitor are as mentioned in the figure. (a) Inhibition of host fed HGP and (b) inhibition of non-host fed HGP. Black bars: 0.032 HGPIUs; Gray bars: 0.124 HGPIUs.

Table 2
Inhibitory activity of tomato PIs against HGPs isolated from larval instar stages of *H. armigera:* Two concentrations of tomato PIs (0.032 HGPIUs and 0.124 HGPIUs) were used to check their inhibitory potential against 0.4U of HGP of various instar stages of *H. armigera*

Source of HGP[a]	% Inhibition	
	0.032 HGPIUs[b]	0.124 HGPIUs[b]
Second instar	63.4 ± 3	73.6 ± 1.76
Third instar	66.9 ± 0.26	82.9 ± 2.16
Fourth instar	48.55 ± 0.27	70.9 ± 0.46
Fifth instar	64.25 ± 0.2	80.3 ± 0.28
Sixth instar	62.15 ± 0.02	81 ± 0.69

The percent inhibition (%) values indicated in the table are the maximum proteolytic activity inhibition by the respective HGPI concentrations. Azocasein was used as a substrate in assays. Each value is shown as mean ± SE ($n = 3$).

[a] HGP: *Helicoverpa* gut proteinase.

[b] HGPIUs: *Helicoverpa* gut proteinase inhibitor units.

$p < 0.05$ and < 0.01 by ANOVA. Fourth instar HGP was found to be more insensitive to inhibition by tomato PIs (48% at 0.032 HGPIUs while 70% at 0.124 HGPIUs), which can be attributed to the fact that the fourth instar stage, the most voracious and devastating phase of *H. armigera*, shows very high proteinase activity and maximum number of proteinase isoforms (Patankar et al., 2001).

To study the stability of PIs towards HGP, 1 unit of tomato PI activity was incubated with each of 0.2 and 1.0, HGP unit for 30 min and 3 h separately. These samples were then resolved on native PAGE and visualized for inhibitory profile (Fig. 5). The presence of three intact isoforms of tomato PIs indicated that they were stable at both the concentrations of HGP even after 3 h of incubation.

2.4. Effect of tomato PIs on H. armigera growth and development

To demonstrate effect of tomato PIs on growth and development of *H. armigera*, three different concentrations of inhibitor, 0.5×(0.289 HGPIU/g), 1× and 2× (1.158 HGPIU/g) were added per gram control diet. 1× (0.579 HGPIU/g) amount of inhibitor is also equivalent to the constitutive level of inhibitor present in the fresh flower tissue (Table 1). Further, half of the optimum (0.289 HGPIUs) and double of the optimum (1.158 HGPIUs) amounts of inhibitor representing 0.5× and 2× concentration were also used to evaluate extent of tomato PI activity against *H. armigera* growth and development (Table 3). Approximately 40–55% larvae were underweight in all the three test populations leading to 17–32% reduction in weight as compared to larvae fed on control diet. The mortality was found to be 5–20% in the PI-fed larvae starting from second instar stage to the sixth instar stage as compared to the control. These effects were proved to be significant at $p < 0.01$ by ANOVA. Weight loss, percentage of pupae showing weight loss, delayed pupations and % mortality was also recorded at pupal stage for all three PI concentrations. Pupae (66–78%) were underweight in all the test populations. Delayed pupation was also significantly higher in case of 2× PI dose fed larvae (53%) ($p < 0.01$). However, % pupal weight loss and pupal mortality were not statistically significant (Table 3). Furthermore, equal number of females and males from the same PI-dose population were kept together to test the egg laying capacity as against the controls. The reduction in egg laying capacity was found to be dose dependent and statistically significant ($p < 0.01$), i.e., it reduced from 63% at 0.5×-PI doses to 23% at 2×-PI doses in test insects as compared to the control.

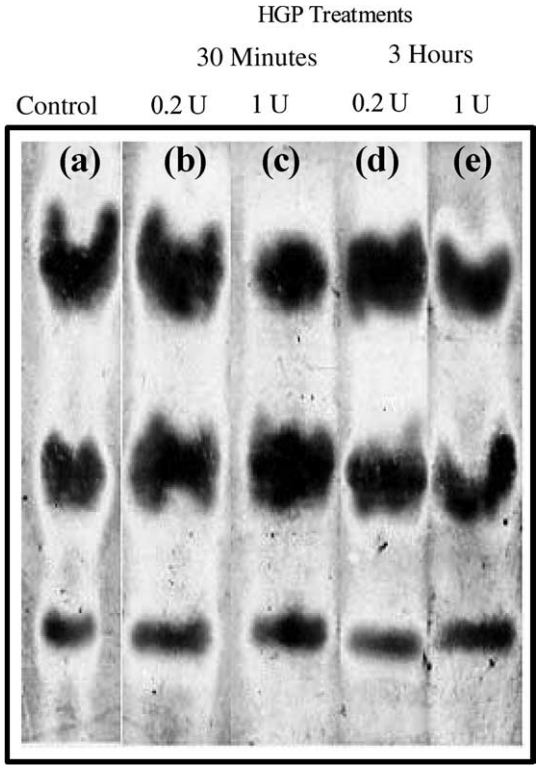

Fig. 5. Stability of tomato PIs to HGPs: Tomato PIs (1 U) was incubated with each of 0.2 U and 1 U HGP for 30 min or 3 h separately, at 37 °C. Mixtures were resolved on 15% PAGE and then visualized for inhibitor activity. Lane a: Untreated HGPI (1 U); Lane b: 1 U HGPI + 0.2 U HGP incubated for 30 min; Lane c: 1 U HGPI + 1 U HGP incubated for 30 min; Lane d: 1 U HGPI + 0.2 U HGP incubated for 3 h and Lane e: 1 U HGPI + 1 U HGP incubated for 3 h.

Table 3
Effect of tomato PIs on growth and development of *H. armigera*: Second instar *H. armigera* larvae were fed on control diet as well as diet supplemented with tomato PI extract

H. armigera development parameters	Control (%)	Different doses of tomato PI/gm diet		
		0.5×(0.289 HGPIU)	1×(0.579 HGPIU)	2×(1.158 HGPIU)
% Larval weight loss	0	17[a]	20[a]	32[a]
% Larvae showing weight loss	0	40[a]	40[a]	55[a]
% Larval mortality	0	5	17[a]	20[a]
% Pupal weight loss	0	10	14	14
% Pupae showing weight loss	0	78[b]	66[b]	75[b]
% Delayed pupation	0	48[b]	47[b]	53[a]
% Pupal mortality	0	6	10	6
% Egg laying capacity of moths	100 (670)	63[b] (425)	50[b] (340)	23[a] (157)

Parenthesis values indicate the number eggs/female. Experiment was repeated twice with 30 larvae in each set.
[a] Values statistically significant at $p < 0.01$.
[b] Values statistically significant at $p < 0.05$.

These results clearly indicated that tomato PIs affect the *H. armigera* growth and development significantly.

3. Discussion

Tomato PIs are extensively studied and used as model to obtain insight of plant defenses against herbivore attack. Plant-mediated interaction of tomato with *H. zea* has been investigated by feeding larvae on tomato leaflets which resulted in systemic accumulation of several pathogenesis related proteins including PIs (Stout et al., 1999). However, biochemical interaction of tomato PIs with *H. armigera* gut proteinases has not yet been investigated. Our studies indicated that tomato PIs are highly stable to gut proteinases, inhibit proteinase activity of different larval instars, possess inhibitory activity against mixture of gut proteinases of larvae feeding on several host and non-host PIs. Furthermore, in feeding experiments tomato PIs showed adverse effects on various developmental parameters of *H. armigera*, most significantly on fecundity (no. of eggs/female) at all the three levels (0.5×, 1× and 2×) of inhibitor where 1× level is equivalent to the constitutive level of PI found in tomato flowers. Low fecundity values symbolize less progeny, which is a direct impact on the subsequent generation of *H. armigera*. Similar observations were previously reported by Telang et al. (2003) and Tamhane et al. (2005); using non-host PIs such as bitter gourd and capsicum PIs, respectively. However, it is interesting to note that host plant PIs such as tomato PIs, also impart adverse effects on growth and development of *H. armigera* and the potential of tomato PIs is comparable to other non-host PI sources which are previously reported as potential tools of defense against *H. armigera* (Telang et al., 2003; Giri et al., 2004; Tamhane et al., 2005).

Qualitative analysis of tomato PIs in leaves, flowers and developing fruits revealed identical inhibitor profiles. However, quantitatively the amount of PI in flower was about 300 and 1000 times higher in case of TI while 700 and 400 times higher for HGPI as compared to those in leaves and fruits, respectively. These observations lead to an important question that does the level of PIs matter in imparting resistance to the pest in a particular tissue in a whole plant? Earlier, contrasting effects of high and low level expression of mustard trypsin inhibitor (*MTI-2*) on insects have been reported (De Leo et al., 1998). The high level of *MTI-2* expression in leaves of tobacco has deleterious effect on larvae of Egyptian cotton worm (*Spodoptera littoralis*), causing mortality and decrease in larval feeding. On the contrary, larvae fed on leaves of transgenic line expressing low levels of *MTI-2* have not shown mortality, but shown net gain in weight and faster development of larvae (De Leo et al., 1998). Differences in growth and feeding pattern have been reported when two-spotted spider mites (*Tetranychus urticae*) were fed on cultivated tomato plant and its isogenic line (*defenseless 1* mutant) lacking PI accumulation mechanisms. In case of normal tomato plant, feeding and fecundity of spider mite were significantly reduced compared with that in *defenseless 1* mutant (Li et al., 2002). Our studies indicating higher constitutive levels of PIs in flower than in leaves and fruits and our field observation that the tomato flowers are less infested by *H. armigera* than its leaves or fruits supplement these reports.

It is further possible that flower being a reproductive organ, plant diverts its metabolic energy into higher production of PIs in flowers for its protection than that in leaves and fruits. It thus explains the economical partitioning of the sink by the plant for its fitness and protection. Therefore, it would be interesting to explore the regulatory mechanisms responsible for high accumulation of PIs in tomato flowers. It has been reported that constitutive over expression of allene oxide cyclase in tomato elevates levels of jasmonates and octadecanoids in flowers but not in leaves indicating that the signaling mechanism to induce PI accumulation might be more active in flower than the other organs of tomato plant (Miersch et al., 2004). Peña-Cortes et al. (1995) have reported constitutive levels of PIs expressed in certain stages of flower development in tomato and potato plants. Abundance of PIs in the reproductive organs of tomato and potato, and in potato tubers thus supports protective role against pathogen and herbivore attack (Peña-Cortes et al., 1995) Constitutive high levels of other pathogenesis related proteins in flowers have been reported in tomato plants, which may have cumulative adverse effect on pest or pathogen thus building more protection (Hause et al., 2000; Miersch et al., 2004). A raw fruit of wild tomato (*L. peruvianum*) contains about 50% total soluble protein as PI proteins, which are involved in defense against insect attack (Pearce et al., 1988; Wingate and Ryan, 1991).

Wound-inducible serine PIs accumulate in storage organs and in leaves in response to herbivore attack, pathogen infection or other environmental stresses (Ryan, 1990). Inhibitory activities of these proteins are directed against wide range of proteinases from animals, insects and microbial origin. It is an established fact that these inhibitors are induced in very high amounts (2–10%) in tissues and are involved in protection of the plants against predators (Ryan, 1990; Boulter, 1993). Three members of Pin II proteins identified in *Nicotiana alata* are expressed at very high levels in stigmas at all stages of development from immature bud to mature receptive flower and are postulated to be involved in protection against pests and pathogens (Atkinson et al., 1993). Based on our present studies it can be suggested that, apart from the inducible PIs; the higher constitutive levels of PIs in tomato flower may play a major role in protecting it from the insect attack.

4. Experimental

4.1. Extraction of tomato PIs

Various organs of tomato plant (*L. esculentum*, Mill; cultivar- Dhanashree) viz., leaves, flowers, two stages of unripe fruit (DFI, DFII) and seeds were procured from

the Division of Horticultural Sciences, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India. The plant materials were harvested, freeze-dried and ground to a fine powder in liquid nitrogen. The PI extraction protocol was same as described by Plunkett et al. (1982). The protein content was determined by Bradford's method (Bradford, 1976) using protein estimation kit (Biogenei, India).

4.2. Preparation of HGP

For HGP extraction, the midguts were isolated by dissecting the larvae and stored at −20 °C till further use. The gut tissue, when required, was mixed with suitable volume of 0.2 M gly-NaOH buffer, pH 10.0, and allowed to stand for 2 h at 4 °C. The gut luminal contents were removed by centrifugation at 14,000*g* for 15 min at 4 °C. The resultant supernatant (mg/μl) was used as a source of HGP. Field collected fourth instar *H. armigera* larvae, growing on various host plants (Tomato, Chickpea, Sweat pea, Pigeon pea and Okra) and fourth instar larvae grown on artificial diets supplemented with combination of non-host proteinase inhibitors viz., winged bean PIs, pinII, and both winged bean PIs and pinII together were used for HGP extraction. These HGPs were used to study interaction of tomato PIs with, Tom-HGP and Cp-HGP, host and non-host PI fed HGP and stability of PIs towards HGP. To evaluate inhibitory potential of tomato PIs against different-instar-specific gut proteinases, larvae were grown on control diet upto desired instar stage (2nd, 3rd, 4th, 5th, and 6th) and respective HGPs were extracted. All the experiments were done in triplicates and the data obtained was analyzed statistically.

4.3. Proteinase and PI assay

Trypsin, chymotrypsin and HGP activities were estimated using the chromogenic substrates benzoyl-arginyl-*p*-nitroanilide (BA*p*NA); succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide (SAA*p*LNA) and azocasein (Sigma Chemicals, USA) respectively, according to modified protocol of Erlanger et al. (1964) as reported earlier (Patankar et al., 2001; Telang et al., 2003; Tamhane et al., 2005). For the enzyme inhibitor assay, a suitable volume of enzyme equivalent to 0.4 U HGP, was mixed with proteinase inhibitor, incubated at 27 °C for 15 min and residual proteinase activity was estimated. 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 given assay conditions.

4.4. Visualization of TIs, CIs and HGPIs

Crude protein extracts were separated on 15% native polyacrylamide gel electrophoresis (PAGE) (Hoefer SE 600, Amersham Pharmacia Biotech Inc., USA) using Davis buffer system (Davis, 1964). Visualization of TIs, CIs and HGPIs, after PAGE was carried out using the gel X-ray film contact print technique (Pichare and Kachole, 1994; Mullimani et al., 2002). To check the stability of tomato PIs against HGP, 1.0 inhibitor unit was incubated with each of 1 and 0.2 HGP units at 37 °C for 30 min and 3 h separately. These treated PIs were resolved on the native gel and processed the gel for HGPI activity visualization.

4.5. Bioassays with H. armigera larvae fed on diet containing tomato PIs

Laboratory cultures of *H. armigera* were maintained in a room (27 ± 2 °C, 60 ± 5% RH) for several generations to ensure genetic homogeneity. The control diet for *H. armigera* was prepared as described by Nagarkatti and Prakash (1974). A pilot experiment was performed using 10 control 4th instar larvae. HGP was extracted from midgut tissue and assayed biochemically against tomato PIs, using azocasein as a substrate. Thereafter the amount of HGPI required to inhibit total proteolytic activity (~5.0 HGPUs) of one insect gut was calculated which was represented as 1×. The control diet was supplemented with appropriate amount of tomato PIs to make the final concentration of tomato PIs to 0.5× (0.289 HGPIUs), 1× (0.579 HGPIUs), and 2× (1.158 HGPIUs) inhibitor units/g of diet/insect. The neonate larvae of *H. armigera* were initially reared for 3 days on control diet upto the first instar, since our earlier research experience revealed that they die on the same day on PI containing diet. Their development was monitored starting from the early second instar by feeding them on diet containing 0.5×, 1× and 2× inhibitor units/g of diet/insect.

The bioassays were conducted in two sets consisting 30 larvae/set along with the control larval population (larvae fed on artificial diet without PIs, i.e. control diet). The larval and pupal weights from all the sets were recorded everyday till 3rd day of pupal stage (~25 days) The average weights of the insects from total larval as well as pupal period, % mortality, % larvae and pupae showing weight loss, were calculated and the data was analyzed statistically. The fecundity (number of eggs/adult female) was recorded and compared with that of the control larval population.

4.6. Statistical analysis

ANOVA was performed for the data of TI and HGPI content from various organs of tomato plant (Table 1), inhibition potential of tomato PIs against various instars (Table 2) and feeding assays (Table 3) using Analyse-it + General 1.71 software.

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