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**Vaijayanti Tamhane**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **AA** | Amino Acids |
| **AI** | Amylase Inhibitor |
| **BA*p*NA** | Benzoyl-DL-Arginyl-*p*-Nitroanilide |
| **BBI** | Bowman-Birk type (proteinase) Inhibitor |
| **BMMY** | Buffered Methanol-complex Medium |
| ***Bt*** | *Bacillus thuringiensis* |
| **CanPI** | *Capsicum annuum* Proteinase Inhibitor |
| **CBB-R250** | Coomasssie Brilliant Blue-R250 |
| **cDNA** | complementary deoxyribonucleic acid |
| **CI** | Chymotrypsin Inhibitor |
| **CP HGP** | HGP from larvae fed on chickpea |
| **DNA** | deoxyribonucleic acid |
| **EDTA** | ethylenediamine tetra acetate |
| **g, mg, µg, ng** | gram, milligram, microgram, nanogram |
| **GXCT** | Gel- X rayfilm Contact-print Technique |
| **HCl** | hydrochloric acid |
| **HGPI** | *Helicoverpa armigera* Gut Proteinase Inhibitor |
| **HGPs** | *Helicoverpa armigera* Gut Proteinases |
| **$IC_{50}$** | Inhibitor Concentration at 50% inhibition |
| **IRD** | Inhibitory Repeat Domain |
| **JA** | Jasmonic Acid, jasmonate |
| **kDa/kD** | kilo Dalton |
| **L, mL, µL** | liter, milliliter, microliter |
| **LB** | Luria Bertonii |
| **M, mM, µM** | molar, millimolar, micromolar |
| **MeJ** | Methyl Jasmonate |
| **MGY** | Minimal Glycerol Medium |
| **MM** | Minimal Methanol |
| **mmole, µmole** | millimole, micromole |
| **mRNA** | messenger RNA |

| | |
|---|---|
| **NaCl** | sodium chloride |
| **NaOH** | sodium hydroxide |
| **OD** | Optical Density |
| **PAGE** | Poly-acrylamide Gel Electrophoresis |
| **PCR** | Polymerase Chain Reaction |
| **PI** | Proteinase Inhibitor |
| **PIN-I/II** | Potato (*Solanum tuberosum*) Proteinase Inhibitor I/II |
| **Pin-II HGP** | HGP from larvae fed on Pin-II PI incorporated diet |
| **PMSF** | phenyl methyl sulphonyl fluoride |
| **PP HGP** | HGP from larvae fed on pigeonpea |
| **RDB** | Regeneration Dextrose Medium |
| **RNA** | Ribonucleic Acid |
| **rpm** | revolutions per minute |
| **RT-PCR** | Reverse Transcriptase-Polymerase Chain Reaction |
| **SA** | Salicylic Acid, Salicylate |
| **SAAAPL*p*NA** | Succinyl-Alanyl-Alanyl-Alanyl-Prolyl-Leucyl-*p*-Nitroanilide |
| **SAAA*p*NA** | Succinyl-Alanyl-Alanyl-Alanyl-*p*-Nitroanilide |
| **SDS** | Sodium Dodecyl Sulphate |
| **TI** | Trypsin Inhibitor |
| **TLCK** | N-*p*-Tosyl-L-Lysine Chloromethyl Ketone |
| **TPCK** | N-*p*-Tosyl-L-Phenylalanine Chloromethyl Ketone |
| **TRIS** | Tris-Hydroxymethyl Aminomethane |
| **WB HGP** | HGP from larvae fed on winged bean PI incorporated diet |
| **WBPI** | winged bean (*Psophocarpus tetragonolobus*) proteinase inhibitor |

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# Thesis Summary

***Helicoverpa armigera*** being a polyphagous devastating pest is responsible for causing sever losses of several crop plants. Many different approaches have been used for its control, but the focus is now shifting towards the use of environment friendly pest control approaches. Use of PIs in developing insect tolerant transgenics is one of them and I have carried out my thesis work on a non-preferred host of *H. armigera*, *Capsicum annuum.* The major objectives and the results obtained are summarized in the following points;

### *To purify proteinase inhibitors from Capsicum annuum and study their basic biochemical characteristics and interactions with Helicoverpa armigera gut proteinases*

Various tissues of *C. annuum* were screened for PI profiles and activity. It was noted that though fruit tissues had diverse forms of PI the leaves had the highest PI specific activity. Leaf tissue was, therefore, used to purify and characterize *C. annuum* PIs. Ion exchange chromatography followed by gel filtration was used to purify two forms of PI's, CapA1 and A2. CapA1 and A2 exhibited molecular weight of around 12 kDa and had trypsin and chymotrypsin inhibitory activities. These two inhibitors also showed inhibition of crude and fractionated forms of gut proteinases of *H. armigera* in solution assays. Large scale purification of CapA1 and A2 was carried out for bioassay studies on *H. armigera*. Several dilutions (0.5X, 1X, 3X and 6X inhibitor concentrations) of CapA1, CapA2 and crude leaf extracts were incorporated in artificial diet of *H. armigera* and the bioassay was carried out for two successive generations. The inhibitor fed insects showed reduction in larval and pupal weights and dramatic reduction in fertility and fecundity in a dose dependent manner. The effects of CapA1 and CapA2 were even more pronounced in the 2$^{nd}$ generation of *H. armigera* indicating the success of PIs to limit *H. armigera* population growth.

### *To clone, characterize CanPI genes and study CanPI expression in planta*

Two full length *CanPI* cDNAs reported in the database were used to design oligo-nucleotide primer pairs for *CanPI* gene isolation. On RT-PCR using one of these primer pairs, four bands of sizes 800, 600, 450, and 300 bp were obtained. Predominance of 800 and 600 bp bands were noted in the fruit tissue while 450 and 300 bp bands were prominent only in the stem tissue and showed homologies to

members of Pin-II family of proteinase inhibitors of Solanaceae family. Characteristic features of Pin-II family PIs like a signal peptide followed by variable number of inhibitory repeat domains (IRD) were revealed while; several novel forms of *CanPIs* were also discovered. 1, 2 and 4 IRD *CanPIs* were not reported earlier. Existence of internal variability in the *CanPIs* was observed. Most notably, all the 4 IRD type *CanPI* genes showed simultaneous presence and variability in expression across the different *C. annuum* tissues screened suggesting their important physiological function *inplanta* apart from plant defense. *C. annuum* plants naturally infested with aphids ( *Myzus persicae*) and lepidopteran pest (*Spodoptera litura*) showed significantly high expression levels of *CanPIs* with specifically induced 4 IRD form other wise absent in steady state leaf tissue. This, not only indicated the involvement of *CanPIs* in plant defense, but also specifically highlighted the importance of 4 IRD *CanPIs* in insect resistance.

***To study properties of recombinant CanPI proteins their effect on H. armigera growth and development***

Based on the sequence analysis results, most divergent forms of 3- and 4- IRD CanPIs were selected for cloning into yeast (*Pichia pastoris*) for expression of the recombinant PIs. Two 3 IRD forms namely CanPI-3 and CanPI-5 and two 4 IRD forms namely CanPI-7 and CanPI-9 were cloned into *P. pastoris* and recombinant PIs were obtained. Cleavage was noted in all the recombinant CanPIs due to extra cellular proteases secreted by *P. pastoris.* The cleaved rCanPIs showed multiple activity bands indicating that the proteases had acted at the linker regions only liberating active PI fragments of 1, 2, and 3 IRDs along with intact uncleaved 3 and 4 IRD CanPI molecules. The cleavage was prevented in an unbuffered medium and uncleaved forms of CanPIs could be procured. The deduced amino acid sequences of CanPIs showed presence of all trypsin inhibitory sites in the 3- IRD CanPIs and two trypsin inhibitory sites in 4 IRD CanPIs. The trypsin and chymotrypsin inhibition by rCanPIs was studied in solution assays and it co-related with the number and type of active sites.

All the four rCanPIs showed inhibition of *H. armigera* gut proteinases in solution assays. Appropriate proportion of CanPIs was incorporated in artificial diet individually and feeding bioassays of *H. armigera* were performed. All the four Can PIs showed anti-metabolic effects on *H. armigera* growth and development with

significant mortality. Of all the four CanPIs, CanPI-7 which had the most diverse IRD combination, turned out to be the best candidate for retarding growth of *H. armigera*.

## Organization of the Thesis

The above work carried out in this thesis has been organized into five chapters and the contents are as follows;

***Chapter 1: Review of Literature***

This section is divided into two parts; **Part I** deals with the description of the polyphagous insect pest *Helicoverpa armigera* with respect to its lifecycle, crop losses, conventional and modern control measures and its adaptive capabilities. **Part II** gives an overview of inducible plant defense mechanisms followed by a detailed description of one of the inducible protein, Pin-II family of proteinase inhibitors and their evolution, structures, endogenous and insect defense functions.

***Chapter 2: In vitro and in vivo effect of Capsicum annuum proteinase inhibitors on Helicoverpa armigera gut proteinases***

This chapter describes purification of two PIs from *C. annuum* leaf tissue, their biochemical characterization and inhibitory activity analysis using standard enzymes and fractionated *H. armigera* gut proteinases. Anti-metabolic effects of the pure *C. annuum* PIs on *H. armigera* are reported as observed by *in vivo* feeding studies.

***Chapter 3: Expression patterns of Pin-II type proteinase inhibitor genes in Capsicum annuum Linn.***

This chapter describes the cloning and details of sequence characterization of several novel proteinase inhibitor genes from *C. annuum (CanPIs)*. The *CanPI* expression patterns *in planta* as well as on wounding and biotic stresses, as revealed by RT-PCR analysis have also been reported.

***Chapter 4: Diverse forms of Pin-II family proteinase inhibitors of Capsicum annuum produce adverse effect on growth and development of Helicoverpa armigera***

This chapter describes the *Pichia pastoris* cloning, of selected *CanPI* genes for recombinant PI expression and their characterization by in gel, *in vitro* and *in vivo*

inhibitory assays with standard enzymes and *H. armigera* gut proteinases has been reported.

***Chapter 5: General discussion and future directions***

This chapter describes important findings of the present research work. Several novel aspects about the diversity in *CanPI* expression have been discussed with respect to the other Pin-II type PIs. The advantages in using *C. annuum* PIs for developing insect tolerant transgenic plants, vis-à-vis some recent and future approaches of insect control have been discussed.

This is followed by ***Bibliography*** and ***Bio-data***

# CERTIFICATE

Certified that the work in the Ph.D. thesis entitled **'Identification and characterization of plant derived proteinaceous inhibitors of gut digestive enzymes of polyphagous insect pest *Helicoverpa armigera* Hübner'** submitted by **Ms. Vaijayanti Tamhane** was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

Date:

Dr. Vidya S. Gupta
(Research Advisor)

# DECLARATION

I hereby declare that the thesis entitled **'Identification and characterization of plant derived proteinaceous inhibitors of gut digestive enzymes of polyphagous insect pest *Helicoverpa armigera* Hübner'** submitted for Ph.D. degree to the **University of Pune** has not been submitted by me for a degree at any other university.

Date:

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# Chapter 1

## Review of Literature

## Part I: Lepidopteran Insect pest *Helicoverpa armigera* and its control

Insects attack almost all the crop plants causing serious economic losses especially in cereals, legumes and cotton. The annual global losses caused by pests (including diseases) amount to as much as US $ 300 billion (Thomas, 1999). A variety of herbivores belonging to different insect orders such as Lepidoptera (butterflies and moths), Coleoptera (beetles), Diptera (true flies) and Hemiptera (true bugs: cicadas, leafhoppers and aphids); especially the first two (Wu *et al.*, 1997), bring about major yield losses by attacking plants in fields or by using the stored grains as a food source. Some of these insects are vectors for transmission of various pathogenic diseases in plants (Hilder and Boulter, 1999).

Lepidoptera (moths and butterflies) is the second largest order in the class Insecta. Most Lepidopteran larvae are herbivorous; some species eat foliage (eg. *Manduca sexta*: tobacco hornworm), some burrow into stems (eg. *Chilo suppressalis*: rice stem borer) or roots (eg. *Vitacea polistiformis*: grape root borer), while some are leaf-miners (eg. *Leucoptera coffeella*: coffee leaf miner). Although many Lepidoptera, especially butterflies, are valued in the aesthetic sense for their beauty and from a horticultural point as pollinators, only a few such as *Bombyx mori*: the silkworm, are exploited commercially. Otherwise, this order represents one of the most destructive groups of insects. The ubiquitous nature of Lepidopteran insect pests is a threat to many agriculturally important crops. Some adult Lepidopteran moths can lay up to 500 eggs at a time, and, upon hatching and emergence, the larvae immediately start feeding voraciously on available food sources (Waterhouse, 1957; Telang *et al.*, 2000). The host plant range for Lepidopteran pests may either be narrow (monophagous), e.g., *Manduca sexta*, which exhibits preference for Solanaceous plants, or diverse (polyphagous), e.g., *Helicoverpa armigera*, which feeds on various legumes, vegetables and fruits. Among the Lepidopteran insect-pests, *Helicoverpa* species have received considerable attention due to their wide geographical presence and heavy economic losses imparted by these insects to a variety of crops.

Members of the Noctuid, genus *Heliothis* are known as agricultural pests of worldwide significance. The four major pest species, *H. armigera*, *H. zea*, *H. virescens* and *H. punctigera* are highly polyphagous and collectively attack a wide range of food, fiber, oil, and fodder crops as well as many horticultural and ornamental crops. On the global scale losses due to *Heliothis*/*Helicoverpa* species in different crops go up to $7.5 billion in addition to insecticides that cost $ 2 billion annually (Gowda and Sharma, 2005). *H. armigera* has been identified as an economically important pest, which spans more than one continent. *H. armigera* has been recorded feeding on 181 cultivated and uncultivated plant species belonging to 45 families in India (Manjunath *et al.*, 1989) with uncovering reports of new plant species as its host **(Fig. 1.1)**. *H. armigera* larvae are extremely damaging because they prefer to feed and develop on the reproductive structures of crops which are rich in nitrogen (Fitt, 1989) and these structures are often part of the crop that is harvested (King, 1994). In India, total losses in cotton and pulses may exceed $ 550 million per annum despite the use of chemical pesticides, the cost of which goes up to another $ 150 million per annum (Gowda and Sharma, 2005). The losses due to *H. armigera* are estimated to be 20-80% in cotton, 14-100% in pigeonpea, 18-26% in sorghum, 30-60% in sunflower, 15-46% in tomato and over 40% in okra (Reddy and Zehr, 2004). As the damage threshold is very low for the high-economic value crops, frequent application of pesticides is necessary for profitable returns. Extensive use of pesticides is detrimental to the environment, human health and the natural enemies of *H. armigera*, which help to maintain the pest population at sub-economic level in most ecosystems.

### *The life cycle of H. armigera*

In the life cycle of *H. armigera*, eggs are laid singly on the lower surface of leaves, flower buds, flowers and young pods, and at times on the shoot tips. They are yellowish white at first and change to dark brown before hatching. The eggs take 3-4 days to hatch in to neonate larvae. First instar larvae have a black to brown head capsule and a yellowish-white body with a spotted appearance. Larval color darkens with successive molts and the larvae typically pass through 5-6 instars in 12-16 days. Fully grown larvae drop to the ground to enter the soil 2-6 cm deep for pupation, which lasts for 7-10 days. Adult moths emerge from just after dark to midnight and crawl onto a plant or vertical substrate where their wings dry (King, 1994). Moths

feed on nectar, females release sex pheromones and mating occurs approximately 4 days after moth emergence. Studies on moth survival and fecundity have indicated that moths can survive up to 9 days (**Fig. 1.2**). Modelers have assumed field fecundities ranging from 500 to 3000 eggs per female, modified by the host plant and the temperature (Fitt, 1989). Developmental duration, color, etc. of *H. armigera* have been found to vary with the host plant.

### *Factors contributing to the major pest status of H. armigera*

Due to its polyphagous nature one or the other host is available throughout the year, allowing *H. armigera* to feed simultaneously on a number of hosts leading to high population buildups (Singh, 2005). The adults are capable of migrating over long distances (up to 155 miles (250 km)) to find suitable weather conditions and host plants, thus surviving under adverse conditions (McCaffery *et al.,* 1989). *H. armigera* exhibits a facultative diapause (110 days of pupal duration) to survive the extremes of both, summer and winter. With the start of warmer weather, the diapause is broken and healthy adult moths emerge out (Ahmad and Rai, 2005). Female moths can lay up to 3,000 eggs each (Shanower and Romeis, 1999) and their fecundity is influenced by temperature, humidity and larval and adult nutrition. The variable development time of *H. armigera* on different host plants (around 28 days), varying number of generations per year (up to 11), strong migratory ability, and co-occurrence of diapausing and nondiapausing individuals together contribute to produce overlapping generations in the field. Due to modern agricultural practices many important plant traits towards biotic and abiotic resistance were not given priority but high yielding and early maturing varieties were emphasized which coupled with better irrigation facilities and application of fertilizers have played a major role in *H. armigera* attaining status of a serious pest (Singh, 2005).

### *Gut proteinase diversity and polyphagous nature of H. armigera*

The digestive complement of *H. armigera* consists of endo-peptidases like serine, metallo- and cathepsin-B like proteinases, and, the exo-peptidases, viz, amino- and carboxypeptidases. The gut proteinase composition varies according to the diet and the developmental stage of the larvae (Patankar *et al.,* 2001; Chougule *et al.,* 2005). Gut proteinase activity increases during larval development with the highest activity seen in the fifth instar larvae, followed by a sharp decline in the sixth instar. Over

90% of the gut proteinase activity of the fifth instar larvae is of the serine proteinase type, however, the second instar larvae show presence of proteinases of other mechanistic classes like metalloproteases, aspartic and cysteine proteases along with serine proteinase activity (Patankar *et al.,* 2001). Trypsin/chymotrypsin-like proteinases from the midgut of *H. armigera* have been purified and characterized by Johnston *et al.* (1991) and Telang *et al.* (2005). A gene coding for Carboxypeptidase A of *H. armigera* has been isolated and expressed as a recombinant protein in insect cells using the baculovirus system (Bown *et al.*, 1998). Screening of the cDNA library prepared from the midguts of *H. armigera* reared on a high-protein and inhibitor free diet has revealed 18 genes encoding trypsin-like proteinases, 14 genes for chymotrypsin-like proteinases and 2 genes for elastase-like proteinases (Gatehouse *et al.*, 1997). Within the chymotrypsin family, there are several distinct subfamilies (Bown *et al.,* 1997; Mazumdar-Leighton *et al.,* 2000). Such a wide array of proteinases as well as their plasticity and variability at different stages of development enable the insect to utilize proteins from a variety of host plants, which may differ in quality and quantity, and explains the polyphagous nature of *H. armigera* (Patankar *et al.*, 2001).

Although the exact nature of the regulatory mechanism governing differential expression of proteinase genes in Lepidopteran insects is not yet known, recent studies have identified neuro-peptide like proteins (Harshini *et al.,* 2002a, 2002b; Davey *et al.,* 2005), which have an ability to "flick the switch of molecular expression". This mechanism allows effective digestion of available plant proteins with a minimal complement of proteinases.

### *Methods to control H. armigera*

Various methods of insect control are, therefore, used to minimize the damage to crop plants in fields as well as the post-harvest produce. Traditional crop husbandry practices that include manipulation of crops such as time of sowing, deep ploughing of soil, intercropping with marigold, linseed, sesame, sorghum, etc as trap crop, hand picking of large sized larvae, spacing and fertilizer application are considered to be useful in reducing crop damage by *H. armigera* (Gowda and Sharma, 2005).

Use of synthetic pesticides for pest control started in 1946 with DDT (dichloro diphenyl trichloroethane) (Elzinga, 1978) followed by chlorinated hydrocarbon,

organophosphate, carbamate and synthetic pyrethroids group. In fact, prolonged exposure of insects to pesticides like organophosphates and synthetic pyrethroids led to resistant pest resurgance and *H. armigera* emerged as 'the most devastating pest' (Armes *et al.,* 1996). Overuse of pesticides has caused the phenomenon of 'pesticide treadmill' which is characterized by increasing use of pesticide, increasing input cost and pesticide-induced outbreak of insect damage resulting in declining crop yield and income of farmers (Soon, 1997). Due to their non-specificity, the chemical pesticides also eliminate other beneficial insects and natural enemies of the pest resulting in a loss of balance in the ecosystem apart from environmental pollution. Contamination of agricultural produce like food grains, fruits and vegetables pose a serious threat to human health. In spite of these shortcomings, synthetic insecticides still remain a popular method of pest control.

A healthier alternative of biological control of *H. armigera* involves deploying the natural enemies i.e. both parasites and predators **(Fig 1.2B)**. The major hurdle in this method of control is mass rearing of the natural enemies at a low cost (Gowda and Sharma, 2005). An egg parasite like *Trichogramma* spp. has been tried out for *H. armigera* control. Some larval and pupal parasitoids like *Goniopthalmus halli* and *Campoletis chlorideae* have been observed to be effective in pigeonpea and chickpea fields, respectively. Encouraging natural predators such as birds by providing bird perches is quite effective but very little success has been achieved so far in utilization of the parasites and the predators. The greatest potential for biological control of *H. armigera* lies in the use of *Bacillus thuringiensis* and *Nuclear Polyhedrosis Virus* (NPV) as microbial insecticides. *B. thuringiensis* synthesizes a family of d-endotoxins (Cry proteins/*Bt* toxins) that form insoluble inclusions during sporulation in the bacterium and exhibit toxicity to a range of invertebrates. After ingestion, the Cry pro-toxin inclusion is solubilized and activated in the insect midgut producing a toxin-induced lysis of midgut epithelial cells followed by death of the insects (Knowles, 1994; Frutos *et al.,* 1999). However, due to photosensitivity, conventional *Bt* sprays lack persistence. NPV belongs to the family of *Baculoviruses* affecting insects. Mode of infection is through ingestion of the virus. The alkaline environment and presence of proteolytic enzymes in *H. armigera* midgut result in dissolution of viral polyhedral wall and coat liberating virions. These virions pass through the midgut walls, enter haemocoel and infect nuclei of the cells in practically all tissues of insect body

leading to death of the insect (http://www.ncipm.org.in). Polyhedral inclusion bodies are extracted from dead insects and formulated as liquid concentrates. Rapid degradation of NPV due to its exposure to UV light is a major problem in fields. Amongst other microbial pathogens, some saprophytic fungi that have been identified for controlling *H. armigera* include *Metarhizhium* (Nahar *et al.,* 2004), *Beauveria* (Hazzard *et al.,* 2003), etc., which secrete enzymes like chitinases that degrade the protective cuticular layer of the insect larvae and lead to innervations of larval mycelia followed, ultimately, by death of the insects. Fungal chitinases and related enzymes are also being evaluated for their standalone effectiveness in controlling insect populations. Culture supernatants of vegetative *Bacillus cereus* or *B. thuringiensis* cells have provided vegetative insecticidal proteins (VIPs) like Vip1, 2 and 3 which have shown acute toxic effects on various pests including *H. armigera* (Hilder and Boulter, 1999; Chen *et al.,* 2003).

Sex pheromone traps for male moths and sprays of natural plant products like Neem oil have come up as semi-modern and alternative technologies. Any of these biological methods of control alone, however, are not as effective as synthetic pesticides.

Unfortunately, level of resistance against *H. armigera* in the available germplasm of host plants like chickpea and pigeonpea has been found to be low to moderate. Frequently, the resistant lines are less preferred in terms of taste, seed color, and/or size and are often susceptible to wilt, sterility mosaic virus, or other diseases. High level of resistance is available in the wild species of cotton, pigeonpea, chickpea, groundnut and tomato but many of these wild species do not cross with the cultivated species with the conventional breeding methodology. Use of tissue culture techniques like embryo rescue or transgenic technology could be used in crop improvement programs to improve resistance of host plants against *H. armigera* (Gowda and Sharma, 2005).

### *Integrated pest management (IPM)*

IPM aims to reduce pesticide inputs and produce a more sustainable farming system (Thomas, 1999). IPM emphasizes the contribution of alternate pest control methods including biological control, host plant resistance breeding and cultural techniques (Oerke and Dehne, 2004). It is generally accepted that under IPM, insecticides should

be applied only when the projected cost of damage from pests is greater than the estimated cost of control measures. IPM practitioners look at the entire range of pests associated with a crop, rather than individual insect species. They devise and implement strategies to keep the pest population below level at which growing the crops become uneconomic, known as the 'economic threshold level'.

### *Transgenic technology*

One of the important aims of research using recombinant DNA technology is to enhance insect resistance of plants by transfer of heterologous genes from various sources (Ferry *et al.,* 2006). Following the initial success of *Bt* toxin against insect pests, it was employed for genetic modification of plants. Development of *Bt*-transgenics in crops like tobacco (Barton *et al.,* 1987) and cotton (Gupta *et al.,* 2000; Carriere *et al.,* 2003) was followed by thorough trials to determine the afforded insect resistance. Commercial introduction of genetically modified maize, potato and cotton plants expressing *Bt* genes in the mid-1990s is the most important landmark in crop improvement. Currently, about 18 million hectares globally are planted with plants expressing *Bt* toxins effective against various insect- pests (Bates *et al.,* 2005). As of today, commercial transgenic crops carry variants of *Bt* including *cry1Aa* (cotton, potato), *cry1Ab* (cotton, maize, potato, rapeseed, tobacco, tomato) c*ry1Ac* (cotton, maize, potato, rapeseed, tobacco, tomato) *and cry3A* (alfalfa, canola, cotton, eggplant, maize, potato, rice, tomato) (http://www.icrisat.org/gt-*Bt*/ResearchBriefs/TABLE2.HTM). Transgenic *Bt* crop plants have revolutionized agriculture by increasing productivity and reducing environmental pollution. Recent reports suggest a 2.4 million tones increase in yields and 21,000 tones pesticide usage cut down due to a shift to *Bt* transgenics crops in the USA accounting to an increase in farm income by $ 1.9 billion (Christou *et al.,* 2006). Initially there was opposition to *Bt* technology, as it involved a toxin for insect control, which probably exerted high selection pressure on insect pests and could lead to pest resurgence, making the toxin technology ineffective. Several laboratory level experiments showed that *Bt* toxins indeed lead to development of *Bt* resistant insect populations (Avisar *et al.,* 2005). But the successful field implementation of *Bt* technology in different crops at field levels for a period of more than 10 years has now changed the views as summarized well in Christou *et al.* (2006). However, in anticipation of insect adaptation to *Bt* (McGaughey *et al.,* 1998; Brousseau *et al.,* 1999) and with the aim to develop a

library of naturally occurring antagonistic agents, attention was also given to other biomolecules.

Inhibitors of amylases and digestive proteinases of insects and lectins are few of the most studied molecules in plant defense (Hilder and Boulter, 1999; Jouanin *et al.*, 1998; Schuler *et al.*, 1998; Sharma *et al.*, 2000; Lawrence and Koundal, 2002; Carlini and Grossi-de-Sa, 2002; Babu *et al.*, 2003; Haq *et al.*, 2004). Lectins disrupt gut cell wall of insects and impair membrane fluidity by binding to the glycoproteins of brush border cells. As an out come of lectin ingestion, nutrient uptake by the insect is hampered. Transgenic expression of snowdrop lectin (*Galanthus nivalis* agglutinin: GNA) has exhibited increased insect tolerance in plants against aphids and some Lepidopteran insects (Down *et al.*, 1996; Gatehouse *et al.*, 1996; Fitches *et al.*, 1997). a-amylase inhibitors (AIs) occur in many plants as part of the natural defense mechanisms. They are particularly abundant in cereals (Franco *et al.*, 2000) and legumes (Ishimoto and Chrispeels, 1996; Grossi-de-sa and Chrispeels, 1997). AIs identified so far are inactive or have very low activity at alkaline pH of the Lepidopteran insect gut. Further research is needed to identify potent a-amylase inhibitors of plant origin or to modify the available inhibitor proteins to have strong activity against Lepidopteran insect pests. AIs, such as the *Hordeum vulgare* (barley) bi-functional a- amylase/trypsin inhibitor are effective against pests of stored grains, especially weevils, which rely on digestive enzymes like a-amylases to meet their nutritional requirements (Garcia-Olmedo *et al.*, 1987). Complete resistance against bruchids was found in transgenic pea, azuki bean and chickpea seeds expressing the amylase inhibitor, aAI-I, from *Phaseolus vulgaris* (common bean) (Shade *et al.*, 1994; Shroeder *et al.*, 1995; Ishimoto and Chrispeels, 1996; Sarmah *et al.*, 2004).

Recently, insecticidal proteins from non-plant sources have also been used to develop transgenic plants. cDNA for bovine spleen trypsin inhibitor (SI) including the natural mammalian presequence was expressed in tobacco using *Agrobacterium tumefaciens*-mediated transformation (Christeller *et al.*, 2002). Expression of modified *SI* in transgenic tobacco leaves at 0.5% of total soluble proteins reduced both, survival and growth of *H. armigera* larvae, feeding on leaves from the late first instar. Use of insect molting hormone expressed in transgenic plants proved to be effective in inducing early molting in insects, rendering them unfit for population development (Christou *et al.*, 2006). In another study, a fusion protein consisting of an insect

neuropeptide (*Manduca sexta* allatostatin) and snowdrop lectin (GNA) was found to inhibit feeding and prevent growth of fifth stadium larvae of the tomato moth *Lacanobia oleracea* (Fitches *et al.,* 2002). Similarly, a fusion protein consisting of GNA and an insecticidal spider venom neurotoxin (*Segestria florentina* toxin 1: SFI 1) also proved insecticidal to the first stadium larvae of *L. oleracea* in feeding studies (Fitches *et al.,* 2004).

## Proteinase inhibitor based approach for insect control

Serine proteinases have been identified in extracts from the digestive tracts of many insects; particularly those of the Lepidoptera (Applebaum, 1985). Studies on the effects of PIs on insects first began in the 1950s when Lipke *et al.* (1954) found that a protein fraction from soybeans inhibited growth, as well as proteolytic activity *in vitro*, of the mealworm, *Tribolium confusum*. A large number of PIs from various families of plants were studied thereafter for their 'antinutritional' effects on insects. PIs function by blocking the digestive proteinases in the larval gut thereby limiting the release of amino acids from food proteins (Hilder and Boulter, 1999). As a consequence, growth and development of the larvae are arrested (De Leo *et al.,* 2001; Damle *et al.,* 2005). PIs do not exert strong selection pressure on insects since they do not cause mass death of the insect populations. PIs reduce overall fitness of the insect populations and thus reduce their impact on crop. PIs also affect a number of vital metabolic processes in insects. They interfere with proteolytic activation of enzymes as well as molting and water-balance of the insects (Hilder *et al.,* 1993; Boulter 1993). Another advantage is that antagonistic effects of PIs on insects act synergistically along with many other components of the ecosystem. In tritrophic interactions (plants, pests and their predators) the retarded insects become easy targets for greater parasitism by natural enemies (Lewis *et al.,* 1997). Their effectivity against insects has been demonstrated by feeding the larvae on artificial diet containing PIs (Broadway and Duffy, 1986b; Johnston *et al.,* 1993; Harsulkar *et al.,* 1999; Bown *et al.,* 2004; Giri *et al.,* 2005) and has been summarized in **Table 1.1.** Success of these experiments has led the way to transgenic expression of PIs in various crop plants.

**Table 1.1: Effects of dietary proteinase inhibitors on insects**

| Insect | PI | Effect | Reference |
|---|---|---|---|
| *H. armigera* | Soybean Kunitz PI | Weight reduction | Johnston *et al.,* 1993 |
| *Heliothis zea* | | Weight reduction | Broadway and Duffey, 1986b |
| *Spodoptera exigua* | | Weight reduction | |
| *Callosobruchus maculantus* | Soybean BBI | Mortality | Gatehouse and Boulter, 1983 |
| *H. armigera* | | Weight reduction | Johnston *et al.,* 1993 |
| *Spodoptera exigua* | Potato PIN-II | Weight reduction | Broadway and Duffey, 1986b |
| *H. armigera* | Groundnut PI | Weight reduction, delayed pupation | Harsulkar *et al.,* 1999 |
| *Spodoptera litura* | Mustard PI | Reduction in fertility | DeLeo and Gallerani, 2002 |
| | Bittergourd | Reduction in weight, fertility and fecundity | Telang *et al.,* 2003 |
| *H. armigera* | Winged bean PI | Weight reduction | Harsulkar *et al.,* 1999 |
| | Bitter Gourd PI | Reduction in weight, fertility and fecundity | Telang *et al.,* 2003 |
| | Tomato PI | Reduction in weight and fecundity | Damle *et al.,* 2005 |
| | Chickpea PI | Weight reduction | Srinivasan *et al.,* 2005a |

Most PIs, being products of individual genes rather than complex biochemical pathways, are easier to manipulate. The first proteinase inhibitor gene of plant origin, which was transferred to tobacco, encoded a double-headed trypsin inhibitor (CpTI) from *Vigna ungiculata* (cowpea). It resulted in enhanced plant resistance towards *H. virescens* (Hilder *et al.,* 1987). Similar results were obtained in field trials in the US against *H. zea* (Hoffmann *et al.,* 1992). Further research and development has led to establishment of many PI-transgenic varieties of plants as elaborated in **Table 1.2**.

**Table 1.2: List of plant proteinase inhibitors used to develop transgenic plants**

| Proteinase Inhibitor Type | Plant Source | Inhibition Specificity | Target Plant | Target Insect Class(es) |
|---|---|---|---|---|
| Kunitz | Giant taro (*Alocasia macrorrhiza)* | Trypsin, Chymotrypsin | Tobacco | Lepidoptera |
| Kunitz | Soybean (*Glycine max)* | Trypsin | Tobacco, Poplar, Rice, Potato | Lepidoptera, Coleoptera |
| Kunitz | Sweetpotato (*Ipomoea batatas*) | Trypsin | Tobacco | Lepidoptera |
| Bowman-Birk | Cowpea (*Vigna unguiculata*) | Trypsin | Tobacco, Rice, Potato, Apple, Sunflower, Strawberry, Cotton, Wheat, Pigeonpea | Lepidoptera, Coleoptera |
| Potato type I PI | Tomato (*Lycopersicon esculentum*) | Chymotrypsin | Tobacco | Lepidoptera |
| Potato type II PI | Potato (*Solanum tuberosum*) | Trypsin, Chymotrypsin | Tobacco | Lepidoptera |
| Potato type II PI | Tomato (*Lycopersicon esculentum*) | Trypsin, Chymotrypsin | Tobacco, Rice | Lepidoptera |
| Potato type II PI | Tobacco (*Nicotiana alata*) | Trypsin, Chymotrypsin | Tobacco, Peas | Lepidoptera |
| Mustard TI | Mustard (*Brassica nigra*) | Trypsin, Chymotrypsin | Tobacco, Arabidopsis, Rapeseed | Lepidoptera |
| Barely TI | Barely (*Hordeum vulgare*) | Trypsin, Amylase | Tobacco | Lepidoptera |
| Squash TI | Squash (*Cucurbita maxima*) | Trypsin | Tobacco | Lepidoptera |
| Cysteine PI | Rice (*Oryza sativa*) | Papain | Arabidopsis, Poplar, Tomato, | Coleoptera |
| Cysteine PI | Maize (*Zea mays*) | Cathepsin L | Rice | Coleoptera |

Adapted from Giri *et al*. (2005).

### *Effect of PI ingestion on insects*

Although most of the plant species, including hosts, express PIs, not all are effective in defense against herbivorous insects, infact; insects thrive on the host plants. The answer to this paradox might be found in adaptation of the insect to its host plant PIs. One such evidence was from chickpea seeds where Bowman-Birk type trypsin inhibitors were inactivated by the digestive action of *H. armigera* gut proteinases (Giri *et al.,* 1998). However, a recent report has stated that chickpea seeds also contain a Kunitz type PI (CaKPI) that exhibits antagonistic effects on growth and development of *H. armigera* (Srinivasan *et al.,* 2005). Low level of expression of CaKPI in chickpea seeds was proposed to be one of the reasons for the success of *H. armigera* in colonizing chickpea as a host plant. Other studies proposed that the insect chooses a developmental stage of plant tissue where host defense is inadequate as in *Cajanus cajan* and tomato (Chougule *et al.,* 2003; Damle *et al.,* 2005). Thus, although the host plants possess effective PIs, their importance in plant protection could be undermined due to low level of expression. This hurdle can be overcome by expressing host-PI genes under a stronger promoter to increase the inhibitor protein content. Another approach to improve PI-based plant defense would be to express potent non-host PI genes in host plants. The PIs are thought to contribute significantly in making the non-host plants unsuitable as food for the insect. PIs from non-host plants, such as winged bean and bittergourd showed inhibition of the gut proteinases of *H. armigera* reared on host plants like chickpea, pigeonpea, tomato, cotton and okra (Patankar *et al.,* 2001). Moreover, these PIs added to larval diet inhibited growth of *H. armigera* and *Spodoptora litura* larvae (Harsulkar *et al.,* 1999; Telang *et al.,* 2003). Thus, the nature of insect response to ingested PIs is implicated in the success or failure of the PI strategy (Jongsma *et al.,* 1995).

Lepidopteran insects are equipped to counteract the deleterious effects of ingested PIs by three possible mechanisms. Firstly, generalized over expression of digestive proteinases by the insect can compensate for the loss of activity due to inhibitor binding (Broadway and Duffey, 1986b; Broadway, 1997; Gatehouse *et al.,* 1997; Chougule *et al.,* 2005). The second option for the insect can be a *de novo* synthesis of proteinases, which are insensitive to inhibition by ingested PIs (Broadway, 1996; Bown *et al.,* 1997; Mazumdar-Leighton and Broadway, 2001a, 2001b; Volpicella *et al.,* 2003). Finally, the insect can synthesize or over express those proteinases,

which can bind and degrade the ingested PI (Giri *et al.,* 1998). These studies have concluded that the changes in the gut proteinase complement are complex, as a subset of proteinase genes (insensitive to proteinase inhibitors) gets up regulated while a subset of proteinase genes (sensitive to proteinase inhibitors) gets down regulated. Appropriate PI(s) need to be selected or modified from the available PI pool to inhibit both constitutively expressed as well as induced proteinases. For sustainable PI-mediated plant resistance against *H. armigera*, Harsulkar *et al.* (1999) have suggested a combinatorial approach that involves successive expression of two non-host PIs. They argue that this strategy would force the insect to change its gut complement at least twice, reducing the possibility of insect adaptation to PIs.

## Part II: Pin-II family of proteinase inhibitors

### *Induced plant defenses*

Competition for survival is continuously going on amongst all organisms. This includes struggle during which interactions of different organisms are inevitable. Some organisms, particularly animals, move when they are in direct competition, but sedentary lifecycle of plants prevent themselves from doing so. They have, thus created structural and biochemical barriers for effective protection. The structural barriers like chitin and suberin prevent exposure of plant tissue to pathogens in addition to prevent water loss. When these barriers are broken the entry of pathogenic fungi/bacteria in the plant is inevitable (Zhou and Thornburg, 1999). To limit these and insect infestations plants have evolved potent phytochemicals in the form of direct and indirect defenses and their complex regulation on induction is governed by important phytohormones like Jasmonic Acid (JA), Salicylic Acid (SA) and its derivatives, ethylene and abscisic acid. The hormones elicit various defense genes through separate signal transduction pathways, which depending on the type of infection/ infestation, cross communicate by either complementing or antagonizing their actions (Karban and Baldwin, 1997). The cross–talk between the defense signaling pathways provides the plant with elaborate regulatory potential that leads to the activation of most suitable defense against the invader encountered (Pieterse *et al.*, 2001; Van Wees *et al.*, 2003).

Many plants respond to herbivore attack by activating defense genes in leaves whose products inhibit digestive proteases of herbivores and reduce the nutritional quality of

the ingested proteins, making the attackers ill. In tomato plants, wounding causes a systemic reprogramming of leaf cells that results in the synthesis of over 20 defense-related proteins (Ryan, 2000). This is analogous to the inflammatory and acute phase responses of animals in response to trauma. Most of the newly synthesized proteins fall into functional groups such as (i) signal pathway components, (ii) antinutritional proteins, including both proteinase inhibitors and polyphenol oxidase (PPO), and (iii) proteinases (Ryan, 2000). Several other genes are also activated, but they do not fall into the above groupings and their roles in defense are obscure.

### *Systemin signaling and activation of the Jasmonate pathway*

Signal transduction pathway appears to be a strategy of the plant to amplify its ability to mount a maximal defense response against the attacking predators. Wound-inducible signal pathway genes in tomato leaves include prosystemin, calmodulin (CaM), lipoxygenase (LOX) and allen oxide synthase (AOS). The latter two enzymes are components of the octadecanoid pathway and they reside in the chloroplast where they participate in the conversion of linolenic acid to signaling molecules, phytodienoic acid (OPDA) and JA, which are oxylipin analogs of prostaglandins.

The mechanism of this systemic signaling was discovered by identification of an 18 AA peptide `systemin' from tomato leaves (Pearce *et al.*, 1991) formed from a larger precursor molecule (200 AA) prosystemin. Antisense prosystemic tomato plants were shown to be deficient in long distance wound signaling (Orozo-Cardenas *et al.*, 1993). On wounding systemin activates defense related genes through the jasmonate pathway. It travels through the phloem to the distal parts of the plant, triggering release of more systemin from prosystemin locally and systemically. However, prosystemin lacks a signal sequence and glycosylation sites and is apparently not synthesized through the secretory pathway, but in the cytoplasm. The polypeptide activates a lipid-based signal transduction pathway in which the 18:3 fatty acid, (linolenic acid), is released from plant membranes and converted to the oxylipin signaling molecule jasmonic acid. A wound-inducible systemin cell surface receptor with MW of 160,000 (Sr160) has recently been identified. The receptor regulates an intracellular cascade including, depolarization of the plasma membrane, the opening of ion channels, an increase in intracellular Ca2+, activation of a MAP kinase activity and a phospholipase A2 activity. These rapid changes appear to play important roles

leading to the intracellular release of linolenic acid from membranes and its subsequent conversion to jasmonic acid, a potent activator of defense gene transcription (**Fig. 1.3**). Systemin is active at extraordinarily low levels (i.e., fmol/plant) and ranks among the most potent gene activators known (Ryan, 2000).

Recently, small, hydroxyprolin rich peptides with defense signaling activities similar to systemin have been identified (Ryan and Pearce, 2003) from tomato and called Hydroxyproline rich systemins (TomHypsys). Systemin is found only in solanae sub tribe of Solanaceae where as hydroxyprolin systemins are broadly distributed and function similarly by amplifying the jasmonate pathway in wounded plant tissues.

Jasmonate and signaling derivatives of oxygenated lipids C16 and C18 play an important role in regulation of plant defense and development. Plant defense against insect herbivores and some microbes are signaled through the biosynthesis and action of JA. JA is the ultimate product of octadecanoid pathway. Linolenic acid is converted to 13 Hydroperoxide by enzyme lipoxygenase. 12 Oxyphytodienoic acid (OPDA) is further formed from it due to action by 2 enzymes, allen oxide synthase and allen oxide cyclase. This is the first cyclopentenone compound formed in the pathway, which is further reduced followed by 3 B oxidation steps in perioxysomes to finally form JA (Stintzi and Browse, 2000) (**Fig. 1.3**). Role of JA in major reprogramming of gene expression on wounding/ insect attack is well demonstrated (Reymond *et al.,* 2000). Several evidences now support the view that OPDA and other cyclopentenone apart from JA also play an important role by eliciting different set of defense genes (Stintzi *et al.,* 2001), mediated by structural feature, a, ß unsaturated carbonyl group of cyclopentenone. Recent findings indicate that the plant cell wall also plays an active role in defense as a source of peptide signals for systemic wound signaling (Narváez-Vásquez *et al.,* 2005).

The signaling molecules induced by wounding or insect attack further elicit signal transduction pathways for local and systemic expression of defense compounds and enzymes, which protect the plant from the invader with their different modes of action. These have been studied extensively in Tomato and are summarized in the **Table 1.3**. Of these different inducible defense molecules my laboratory focuses on the study of PI mediated plant defenses. The following paragraphs give a brief description of PIs and their types.

**Table 1.3: Inducible anti-nutritional proteins of the Tomato plant**

| Plant Protein | Putative mode of action | Stability in insect gut | Herbivory/ wounding induced |
|---|---|---|---|
| ***Signal pathway genes*** | | | |
| Prosystemin<br>Systemin receptor | Involved in initiation of defense signaling | Unknown | Yes |
| ACC synthase | A key enzyme in ethylene biosynthesis | Unknown | Yes |
| NADPH oxidase | An enzyme involved in the production of active oxygen species | - | Yes |
| ***Defense genes*** | | | |
| Lipoxygenase | Formation of lipid peroxides and breakdown products with strong electrophilic action. Potential loss of amino acids. | Unknown | Yes |
| Allene oxide synthase | Participate in the conversion of linolenic acid to signaling molecules, phytodienoic acid and jasmonic acid. | - | Yes |
| Calmodulin<br>Polygalacturonas e | Calcium binding protein<br>Secondary messengers for the production of PIs | -<br>Unknown | Yes<br>Yes |
| Cysteine PI | Inhibition of cysteine proteases and overproduction of proteases. Likely targets are insects with acidic digestive systems (e.g., beetles). | Yes | Yes |
| Aspartic PI,<br>Metallo/ carboxypeptidase I | Inhibition of respective proteinases | Unknown | Yes |
| Serine PI-I<br>Serine PI-II | Inhibition of serine protease; hyper accumulation of inhibitor-insensitive proteases; amino acid deprivation. | Yes | Yes |
| Polyphenol oxidase | Formation of quinones and subsequent reactions with nucleophilic side chains of amino acids (e.g., lysine, histidine, cysteine). | Yes | Yes |
| Peroxidase | Formation of quinones and subsequent reactions with nucleophilic side chains of amino acids. Protein cross-linking | Possible | Yes |
| Phenylalanine ammonia lyase (PAL) | Enzymatic removal of phenylalanine. | Unknown | Primarily pathogen-induced |
| Cathepsin D | Inhibition of aspartyl proteases and overproduction of proteases. Likely targets are insects with acidic digestive systems (e.g., beetles). | Yes | Yes |
| ***Proteinase genes*** | | | |
| Leucine amino peptidase | Liberation of arginine from N terminus of peptides; possibly acts in tandem with ARG. | Yes | Yes |
| Aspartic proteinase<br>Cysteine proteinase | Regulation of endogenous proteinases | | Yes |
| Arginase | Enzymatic removal of arginine | Yes | Yes |
| Threonine deaminase | Enzymatic removal of threonine. | Yes, activated in insect gut | Yes |

(Modified from Felton, 2005; Ryan, 2000)

### *PIs of plant origin*

Proteinase inhibitor is one of the most studied classes of plant defense proteins. PIs are abundantly present in the storage tissues of plants and can represent up to 10% of total protein (Ussuf *et al.,* 2001). Their gross physiological function is the prevention of unwanted proteolysis (Laskowski and Kato, 1980). PIs play key regulatory roles in many biological processes, including the blood coagulation system, the complement cascade, apoptosis and the hormone processing pathways (Laskowski and Kato, 1980; Christeller, 2005). Majority of plant PIs are in the range of 8 to 20 kDa. These PIs usually have a high content of cysteine residues that form disulfide bridges and confer resistance to heat, extreme pH and proteolysis. Studies on the biosynthesis of several plant PIs has demonstrated that these PIs are synthesized as either prepro-proteins or pre-proteins that are processed either during or after synthesis to produce the native PIs. Many PIs are produced in response to various biotic and abiotic stress conditions, e.g. pathogen invasion, insect attack, wounding, and environmental stress such as salinity and dehydration (Koiwa *et al.,* 1997; Ussuf *et al.,* 2001). PIs are broadly classified on the basis of type of proteinases they inhibit. Inhibitors of serine, cysteine, aspartic and metallo proteinases are reported in various plant species. By far, most of the known PIs are specific for serine proteinases (Garcia-Olmedo *et al.,* 1987; Ryan, 1990). Serine proteinase inhibitors are further classified in to different families such as Kunitz, Bowman-Birk, Wound-inducible (potato proteinase inhibitor {PIN} type I and II), Squash, Cereal trypsin /α-amylase inhibitor, Mustard seeds trypsin inhibitor, etc, depending on their molecular mass, sequence homology and pattern of expression in plant tissue, etc (Garcia-Olmedo *et al.,* 1987; Ryan, 1990; Christeller and Laing, 2005) **(Table 1.4)**. All the serine PIs seem to obey a standard 'lock and key' mechanism of inhibition, which has been thoroughly studied with the help of X-ray crystallography of the three-dimensional structure of several enzyme-inhibitor complexes. The inhibitors are highly specific substrates for their target enzymes, which undergo a limited and extremely slow proteolysis, so that the system behaves as if the free enzyme and the inhibitor are in simple equilibrium with the enzyme/inhibitor complex.

**Table 1.4: Serine proteinase inhibitor families and some of their characteristic features**

| PI family | Characteristic features | | |
|---|---|---|---|
| | Molecular weight (kDa) | Number of disulfide bridges | Active site |
| Soybean trypsin inhibitor (Kunitz) | 20 | 2 | Majority-1, TI or CI |
| Bowman-Birk | 8-16 | 7 | 2, TI and CI |
| Potato inhibitor-I | 8 | 1 or none | 1, CI |
| Potato inhibitor-II | 12 | 5 | 2, CI and TI |
| Squash inhibitor | 3 | 3 | 1, TI or EI |
| Cereal trypsin /α-amylase inhibitor | 13 | 5 | TI and/or AI |
| Mustard seed trypsin inhibitor | 7 | 4 | 2, TI |

TI=trypsin inhibitor; CI=chymotrypsin inhibitor; EI=elastase inhibitor

On the surface of each inhibitor molecule, there is at least one reactive bond (P1-P1'), which interacts with the active site of the enzyme. An inhibitor molecule, which has undergone hydrolysis of its reactive bond is as active as the unhydrolyzed inhibitor and is able to form a stable complex with the enzyme. In most, but not all, of these inhibitors the reactive site peptide bond is encompassed in at least one disulfide loop which ensures that during conversion of original to the modified inhibitor the two peptide chains can not dissociate. The nature of the amino acid residue at the P1 position generally determines the proteinase inhibited. Lys or Arg for trypsin-like enzymes, Phe, Tyr or Leu for chymotrypsin and Ala for elastase are usually present at the P1 position of the inhibitor. P1' position does not seem to be involved in determining specificity. More than one reactive site is present occasionally in a single polypeptide chain, in which case more than one enzyme molecule of the same or different specificity can be simultaneously inhibited by a single inhibitor molecule

(Laskowski and Kato, 1980; Garcia-Olmedo *et al.,* 1987). The inhibitory loops of serine protease inhibitors have a characteristic conformation, defined by the torsion angles of the P3–P3' segment, irrespective of the family they belong to, while the remaining part of the molecule, known as scaffold, has widely different folds in different families of inhibitors. The amino acid sequence of the inhibitory loop shows high degree of variability except at the P1 recognition site and yet the conformation of the loop is strikingly similar. Earlier studies have also indicated a strong scaffolding contribution towards the rigidity of the reactive site loop (Khamrui *et al.,* 2005).

Proteinase inhibitors belonging to the family of Potato inhibitor II type or Pin-II type have been studied extensively with respect to their gene evolution, protein structures, endogenous functions and insect defense functions. The following sections give an overview of various structural and functional aspects of this PI family.

## Pin-II Family of Proteinase inhibitors

### *Occurrence*

Pin-II type PIs are one of the important serine PIs and the only wound inducible type PIs which have been extensively studied. They were initially found only in Solanaceae where wound induced up regulation of Pin-II PI proteins and gene was demonstrated and their functional co-relation to insect defense was established (Green and Ryan, 1972). EST and genomic database screening led to the identification of many homologous genes dispersed throughout the whole range of mono and dicotyledonous plants indicating more widespread occurrence of Pin-II family. The striking feature of Pin-II family is the presence of variable number of inhibitory repeats, structurally forming multi-domain protein. The number of inhibitory repeats varies from 1 to 8 repeat domains amongst different members of Solanaceae screened (**Fig. 1.4** ). The occurrence and development of multiple repeat Pin-II PIs have been found only in Solanaceae where as single repeat PIs of this family are found among other families (**Fig. 1.5**). These PIs are thought to be the ancestral members that have given rise to the other forms by series of domain duplication events (Barta *et al.,* 2002) **(Fig. 1.6)**.

### *Gene structure and evolution of Pin-II PIs*

The gene structure of Pin-II family is conserved. It consists of an exon encoding the N terminus of the signal peptide followed by second main exon encoding the C-terminus of the signal peptide and variable number of repeats that are always separated by an intron of 100-200bp (Barta *et al.*, 2002) **(Fig. 1.4)**. The repeats are linked to each other by a small stretch of 5 AA residues. The sequence of these linker regions is almost conserved in *Nicotiana* sp (EEKKN), whereas in Pin-II PIs of other genera it is different in AA sequence though it functions similarly. The linker region is very sensitive to proteases and is cleaved by endogenous proteinases *in planta* (Heath *et al.*, 1995). The sequence of the repeats with ~ 55 amino acids is highly variable, however, presence of eight cysteines and a single proline residue in a repeat are conserved throughout. The cysteines are involved in formation of four di-sulphide bonds, which stabilize the repeat structure. An endoplasmic reticulum signal peptide is present at N terminus of all the Pin-II PIs and a C terminal peptide that functions, as vacuolar-targeting signal is present in some Pin-II PIs.

The striking feature of multi-domain Pin-II PIs is that the sequence repeat does not correspond to the structural repeat. It has been postulated that gene duplication took place in an ancestral aPI1 (from *N. alata*) or PSI1.2 (from *C. annuum*) like IRD in which sequence repeat corresponds to structural repeat. This ancestral sequence, containing EEKKN linker would have led to the random incorporation of amino acids between the two repeats. After inclusion of the DPRNP linker via this mechanism, the linker may have caused the resulting two repeat protein to domain swap from the ancestral domain structure to modern domain structure to attain a thermodynamically more stable conformation **(Fig. 1.6 and 1.7)**. The Pin-II PIs with higher number of repeats could have been produced by additional events of gene duplication as well as by unequal crossing over or repeat insertion and truncation events (Schirra and Craik, 2005) **(Fig. 1.6).** Unequal crossing over is presumed to be responsible for the expansion of the repeated domains.

Pin-II PIs are coded by one, two or multigene family in *Nicotiana, Solanum* and *Capsicum*, respectively. In *Nicotiana* most of the members show presence of single functional genomic copy of Pin-II PIs, though in its various species the number of IRDs might vary from 2 to 8 **(Fig. 1.8)**. It was suggested by Wu *et al.* (2006) that the number of IRDs in different *Nicotiana* taxa was independent of phylogenetic

associations. The repeat expansion events appeared to be haphazard, since plants with close phylogenetic relationships had different repeat numbers as in case of *N. acuminata* and *N. corymbosa* with 7 and 2 IRDs, respectively (Wu *et al.,* 2006). The evolutionary advantages of repetitive IRDs have not been established, but it is reasonable to assume that repetitive domains provide plants with a more efficient use of transcription, translation and cell compartment targeting (Heath *et al.,* 1995).

In case of tomato, a genomic clone of Pin-II type PI gene showed regulatory region containing two wound responsive elements similar to box-WUN-motif that may account for the wound inducible expression of the PI. In addition, ELI-box3, TCA-element and ABRE were possibly involved in regulatory expression of tomato PI (Zhang *et al.,* 2004).

### *Pin-II protein structures*

Precursor proteins of Pin-II family are found to have very peculiar structures. The structures of single domain Pin-II PIs of *N. alata*, precursor PIs with two domains from potato and tomato and 6 IRD PIs of 43 kDa from *N. alata* have been studied.

The NaProPI precursor forms a circular 'clasped bracelet' conformation as a result of formation of disulphide bridges between the partial repeat regions at the N and C terminal of the precursor **(Fig. 1.9)**. Single domain PIs having either TI or CI active sites are formed by proteolytic cleavage at the linker regions of the 6-domain *N. alata* precursor (4-TIs and 2-CIs) (Heath *et al.,* 1995; Lee *et al.,* 1999). Each of the single domain PIs comprises 53 AA, including 8 cysteine residues, which are linked to form 4 disulphide bridges (Nielsen *et al.,* 1995). The single IRD CI protein contains a triple stranded B-sheet as the dominant secondary structural element, with several turns and a short region of helix. The putative chymotrypsin-reactive site is present on an exposed loop, which is less defined, than the rest of the protein. The overall shape of CI is disk like and the N and C termini are exposed, consistent with the proposal that this protein results from post-translational processing of the precursor protein (Nielsen *et al.*, 1994, 1995; Greenblatt *et al.,* 1989). Due to the high sequence identity between TI and CI domains it has been anticipated that the trypsin inhibitors adopt similar 3D structures to CI **(Fig. 1.9B and 1.9C)**.

The 43 kDa precursor PI of *N. alata* NaProPI has N terminal endoplasmic reticulum signal peptide followed by a series of six highly homologous IRDs and a 25 AA C

terminal vacuolar targeting signal. Processing of the NaProPI in the secretory pathway removes the signal peptide and the vacuolar sorting signal and further leads to release of single IRDs, two of which are CIs and four are TIs. It has been demonstrated that the sequence repeat in NaProPI does not correlate with the structural repeat in this 43 kD inhibitor. An active two-chain domain (C2) is formed by joining two partial domains in addition to five single chain domains (TI1-TI4 and CI1) (**Fig. 1.9B**).

Further studies have been conducted to find the structure and possible folding mechanism of single domains corresponding to sequence repeat and two contiguous structural domains, CI and TI, of the same precursor (Scanlon *et al.,* 1999; Schirra *et al.,* 2001).

aPI1 for example, a recombinant protein corresponding to a single repeat, adopts a stable three dimensional structure representing a circular permutation of the usual PI fold, but still shows CI activity similar to the wild type IRDs (Scanlon *et al.,* 1999) (**Fig. 1.9C**). However duplication of the repeat sequences to form aPI2, a recombinant two-repeat inhibitor, results in a protein that folds like the naturally occurring PIs across the repeats, by forming a circularized clasped bracelet fold. The fold across the repeats is thermodynamically more stable than the fold along the repeats when multiple repeats are present in the inhibitor. This behavior is reminiscent of `domain swapping' in the multidomain proteins (Lee *et al.*, 1999) (**Fig. 1.6 and 1.7**).

The first two single chains IRDs (CI-TI) also adopt the same consensus structure although five residues from the active site loop of the contiguous inhibitors are missing. Even in the absence of the six domains together the CI-TI two domain PIs (CI-TI) acquire a similar conformation as in a complete 6-domain precursor. Individually each domain has identical secondary structure and the linker region connecting the two domains acquires form of a distorted loop. It has been clearly shown that the CI-TI domains are essentially independent of each other and have no long-lived and highly specific interactions between them. The lack of strong inter domain association is likely to be important for individual inhibitors to ensure that there is no masking of reactive sites. In all precursor Pin-II PI sequences, two types of linkers EEKKN and DPRNP were detected. Due to presence of prolines in the DPRNP linker and none in EEKKN linker, the former type can adopt a smaller conformational range. EEKKN linker has no conformational preference, while

DPRNP prefers its own incorporation into the structure of PI domain to an extended conformation (Schirra and Craik, 2005) (**Fig. 1.7** ).

The crystal structure of unbound form of two IRD PI of tomato inhibitor II by Barette-Ng *et al.* (2003a, b.) reveals significant conformational flexibility in the absence of bound proteinases. Each individual IRD adopts the fold determined previously for the single domain Pin-II inhibitors. The N terminus of the TI-II initiates the folding of domain I and then completes the folding of domain II before coming back to complete the rest of domain I. Four copies of the unbound inhibitor within the asymmetric unit of crystalline unit cell, provids a unique opportunity to examine significant range of conformational flexibility present in the global structures of the inhibitor and flexibility within reactive site loops. Conformational flexibility seen in the reactive site loops of unbound TI-II suggests a mechanism by which the inhibitor can balance the need for tight binding required for broad inhibitory function (Barette-Ng *et al.*, 2003a). The crystal structures of TI-II show dramatic change in conformational flexibility in the bound and unbound forms (Barette-Ng *et al.*, 2003b). (**Fig. 1.10**)

The structural basis of inhibition of a multidomain inhibitor from Pin-II family of PI (TI-II) has been shown by its ternary complex with two subtilisin Carlsberg molecules and revealed how it can bind to and simultaneously inhibit two enzyme molecules within a single ternary complex (Barette-Ng *et al.,* 2003a). An inhibitory reactive site loop is found in each domain of TI-II and these loops are positioned at opposite ends of the elongated molecules there by allowing a single inhibitor to bind to two proteinase molecules simultaneously (**Fig. 1.11**). There is a considerable reduction in flexibility of the loop on binding to proteinases and no inhibitor cleavage is observed. Remarkable distortion of the active site of subtilisin is induced by the presence of phenylalanine in the P1 position of the reactive site of domain II of the TI-II.

### *Potential of Pin-II PIs to inhibit proteinases*

Each inhibitory repeat of the Pin-II precursor contains a single reactive site. The P1 residue of the reactive site, which reacts with proteinase active site, determines its specificity. Presence of lysine 'L' or argenine 'R' in the P1 position confers the inhibitor with either chymotrypsin or trypsin inhibitory potential. P3 to P2' a stretch of 5 AA's close to the reactive site is important in determining enzyme specificity of

the inhibitor. Most of the variations in the IRD sequences are found in or close to this reactive site loop, thus conferring a range of varied inhibitory specificities to the Pin-II PIs. The reactive site loop comprising residues P4 to P3' of the inhibitor domain interact with S6 to S2' of proteinase pocket to bring about its inhibition by mimicking a substrate. Unbound inhibitors display a considerable conformational variability in the reactive site loop (Lee *et al.*, 1999; Nielsen *et al.*, 1995). The core reactive site loop i.e. ($P_3$-$P_2$) does not show very high sequence variability. It is bound by two disulphide bonds (eg. **C**PRN**C, C**TLN**C**,) which gives the reactive site a considerable rigidity, while the AA in the adjoining region of this core segment show a very high sequence variability, conferring flexibility of broad inhibitory potential. In PIs with broad inhibitory potential the reactive site loop must retain a certain degree of flexibility in order to allow binding of the PI to the binding sites of a variety of different proteinases (Barret-Ng *et al.*, 2003a).

Study by Bryant *et al.* (1976) was one of the pioneering reports on the purification and characterization of Pin-II PIs from potato. Proteinase inhibitor II, an inhibitor of chymotrypsin and trypsin, is found to be a heat-stable protein with a dimeric molecular weight of 21,000. Four monomeric isoinhibitor species of molecular weight 10,500 comprising inhibitor II have also been isolated. Reconstituted dimmers from these possess two binding sites for bovine alpha-chymotrypsin, indicating that each monomer possesses one binding site for this enzyme. Significant differences have been noted among the reconstituted dimers in their isoelectric points, immunoelectrophoretic mobilities, ion-exchange properties, and their inhibitory activities against trypsin. The properties of the inhibitor II dimeric species are similar but not identical to inhibitors IIa and IIb reported from Japanese potatoes (variety "Danshaku-Imo"), indicating the existence of intervarietal, as well as intravarietal, differences among potato tuber inhibitor II isoinhibitors. Further the amino acid sequence of two low molecular weight proteinase inhibitors from Russet Burbank potatoes have shown difference only at nine positions but specificity towards chymotrypsin and trypsin inhibition (Hass *et al.*, 1982).

In various members of Pin-II precursors studied there is a combination of trypsin/ chymotrypsin inhibitory domains. For example the six domain *N. alata* PI (NaProPI) possesses four TI domains and two CI domains. In potato PI (PotII PI), which has two IRD's, one is TI specific and the other is CI specific. Single IRD PI of Pin-II family

can bind to a single proteinase, while two domain Pot II PIs of tomato and potato can simultaneously inhibit two proteinase molecules. However, the NaProPI with 6 IRDs cannot bind to six proteinases simultaneously because of steric interference. This six IRD molecule of *N. alata* could inhibit maximum of four chymotrypsin or 2.6 trypsin molecules. It is thus important to have a proteolytically processed precursor for maximum protease inhibition (Heath *et al.,* 1995). However, interchange of reactive site of domain I (L) to domain II (R) in a two domain Pot II PI, did not result in exact inhibitory specificity transfer (Beekwilder *et al.,* 2000).

Six small (5.5 to 5.8 kDa) wound induced Pin-II family PIs from *N. tabaccum* show homologies to small molecular mass members of this family and show inhibition of trypsin and chymotrypsin. (Pearce *et al.,* 1993). Two PIs of around 50 amino acids, PSI1.1, PSI1.2, related to each other by circular permutation from *C. annuum* seeds have been found to inhibit trypsin, chymotrypsin, thrombin and factor Xa with different specificities. The PSI 1.2 corresponds to a complete repeat that was predicted as a putative ancestral protein of Pin-II family (Antcheva, *et al.,* 2001). Two IRD PIs from *Solanum tuberosum* and *Lycopersicum esculantum* have been shown to inhibit chymotrypsin, subtilisin and chymotrypsin, su*bt*ilisin and trypsin respectively (Eddy *et al.,* 1980; Plunkett *et al.,* 1982).

### *Effects of Pin-II type PIs on insect proteases - in vitro and in vivo*

Wound induction of Pin-II type PIs and their role in anti-herbivory defense was correlated by the pioneering work of Green and Ryan (1972), which later led to the discovery of several types of PIs and their activities to retard growth and development of insects. Leaves from wounded tomato plants have been shown to accumulate over 200 µg of potato inhibitors I and II /g of leaf tissue and to reduce the growth of larvae of *S. exigua*, the beet armyworm severely (Jongsma *et al.,* 1995). The addition of soybean trypsin inhibitor (S*BTI*) and potato inhibitor II to artificial diets of larvae of *Heliothis zea* and *Spodoptora exigua* has shown an elevation of trypsin like activities in their digestive tracts and inhibition of growth of the larvae at about 10% of the proteins in the artificial diets (Broadway and Duffy, 1986). Three steps have been used to assess the potential of ornamental tobacco (*Nicotiana alata*) PIs in insect control (Heath *et al.,* 1997). In an *in vitro* approach all five inhibitors (one 6-kDs chymotrypsin inhibitor and four 6-kDs trypsin inhibitors from a single

40.3-kDs precursor protein) were tested for their ability to inhibit gut protease activity in insects representing four orders. In most cases the pooled inhibitors and the trypsin inhibitors inhibited the gut proteinase activity ranging from 37-79% depending on the insect tested. The CI was less effective than TIs. Secondly, the *N. alata* PIs in the artificial diet of the native budworm (*Helicoverpa punctigera*) and the black field cricket (*Teteogryllus commodus*) revealed a significant ($P < 0.01$) reduction in growth and were more lethargic behaviour than insects on the control diet. Several of the *H. punctigera* larvae also failed to complete moulting at the third or fourth instar. The third step was to express the *N. alata* PIs in transgenic tobacco under the control of the 35S CaMV promoter. *H. punctigera* larvae fed on transgenic tobacco leaves depicted significant ($P < 0.01$) differences in mortality and/or growth rate at 0.2% soluble protein.

*Manduca sexta* larvae grown on transgenic tobacco plants expressing inhibitor II proteins from the tomato and potato showed severe inhibition at 50 µg inhibitor/ g of tissue while still more inhibition and mortality at 100 µg/g tissue level. Comparisons with inhibitor I and II showed that the trypsin inhibitor activity of Pin-II PI was largely responsible for inhibition of growth (Johnson *et al.,* 1995) while according to Ryan (1990), the presence of the CI site in Pin-II PIs along with TI site might have contributed to the anti-nutritive effects. Interestingly, greater insecticidal effect was observed in tobacco plants transformed with the genomic sequence of the tomato PI-II than in those transformed with the cDNA sequence indicating the presence of intron responsible for its enhanced expression and appropriate splicing of exogenous sequences in the transgenic plants to obtain the active protein (Zhang *et al.,* 2004).

Pin-II PIs of potato have also been used in transgenic rice and wheat plants to control biotic infestations by *Sesmia inferens* in case of rice (Duan *et al.,* 1996) and *Heterodera avenae* a nematode in wheat (Vishnudasan *et al.,* 2005). Interestingly, a direct positive correlation of PI level with plant height, seed weight and seed number was shown in wheat (Vishnudasan *et al.,* 2005). Combined leaf-specific over expression of potato PI-II and carboxypeptidase inhibitors in transgenic tomato resulted in increased resistance to *Heliothis obsolete* and *Liriomyza trifolii* larvae. However, a compensatory response of the larvae to the lower PI concentrations was noted in these plants indicating that the combined expression of defense genes with different mechanisms of action rather than combination of inhibitors might offer a better strategy in pest management (Abdeen *et al.,* 2005).

The cost benefit studies of PI strategy for insect tolerance performed by Zavala *et al.* (2004a, b) demonstrated that the fitness benefits of TPI production outweigh their costs in greenhouse conditions, when *N. attenuata* plants are attacked despite the ongoing evolutionary interactions between plant and herbivore *M. sexta* .

An approach other than over expression of PI genes has also been studied to analyse potential of PI in insect tolerance. JA biosynthesis involves the action of enzyme lipoxygenase on linolenic acid. Anti-sense mediated depletion of lipoxygenase gene in potato plants largely abolished the accumulation of PIs on wounding. As a consequence the weight gain of Colorado potato beetles fed on anti-sense plants was found to be significantly larger than those fed on wild type plants. Similarly, the polyphagous insect pest beet armyworm showed a 57% higher weight when reared on anti-sense lines (Royo *et al.*, 1999).

Novel exploitation of plant TPI activities- herbivore interactions has been done by Wu *et al.* (2006), in commenting phylogenetic regulation of TPI in *Nicotiana* spp. The response to herbivory was studied in 2 diploid (*N. attenuata (Na), N. obtusifolia (No)*) and 2 allotetraploid *(N. clevelandii (Nc), N. quadrivalvis (Nq)*) species, which are the descendents of the former. *Na, Nq* and *Nc* elicited higher TPI activity while No elicited suppressed activity in response to application of oral secretion. It is shown that a network composed of an upstream signaling system, downstream interactions between cis and trans elements and posttranscriptional regulators, probably regulates the PI expression. It was suggested that both the tetraploids probably retained the upstream signaling network from the diploid *Na* but abandoned those from *No* or although both the systems might co-exist, tetraploids still possess the ability to recognize attack from *M. sexta* larvae (**Fig. 1.8**).

### *Endogenous functions of Pin-II PIs*

Proteases are wide spread in plants, animals and microorganisms and comprise approximately 2% of encoded proteins, which are involved in physiological functions in the regulation of protein synthesis and turnover. Their corresponding PI's are also abundant in nature (Fritz, 2000). Initially thought of plant serine PI's having inhibition specificity for animal or microbial enzymes alone and not against plant proteases led to demonstration of anti-metabolic effects of plant serine PIs on insects by inhibiting the gut proteinases (Hilder *et al.*, 1993; Gatehouse *et al.*, 1999).

However, subsequently evidences towards developmental regulation and tissue specific accumulation PIs assigned endogenous functions to them. Plant organs that express Pin-II protein include flowers (Pena-Cortes *et al.*, 1991; Atkinson *et al.*, 1993; Pearce *et al.*, 1993; Damle *et al.*, 2005; Sin and Chye, 2004), stem (Xu *et al.*, 2001), tubers (Sanchez - Serrano *et al.*, 1986), and roots (Taylor *et al.*, 1993). It is suggested that they can regulate cell proteolysis by their action on endogenous proteinases, there by controlling protein turnover and metabolism (Ryan, 1989).

Most of the studies elucidating the endogenous functions of Pin-II PIs are best illustrated in weed plant *Solanum americanum* that expressed SaPin-IIa and b proteins having 74% identity and are summarized in the following points:

*SaPin-II PIs in regulating proteolysis in stem*

Unlike the previously characterized plant Pin-II proteins and Sa Pin-IIb, SaPin-IIa is abundantly expressed in stems especially in companion cells (CC) and sieve elements (SE) of phloem as shown by *in situ* hybridization studies **(Fig 1.12 D)** (Xu *et al.*, 2001).

The CC and SE complex in phloem are involved in macromolecular trafficking and the specific expression of SaPin-IIa in this tissue probably suggests its role in regulating proteolysis in SE as well as in phloem development. Transfer of gene to lettuce plants, which lack their own PI activity showed constitutive expression of SaPin-IIa, the completely inhibition of endogenous trypsin like activity and moderate inhibition of chymotrypsin activity there by suggesting endogenous regulation of proteolysis by SaPin-IIa (Xu *et al.*, 2004).

*SaPin-II PIs in floral development*

*In situ* hybridization localized SaPin-IIa and b more to floral buds that are destined to undergo developmental programmed cell death (PCD) (stigma, stylar transmitting tissue, vascular bundles, nuclear cells of the ovule and the outermost cell layer of the placenta) then the open flowers **(Fig. 1.12A)** (Sin and Chye, 2004). Differential expression profiles of SaPin-IIa and b, suggest their differential regulation and probably overlapping and complementary roles in floral developments. Their expression in layers surrounding the transmitting tissue of the style would confine PCD triggered due to penetration by pollen tube within the transmitting tissue specific accumulation of Pin-II type PIs of tomato as well as SaPin-IIa and b in developing

ovules and in the adjacent layers of placenta of young floral buds (Pena-Cortes *et al.*, 1991; Sin and Chye, 2004) suggests their function in impending PCD during floral development. Interestingly, the expression patterns of SaPin-IIa and b resemble those of cysteine proteinases and their inhibitors.

In *N. alata* , NaPIs account for up to 30% of soluble protein in stigma cells (Atkinson *et al.*, 1994) and are present in its vacuoles. It has been recently observed by Johnson *et al.* (2006) that the high levels of NaPI synthesized in maturing stigmas produces two populations of PI; one in precursor form retaining its targeting information (in the form of a C terminal vacuolar sorting signal) and destined for the vacuole and a second small population of mature PI released from the precursor in the ER (by its proteolytic cleavage) and trafficked to the cell surface giving the first extra cellular line of defense to the stigma, which at maturity lacks the barrier of a waxy cuticle (Johnson *et al.*, 2006).

*Sa Pin-II PIs in seed development*

SaPin-IIa is detected in the innermost layer of the ovule and the developing endothelium, while SaPin-IIb is detected in the layers immediately adjacent to the developing endothelium (nucellus). RNAi mediated silencing of SaPin-II PIs adversely affected nutritional support to the endosperm and embryo. Normal embryogenesis was absent in these lines. Majority of seeds in the silenced lines were aborted due to defective seed coat that lead abnormal endosperm development (Sin *et al.*, 2006) (**Fig. 1.12B**). The seed coat needs to transport metabolites to the developing embryo while proteinases play a role in embryo nutrition by participating in the breakdown or modification of macromolecules. Hence, the PIs in developing seeds of *S. americanum* could play a role in protection of the endosperm and embryo by regulating proteinases generated within the seed. SaPin-IIa and b are strategically located in protecting the embryo sac from PCD – associated proteinases generated from the seed coat.

The applications of Pin-II proteinases in regulating endogenous proteins need further optimization as plant transformation techniques are progressively being exploited in molecular farming for the production of desirable proteins including biopharmaceuticals. *In vivo* systems may serve as excellent model systems to study the endogenous functions of Pin-II PIs and regulation of these proteins (Chye *et al.*, 2006).

*Pin-II PIs in glandular trichomes*

Liu *et al.* (2006) have shown that SaPin-IIb is also constitutively expressed in glandular trichomes. Over expression of SaPin-IIb in tobacco resulted in a significant increase in glandular trichome density and promotion of trichome branching. These results suggest that SaPin-IIb could play roles in trichome-based defense by functioning as a constitutive component of trichome chemical defense and/or by regulating the development of glandular trichomes.

Pin-II PIs thus span the gap between basic and applied sciences with their important endogenous functions in plants and have obvious potential applications in pest control. There is much potential for future research and applications on this fascinating class of proteins.

## About *Capsicum annuum*

*Capsicum* is a genus of plants from the nightshade family (Solanaceae) and has more than 45 species. Some of the *Capsicum* species are used as spices, vegetables, and medicines. The fruit of *Capsicum* plant has a variety of names depending on place and type. They are commonly called 'chili pepper' or just `pepper' in Britain and the US; the large mild form is called `bell pepper' in the US, `capsicum' in Australian English, and `paprika' in some other countries (though paprika can also be of different types, or the powder of dried capsicum fruits). Hot varieties are called `chillis' in Australia.

The fruit of most species of *Capsicum* contains capsaicin (methyl vanillyl nonenamide), a lipophilic chemical that can produce a strong burning sensation in the mouth (and, if not properly digested, anus) of the unaccustomed eater. Most mammals find this unpleasant; however, birds are unaffected. Apparently, the secretion of capsaicin is an adaptation to protect the fruit from consumption by mammals while the bright colors attract birds that spread the seeds. The amount of capsaicin in peppers is highly variable and dependent on genetics, giving almost all types of peppers varied amounts of perceived heat. The only pepper without capsaicin is the bell pepper. Chilli peppers are of great importance in Native American medicine, and capsaicin is used in modern Western medicine mainly in topical preparations as a circulatory stimulant and pain reliever and it stimulates thermo receptor nerve endings in the skin, especially the mucus membranes. The Scoville scale , named after its creator, chemist Wilbur Scoville, is a measure of the "hotness" of a chili pepper, which indicates the amount of capsaicin present. Many hot sauces use their Scoville rating in advertising as a selling point. Scoville Organoleptic Test, developed in 1912, for testing hotness, used solution of the pepper extract diluted in sugar water until the "heat" was no longer detectable to a panel of (usually five) tasters and the degree of dilution was the measure of the Scoville scale. A bell pepper with no capsaicin has a Scoville rating of zero while the hottest chillis (habaneros) have a rating of 300,000 or more.

The genus *Capsicum* originated in the central and South American tropics. Hot peppers have now spread throughout the American, African and Asian tropics, where their fruits are valued for the flavour they add to the local diet. Nonpungent, 'sweet'

or vegetable peppers are preferred by consumers in temperate regions such as Europe and North America, but have spread to the tropics as diets in these countries have become more westernized. Five species of *Capsicum* were cultivated in different parts of the American tropics by the time of the European Conquests. Today, the *C. annuum* complex, which includes three closely related species, *C. annuum*, *C. chinense* and *C. frutescens*, is grown most widely all over the world. Among these three species *Capsicum annuum* was domesticated in highland Mexico. It includes most of the Mexican chilli peppers, most of the hot peppers of Africa and Asia, and various cultivars of sweet pepper grown in temperate countries (Pickersgill *et al.*, 1997). Classification of *Capsicum annuum* is as given below.

**Classification of *Capsicum annuum***

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Asteridae
Order: Solanales
Family: Solanaceae
Genus: *Capsicum*
Species: *C. annuum*

*Capsicum annuum* has the lowest number of chromosomes amongst different members of Solanaceae i.e. (2X = 24). It is relatively easy to increase or decrease ploidy levels artificially in *Capsicum.* Somatic doubling can be achieved by treating wounded leaf axils with colchicine. However, synthetic autotetraploids seem to have no agronomic or breeding advantages over diploids **(Fig. 1.13)**.

***Genesis of this work***

*C. annuum* is one of the non-preferred hosts of *H. armigera*. Initial elementary screening experiments detected *H. armigera* growth retardation when fed with *C. annuum* seed extract. It was thus thought that like winged bean and bittergourd earlier in my lab, *Capsicum* might also contain PIs that could be possibly used in insect control. Further work about isolating PI proteins and genes from *C. annuum* was, therefore, taken up.

# Chapter 2

## *In vitro* and *in vivo* effect of *Capsicum annuum* proteinase inhibitors on *Helicoverpa armigera* gut proteinases.


### Abstract

Two proteinase inhibitors (PIs), CapA1 and CapA2 were purified from *C. annuum* Linn. Cv. Phule Jyoti, leaves and assessed for their *in vitro* and *in vivo* activity against *Helicoverpa armigera* gut proteinases (HGPs). Both the inhibitors exhibited molecular weights of about 12 kDa with inhibitory activity against bovine trypsin and chymotrypsin indicating presence of probable two inhibitor repeats of PIN-II family. CapA1 and CapA2 inhibited 60-80% HGP (azocaesinolytic) activity of fourth instar larvae feeding on various host plants while 45–65% inhibition of HGP activity of various instars ($II^{nd}$ to $VI^{th}$) larvae reared on artificial diet. Partial purification of HGP isoforms, their characterization with synthetic inhibitors and inhibition by *C. annuum* PIs revealed that most of the trypsin-like activity (68-91%) of HGPs was sensitive to *C. annuum* PIs while 39-85% chymotrypsin-like activity of HGPs was insensitive to these inhibitors. Feeding of *C. annuum* leaf extracts and two purified PIs in various doses to *H. armigera* larvae for two successive generations through artificial diet demonstrated their potential in inhibiting larval growth and development, delay in pupation period and dramatic reduction in fecundity and fertility. This is the first study demonstrating efficacy of *C. annuum* PIs against insect gut proteinases as well as larval growth and development of *H. armigera*.

## 1. Introduction

*Helicoverpa armigera* is one of the devastating field pests of many important crops causing severe economic losses (Manjunath *et al.,* 1989). Analysis of digestive proteinases of *H. armigera* has revealed presence of serine proteinases, predominantly trypsin and chymotrypsin-like enzymes (Johnston *et al.,* 1991; Xu and Qin, 1994; Bowen *et al.,* 1997; Bowen *et al.,* 1998; Harsulkar *et al.,* 1998 and Patankar *et al.,* 2001). Inhibitors of these proteinases bind to midgut proteinases thereby reducing the digestive capabilities of the insects (Broadway and Duffy, 1986a, b). In polyphagous insects such as *H. armigera* diverse specificities and intricate changes in expression of proteinases are responsible for the inactivation of host plant and newly exposed PIs (Bown *et al.,* 1997; Patankar *et al.,* 2001; Jongsma *et al.,* 1995a; Broadway, 1996; Michaud, 1997; Wu *et al.,* 1997; Giri *et al.,* 1998, 2003; Harsulkar *et al.,* 1999 and Gruden *et al.,* 2004). Hence, identification of PIs having specificities towards different insect gut proteinases with high binding efficiency is necessary for effective inhibition of midgut proteinases of *H. armigera*. Such PI(s) may have direct relevance and application in development of transgenic plants with insect tolerance trait (Jouanin *et al.,* 1998, Lawrence and Koundal, 2002). Several plant species have been reported to contain high levels of serine proteinase inhibitors (PIs) in leaves, flowers, seeds and tubers as their defense tools against insects (Gracio-Olmedo *et al.,* 1987; Ryan, 1990). Wound inducible PIs of tomato or potato, sub-type I and II (PIN-II family) are of special importance in plant defense because of their expression at the time of or immediately after damage which reduces burden of plant to maintain the PI level throughout the life and flexibility in their activities and specificities against proteinases (Zavala *et al.,* 2004a).

Previous studies carried out in my laboratory towards understanding interaction between host-plant PIs and *H. armigera* gut proteinases (HGPs) have explained biochemical mechanism for susceptibility of host plants to insect infestation (Giri *et al.,* 1998; Harsulkar *et al.,* 1999; Chougule *et al.,* 2003). Extensive screening of several non-host plants for potential insect gut proteinase inhibitor has resulted in identification of winged bean (*Psophocarpus tetragonolobus*), potato (*Solanum tuberosum*), bitter gourd (*Momordica charantia*), capsicum (*C. annuum* L.) and groundnut (*Arachis hypogea*) as good sources. PIs of winged bean, bitter gourd, groundnut and potato (PIN II) have revealed adverse effects on larval growth, egg

laying capacity of *H. armigera moth* and hatchability of eggs (Harsulkar *et al.,* 1999; Telang *et al.,* 2003). I have selected *Capsicum* as a system to purify and characterize proteinase inhibitors with high potency as antifeedants for my thesis work.

*C. annuum* leaves contain seven wound inducible PI proteins of which four inhibit trypsin and chymotrypsin whereas three inhibit only chymotrypsin (Antcheva *et al.,* 1996, 2001; Shin *et al.,* 2001; Kim *et al.,* 2001). In this chapter purification of two *C. annuum* PIs, CapA1 and CapA2 and their inhibitory potential against mixture of HGPs (crude gut extract) and partially purified HGP isoforms(s) have been described. Inhibition potential of purified PIs has been tested against gut proteinases of *H. armigera* larvae feeding on artificial diet at various larval stages (from two to six), against mid fourth instar larvae feeding on various host plants and against mid fourth instar larvae exposed to non-host plant PIs. *H. armigera* feeding assays have also been carried out using *C. annuum* leaf extract and two purified PIs to demonstrate their *in vivo* efficacy to inhibit larval growth and development.

## 2. Materials and Methods

### *2.1 Plant material and PI extraction*

Leaves of *C. annuum* Var. Phule Jyoti were collected from Agriculture College (Mahatma Phule Agricultural University), Pune, India. Around 400g leaves were blended in distilled water containing 0.1% PVPP and centrifuged at 14,230 g at 4$^{o}$C for 10 min. Clear supernatant containing PIs was collected while cake was treated with 1.0 M KCl for 15 min to remove cell wall bound PIs. Suspension was again centrifuged and supernatant was collected. This was repeated twice for complete extraction of PIs. All the supernatants were pooled together (1800 ml) and stored at –20$^{o}$C till further use (Plunkett *et al.,* 1982). Protein content of the extract was determined by Bradford's method (Bradford, 1976).

### *2.2. Analysis of proteinase and PI assay*

Total HGP activity was measured by azocaesinolytic assay (Brock *et al.,* 1982). For azocaesinolytic assay 60 µl of diluted enzyme was added to 200 µl of 1% azocaesin (in 0.2 M glycine-NaOH, pH 10.0) and incubated at 37$^{o}$C for 30 min. The reaction was terminated by the addition of 300 µl of 5% trichloroacetic acid. After

centrifugation at 14,230 g for 10 min, an equal volume of 1 M NaOH was added to the supernatant and absorbance was measured at 450 nm. One proteinase unit was defined as the amount of enzyme that increased the absorbance by 1.0 OD under the given assay conditions. For inhibitor assay suitable amount of inhibitor and enzyme were pre-incubated at room temperature for 20 min and the residual enzyme activity was assayed as above. One PI unit is defined as amount of inhibitor required for inhibiting one proteinase activity unit. Bovine trypsin and trypsin/chymotrypsin/elastase like activity of HGPs were estimated using enzyme-specific chromogenic substrates, BApNA and SAAPLpNA as reported earlier. 150 µl diluted enzyme for BApNA assay and 60 µl for SAAPLpNA, assay was added to 1 ml of 1 mM substrate solution and incubated at 37°C for 10 min. The reaction was terminated by addition of 200 µl of 30% acetic acid and absorbance was checked at 410 nm and 405nm for BApNA and SAAPLpNA assays, respectively. One proteinase unit was defined as the amount of enzyme that increased the absorbance by 1.0 OD under the given assay conditions. For enzyme inhibitor assay, the inhibitor was mixed with the enzyme and premix was incubated at 25-27°C for 30 min. The residual enzyme activity was then estimated as above. One PI unit is defined as amount of inhibitor required for inhibiting one proteinase activity unit.

*C. annuum* leaf extract and purified PIs were separated on native-PAGE (Davis 1964) and the gel was further processed for trypsin inhibitor (TI) activity visualization by gel X-ray film contact-print technique (GXCT) (Pichare and Kachole 1994). This involves the resolution of PIs in native or SDS PAGE gels followed by their incubation in buffer (Tris- HCl pH 7.8) containing trypsin (0.03% w/v). These gels are further placed on X-ray films, which have a gelatin coating. Due to the action of the proteinases imbibed in the gel, the gelatin coating on the X-ray film was degraded, except for the corresponding region in the gel where the PIs were present. The activity of PIs could thus be indirectly visualized in the form of an undegraded gelatin band. The same technique was used to visualize the HGP activity (Harsulkar *et al.*, 1998).

### *2.3. Purification of C. annuum PIs*

Ammonium sulphate precipitated (65% saturation) and heat treated (65°C) leaf extract (240 TI units, 42 mg protein) was loaded on 50 ml capacity DEAE Fast Flow ion exchange column equilibrated with 50 mM Tris-HCl, pH 8.0 buffer. The column was

washed with 350 ml of 50 mM Tris-HCl, pH 8.0 and 3 ml fractions (fraction no.1 to 116) were collected. About 30 ml of 0.25 M NaCl in 50mM Tris-HCl pH 8.0 (fraction no. 117 to 136) was then passed through the column followed by fine step gradient of 0.25-0.4 M in 50mM Tris-HCl, pH 8.0 (60 ml) (fraction no. 137 to 176) and 25 ml wash of 0.4 M NaCl in 50 mM Tris-HCl, pH 8.0 (fraction no. 177 to 192) and fractions of 1.5 ml per tube were collected. Dot-blot assay of each fraction was carried out to identify the presence of inhibitory activity using undeveloped X-ray film (Pichare and Kachole 1994). Dialyzed and concentrated inhibitor fractions were further separated on native-PAGE followed by TI activity visualization. The fractions containing same inhibitor bands were pooled together. The pooled fractions of CapA1 and CapA2+CapB1 were reloaded individually on DEAE fast flow matrix (5 ml capacity column) followed by individual separations on Sephadex G-75 gel filtration column to remove co-eluted proteins. Inhibitor fractions were pooled together, concentrated and stored at -20$^0$C till further use.

### *2.4. HGP extraction and partial purification*

Midgut tissue was dissected from fourth instar larvae of *H. armigera* and immediately frozen in liquid nitrogen and stored at –80$^o$C. For extraction of HGPs, midgut tissue was homogenized in 0.2 M Glycine-NaOH buffer, pH 10.0 in 1:1 ratio and kept at 10$^o$C for 2 h. Suspension was centrifuged at 4$^o$C for 20 min at 14,230 g and resulting supernatant was used as a source of HGPs.

Partial purification of HGPs was carried out on 5 ml capacity DEAE Fast Flow ion exchange column equilibrated with 50 mM Tris-HCl, pH 8.0 buffer. HGP extract (750 µl) was mixed with 50 µl of 0.5 M Tris-HCl, pH 8.0 buffer and loaded on column. The column was washed with 25 ml of equilibration buffer to remove the unbound proteins. NaCl gradient of 0.1 M (6 ml), 0.2 M (6 ml), 0.3 M (11 ml), 0.4 M (6 ml), 0.44 M (6 ml), 0.48 M (6 ml), 0.5 M (6 ml), 0.7 M (10 ml) and 1 M (10 ml) in 50 mM Tris-HCl, pH 8.0 buffer was applied and fractions were collected at 4$^o$C. Dialyzed and concentrated fractions were further separated on native-PAGE followed by proteinase activity band visualization. The fractions were pooled in three groups according to the presence of proteinase isoforms and used for further analysis.

### *2.5. Feeding assay*

*In vivo* efficacy of *C. annuum* inhibitors was studied by feeding assays using laboratory-established culture of *H. armigera*. The artificial diet as suggested by Nagarkatti and Prakash (1974) was modified for my laboratory conditions (Gupta *et al.*, 2000). Composition of the diet for 650 ml was as follows: 77.7 g chickpea seed meal, 5.6 g of wheat germ, 19.2 g of dried yeast powder, 12.8 g of casein, 4.6 g of ascorbic acid, 1.5 g of methyl-p-hydroxybenzoate, 0.8 g of sorbic acid, 0.2 g of streptomycin sulfate, 0.2 g of cholesterol, one capsule of vitamin B complex (approximately 0.2 g) (Emcure, India), 1.0 ml of formaldehyde (40%), 0.8 ml multivitamine drops (Pharmpak, India), 0.8 ml vitamin E (MERCK, India) and 12.0 g of agar-agar (Sisco Research Laboratory, India). This basic diet was supplemented with appropriate amounts of inhibitor. Total gut proteolytic activity of a single gut of the fourth instar larva was estimated. Minimum inhibitor amount of purified as well as *C. annuum* leaf extract required to inhibit maximum total proteolytic activity present in single gut was calculated and identified inhibitor amount was incorporated per gram of the artificial diet (1X PI). The assay was carried out for two successive generations. In the first generation two inhibitor concentrations (0.5X and 1X) of CapA1, CapA2 and *C. annuum* leaf extract were fed to the larvae. Four concentrations (0.5X, 1X, 3X, and 6X) of each PI were used for feeding assays of *C. annuum* PI exposed second-generation larvae along with appropriate control. Thirty early second instar larvae were analyzed for each concentration of PI containing diet and in the control group (artificial diet without PI). Larval weights were taken everyday and % weight reduction in the PI fed larvae was compared to control group. In the pupal stage, sex of the insects was determined by observing the sexual dimorphism in the last two abdominal segments. Larval mortality, pupation period, pupae weight, number of malformed pupae, fertility and fecundity of adults were recorded and compared with that of the control group in both the generations to estimate the adverse effects of *C. annuum* PIs on growth, development and reproductive capabilities of *H. armigera*.

### *2.6 Statistical analysis*

ANOVA analysis has been carried out for HGP-inhibitor interaction results and feeding assays data using SYSTAT11 software.

## 3. Results

### *3.1. Purification and characterization of C. annuum PIs*

The purification steps employed increase specific activity of PIs from 1.75 (leaf extract) to 18.74 and 18.76 for purified CapA1 and CapA2, respectively. The protein content and PI activity analysis of each step of the purification are as given in **Table 2.1**. CapA1 and CapA2+CapB1 were eluted as unbound fractions from DEAE-Sepharose in two major peaks **(Fig. 2.1A and 2.1B)**. CapA1 and CapA2 activity bands having similar mobility on native PAGE were considered as different inhibitor proteins because of their elution in two peaks separated by few fractions having no inhibitor activity **(Fig 2.1A)**. Other PI isoforms were eluted in fine step gradient of NaCl in buffer. CapB2 eluted at 0.25 to 0.33 M NaCl while other three forms of PI were eluted in three different combinations, CapB2+CapC, CapC+CapD and CapD+CapE at slightly higher NaCl concentrations (0.29 to 0.4 M) **(Fig. 2.1A and 2.1B)**. Two *C. annuum* PI isoforms, CapA1 and CapA2, were selected for further purification. Fractions containing CapA1 and CapA2+CapB1 were pooled separately and further resolved on ion exchange matrix, which resolved CapA2 and CapB1 and eliminated the non-inhibitor proteins completely except one high molecular weight protein of ~96 kDa in CapA1 and CapA2 preparations. Further purification on Sephadex G-75 gel filtration column subsequently eliminated contaminating ~96 kDa protein.

**Table 2.1: Summary of *C. annuum* proteinase inhibitor purification**

| Steps involved | Total trypsin inhibitor unit | Total protein (mg) | Specific activity | Fold purity | % HGP inhibition (trypsin-like activity) |
|---|---|---|---|---|---|
| **Crude leaf extract** | 916.30 | 521.73 | 1.75 | - | 82.60 |
| **Ammonium sulfate precipitation** | 666.60 | 294.00 | 2.26 | 1.29 | 84.89 |
| **Heat treatment (65 °C)** | 594.90 | 146.40 | 4.06 | 2.32 | 85.09 |
| **DEAE column** | | | | | |
| **Pool I-Cap A1** | 296.00 | 39.90 | 7.42 | 4.23 | 80.15 |
| **Pool II-Cap A2** | 180.00 | 31.80 | 5.65 | 3.22 | 72.30 |
| **Reloading on DEAE column** | | | | | |
| **Cap A1** | 80.49 | 7.26 | 11.09 | 6.33 | 80.61 |
| **Cap A2** | 72.96 | 6.40 | 11.35 | 6.48 | 73.54 |
| **Gel filtration on Sephadex G-75** | | | | | |
| **Cap A1** | 15.01 | 0.80 | 18.76 | 10.72 | 81.00 |
| **Cap A2** | 10.12 | 0.54 | 18.74 | 10.70 | 74.00 |

**Figure 2.2A** represents activity bands of purified CapA1 and CapA2 inhibitor proteins resolved on native PAGE. The molecular weight of CapA1 and CapA2 determined on SDS-PAGE, in the absence of reducing agent, appeared to be 28 kDa and 29 kDa, respectively **(Fig. 2.2B)**. These profiles were corroborated with inhibitor activity visualization of CapA1 and CapA2 using GXCT (Compare **Fig. 2.2B and 2.2C**). However, reduction of disulfide bonds of purified CapA1 and CapA2 with β-mercaptoethanol followed by their separation on SDS-PAGE indicated ~12 kDa molecular mass of both the inhibitors **(Fig. 2.2D).**

### *3.2. Interaction of CapA1 and CapA2 with HGPs of larvae growing on host plants, fed on non-host plant PI and of various larval stages*

In order to assess the inhibition potential of CapA1 and CapA2against various blends of HGPs, three-host plants (chickpea, pigeonpea and cotton) fed larval HGPs, two non-host plants (winged bean and potato inhibitor II PI) fed larval HGPs and HGPs of artificially reared larvae were used. Along with CapA1 and CapA2, PIN I, soybean trypsin inhibitor (S*BT*I) and bitter gourd PI was included as controls in the inhibition analysis as they were characterized for their activities against insect proteinases (Johnston *et al.,* 1991; Bown *et al.,* 1997; Telang *et al.,* 2003)**.** Minimum inhibitor amounts required to attain the maximum inhibition of HGPs of chickpea fed larvae was calculated for each inhibitor and was used for inhibition assays with other HGPs. *C. annuum* PIs and bitter gourd PIs exhibited differential inhibition of total HGP activity (azocaesinolytic). *C. annuum* PIs exhibited stronger inhibition of HGPs of larvae grown on chickpea, pigeonpea, cotton and artificial diet whereas bitter gourd PIs indicated higher inhibition of winged bean and PIN II fed larval HGPs. ANOVA indicated that CapA1 and CapA2 inhibition of HGP azocaesinolytic activity of chickpea fed larvae was comparable with PIN I and BG crude while higher than S*BT*I, whereas cotton fed larval azocaesinolytic HGP activity inhibition by CapA1 and CapA2 was significantly higher than PIN I and S*BT*I **(Fig. 2.3A)**. Significantly lower inhibition of HGP activity of larvae growing on pigeonpea, artificial diet and larvae fed on winged bean and PIN-II PI was obtained by CapA1 and CapA2 than PIN I and SB-TI **(Fig. 2.3A)**. CapA2 demonstrated significantly higher HGP azocaesinolytic activity inhibition than CapA1 of fourth and fifth instar larvae. CapA2 and Cap crude PIs inhibited fourth instar HGPs significantly higher than PINI, S*BT*I and BG crude PIs. CapA2 inhibition of second, third, fifth and sixth instar larval HGP was comparable with that of S*BT*I and PINI **(Fig. 2.3B)**. *C. annuum* PIs also exhibited significantly higher HGP inhibition than bitter gourd PIs through out the larval development, except in second and fifth instar where the difference was insignificant **(Fig. 2.3B)**. Using equalized trypsin inhibitor units of CapA1, CapA2 and Pin I against chickpea HGP, PIN I indicated stronger inhibition than CapA1 and CapA2 **(Fig. 2.4)**. This could be because of the multiple inhibitor activity bands in PIN I and single inhibitory band in CapA1 and CapA2.

### *3.3. Partial purification of HGPs, their characterization and interaction with purified C. annuum PIs*

Gut proteinases of chickpea fed *H. armigera* larvae, which contained at least eight major proteinase isoforms **(Fig. 2.5A)** were separated into three fractions using ion exchange column chromatography. Fraction I and II contain equal amounts of proteinase activity, however, fraction III contains minor proteinase activity **(Fig. 2.5B)**. Fraction I revealed a slow moving major activity band (HGP-1) with minor bands of HGP-3, -4 and -8 on electrophoretic gel **(Fig. 2.5A)**. HGP-3, -4, -7 and -8 were the major activity bands in the fraction II, while HGP-5 was in trace amounts. HGP-2 was eluted as a major activity band in fraction III with minor amounts of HGP-4, -5, -6, -7 and -8. Analysis of HGPs using synthetic inhibitors of various proteinase specificities indicated presence of 49% trypsin-like, 44% chymotrypsin-like and 18% elastase-like activity **(Fig. 2.5B)**. The highest trypsin-like activity (53%) was observed in fraction III and the lowest in fraction I (37%). Almost similar chymotrypsin activity (33-35%) was found in all the three fractions. Elastase-like activity in fraction I and II, was 18 to 20%, respectively and in fraction III, it was only 13%.

Equalized proteolytic activity units of crude and fractionated HGPs were tested against CapA1, CapA2, PIN I and SBTI using substrates of various proteinase specificities. HGP azocaesinolytic activity inhibition of crude and partially purified HGPs by CapA1 and CapA2 was in the range of 40-61%, which was significantly lower than S*BT*I and PIN I, except CapA1 against fraction I and II and CapA2 against fraction III where the difference was comparable with S*BT*I **(Table 2.2)**. CapA1 and CapA2 inhibited trypsin-like activity of HGPs and partially purified HGPs significantly higher than PIN SBTI, and I except fraction II where CapA1 and PIN I inhibition difference was insignificant. Chymotrypsin/elastase-like activity of crude and fractionated HGPs was weakly inhibited by CapA1 and CapA2 than PIN I, while significantly higher than S*BT*I of fraction II and fraction III. Most of the CapA1 and CapA2 insensitive chymotrypsin/elastase like activity was present in fraction I, where CapA1 could inhibit 15% and CapA2 could inhibit 51%. On the other hand, PIN I inhibited 77% of the chymotrypsin/elastase-like activity of the fraction I. Azocaesinolytic HGP activity inhibition by CapA1 and CapA2 was similar except fraction II, however, variation was observed in their specificities towards trypsin and

chymotrypsin-like activities. This difference in activities was magnified in inhibition studies with partially purified HGPs. CapA2 inhibited chymotrypsin-like activity of fraction I and III more than CapA1. Trypsin-like activity of fraction I and III was equally inhibited by CapA1 and CapA2 while CapA2 exhibited significantly higher inhibition of trypsin-like activity of fraction II than CapA1 **(Table 2.2)**.

### *3.4. Effect of C. annuum PIs on growth and development of H. armigera*

Artificial diets containing purified *C. annuum* inhibitors and *C. annuum* leaf extracts were fed to insects for two successive generations. In the first generation two concentrations (0.5X and 1X) and in the second generation four concentrations (0.5X, 1X, 3X and 6X) were used to study their effect on *H. armigera* larval growth and development. In the first generation, 12 to 24% weight reduction was observed in larvae fed on PI containing diet. Pupation of 32 to 44% larvae was delayed by minimum three days due to PI exposure. Egg-laying capacity of adults and egg hatching was reduced by 60% and 40%, respectively.

In the second generation, maximum larval weight reduction was 55, 42 and 53% for CapA1, CapA2 and *C. annuum* PIs, respectively (**Table 2.3**). Statistical analysis revealed that effect of PIs on larval growth inhibition was dose dependent, excluding 0.5X and 1X crude Cap PIs where the difference was insignificant. Around 40% larval mortality was observed in 6X CapA2 containing diet, and 30% and 23% larval mortality in 3X and 1X PI containing diet fed group, respectively which was statistically dose dependent. However, in other two PI fed groups the mortality was not dose dependent. Pupa formation rate was significantly lower in all fed groups, than control group, except 0.5X of CapA1. These results indicated delay in pupae formation, which was accounted to be three to eight days. In addition to this, significant decrease in pupal weight was observed in PI fed groups compared to control. However, the reduction in pupal weight was not dose dependent. Increased malformed pupae were observed in the PI-fed groups compared control; however, it was not significant (**Fig. 2.6**). *C. annuum* PIs exhibited adverse effect on egg laying and egg hatching capacities of *H. armigera* moth. As compared to control, significantly lower eggs were laid by female moths and the effect was dose dependent (**Table 2.3**). Egg hatching was also dramatically reduced in PI-fed groups (**Table 2.3**).

## 4. Discussion

### *4.1 Properties of C. annuum PIs and their significance*

In the present study, two PIs from *C. annuum* of molecular weight 12 kDa were purified and characterized. Inhibitor protein of 12.3 kDa MW possessing two-inhibitor repeats was reported from potato (Schaller and Ryan, 1995; Barta *et al.*, 2002). A typical characteristic of PIN II family inhibitors is presence of a precursor containing 6 to 8 inhibitor repeats, which upon cleavage results in forming active fragments of single or multi-inhibitor repeats. Single inhibitor repeat is generally of 6-kDa MW protein. Earlier studies on *C. annuum* PIs have revealed presence of six inhibitors (6 kDa) in leaves (Moura and Ryan, 2001). A *C. annuum* wound induced inhibitor, having three-inhibitor repeats was also reported to have MW 22-kDa (Kim *et al.*, 2001). Based on this information, it is speculated that the inhibitors purified from *C. annuum* are having two-inhibitor repeats, as their MWs are around 12 kDa. Inhibitors of PIN II family from other plants such as potato and tomato have around 50% amino acid diversity between the two repeats (Atkinson *et al.*, 1993; Barta *et al.*, 2002). Such variation in amino acids may be responsible for the diverse specificities and activities of inhibitors against various proteinases. Strong activity of *C. annuum* inhibitors similar to potato inhibitors against HGP can be attributed to characteristic of PIN II family. However, this needs to be further supported by N-terminal amino acid sequencing.

### *4.2 Complexity and flexibility in HGPs expression can be controlled through diversity in plant serine PIs*

Considerable plasticity exists in the digestive physiology and feeding behavior of insects in response to PIs, which is a main reason for limitation of PI-based insect resistance development strategy. It is more severe in the insects of polyphagous nature like *H. armigera*. To cope up with such diverse proteinases, multiple and strong PIs with unique pattern of expression are essential (Harsulkar *et al.*, 1999). The presence of multi-domain inhibitors, high-level expression in target organ and wound inducible expressions are the unique features of plant defense system against herbivore attack (Atkinson *et al.*, 1993, Green and Ryan, 1972). However, use of single inhibitor protein and its constitutive expression under universal promoter to control insects like

*H. armigera* have less feasibility of success (Murdock and Shade, 2002). In depth analysis of gut proteinases of *H. armigera* larvae has revealed the fact that they are very complex in their specificities and expression depending upon the ingested PIs (Bown *et al.*, 1997; Patankar *et al.*, 2001; Gatehouse *et al.*, 1997).

In the present study *C. annuum* PIs inhibited more than 60% total proteolytic (azocaesinolytic) activity of larvae fed on cotton and chickpea. However, less than 50% HGP activity of larvae collected from pigeonpea and larvae fed on artificial diet with or without added PIs was inhibited by *C. annuum* PIs. This could be due to very diverse forms of proteinases expressed by larvae grown on pigeonpea (Patankar *et al.*, 2001). Winged bean PIs and PIN II fed larvae induced proteinases of different specificity that are insensitive to winged bean and PIN II inhibitors. *C. annuum* PIs exhibited high inhibition of HGPs expressed throughout the larval developmental stages.

Interactions of purified *C. annuum* PIs with HGPs (mixture of several isoforms of HGP) and partially purified HGP were also carried out in the present study. Both the purified inhibitor isoforms demonstrated promising *in vitro* inhibition of gut proteinase activity of *H. armigera* larvae exhibiting more affinity towards trypsin-like HGPs than chymotrypsin / elastase like HGPs. Using synthetic substrate it was not possible to discriminate between chymotrypsin and elastase-like activity, because both the enzymes act on the same substrate. However, RT-PCR analysis revealed no expression of elastase-like proteinases of *H. armigera* larvae fed with *C. annuum* PIs indicating its sensitivity to these PIs. On the other hand, few chymotrypsin-like forms revealed high-level expression in *C. annuum* PIs fed larvae as compared to control diet fed larvae (Chougule *et al.*, 2005). In order to comment conclusively it is necessary to carryout inhibition studies with purified chymotrypsin like and elastase like gut proteinases of *H. armigera*.

### *4.3. C. annuum PIs arrest larval growth and development, delays pupation, and reduces fecundity and fertility of H. armigera*

Disruption of amino acid metabolism by inhibition of protein digestion through PI is the basis of PI-based defense in plants; however, in nature it might be coupled with other factors. To evaluate *in vivo* effects of *C. annuum* PIs on *H. armigera*, feeding assays were conducted with added inhibitor protein in the artificial diet. Larval

growth and development were dramatically reduced when insects were fed on *C. annuum* PI diet. Reduced feeding of larvae was observed in the case of PI-incorporated diet as compared to control diet. The adverse effects were significant at higher concentration of PI doses. Significant mortality of larvae was also evident. This can be explained, as larval stage is very crucial for accumulating nutrients and energy, which is used for pupal, adult development, fecundity and fertility. Starvation and added stress on gut proteinase expression system to synthesize new and higher amounts of proteinases could be the possible reasons for arrested growth and mortality of *H. armigera* larvae. Other researchers also observed growth retardation and mortality with high PI doses to various insects (Murdock and Shade, 2002; Zavala *et al.*, 2004b). Successive exposure of *C. annuum* inhibitors to two generations of *H. armigera* exhibited antibiosis, which was more pronounced in second generation.

At 0.5X concentration of CapA1 and CapA2 inhibitors, the protein amounts were 5µg/gm of diet and 4µg/gm of diet while at 6X concentration, it was 60 µg/gm of diet and 48 µg/gm of diet, respectively. Requirement of lower protein amount of CapA2 than that of CapA1 for maximum effect on *H. armigera* growth retardation indicates its high specificity towards HGPs. The inhibitor amounts used in terms of protein concentration are well within the expression limits in transgenic plants and lower than that of other inhibitors expressed in plants for insect resistance. For example, tobacco plants expressing 1% cowpea trypsin inhibitor of total leaf soluble protein was reported to be resistant to insects (Hilder *et al.*, 1993). Transgenic tobacco plants resistant to *M. sexta* expressed 332 µg PIN II protein per gram of tobacco leaf (Johnson *et al.*, 1989). Almost 7% of partially functional equistatin inhibitor of the total soluble proteins was expressed in potato (Outchakourov *et al.*, 2003). Based on this information and present study, CapA1 and CapA2 can be considered as suitable candidates for developing insect resistant transgenic plants.

In summary, this chapter describes the purification of two *C. annuum* leaf proteinase inhibitors, their biochemical characterization and inhibitory properties towards standard enzymes, crude and fractionated HGP's. The *in vivo* effect of the purified PIs and crude leaf extracts of *C. annuum* on growth and development of *H. armigera* for two consecutive generations has also been studied.

# Chapter 3

## Expression patterns of Pin-II type proteinase inhibitor genes in *Capsicum annuum* Linn.


### Abstract

Pin-II type proteinase inhibitor (PI) genes were cloned from fruit and stem tissues of *Capsicum annuum* L. var Phule Jyoti using primers designed from reported *CanPI* gene sequence (AF039398). Newly isolated *CanPI* genes sequences did not match with any of the reported *CanPIs* but belonged to the Pin-II PI family and showed the presence of characteristic inhibitory repeat domains (IRDs). Total of 21 novel *CanPIs* were identified in the study, with three subtypes of 1 IRD type, eight subtypes of 2 IRD type, three subtypes of 3 IRD type, five subtypes of 4 IRD type and two partial *CanPI* sequences. Most of the sequences showed variation in the deduced amino acid sequences close to the reactive site loop and accounted for an overall sequence divergence of around 2 - 20 % amongst them. Semi-quantitative RT-PCR expression analysis of *CanPIs* showed qualitatively and quantitatively different expression patterns in the tissues of mature *C. annuum* plants. The fruit tissue showed the maximum *CanPI* expression and presence of all the four types of *CanPIs* expressed simultaneously. Stem tissue depicted a unique pattern of *CanPI* expression with the maximum expression of 1- and 2- IRD type *CanPIs*. *CanPI* expression was up regulated upon wounding and insect attack. Differential expression patterns of *CanPIs* were observed in wounded local and systemic tissues. 4- IRD type *CanPIs* were specifically induced due to aphid (*Myzus persicae*) and lepidopteran pest (*Spodoptera litura*) infestation. Virus infected leaves on the other hand did not show any effect on *CanPI* expression. The analysis demonstrated spatial, temporal as well as qualitative and quantitative regulation of *CanPI* expression.

## 1. Introduction

Sedentary lifestyle of plants has given rise to evolution of very potent biochemical responses on insect / pathogen attack or wounding. Biochemical defenses can be broadly classified into two types, namely direct defenses and indirect defenses such as volatiles. Complex blends of different metabolites like alkaloids, glucosonolates, enzyme inhibitors, enzymes, defense related proteins, and pathogenesis related proteins are induced as direct defense of plants against the invading pest/pathogen. In the course of co-evolution, plants have developed pathways for regulating the defense at systemic and local level through a signal transduction pathway, leading to the synthesis of defense phytochemicals (Green and Ryan, 1972; Agarwal, 1998). This process is governed by hormonal action of Jasmonic acid (JA), salicylic acid (SA), ethylene and abscesic acid.

JA pathway and its cyclopentenone derivatives are of prime importance in plant defense signaling against a range of different insects apart from their roles in plant reproduction and metabolite regulation (Walling *et al.,* 2000; Thaler *et al.,* 2002). The SA pathway is primarily induced in defense against pathogens like bacteria, fungi and viruses (Ryals *et al.,* 1996). Though JA and SA operate via distinct pathways, they interact through NPR1, which regulates the downstream gene expression (Pieterse *et al.,* 1998; Spoel *et al.,* 2003). Wounding results in marked changes in gene expression that contributes to tissue defense and repair (Reymond *et al.,* 2000). Several signals are involved in wound induced gene expression in tomato, but their expression is blocked by application of salicylic acid (Pena-Cortes *et al.,* 1993; 1995). In Solanaceae, wounding and insect attack releases systemin (polypeptide hormone) from its precursor (Pearce *et al.,* 1991), which induces JA metabolites through octadecanoid pathway, that play a major role in signaling defense by expression of a long-distance signal in the form of proteinase inhibitors (PIs) (Ryan, 2000; Kessler and Baldwin, 2002; Howe *et al.,* 1999; Lee and Howe, 2003). Systemin rapidly travels to the distal parts of the plant and brings about the amplification of JA pathway systemically (Ryan and Pearce, 2003; Narvaez-Vasquez and Ryan, 2004; Li *et al.,* 2002a). JA independent genes are also induced on wounding in Solanaceae as well as in *Arabidopsis* (Howe *et al.,* 2000; Ryan, 2000; Lee and Howe, 2003) presenting a more complex view of wound signaling in plants. Several genes related

to photosynthesis and growth are also shown to be down regulated upon insect feeding / wounding (Hui *et al.,* 2003).

Dynamics of temporal and spatial regulation of gene expression of distinct set of genes in response to wounding, insect feeding and pathogen attack studied by microarray based technology (Halitschke *et al.,* 2003; Korth and Dixon, 1997; Voelckel *et al.,* 2004a) have further shown only a few genes specifically induced on plant-insect interaction (Reymonds *et al.,* 2000; Korth, 2003; Kenneth *et al.,* 1997). Molecular responses of *Nicotiana attenuata* to feeding by the specialist herbivore *Manduca sexta* were studied by differential display and uncovered 16 upregulated and 9 downregulated cDNAs (Hermsmeier *et al.,* 2001). Similarly, fatty acid - amino acid conjugates (FACs) and some enzymes in the insect saliva are specifically recognized by plants, which elicit an insect feeding specific response via jasmonate pathway (Alborn *et al.,* 1997). Thus, the transcriptional host responses are remarkably similar to both, the specialist as well as polyphagous types of insect pests. This is supported by the studies in case of *Nicotiana* infested by generalist *Spodoptera* and specialist *Pieris* herbivores (both Lepidopteran) (Reymonds *et al.,* 2004). Furthermore, a coordinated signaling between JA, SA and ethylene pathways for down stream defense gene regulation has also been proved during interaction of *Spodoptera* and *Arabidopsis* (Stotz *et al.,* 2000, 2002; Li *et al.,* 2002b).

Aphids, the largest group of phloem feeders, feed by inserting their stylets in the phloem sieve elements through epidermal and mesophyll cells and suck phytoassimilate sap as their food source. Phloem sap is rich in sugars and has a relatively low proportion of free amino acids. The excess of sap taken up by aphids to receive adequate supply of amino acids is given out which attracts ants (Pollard *et al.,* 1972). It is further demonstrated that the aphid-infested plants very peculiarly regulate genes involved in manipulation of phloem sap like glutamate synthase upregulation and of germin ($H_2O_2$ generating enzyme) downregulation, which are nutritionally advantageous to aphid feeding (Voelckel *et al.,* 2004b). Some completely novel genes are also elicited only by aphid attack with a weak elicitation of jasmonate pathway dependent defenses (Walling, 2000) probably due to the stealthy feeding behavior of the aphids (Voelckel *et al.,* 2004b). Distinct difference in expression patterns has been observed in tomato plant induced by feeding of different types of insects, namely leaf chewing insect *Manduca sexta*, cell content feeder *Tupiocoris notatus* and phloem

feeder *Myzus nicotinae* (Voelckel *et al.,* 2004b). Thus, effect of multiple herbivores feeding can differentially affect different components of the plant defense system (Rodriguez-Saona *et al.,* 2005). The mechanism underlying the induction and signaling of the hormonal actions, which leads to realization of a peculiar defense response is not yet completely understood.

Presence of prosystemin and its involvement in systemic wound signaling has been shown in case of *Capsicum annuum*. *C. annuum* prosystemin cDNA shows 73% sequence identity but is the most divergent sequence amongst tomato, potato and nightshade prosystemin sequences (Constable *et al.,* 1998). During a hypersensitive response to TMV in *C. annuum,* several mRNAs were differentially expressed including proteinase inhibitors, which expressed maximally up to 72 hours post infection (Shin *et al.,* 2001). Regulation of *C. annuum* PIs (both proteins and cDNAs) expression has been studied on inductions like wounding, systemin, Methyl Jasmonate (MJa), Poly Ethylene Glycol, salt, ABA, cold stress and electric current application (Kim *et al.,* 2001; Moura and Ryan, 2001). *C. annuum* PIs show steady state expression levels which are elevated on MJa application and wounding differentially (Moura and Ryan, 2001).

The present study includes the expression profiles of PIs in different varieties of *C. annuum* and PI expression patterns in different organs of mature *C. annuum* var Phule Jyoti. The PI expression levels in *C. annuum* plants naturally infested with aphids, virus and chewing insects were investigated. Changes in PI expression on induced wounding was also studied. A direct correlation in the quantity of expressed PIs and systemin expression levels in steady state and induced *C. annuum* has been observed. Results indicate that *C. annuum* has highly diverse PI genes and their expression is regulated temporally and spatially on various inductions.

## 2. Materials and methods

### *2.1 Plant materials and tissue collection*

Green fruits of ten varieties of *C. annuum* namely, Phule Jyoti, Phule Jwala, Pant C1 and AVT (advanced varietal trials) varieties were collected from the fields of Vegetable Section, Mahatma Phule Agricultural University, Rahuri.

Mature healthy plants of *C. annuum* var Phule Jyoti (~ 5 months old), bearing flowers and fruits of all stages, maintained in the green house were used for collecting tissue from different plant parts individually. Leaf, flower, fruit stages (early, mid, late, turning) and stem tissue were collected. Similar tissue type from three individual plants was collected, pooled, labeled and flash frozen in liquid nitrogen and was stored in –80$^{o}$C till further use.

*Capsicum annuum* var Phule Jyoti seeds were grown in green house conditions. Seedlings were raised in the seedbeds for 25-30 days and then transplanted individually into pots, which were kept in either green house or in open garden.

Induction by wounding was carried out on ~2 months old, healthy plants, which were kept in the green house. Tissue was collected at about 8-10 leaf stage separately from local and systemic regions of wounded plants after 3h, 6h, 9h, 12h and 24h respectively. Unwounded plants were used as control and tissue was collected at the same intervals.

Some *C. annuum* plants (around 5-6 months old) were kept in open conditions for natural aphids (*Myzus persicae*) and virus infection. These infections were persistent for more than 30 days. Leaf and fruit tissues from such plants were collected. Aphids were removed from the under surface of the leaves by cleaning them with a wet tissue paper. Healthy plants maintained in the open conditions were used for collecting control plant material.

### *2.2 Isolation of total RNA form C. annuum*

Tissue stored in –80$^{o}$C freezer was ground to a fine powder in liquid nitrogen and ~50 mg of each of the tissues was used for isolation of RNA. Total RNA was isolated by using TRIZOL (Invitrogen, USA) reagent and was given DNAse treatment at 37$^{o}$C for 30 min. DNAse in the RNA solution was inactivated by addition of Stop solution (Promega, USA) and further incubated at 65$^{o}$C for 15 min. The RNA quality of DNAse treated and untreated samples were checked on 1.5% agarose gel, to ensure no RNA degradation after DNAse treatment. The DNAse treated RNA was quantified by determining the OD at 260 nm.

### *2.3 Primer designing*

Full length cDNA sequences of *Capsicum annuum* proteinase inhibitor, prosystemin and 18s rRNA sequence were downloaded from the NCBI database (www.ncbi.nlm.nih.gov). Forward and reverse primers for the amplification of full length *C. annuum* PIs were designed and synthesized. Highly conserved regions of prosystemin and 18s rRNA were determined using NCBI blast software. Primers were designed to amplify at least 250 bases from these conserved regions. Details of the accession numbers, names of the cDNA sequences, primer sequences and the expected band size are given in **Table 3.1**.

### *2.4 First strand (cDNA) synthesis and RT-PCR analysis using equalized cDNA*

Around 1.5 µg total RNA was used to carry out first strand synthesis using reverse transcriptase (RT) kit (Promega, USA) as per manufacturer's protocol. Initially a RT-PCR was carried out using primers for amplification of 18s rRNA to optimize the cDNA concentration. The PCR was carried out using 1µl of undiluted and 1:10 diluted single stranded cDNA for 25 cycles (95 $^{o}$C, 30 s for denaturation, 60$^{o}$C, 45 s primer annealing, 70$^{o}$C, 90 s for extension) and intensity of the amplified band was checked on a 2% agarose gel stained with ethidium bromide. cDNA amounts used in the PCR were modified to match the intensities of the amplified bands in all the samples of each experiment. The amount of cDNAs showing uniform amplification with18s rRNA primers were determined and the same were used for amplification with the gene specific primers. PCR conditions for specific gene amplification were as follows, 95$^{o}$C for 5 min for initial denaturation, followed by 34 cycles of 95$^{o}$C for 30 s, 45$^{o}$ / 65$^{o}$C for 45 s and 70$^{o}$C for 90 s, with final extension of 70$^{o}$C for 10 min.

### *2.5 Cloning PCR products, sequencing and sequence analysis*

cDNA of the stem tissue was amplified in two independent PCRs with a proof reading enzyme (Accuprime Pfx – Invitrogen, USA) using the PI gene specific primer pair. The aliquot of the reaction was checked and quantified on 2 % agarose gel. The remaining reactions were A-tailed at 70 $^{o}$C for 45 minutes by addition of Taq polymerase enzyme (Banglore Genie, India). The amplified products were separately ligated with pGEM-T Easy vector (Promega, USA) as per manufacturer's

instructions. The ligation reactions were stored at 4 °C overnight and then used to carry out the transformation of competent *E. coli* Top10 cells (Invitrogen, USA) (Sambrook *et al.*, 1989). Transformed cells were selected on ampicilin containing LB medium (0.7 µg/ml) and confirmed by carrying out colony PCR with the same gene specific primers. Colony PCR positive clones were grown in 3 ml LB broth with ampicilin overnight and plasmids isolated by alkali lysis method, followed by RNAse treatment and quantification. Presence of inserts in the plasmids was confirmed by carrying out restriction digestions with *EcoR*1 enzyme.

Sequencing of the plasmids was carried out using M13 forward and reverse primers in the MEGA Base sequencer (Amersham, Sweden). Sequence editing was done by NCBI Blast tools. Full-length cDNA clones confirmed after sequence editing, were then analysed. Predicted amino acid sequences of the cDNAs were determined using the Expassy Translate Tool software. Multiple sequence alignments, dendrograms and phyllogenetic trees of the nucleotide and predicted amino acid sequences were constructed using Clustal-X and DNA STAR software.

**Table 3.1: Oligonucleotide primers used for RT-PCR analysis**

| Gene name | Accession No. | Primer sequence | Expected band size bp. |
|---|---|---|---|
| **CanPI-1** | AF039398 | F 5' ATGGCTGTTCCCAAAGAA 3'<br>R 5' CTGTTCATGCTTTTACTTTTC 3' | 614 |
| **CanPI-2** | AF221097 | F 5' ATGGCTGTTCACAAAGAAGTT 3'<br>R 5' GACACTGTTCATGCTTTTATTTTT C 3' | 614 |
| ***C. annuum* Prosystemin *peppro*** | AF000376 | F 5'CCAAAGGTGGAACGTGAAGAGGGAGG 3'<br>R 5' GCAACAAACCCCGACTTCTGGAAGGG 3' | 375 |
| ***C. annuum* 18s rRNA gene** | AA840641 | F 5' CCGGTCCGCCTATGGTGTGCACCGG 3'<br>R 5' CCTCTGACTATGAAATACGAATGCCCC 3' | 245 |

## 3. Results

### *3.1 Novel CanPI genes having varied number of inhibitory repeat domains- Identification and characterization*

Good quality RNA from stem tissue of *C. annuum* plants as well as mid stage green fruits were used for RT-PCR analysis using the F and R primers designed based on the reported *CanPin-II* PI sequence (AF039398, CanPI-1). On RT-PCR amplification, four bands having molecular weights of approximately 800bp, 600bp, 450bp and 300bp were obtained in the fruit and stem tissues (**Fig. 3.1**). In case of stem tissue the bands of sizes 600bp, 450bp and 300bp were prominent (**Fig 3.1, lane 2**), while in the case of fruit tissue bands of size 800bp and 600bp were prominent (**Fig. 3.1, lane 1**). These bands were eluted and cloned into pGEM-T Easy vector as detailed in materials and methods section. About 60 and 40 representative clones from stem and fruit tissue from three independent RT-PCR reactions were sequenced. The sequence analysis of these clones revealed that they all have homology to Pin-II family proteinase inhibitors. The sequence alignment further indicated the prominence of 441 bp and 267bp clones in stem RT-PCR while, predominance of 789bp and 614bp in the fruit RT-PCR. The sequence alignments of the clones with size 441bp and 267bp from stem and the size 789bp and 614bp from fruit tissue with those of reported *CanPIs* genes are depicted in **Fig. 3.2A and 3.2B**, respectively. Thus, the stem and fruit tissue represented 1, 2, 3 and 4 repeat domain PI genes corresponding to the four amplicons in the RT-PCR. These *CanPI* genes had a typical Pin-II family gene structure with a signal peptide sequence of ~25 amino acids (75 bp) followed by one to four inhibitory repeat domain (IRD(s)) each of 55 amino acid (166 bp) long, joined together by linker region of ~5 amino acids (**Fig. 3.2A amd 3.2B**). On screening sequences of several clones of one to four IRDs, it was noted that all the clones did not show exact identical DNA or amino acids sequences. Considerable sequence variations were noted in both DNA and amino acid sequences, which led to the identification of three 1- IRD type, seven 2- IRD type, three 3- IRD type and five 4- IRD type *CanPIs* (**Fig. 3.2A and 3.2B**). The details of these sequences have been deposited in the NCBI database and are provided in **Table 3.2.** However, *CanPIs* with sequence similar to reported *CanPIs* of 3- IRD type (AF039398 and AF221097) could not be isolated,

which might be attributed to the use of developing fruits and stem rather than leaves and/or different genotypes of *C. annuum* used in the present study (Kim *et al.*, 2001, Shin *et al.*, 2001). The 1-, 2- and 4- IRD type *CanPIs* isolated, are however, novel and not reported earlier. The variations were particularly noted at one or two amino acids in the signal sequence, within the linker sequence of 5 amino acids, around three amino acids C terminal to the linker, within the reactive site and the reactive site loop comprising around 10 amino acids on the N and C terminal side of the reactive site residue P1 (P5 to P7'). Apart from the major variations in amino acid sequence restricted to these locations, some other random variations were also observed in the IRDs.

Pair wise comparison of dedused AA sequences of *CanPIs* showed 86 - 97% amino acid identity in 1- IRD CanPIs, 82 - 99% in 2- IRD CanPIs, 84 - 99% in 3- IRD CanPIs and 93 - 99% in 4- IRD CanPIs (**Fig. 3.3**). Based on the deduced amino acid sequence of these CanPIs, 1- and 3- IRD type CanPIs had only trypsin inhibitory site while 2- and 4- IRD type CanPIs have trypsin and chymotrypsin inhibitory sites. Dendrogram of all full length CanPI precursors showed clustering mostly on the basis of IRD types i.e. 2- IRD, 4- IRD and 1- IRD type *CanPIs* were grouped together(**Fig. 3.4**). 1- IRD type *CanPIs* formed a distinct cluster away from all the other PIs. Alignments of signal sequences, IRDs and linker regions of all the 20 CanPI amino acid sequences were carried out to detect the variability amongst them. On analyzing all the IRD type CanPIs (total 20 genes and 52 IRDs) in the present study unique IRDs were discovered with their total number being 17 (**Fig. 3.5**). Of all the 17 unique repeats three repeats had `L' in the P1 position of the active site and represented a chymotrypsin inhibitor. Five repeats had `K' at P1 position, which most likely represented a trypsin inhibitor. All the remaining repeats were trypsin inhibitors as `R' was present in the P1 position (**Fig. 3.5A**). Amino acid substitutions in remaining part of the inhibitory repeat made them different from each other. The dendrogram very clearly shows the separate clustering of chymotrypsin inhibitor repeats with `L' in the P1 position and trypsin inhibitory repeats with `K' in the P1 position (**Fig. 3.5B**). The remaining repeats, though trypsin inhibitory in nature, have very divergent sequences as indicated by the dendrogram.

**Table 3.2: *Capsicum annuum* Pin-II type PI genes**

| Name | Accession No. | IRDs | Full length/ Partial | Size (bp) | AA (Mature PI) | Reference |
|---|---|---|---|---|---|---|
| *CanPI-1* | AF039398 | 3R -1 | FL | 614 | 179 | Kim *et al.*, 2001 |
| *CanPI-2* | AF221097 | 3R -2 | FL | 614 | 179 | Shin *et al.*, 2001 |
| ***CanPI-3*** | **AY986465** | 3R -3 | FL | 614 | 179 | **Present Study** |
| ***CanPI-4*** | **AY986466** | 3R -4 | FL | 614 | 179 | |
| ***CanPI-5*** | **DQ005912** | 3R -5 | FL | 614 | 179 | |
| ***CanPI-6*** | **DQ008951** | -- | Partial | 613 | -- | |
| ***CanPI-7*** | **DQ005913** | 4R-1 | FL | 789 | 273 | |
| ***CanPI-8*** | **DQ005914** | 4R-2 | FL | 789 | 273 | |
| ***CanPI-9*** | **DQ005915** | 4R-3 | FL | 789 | 273 | |
| ***CanPI-10*** | **DQ005916** | 4R-4 | FL | 789 | 273 | |
| ***CanPI-11*** | **DQ008950** | 4R-5 | FL | 789 | 273 | |
| ***CanPI-12*** | **DQ008952** | -- | Partial | 660 | -- | |
| ***CanPI-13*** | **EF136387** | 1R-1 | FL | 267 | 63 | |
| ***CanPI-14*** | **EF136388** | 1R-2 | FL | 267 | 63 | |
| ***CanPI-15*** | **EF136389** | 1R-3 | FL | 267 | 63 | |
| ***CanPI-16*** | **EF125182** | 2R-1 | FL | 441 | 121 | |
| ***CanPI-17*** | **EF136381** | 2R-2 | FL | 441 | 121 | |
| ***CanPI-18*** | **EF136382** | 2R-3 | FL | 441 | 121 | |
| ***CanPI-19*** | **EF136383** | 2R-4 | FL | 441 | 121 | |
| ***CanPI-20*** | **EF136384** | 2R-5 | FL | 441 | 121 | |
| ***CanPI-21*** | **EF136385** | 2R-6 | FL | 441 | 121 | |
| ***CanPI-22*** | **EF136386** | 2R-7 | FL | 441 | 121 | |
| ***CanPI-23*** | **EF144129** | 2R-8 | FL | 402 | 117 | |

On analyzing the signal sequences of 25 amino acids at the N terminal of all the CanPIs, five variants were detected and named as SP-1 to SP-5 (**Fig. 3.6A**). Of the 20 CanPIs with varied number of IRDs, 11 CanPIs showed the presence of SP-1, 7 CanPIs had SP-5, where as SP-2, SP-3 and SP-4 were represented by single *CanPI* genes namely AF221097, CanPI-23 and CanPI-5, respectively. SP-3, a signal peptide

of CanPI-23 was different from the other sequences in having only 16 amino acids (**Fig. 3.6B**).

Comparison of Pin-II type PIs from other Solanaceae members with these CanPIs showed clustering of CanPIs (**Fig. 3.7**). All the CanPIs except 1 IRD type CanPIs, showed distinct clusters away from the other Solanaceae members. Within CanPIs, sub clustering was observed based on the number of IRDs in the sequences (**Fig. 3.7**). CanPI-8 is the only exception, which grouped with the other Solanaceae PIs.

In all Pin-II type PIs of other members of Solanaceae precursor molecule showed presence of a partial IRD (total 19 Amino acids + 5 amino acids linker region) containing the active site just towards C terminal to the signal sequence of 25 amino acids. In contrast to this feature, in all the *CanPI* genes studied previously as well as here, the partial IRD at the N terminal of the precursor is represented by four amino acids (KACS) only, followed by a linker sequence. An active site is clearly absent from the partial IRD at the N terminal region of the CanPIs (**Fig. 3.8**).

Considerable sequence variation was also noted in the linker sequence. EENAE, EGNAE, EASAE and QRNAK linkers were detected either joining the IRDs or joining the signal sequence to the IRD, in CanPIs.

### *3.2 Mature plant tissue screening shows differential expression pattern of Pin-II type PIs*

Fruit tissues from 10 varieties of *C. annuum* were screened by semi-quantitative RT-PCR for the expression of *CanPIs* and prosystemin. It was observed that all the varieties showed presence of four bands corresponding to the 1 to 4 IRD type PI genes. Relative expression of the PIs differed amongst the varieties but it correlated to the expression of the prosystemin gene (**Fig. 3.9**).

Leaf, flower, early stage fruit, mid stage fruit, late stage fruit, turning fruit and stem tissues from healthy mature plants of *C. annuum* var Phule Jyoti were analysed by semi-quantative RT-PCR (**Fig. 3.10**). Equalized cDNAs were used to carry out RT-PCRs with (1) *CanPI* gene specific primer pairs designed from reported sequences, AF039398 and AF221097 and (2) prosystemin specific primers (**Table 3.1**). Primer pair for AF039398 amplified four types of *CanPI* genes (from 4- to 1- IRD type), whereas primer pair for AF221097 amplified only two types of *CanPI* genes (2- and

3- IRD). In the leaf and flower tissue, expression of the *CanPI* genes was very low and only two weak bands of 614 bp and 441 bp equivalent to three and two IRD were observed. In all the stages of fruit tissue and stem, 4 bands of size 789 bp, 614 bp, 441 bp and 267 bp representing full-length Pin-II type PI precursor genes of 4-, 3-, 2- and 1- IRD(s), respectively, were observed. Though the presence of all the four bands was noted in the above tissues, level of expression varied. In the fruit tissues, early stage fruit showed less amplification of four IRD PI band, but with the advancement in the fruit development, the expression level of this PI type increased in the mid and late stage fruit, being maximum in turning fruit. In contrast to the 4- IRD PI, the 3- IRD PI showed maximum expression in the early stage and mid stage fruit and its expression decreased in the further stages of fruit development, with the least expression in the turning fruit (**Fig. 3.10**). The two and one IRD PI bands showed a consistent presence in all the fruit tissue types. In the stem tissue a completely different expression pattern of all the four *CanPIs* was observed, most notably it depicted the highest expression of two and one IRD *CanPIs* (**Fig. 3.10**). The amplification with primer pair for AF221097 showed similar expression pattern for the three IRD *CanPI.*

The prosystemin expression also revealed variation in different tissues (**Fig. 10**). Negligible expression of prosystemin was observed in flower tissue. Though, the leaf showed the lowest PI expression with previous primer pairs, there was some expression of prosystemin in leaf tissue. In the fruit tissues, early stage fruit showed low expression, which increased in the mid stage fruit, again reduced in the late stage fruit and was hardly detectable in the turning stage fruit. Stem tissue indicated low prosystemin expression. Thus, the expression of prosystemin correlated with the *CanPI* expression in all the tissue types studied with exception of leaf and turning fruit stage.

### *3.3 Mechanical wounding of leaves of young plants elevates the PI expression levels*

In the present study, mechanical wounding was brought about by making cuts on the upper surface of two leaves of each *C. annuum* plant and tissue was collected from five plants after every three hours. The actually wounded leaves of the plant were considered as local and the leaves above it as systemic tissue. In the local control tissue *CanPI* expression was almost absent. Detectable expression of *CanPI*s was

observed at 6 h after wounding which increased till 9 hours after wounding. *CanPI* expression declined considerably in the tissue which was collected 12 hours after wounding, but increased substantially again in the next 12 hours (24 hours after wounding) (**Fig. 3.11**). This pattern of *CanPI* expression in wounded local tissue was consistent with both the PI primer pairs tested as well as primer pair for systemin although the overall expression of systemin was low. Secondly, only in the local tissues a two IRD PI (441 bp) band was noted in addition to the 3- IRD PI expressed. This phenomenon was consistent in both the *CanPI* primer pairs used (AF039398 and AF221097). In the systemic tissue, however, a different pattern of *CanPI* expression was observed. Here, the *CanPI* expression in the form of a band of 3- IRD PI (614 bp) was detected 3 hours after wounding which enhanced till 12 hours after wounding and declined 24 hours after wounding. A rise and fall in the *CanPI* expression, in the first 24 hours, as in the local wounded tissue was not detected in the systemic tissue.
Pattern of *CanPI* expression in both, the local and systemic tissues, correlated directly with the expression of prosystemin.

### *3.4 Natural infestation by Myzus persicae (aphid) and Spodoptora litura (Lepidopteran pest) significantly enhances PI expression levels in mature plants*

*CanPI* expression in leaf and fruit tissue infected with virus and aphid and healthy control plants was studied by the semi-quantitative RT-PCR (**Fig. 3.12A**). It was observed that the control and virus infected leaf tissue showed weak presence of two *CanPI* bands corresponding to 3 and 2- IRD PIs, where as the aphid infected leaf tissue showed very strong expression of *CanPIs*. It also revealed additional expression of a band of 789bp (4- IRD PI band), which was absent in control leaf tissue. However, the control, virus infected and aphid infested fruit tissue showed a similar pattern of expression with 4-, 3-, and 2- IRD PI bands in all the tissues. In field, the infection by aphids was predominantly observed on the leaves and stems of *C. annuum* plants and aphids did not locally infest fruits. Therefore, it can be suggested that the aphid infested *C. annuum* fruit tissue was a systemic tissue of plants with prolonged aphid infestations.

Similar to the aphid infested tissue, the *Spodoptora litura* infested *C. annuum* leaves, also showed upregulation of *CanPIs* with specifically induced four IRD type *CanPIs*

which were not detected in uninfested control plants (**Fig. 3.12B**). The prosystemin expression also correlated with the *CanPI* expression in both, *M. persicae* and *S. litura,* infested tissues.

## 4. Discussion

### *4.1 Spatial regulation of CanPIs*

PI gene regulation of Solanaceous plants is governed by systemin signaling induced by wounding and attack by pest/pathogens (Orozco-Cardenas *et al.,* 1993). Apart from this, there exists a steady state level of PIs in various plant tissues also. Plant part(s) that have a considerable amount of stored food, like fruits, tubers and seeds need the highest protection from pests. High PI content in these organs protects them against herbivores and pathogens (Ryan, 1990; Pearce *et al.,* 1993; Karaban and Baldwin, 1997; Koiwa *et al.,* 1997; Damale *et al.,* 2005).

Various plant parts of *C. annuum* were, therefore, analysed in the present study using semi-quantitative RT-PCR approach to elucidate the spatial changes in PI gene expression, if any. Organ-specific expression of the *CaPin-II* gene (AF221097) in *C. annuum* L var VK-1 was reported to be the highest in the flower and green fruit tissue (Shin *et al.,* 2001). In my study, qualitatively as well as quantitatively different patterns of PI expression, were observed in different organs. Leaf and flower tissues showed the least *CanPI* expression while the fruit tissue not only depicted the maximum quantities of *CanPIs* in a steady state level but also had the highest diversity in the type of PIs by expressing all the four *CanPI* forms simultaneously. The relative expression of four IRD *CanPIs* increased while that of the three IRD *CanPIs* decreased with progression in the developmental stages of the fruit. To our knowledge this is the first report discovering simultaneous expression of four *CanPI* cDNA forms which show spatial regulation in mature *C. annuum* plant. These results strongly suggest defense as well as other physiological role of *CanPIs* in the fruit. There have been limited but recent reports to support this view. Sin *et al.* (2006) showed that Pin-II type PIs expressed in the seeds and fruits of *Solanum americanum* protect the developing endosperm and embryo from programmed cell death associated proteases. The plants in which PI expression was blocked by RNAi mediated silencing were found to be deficient in producing fertile seeds proving that the PIs

play a definite and important role in fruit development and in maintaining viability of seeds during the process of maturation.

Phloem tissue in stems needs to regulate the trafficking of macromolecules passing through it (Oparkaa *et al.,* 1999). Interestingly, the stem tissue of *C. annuum* showed unique pattern of PI expression with predominance of 1- and 2- IRD type PIs having numerous sub types based on varied amino acid sequences, suggesting their stem specific physiological role. Xu *et al.* (2001) isolated Pin-II type PI genes, *SaPin2a* and *SaPin2b* from stem of *Solanum americanum* and immunolocalisation experiments revealed their expression specifically in the phloem sieve elements. Further experiments on over-expressing these genes in lettuce demonstrated their function in prevention of endogenous proteolysis (Zhang *et al.,* 2004). Xu *et al.* (2004) further demonstrated tissue specific expression of SaPin2b in various stages of floral development while that of SaPin2a in glandular trichomes of *S. americanum.* Its high expression in trichomes was also correlated with trichome density and branching in transgenic *Nicotiana* (Liu *et al.,* 2006). However, the variability in *CanPI* expression in different tissues and the variability within the *CanPI* forms (such as within 1- IRD to 4- IRD forms) observed in the present study, were not detected in the previous reports. It is surprising to note that other researchers who have isolated PI genes from *C. annuum* cDNA libraries made from TMV infected leaves (AF221097) and pericarp (AF039398) (Shin *et al.*, 2001; Kim *et al.,* 2001) have not come across such high diversity in the *CanPI* genes. It would be interesting to investigate the changes in proportion of a particular *CanPI* of a specific IRD type in various tissues and to understand its role specific to that stage.

Variability in Pin-II type PI genes is a common feature of family Solanaceae. On southern hybridization in *C. annuum* varieties VK-1 and Nockwang with *CanPin -II* (AF221097) and *CaPI-2* (AF039398) as probes, multiple banding pattern was obtained indicating the existence of a multi-gene family of PIs in hot pepper genome (Shin *et al.*, 2001; Kim *et al.,* 2001). A single copy of Pin-II type PI is present in the genome of *N. attenuata*, while *S. americanum* shows presence of two genes. In the present study various forms of *CanPI* genes with sequence divergence in each of the form were observed in *C. annuum* var. Phule Jyoti as detailed in the results section.

To rule out the possibility that this variability in *CanPI* sequences is variety specific, ten cultivars of *C. annuum* from different regions of India were screened for the PI

diversity in fruits. All of them showed similar pattern of *CanPI* expression in fruit tissue. It thus showed that the diverse expression pattern obtained in *C. annuum* var Phule Jyoti, is not variety specific but could be ubiquitously found amongst *Capsicum* varieties.

### *4.2 Wound induced temporal and qualitative changes in CanPI expression*

Wounding results in release of cell-wall oligosaccharides and certain endopeptidases at the site of injury. In Solanaceae, this brings about the release of polypeptide systemin (18-20 AA) from the larger prosystemin (200 AA). Systemin leads the activation of the octadecanoid pathway for release of jasmonates, which act as an important signal for activation of several defense related pathways including the PI expression. Unlike cell-wall oligosaccharides, systemin moves through the phloem element and is transported to plant parts distal to the wound site where it acts to release more systemin molecules in turn amplifying the jasmonate pathway systemically. Thus the wounded local tissue as well as unwounded plant parts exhibits upregulation of wound responsive genes.

In the present study the *CanPI* expression was analysed in wounded leaf tissues of *C. annuum* plants at regular time intervals, till 24 h after wounding. Local and systemic responses to wounding differed in levels as well as pattern of *CanPI* expression. A rise up to 9 h, fall between 9-12 h and again a rise up to 24 h in *CanPI* expression was noted in the local tissue unlike the gradual rise till 12 h and fall at 24 h in systemic expression of *CanPIs*. Qualitatively, different transcripts of both, 3- and 2- IRD PIs were upregulated locally, where as only the 3- IRD PIs were upregulated systemically.

This response differs than that observed by Shin *et al.* (2001), which may be because of the difference in the method of wounding, extent of wounding and age of the plant. In a similar experiment, *CaPin-II* (AF221097) expression went on increasing till 12 h after wounding in local and till 6 h after wounding in systemic tissue. Expression decreased to undetectable levels in the next 12 h and no further change was noticed even up to 72 h after wounding.

A cross cut wounding performed in the present study to ensure the effect of wounding might have been a harsh condition to the plant as the wounded leaf tissue was observed to be dehydrated and drooping progressively till 12 h. However, these plants

recovered and regained turgidity by 24 h. This change in the leaf physiology might have reflected in the PI expression pattern by showing a short rise and fall in local PI expression. In an another study by Moran and Thompson (2001), wounded *C. annuum* plants were supplied with systemin, water or MeJ which resulted in accumulation of PI transcripts till 12 h, the effect being the highest in MeJ supplied plants. Constitutive expression of a single PI (3- IRD) gene band (`F band') in the northern analysis changed to induced expression of two, `F' band and an additional novel `S' band only in the wounded tissues. The molecular weight of the S band was almost identical to that of F band. The effect also reflected in higher PLPI (Pepper Leaf PI protein) accumulation in the leaves. Presence of such a slow moving band, although not observed in wounded tissue in the present study, was noted in case of 3 and 4 IRD bands expressed in the fruit tissue under steady state condition.

### *4.3 Regulation of multiple IRD CanPIs expression induced by biotic stresses*

Expression of gene regulation in plant-pathogen/pest interactions is very complex and several proteins with varied functions *in planta* have been detected to be up or down regulated in the interactions using microarray technology (Hermsmeirer *et al.,* 2001; Voelkel *et al.,* 2004b). It has been further reported that herbivore feeding causes biochemicals and morphological changes in the plant, which are specific to the type of herbivory (Walling *et al.,* 2000) and distinctly different imprints are left by attack from different herbivores (Voelckel *et al.*, 2004b). Change in expression pattern of the PIs, when the plant is infected by different pests and pathogens has also been documented (Turlings *et al.,* 1991; Thaler, 1999; Kessler and Baldwin, 2001). Variation in the expression patterns of *CanPIs* was therefore, studied when the *C. annuum* plants were naturally infested with *Spodoptera litura* , *Myzus persicae* and viruses.

*1. Spodoptora litura (Lepidoptera) infestation*

In the present study, long duration feeding of *Spodoptera litura* on *C. annuum* leaves showed strong up regulation of *CanPIs*. Specifically four IRD type *CanPIs* were found to be up regulated in insect-infested plants which were not observed in control leaves. This response in *CanPI* expression profile was distinctly different than that caused by wounding in systemic and local tissues. In the previous reports, molecular responses of *Nicotiana attenuata* to feeding by the specialist herbivore *Manduca*

*Sexta* were studied by differential display and 16 upregulated and 9 downregulated cDNAs were identified (Hermsmeier *et al.,* 2001). In another study, antiherbivore defense function of endogenous trypsin proteinase inhibitors of *N. attenuata* was well demonstrated (Zavala *et al.,* 2004a). Although, production of this TPI incurs costs on the plant but the fitness benefits offered by TPIs during infestation of the plant by herbivores, are more than the costs (Zavala *et al.,* 2004b). When oral secretions from *Manduca Sexta* were introduced into wounds, TPI activity and mRNA transcript accumulation as well as JA levels increased dramatically over and above only wound induced levels in *N. attenuata* (Wu *et al.,* 2006). This once again established the importance of the plant's reliance on proteinase inhibitor mediated defense.

*2. Myzus persicae (aphid) infestation*

Mode of feeding by aphids imparts minimum tissue damage (compared to that caused by chewing insects) though, they inflict considerable fitness costs and also transmit viral pathogens to the plants (Dixon, 1998). Secondly, this type of host tissue damage caused by inserting aphid stylets is very similar to insertion of fungal hyphae (Fidantsef *et al.,* 1999). Very less molecular information about the induction by the aphid feeding is available. The feeding by aphids is thought to induce SA pathway for the expression of pathogenesis related genes as induced by the bacterial and fungal pathogens (Moran and Thompson, 2001; Moran *et al.,* 2002). Elicitation of PIs, the `signal signature of jasmonate pathway' is also induced by aphid attack but studies in different systems reveal the importance of right time point for such elicitation (Fidantsef *et al.,* 1999; Zhu-Salzman *et al.,* 2004; de Ilarduya *et al.,* 2003). Thus, aphid feeding displays a smaller transcriptional response both qualitatively and quantitatively (Voelckel *et al.,* 2004b), with a cross talk between the major signaling molecules JA and SA. In particular, genes involved in cell wall modification, water transport, vitamin biosynthesis, photosynthesis, carbon assimilation and nitrogen and carbon mobilization were up regulated in the phloem of *Myzus persicae* infested celery plants (Divol *et al.,* 2005).

Aphid *Myzus persicae* infested *C. annuum* leaves in the present study showed a substantially higher expression of *CanPIs* (**Fig. 3.12**) especially the four IRD *CanPIs* which are otherwise expressed in the fruit tissue at steady state. Interestingly this profile was similar to that induced by the lepidopteran insect, *Spodoptera litura*. Upregulated *CanPIs* in *C. annuum* plants with sustained long term aphid infestations

might be involved in a significant role in limiting the growth of aphids and controlling their fecundity, since, the infested plants also managed to produce fruits, though less than the control plants. Despite the lack of cysteine protease and chymotrypsin activity in aphid gut, oil seed rape and pea, expressing cysteine PI (oryzacystatin) and BBI (with TI and CI activity), respectively, were reported to be toxic and showed weight and fecundity reduction in the feeding aphids (Rahbe *et al.,* 2003a, b).

*3.* Virus *infection*

Virus infected leaves did not show up regulation of PIs in the present study. Shin *et al.* (2001) have reported that an avirulent strain of TMV showed development of a hypersensitive response in *C. annuum* and led to the upregulation of several defense related proteins including PIs. However, a virulent strain of TMV, on the other hand was incapable to show such a response. Our observation was in agreement with this suggesting that the virus strain infecting *C. annuum* leaves might be virulent in nature. These results indicate diverse and complex function of the *CanPIs* under various physiological conditions. Detailed investigations by isolating and sequencing tissue specific and induction specific *CanPI* genes expressed under various conditions and correlating them to the regulation of the respective function is indeed needed to describe the precise role of *CanPIs.*

### *4.4 Sequence variability in CanPIs*

PIs from the Pin-II family are known for variability in the number of inhibitory repeat domains per gene, from single IRD genes to 8 IRD genes distributed mostly in Solanaceous plants (Barta *et al.,* 2002). Pin-II type PI genes from Solanaceae have been observed to contain single domain in eggplant (*Solanum melongena*) and tobacco (*Nicotiana tabacum*) (Pearce, 1993), two in potato (*Solanum tuberosum*) and tomato (*Lycopercisum esculantum*) (Choi *et al.,* 1990; Graham *et al.,* 1985; Bryant *et al.,* 1976; Plunkett *et al.,* 1982; Sanchez-Serrano *et al.,* 1986), three in *Nicotiana alata* , tomato and pepper (*Capsicum annuum)* (Taylor *et al.,* 1993; Baladin *et al.,* 1995; Shin *et al.,* 2001; Kim *et al.,* 2001), four in *N. alata* (Miller *et al.,* 2000), six in *N. alata* and *N. glutionasa* (Atkinson, 1993; Choi D *et al.,* 2000), seven in *N. attenuata* (Zavala *et al.,* 2004a) and eight in *N. glutionasa* (Choi D *et al.,* 2000).

In the present study, 10 variants of 3- and 4- IRDs of Pin-II type genes from the developing fruits of *C. annuum* and ten more *CanPIs* of 1- and 2- IRD from the stem

tissue of *C. annuum* were identified and characterized. 3- and 4- IRD PIs from the stem tissue were also sequenced but did not show diversity as obtained in the fruit tissue. Only one type each of 3- and 4- IRD *CanPI* was expressed in stem. This clearly highlights that the spatial regulation of *CanPI* expression is not just limited to the different IRD types but also specifically governs the PI diversity within an IRD type.

3- IRD genes have also been reported previously from *C. annuum*. However, the sequence analysis showed that the genes isolated in the present study are different (3-16%) from the reported ones (Kim *et al.,* 2001; Shin *et al.,* 2001). Two classes of genes of Pin-II family having 4- and 6- IRDs were identified earlier from a stigma cDNA library of *Nicotiana alata* (Miller *et al.,* 2000). Screening of a subtractive cDNA library prepared from *N. glutinosa* to isolate TMV-responsive genes also resulted in the identification of two types of Pin-II type cDNAs, NGPI-1 and NGPI-II, having 6- and 8- IRDs, respectively (Choi *et al.,* 2000).

The variability in the Pin-II family is not only restricted to the number of IRDs per gene but also to the sequence of individual IRD. Around 77 different IRDs in the Pin-II family have been identified so far (Barta *et al.,* 2002). In the present study 18 precursor genes (from 1- to 4- IRDs) contributing 17 unique IRDs have been identified. Moura and Ryan (2001) identified, characterized and determined N-terminal sequence of seven PIs from *C. annuum* leaves. These represented individual IRDs formed by cleavage of larger precursor forms and all of them showed homologies to the two earlier reported cDNAs of *C. annuum* (Kim *et al.*, 2000; Shin *et al.*, 2000). The deduced AA sequence of the IRDs from the *CanPI* genes in the present study however, showed 2 to 25% sequence divergence in the vicinity of reactive site loop similar to the 15% sequence divergence reported in the AA sequence of 5 mature PIs formed after cleavage of a larger precursor NaProPI from *N. alata* (Nielsen *et al.,* 1995). All the CanPIs isolated here showed presence of a 5 AA (EGNAE, QRNAK, EASAE or EENAE) region corresponding to the EEKKN linker region of *N. alata* PIs (Heath *et al.,* 1995; Nielsen *et al.,* 1996), which probably functions in a similar way. Though these linker regions are different than the *N. alata* linker sequences (Moura and Ryan, 2001) they are most likely processed in a similar way by cleavage at or within the linker followed by terminal proteolysis (Heath *et al.,* 1995; Horn *et al.,* 2005). Presence of a number of reactive sites per precursor

molecule as in case of CanPIs in my study and in case of NaProPI (Atkinson 1993) is advantageous, as it represents efficient molecular design for transcription, translation and localization of several PIs (Nielsen *et al.,* 1995). The sequence diversity, the repeat number diversity within *C. annuum* Pin-II PIs and their dissimilarity to *N. alata* linker sequence but potential to inhibit proteinase make it important to study the structural features of CanPI proteins.

In the present study several *CanPI* genes have been isolated from developing green fruit and stem tissues, which were healthy and not induced by any chemical treatment or wounding or insect chewing. This indicated steady state expression pattern of these forms of PIs, correlating to their endogenous functions *in planta,* in addition to their role in insect defense in *C. annuum* as discussed in the previous paragraphs. This necessitates further investigation of simultaneous expression of many variants of PIs in fruit tissue of *C. annuum.*

A partial sequence at N terminal of *Nicotiana* precursor Pin-II type PI is involved in the formation of a complete functional IRD by circularization of the precursor molecule achieved by joining of the N and C terminals of the precursor by disulphide bonds (Lee *et al.,* 1999) giving rise to a double chain IRD. In all the *CanPI* precursors (from 1 to 4 IRD type) there is a clear absence of partial IRDs at the N and C terminal of the precursors. Thus, all single chain IRDs are expected from these *CanPIs* in *C. annuum,* probably representing ancestral types of Pin-II PIs. aPI1 and PSI1.2 are the single domain PI proteins from *N. alata* and *C. annuum,* respectively, and are thought to be different than the other Pin-II PIs being formed from a single sequence repeat (Scanlon *et al.,* 1999; Antcheva *et al.,* 2001). They are probably formed from ancestral Pin-II precursors where domain swapping has not occurred and so the sequence repeat forms a structural repeat. All the *CanPI* precursors most probably show such ancestral precursor type.

The signal sequence also showed variations in AA sequences and a small signal sequence of 17 AA was detected as against the normal 25 AA signal sequences. Ryan and Pearce (2003) have identified several hydroxyprolin systemin like peptides (HypSys) from tobacco and tomato, different than the systemins, but functionally related to them. They had raised several questions about the wound signaling of the HypSys and the compliment of defense genes they could activate. There is a possibility of finding such peptides in *C. annuum.* Secondly, cyclopentenone

derivatives like OPDA other than JA in combination are involved in modulating the defense gene expression through the JA pathway (Stinzi *et al.*, 2001). It could be hypothesized that such variability in the signaling molecules themselves may reflect in expression of diverse *CanPI* genes.

The present study also raises several questions about the functional significance of spatially and temporally regulated, different IRD type *CanPIs*. Further more, the specific role of *CanPI* diversity in an IRD types needs to be investigated. Precursor of multiple IRDs of Pin-II type, are more advantageous to the plant, as a single machinery of transcription and translation is required to generate several active PI molecules. Even then *C. annuum* has, not only maintained smaller precursor molecules having 1- and 2-IRDs, but also expressed them specifically in particular tissues and on wounding as discussed previously. This necessitates more in depth studies regarding the functional role of various IRD Pin-II PIs in *C. annuum*.

In summary, this chapter describes the cloning and sequence characterization of different IRD type (1- to 4-) PI genes from stem and fruit tissues of *C. annuum*, belonging to the Pin-II family. The tissue specific, wounding specific and abiotic stress specific induction of PIs and its correlation with systemin expression as revealed by semi-quantitative RT-PCR has also been described.

# Chapter 4

## Diverse forms of Pin-II family proteinase inhibitors of *Capsicum annuum* produce adverse effect on growth and development of *Helicoverpa armigera.*


### Abstract

Deduced amino acid sequences of the *CanPIs* showed up to 15% sequence divergence among each other and with reported inhibitor. Four *CanPIs*, two having three IRD (*CanPI-3* and *CanPI-4*) and the other two having four IRD (*CanPI-7* and *CanPI-9*) from *C. annuum* fruit and showing deduced amino acid sequence divergence among each other and with the reported inhibitor, were cloned in *Pichia pastoris* for expression of recombinant CanPIs. Amino acid sequence analysis of these CanPIs revealed that the three IRD PIs have trypsin inhibitory sites, while four IRD CanPIs have both trypsin and chymotrypsin inhibitory sites. Recombinant CanPIs showed up to 90% inhibition of bovine trypsin while chymotrypsin inhibition varied with the number of chymotrypsin inhibitory sites present in the CanPIs. Recombinant inhibitors exhibited over 70% inhibition of gut proteinases of *Helicoverpa armigera*. *H armigera* larvae fed with recombinant CanPIs incorporated in artificial diet individually, showed 35% larval mortality, severe larval weight reduction and up to 24% pupal weight reduction as compared to larvae fed on control diet. Of the four CanPIs, CanPI-7, with two sites for TI and two sites for CI, showed consistent antagonistic effect on *H. armigera* growth and development. Among all the CanPIs, CanPIs containing diverse IRDs are the best suited for developing insect tolerant transgenic plants.

## 1. Introduction

Plant proteinase inhibitors (PIs) of Pin-II type are of interest due to their large structural and functional diversity in numerous plant species. Several fold higher accumulation of inhibitor protein in insect damaged tissues categorized them as plant defensive proteins (Green and Ryan, 1972; Ryan, 1990). Precursor protein of Pin-II type inhibitors in various plants ranges from 2 to 8 IRD, which upon cleavage by plant proteases release single inhibitor protein active against serine proteinases (Heath *et al.*, 1995; Horn *et al.*, 2005). The ancestral Pin-II inhibitors of single inhibitory repeat are distributed in several mono- and dicotyledonous plants whereas the multiple inhibitor repeat precursors arising due to a series of gene duplication and/or domain duplication events are restricted to Solanaceae (Barta *et al.*, 2002). The precursor inhibitor proteins have a conserved structure with putative endoplasmic reticulum signal peptide at N-terminal followed by inhibitory repeat domains (1 to 8) and a C-terminal region which probably functions as a vacuolar sorting signal (Miller *et al.*, 2000). Pin-II type PIs have been isolated and characterized from *N. tabacum*, *L. esculantum* and *C. annuum* (Plunkett *et al.*, 1986; Pearce *et al.*, 1993; Antcheva *et al.*, 1996). A circular conformation of 43 kDa inhibitory precursor of *N. alata* acquired by joining the partial repeats at the N- and C-terminal generated the sixth functional IRD. This type of `clasped bracelet' fold is adopted by the members of Pin-II family regardless of the number of inhibitory repeats (Lee *et al.*, 1999). Potential of these proteins for inhibiting insect growth and development has been demonstrated in several transgenic plants (Johnson *et al.*, 1989; Heath *et al.*, 1995; Duan *et al.*, 1996; Zavala *et al.*, 2004b).

Pin-II type PI proteins and genes have been identified and characterized from *Capsicum annuum.* A 6 kDa protein PSI1.1 purified from seeds has shown potential to inhibit trypsin and chymotrypsin (Antcheva *et al.*, 1996). In another study, a protein PSI1.2 from seeds has been reported to be a circularly permuted, ancestral member of Pin-II family (Antcheva *et al.*, 2001). Seven small 6 kDa PIs from leaves of *C. annuum* have been purified and N-terminal sequenced to show homologies to various repeat regions of previously reported *C. annuum* PI genes (Moura and Ryan, 2001). Three repeat PI genes with NCBI accession numbers AF221097 (Shin *et al.*, 2001) and AF039398 (Kim *et al.*, 2001) have also been isolated from cDNA libraries constructed from Tobacco Mosaic Virus infected leaves and pericarp of *C. annuum*,

respectively. These *CanPI* genes are expressed constitutively as well as in significantly high amounts upon induction by various elicitors.

Two PIs namely CapA1 and CapA2 have been purified and characterized from *C. annuum* leaves having a molecular weight of about 12 kDa with inhibitory activity against bovine trypsin and chymotrypsin as detailed in Chapter 2. These PIs showed anti-metabolic effects on *H. armigera* by inhibiting larval growth and development and severe reduction in fecundity and fertility of adults (Chapter 2). In the Chapter 3 cloning and characterization of novel CanPI genes with one to four IRDs have been detailed. Specific upregulation of the 3 and 4 IRD CanPIs were observed in aphid and lepidopteran infested *C. annuum* plants, suggesting their involvement in resisting insect attack. Based on these observations two diverse forms of 3 IRD and 4 IRD CanPIs each were selected for further investigations. Present chapter commits of characterization of these 4 novel Pin-II PI genes from *C. annuum* having considerable sequence variation compared to the reported *CanPI* genes. These genes have been expressed in *Pichia pastoris* and the recombinant PI proteins analyzed *in vitro* and *in vivo* for their inhibitory activity against *H. armigera* gut proteinases.

## 2. Materials and Methods

### *2.1 Sub-cloning of the CanPI genes in yeast expression vector pPIC9*

Primers for amplification of the 3- and 4-IRD *CanPI* gene insert in pGEM-T vectors were designed such that they included the *Xho*I site followed by a region from the mature peptide in the forward primer sequence. The signal sequence portion which was lost due to cleavage by *Xho*I enzyme from the pPIC9 vector was reconstructed in the forward primer. The reverse primer was designed to include a *Not*I site followed by the reverse complement of the 3'region of the mature peptide. Forward primer sequence was 5' AAA AAA CTC GAG **AAA AGA GAG GCT GAA GCT** AAG GCT TGT TCA CAA AG 3' while reverse primer sequence was 5' AAA AAA GCG GCC GC CTG TTC ATG CTT TTA C 3' Underlined region of the primer indicates *Xho*I site in F primer and *Not*I site in the reverse primer, respectively; bold region in the F primer indicates the lost region from the vector and the remaining region in both the primers is of the mature PI. These primers were used to carry out amplification of the mature peptide regions from the full-length gene cloned in the pGEM-T vector.

The amplification was carried out using Accuprime Pfx polymerase (Invitrogen, USA). The amplified product was digested with *Xho*I and *Not*I and purified using gel elution kit (Gen Gel elution kit) (Sigma, USA). Yeast expression vector pPIC9 was prepared by digesting it with the same restriction enzymes. The digested insert and the vector were quantified and ligated in 1:3 ratio using T4 ligase (Promega, USA). The ligated product was used for transformation of competent cells of *E. coli* strain Top10. Positive clones were identified by colony PCR, plasmids were extracted from them as detailed in chapter 3 (Materials and methods section) and checked by restriction digestion with *Xho*I and *Not*I enzymes. Sequencing of the inserts in pPIC9 vector was carried out to confirm whether the signal sequence from the vector and the initiation of the CanPI mature peptide cloned are in frame.

### *2.2 Cloning of CanPI genes in Pichia pastoris*

Plasmids (~2 mg) of CanPI mature peptides cloned in pPIC9 vector were isolated, digested with *Sal*I for linearization and checked on gel to ensure complete digestion of the plasmid. The linearised plasmid was purified by phenol: chloroform and quantified. Around ~2 mg of the linearised pPIC9 plasmids with the cloned *CanPIs* were used for *P. pastoris* transformation.

*P. pastoris* GS115 cells were grown and competent cells were prepared using Easy Comp Kit (Invitrogen, USA). Transformation of the competent GS115 cells was also carried out using reagents from the same kit and the transformed *P. pastoris* colonies were selected on histidine deficient medium RDB (Invitrogen, USA) which selectively allows the growth of the cells having the integrated pPIC9 vector. The untransformed *P. pastoris* lacks the histidine biosynthesis gene and is not able to grow on the auxotrophic medium. The colonies appearing on the auxotrophic medium were checked by performing colony PCR and positive clones were further used for protein expression analysis.

### *2.3 Yeast expression of recombinant CanPIs*

The single recombinant *P. pastoris* colonies were initially grown at 30 $^{o}$C in 25 ml MGY medium (Invitrogen, USA) for ~24 hours till the growth reaches ~1 OD at 600 nm. The cell pellet was collected by centrifugation at 3000 g, 10 min at RT and was transferred to 100 ml of BMMY medium as per the instructions of the manufacturer

(Invitrogen, USA). Methanol (1%) was supplemented every 24 h and the culture was allowed to grow for ~96 h. It was then centrifuged at 14, 000 g, 10 min at 10 $^{o}$C to obtain the supernatant, which was precipitated by ammonium sulphate (90%). The precipitate was dissolved in minimum water and dialyzed against water and concentrated to ~15 fold. Protein estimation was carried out spectrophotometrically using Bradford reagent (Bradford, 1976). These expressed proteins were assessed for their inhibitory potential against trypsin by solution assays (Chapter 2). The clones expressing maximum amount of inhibitor were used to carry out large-scale expression of the recombinant CanPIs. To get rid of extra cellular proteases secreted by *P. pastoris* the expression of the recombinant CanPIs was carried out in MM medium (Invitrogen, USA) which is an unbuffered medium wherein the pH of the medium drops down to 2 or less during growth of *P. pastoris*, inactivating the extra cellular proteases secreted by *P. pastoris.*

### *2.4 Proteinase and recombinant PI assay*

Total HGP, trypsin and chymotrypsin activities were measured by azocaesinolytic assay (Brock *et al.,* 1982). For enzyme inhibitor assay, the inhibitor was mixed with the enzyme and premix was incubated at 25-27$^{o}$C for 30 min. The residual enzyme activity was then estimated. One proteinase unit was defined as the amount of enzyme that increased the absorbance by 1.0 OD under the given assay conditions. One PI unit is defined as amount of inhibitor required for inhibiting one proteinase activity unit.

Recombinant CanPIs were separated on native-PAGE (Davis, 1964) and the gel was further processed for trypsin inhibitor (TI) activity visualization by GXCT as described in Chapter 2 (Materials and methods section).

### *2.5 HGP extraction*

Midgut tissue was dissected out from the fourth instar larvae of *H. armigera* and immediately frozen in liquid nitrogen and stored at –80 $^{o}$C. For extraction of HGPs, midgut tissue was homogenized in 0.2 M glycine-NaOH buffer, pH 10 in 1:1 ratio and kept at 10 $^{o}$C for 2 h. Suspension was centrifuged at 4 $^{o}$C for 20 min at 7500 g and resulting supernatant was used as a source of HGPs.

### *2.6 H. armigera feeding assay*

*In vivo* efficacy of recombinant *C. annuum* inhibitors was studied by feeding assays using laboratory-established culture of *H. armigera*. The artificial diet was prepared as reported by Nagarkatti and Satyaprakash (1974) and detailed in Materials and methods section in chapter 2. Total gut proteolytic activity of a single gut of the fourth instar larva was estimated, recombinant CanPIs required to inhibit maximum proteolytic activity present in single gut was calculated and that amount of CanPI when incorporated per gram of the artificial diet gave1X inhibitor diet. 3X inhibitor diets of all the four CanPIs were used for the present study.

Initially, the *H. armigera* larvae were collected from the pigeonpea fields of Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, Maharashtra, India. The larvae were cultured in laboratory for two generations on artificial diet. The neonates that hatched from the eggs laid by the 2$^{nd}$ generation moths were reared for the first 3-4 days on control diet and then transferred to CanPI containing diet and control diet (artificial diet without PI) in separate sets of 30 larvae each and two replicates of each set were performed. Each larva was maintained in an individual vial containing inhibitor or control diet. All the vials were checked daily, for cleaning and also for addition of diet, as per the consumption by the insect. Larval weights of all the sets were taken on every alternate day and % weight reduction in the PI fed larvae was compared to the control group. Larval mortality, pupation period, pupal weight, maximum larval weight were recorded and compared with that of the control group to estimate the adverse effects of CanPIs on growth, development and reproductive capabilities of *H. armigera*.

## 3. Results

### *3.1 Sequence characterization of novel Pin-II family inhibitor genes from developing fruits of C. annuum selected for cloning into Pichia pastoris*

An oligonucleotide primer pair from the reported *C. annuum* Pin-II family PI sequence (Accession number AF039398; Kim *et al.*, 2001) was used to isolate *CanPI* genes from developing green fruit and stem mRNA by RT-PCR as described in the previous chapter 3. The study could identify totally 18 different full-length *CanPI*

genes. Of these, 2 CanPIs with 3 inhibitory repeats and 2 CanPIs with 4 inhibitory repeats with maximum sequence diversity were selected for further analysis. The alignment of deduced amino acid sequence of these CanPIs and two reported CanPIs (Kim *et al.,* 2001; Shin *et al.,* 2001) shows the amino acid substitution at several places particularly within and close to the reactive site loop. The fourth IRD of the CanPI-7 and CanPI-9 showed alignment with the third IRD of CanPI-1 (AF039398) **(Fig. 4.1)**. Sequence alignment of only repeat region (50 amino acid) from each of these CanPIs revealed total 9 unique repeat domains from these six genes **(Fig. 4.2A)**, which were present in various combinations in these genes. The pair wise comparison of these repeats showed a minimum of 2% and maximum of 25.5% amino acid variation **(Fig. 4.2B)** within these repeats. However, all these repeat sequences possessed 8 conserved cysteine residues and a single reactive site, characteristic of the Pin-II repeat sequences **(Fig. 4.2A)**. CanPI-3 and CanPI-5 had inhibitory repeats no. 4, 4 and 5 in combination, while CanPI-7 and CanPI-9 had repeats 1, 6, 2, 3 and 4, 7, 2, 3, respectively, joined with the linker regions. Earlier reported CanPI-1 and CanPI-2 had inhibitory repeat no. 1, 2, 3 and 4, 8, 9 respectively.

### *3.2 Expression and cleavage of 3- and 4- repeat CanPIs in Pichia pastoris generate active inhibitors*

Four *CanPI* genes namely *CanPI-3* and *CanPI-5* representing 3 IRD and *CanPI-7* and *CanPI-9* representing 4 IRD with maximum amino acid variations were selected for cloning in yeast expression vector pPIC9 **(Fig. 4.3)** to obtain recombinant inhibitor proteins for further biochemical characterization. Recombinant CanPIs were expressed in a buffered medium (BMMY) at pH 6 and the unbuffered medium (MM) where pH dropped to 2 or less as the culture growth progressed. The protein obtained in growth medium was partially purified and analysed on native and SDS PAGE for protein and inhibitory activity profiles.

Partially purified recombinant protein preparations from BMMY **(Fig. 4.4A and 4.4B)** and MM **(Fig. 4.4C and 4.4D)** cultured *P. pastoris* transformed cells exhibited different profiles. Multiple recombinant proteins expressed with BMMY medium either treated with trypsin **(Fig. 4.4A)** or stained with CBB R-250 **(Fig. 4.4B)** suggested cleavage of proteins probably due to action of extra cellular proteases secreted by *P. pastoris.* Two bands of size 19 kDa and 12 kDa in case of CanPI-3 and

CanPI-5, while three bands of size 26 kDa, 19 kDa and 12 kDa in case of CanPI-7 and CanPI-9 were predominant with a faint band of around 6 kDa **(Fig. 4.4B)**. This could be because of the two linker regions in case of CanPI-3 and CanPI-5 and three linker regions in CanPI-7 and CanPI-9 sensitive to proteolysis resulting into processed PI repeats. These repeat units, however, could reveal their respective trypsin inhibitory activity **(Fig. 4.4A)**. Recombinant proteins obtained from MM medium grown *P. pastoris* transformed cells resulted into different profiles **(Fig. 4.4C and 4.4D)** as compared to BMMY harvested recombinant proteins **(Fig. 4.4A and 4.4B)**. Major bands of size ~19 kDa for CanPI-3 and CanPI-5, and ~26 kDa for CanPI-7 and CanPI-9 equivalent to their respective uncleaved sizes were detected in both the cases **(Fig. 4.4D)**. The acidic pH of MM medium in which the *P. pastoris* was grown, was probably responsible to suppress protease activity on recombinant proteins.

These recombinant protein preparations were further resolved on native PAGE to visualize the inhibitor profiles against trypsin, chymotrypsin and *Helicoverpa armigera* gut proteinases (HGP). Recombinant protein preparations harvested from BMMY medium grown cells showed more number of trypsin inhibitor activity bands (about 8-9 bands) **(Fig. 4.5A)** than those in the MM harvested recombinant preparations (about 2-3 bands) **(Fig. 4.5B)**. Visualization of the BMMY harvested CanPI preparations with chymotrypsin and HGP showed many bands **(Fig. 4.6A, B)** indicating inhibitory activities of all the CanPI fragments with the enzymes tested. The chymotrypsin and HGP inhibitory activity of the inhibitors extracted from MM medium grown *P. pastoris* cells showed only a few bands as in case of trypsin inhibitor (Figure not shown). Interestingly, all these inhibitor proteins were highly stable to further proteolysis even upon long storage.

Amino acids sequence prediction of these *CanPI* genes indicated that these inhibitors have reactive sites for trypsin and/or chymotrypsin. All the three repeats of CanPI-3 and CanPI-5 have site for trypsin inhibition whereas CanPI-7 has two repeats each with site for trypsin and chymotrypsin inhibition. Interestingly, CanPI-9 has three sites for trypsin inhibition and one for chymotrypsin inhibition. In order to confirm this, recombinant CanPI protein preparations extracted from BMMY and MM medium grown cells were used for trypsin, chymotrypsin and HGP inhibition studies in solution assays**. Figure 4.7** depicts the inhibition potential of CanPIs against trypsin and chymotrypsin. Maximum inhibition (around 85% or more) of bovine

trypsin required ~1.5 and 1.0 µg of BMMY and MM extracted recombinant CanPI proteins, respectively. In case of chymotrypsin inhibition, using the same amounts of the recombinant PIs, as expected, CanPI-3 and CanPI-5 inhibited only 30 to 70% of chymotrypsin activity even at high inhibitor concentration. On the contrary, CanPI-7 and CanPI-9 recombinant proteins inhibited more than 85% of chymotrypsin activity **(Fig. 4.7)**.

The amount of inhibitor (IC50) for standard trypsin and chymotrypsin activity (0.45 U $ml^{1}$) was estimated for all the CanPIs expressed in BMMY medium. **Table 4.1** shows molarity of the enzyme and the inhibitor at IC50. Even after correction of the expected molarity value to suit the number of trypsin and chymotrypsin inhibitory sites in the recombinant inhibitors the observed molarities of the CanPIs at IC50 are several fold less. It is interesting to note that the recombinant CanPIs do not show stoichiometric inhibition of the two standard enzymes. The details of the calculations about $IC_{50}$ with trypsin and chymotrypsin are given in **Table 4.1.**

**Table 4.1: Fold differences in PI molarity at $IC_{50}$ of Trypsin and Chymotrypsin inhibition by CanPIs**

| PI | TI sites | Molarity of PI at $IC_{50}$ Trypsin $6.78x10^{-4}$(M) | | Fold difference | CI sites | Molarity of PI at $IC_{50}$ Chymotrypsin $5.5x10^{-4}$ (M) | | Fold difference |
|---|---|---|---|---|---|---|---|---|
| | | Expected | Observed | | | Expected | Observed | |
| ***CanPI-3*** | **3** | $2.26x10^{-4}$ | $0.54x10^{-4}$ | **4.2** | **0** | Not expected | $1.2x10^{-4}$ | - |
| **CanPI-5** | **3** | $2.26x10^{-4}$ | $0.71x10^{-4}$ | **3.2** | **0** | Not expected | $1.1x10^{-4}$ | - |
| **CanPI-7** | **2** | $3.39x10^{-4}$ | $0.18x10^{-4}$ | **18.8** | **2** | $2.8x10^{-4}$ | $0.3x10^{-4}$ | **9.2** |
| **CanPI-9** | **3** | $2.26x10^{-4}$ | $0.37x10^{-4}$ | **6.1** | **1** | $5.5x10^{-4}$ | $0.6x10^{-4}$ | **9.2** |

Recombinant CanPIs expressed in BMMY and MM medium, were also analyzed for the inhibition of gut proteinases of chickpea grown *H. armigera* (CP-HGP) using azocaesin as a substrate **(Fig. 4.8A, B)**. Increasing amount of the recombinant inhibitors was used to estimate maximum inhibition of CP-HGP, to be obtained with individual CanPI. About 60 to 70% HGP inhibition was obtained with ~0.5 to 1 µg of

CanPI proteins expressed in the BMMY medium while only ~0.1 to 0.2 µg CanPI protein is required to obtain similar inhibition with the recombinant protein harvested from MM medium. Further increase in inhibitor concentration only marginally increased the inhibition values. These amounts of recombinant proteins were further used for analyzing inhibition of HGP from larvae fed on pigeon pea (PP-HGP) or larvae fed on artificial diet containing winged bean proteinase inhibitors (WB-HGP) **(Fig. 4.9)**. About 65 to 70% inhibition of CP-HGP, 50-60% inhibition of PP-HGP and 20-40% inhibition of WB-HGP were observed with all the four CanPIs. At these just-saturating amounts of the CanPI, the chymotrypsin inhibition was very low as compared to the trypsin inhibition **(Fig. 4.9)**.

### *3.3 CanPIs retard the growth and development of H. armigera and also lead to significant larval mortality*

Recombinant CanPIs were incorporated in artificial diet for testing their *in vivo* potential against *H. armigera*. In the present bioassays ~ 70 to 80 µg of all the four CanPI inhibitors individually per gram of diet along with control (without inhibitor) were used. Second instar lab-reared *H. armigera* larvae were released on experimental diet and the larval mass was monitored every alternate day. The larval and pupal mass and the larval deaths were recorded and compared with the control. The results showed that the larval growth in the 3$^{rd}$ and the 4$^{th}$ instar larvae was severely reduced by CanPIs (19 to 40%) as compared to larvae fed on control diet **(Table 4.2)**. Maximal larval mass was also reduced (10 to 25%). Pupa formed from the CanPI fed insects exhibited 12 to 24 % less weight than the controls. Significant mortality (~30%) was also observed in the CanPI fed insects as compared to the control diet fed larvae, particularly during the transition from larva to pupa. Although, all the four CanPIs from the present study showed significant effect in retarding larval growth and development, CanPI-3 (three repeat PI) and CanPI-7 (four repeat PI) were more effective and showed a higher reduction in maximal larval and pupal mass gain than CanPI-5 and CanPI-9. This reduction in weight directly contributes to less fertility and fecundity of the moths emerging from the inhibitor fed diets as reported earlier in Chapter 2.

**Table 4.2: Anti-metabolic effect on *Helicoverpa armigera* fed with CanPIs.**

| PI | Larval wt. reduction (%) | | Max. larval wt. reduction (%) | Pupal wt. reduction (%) | Larval deaths (%) | mg/g of CanPI in diet |
|---|---|---|---|---|---|---|
| | 3rd Instar | 4th Instar | | | | |
| **CanPI-3** | 40.2 | 10.8 | 18.60 | 19.62 | 43.33 | 0.08 |
| **CanPI-5** | 29.9 | 18.7 | 24.64 | 24.28 | 35.00 | 0.07 |
| **CanPI-7** | 39.7 | 29.7 | 25.32 | 20.41 | 30.00 | 0.07 |
| **CanPI-9** | 31.7 | 22.2 | 10.98 | 12.57 | 36.67 | 0.08 |
| **Control** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

## 4. Discussion

### *4.1 Extra cellular proteases of P. pastoris cleave the recombinant CanPIs to generate variable IRD active CanPI fragments*

The recombinant CanPIs expressed in a buffered medium (BMMY) were found to be cleaved as indicated by activity bands corresponding to the size of 1 to 4 IRDs which was prevented by expression of the CanPIs in an unbuffered medium. All the CanPIs isolated including the 4 IRDs showed presence of a 5 AA (EGNAE or EASAE) region corresponding to the EEKKN linker region of *N. alata* PIs (Heath *et al.*, 1995; Nielsen *et al.*, 1996) which probably functions in a similar way.

Though these linker regions are different than the *N. alata* linker sequences (Moura and Ryan, 2001) they are most likely processed in a similar way by cleavage at or within the linker followed by terminal proteolysis (Heath *et al.*, 1995; Horn *et al.*, 2005). This has been supported by appearance of active CanPIs corresponding to the sizes of 1, 2, 3 and 4 repeat units upon cleavage by extra-cellular proteases expressed by *P. pastoris* in the present study **(Fig. 4.4)**. Although 2 IRD PI-II have been reported in potato and their various combinations have been cloned and expressed in a similar system of *P. pastoris*, their cleavage has not been discussed (Beekwilder *et al.*, 2000). Presence of a number of reactive sites per precursor molecule as in case of CanPIs in present study and in case of NaProPI (Atkinson *et al.*, 1993) is advantageous, as it represents efficient molecular design for transcription, translation and localization of several PIs (Nielsen *et al.*, 1995). However, there are only few reports about the structural studies of these precursor proteins. In case of *N. alata*,

circular conformation has been assigned to the precursor PI which gets cleaved at linker to release single domain Pin-II PI (Scanlon *et al.,* 1999). In case of tomato and potato, Pin-II PIs with 2 IRDs have been analyzed for their structural information as well as inhibitory potential. In case of *C. annuum* there are reports of isolation of several single domain PIs as well as multiple IRD PIs (Chapter 3). However, structural studies of these have not been performed. The sequence diversity and the repeat number diversity within *C. annuum* Pin-II-PIs, their dissimilarity to *N. alata* linker sequence but potential to inhibit proteinases make them important to study the structural features.

### *4.2 Inhibition of bovine trypsin and chymotrypsin by CanPIs correlates to the number and type of active sites*

Both the, cleaved and uncleaved, CanPIs in buffered and unbuffered medium, respectively, showed strong inhibition of trypsin **(Fig. 4.7)**, whereas chymotrypsin inhibition differed on the basis of number of CI sites present in each inhibitor. Residue(s) at the P1 position of the inhibitor is of importance in determining the specificity of inhibition while residues P4 to P2' of inhibitor interact with binding pocket S4 to S2' of the proteinase. Accordingly, Arg (R) at the P1 position makes a typical TI while Leu (L) at the P1 position shows a typical CI activity. Deduced AA sequence of the CanPIs indicated that 3 IRD CanPIs have only TI active sites whereas 4 IRD CanPIs have one or two CI active sites and remaining TI active sites. Recombinant CanPIs of 3 IRD with all these TI active sites in the present study showed weaker inhibition of chymotrypsin also. Similar observation has been reported by Antcheva *et al.* (1996) for the purified *C. annuum* PI with an active site for TI. On the other hand, the single domain PIs from *C. annuum* leaves with homologies to domains having CI/TI site have shown strong inhibition of both, trypsin and chymotrypsin (Moura and Ryan, 2001), whereas, replacing the reactive site of CI (domain 1) with TI (domain 2) in a 2 domain PI of potato, could not transfer inhibitory activity of domain II to domain I (Beekwilder *et al.,* 2000). The involvement of entire inhibitor structure along with the adventitious contacts is thus important in an enzyme inhibitor interaction (Komiyama *et al.*, 2003). In the present study, several folds less $IC_{50}$ values were depicted for the cleaved inhibitors than the theoretical $IC_{50}$ value expected for the uncleaved intact inhibitors. This could be because of cumulative effect of various factors which include cocktail of various

cleaved and uncleaved forms of PIs, variation in the combinations of the repeat domains in the cleaved form and sequence variation in each repeat domain leading to a pool of various specificities of protease inhibition. Simultaneous binding of multiple domain inhibitors to various proteinases and variation in the orientation of multiple domains with respect to each other are additional factors, which can modify the specificities of the inhibitors. The crystal structure of the tomato TI-II in complex with two molecules of su*bt*ilisin as well as unbound tomato TI-II reported earlier indicated the conformational variation of the repeat domains as a need of the inhibitor to balance between tight binding to the proteinase enzymes and broader specificity of the inhibitory function (Barrette-Ng *et al.*, 2003a, b).

### *4.3 Recombinant CanPIs retard growth and development of H. armigera*

*H. armigera*, a polyphagous pest, is known to express several different types of serine proteinases (Patankar *et al.*, 2001) and has an ability to modify the gut complement on exposure to different dietary components (Gatehouse *et al.*, 1997; Chougule *et al.*, 2005). In the present study, all the four types of CanPIs could efficiently inhibit (70%) HGP activity of larvae fed on chickpea and pigeonpea. It is interesting to note that the CanPI-7 and CanPI-9 with 4 IRD having both trypsin and chymotrypsin inhibitory sites could inhibit HGP activities better than the 3 IRD forms (CanPI-3 and CanPI-5) which have only trypsin inhibitory active sites. WB-HGPs isolated from insects fed on artificial diet containing non-host (winged bean) seed extract are likely to contain novel induced proteinases (Patankar *et al.*, 2001) and are also inhibited by CanPIs.

PIs in general (Johnston *et al.*, 1993) and Pin-II type PIs in particular play an important role in plant defense. However, the natural host PI may be ineffective or moderately effective to resist pest infestation due to a co-evolutionary interaction between the two (Broadway, 1996, Jongsma *et al.*, 1995a; Giri *et al.*, 1998), although if expressed in an appropriate proportion, can confer resistance against insect pests (Zavala *et al.*, 2004b; Srinavasan *et al.*, 2005). PIs from several non-host species have been tested to retard *H. armigera* growth and development (Harsulkar *et al.*, 1999), such as PIs from winged bean (Giri *et al.*, 2003) and bittergourd (Telang *et al.*, 2003). *C. annuum*, which was formerly a non-preferred host of *H. armigera*, is presently acquiring a pest status by causing field losses. Even then, the *C. annuum* PIs tested in the present study are effective to control *H. armigera* at the moderate concentration of

the PIs tested. Though the effect of recombinant CanPIs was tested starting from the late 2nd instar onwards, the PIs were observed to be effective growth retardants. Feeding of the larvae on CanPI inhibitor diets showed 30% mortality while the survived showed up to 40% reduction in larval weights in the early instars (3rd and 4th). This reduction in larval weight is further reflected in reduced pupal weight by 12%-25%, which ultimately results in decreased fecundity (De Leo and Gallerani, 2002; Zavala *et al.*, 2004b; Telang *et al.*, 2002; Damale *et al.*, 2005).

Alterations in the expression levels of endogenous PIs (Pin-II type) of *Nicotiana attenuata* have been demonstrated to proportionately affect the growth of the pest *Manduca sexta* and *Tupiocoris notatus* (Zavala *et al.*, 2004b). The insect can overcome the effect of the PI, if its amount in the diet is less than the sensitivity threshold of the insect, and vice versa. If the amount is higher than sensitivity threshold, the inhibitor proves to be effective (Johnston *et al.*, 1993; Wu *et al.*, 1997). In the chapter 2 efficacy of purified *C. annuum* leaf PIs (CapA1 and CapA2) was analysed at 0.5X, 1X, 3X and 6X inhibitor concentrations, which showed that even the lowest PI levels affected fertility and fecundity of *H. armigera* and revealed a dose dependent effect on reduction of larval weight, fecundity and fertility of *H. armigera*. Based on this observation, 3X concentration of CanPIs was used for the insect bioassays in the present study.

Lepidopteran pests react to the ingested PIs by i) over-expressing the inhibited proteinases ii) by producing inhibitor insensitive or inhibitor degrading proteinases and there by overcoming the effects of the ingested PIs (Broadway 1996, Bown *et al.*, 1997). In case of *H. armigera* cultures fed with TI/CI, trypsin and chymotrypsin specific enzymes were shown to be down regulated; where as other classes of proteases were overexpressed (Gatehouse *et al.*, 1997). In the present study the surviving larvae may have adapted to such mechanisms as they have both TI/CI in their CanPI inhibitor diets. But in more than 30% larvae tested the shift to other probable mechanisms for adaptation proved to be lethal at the amount of the inhibitor fed. Similar observations of increase in the mortality rate of herbivores feeding on PI transgenics as compared to control have been made. The mortality was even more when neonates were exposed to the inhibitor in the diet (McManus and Burguess, 1995; Heath *et al.*, 1997; Charity *et al.*, 1999; De Leo and Gallarani, 2002; Zavala *et al.*, 2004b). Based on these studies, the *CanPIs* can be considered as potential candidates to develop and test transgenic plants for improved tolerance to *H.*

*armigera*. To the best of our knowledge this is the first report where 4 repeat Pin-II type inhibitor genes have been cloned and characterized from *C. annuum.*

In summary, this chapter describes the cloning of selected *CanPI* genes in *Pichia pastoris* for recombinant PI protein expression. The expressed proteins were characterized for their biochemical properties and inhibitory potential of standard enzymes as well as HGPs. The chapter further describes the, in vivo effect of the CanPI proteins on *H. armigera* growth and development as studied by performing the feeding bioassays.

# Chapter 5

## General discussion and future directions

### *5.1 Unique features of C. annuum PIs*

The study of host-pest interactions has become very important as revealed by new discoveries of host resistance and pest adaptations growing at a fast rate. They have co-evolved for the past millions of years and have developed unique strategies to overcome each other's offence/resistance mechanisms and survived. Of the several means of defense, plants have relied upon 'indigestion' as an important phenomenon for resisting insects. Plants express and store an array of constitutive and inducible phytochemicals that bring about indigestion in the insects, which deprives them of nutrients in turn affecting their growth and development. Inhibitors of digestive enzymes like proteinase inhibitors act by blocking gut proteinases and affecting protein metabolism thus causing indigestion in the insect gut. Although several PIs have been studied that show insect antibiosis, the search for novel PIs from different plant sources continues to contribute to the virtual repository of antagonistic/defensive phytochemicals.

*Capsicum annuum* (Solanaceae), which showed promising control on *Helicoverpa armigera* (Lepidoptera) in preliminary studies, was selected for screening and detailed investigation on its PI proteins and genes. Various tissues of *C. annuum* were studied for PI profiles and activity. It was noted that though fruit tissues had diverse forms of PI(s), the leaves had the highest PI activity. Leaf tissue was, therefore, selected and used to purify and characterize *C. annuum* PI isoforms. By a combination of ion exchange chromatography and gel filtration, two forms of PIs, *viz*. CapA1 and A2 were successfully purified to relative homogeneity.

CapA1 and A2 exhibited molecular weights of around 12 KDa and had trypsin and chymotrypsin inhibitory activities. These two inhibitors also showed inhibition of gut proteinases of *H. armigera* in crude and fractionated forms during solution assays. Large-scale purification of CapA1 and A2 was carried out for bioassays studies on *H. armigera*. Several dilutions (0.5X, 1X, 3X and 6X inhibitor concentrations) of CapA1, CapA2 and crude leaf extracts were incorporated in artificial diet of *H. armigera* and the bioassay was carried out on two successive generations of the

insect. The inhibitor fed insects showed not only reduction in larval and pupal weights but also dramatic reduction in fertility and fecundity in a dose dependent manner. The effects of CapA1 and CapA2 were even more pronounced in the second generation of *H. armigera* indicating the success of PIs to limit *H. armigera* population growth. This promising result of *C. annuum* PI proteins encouraged us to isolate the respective CanPI genes.

Two full-length *CanPI* cDNA sequences reported in the database were used to design oligo-nucleotide primer pairs for *CanPI* gene isolation. On RT-PCR using one of these primer pairs, four bands of sizes 800, 600, 450, and 300 bp were obtained. Predominance of 800 and 600 bp bands was noted in the fruit tissue while 450 and 300 bp bands were prominent only in the stem tissue. These amplicons were cloned and further sequence analysis revealed homology to members of Pin-II family of proteinase inhibitors from Solanaceae. The other genera of Solanaceae except *C. annuum* have been reported for the presence of Pin-II family PIs with characteristic features like a signal peptide followed by variable number (one to eight) of inhibitory repeat domains (IRD). In the present study several novel forms of *CanPIs* were discovered, *e.g.*, the 1, 2 and 4 IRD *CanPIs*, which were not reported earlier. Existence of internal variability in the *CanPIs* was a further striking observation. Most notably, all the 4 IRD type *CanPI* genes showed simultaneous presence and variability in expression across the different *C. annuum* tissues screened.

*C. annuum* plants naturally infested with aphids (*Myzus persicae*) and lepidopteron pest (*Spodoptera litura*) showed significantly high expression levels of *CanPIs* with specifically induced 4 IRD form, which is otherwise absent in uninfested leaf tissue. This not only indicated the involvement of *CanPIs* in plant defense but also specifically emphasized on the importance of 4 IRD *CanPIs* in insect resistance. The expression variability in *CanPIs* and their correlation with the tissue types, inducibility etc. provide an excellent example of temporal, spatial, qualitative and quantitative gene regulatory mechanism (s) operating in *C. annuum* plants.

The present study on *C. annuum* PIs has shed light on some novel aspects and raised several questions about the regulatory mechanisms operating in plants, especially those that come into significance during infestation. A growing body of evidence supports the view that multiple hormonal actions and cross talk between signaling molecules are responsible for realization of a particular response. The very high

diversity in CanPIs expressed simultaneously probably represents the ultimate result of the multi faceted cross talk.

The expression analysis suggested the role of 3 and 4 IRD CanPIs in insect resistance. Based on the sequence analysis results we selected most divergent forms of 3 and 4 IRD CanPIs for cloning into a yeast (*Pichia pastoris*) expression vector for recombinant expression of the PIs. Two 3- IRD forms namely, CanPI-3 and CanPI-5 and two 4- IRD forms namely, CanPI-7 and CanPI-9 were cloned into *P. pastoris* and recombinant PIs were obtained.

Fragmentation was noted in all the recombinant CanPIs which possibly resulted due to cleavage by the extra cellular proteases secreted by *P. pastoris*. The cleaved rCanPIs showed multiple activity bands indicating that the proteases had acted at the linker regions thus liberating active PI fragments of 1-, 2-, and 3- IRDs along with intact uncleaved 3- and 4- IRD CanPI molecules. This cleavage was effectively prevented in an unbuffered medium and intact forms of CanPIs were procured. The deduced amino acid sequences of CanPIs showed presence of all trypsin inhibitory sites in the 3- IRD CanPIs and both, trypsin and chymotrypsin inhibitory sites in 4 IRD CanPIs. The trypsin and chymotrypsin inhibition by rCanPIs was studied by *in vitro* assays and it co-related with the number and type of active sites.

All the four rCanPIs inhibited *H. armigera* gut proteinases during *in vitro* assays. Appropriate proportion of CanPIs was incorporated in artificial diet individually and feeding bioassays of *H. armigera* were performed. All the recombinant-expressed CanPIs exerted anti-metabolic effects on *H. armigera* growth and development, which also led to a significant level of insect-mortality. It appears that presence of multiple isoforms/variants of TI and CI IRDs in this 4 IRD member must have contributed to its high anti-insect character. Alterations in local and/or global structures in the variedly cleaved rCanPI isoforms may contribute to changes in binding and inhibition specificities of the PI. Of all the four CanPIs, CanPI-7, which has most diverse IRD combination, turned out to be the best candidate for retarding *H. armigera*. The PIs from other plants in general and Solanaceae specifically need to be analysed for presence of such diversity, if it does exist, in order to bestow the functional diversity to the unique observation of high diversity in *C. annuum* PIs.

### *5.2 Diversity: basis of evolution*

Diversity is the basis of life. All organisms in the process of evolution show variation amongst their populations; further accumulation of these variations followed by natural selection finally leads to speciation. Thus, presence of variation/diversity is of prime importance since it forms the platform on which the force of natural selection acts by giving way to the survival of ecologically best-suited phenotype/genotype as per the principle of `survival of the fittest'

Evolution is also guided by interactions of the living organisms with biotic and abiotic components of the ecosystem. The biotic interactions result in complicated co-evolutionary phenomena, because in this case the adaptations are mutual. Plant-insect interactions represent a very ancient relationship, which has existed from even before the Carboniferous era and has mutually affected evolution, speciation and spread of each other. These taxa have co-evolved initially in aquatic environments and later in terrestrial habitats too. Ancient, interdependent relationships between these two taxa, led to evolution of molecular defense mechanisms in plants, whereas insects evolved by developing adaptive or alternative strategies to overcome host defense. Thus the ecological studies in the plant-insect pest interactions elucidate these basic natural mechanisms and also provide insights in designing plant defense for present agricultural situations. Studies by Prof. Ian Baldwin's group (Max Plank Institute of Chemical Ecology, Jena, Germany), using *Nicotiana attenuata* and its natural pest *Manduca sexta* have revealed several novel aspects of the plants defense strategies using volatiles, secondary metabolites, PIs etc. which are strictly regulated (Xu *et al.*, 2006; Kessler *et al.*, 2004; Steppuhn *et al.*, 2004 etc.). Due to the presence of wound/insect induced defense responses in Solanaceae, this plant family provides a very good system to study insect pest-plant interactions.

### *5.3 Why so much of PI gene diversity exists in C. annuum?*

Solanaceae members show high diversity in copy number of PI genes eg. *Nicotiana* species shows single or rarely two genomic copies of Pin-II type PIs, *Solanum nigrum* and *Solanum americium* show two copies of different Pin-II PIs (Zavala *et al.*, 2004a; Xu *et al.*, 2001) while *Capsicum annuum* shows a small multigene Pin-II family of PIs in its diploid genome (Shin *et al.*, 2001; Kim *et al.*, 2001).

Pin-II genes in Solanaceae are important due to their evolution into multiple inhibitory repeat types from the ancestral single repeat Pin-II PI precursor (Barta *et al.*, 2002). *Solanum* species express two repeat Pin-II PIs, tomato posses both two and three repeat Pin-II PIs, while different species of *Nicotiana* show expression of Pin-II PIs of two or more repeats and some species express even 6 and 8 repeat Pin-II PIs (Xu *et al.*, 2001; Wu *et al.*, 2006; Kim *et al.*, 2001). Pin-II PIs with higher IRDs have not been found in tomato and potato. *C. annuum* on the other hand shows presence of 1 to 4 repeat Pin-II PIs as revealed from the present study. Furthermore, significantly high diversity in precursor Pin-II PIs, giving rise to many su*bt*ypes of each type of precursor is particularly found only in *C. annuum*. Other species of Solanaceae have not shown such a high diversity in the precursor su*bt*ypes except for few representatives like SaPin-II a and b from *S. americanum*, where in both are two repeat Pin-II precursors but show more than 20% sequence divergence (Xu *et al.*, 2001). The co-relation of phylogeny of various genera in Solanaceae with the diversity of Pin-II type PIs may provide insights into evolutionary mechanisms acting in the family.

In the present study we aimed to clone the CanPI genes that were already reported in the database. However, our attempts led to the discovery of a series of varied Pin-II precursors, which not only showed diverse types of number of repeats per gene but also depicted further variation within each su*bt*ype. Initial assumption of these variations to be cloning artifacts was ruled out when, sequence analysis displayed that the newly identified PIs, like other Pin-II PIs, showed highly conserved repeat elements in inhibitors (such as presence of eight cysteines per repeat) although variations were observed at regions associated with proteinase –binding and -inhibition; the variation at activity-related residues are not expected to have any impact on the global structure of the repeat domain. It was further noted that, various combinations of IRDs were present in the precursor molecules and several unique IRD sequences were also discovered. Therefore, the possibility of 1- and 2- IRD type precursor PIs getting amplified from the 3- or 4- IRD type precursors was ruled out. Amplification of all IRD type CanPI forms with the N terminal ~25 AA signal sequence and a 'C' terminal stop codon further strengthened the presence of diverse full length CanPI genes in *C. annuum*, indicating that activity isoforms of CanPIs arise not only from post-translational modifications, but also due to presence of

multiple gene-copies. Of course, confirmation of copy number of these CanPIs by Southern analysis and the transcriptional confirmation by Northern analysis would serve to validate this hypothesis.

This extraordinarily high gene diversity in Pin-II PIs (CanPIs) has raised many questions; to list a few; (i) why this high diversity is found only in *C. annuum*? (ii) or conversely why has it not yet been detected in other members of Solanaceae? Probable clues to these questions may lie specifically in the evolutionary divergence of *C. annuum* as compared to other members of Solanaceae.

### *5.4 How is the CanPI gene diversity regulated?*

Cloning and sequence analysis of *CanPIs* from fruit and stem tissue of *C. annuum* appeared to indicate that the expression of highly diverse *CanPI* precursor sequences (1 to 4 IRD type and their subtypes) was not random; all the *CanPI* forms were not ubiquitously found in all tissue types but, rather, their expression was regulated spatially at steady state levels and also by inducers like wounding and biotic stresses. The regulatory mechanisms in *C. annuum* acted on the PI expression for induction of specific IRD types in different tissues and stresses, respectively and further extended to the proportion of different CanPI precursors of an IRD type. Interestingly, the 3 and 4 IRD precursors of stem did not show presence of subtypes as detected in the 3 and 4 IRD precursors of fruits. It is quite likely that further diverse forms of CanPI precursors might be observed in different tissues under different physiological conditions. Biotic stress to *C. annuum* in the form of naturally occurring and long-term insect infestation showed induction of CanPIs of higher IRD type, which were otherwise not expressed at steady state levels. All these observations undoubtedly show the involvement of CanPIs in tissue-specific endogenous physiological functions and also in induced defense against insects. However, many important questions related to the physiological role of CanPIs arise, such as, (i) when the multiple IRD PIs are advantageous and can serve similarly after precursor cleavage, why does the plant maintain so many smaller precursor forms? (ii) why are one and two IRD CanPIs diverse in the stem tissues?, (iii) why do the three and four IRD CanPIs show high subtype diversity and variability in expression during stages of fruit development?, and perhaps most importantly, (iv) what is the mechanism by which *C.*

*annuum* brings about the co-ordinated regulation of CanPI expression for endogenous and induced/defense functions?

### *5.5 CanPin-II PI gene diversity further amplified in* Pin-II *proteins*

It would be important and interesting to understand the cumulative and ultimate effect of plants regulatory mechanisms governing the expression of the CanPI genes on their translational products. In an attempt towards elucidating this, we developed individual recombinant CanPIs and studied their biochemical properties. It led to discovery of further complexity in the protein products of these genes. Multiple, active inhibitory fragments were o*b*tained from the single, three and four IRD precursors, various combinations of which drastically changed (increased) the overall inhibition specificities of the precursors. The type of PI protein fragmentation o*b*tained in the present study as detailed in Chapter 4, is probably very similar to the one that occurs *in planta*, by action of plant proteinases on the linker regions (Heath *et al.,* 1995, Horn *et al.,* 2005). It has been demonstrated that the action of endogenous plant proteinases, on the precursors, generated IRDs with ragged ends, which, in turn, generated variability for defense functions (Horn *et al.,* 2005).

From all the reported Pin-II PIs a total of 77 different IRDs have been reported (Barta *et al.,* 2002). The diversity in unique IRDs from *C. annuum* that are expressed simultaneously is very high and contributes to a diverse inhibitor cocktail in the plant. Interestingly, two single IRD PIs expressed simultaneously from *C. annuum* (PS I 1.1 and PS I 1.2) represented polypeptides that were circularly permuted with respect to each other, and both showed varied inhibitory activities (Antcheva *et al.,* 2001) towards proteinases. One form was considered as the ancestral type (PSI 1.2) and the other (PSI 1.1) as an IRD formed from domain swapped Pin-II precursor CanPI. This suggests existence and expression of both the precursor types in *C. annuum* although the gene structure for domain swapped CanPI has not been observed so far. In our studies we probably identified the ancient type PI precursors but not the domain swapped CanPI precursor genes.

It will be interesting to find out how much of the diversity in CanPI mRNAs actually translates into CanPI proteins *in situ*. But it is certain that in the translated CanPI protein form(s) the diversity will be many folds higher than that observed in the mRNA. Unlike mRNAs, the CanPI proteins are exposed to varying levels of

endogenous proteinases, which act on the linker sequence connecting the IRDs to release active CanPI fragments with variable number of IRDs. Present experiments with recombinant cleaved CanPIs demonstrated non-stoichiometric inhibition of trypsin and chymotrypsin. This suggested the cumulative contribution of variation in IRDs, linker sequences and structures of individual CanPI fragments, in modifying their inhibition specificities. In many Solanaceous plants such as *Nicotiana* 4 and 6 IRD PIs (Miller, 2000) the Pin-II PIs amounts in precursor form of protein are less than their cleaved single domain counterparts. Moreover, in *C. annuum* leaves single and two domain PI proteins have been detected (present study and Antcheva, *et al.*, 2001). Presently, it is difficult to explain the requirement of such high diversity of CanPIs at steady state for various endogenous proteinase regulatory activities as against the plants machinery required to maintain this diversity, except for the significance of such diverse inhibitor cocktails for defense purposes.

It has been reported that the expression levels of the precursor PIs as well as the plant proteinases increase due to insect attack, the ultimate effect of which directed towards making a diverse inhibitor cocktail best suited for the infestation (Horn *et al.*, 2004).

In the present study, Pin-II PIs have IRDs for both trypsin and chymotrypsin inhibition and the variations close to the reactive site loop are responsible for changed/modified specificities of proteinase inhibition. The consistent anti-metabolic effect of CanPI-7 on *H. armigera* has demonstrated the importance of simultaneous action of varied activity domains as in CanPI-7, in affecting the insect metabolism severely. It has been suggested that, although not such a high diversity as in our case, the diversity within cyclotides represents a significant potential resource for the development of environment friendly crop protection agents that might avoid the development of resistance by insects (Craik *et al.*, 2004).

### *5.6 CanPIs in insect resistant transgenic plants - will they really work?*

PI based approaches for insect control has its own pros and cons however, large-scale field experimentation of PI transgenics has not been performed and reported, thus questioning the applicability of PIs for insect pest control. Laboratory level experiments using PIs of different types for *in vitro* and *in vivo* assays have shown PIs to be effective growth retardants. Though a killing (wipe out) effect brought about by toxins, is rarely shown by PIs, they do effectively impede insect growth and

development, thus affecting the population dynamics of subsequent insect generations. The effects of PIs on insects are very much dependent on the amount of PI ingested by the insect. To exert maximum effect on the insect, the PI amounts must be higher than the threshold values (Johnston *et al.,* 1993; Wu *et al.,* 1997). For the success of PI transgenics in insect defense, it is, therefore, necessary that the plants express the PIs at the effective levels. However, it has been observed in several studies that in an attempt to express high PI levels in transgenic plants, productivity and other characters, which may be of agronomic importance in crop plants, are severely affected, though the plants become tolerant to insects (Zavala *et al.,* 2004a, b; Glawe *et al.,* 2003). Thus, the field application of PI transgenic may suffer due to this very important drawback not present in *Bt* transgenics.

Our present study on *C. annuum* PI diversity and expression sheds light on the steady state levels of the CanPI expression. Even in absence of any inducers, diverse and high amounts of CanPIs are expressed leading to their probable involvement in important endogenous function *in planta*. Recent reports on Pin-II PIs of *Solanum americanum* (SaPin-II a, b) have shown them to be integral proteins in regulating endogenous plant serine proteinases (Xu *et al.,* 2001; Xu and Chye, 2004; Sin *et al.,* 2004, 2006; Liu *et al.,* 2006). Based on this it could be speculated that the plant has a machinery to regulate the expression of PIs as per the requirement of both, endogenous and defense, functions thus exerting minimum effects, if any, on plant productivity. Future research in elucidating these mechanisms and their application in PI transgenics would probably increase their acceptability. In this regards it is advantageous to make use of tissue specific/induction via specific promoters for PI expression. This would reduce the burden of constitutive PI expression and will in turn avoid effect on crop productivity.

Growing human population has put tremendous stress on agriculture and to match the needs more and more landmasses have been cultivated. With the intensive agricultural practices on a rise the natural / wild plant varieties are becoming rare and natural forces controlling insect populations are getting abolished. Thus the present agricultural scenario is very unnatural and has converted insects into pests (Lewis *et al.,* 1997). The practice of "mono-culture" has led to large availability of host crop(s), which in turn causes a localized abundance in the insect population. Insects thrive because of readily available food-source in large amounts; they compete with

us for this food source, hence we consider them pests. These plants have been a food source for the insects for a long time, but natural sparse (uncultivated) populations of these plants may have limited insect populations. Intensive farming just provides more food source to the insects too! So humans may be partially responsible for this increase in insect population.

Probably due to this reasons the plant's natural modes of defense are falling back and are proving to be ineffective in agricultural fields, though their importance in natural system is undou*bt*edly accepted (Chen *et al.*, 2005). Under the present unnatural expanse of agriculture, probably toxins would play a major role to control pests. Gene pyramiding using a toxin in combination with another defense protein would prove to be effective. With the success of *Bt* technology, other toxins also have bright chances in insect resistance transgenic technology. There are repor ts of using combination of different defense molecule simultaneously in transgenic plants (as summarized in Christou *et al.*, 2006). Use of toxin from spider venom, vegetative inducible proteins and lectins like GNA, which recognize target receptors in insect gut are potential candidate toxins for use in crop protection against insect pests.

One of the plants' natural defense mechanism relies on causing indigestion in the infesting insects (Felton, 2005). Several phytochemicals other than PIs also act on insect and cause a similar 'indigestion' effect. So in a long lasting co-evolutionary interaction between the two, plants appear have depended on and developed different strategies to bring about 'indigestion' mediated defense. Thus the choice of PIs for insect defense is certainly a long lasting and sustainable approach of plant defense, if proper expression is ensured. Due to a high diversity in CanPI IRDs it is possible to identify combinations of IRDs, which are best suited for a particular insect infesta tion. CanPI-7 with all the varied IRDs and with specificity to both trypsin and chymotrypsin showed the highest effect on *H. armigera* inhibition during *in vivo* assay in the present study. Lack of natural diversity in some PIs has led researchers to induce the variations artificially using tools like phage display (Jongsma *et al.*, 1995b). As per our judgment many CanPI genes are still waiting to be discovered from *C. annuum*, which will lead to increase in the number of unique IRDs. Synthetic constructs could be designed by selecting and combining novel IRDs and increasing the precursor PI size by fusing selected IRDs to achieve maximum plant protection against insects. The naturally occurring gene diversity in CanPIs provides a very

effective / elaborate starting material to select the best insect defensive combinations as provided by the natural/wild plant populations in previous times, which could be further modified using modern genetic engineering tools ultimately trying to reach to the goal of crop protection through productive, sustainable, but environmentally friendly insect resistant strategies.

# Bibliography

## List of references cited in the thesis


Abdeen A, Virgos A, Olivella E, Villanueva J, Avile´s X, Gabarra R, Prat S (2005) Multiple insect resistance in transgenic tomato plants over-expressing two families of plant proteinase inhibitors. Plant Molecular Biology 57: 189–202.

Agrawal AA (1998) Induced responses to herbivory and increased plant performance. Science 279: 1201-1202.

Ahmad R, Rai AB (2005) 25 Years of Research on *Helicoverpa* at IIPR. Indian Institute of Pulse Research, Kanpur, India.

Alborn HT, Turlings TCJ, Jones TH, Stenhagen G, Loughrin JH, Tumlinson JH (1997) An elicitor of plant volatiles from beet armyworm oral secretions. Science 267: 945-949.

Antcheva N, Patthy A, Athanasiadis A, Tchorbanov B, Zakhariev S, Pongor S (1996) Primary structure and specificity of serine proteinase inhibitor from paprika (*Capsicum annum*) seeds. Biochimica et Biophysica Acta 1298: 95-101.

Antcheva N, Pintar A, Patthay A, Simoncsits A, Barta E, Tchorbanov B, Pongor S (2001) Proteins of circularly permuted sequence present within the same organism: The major serine proteinase inhibitor from *Capsicum annum* seeds. Protein Science 10: 2280-2290.

Applebaum SW (1985) Biochemistry of digestion. In Comprehensive Insect Physiology, Biochemistry and Pharmacology, ed. G. A. Kerkut, L. I., Gilbert, 4:279-311. New York: Pergamon.

Armes NJ, Jadhav DR, DeSouza KR (1996) A survey of insecticide resistance in *Helicoverpa armigera* in the Indian subcontinent. Bulletin Entomological Research 86: 499-514.

Atkinson AH, Heath RL, Simpson RJ, Clarke AE, Anderson MA (1993) Proteinase inhibitors in *Nicotiana alata* stigmas are derived from a precursor protein, which is processed into five homologous inhibitors. Plant Cell 5: 203-213.

Avisar D, Segal M, Sneh B, Zilberstein A (2005) Cell-cycle-dependent resistance to *Bacillus thuringiensis* Cry1C toxin in Sf9 cells. Journal of Cell Science 118: 3163-3171.

Babu RM, Sajeena A, Seetharaman K, Reddy MS (2003) Advances in genetically engineered (transgenic) plants in pest management–an overview. Crop Protection 22: 1071-1086.

Balandin T, van der Does C, Albert JM, Bol JF, Linthorst HJ (1995) Structure and induction pattern of a novel proteinase inhibitor class II gene of tobacco. Plant Molecular Biology 27: 1197–1204.

Barrette-Ng IH, Kenneth KSN., Cherney MM, Pearce G, Ghani U, Ryan CA, James MNG (2003a) Unbound form of tomato inhibitor-II reveals inter-domain flexibility and conformational variability in the reactive site loops. Journal of Biological Chemistry 278: 31391-31400.

Barrette-Ng IH, Kenneth KSN, Cherney MM, Pearce G, Ryan CA, James MNG (2003b). Structural basis of inhibition revealed by 1:2 complex of the two headed tomato inhibitor-II and subtilisin Carlsberg. Journal of Biological Chemistry 278: 24062-24071.

Barta E, Pintar A, Pongor S. (2002) Repeats with variations: accelerated evolution of the Pin2 family of proteinase inhibitors. Trends Genetics 18: 600-603.

Barton KA, Whiteley HR, Yang NS (1987) *Bacillus thuringiensis* delta-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. Plant Physiology 85: 1103-1109.

Bates SL, Zhao JZ, Roush RT, Shelton AM (2005) Insect resistance management in GM crops: present and future. Nature Biotechnology 23: 57-62.

Beekwilder J, Schipper B, Bakker P, Bosch D, Jongsma M (2000) Characterisation of potato proteinase inhibitor II reactive site mutants. European Journal of Biochemistry 267: 1975-1984.

Boulter D (1993) Insect pest control by copying nature using genetically engineered crops. Phytochemistry 34: 1453-1466.

Bown DP, Wilkinson HS, Gatehouse JA (1997) Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families. Insect Biochemistry and Molecular Biology 27: 625-638.

Bown DP, Wilkinson HS, Gatehouse JA (1998) Midgut carboxypeptidase from *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae: enzyme characterisation, cDNA cloning and expression. Insect Biochemistry and Molecular Biology 28: 739–749.

Bown DP, Wilkinson HS, Gatehouse JA (2004) Regulation of expression of genes encoding digestive proteases in the gut of a polyphagous lepidopteran larva in response to dietary protease inhibitors. Physiological Entomology 29:278–290.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Broadway RM (1996) Plant dietary proteinase inhibitors alter complement of midgut proteases. Archives of Insect Biochemistry and Physiology 32: 39–53.

Broadway RM (1997) Dietary regulation of serine proteinases those are resistant to serine proteinase inhibitors. Journal of Insect Physiology 43: 855–874.

Broadway RM, Duffey SS (1986a) The effect of dietary protein on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. Journal of Insect Physiology 32: 673-680.

Broadway RM, Duffey SS (1986b) Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. Journal of Insect Physiology 32: 827–833.

Brock RM., Forsberg CW, Buchanan-Smith JG (1982) Proteolytic activity of rumen microorganisms and effect of proteinase inhibitors. Applied Environmental Microbiology 44- 561–569.

Brousseau R, Luke M, Dwayne H (1999) Insecticidal transgenic plants: are they irresistible? AgBiotechNet 1 ABN 022:1-9

Bryant J, Green TR, Gurusaddaiah T, Ryan CA (1976) Proteinase inhibitor II from potatoes: isolation and characterization of its promoter components. Biochemistry 15: 3418–3424.

Carlini CR, Grossi-de-Sa MF (2002) Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. Toxicon 40: 1515-1539.

Carriere Y, Ellers-Kirk C, Sisterson M, Antilla L, Whitlow M, Dennehy TJ, Tabashnik DE (2003) Long-term regional suppression of pink bollworm by *Bacillus thuringiensis* cotton. Proceedings of the National Academy of Sciences, USA 100: 1519-1523.

Charity JA, Anderson MA, Bittisnich DJ, Whitecross M Higgins TJV (1999) Transgenic tobacco and peas expressing a proteinase inhibitor from *Nicotiana alata* have increased insect resistance. Molecular Breeding 5: 357-365.

Chen H, Wilkerson CG, Kuchar JA, Phinney BS, Howe GA (2005) Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut Proceedings of the National Academy of Sciences, USA 102:19237-19242.

Chen J, Yu J, Tang L, Tang M, Shi Y, Pang Y (2003) Comparison of the expression of *Bacillus thuringiensis* full-length and N-terminally truncated *vip3A* gene in *Escherichia coli*. Journal of Applied Microbiology 95: 310–316.

Choi D, Park J, Seo YS, Chun YJ, Kim WT (2000) Structure and stress-related expression of two cDNAs encoding proteinase inhibitor II of *Nicotiana glutinosa*. Biochimica et Biophysica Acta 1492: 211–215.

Choi Y, Moon Y, Lee JS (1990) Primary structure of two proteinase inhibitor II genes closely linked in the potato genome. Korean Journal of Biochemistry 23: 214–220.

Chougule NP, Giri AP, Sainani MN, Gupta VS (2005) Gene expression patterns of *Helicoverpa armigera* gut proteases. Insect Biochemistry and Molecular Biology 35: 355-367.

Chougule NP, Hivrale VK, Chhabda PJ, Giri AP, Kachole MS (2003) Differential inhibition of *Helicoverpa armigera* gut proteinases by proteinase inhibitors of pigeonpea (*Cajanus cajan*) and its wild relatives. Phytochemistry 64: 681-687.

Christeller J, Laing W (2005) Plant serine proteinase inhibitors. Protein and Peptide Letters 12: 1-9.

Christeller JT (2005) Evolutionary mechanisms acting upon proteinase inhibitor variability. FEBS Journal 272 : 5710–5722

Christeller JT, Burgess EP, Mett V, Gatehouse HS, Markwic k NP, Murray C, Malone LA, Wright MA, Philip BA, Watt D, Gatehouse LN, Lovei GL, Shannon AL, Phung MM, Watson LM, Laing WA (2002) The expression of a mammalian proteinase inhibitor, bovine spleen trypsin inhibitor in tobacco and its effects on *Helicoverpa armigera* larvae. Transgenic Research 11 (2): 161-173.

Christou P, Capell T, Kohli A, Gatehouse JA, Gatehouse AMR (2006) Recent developments and future prospects in insect pest control in transgenic crops. Trends in Plant Science 11: 302-308.

Chye ML, Sin SF, Xu ZF, Yeung C (2006) Serine proteinase inhibitor proteins: exogenous and endogenous functions. In Vitro Cell Developmental Biology Plant 42: 100–108.

Constabel CP, Yip L, Ryan CA (1998) Prosystemin from potato, black nightshade, and bell pepper: primary structure and biological activity of predicted systemin polypeptides. Plant Molecular Biology 36: 55-62.

Craik DJ, Daly NL, Mulvenna J, Plan MR, Trabi M (2004) Discovery, structure and biological activities of the cyclotides. Current Protein and Peptide Science 5: 297-315.

Damle MS, Giri AP, Sainani MN, Gupta VS (2005) 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). Phytochemistry 66: 2659-2667.

Davey M, Duve H, Thorpe A, East P (2005) Helicostatins: brain-gut peptides of the moth, *Helicoverpa armigera* (Lepidoptera: Noctuidae). Archives of Insect Biochemistry and Physiology 58: 1-16.

Davis BJ (1964) Disc electrophoresis: II. Methods and application to human serum. Ann. N.Y. Acad. Sci. 121: 404– 429.

de Ilarduya OM, Xie QG, Kaloshian I (2003) Aphid-induced defense responses in Mi-1-mediated compatible and incompatible tomato interactions. Molecular Plant-Microbe Interactions 16: 699-708.

De Leo F, Bonade-Bottino M, Ceci LR, Gallerani R, Jouanin L (2001) Effects of a mustard trypsin inhibitor expressed in different plants on three lepidopteran pests. Insect Biochemistry and Molecular Biology 31: 593-602.

De Leo F, Gallerani R (2002) The mustard trypsin inhibitor 2 affects the fertility of *Spodoptera littoralis* larvae fed on transgenic plants. Insect Biochemistry and Molecular Biology 32: 489-496.

Divol F, Vilaine F, Thibivilliers S, Amselem J, Palauqui J, Kusiak C, Dinant S (2005) Systemic response to aphid infestation by *Myzus persicae* in the phloem of *Apium graveolens*. Plant Molecular Biology 57: 517–540.

Dixon AFG (1998) Aphid Ecology: An optimization approach, Ed 2. Chapman and Hall, New York.

Down RE, Gatehouse AMR, Hamilton WDO, Gatehouse JA (1996) Snowdrop lectin inhibits development and decreases fecundity of the glasshouse potato aphid (*Aulacorthum solani*) when administered *in vitro* and via transgenic plants both in laboratory and glasshouse trials. Journal of Insect Physiology 42: 1035-1045.

Duan X, Li X, Xue Q, Abo el Saad M, Xu D, Wu R (1996) Transgenic rice plants harbouring an introduced potato proteinase inhibitor II gene are insect resistant. Nature Biotechnology 14: 494-498.

Eddy JL, Derr JE, Hass GM (1980) Chymotrypsin inhibitor from potatoes: interaction with target enzymes. Phytochemistry 19: 757-761.

Elzinga RJ (1978) Fundamentals of entomology. Prentice Hall of India Pvt Ltd., New Delhi.

Felton GW (2005) Indigestion is a plant's best defense. Proceedings of the National Academy of Sciences, USA 102: 18771–18772.

Ferry N, Edwards MG, Gatehouse J, Capell T, Christou P, Gatehouse AMR (2006) Transgenic plants for insect pest control: a forward looking scientific perspective. Transgenic Research 15:13–19.

Fidantsef AL, Stout MJ, Thaler JS, Duffey SS, Bostock RM (1999) Signal interactions in pathogen and insect attack: expression of lipoxygenase, proteinase inhibitor II, and pathogenesis-related protein P4 in the tomato,

*Lycopersicon esculentum*. Physiological and Molecular Plant Pathology 54: 97-114.

Fitches E, Audsley N, Gatehouse JA, Edwards JP (2002) Fusion proteins containing neuropeptides as novel insect control agents: snowdrop lectin delivers fused allatostatin to insect haemolymph following oral ingestion. Insect Biochemistry and Molecular Biology 32: 1653-1661.

Fitches E, Edwards MG, Mee C, Grishin E, Gatehouse AMR, Edwards JP, Gatehouse JA (2004) Fusion proteins containing insect-specific toxins as pest control agents: snowdrop lectin delivers fused insecticidal spider venom toxin to insect haemolymph following oral ingestion. Journal of Insect Physiology 50: 61-71.

Fitches E, Gatehouse AMR, Gatehouse JA (1997) Effects of snowdrop lectin (GNA) delivered via artificial diet and transgenic plants on the development of tomato moth (*Lacanobia oleracea* ) larvae in laboratory and glasshouse trials. Journal of Insect Physiology 43: 727-739.

Fitt GP (1989) The ecology of *Heliothis* in relation to agroecosystems. Annual Review of Entomology 34: 17-52.

Franco OL, Rigden DJ, Melo FR, Bloch C Jr, Silva CP, Grosside-Sa´ MF (2000) Activity of wheat alpha-amylase inhibitors towards bruchid alpha-amylases and structural explanation of observed specificities. European Journal of Biochemistry 267: 2166–2173.

Fritz H (2000) Foreword In: von der Helm, K., Korant BD, Cheronis JC eds. Proteases as targets for therapy. Berlin: Springer-Verlag: 5–6.

Frutos R, Rang C, Royer M (1999) Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. Critical Reviews in Biotechnology 19: 227–276.

Garcia-Olmedo F, Salcedo G, Sanchez-Monge R, Gomez L, Roys J, Carbonero P (1987) Plant proteinaceous inhibitors of proteases and amylases. Oxford Survey of Plant Molecular and Cellular Biology 4: 275–334.

Gatehouse AMR, Down RE, Powell KS, Sauvion N, Rahbe Y, Newell CA, Merryweather A, Hamilton WDO, Gatehouse JA (1996) Transgenic potato plants with enhanced resistance to the peach-potato aphid *Myzus persicae*. Entomologia et Experimentlis Applicata 79: 295-307.

Gatehouse AMR, Norton E, Davison GM, Babbe´ SM, Newell CA, Gatehouse JA (1999) Digestive proteolytic activity in larvae of tomato moth, *Lacanobia oleracea*, effects of plant protease inhibitors *in vitro* and *in vivo*. Journal of Insect Physiology 45: 545-558.

Gatehouse LN, Shannon AL, Burgess EPJ, Christeller JT (1997) Characterization of major midgut proteinase cDNAs from *Helicoverpa armigera* larvae and

changes in gene expression in response to four proteinase inhibitors in the diet. Insect Biochemistry and Molecular Biology 27: 929-944.

Giri AP, Chougule NP, Telang MA, Gupta VS (2005) Engineering insect tolerant plants using plant defensive proteinase inhibitors. In: Recent research developments in phytochemistry, (Pandalai, S. G., Ed) Research Signpost, India, 8: 117-137.

Giri AP, Harsulkar AM, Deshpande VV, Sainani MN, Gupta VS, Ranjekar PK (1998) Chickpea defensive proteinase inhibitors can be inactivated by podborer gut proteinases. Plant Physiology 116: 393-401.

Giri AP, Harsulkar AM, Ku MSB, Deshpande VV, Gupta VS, Ranjekar PK, Franceschi VR (2003) Identification of potent inhibitors of *Helicoverpa armigera* gut proteinase in winged bean seeds. Phytochemistry 63: 523-532.

Giri AP, Kachole MS (1998) Amylase inhibitors of pigeon pea (*Cajanus cajan* L.) seeds. Phytochemistry 47: 197-202.

Glawe AG, Zavala JA, Kessler A, Van Dam NM, Baldwin IT (2003) Ecological costs and benefits correlated with trypsin protease inhibitor production in *Nicotiana attenuata* . Ecology 84:79-90.

Gowda CLL, Sharma HC (2005) Legume pod borer/cotton bollworm, *Heliothis*/*Helicoverpa* - the global problem. National symposium on *Helicoverpa* management - a national challenge, Kanpur, India. pp 1-9.

Graham JS, Pearce G, Merryweather J, Titani K, Ericsson L, Ryan, CA (1985b) Wound-induced proteinase inhibitors from tomato leaves, II: The cDNA-deduced primary structure of pre-inhibitor II. Journal of Biological Chemistry 260: 6561-6564.

Green TR, Ryan CA (1972) Wound induced proteinase inhibitor in plant leaves: A possible defence mechanism. Science 175: 776-777.

Greenblatt H M, Ryan CA, James MNG (1989) Structure of the complex of *Streptomyces griseus* proteinase B and polypeptide chymotrypsin inhibitor-1 from Russet Burbank potato tubers at 2.1 Å resolution. Journal of Molecular Biology 205: 201-228.

Grossi De Sa MF, Chrispeels MJ (1997) Molecular cloning of bruchid (*Zabrotes subfasciatus*) a-amylase cDNA and interactions of the expressed enzyme with bean amylase inhibitors. Insect Biochemistry and Molecular Biology 27: 271-281.

Gruden K, Kuipers AGJ, Guncar G, Slapar N, Strukelj B, Jongsma MA (2004) Molecular basis of Colorado potato beetles adaptation to potato plant defense at the level of digestive cysteine proteinases. Insect Biochemistry and Molecular Biology 34: 365-375.

Gupta GP, Mahapatro GK, Chandra A (2000) Bio-potency of insecticidal crystal proteins of *Bacillus thuringiensis* against cotton (*Gossypium hirsutum*) bollworms. Indian Journal of Agricultural Sciences 70: 194-196.

Halitschke R, Gase K, Hui DQ, Schmidt DD, Baldwin IT (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. Plant Physiology 131: 1894–1902.

Halitschke R, Schittko U, Pohnert G, Boland W, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata* III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. Plant Physiology 125: 711-717.

Haq SK, Atif SM, Khan RH (2004) Protein proteinase inhibitor genes in combat against insects, pests and pathogens: natural and engineered phytoprotection. Archives of Biochemistry and Biophysics 431: 145-159.

Harshini S, Nachman RJ, Sreekumar S (2002a) Inhibition of digestive enzyme release by neuropeptides in larvae of *Opisina arenosella* (Lepidoptera: Cryptophasidae). Comparative Biochemistry and Physiology Part B 132: 353-358.

Harshini S, Nachman RJ, Sreekumar S (2002b) *In vitro* release of digestive enzymes by FMRF amide related neuropeptides and analogues in the lepidopteran insect *Opisina arenosella* (Walk.). Peptides 23: 1759-1763.

Harsulkar AM, Giri AP, Gupta VS, Sainani MN, Deshpande VV, Patankar AG, Ranjekar PK (1998) Characterization of *Helicoverpa armigera* gut proteinases and their interaction with proteinase inhibitors using gel -X-ray film contact print technique. Electrophoresis 19: 1397-1402.

Harsulkar AM, Giri AP, Patankar AG, Gupta VS, Sainani MN, Ranjekar PK Deshpande VV (1999) Successive use of non-host plant proteinase inhibitors required for effective inhibition of gut proteinases and larval growth of *Helicoverpa armigera*. Plant Physiology 121: 497-506.

Hass GM, Hermodson MA, Ryan CA, Gentry L (1982) Primary structures of two low molecular weight proteinase inhibitors from potatoes. Biochemistry 21: 752-756.

Hazzard RV, Schultz BB, Groden E, Ngollo ED, Seidlecki E (2003) Evaluation of oils and microbial pathogens for control of lepidopteran pests of sweet corn in New England. Journal of Econonomic Entomology 96: 1653-1661.

Heath RL, Barton PA, Simpson RJ, Reid GE, Lim G, Anderson MA (1995) Characterization of the protease processing sites in a multidomain proteinase inhibitor precursor from *Nicotiana alata*. European Journal of Biochemistry 230: 250–257.

Heath RL, McDonald G, Christeller, JT, Lee M, Bateman K, West J, Heeswijck VR, Anderson MA (1997) Proteinase inhibitors from *Nicotiana alata* enhance plant resistance to insect pest. Journal of Insect Physiology 43: 833-842.

Hermsmeier D, Schittko U, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*: III. Fatty acid-amino acids conjugates in herbivore oral secretion are necessary and sufficient for hebivore-specific plant responses. P lant Physiology 125: 683-700.

Hilder VA, Boulter D (1999) Genetic engineering of crop plants for insect resistance - a critical review. Crop Protection 18: 177-191.

Hilder VA, Gatehouse AMR, Boulter D (1993) Transgenic plants conferring insect tolerance. Proteinase inhibitor approach. In: Transgenic plants vol. 1. Engineering and utilization. (Eds. SD Kung and R Wu). Academic Press, Inc.California, 317-338.

Hilder VA, Gatehouse AMR, Sherman SE, Barker RF, Boulter D (1987) A novel mechanism for insect resistance. Nature 330: 160–163.

Hoffmann MP, Zalom FG, Wilson LD, Smilanick JM, Malyj LD, Kiser J, Hilder VA, Barness WM (1992) Field evaluation of transgenic tobacco containing genes encoding *Bacillus thuringiensis* endotoxin or cowpea trypsin inhibitor: efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae). Journal of Economical Entomology 85: 2516-2522.

Horn M, Patankar AG, Zavala JA, Wu J, Doleèková-Marešová L, Vůjtěchová M, Mareš M, Baldwin IT (2005) Differential elicitation of two processing proteases controls the processing pattern of the trypsin proteinase inhibitor precursor in *Nicotiana attenuate*. Plant Physiology 139: 375-388.

Howe GA, Lee GI, Itoh A, Li L, DeRocher A (2000) Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. Plant Physiology 123: 711-724.

Howe GA, Ryan CA (1999) Suppressors of systemin signaling identify genes in the tomato wound response pathway. Genetics 153: 1411-1421.

Hui DQ, Iqbal J, Lehmann K, Gase K, Saluz HP, Baldwin HP (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. V. Microarray analysis

and further characterization of large-scale changes in herbivore-induced mRNAs. Plant Physiology 131: 1877-1893. ACLE

Ishimoto M, Chrispeels MJ (1996) Protective mechanism of the Mexican bean weevil against high levels of a-amylase inhibitor in the common bean. Plant Physiology 111: 393-401.

Johnson ED, Miller E A, Anderson MA (2006) Dual location of a family of proteinase inhibitors within the stigmas of *Nicotiana alata*. Planta. Epub ahead of print DOI 10.1007/s00425-006-0418-6.

Johnson R, Narvaez J, An G, Ryan C (1989) Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. Proceedings of National Academy of Science USA 86: 9871-9875.

Johnston KA, Gatehouse JA, Anstee JH (1993) Effect of soybean protease inhibitors on the growth and development of larval *Helicoverpa armigera*. Journal of Insect Physiology 39: 657-664.

Johnston KA, Lee MJ, Brough C, Hilder VA, Gatehouse AMR, Gatehouse JA (1995) Protease activities in the larval midgut of *Heliothis verescens*: evidence for trypsin and chymotrypsin-like enzymes. Insect Biochemistry and Molecular Biology 25: 375-383.

Johnston KA, Lee MJ, Gatehouse JA, Anstee JH (1991) The partial purification and characterization of serine protease activity in midgut of larval *Helicoverpa armigera*. Insect Biochemistry 21: 389-397.

Jongsma MA, Bakker PL, Peters J, Bosch D, Stiekema WJ (1995a) Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. Proceedings of the National Academy of Sciences, USA 92: 8041-8045.

Jongsma MA, Bakker PL, Stiekema WJ, Bosch D (1995b) Phage display of a double-headed proteinase inhibitor: Analysis of the binding domains of potato proteinase inhibitor II. Molecular Breeding 1: 181-191.

Jouanin L, Bonade-Bottino M, Girard C, Morrot G, Giband M (1998) Transgenic plants for insect resistance. Plant Science 131: 1–11

Karban R, Baldwin IT (1997) Induced Responses to Herbivory. The University of Chicago Press p319.

Kenneth, Korth, Dixon (1997) Evidence for chewing insect-specific molecular events distinct from a general wound response in leaves. Plant Physiology 115: 1299-1305.

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science 291:2141-2144.

Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: The emerging molecular analysis. Annual Reviews in Plant Biology 53: 299–328.

Kessler A, Halitschke R, Baldwin IT (2004) Silencing the jasmonate cascade: induced plant defenses and insect populations. Science 305: 665-668.

Khamrui S, Dasgupta J, Dattagupta JK, Sen U (2005) Single mutation at P1 of a chymotrypsin inhibitor changes it to a trypsin inhibitor: X-ray structural (2.15 A) and biochemical basis. Biochimica et Biophysica Acta 1752: 65-72.

Kim S, Hong Y, An CS, Lee K (2001) Expression characteristics of serine proteinase inhibitor II under variable environmental stresses in hot pepper (*Capsicum annum* L.). Plant Science 161: 27-33.

King ABS (1994) *Heliothis/Helicoverpa* (Lepidoptera: Noctuidae). In Insect pests of cotton, Mathews GA and Turnstall JP (*eds.*). CAB International. Wallingford, U.K. pp. 39-106.

Knowles BH (1994) Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxins. Advances in Insect Physiology 24: 275–308.

Koiwa H, Bressan RA, Hasegawa PM (1997) Regulation of protease inhibitors and plant defense. Trends in Plant Science 2: 379-384.

Komiyama T, VanderLugt B, Fugere M, Day R, Kaufman RJ, Fuller RS (2003) Optimization of protease-inhibitor interactions by randomizing adventitious contacts. Proceedings of National Academy of Sciences, USA 100: 8205-8210.

Korth K, Dixon RA (1997) Evidence for chewing insect-specific molecular events distinct from a general wound response in leaves. Plant Physiology 115: 1299-1305.

Korth KL (2003) Profiling the response of plants to herbivorous insects. Genome Biology 4: 221.1-222.4.

Laskowski M Jr, Kato I (1980) Protein inhibitors of proteinases. Annual Reviews in Biochemistry 49: 593-626.

Lawrence PK, Koundal KR (2002) Plant proteinase inhibitors in control of phytophagous insects. Electronic Journal of Biotechnology 5: 93-109.

Lee GI, Howe GA (2003) The tomato mutant spr1 is defective in systemin perception and the production of a systemic wound signal for defense gene expression. The Plant Journal 33: 567–576

Lee MCS, Scalon MJ, Craik DJ, Anderson MA (1999) A novel two-chain proteinase inhibitor generated by circularization of a multidomain precursor protein. Nature Structural Biology 6: 526-530.

Lewis WJ, van Lenteren JC, Phatak SC, Tumlinson III JH (1997) A total system approach to sustainable pest management. Proceedings of the National Academy of Sciences, USA 94: 12243-12248.

Li C, Williams M, Loh Y.-T., Lee, G.I. and Howe, G.A. (2002b) Resistance of cultivated tomato to cell-content feeding herbivores is regulated by the octadecanoid signaling pathway. Plant Physiology 130: 494–503.

Li, L, Li C, Lee GI, Howe GA (2002a) Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. Proceedings of the National Academy of Sciences, USA 99: 6416-6421.

Lipke H, Fraenkel GS, Liener IE (1954) Effects of soybean inhibitors on growth of *Tribolium confusum*. Journal of the Science of Food and Agriculture 2: 410-415.

Liu J, Xia K, Zhu J, Deng YG, Huang XL, Hu BL, Xu X, Xu ZF (2006) The Nightshade Proteinase Inhibitor IIb Gene is constitutively expressed in glandular trichomes. Plant Cell Physiology 47: 1274–1284.

Manjunath TM, Bhatnagar VS, Pawar CS, Sithanatham S (1989) Economic importance of *Heliothis* spp. in India and assessment of their natural enemies and host plants. In: King, E.G., Jackson, R.D. (Eds.), Proceedings of the Workshop on Biological Control of Heliothis: Increasing the effectiveness of Natural Enemies, Far Eastern Regional Office, US Department of Agriculture, New Delhi, India, pp. 197-228.

Mazumdar-Leighton S, Babu CR, Bennett J (2000) Identification of novel serine protease gene transcripts in the midguts of two tropical insect pests, *Scirpophaga incertulas* (Wk.) and *Helicoverpa armigera* (Hb.). Insect Biochemistry and Molecular Biology 30: 57-68.

Mazumdar-Leighton S, Broadway RM (2001a) Transcriptional inductioron of diverse midgut trypsins in larval *Agrotis ipsilon* and *Helicoverpa zea* feeding on the soybean trypsin inhibitor. Insect Biochemistry and Molecular Biology 31: 645-657

Mazumdar-Leighton S, Broadway RM (2001b) Identification of six chymotrypsin cDNAs from larval mid guts of *Helicoverpa zea* and *Agrtis ipsilon* feeding on the soybean (Kunitz) trypsin inhibitor. Insect Biochemistry and Molecular Biology 31: 633-644

McCaffery AR, King ABS, Walker AJ, El-Nayir H (1989) Resistance to synthetic pyrethroids in the bollworm, *Heliothis armigera* from Andhra Pradesh, India. Pesticide Science 27: 65-76.

McGaughey WH, Gould F, Gelernter W (1998) *Bt* resistance management: a plan for reconciling the needs of the many stakeholders in *Bt*–based products. Nature Biotechnology 16:144–146.

McManus MT, Burguess EPJ (1995) Effects of the soybean (Kunitz) trypsin inhibitor on growth and digestive proteases of larvae of *Spodoptera litura*. Journal of Insect Physiology 41: 731-738.

Michaud D (1997) Avoiding protease-mediated resistance in herbivorous pests. Trends in Biotechnology 15: 4-6.

Miller EA, Lee MC, Atkinson AH, Anderson MA (2000) Identification of a novel four-domain member of the proteinase inhibitor II family from the stigmas of *Nicotiana alata*. Plant Molecular Biology 42: 329–333.

Moran PJ, Cheng YF, Cassell JL, Thompson GA (2002) Gene expression profiling of *Arabidopsis thaliana* in compatible plant–aphid interactions. Archives of Insect Biochemistry and Physiology 51: 182–203.

Moran PJ, Thompson GA (2001) Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. Plant Physiology 125: 1074-1085.

Moura DS, Ryan CA (2001) Wound-inducible proteinase inhibitors in pepper. Differential regulation upon wounding, systemin and methyl jasmonate. Plant Physiology 126: 289-298.

Murdock LL, Shade RE (2002) Lectins and protease inhibitors as plant defenses against insect pests. Journal of Agricultural and Food Chemistry 50: 6605-6611.

Nagarkatti S, Prakash A (1974) Rearing *Heliothis armigera* (Hubn) on artificial diet Technical Bulletin 17. Commonwealth Institute of Biological Control, Banglore. pp 169-173.

Nahar P, Ghormade V, Deshpande MV (2004) The extracellular constitutive production of chitin deacetylase in *Metarhizium anisopliae*: possible edge to entomopathogenic fungi in the biological control of insect pests. Journal of Invertebrate Pathology 85: 80-88.

Narváez-Vásquez J and Ryan CA (2004) The cellular localization of prosystemin: a functional role for phloem parenchyma in systemic wound signaling. Planta 218: 360–369

Narváez-Vásquez J, Gregory Pearce, Clarence A. Ryan (2005) The plant cell wall matrix harbors a precursor of defense signaling peptides Proceedings of the National Academy of Sciences, USA 102: 12974–12977

Nielsen KJ, Heath RL, Anderson MA, Craik DJ (1994) The three-dimensional solution structure by 1H-NMR of a 6-kDa proteinase inhibitor isolated from the stigma of *Nicotiana alata*. Journal of Molecular Biology 242: 231–243.

Nielsen KJ, Heath RL, Anderson MA, Craik DJ (1995) Structures of a series of 6-kDa trypsin inhibitors isolated from the stigma of *Nicotiana alata*. Biochemistry 34: 14304–14311.

Nielsen KJ, Hill JM, Anderson MA, Craik DJ (1996) Synthesis and structure determination by NMR of a putative vacuolar targeting peptide and model of a proteinase inhibitor from *Nicotiana alata*. Biochemistry 35: 369–378.

Oerke EC, Dehne H (2004) Safeguarding production-losses in major crops and the role of crop protection. Crop Protection 23: 275-285.

Oparkaa KJ, Turgeon R (1999) Sieve elements and companion cells—traffic control centers of the phloem. The Plant Cell 11: 739–750.

Orozco-Cardenas M, McGurl B, Ryan CA (1993) Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae. Proceedings of the National Academy of Sciences, USA 90: 8273–8276.

Outchkourov NS, Peters J, de Jong J, Rademakers W, Jongsma MA (2003) The promoter-terminator of *chrysanthemum* rbdS1 directs very high foreign gene expression levels in dicotyledonous plants. Planta 216: 1003-1112.

Patankar AG, Giri AP, Harsulkar AM, Sainani MN, Deshpande VV, Ranjekar PK, Gupta VS (2001) Complexity in specificities and expression of *Helicoverpa armigera* gut proteinases explains polyphagous nature of the insect pest. Insect Biochemistry and Molecular Biology 31: 453-464.

Pearce G, Johnson S, Ryan CA (1993) Purification and characterization from tobacco (*Nicotiana tabacum*) leaves of six small, wound-inducible, proteinase isoinhibitors of the potato inhibitor II family. Plant Physiology 102: 639–644.

Pearce G, Strydom D, Johnson S, Ryan CA (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science 253: 895–898.

Pena-Cortes H, Albrecht T, Prat S, Weiler E Willmitzer L (1993) Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta 191: 123-128.

Pena-Cortes H, Fisahn J, Willmitzer L (1995) Signals involved in wound-induced proteinase inhibitor II gene expression in tomato. Proceedings of the National Academy of Sciences, USA 92: 4106-4113.

Pena-Cortes H, Willmitzer L, Sánchez-Serrano JJ (1991) Abscisic acid mediates wound induction but not developmental-specific expression of the proteinase inhibitor II gene family. Plant Cell 3: 963-972.

Pichare MM, Kachole MS (1994) Detection of electrophoretically separated proteinase inhibitors using X-ray film. Journal of Biochemical and Biophysical Methods 28: 215-224.

Pickersgill B (1997) Genetic resources and breeding of *Capsicum* spp. Euphytica 96: 129–133.

Pieterse CMJ, Ton J, Van Loon LC (2001) Cross-talk between plant defense signaling pathways: boost or burden? AgBiotechNet 3: 1-8.

Pieterse CM, Van Wees SC, Van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ, Van Loon LC (1998) A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell. 10: 1571-1580.

Plunkett G, Senear DF, Zuroske G, Ryan CA (1982) Proteinase inhibitors I and II from leaves of wounded tomato plants: purification and properties. Archives of Biochemistry and Biophysics 213: 463-472.

Pollard DG (1972) Plant penetration by feeding aphids (*Hemiptera, aphidoidea*): a review. Bull Entomol Res 62: 631–714.

Rahbe Y, Deraison C, Bonade-Bottino M, Girard C, Nardon C, Jouanin L, (2003a) Effects of the cysteine protease inhibitor oryzacystatin (OC-I) on different aphids and reduced performance of *Myzus persicae* on OC-I expressing transgenic oilseed rape. Plant Science 164: 441-450.

Rahbe Y, Ferrasson E, Rabesona H, Quillien L (2003b) Toxicity to the pea aphid *Acyrthosiphon pisum* of anti-chymotrypsin isoforms and fragments of Bowman-Birk protease inhibitors from pea seeds. Insect Biochemistry and Molecular Biology 33: 299–306.

Reddy KVS, Zehr UB (2004) In New directions for a diverse planet. Proceedings of the 4th International Crop Science Congress. Brisbane, Australia. pp 1-8.

Reymond P, Bodenhausen N, Van Poecke RM, Krishnamurthy V, Dicke M, Farmer EE (2004) A conserved transcript pattern in response to a specialist and a generalist herbivore. Plant Cell 16: 3132–3147.

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell 12: 707–719.

Rodriguez-Saona, Chalmers JA, Raj S, Thaler JS (2005) Induced plant responses to multiple damagers: differential effects on an herbivore and its parasitoid. Oecologia 143: 566–577.

Royo J, Leo J, Vancanneyt G, Albar JP, Rosahl S, Ortego F, Castan EP, Sanchez-Serrano J (1999) Antisense-mediated depletion of a potato lipoxygenase reduces wound induction of proteinase inhibitors and increases weight gain of insect pests. Proceedings of the National Academy of Sciences, USA 96: 1146-1151.

Ryals JA, Neuenschwander UH, WillitsMG, Molina A, Steiner HY, Hunt MD (1996) Systemic acquired resistance. Plant Cell 8: 1809–1819

Ryan CA (1989) Proteinase inhibitor gene families: strategies for transformation to improve plant defenses against herbivores. Bioessays 10: 20-24.

Ryan CA (1990) Proteinase inhibitors in plants, genes for improving defenses against insects and pathogens. Annual Reviews on Phytopathology 28: 425-449.

Ryan CA (2000) The systemin signaling pathway: differential activation of plant defensive genes. Biochimia et Biophysicia Acta 1477: 112-121.

Ryan CA, Pearce G (2003) Systemins: a functionally defined family of peptide signals that regulate defensive genes in Solanaceae species. Proceedings of the National Academy of Sciences, USA 100: 14577-14580.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, second edition, C old Spring Harbor Laboratory Press, Plainview, New York

Sanchez-Serrano J, Schmidt R, Schell J, Willmitzer L (1986) Nucleotide sequence of proteinase inhibitor II encoding cDNA of potato (*Solanum tuberosum*) and its mode of expression. Molecular and General Genetics 203: 15-20.

Sarmah BK, Moore A, Tate W, Molvig L, Morton RL, Rees DP, Chiaiese P, Chrispeels MJ, Tabe LM, Higgins TJV (2004) Transgenic chickpea seeds expressing high levels of a bean a amylase inhibitor. Molecular Breeding 14: 73-82.

Scanlon MJ, Lee MC, Anderson MA, Craik DJ (1999) Structure of a putative ancestral protein encoded by a single sequence repeat from a multidomain proteinase inhibitor gene from *Nicotiana alata* . Structure, Folding, and Design 7: 793-802.

Schaller A, Ryan CA (1995) Systemin-a polypeptide defense signal in plants. Bioessays 18: 27-33.

Schirra HH, Scanlon MJ, Lee MCS, Anderson MA, Craik DJ (2001) The solution structure of C1–T1, a two-domain proteinase inhibitor derived from a circular

precursor protein from *Nicotiana alata*. Journal of Molecular Biology 306: 69–79.

Schirra HJ, Craik DJ (2005) Structure and folding of potato type II proteinase inhibitors: circular permutation and intramolecular domain swapping. Protein and Peptide Letters 12: 421-431.

Schroeder HE, Gollasch S, Moore A, Tabe LM, Craig S, Hardie DC, Chrispeels MJ, Spencer D, Higgins TJV (1995) Bean alpha amylase inhibitor confers resistance to the pea weevil (*Bruchus pisorum)* in transgenic peas (*Pisum sativum* L.). Plant Physiology 107: 1233-1239.

Schuler TH, Poppy GM, Kerry BR, Denholm I (1998) Insect resistant transgenic plants. Trends in Biotechnology 16: 168–175.

Shade RE, Shroeder HE, Pueyo JJ, Tabe LM, Murdock LL, Higgins TJV, Chrispeels MJ (1994) Transgenic pea seeds expressing alpha-amylase inhibitor of the common bean are resistant to bruchid beetles. Biotechnology 12: 793-796.

Shanower TG, Romeis J (1999) Insect pests of pigeonpea and their management. Annual Review of Entomology 44: 77-96.

Sharma HC, Sharma KK, Seetharam N, Ortiz R (2000) Prospect for using transgenic resistance to insects in crop improvement. Electronic Journal of Biotechnology 3: 76-95.

Shin R, Lee G, Park C, Kim T, You J, Nam Y, Paek K (2001) Isolation of pepper mRNAs differentially expressed during the hypersensitive response to tobacco mosaic virus and characterization of a proteinase inhibitor gene. Plant Science 161: 727-737.

Sin S, Yeung EC, Chye M (2006) Down regulation of *Solanum americanum* genes encoding proteinase inhibitor II causes defective seed development. The Plant Journal 45: 58-70.

Sin SF, Chye ML (2004) Expression of proteinase inhibitor II proteins during floral development in *Solanum americanum*. Planta 219: 1010–1022.

Singh TVK (2005) Ecology of *Helicoverpa* species in relation to agro-ecosystem. National Symposium on *Helicoverpa* Management - A National Challenge, Kanpur, India. pp 1-9.

Soon LG (1997) Integrated pest management in developing Asia. In Proceedings of the International Conference on Ecology and Agriculture: Towards Sustainable Development (Eds. Dhaliwal GS, Randhawa NS, Arora R, Dhawan AK) Chandigarh, India pp 3-16.

Spoel S, Koornneef A, Claessens SMC, Korzelius JP, Van Pelt JA, Mueller MJ, Buchala AJ, Metraux JP, Brown R, Kazan K (2003) NPR1 Modulates cross-

talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell 15: 760–770.

Srinivasan A, Giri AP, Harsulkar AM, Gatehouse JA, Gupta VS (2005) A Kunitz trypsin inhibitor from chickpea (*Cicer arietinum* L.) that exerts anti-metabolic effect on podborer (*Helicoverpa armigera*) larvae. Plant Molecular Biology 57: 359-374.

Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine's defensive function in nature. PLoS Biology 2 (8):E217.

Stintzi A, Browse J (2000) The *Arabidopsis* male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proceedings of the National Academy of Sciences, USA 97: 10625–10630.

Stintzi A, Weber H, Reymond P, Browse J, Farmer EE (2001) Plant defense in the absence of jasmonic acid: The role of cyclopentenones. Proceedings of the National Academy of Sciences, USA 98: 12837–12842.

Stotz HU, Koch T, Biedermann A, Weniger K, Boland W, Mitchell-Olds T (2002) Evidence for regulation of resistance in *Arabidopsis* to Egyptian cotton worm by salicylic and jasmonic acid signaling pathways. Planta 214: 648–652.

Stotz HU, Pittendrigh BR, Kroymann J, Weniger K, Fritsche J, Bauke A, Mitchell-Olds T (2000) Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of *Arabidopsis* against Egyptian cotton worm but not diamondback moth. Plant Physiology 124: 1007-1019.

Taylor BH, Young RJ, Scheuring CF (1993) Induction of a proteinase inhibitor II-class gene by auxin in tomato roots. Plant Molecular Biology 23: 1005-1014.

Telang A, Booton V, Chapman RF, Wheeler DE (2000) How female caterpillars accumulate their nutrient reserves? Journal of Insect Physiology 47: 1055-1064.

Telang A, Buck NA, Wheeler DE (2002) Response of storage protein levels to variation in dietary protein levels. Journal of Insect Physiology 48: 1021-1029.

Telang MA, Giri AP, Sainani MN, Gupta VS (2005) Characterization of two midgut proteinases of *Helicoverpa armigera* and their interaction with proteinase inhibitors. Journal of Insect Physiology 51: 513–522.

Telang MA, Srinivasan A, Patankar A, Harsulkar A, Joshi V, Damle A, Deshpande V, Sainani MN, Ranjekar PK, Gupta G, Birah A, Rani S, Kachole M, Giri AP, Gupta VS (2003) Bitter gourd proteinase inhibitor: potential growth inhibitors of *Helicoverpa armigera* and *Spodoptera litura*. Phytochemistry 63: 643-652.

Thaler JS (1999) Jasmonate-inducible plant defences cause increased parasitism of herbivores. Nature 399:686–688.

Thaler JS (2002) Effect of jasmonate-induced plant responses on the natural enemies of herbivores. Journal of Animal Ecology 71:141–150.

Thaler JS, Fidantsef AL, Duffey SS, Bostock RM (1999) Tradeoffs in plant defense against pathogens and herbivores: a field demonstration of chemical elicitors of induced resistance. Journal of Chem Ecology 25: 1597–1609.

Thomas MB (1999) Ecological approaches and the development of 'truly integrated' pest management. Proceedings of the National Academy of Sciences, USA 96: 5944-5951.

Turlings TCJ, Tumlinson JH, Eller FJ, Lewis WJ (1991) Larval damaged plants: source of volatile synomonesthat guide the parasitoid *Cotesia marginiventris* to the microhabitat of its hosts. Entomologia Experimentalis Et Applicata 58: 75-82.

Ussuf KK, Laxmi NH, Mitra R (2001) Proteinase inhibitors: Plant-derived genes of insecticidal protein for developing insect-resistant transgenic plants. Current Science 80: 847-853.

Van Wees SCM, Chang HS, Zhu T, Glazebrook J (2003) Characterization of the early response of *Arabidopsis* to *Alternaria brassicicola* infection using expression profiling. Plant Physiology 132: 606–617.

Vishnudasan D, Tripathi MN, Rao U, Khurana P (2005) Assessment of nematode resistance in wheat transgenic plants expressing potato proteinase inhibitor (PIN2) gene. Transgenic Research 14:665–675.

Voelckel C, Baldwin IT (2004a) Herbivore-induced plant vaccination. Part II. Array-studies reveal the transience of herbivorespecific transcriptional imprints and a distinct imprint from stress combinations. Plant Journal 38: 650–663.

Voelckel C, Weisser WW, Baldwin IT (2004b) An analysis of plant–aphid interactions by different microarray hybridization strategies. Molecular Ecology 13: 3187–3195. Blackwell Publishing, Ltd.

Volpicella M, Ceci LR, Cordewener J, America T, Gallerani R, Bode W, Jongsma MA, Beekwilder J (2003) Properties of purified gut trypsin from *Helicoverpa zea* adapted to proteinase inhibitors. European Journal of Biochemistry 270: 10-19.

Walling LL (2000) The myriad plant responses to herbivores. Journal of Plant Growth Regulation 19:195–216.

Waterhouse DF (1957) Digestion in insects. Annual Review of Entomology 2: 1-18.

Wu J, Hettenhausen C, Baldwin IT (2006) Evolution of proteinase inhibitor defenses in North American allopolyploid species of *Nicotiana*. Planta 224: 750–760.

Wu Y, Llewellyn D, Mathews A, Dennis ES (1997) Adaptation of *Helicoverpa armigera* (Lepidoptera, Noctuidae) to a proteinase inhibitor expressed in transgenic tobacco. Molecular Breeding 3: 371-380.

Xu ZF, Qi WQ, Ouyang XZ, Yeung E, Chye ML (2001) A proteinase inhibitor II of *Solanum americanum* is expressed in phloem. Plant Molecular Biology 47: 727–738.

Xu ZF, Teng WL, Chye ML (2004) Inhibition of endogenous trypsin- and chymotrypsin- like activities in transgenic lettuce expressing heterogeneous proteinase inhibitor SaPIN2a. Planta 218: 623–629.

Zavala JA, Patankar AG, Gase K, Baldwin IT (2004a) Constitutive and inducible trypsin proteinase inhibitor production incurs large fitness costs in *Nicotiana attenuata* . Proceedings of National Academy of Science USA 101: 1607-1612.

Zavala, JA, Patankar AG, Gase K, Hui D, Baldwin IT (2004b) Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as antiherbivore defenses. Plant Physiology 134: 1181-1190.

Zhang HU, Xie X, Xu Y, Wu N (2004) Isolation and functional assessment of a tomato proteinase inhibitor II gene. Plant Physiology and Biochemistry 42: 437–444.

Zhou L, Thornburg R (1999) Wound-inducible genes in plants. Department of Biochemistry and Biophys ics, Iowa State University, Ames, IA 50011, USA, 8:127-158 © CAB International 1999.

Zhu-Salzman K, Salzman RA, Ahn JE, Koiwa H (2004) Transcriptional regulation of *Sorghum* defense determinants against a phloem-feeding aphid. Plant Physiology 134: 420- 431.

# Bio data

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| Exam | Subject | University/ Board | Percentage |
|---|---|---|---|
| Ph.D. (pursuing) | Biotechnology | University of Pune | - |
| M.Sc. | Botany- Spl. in Plant Biotechnology | University of Pune | 80% |
| B.Sc. | Botany | S. P. College, University of Pune | 75% |
| H.S.C. | P, C, M, B. | Maharashtra State Board | 75% |
| S.S.C. | - | Maharashtra State Board | 85% |

**Special achievements:**

1. Received the Best Poster Award (Jan 2005), in the area of Biological Sciences, at the Science day poster presentation session organized at National Chemical Laboratory, Pune.
2. Cleared the NET examination conducted by the Council of Scientific and Industrial Research (CSIR, New Delhi) in the area of Life Sciences and received the CSIR-JRF and SRF fellowships (July 2001 to June 2006).
3. Received the `Late Dr. T.S. Mahabale Gold Medal' and `Late Prof. V.V. Apte Memorial Prize' for securing highest number of marks and standing first at the M.Sc. (Botany) Exam held in May 2000.

**Publications:**

***Publications arising from the current research work***:

1. **Tamhane VA**, Chougule NP, Giri AP, Dixit AR, Sainani MN, Gupta VS (2005) *In vivo* and *in vitro* effect of *Capsicum annum* proteinase inhibitors on *Helicoverpa armigera* gut proteinases. Biochimica et Biophysica Acta 1722: 156-167.
2. **Tamhane VA,** Giri AP, Gupta VS (2005) *Helicoverpa armigera*: Ecology and control using novel biotechnological approaches. In online Encyclopedia of Pest Management, Ed. David Pimintel, DOI: 10.1081/E-EPM-120041174 Taylor and Francis Books, Dekker Encyclopedias

3. **Tamhane VA**, Giri AP, Sainani MN, Gupta VS Pin-II family proteinase inhibitors of *Capsicum annum* expressed in *Pichia pastoris* produce adverse effect on *Helicoverpa armigera* larval development by interfering with midgut digestive proteinases. (Communicated to Plant Molecular Biology).

4. **Tamhane VA**, Giri AP, Gupta VS Identification of novel CanPI genes from *Capsicum annum* and the *inplanta* expression pattern of the CanPI genes in infested and wounded tissues. (manuscript under preparation).

***Other Publications and participations:***

1. ***Oral presentation*** - **Tamhane VA** (2006) `*Capsicum annum* PI genes: Clonig and Expression' in the workshop on `Proteomic insights into plant-insect interactions' jointly organized by Max Plank Institute for Chemical Ecology, Jena, Germany and PMB Unit, Division of Biochemical Sciences, NCL, Pune, India during December 13$^{th}$ to 15$^{th}$.

2. Gupta VS, Giri AP, Sainani MN, Chougule NP, Srinavasan A, Telang MA, **Tamhane VA**, Damale MS, Tegeder M, Franceschi V, Rajesh PN, Muehlbauer Fred, (2005) `Development of *Helicoverpa armigera* Hubner resistance in crop plants using proteinase inhibitor approach' In: Proceedings of International Conference on Plant Genomics and Biotechnology: Challenges and Opportunities, IGAU, Raipur, Chattisgarh, India, Pg. 29.

3. ***Poster presentation*** - **Tamhane VA**, Giri AP, Sainani MN, Gupta VS (2005) Molecular diversity of *Capsicum annum* proteinase inhibitor genes and their potential against major legume pest *Helicoverpa armigera* In: International Food Legumes Research Conference IV held at IARI, New Delhi, during October 22-24.

4. Mathur G, **Alkutkar VA**, Nadgauda RS (2003) Cryopreservation of embryogenic culture of *Pinus roxburgii*. Biologia Plantarum 46: 405-410.

5. Gole P, **Tamhane VA** `Changing ecology of the fringe areas', and Gole SP, Haval G, **Tamhane VA (**2002) `Ecological restoration of quarried wasteland in Pune city's fringe' In: National Seminar on `Managing the urban fringe of Indian cities' held during November 27-29, by the Institute of Indian Geographers, Department of Geography, University of Pune, India, 411007.

6. **Alkutkar VA,** Athyle P, Adhikari SV, Ranade AS, Patwardhan M, Kunte K and Patwardhan A (2001) `Diversity of trees and butterflies in forest fragments around Pune city'(2001) In: Tropical Ecosystems: Structure, Diversity and Tropical Ecosystems, Proceedings of the International Conference on Tropical Ecosystems, K.N.Ganashaian, R. Uma Shanker and K.S.Bawa (eds), 685-688.

# Identification and characterization of plant derived proteinaceous inhibitors of gut digestive enzymes of polyphagous insect pest *Helicoverpa armigera* Hübner

A thesis submitted to the
University of Pune
For the degree of
DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

**VAIJAYANTI TAMHANE**

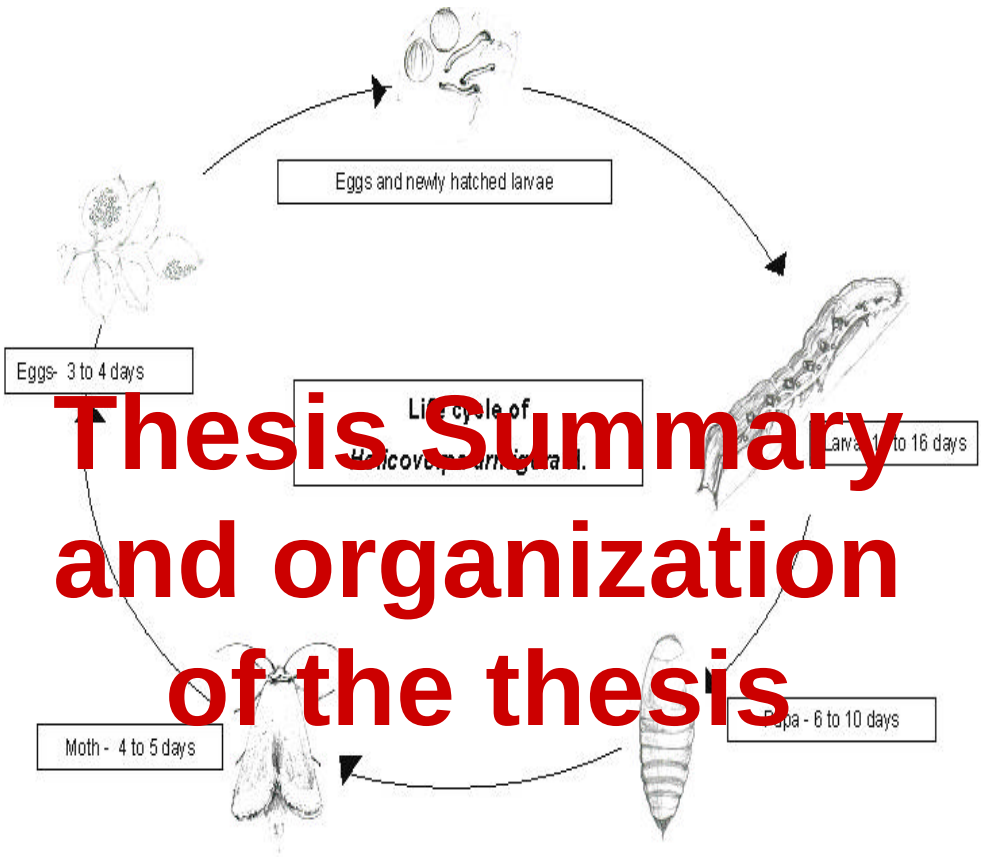
Plant Molecular Biology Unit

Division of Biochemical Sciences

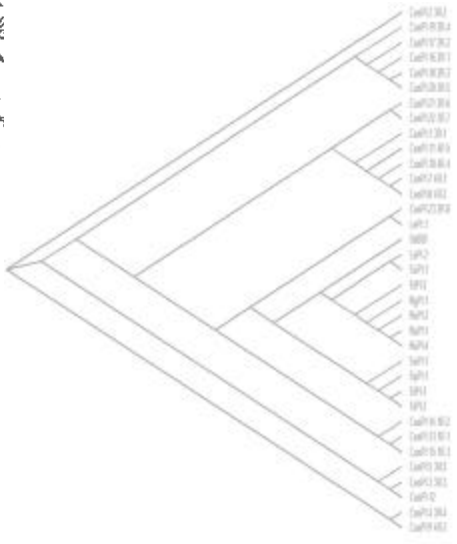
National Chemical Laboratory

Pune-411008 (India)

**January 2007**

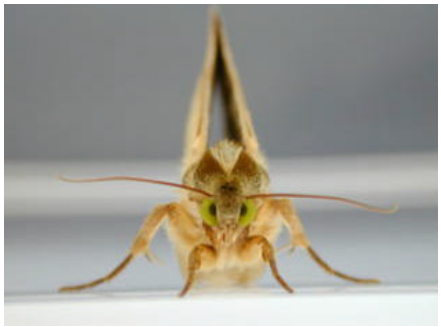



# Thesis Summary and organization of the thesis



# Chapter 1

# Review of Literature



**Part I: Lepidopteran insect pest *Helicoverpa armigera* and its control**

**Part II: Pin-II family of proteinase inhibitors**

# Chapter 2

## *In vitro* and *in vivo* effect of *Capsicum annuum* proteinase inhibitors on *Helicoverpa armigera* gut proteinases

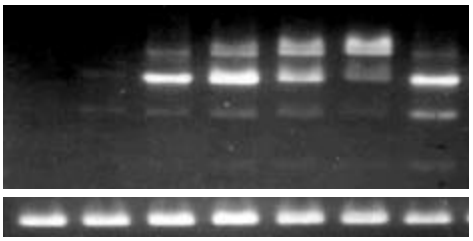
**The research work described in this chapter is a full-length paper, which has been published in BBA General Subjects**

**(Tamhane *et al*., 2005)**

# Chapter 3

## Expression patterns of Pin-II type proteinase inhibitor genes in *Capsicum annuum* Linn.



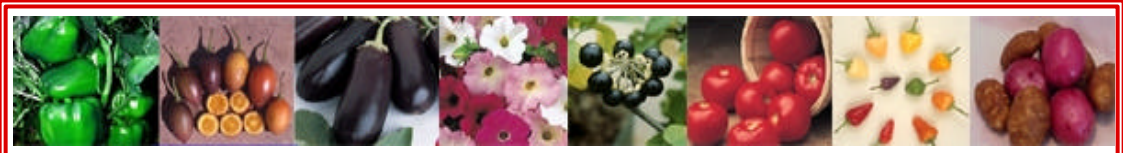


**The research work described in this chapter is part of a full-length paper, which has been communicated to Plant Molecular Biology**

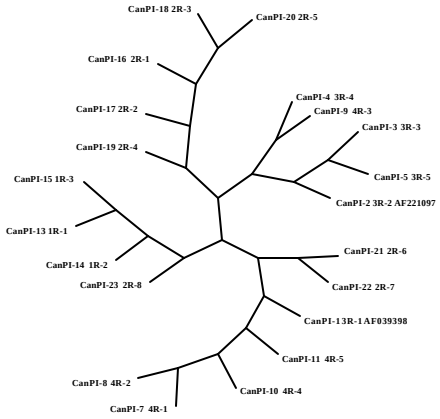
# Chapter 4

## Diverse forms of Pin-II family proteinase inhibitors of *Capsicum annuum* produce adverse effect on growth and development of *Helicoverpa armigera*

**The research work described in this chapter is part of a full-length paper, which has been communicated to Plant Molecular Biology**



# Chapter 5




# General discussion and future directions

# Bibliography

**Figure 1.1: Polyphagous insect pest *Helicoverpa armigera***

**Cotton**

**Piegonpea**

**Strawberry**

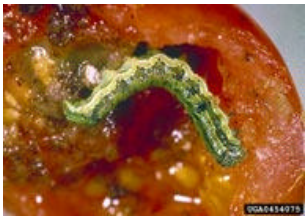
**Chickpea**

**Highly polyphagous nature of *H. armigera***

**Sorghum**

**Capsicum**

**Tomato**



**Corn**

**Flower of Asteraceae**

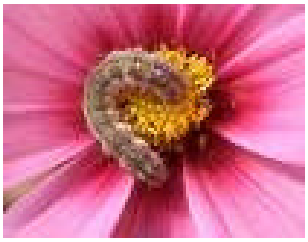
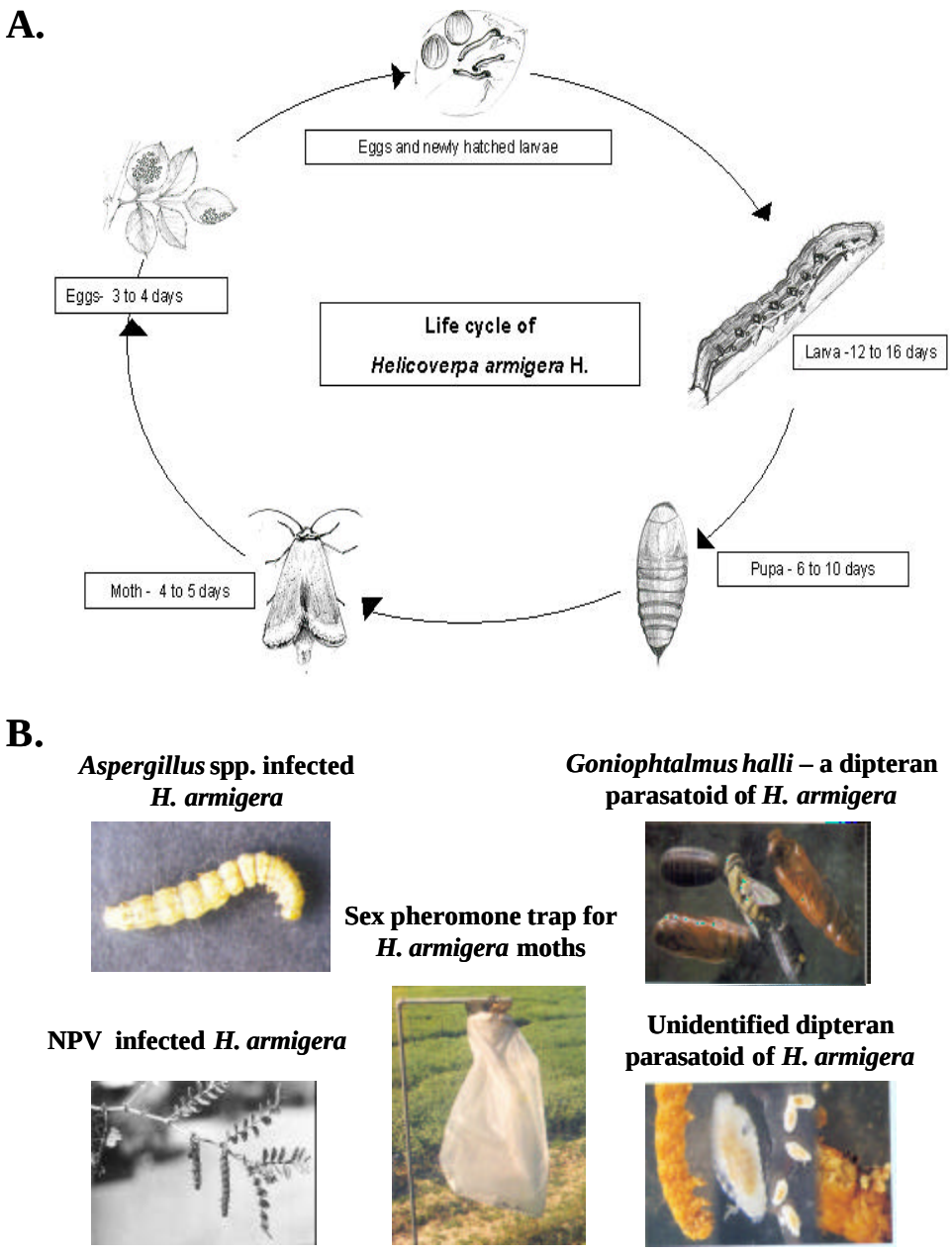


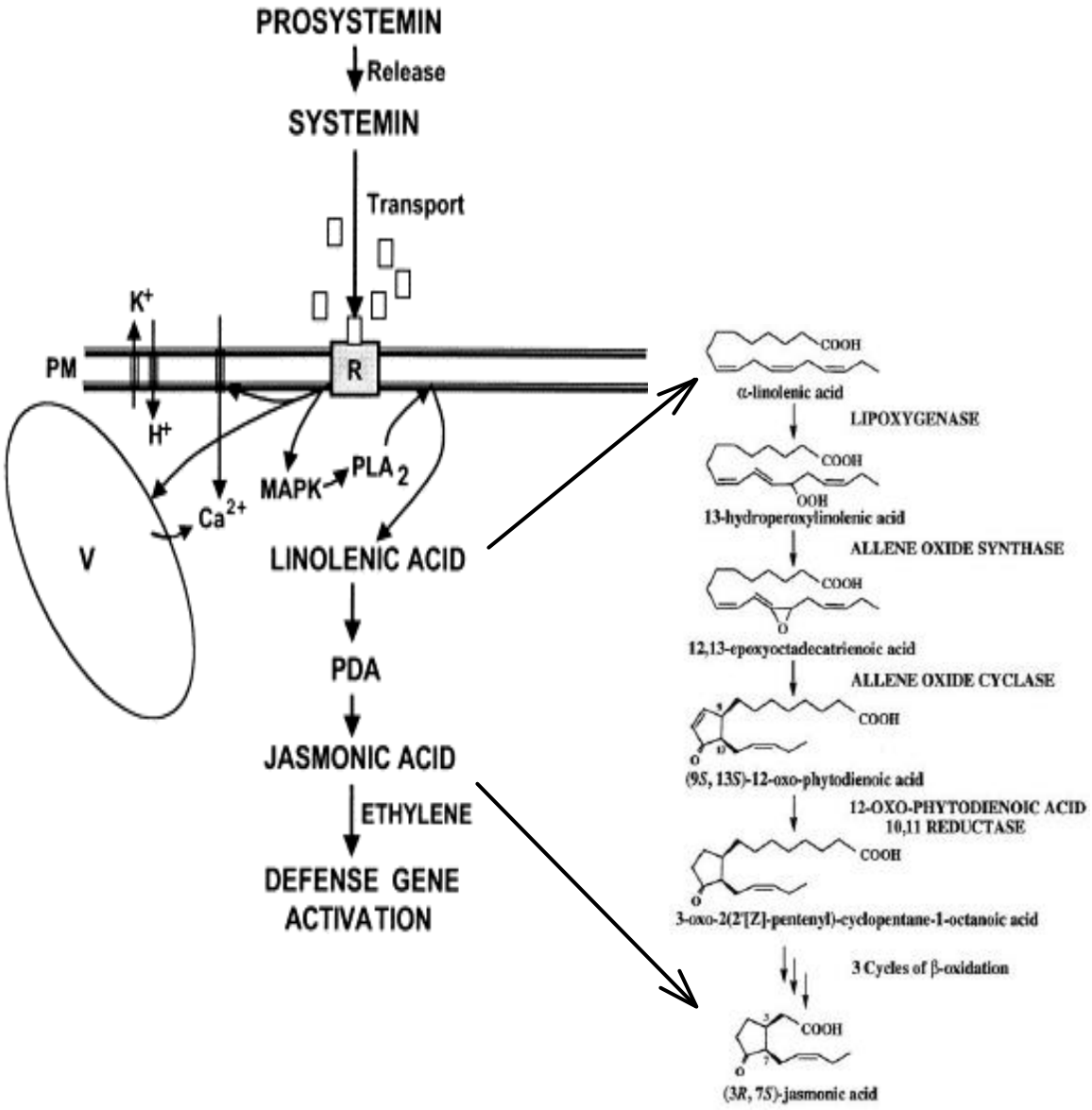
**Figure 1.2: Lifecycle and biological control measures of *H. armigera***




A. Lifecycle of *H. armigera*,

B. Representative biological control measures of *H. armigera*.

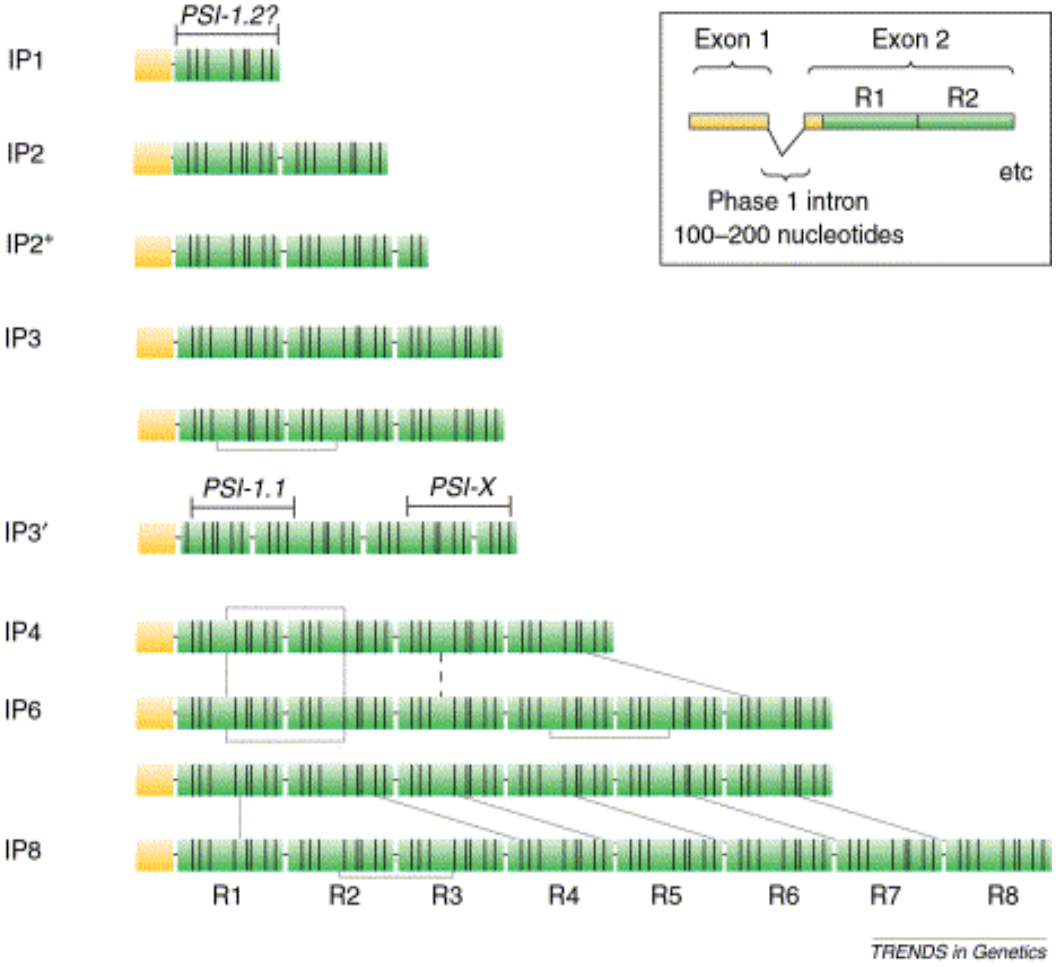
**Figure 1.3: Systemin signaling and biosynthesis of Jasmonic Acid**




A current model for the systemic signaling pathway for defensive genes in tomato plants that are activated by herbivore attacks (wounding). The interaction of systemin with its membrane receptor initiates intracellular events that activate a $PLA_2$. The phospholipase releases LA from membranes, leads to the production of JA, and the activation of defensive genes.

*Modified from Ryan 2000 and Stintzi* et al.*, 2001*.

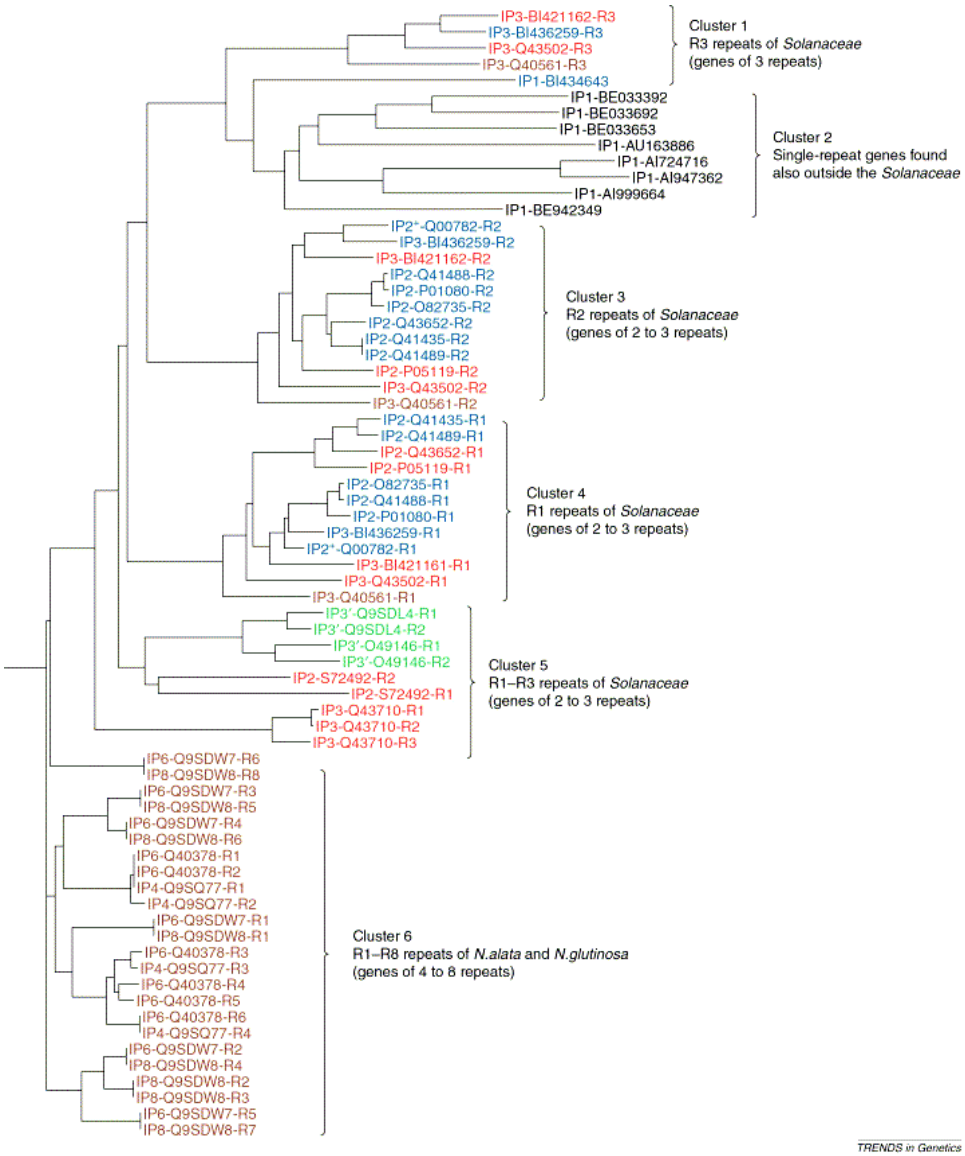
**Figure 1.4: Pin-II PI precursors of variable IRDs**




The domain structure of the potato type II proteinase inhibitor family (Pin-II) precursors. The inset shows the consensus protein structure. IP1...IP8 designate the total number of IP repeats (green boxes) within each precursor. Yellow box, signal peptide; black vertical lines, Cys residues; gray lines, sequence identity (>98%). The presence of adjacent, identical repeats is a recurrent pattern. PSI-1.1, PSI-X and PSI-1.2 are paprika seed inhibitors.

*From Barta* et al., *2002*

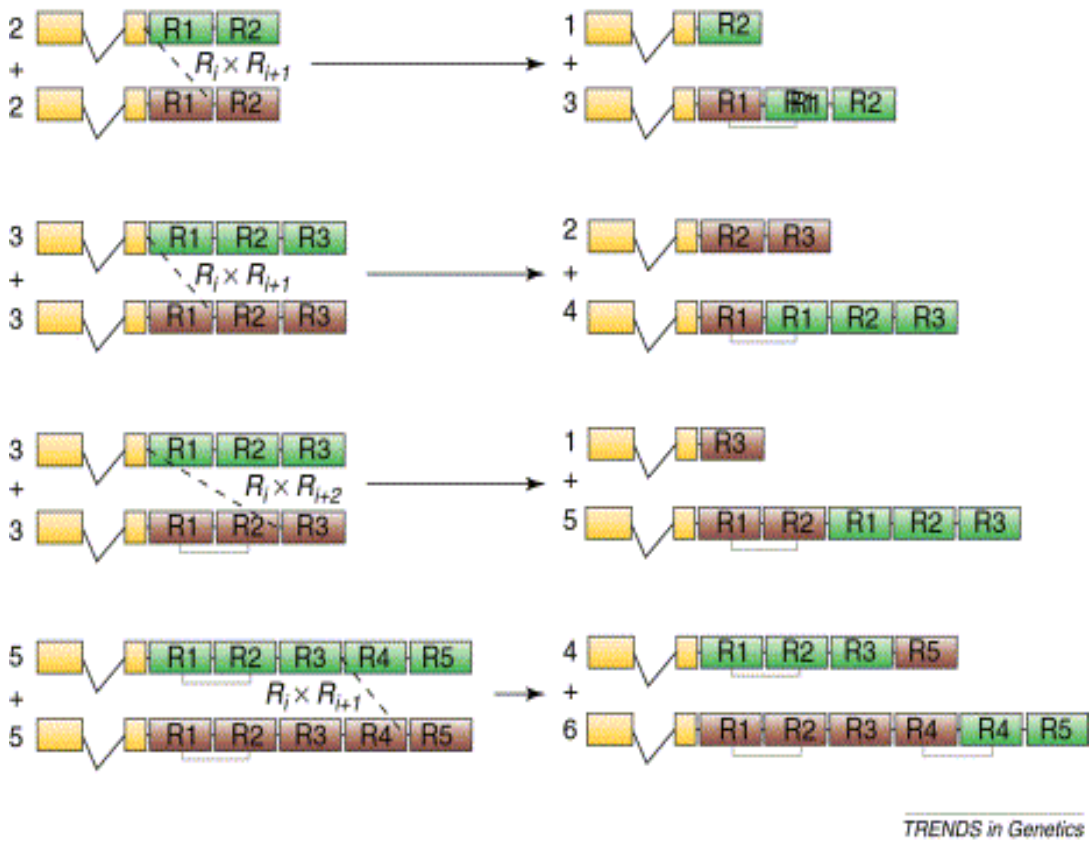
**Figure 1.5: Dendrogram of IP repeats**




Clustering of the DNA sequences coding for IP repeats. The sequences were clustered using the CLUSTAL program. IP indicates the total number of repeats within the gene, R indicates the serial order of the repeat starting from the N-terminus. Brown, tobacco (*N. tabacum*; IP3; *N. alata*: one IP4, one IP6; *N. glutiniosa*: one IP6, one IP8); blue, potato (one IP1, six IP2, one $IP2^{+}$); red, tomato (two IP2 three IP3); green, paprika (two IP3', two IP3); black, non-solanaceous plants (three IP1 in ice-plant, one IP1 in the others).

*From Barta et al., 2002*

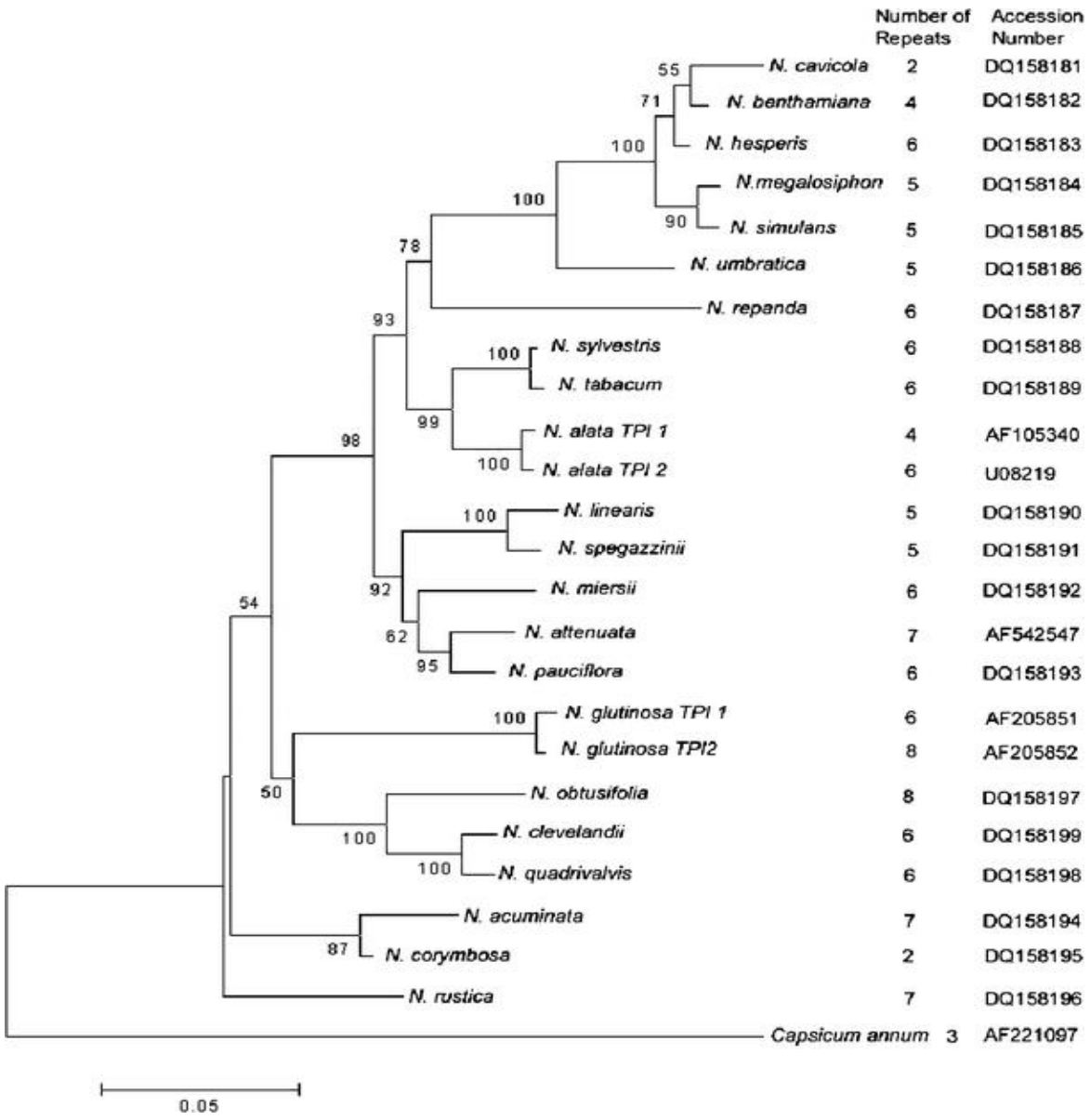
**Figure1.6: Unequal crossover events in Pin-II PI family**




Some of the potential unequal cross-over (UECO) events that explain the emergence of sequence identity patterns of the Pin-II family precursors. The two partners are colored green and brown. Two types of UECO event involving either adjacent ($R_i \times R_{i+1}$) or nonadjacent ($R_i \times R_{i+2}$) repeats are shown by dashed lines. Gray lines indicate sequence identity (>98%).

*From Barta* et al., *2002*

**Figure 1.7: Neighbor-joining tree of *Nicotiana* trypsin proteinase inhibitors (TPIs) based on cDNA sequences**




*From Wu* et al.,*2006*.

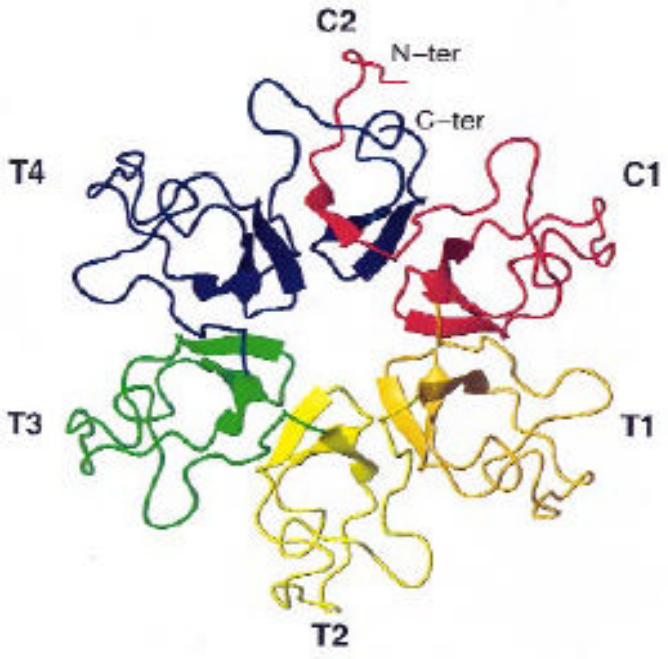
**Figure 1.8: Structures of precursor Pin II PI of *N. alata* and its cleaved IRDs**

**A.** Model of Na-PI structure. The six PI domains are in different colors, with the N-ter and C-ter fragments of C2 indicated. The PI domains are modeled on the three-dimensional structures of the individual PIs. The linker sequences are predicted to be hydrophilic and relatively unstructured. The C-terminal putative targeting domain has been omitted for clarity. **From Lee *et al.*, 1999.**

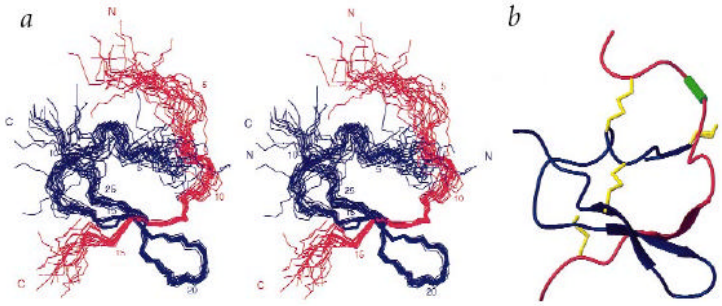
**B.** NMR structure of C2 ***a***, Solution structure of C2 showing backbone atoms (N, Ca and C) of N-ter (red) and C-ter (blue), with every fifth residue of N-ter and C-ter numbered. ***b***, Ribbon representation of the C2 structure. The peptide chains are colored as in (*a*), except that the reactive site is green and the four disulfides are yellow in a ball- and stick representation. Figures were generated using MOLMOL18. **From Scanlon *et al.*, 1999.**

**C.** A comparison of the structures of the contiguous native inhibitor T1, the two-chain inhibitor C2 and aPI1. **(a)** The molecules are shown as Ca ribbons of the mean structure from each NMR ensemble superimposed over the backbone heavy atoms (N, C, Ca) of their b-hairpin regions. The residues are color coded: residues Lys35–Ser53 of T1, Lys1A–Ser19A of C2 and Met1–Ser20 of aPI1 are colored red; Asp1–Ser29 of T1, Arg1B–Ser28B of C2 and Asp26–Ser54 of aPI1 are colored blue; residues Asp30–Pro34 of T1 and the linker region Glu21–Asn25 of aPI1 are colored green; and the protease-reactive sites of the inhibitors are shown in purple. **From Scanlon *et al.*, 1999.**

**A.**




**B.**




**C.**

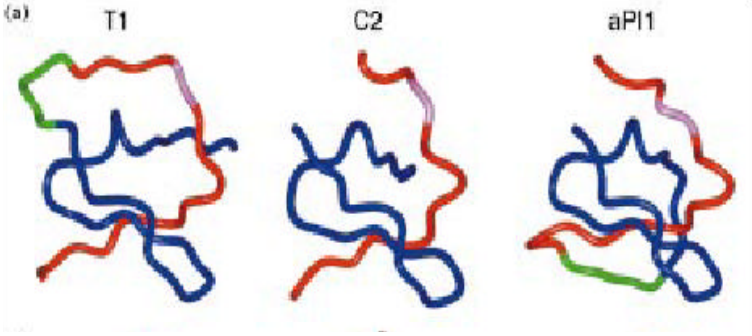
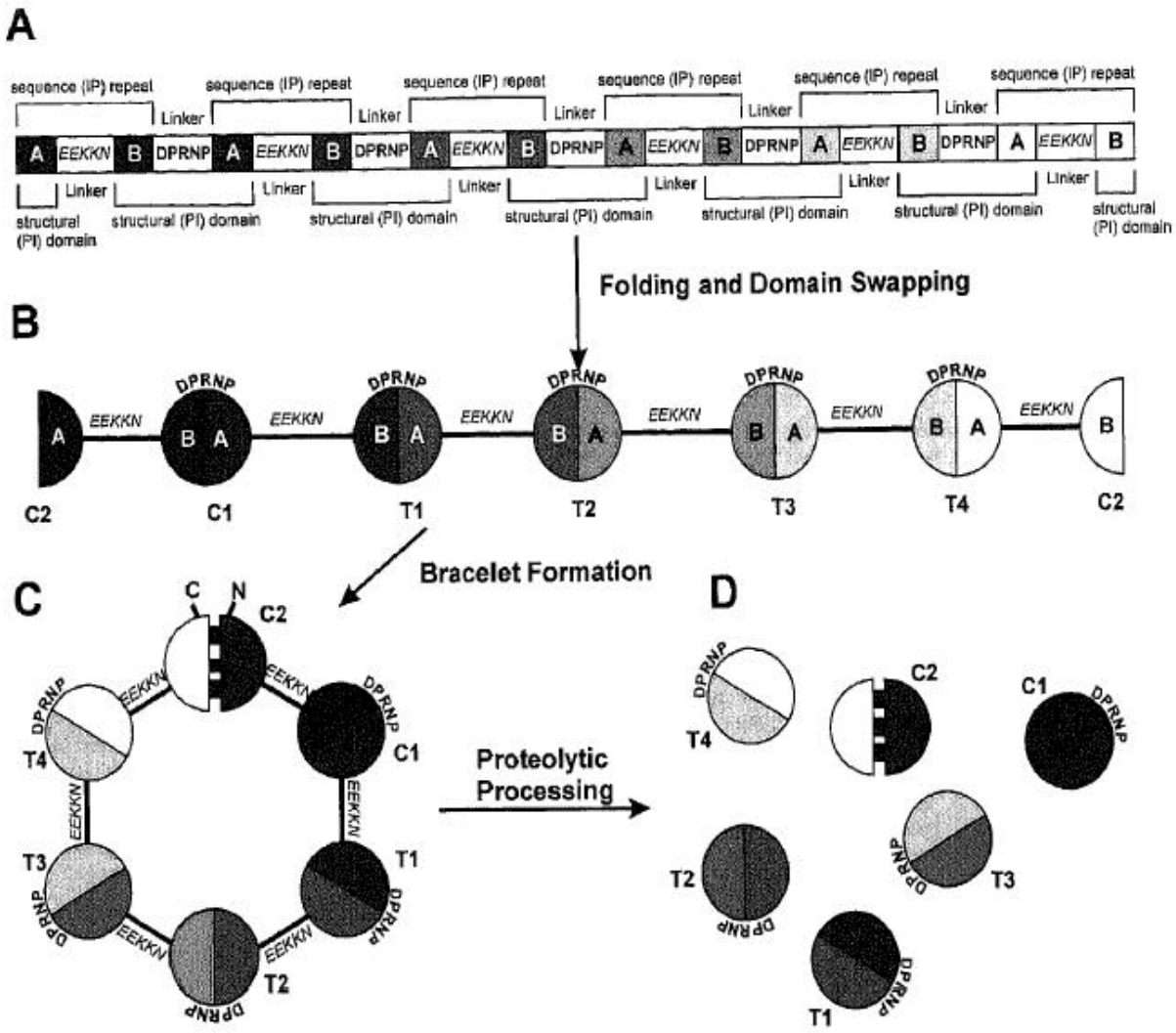


**Figure 1.9: Biosynthesis of *Nicotiana alata* PI**

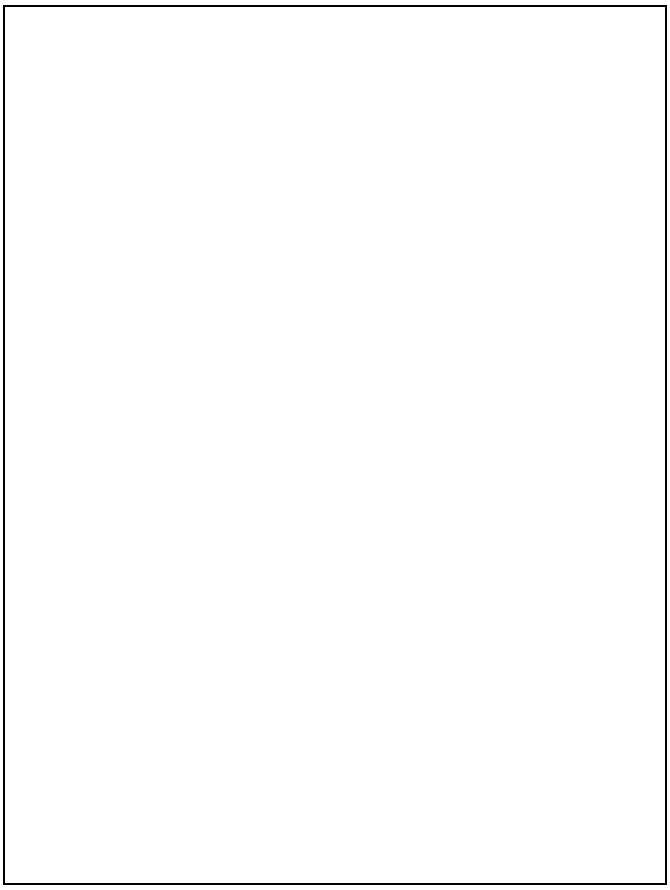



A. Gene arrangement of NaProPI. B. Incorporation of DPRNP linker region and exposure of the EEKKN region to protease action. C. Formation of a circular bracelet fold. D. Proteolytic cleavage of the EEKKN linker to release individual inhibitory repeat domains.

From ***Schirra and Craik et al.,* 2005**.

**Figure 1.10:**

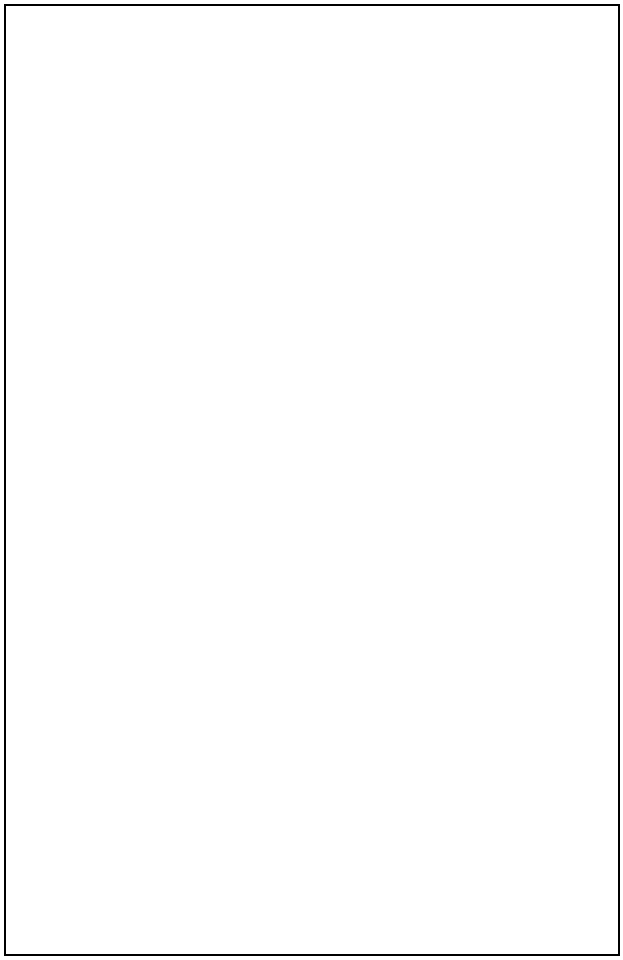
**Least-squares superpositions of unbound TI-II onto bound TI-II**



A stereoscopic view of a least-squares superposition of copies A and B (*A*) and copies C and D (*B*) onto the structure of TI-II from the TI-II:$(subtilisin)_2$ complex is shown. The bound form of TI-II is drawn in *magenta*, whereas the unbound forms are shown in *red* in Domain I and *blue* in Domain II.

From **Barrette Ng *et al.*, 2003 b**

**Figure 1.11: Structure of tomato Pin II type PI in complex with two enzyme molecules**



*A*, structure of the TI-II·(subtilisin)$_2$ complex. Subtilisin molecules are drawn in *yellow*, and the two domains of TI-II are drawn in *red* and *blue*. *B*, stereoscopic view of the structure of TI-II. In the $C_\alpha$ trace of TI-II, cysteine residue side chains and disulfide bonds are drawn in *yellow* and given residue numbers. Residues 74 to 85 and 117 to 123 are missing from the final model, because these portions of the structure were not defined in electron density maps. The approximate location of these residues is designated by magenta dots.

From **Barrette Ng *et al.*, 2003 a**

**Figure 1.12: Endogenous functions of Pin II PIs**

**A.** Immunolocalisation of SaPinIIb in Longitudinal sections of mature buds of *Solanum americanum*. L Longitudinal sections of young .oral bud. M. Transverse section of mature bud. n longitudinal section of stigma and style in young bud.

**B.** In a wild-type developing fruit of *S. americanum* (i), a majority of ovules develop into seeds (arrows). Most of seeds are aborted in young fruit of PIN2-RNAi line (ii). The aborted (black arrowheads) and viable seed (white arrow) are easily recognized.

**C.** Histochemical determination of the expression of SaPin2b promoter- GUS fusion genes in glandular trichomes of transgenic tobacco plants.

**D.** Immunolocalisation of SaPin2a in *S. americanum* stem by light microscopy.

**From Sin *et al.*, 2004, 2006; Xu *et al.*, 2001; Liu *et al.*, 2006.**

**Figure 1.12: Endogenous functions of Pin II PIs**

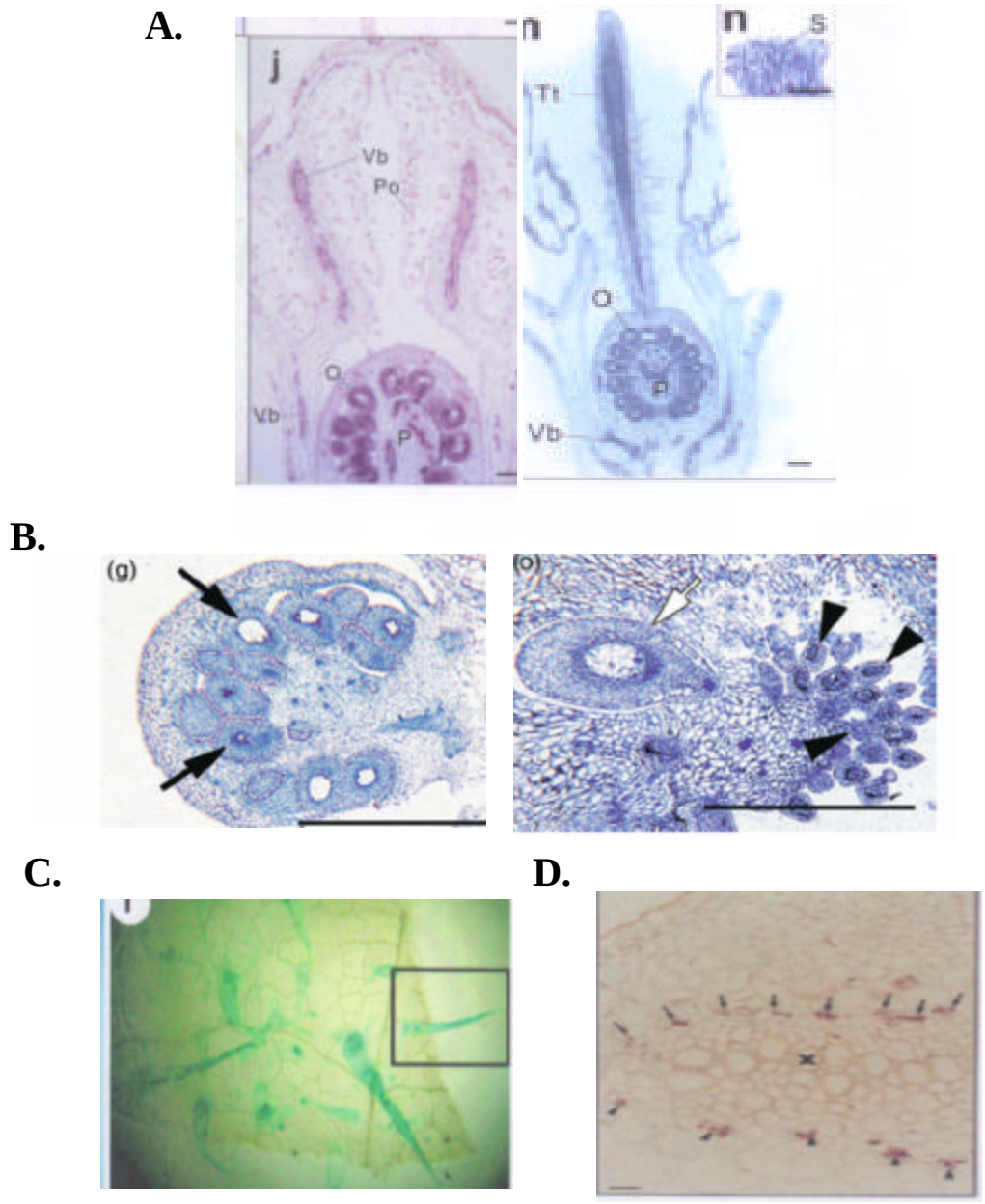


**Figure 1.13: Morphological features of *Capsicum annuum* L.**

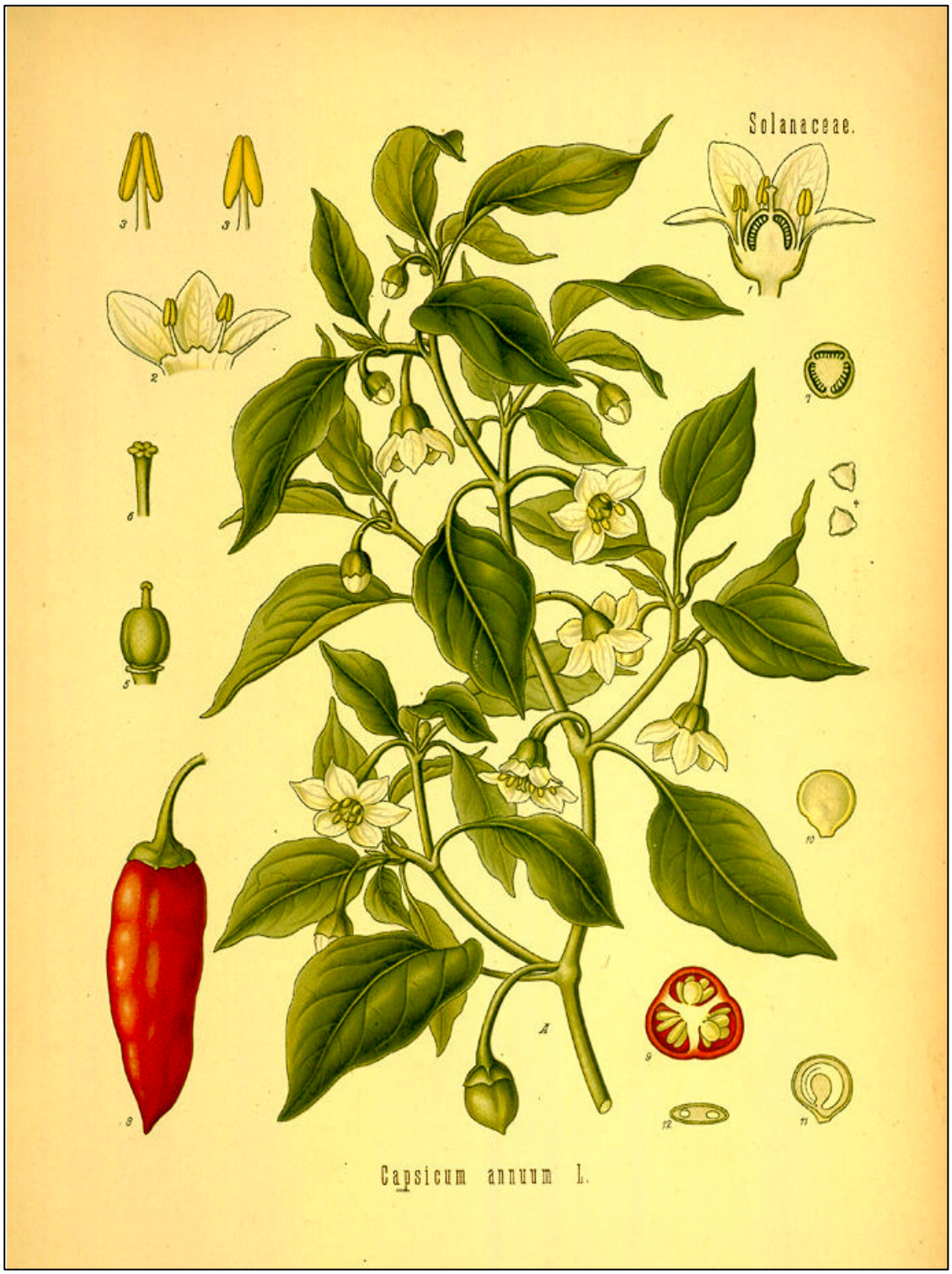
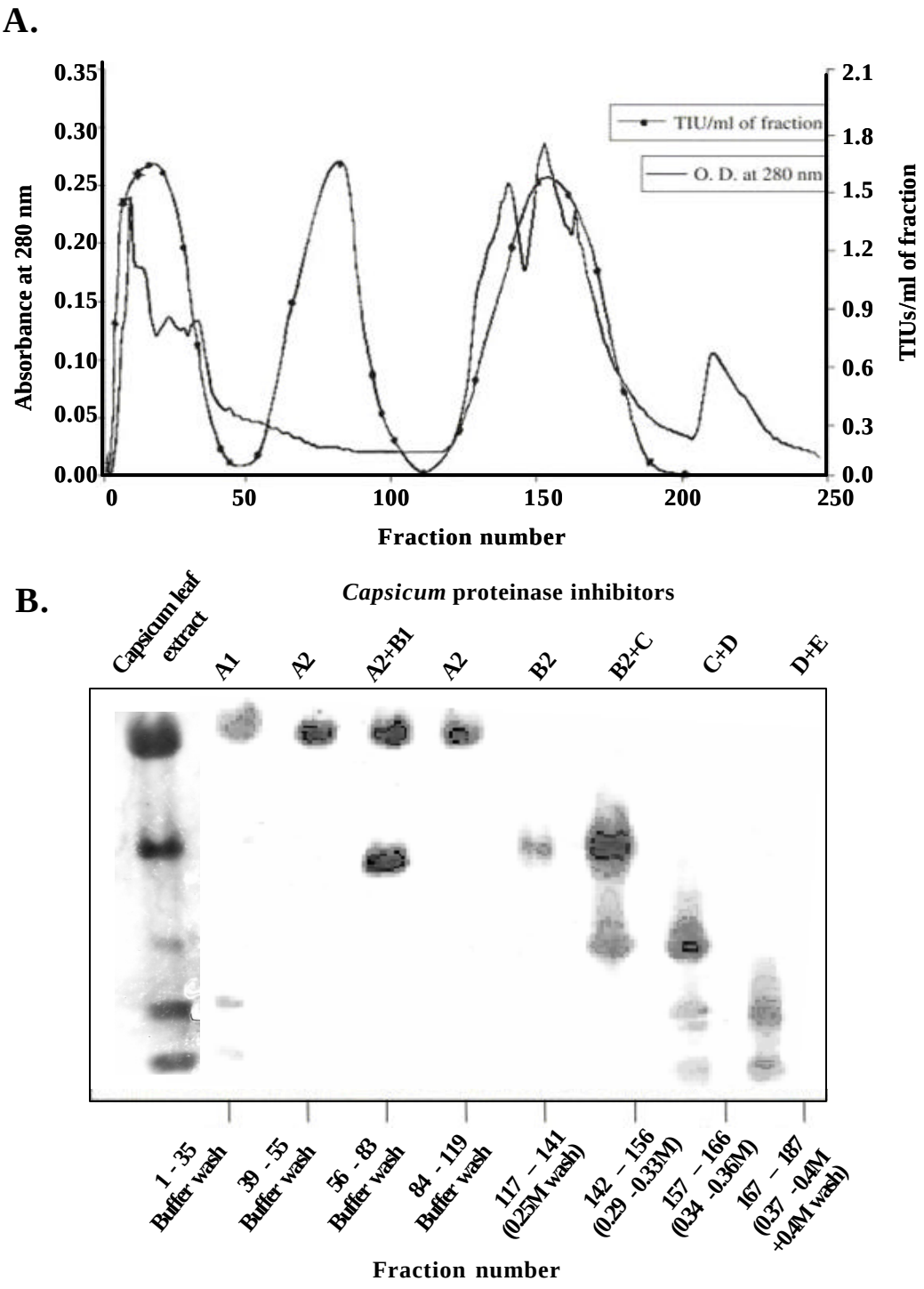
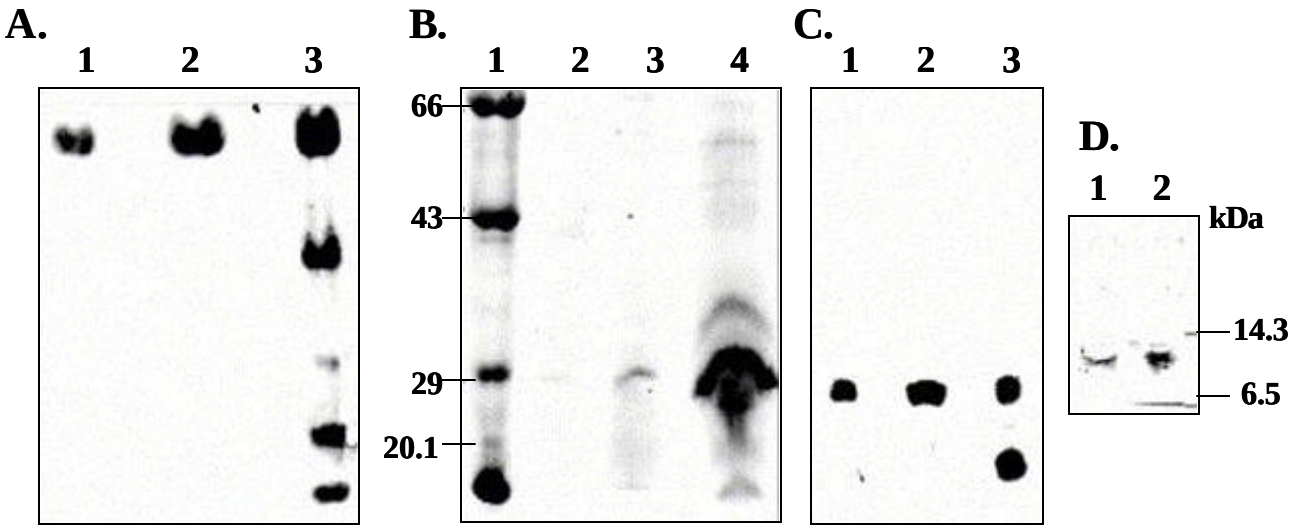


**Figure 2.1: Purification of *C. annuum* inhibitors**




(A) Elution profile of *C. annuum* inhibitors on DEAE-Sepharose ion exchange column. (B) Activity visualization of the pooled fractions of *C. annuum* inhibitors. Detail PI elution protocol and steps in activity visualization of inhibitor are given in the materials and method section.

**Figure 2.2: Electrophoretic characterization of purified *C. annuum* PIs**




(A) Activity visualization of purified inhibitors. Inhibitors were separated on 12% native-PAGE and visualized by gel X-ray film contact print method. Lane 1, CapA1; Lane 2, CapA2; Lane 3, *C. annuum* leaf extract.

(B) Molecular weight determination of purified inhibitors without a reducing agent. Inhibitors were separated on 15% SDS-PAGE and stained with CBB R-250 stain. Lane 1, molecular weight marker; Lane 2, CapA1; Lane 3, CapA2; Lane 4, *C. annuum* leaf extract.

(C) SDS-PAGE activity visualization of *C. annuum* PIs. After the separation of inhibitor proteins on 15% SDS-PAGE, the gel was washed 2.5% Triton X-100 solution to remove SDS and inhibitors were visualized by gel X-ray film contact print method. Lane1, CapA1; Lane 2, Cap A2; Lane 3, *C. annuum* leaf extract.

(D) Molecular weight determination of purified inhibitors with ß-ME. Lane 1, CapA1; Lane 2, CapA2. Inhibitor proteins were separated on 12% SDS-PAGE and silver stained.

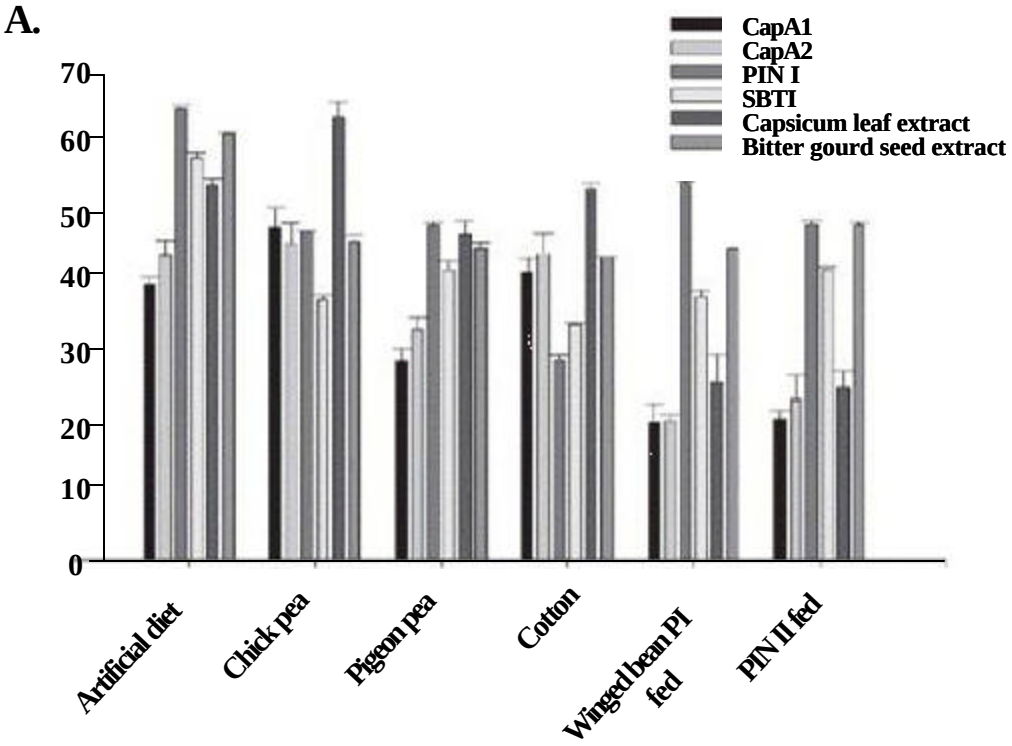
**Figure 2.3:**

**Maximum inhibition of total gut proteolytic activity (azocaesinolytic) of *H. armigera* larvae of different developmental stages, grown on host plants and fed on non-host plant PIs**

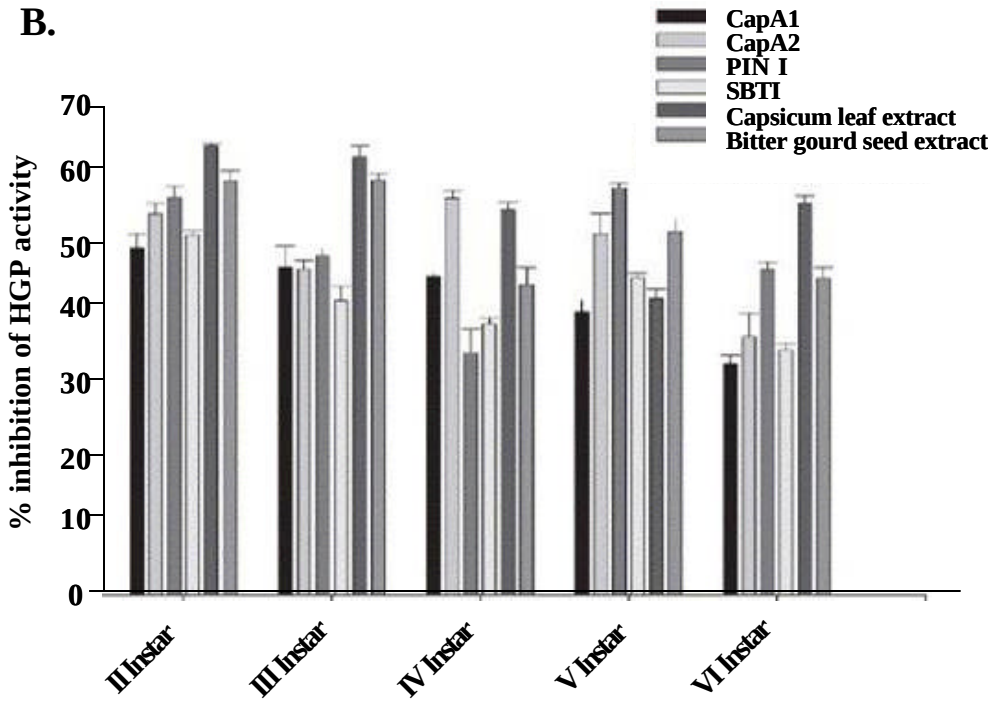
Four purified inhibitors (CapA1, 2.14 µg; CapA2, 0.46 µg; SBTI, 2.67 µg; and PIN I, 2.17 µg) and two PI extracts (*C. annuum* 19.8 µg and bitter gourd 83.34 µg) were used for the inhibition studies. Minimum inhibitor amounts required for maximum inhibition of chickpea HGPs were used. Each value is an average of three replications and standard error is indicated by error bars.

(A) Inhibition of artificial diet, chickpea, pigeon pea, cotton plants grown and winged bean, PIN II fed larval gut proteolytic activity.

(B) Inhibition of II, III, IV, V, and VI instar larval gut proteolytic activity. Azocaesin was used as a substrate for the analysis. Each experiment was repeated thrice.




**Gut proteinases of insects fed on various host and non-host plant PIs**




**Developmental stages of *H. armigera***

**Figure 2.4: Inhibition of *H. armigera* gut proteinase activity by purified CapA1, CapA2 and PIN I inhibitors**

Equal trypsin inhibitor units of individual inhibitors were tested against HGPs. Azocaesin was used as a substrate for the determination of total proteolytic activity and its inhibition by PIs. Each experiment was repeated thrice.

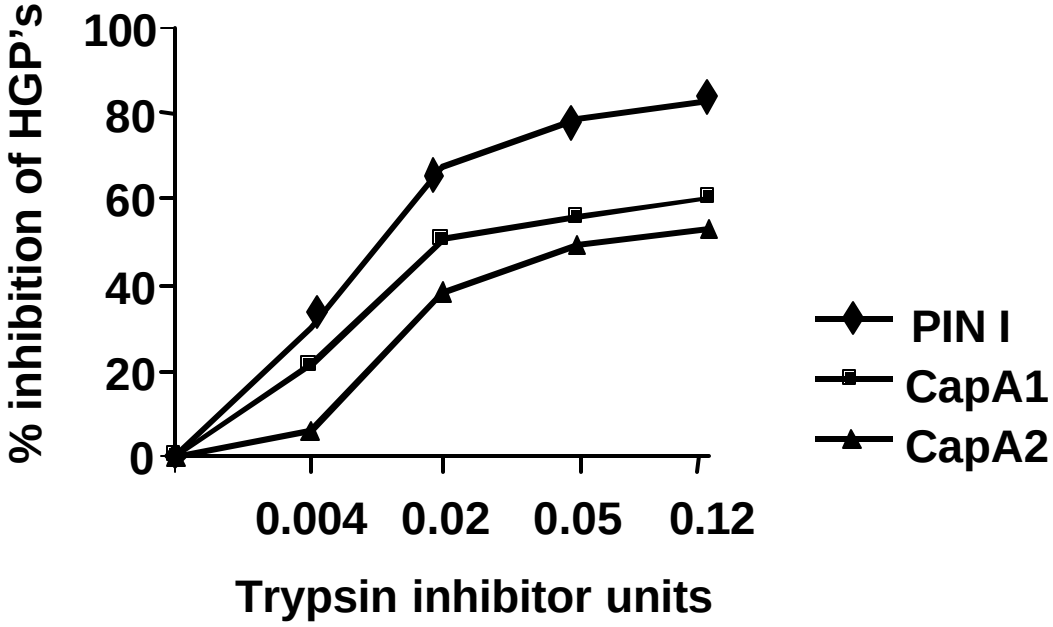


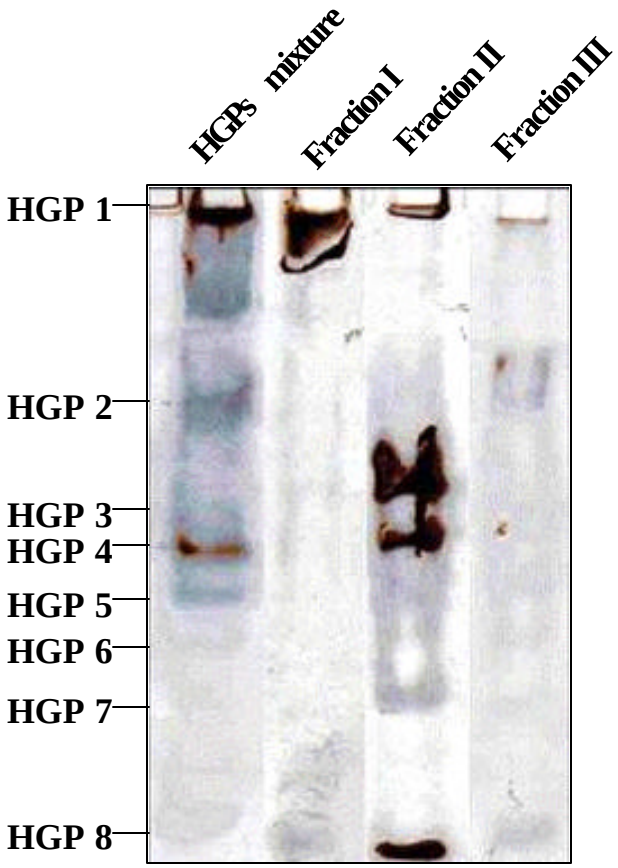
**Figure 2.5: Partial purification of *H. armigera* gut proteinases**

DEAE-Sepharose column was used for the fractionation of HGPs. The details of elution protocol are given in the materials and methods section.

(A) Visualization of proteinase isoforms. Fractions were separated on 10% native-PAGE and proteinase activity bands were visualized by gel X-ray film contact print method.

(B) Quantitative estimation of trypsin-like, chymotrypsin-like and elastase-like activity in the HGP extract and fractions I, II and III. Synthetic inhibitors of particular proteinase types were used for the inhibition of proteinases and residual proteolytic activity was calculated by azocaesinolytic assay. TLCK, chymostatin and elastatinol were used for the maximum inhibition of trypsin-like, chymotrypsin-like and elastase-like proteolytic activity, respectively.

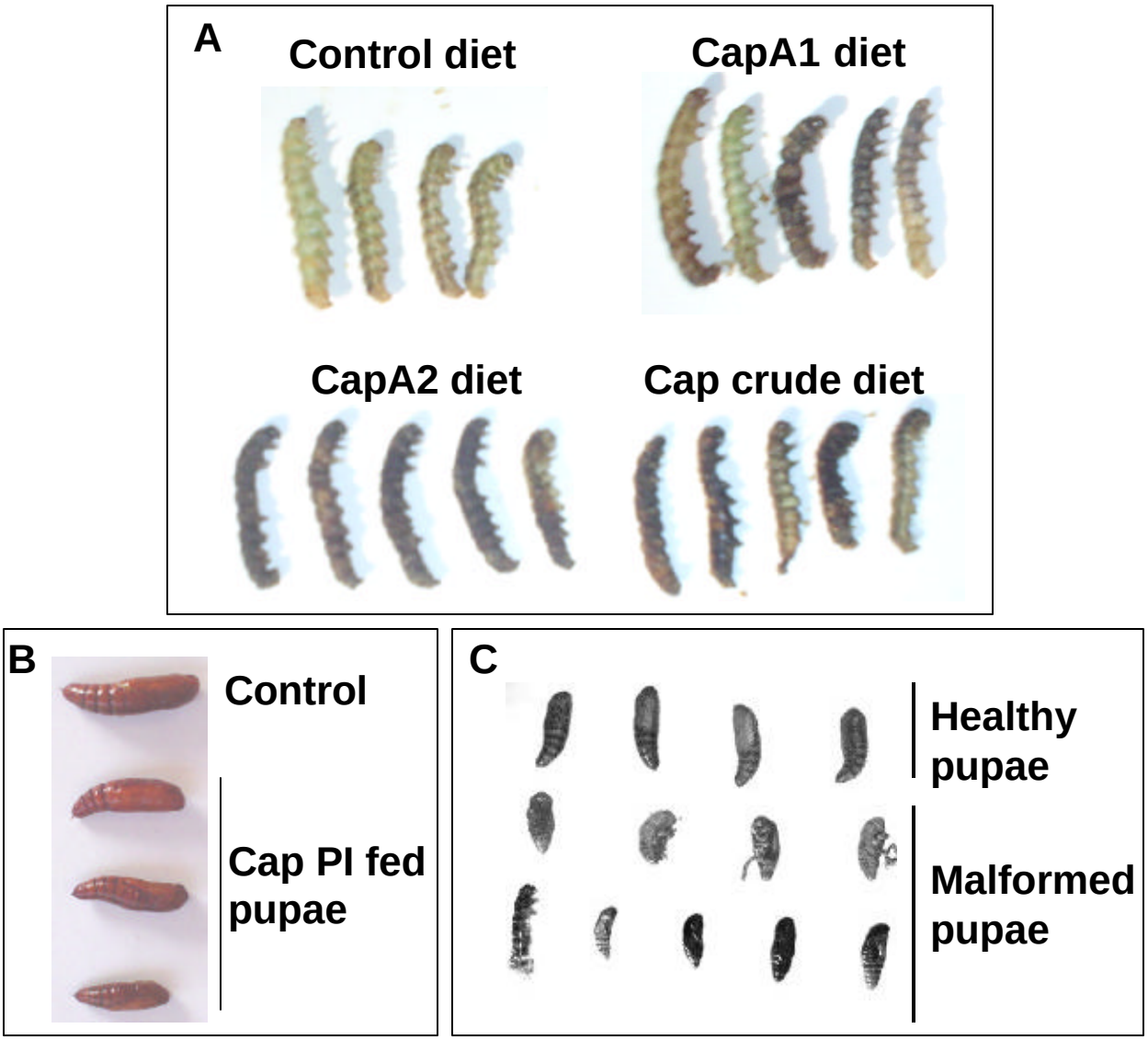
**A.**




**B.**

| | | | | |
|---|---|---|---|---|
| **% trypsin-like activity (TLCK)** | **49** | **37** | **46** | **53** |
| **% chymotrypsin-like activity (Chymostatin)** | **44** | **34** | **35** | **33** |
| **% elastase-like activity (Elastatinol)** | **18** | **20** | **18** | **13** |

**Figure 2.6: Representative picture of effect of *C. annuum* inhibitors on *H. armigera* larvae and pupae**




(A) Effect on larval growth, (B) Effect on pupal weight, (C) Malformed pupae.
Four different concentrations of purified and *C. annuum* leaf extract (0.5×, 1×, 3× and 6× per g of diet) were used for the feeding assays. 1× PI concentration is the minimum inhibitor amount required to inhibit the maximum possible proteolytic activity of whole insect gut.

**Figure 3.1: RT-PCR amplification of *CanPIs***

RT-PCR amplification, with AF039398 primers of mid stage fruit tissue (lane 1), stem tissue (lane 2) of the mature *C. annuum* plants and molecular weight marker (lane 3).

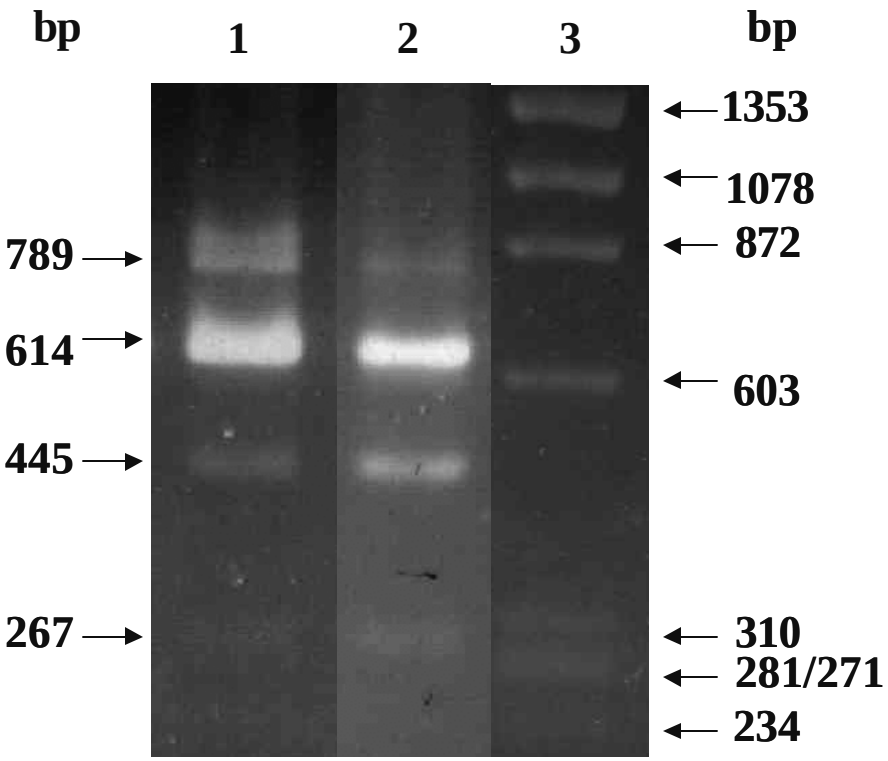


**Figure 3.2: Multiple sequence alignment of deduced amino acid sequences of CanPIs**

A. 1 and 2 IRD type CanPIs (CanPI-13 to CanPI-15 and CanPI-16 to CanPI-22, respectively)

B. 3 and 4 IRD type CanPIs (CanPI-3 to CanPI-5 and CanPI-7 to CanPI-11, respectively) and earlier reported sequences CanPI-1(AF039398) and CanPI-2 (AF221097).

## Figure 3.2A.

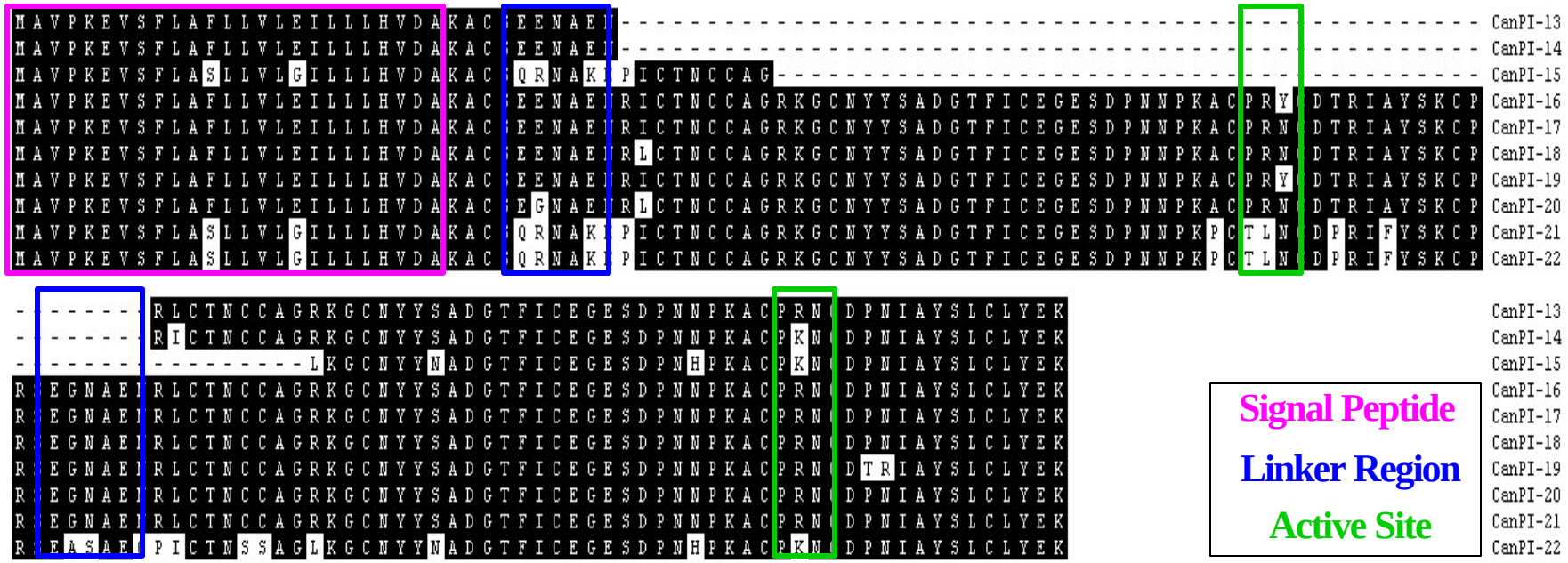


**Figure 3.2B.**

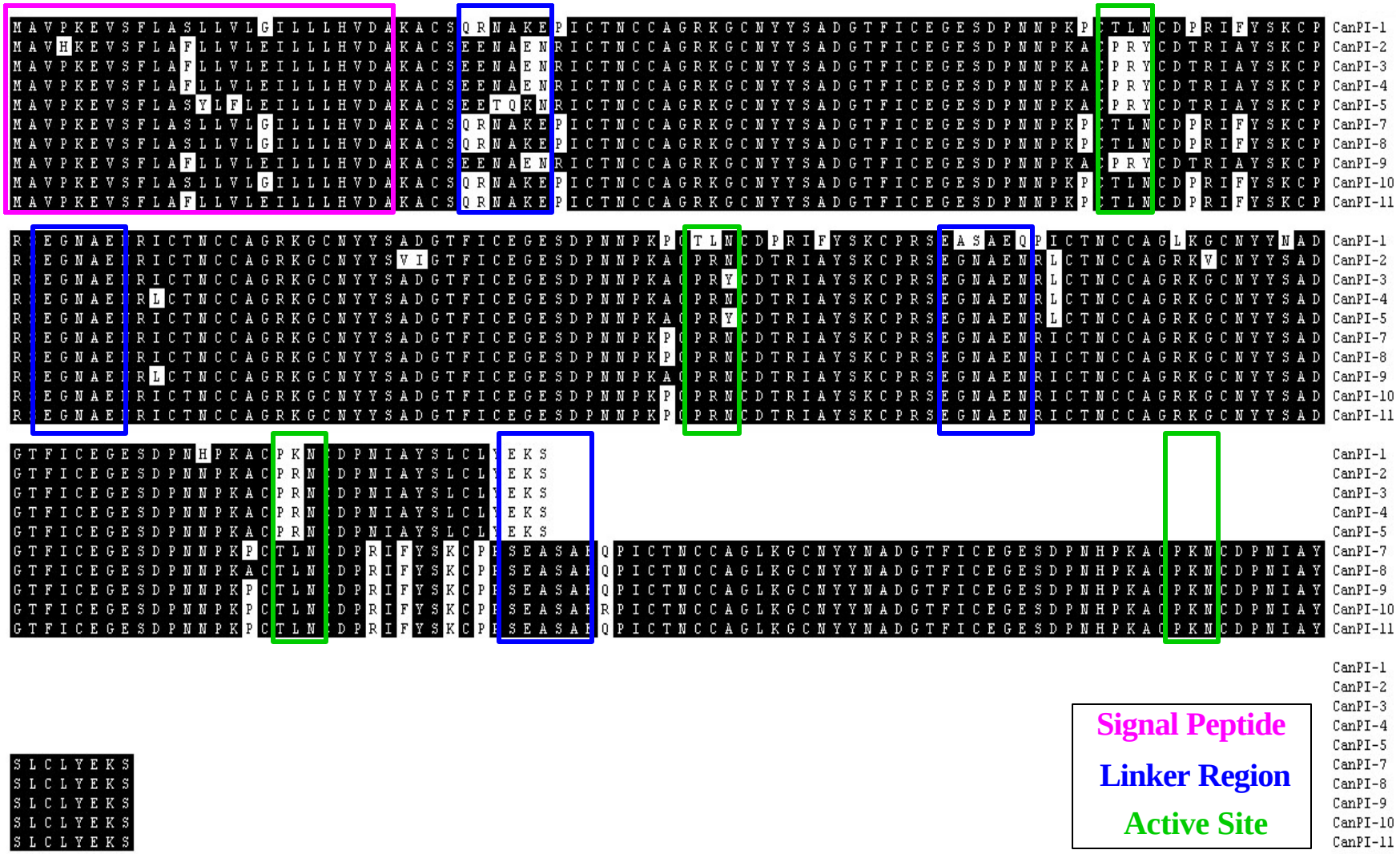

CanPI-1
CanPI-2
CanPI-3
CanPI-4
CanPI-5
CanPI-7
CanPI-8
CanPI-9
CanPI-10
CanPI-11
Signal Peptide
Linker Region
Active Site

**Figure 3.3: Sequence similarity matrices of CanPIs**

A. 1- IRD type CanPIs, B. 2- IRD type CanPIs, C. 3- IRD type CanPIs, D. 4- IRD type CanPIs.

**A.**

**1 IRD *CanPIs***

**CanPI-13**(1R-1) ID

**CanPI-14**(1R-2) 0.977 ID

**CanPI-15**(1R-3) 0.863 0.886 ID

**B.**

**2 IRD *CanPIs***

**CanPI-16**(2R-1) ID

**CanPI-17**(2R-3) 0.993 ID

**CanPI-18**(2R-3) 0.986 0.993 ID

**CanPI-19**(2R-4) 0.986 0.979 0.972 ID

**CanPI-20**(2R-5) 0.979 0.986 0.993 0.965 ID

**CanPI-21**(2R-6) 0.910 0.917 0.910 0.897 0.910 ID

**CanPI-22**(2R-7) 0.835 0.842 0.835 0.821 0.835 0.924 ID

**CanPI-23**(2R-8) 0.157 0.157 0.157 0.157 0.164 0.130 0.123 ID

**C.**

**3 IRD *CanPIs***

**CanPI-1**(3R-1) ID

**CanPI-2**(3R-2) 0.848 ID

**CanPI-3**(3R-3) 0.863 0.975 ID

**CanPI-4**(3R-4) 0.863 0.975 0.990 ID

**CanPI-5**(3R-5) 0.853 0.946 0.970 0.960 ID

**D.**

**4 IRD *CanPIs***

**CanPI-7**(4R-1) ID

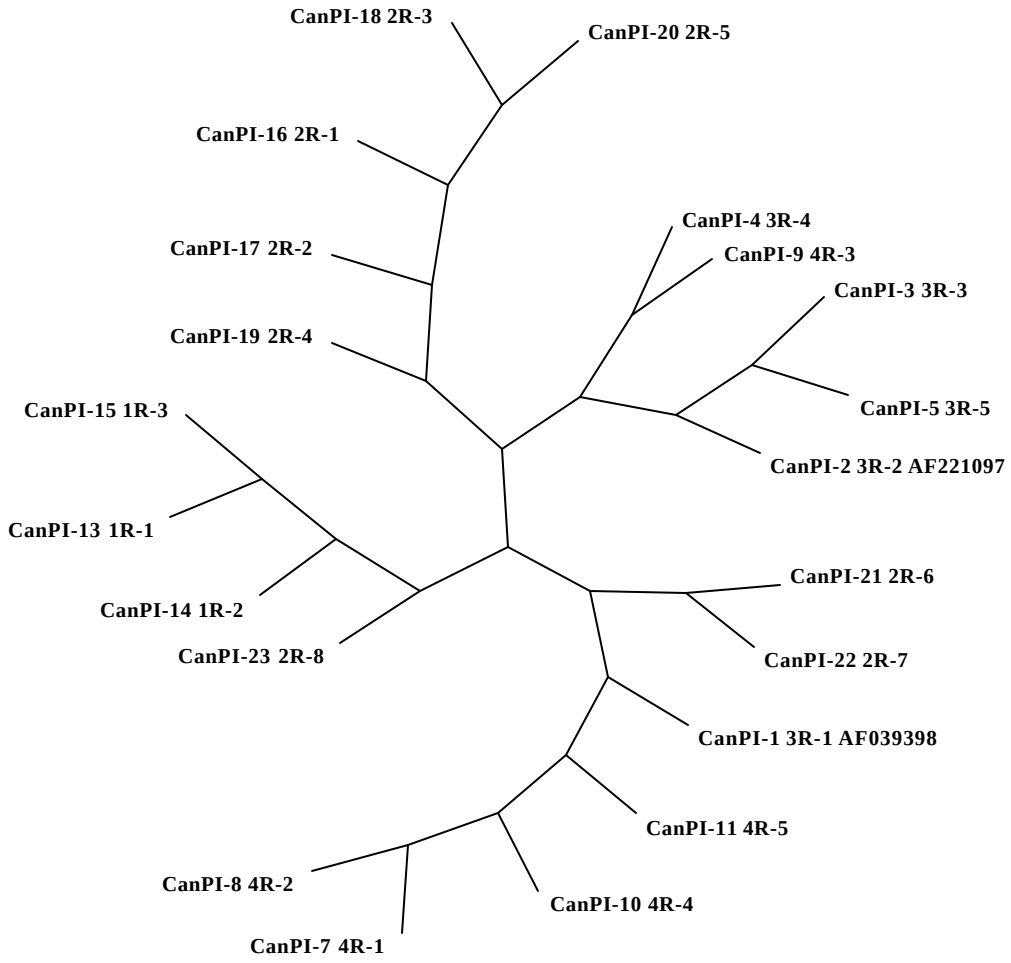
**CanPI-8**(4R-2) 0.996 ID

**CanPI-9**(4R-3) 0.942 0.939 ID

**CanPI-10**(4R-4) 0.996 0.992 0.939 ID

**CanPI-11**(4R-5) 0.992 0.988 0.950 0.988 ID

**Figure 3.4: Radial dendrogram of full length deduced AA sequences all CanPIs**



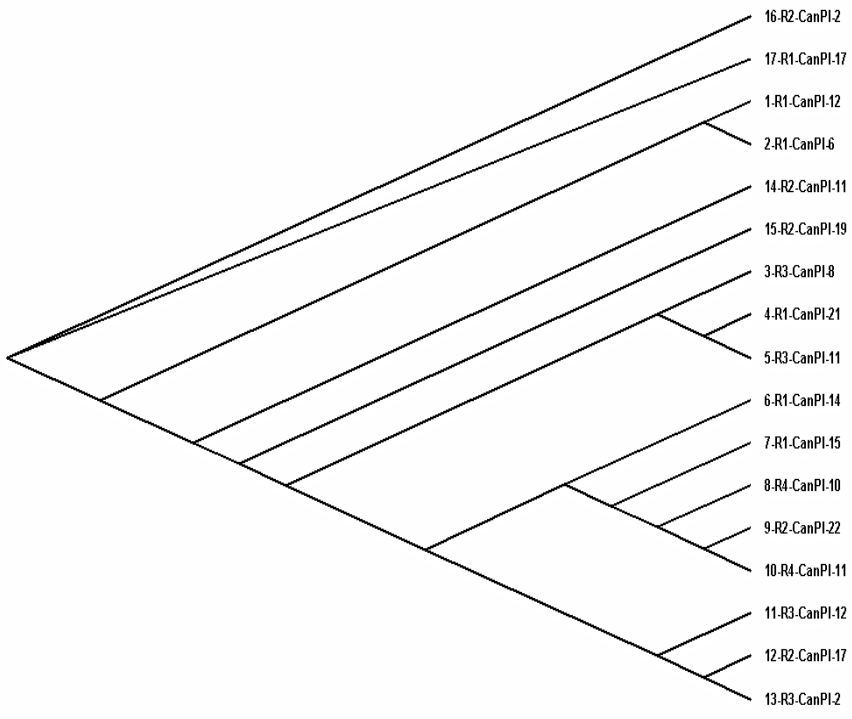

All the CanPIs (1 to 23) of 1 to 4 IRD types have been used to construct the dendrogram. Details of the CanPIs are as detailed in Table 3.2. IRD type of each CanPI is indicated after its name.

**Figure 3.5: Unique AA sequences of CanPI Inhibitory Repeat Domains (IRDs)**

**A.**

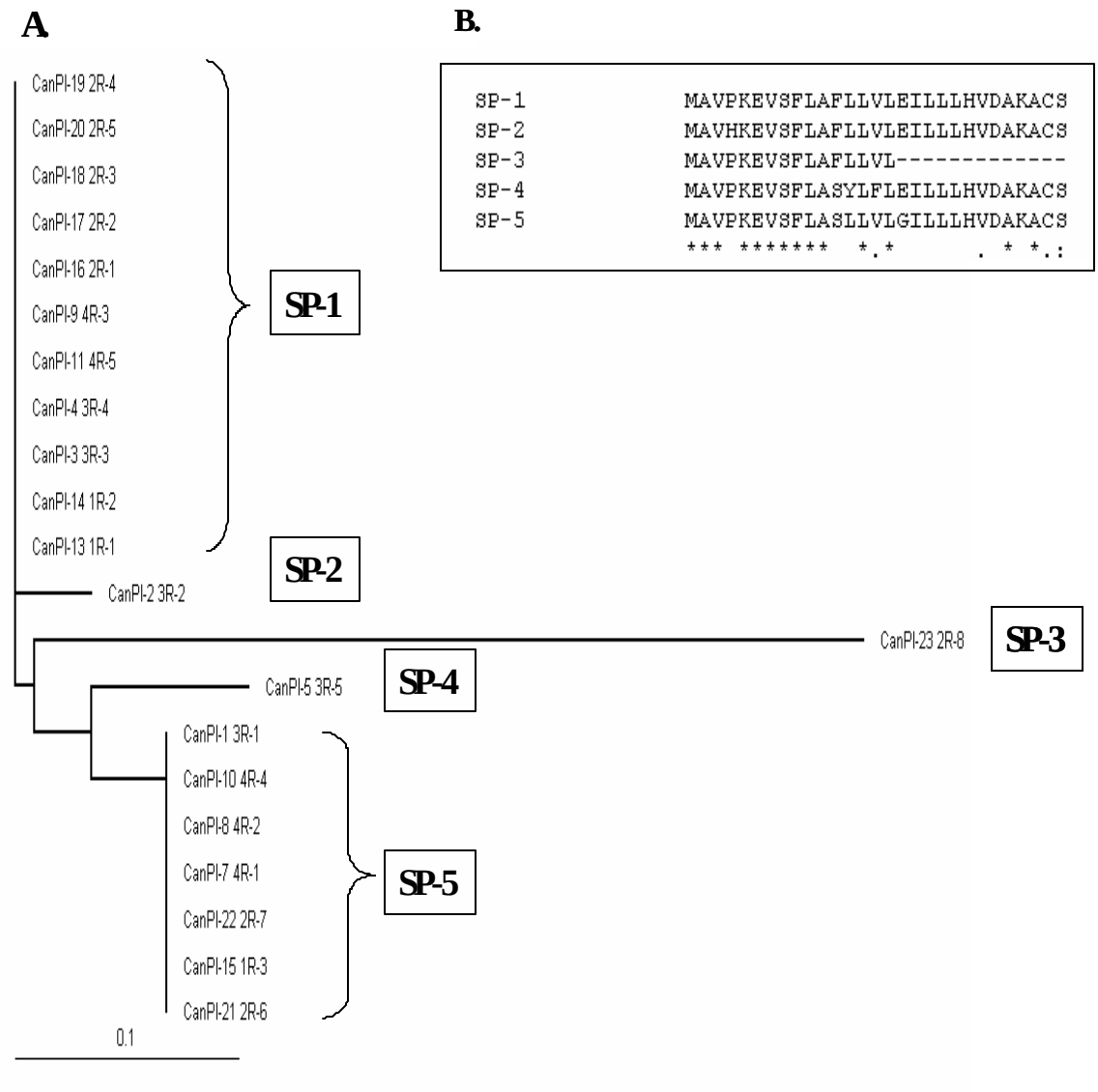
```
1-R1-CanPI-12       NRICTNCCAGRKGCNYYSADGTFICEGESDPNNPKACPRYCDTRIAYS
2-R1-CanPI-6        EPICTNCCAGRKGCNYYSADGTFICEGESDPNNPKACPRYCDTRIAYS
3-R3-CanPI-8        NRICTNCCAGRKGCNYYSADGTFICEGESDPNNPKACTLNCDPRIFYS
4-R1-CanPI-21       EPICTNCCAGRKGCNYYSADGTFICEGESDPNNPKPCTLNCDPRIFYS
5-R3-CanPI-11       NRICTNCCAGRKGCNYYSADGTFICEGESDPNNPKPCTLNCDPRIFYS
6-R1-CanPI-14       NRICTNCCAGRKGCNYYSADGTFICEGESDPNNPKACPKNCDPNIAYS
7-R1-CanPI-15       EPICTNCCAGLKGCNYYNADGTFICEGESDPNHPKACPKNCDPNIAYS
8-R4-CanPI-10       RPICTNCCAGLKGCNYYNADGTFICEGESDPNHPKACPKNCDPNIAYS
9-R2-CanPI-22       QPICTNSSAGLKGCNYYNADGTFICEGESDPNHPKACPKNCDPNIAYS
10-R4-CanPI-11      QPICTNCCAGLKGCNYYNADGTFICEGESDPNHPKACPKNCDPNIAYS
11-R3-CanPI-12      NRLCPNCCAGRKGCNYYSADGTFICEGESDPNNPKACPRNCDPNYCYL
12-R2-CanPI-17      NRLCTNCCAGRKGCNYYSADGTFICEGESDPNNPKACPRNCDPNIAYS
13-R3-CanPI-2       NRLCTNCCAGRKVCNYYSADGTFICEGESDPNNPKACPRNCDPNIAYS
14-R2-CanPI-11      NRICTNCCAGRKGCNYYSADGTFICEGESDPNNPKPCPRNCDTRIAYS
15-R2-CanPI-19      NRLCTNCCAGRKGCNYYSADGTFICEGESDPNNPKACPRNCDTRIAYS
16-R2-CanPI-2       NRICTNCCAGRKGCNYYSVIGTFICEGESDPNNPKACPRNCDTRIAYS
17-R1-CanPI-17      NRICTNCCAGRKGCNYYSADGTFICEGESDPNNPKACPRNCDTRIAYS
                    . :*.*..** * ****.. ************:**.*.  **..  *
```

**B.**




Multiple sequence alignment of deduced amino acid sequences of unique IRDs from 23 different CanPIs as detailed in Table 3.2. B. Dendrogram of the above IRDs, showing clustering of the closely related unique IRD sequences.

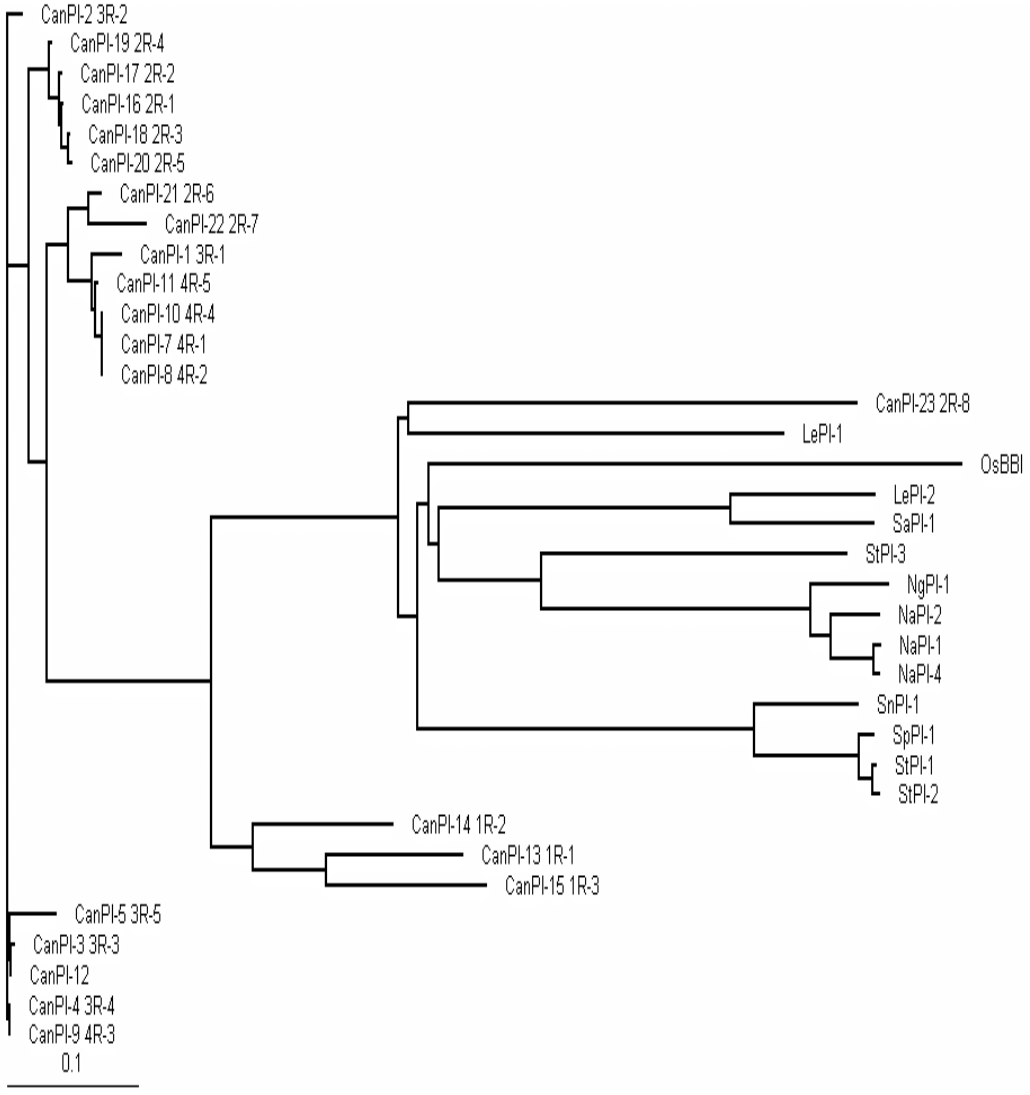
**Figure 3.6: Variations in the Signal Peptide (SP) sequences of CanPIs**




A. Dendrogram of 25 AA signal sequences from the full- length CanPIs, displaying the variability in the 5 unique sequences and the CanPIs representing them.

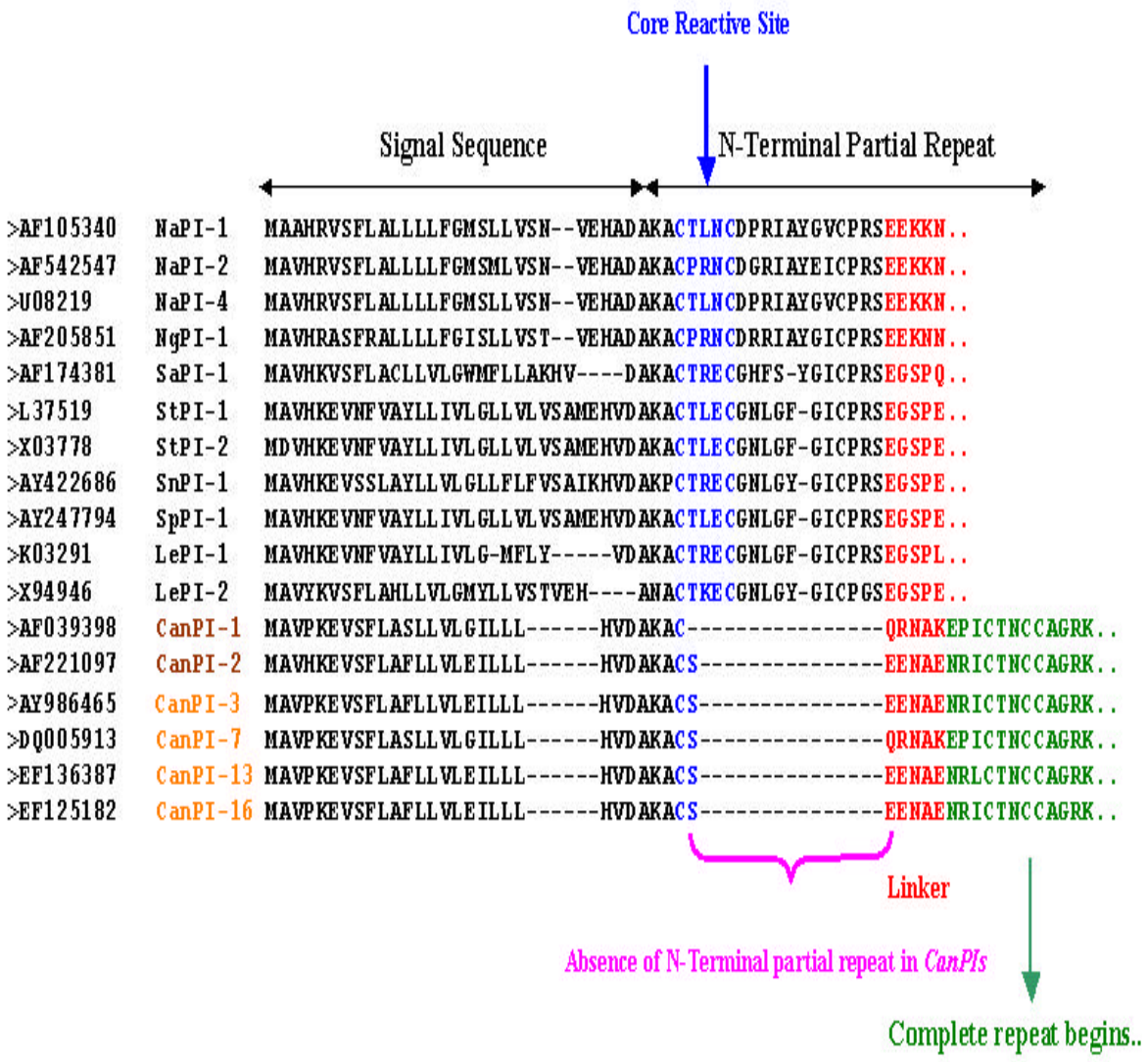
B. Multiple sequence alignment of the deduced AA sequences of the unique signal sequences (SP-1 to SP-5).

**Figure 3.7: Dendrogram of full- length *CanPI* genes and Pin -II type PIs from Solanaceae**



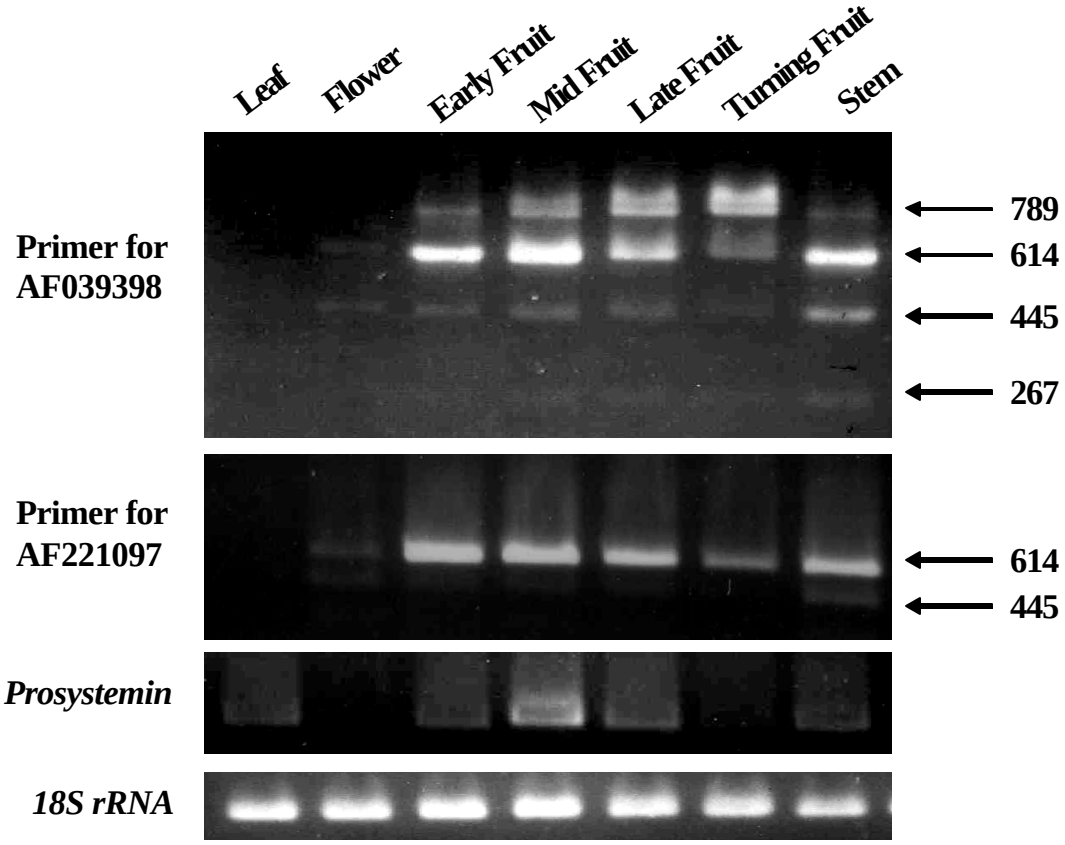

cDNA sequences of the PIs were used to construct the dendrogram. Details of *CanPI*s (CanPI-1 to CanPI-23 as per Table 3.2), LePI-1 (*Lycopersicum esculantum*, K03291, WIPII mRNA), LePI-2 (*Lycopersicum esculantum*, X94946, cevi57), StPI-1 (*Solanum tuberosum*, L37519), StPI-2 (*Solanum tuberosum*, X03778), StPI-3 (*Solanum tuberosum*, X99095, pin2), NgPI-1 (*Nicotiana glutiniosa*, AF205851, NgPI-1), NaPI-1 (*Nicotiana alata*, AF105340, Na-PI IV), NaPI-2 (*Nicotiana alata*, AF542547, 7-DomainTI), NaPI-4 (*Nicotiana alata*, U08219, Na-PI-II), SnPI-1 (*Solanum nigrum*, AY422686, PIN2b), SaPI-1 (*Solanum americanum*, AF174381, PIN2a), SpPI-1 (*Solanum phureja*, AY247794, PIN-II2x), OsBBI (*Oryza sativa*, U76004, BBI type PI) as out-group.

**Figure 3.8: Absence of a stretch of AA from *CanPIs***



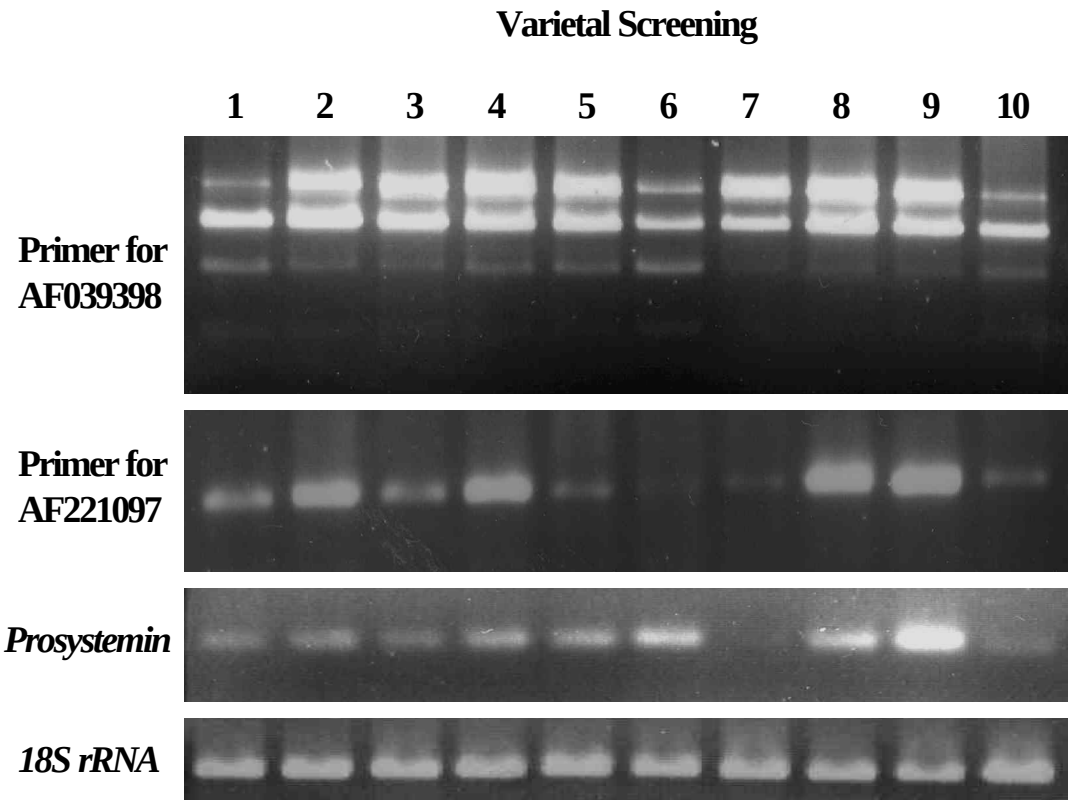

Multiple sequence alignment of N terminal region from the deduced AA sequences of Pin II PI precursors of representative members of Solanaceae and different CanPIs (1 to 4 IRD) representatives. The absence of a partial repeat (19 AA) at the N terminal of the CanPIs is evident. Details of CanPIs are as per Table 3.2.

**Figure 3.9: Organ specific *CanPI* and systemin expression pattern**



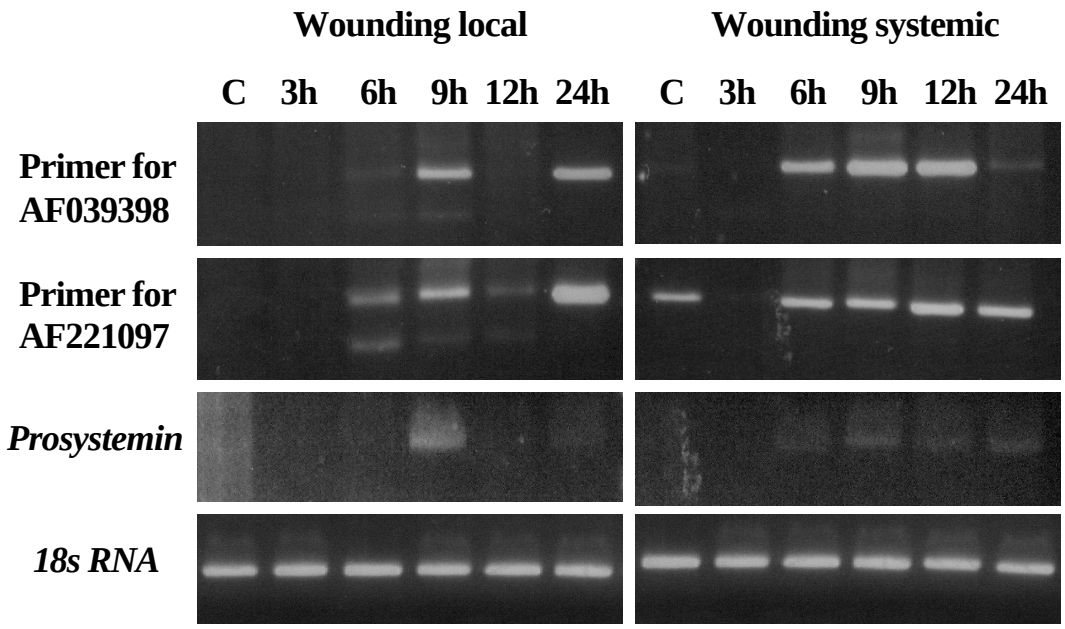

RT-PCR analysis of *CanPI* and systemin expression in different tissues of mature *C. annuum* plants performed using cDNA derived from equal quantities of total RNA and gene specific primers (*18s rRNA* amplification as in Row 4 as control).

**Figure 3.10: *C. annuum* varietal screening for *CanPI* expression**



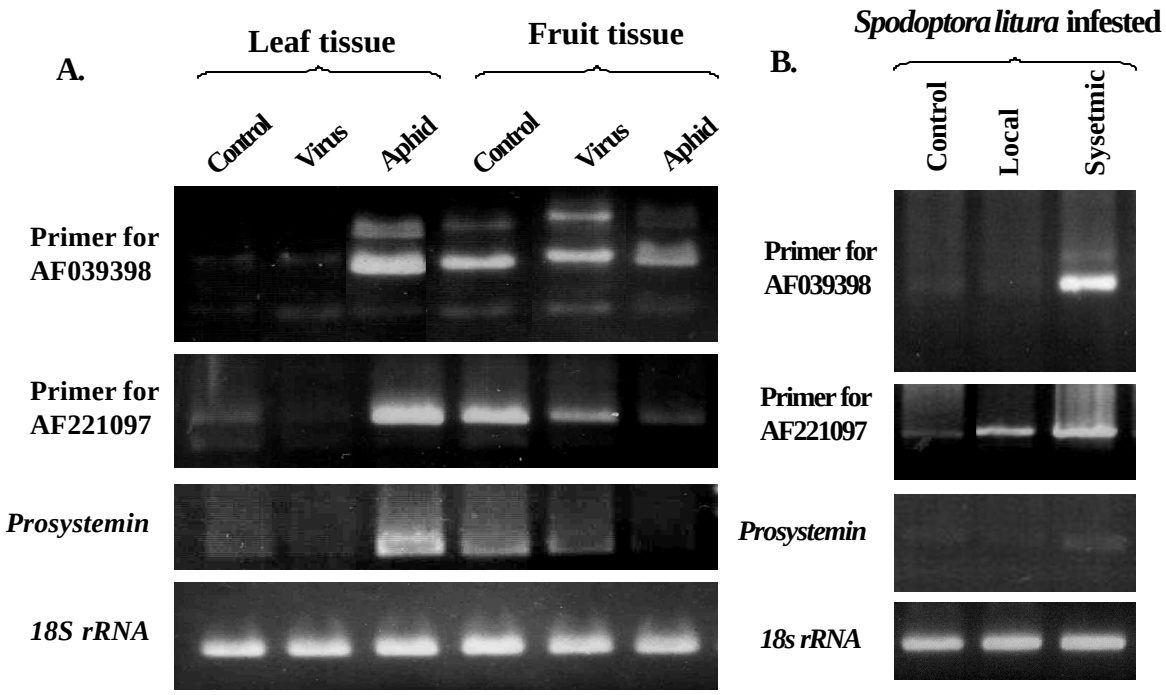

Semi-quantative RT-PCR amplification of fruit tissues from ten different varieties of *C. annuum*, screened using *CanPI* and *prosystemin* specific primers. *18s rRNA* was used as control.

**Figure 3.11**: **Changes in *CanPI* expression pattern on wounding**




Semi-quantative RT-PCR amplification of wounded *C. annuum* local and systemic tissues after 3, 6, 9, 12, and 24 hours after wounding, with *CanPI* and *prosystemin* specific primer pairs. *18s rRNA* was used as control.

**Figure 3.12: Changes in *CanPI* expression due to different biotic stresses**

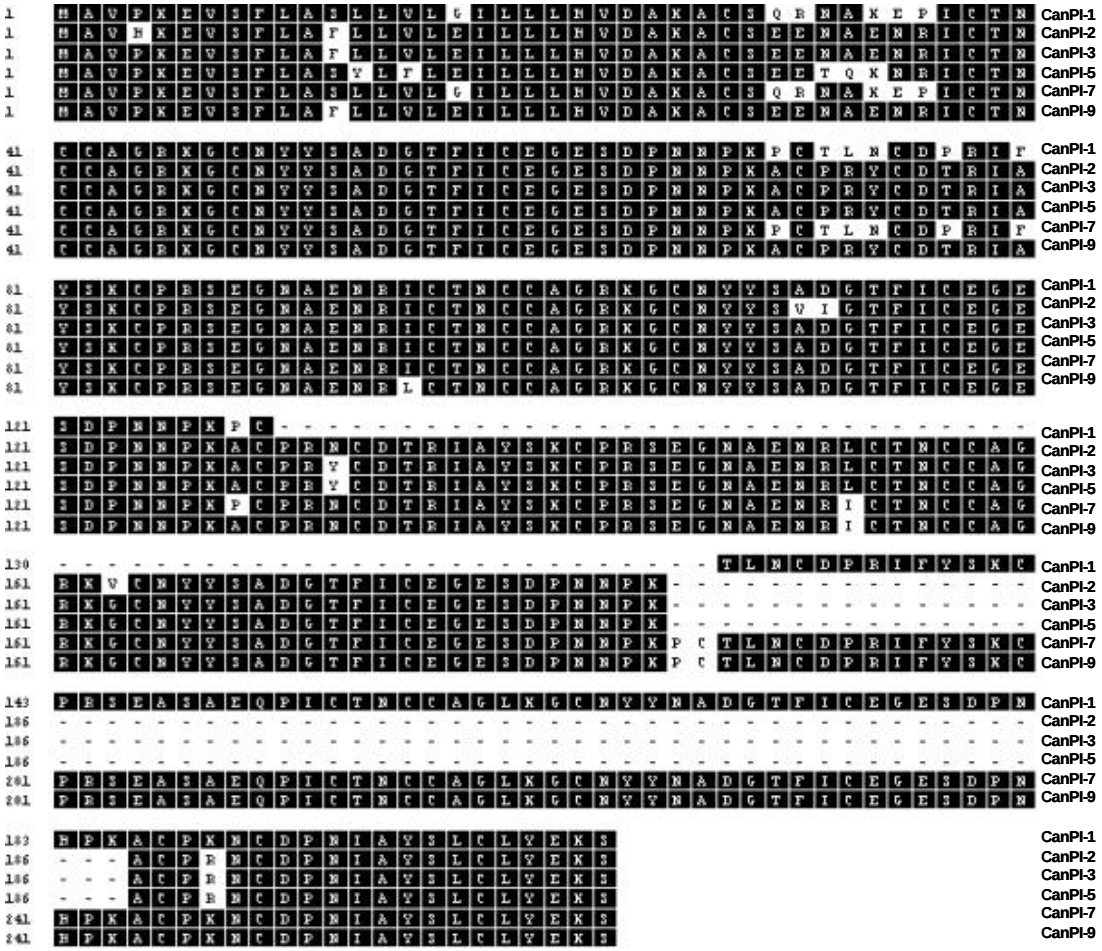



Semi-quantative RT-PCR amplification with *CanPI* and *prosystemin* specific primer pairs of *C. annuum* under various biotic stresses;

A. *C. annuum* leaf and fruit tissues of virus infected and aphid (*Myzus persicae*) infested plants. Leaf and fruit tissues from healthy plants were used as controls.

B. *Spodoptera litura* infested *C. annuum* local and systemic leaf tissues. Leaf tissue from healthy plants was used as control.

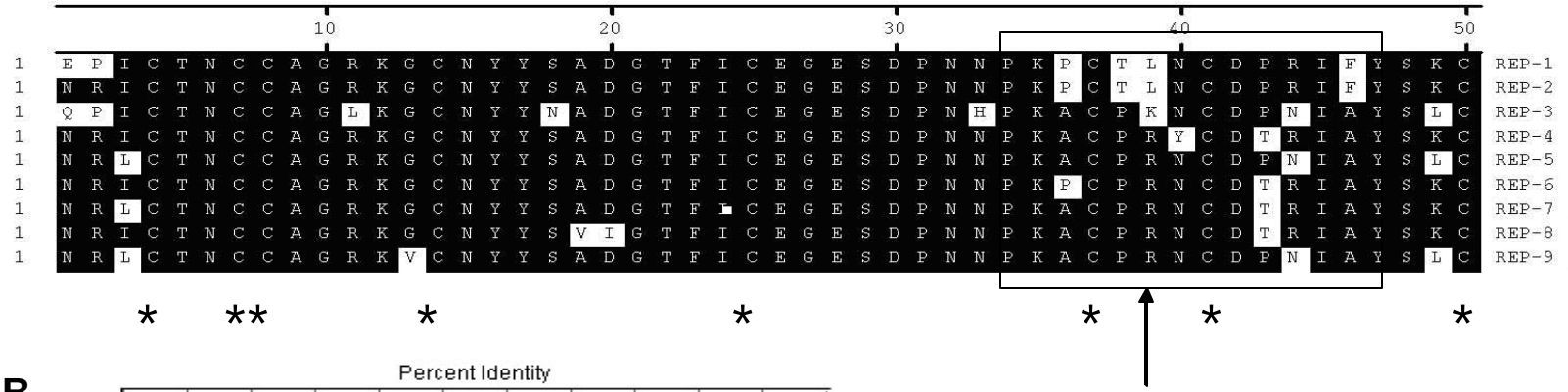
**Figure 4.1: Multiple sequence alignment of CanPIs selected for *Pichia pastoris* cloning**




Multiple sequence alignment of deduced amino acid sequences of all full length CanPIs having maximum divergence CanPI-3, CanPI-5, CanPI-7 and CanPI-9 and two earlier reported *C. annuum* PIs (CanPI-1, CanPI-2).

**Figure 4.2: Unique IRD diversity in the CanPIs selected for *P. pastoris* cloning**

**A.**




**B.**

Percent Identity

Divergence

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 96.0 | 80.0 | 84.0 | 82.0 | 88.0 | 84.0 | 82.0 | 80.0 | 1 |
| 2 | 4.1 | | 78.0 | 88.0 | 86.0 | 92.0 | 88.0 | 86.0 | 84.0 | 2 |
| 3 | 23.3 | 26.1 | | 80.0 | 86.0 | 80.0 | 80.0 | 78.0 | 84.0 | 3 |
| 4 | 18.0 | 13.1 | 23.3 | | 90.0 | 96.0 | 96.0 | 94.0 | 88.0 | 4 |
| 5 | 20.6 | 15.5 | 15.5 | 10.8 | | 90.0 | 94.0 | 88.0 | 98.0 | 5 |
| 6 | 13.1 | 8.5 | 23.3 | 4.1 | 10.8 | | 96.0 | 94.0 | 88.0 | 6 |
| 7 | 18.0 | 13.1 | 23.3 | 4.1 | 6.3 | 4.1 | | 94.0 | 92.0 | 7 |
| 8 | 20.6 | 15.5 | 26.1 | 6.3 | 13.1 | 6.3 | 6.3 | | 86.0 | 8 |
| 9 | 23.3 | 18.0 | 18.0 | 13.1 | 2.0 | 13.1 | 8.5 | 15.5 | | 9 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |

REP-1
REP-2
REP-3
REP-4
REP-5
REP-6
REP-7
REP-8
REP-9

(A) Multiple sequence alignment of deduced amino acid sequences of unique repeat regions from the CanPIs selected for *Pichia pastoris* cloning (CanPI-3, CanPI-5, CanPI-7 and CanPI-9) and two earlier reported *C. annuum* PIs (CanPI-1, CanPI-2). 8 conserved cystines marked by (*), the reactive site (P1) is marked by an arrow and the region of repeats close to the active site showing major sequence variation is marked by a box.

(B) Table showing the percent similarity in the upper triangle and percent divergence in the lower triangle of unique inhibitory repeat domains from the earlier reported CanPIs and the CanPIs selected for *P. pastoris* cloning.

**Figure 4.3: Cloning of CanPIs in pPIC9 vector for *Pichia pastoris* transformation**

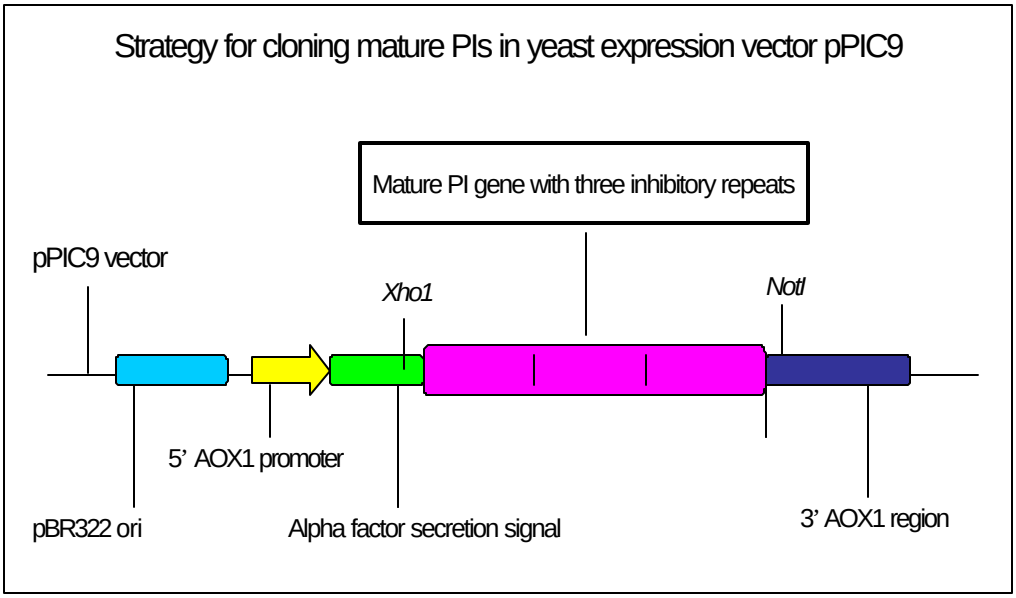
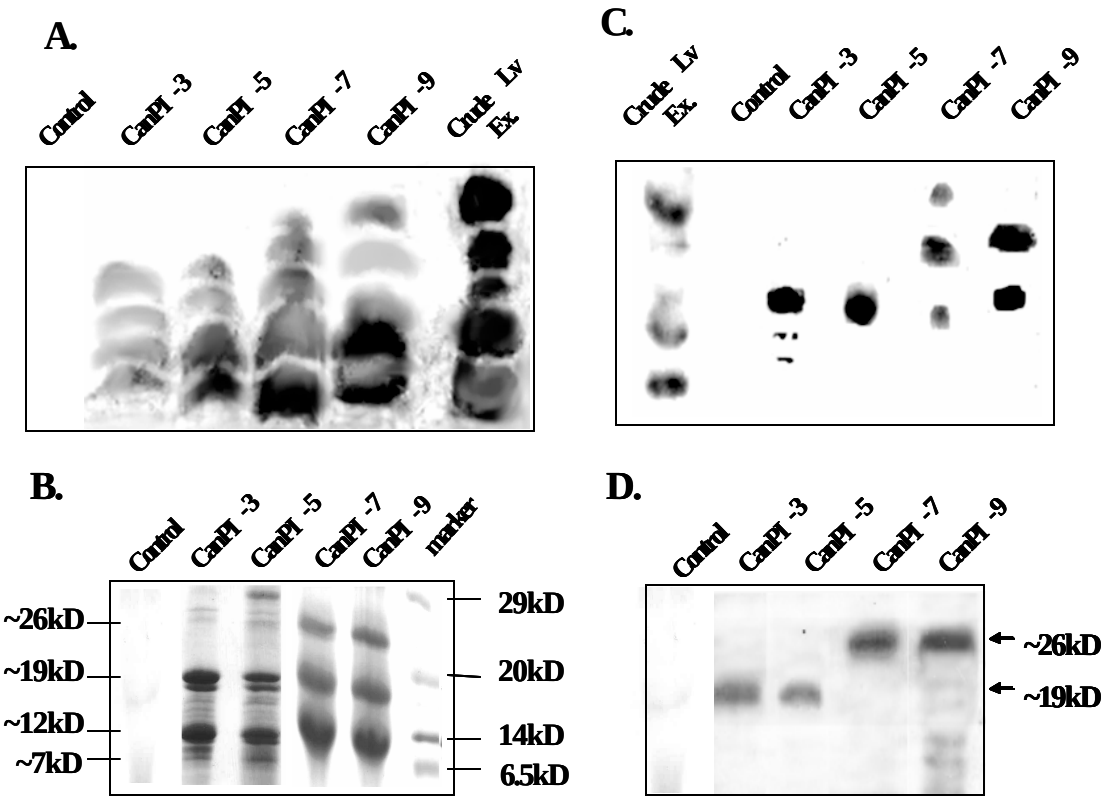


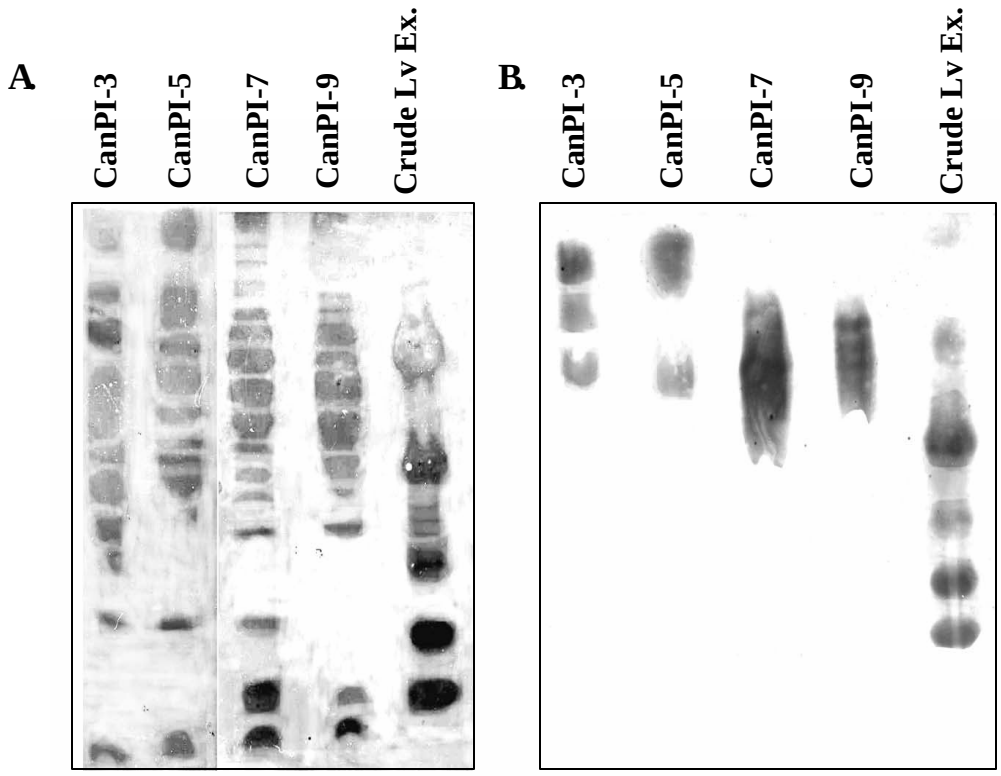

Figure showing the cloning of mature protein region of CanPIs in yeast expression vector pPIC9.

**Figure 4.4: In gel activity visualization and protein staining of recombinant CanPIs**




In gel trypsin inhibitory activity visualization of recombinant CanPIs run in SDS-PAGE gel. When *P. pastoris* cultured in BMMY medium (A) and in MM medium (C). Recombinant CanPI proteins resolved on SDS-PAGE and stained with CBB when *P. pastoris* cultured in BMMY medium (B) and in MM medium (D).

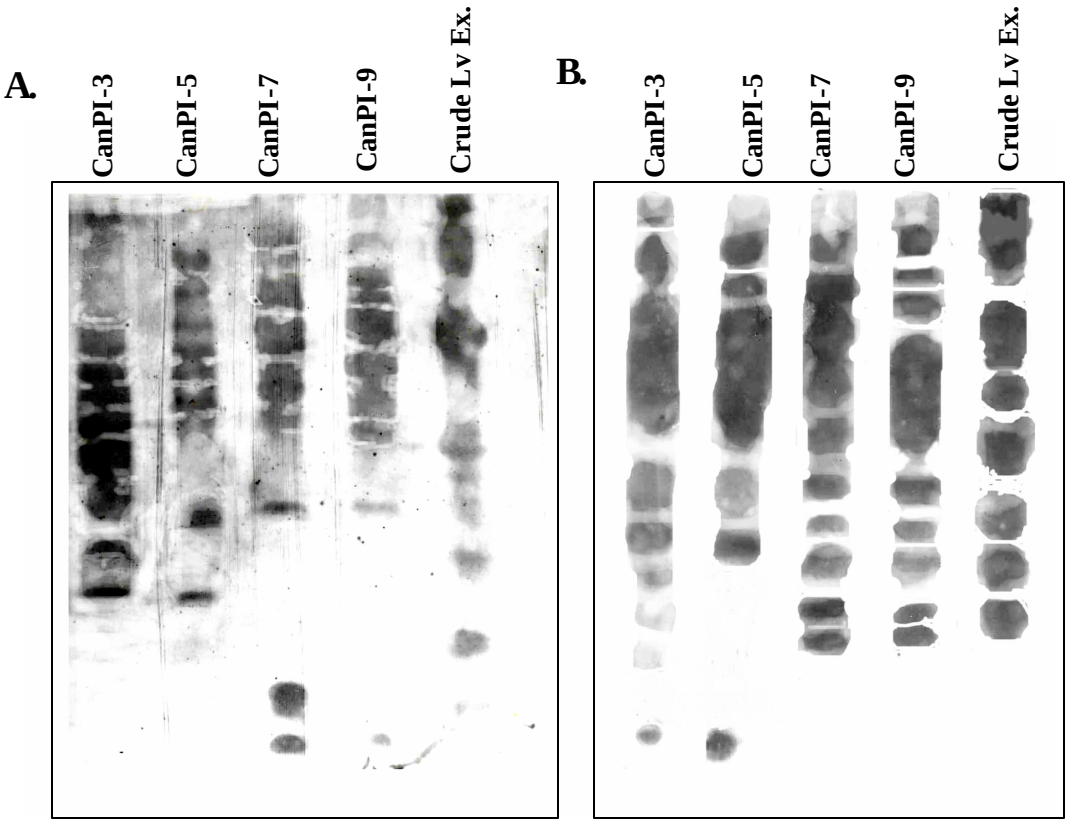
**Figure 4.5: Activity visualization of CanPIs resolved in Native PAGE by trypsin**




A. CanPIs expressed in BMMY medium,

B. CanPIs expressed in MM medium.

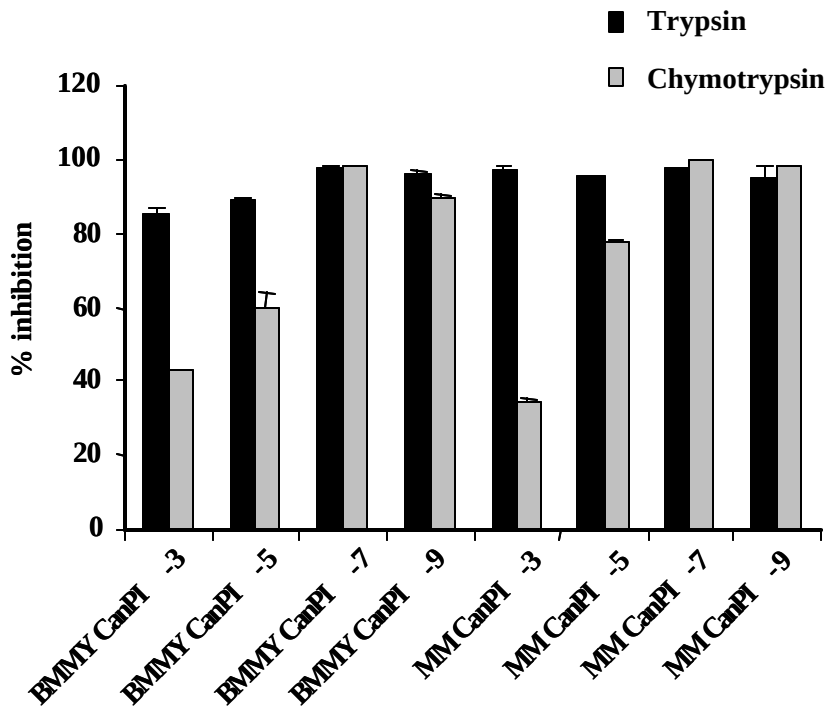
**Figure 4.6: Activity visualization of BMMY expressed CanPIs resolved in Native PAGE**




A. By bovine chymotrypsin

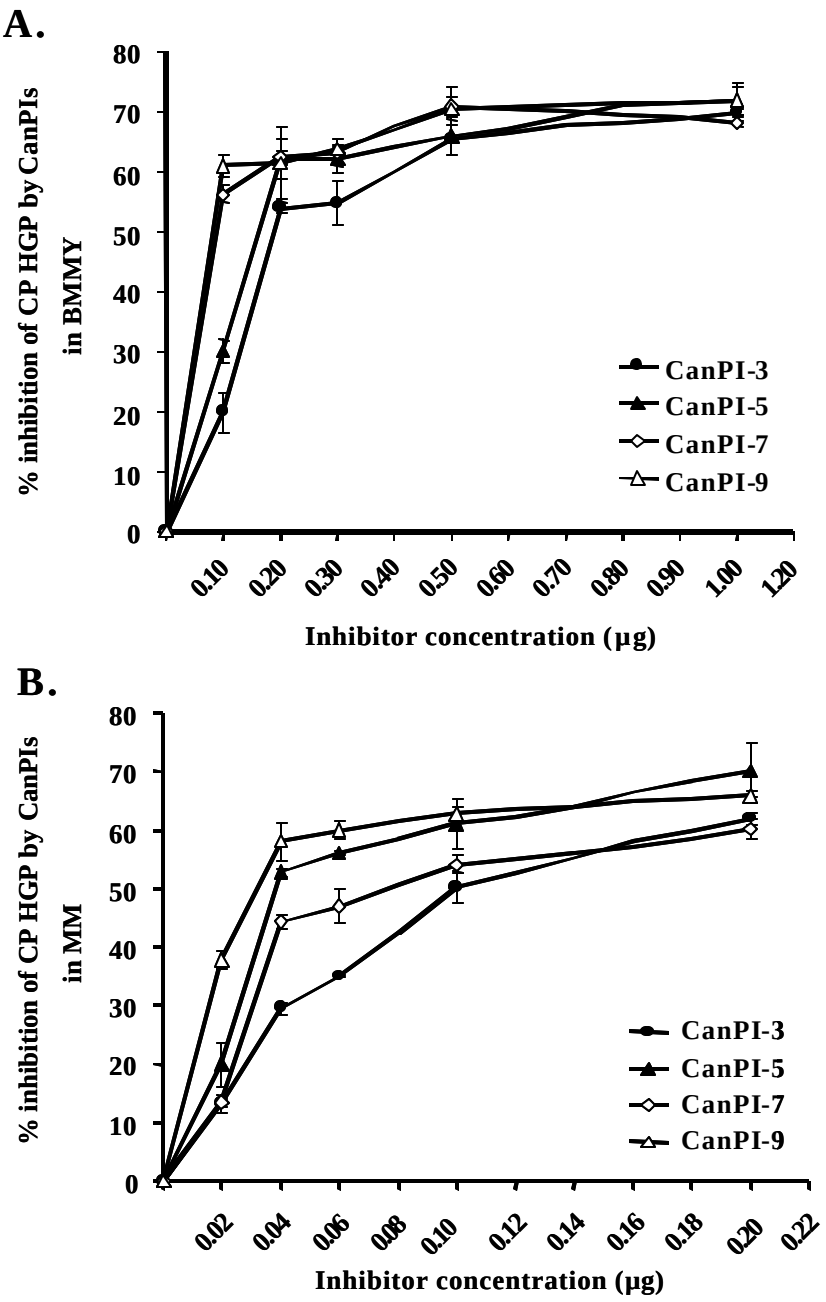
B. By *H. armigera* gut proteinases (HGPs)

**Figure 4.7: Inhibition of Trypsin and Chymotrypsin by recombinant CanPIs**



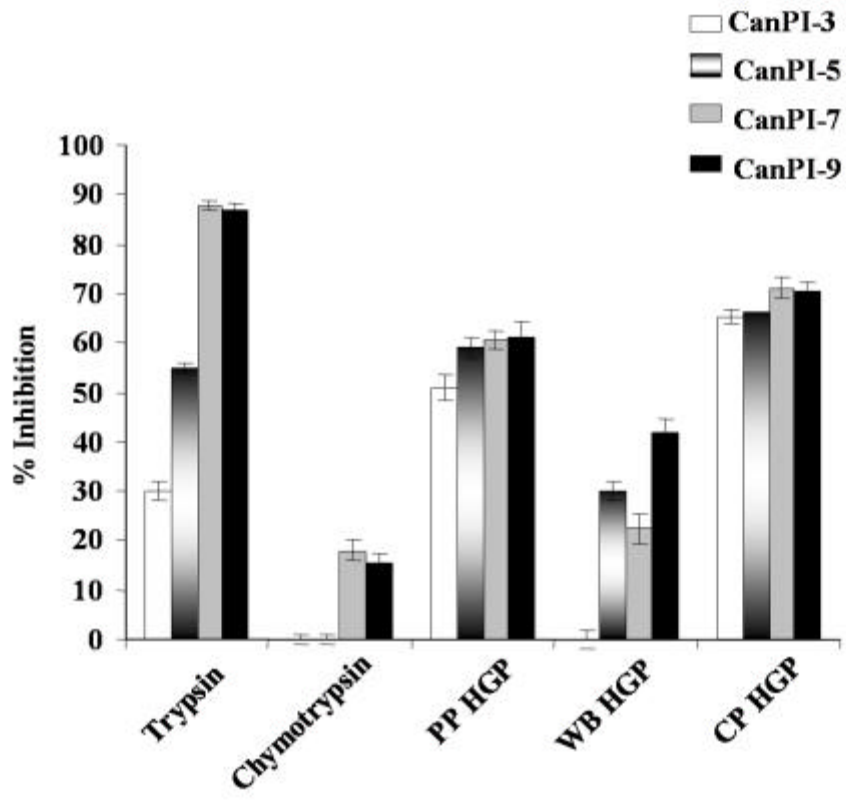

Maximum percent inhibition of bovine trypsin and chymotrypsin by CanPIs expressed in BMMY medium (1.5 µg) and MM medium (1.0 µg). Each value is an average of three replications and standard error is indicated by error bars.

**Figure 4.8: Inhibition of chickpea HGP by recombinant CanPIs**




Graphs showing the inhibition of chickpea HGP by increasing amounts of recombinant CanPIs (A) CanPIs expressed in BMMY medium (B) CanPIs expressed in MM medium. Each value is an average of three replications and standard error is indicated by error bars.

**Figure 4.9: Comparative inhibition of different enzymes by recombinant CanPIs**




Graph showing the inhibition of Bovine trypsin, chymotrypsin and HGPs obtained from Pigeon pea fed larvae (PP HGP), Winged bean inhibitor fed larvae (WB-HGP) and chickpea fed larvae (CP-HGP) with 0.5 µg CanPIs (BMMY expressed) each.

**Table 2.2: Interactions of purified form of C *apsicum annuum* PIs with the fractionated *H. armigera* gut proteolytic activity**

Total proteolytic activity, trypsin-like and chymotrypsin/elastase-like activity in fraction I, II and III was estimated by using Azocaesin, BApNA, and SAAPLpNA as substrates respectively. Equalized units (0.4 U) of each type of proteolytic activity were used in the assays. Purified CapA1, CapA2, PIN I and SBTI were used for inhibition of these proteolytic activities. % values indicated in the table are the maximum proteolytic activity inhibition by respective PIs. Each value is shown as mean ± S. E. (n=3). Values in the parentheses are the protein amounts in µg required for the maximum inhibition of HGPs

| Proteinase inhibitor | *H. armigera* gut proteinases | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Crude HGP | | | Fraction I | | | Fraction II | | | Fraction III | | |
| | Total activity | Trypsin-like activity | Chymotrypsin/elastase-like activity | Total activity | Trypsin-like activity | Chymotrypsin/elastase-like activity | Total activity | Trypsin-like activity | Chymotrypsin/elastase-like activity | Total activity | Trypsin-like activity | Chymotrypsin/elastase-like activity |
| Cap A1 | 59 ± 0.75 (9) | 81 ± 0.09 (8.2) | 10 ± 0.71 (11) | 40 ± 1.07 (7.9) | 86 ± 0.26 (1.7) | 15 ± 0.33 (4.2) | 55 ± 1.79 (7.9) | 82 ± 0.55 (3) | 60 ± 0.05 (4.2) | 49 ± 1.79 (7.9) | 70 ± 0.33 (3.15) | 31 ± 1.12 (4.2) |
| Cap A2 | 61 ± 0.22 (5) | 74 ± 0.44 (6.5) | 40 ± 0.09 (4) | 38 ± 1.22 (3.5) | 86 ± 0.04 (0.9) | 51 ± 0.08 (3.5) | 49 ± 0.42 (3.5) | 91 ± 0.42 (2.8) | 60 ± 0.51 (3.5) | 54 ± 2.84 (3.5) | 68 ± 0.28 (1) | 61 ± 0.83 (3.5) |
| PIN I | 83 ± 1.80 (15) | 65 ± 1.11 (15) | 82 ± 0.43 (12.5) | 82 ± 1.00 (11.5) | 79 ± 0.38 (3.9) | 77 ± 1.18 (2) | 98 ± 0.21 (11.5) | 84 ± 0.39 (3.9) | 71 ± 1.07 (2) | 66 ± 0.20 (11.5) | 62 ± 0.59 (3.9) | 63 ± 0.73 (2) |
| SBTI | 65 ± 1.19 (5) | 46 ± 0.37 (5) | 40 ± 0.82 (12.5) | 43 ± 0.89 (12) | 85 ± 0.36 (4.5) | 56 ± 0.74 (10) | 55 ± 1.46 (12) | 79 ± 1.42 (4.5) | 56 ± 0.62 (10) | 57 ± 1.72 (12) | 15 ± 0.39 (9) | 6 ± 0.41 (4) |

**Table 2.3: Effect of purified and crude inhibitor extract of *Capsicum annuum* leaves on growth and development of *Helicoverpa armigera***

Values represented in % for various parameters of larvae fed on *C. annuum* inhibitor diet compared to larvae fed on control diet. Values in the parentheses are µg of protein per gram of diet.

| | **Proteinase inhibitor** | | | | | | | | | | | | **Control** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Cap crude** | | | | **Cap A1** | | | | **Cap A2** | | | | |
| **Larval weight reduction (%)** | | | | | | | | | | | | | |
| a. III Instar | 18 | 19 | 24 | 35 | 12 | 12 | 34 | 38 | 7 | 12 | 42 | 40 | |
| b. IV Instar | 3 | 4 | 53 | 18 | 3 | 12 | 14 | 55 | 2 | 12 | 39 | 15 | |
| c. V Instar | 3 | 2 | 7 | 11 | 4 | 4 | 3 | 24 | 7 | 1 | 14 | 2 | |
| d. VI Instar | 1 | 3 | 2 | 11 | 2 | 4 | 7 | 11 | 4 | 3 | 14 | 2 | |
| **Reduction in maximum larval weight (%)** | 4 | 5 | 14 | 10 | 3 | 3 | 3 | 6 | 2 | 4 | 7 | 7 | |
| **Larval mortality (%)** | - | 30 | 27 | 23 | - | 17 | 17 | 20 | - | 23 | 30 | 40 | |
| **Pupation (%)** | | | | | | | | | | | | | |
| a. First two days | 78 | 56 | 40 | 22 | 81 | 63 | 37 | 50 | 58 | 68 | 40 | 30 | 88 |
| b. Last three to eight days | 22 | 44 | 60 | 79 | 19 | 37 | 63 | 50 | 42 | 32 | 60 | 70 | 11 |
| **Reduction in pupal weight (%)** | 3 | 6 | 8 | 9 | 5 | 0.3 | 5 | 5 | 7 | 5 | 13 | 13 | |
| **Malformed pupae (%)** | | 10 | 7 | 7 | - | 7 | 7 | 0 | - | 7 | 10 | 7 | 3 |
| **Reduction in egg laying/female (%)** | 70 | 53 | 68 | 74 | 35 | 58 | 69 | 71 | 56 | 52 | 64 | 79 | |
| **Egg hatching (%)** | 26 | 29 | 25 | 26 | 26 | 23 | 20 | 20 | 13 | 56 | 42 | 28 | 63 |