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Plant Pin-II Family Proteinase Inhibitors: Structural and Functional Diversity

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ABSTRACT

Potato inhibitor II (Pin-II) proteins are plant serine proteinase inhibitors (PIs) that occur in various plant species. Pin-II PIs are characterized by inhibitory repeat domains (IRDs) which form their functional units and show extensive sequence variation. Various studies have been conducted to gauge the occurrence (spatial and temporal) of Pin-II PIs and their appearance in response to biotic stresses mainly herbivore attack. Many hypotheses have been proposed to justify the mechanism which has led to the evolution of Pin-II PIs as well as the selection pressure in force. In spite of ample diversity, these molecules are highlighted by their conserved features with respect to their gene and protein sequence and structure. The structural studies highlight the crucial role of conserved residues in stabilizing the reactive loops and the three-dimensional conformation of Pin-II PIs. The remarkable flexibility of reactive loops allows their binding to a wide range of proteinases (either endogenous or from the pest). Apart from defense, Pin-II PIs have also been speculated to have a significant role in endogenous functions, namely regulation of proteolysis, macromolecular trafficking, programmed cell death and consequently aid the plant growth and development in respective tissue. There have been attempts to test these candidates for their potential in insect control. *In vitro* experiments and insect bioassays at laboratory scale have given encouraging results and led to the field experiments in order to develop transgenic plants fortified with Pin-II PIs. This has also helped judging the fitness costs that usually transgenic plants have to pay in return of incorporating the foreign trait.

Keywords: herbivores, inhibitory repeat domain, plant defense, proteinase inhibitors, Solanaceae, stress-induced regulation
Abbreviations: aa, amino acid; ***Can*PI**, *Capsicum annuum* proteinase inhibitor; **CI**, chymotrypsin inhibitor; **HGP**, *H. armigera* gut proteinase; **IF-MALDI-TOF**, Intensity fading matrix assisted laser desorption/ionisation time of flight; **IRD**, inhibitory repeat domain; **PI**, proteinase inhibitor; **Pin-II PIs**, Potato inhibitor II type proteinase inhibitors; **RSL**, reactive site loop; **TI**, trypsin inhibitor

CONTENTS

INTRODUCTION

Plant proteinase inhibitors (PIs) are proteins which form complexes with proteinases thereby inhibit their proteolytic activity. Several families of PIs have been reported depending on specificity towards target proteases their molecular mass and structure. Some of the serine PI families are Kunitz, Bowman-Birk, Squash and Wound-inducible (Pin-II) (Garcia-Olmedo *et al*. 1987; Ryan 1990). The constitutive expression of PIs in plants has been correlated with their role *in planta* whereas their wound-inducibility links them to a defense related role against insect pests. Both PIs and proteases are ubiquitously found in plants and are involved in regulation of the cellular and metabolic functions (Gomes *et al*. 2011). The co-occurrence of PIs and proteases in plants indicates their role in mutual control and fine tuning of each other's activities. In herbivores, proteases comprise a diverse group of digestive enzymes. Herbivore challenged plants upregulate the expression of PIs as a defense mechanism to overcome/restrict the attack. Interestingly some specific proteases are also up-regulated and they are responsible for enhancing the PI activity *in planta* (Horn *et al*. 2005). Upon ingestion by the insects, PIs inhibit the gut proteinases to bring about their starvation and lead to an anti-metabolistic effect in the insect. Several PIs have been isolated and characterized for their potential to inhibit the insect gut proteases and their effects on the overall growth and development of insect (Giri *et al*. 2005; Tamhane *et al*. 2007). Pin-II or Pot-II family of serine PIs is interestingly explored at gene, protein and functional level.

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Invited Review

These Pin-II PIs have a unique single or multi domain repeat structure with variations, also a wound/insect infestation induced up-regulation and expression, post-translational interactions with proteases leading to modification in PI protein structure, activity and function. Together all these features make them an intriguing subject area for plant and insect biologists as well as physiologists. In this review, we focus on this potato inhibitor-II (Pin-II) family of serine PIs which display a striking genetic and molecular diversity and a significant plant defense related role. The origin, evolution, diversity at gene and protein level, their interaction with proteinases, their influence on insect metabolism and their endogenous functions in the plant which remain rather unexplored is described and discussed.

OCCURRENCE

Pin-II PIs are one of the important plant serine PIs which have been studied extensively. They were initially found only in Solanaceae where wound induced up regulation of Pin-II PI genes and proteins was demonstrated and their functional co-relation to insect defense was established (Green and Ryan 1972). Expressed sequence tags and genomic database screening has led to the identification of many Pin-II homologs dispersed throughout the whole range of mono- and dicotyledonous plants, indicating more widespread occurrence of this family (Barta *et al*. 2002) (**Table 1**). The striking feature of Pin-II PIs is the presence of variable number of inhibitory repeat domains (IRDs), structurally forming multi-domain proteins. The number of inhibitory repeats varies from 1- to 8-IRDs amongst different members of Solanaceae.

In Solanaceae, wounding and insect attack lead to the release of a polypeptide hormone 'systemin' from its precursor (prosystemin) (Pearce *et al*. 1991). In most cases, systemin induces jasmonic acid metabolites through the octadecanoid pathway. The octadecanoid pathway metabolites play a major role in plant defense through the expression of long-distance defense signals, one of them being PIs (Howe and Ryan 1999; Ryan 2000; Kessler and Baldwin 2002; Lee and Howe 2003; Schmidt and Baldwin 2006). Systemin travels rapidly to the distal parts of the plant where it induces the JA pathway and regulates the expression of defense molecules including PIs (Li *et al*. 2002; Ryan and Pearce 2003; Narváez-Vasquez and Ryan 2004). Induced up regulation of Pin-II PIs has been noted in various Solanaceous plants like *Nicotiana* sp., *Capsicum annuum*, *Solanum* sp. in response to insect attack, wounding, systemin, methyl jasmonate, polyethylene glycol, salt, abscisic acid, cold stress and application of electric current (Kim *et al*. 2001; Moura and Ryan 2001; Tamhane *et al*. 2009). Experiments with JA biosynthesis and signaling defective tomato mutants provide new evidence that JA, rather than systemin, functions as the systemic wound signal, and that the biosynthesis of JA is regulated by the peptide systemin (Sun *et al.* 2011). The downstream mechanism that leads to regulated PI expression upon JA or systemin signaling remains relatively unexplored. Co-receptors of brassinosteroid receptor BK1 is shown to be involved in transducing JA levels to PI levels in *N. attenuata* (Yang *et al.* 2011)

The Pin-II PIs have been classified under I20 ('I' stands for inhibitor and '20' denotes the serial number) in the MEROPS database (http://merops.sanger.ac.uk) (Rawlings *et al*. 2004, 2008). The MEROPS database aims to "create an integrated source of information about peptidases" i.e. proteolytic enzymes or proteases, which has also been extended to include PIs.

Pin-II PIs are coded by one, two or multi-gene families in *Nicotiana*, *Solanum* and *Capsicum* sp., respectively. In *Nicotiana* most of the members show presence of single functional genomic copy of Pin-II PIs, though in its various species the numbers of IRDs vary from 2 to 8. 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) (**Fig. 1**).

Expression patterns of Pin-II PIs in the different tissues vary qualitatively, quantitatively as well as spatially and temporally. No inter-varietal difference could be observed in the expression patterns of *C. annuum* PIs. However, there are no reports available where the PI expression patterns have been studied among different genus and species. The flower tissue has significantly higher level of PI activity compared to the leaf, stem and fruit tissues (Damle *et al*. 2005; Tamhane *et al*. 2009) (**Table 1**).

Several novel and diverse Pin-II PIs having 1- to 4-IRDs (*CanPIs*) were isolated from developing fruit and stem tissues of *C. annuum.* Though all the four IRD forms were represented in both the tissues, stem tissue showed higher proportion of expression of 1- and 2-IRD *CanPIs* while fruit tissue showed higher expression of 3- and 4-IRD *CanPIs* (Tamhane *et al*. 2009). Wounding and biotic (virus, aphid and lepidopteran insect) stress to the plant, induced variable *CanPI* profiles. Wounding alone was insufficient to induce the up regulation of 4-IRD *CanPI* (**Fig. 2**). The evolutionary advantages of repetitive IRDs have not been established, but it can be predicted that repetitive domains provide plants with a more efficient use of transcription, translation and cell compartment targeting mechanisms (Heath *et al.* 1995). Constitutive and wound-induced accumulation of Pin-II mRNA has been observed in aerial parts of *Lycopersicum* (Peña Cortés *et al*. 1989). A 3-IRD Pin-II class transcript induced by auxin in tomato roots was characterized by Taylor *et al*. (1993). However, the detailed analysis of spatial and temporal expression patterns of various PI isoforms in Solanaceae members has not been made.

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 IRDs that are always separated by an intron of 100-200 bp (**Fig. 3A**). Rigorous analysis of gene structure revealed that the splicing motif is also conserved and found to be GT...AG (Kong and Ranganathan 2008). The last nucleotide of the exon 1 and the first two nucleotides of exon 2 always encode a Gly residue. This Gly (formed by the boundaries of two exons) in signal peptide is a conserved feature of Pin-II family PIs. Putative Pin-II genes from *A. thaliana* and *O. sativa* also show similar features in their gene structure. In case of tomato, a genomic clone of Pin-II PI gene showed regulatory region containing two wound responsive elements similar to box-WUN-motif. These may account for the wound inducible expression of the PI. In addition, ELI-box3 (Elicitor-box3), TCA-element (promoter) and ABRE (abscisic acid (ABA)–responsive element) were possibly involved in regulatory expression of tomato PI (Zhang *et al.* 2004).

The conserved Pin-II PI protein consists of an endoplasmic reticulum signal peptide of 25 amino acids (aa) followed by variable number of IRDs of ~55 aa. The IRDs are separated by 5 aa linker regions. In some Pin-II PIs, a vacuolar sorting signal is present at the C terminal region. In some PIs there are partial IRDs at the N- and C- terminal of the Pin-II PIs form a covalent bond to generate a functional IRD. Exceptionally, *C. annuum* PIs do not possess the N- and C- terminal partial IRDs. For multi-IRD Pin-II protein, there are two possible domain organizations: (1) tandem repeat domain organization where domains are arranged in beads-on-a-string way; or (2) circularly permuted domain organization which is formed by the association between two terminal half-repeats to form a PI domain (Schirra and Craik 2005). The 43 kDa precursor PI of *N. alata* NaProPI forms a circular 'clasped bracelet' conformation as a result of formation of disulfide bridges between the partial

Table 1 Occurrence and diversity of Pin-II PIs (Information presented here has been compiled from NCBI nucleotide database: Only full-length nucleotide sequences have been considered); "*" indicates name of the researcher who reported the sequence in NCBI database)

Genus	Species	Number of PIs	Number of IRDs	Tissue	Accession number	Reference
Nicotiana	*attenuata*	4	2, 6, 7	Leaf	AY426751	Lou *et al.* 2004
					DQ158200	Wu *et al.* 2006
					AY297103	*Patankar 2004
					AF542547	Zavala *et al.* 2004
	alata	2	6,4	Leaf	AF105340	Miller *et al.* 2000
				Flower	U08219	Atkinson *et al.* 1993
	clevelandii	2	2, 6	Leaf	DQ158203	Wu *et al.* 2006
					DQ158199	
	quadrivalvis	2	6	Leaf	DQ158202	Wu *et al.* 2006
					DQ158198	
	obtusifolia	2	6	Leaf	DQ158201	Wu *et al.* 2006
					DQ158197	
	rustica	1	7	Leaf	DQ158196	Wu *et al.* 2006
	corymbosa	1	2	Leaf	DQ158195	Wu *et al.* 2006
	acuminata	1	7	Leaf	DQ158194	Wu *et al.* 2006
	pauciflora	1	6	Leaf	DQ158193	Wu *et al.* 2006
	miersii	1	6	Leaf	DQ158192	Wu *et al.* 2006
	spegazzinii	1	5	Leaf	DQ158191	Wu *et al.* 2006
	linearis	1	5	Leaf	DQ158190	Wu *et al.* 2006
	tabacum	3	6	Leaf	DQ158189	Wu *et al.* 2006
					EF408803	Srinivasan *et al.* 2009
					Z29537	Balandin *et al.* 1995
	sylvestris	1	6	Leaf	DQ158188	Wu *et al.* 2006
	repanda	1	6	Leaf	DQ158187	Wu *et al.* 2006
	umbratica	1	5	Leaf	DQ158186	Wu *et al.* 2006
	simulans	1	5	Leaf	DQ158185	Wu *et al.* 2006
	megalosiphon	1	5	Leaf	DQ158184	Wu *et al.* 2006
	hesperis	1	6	Leaf	DQ158183	Wu *et al.* 2006
	benthamiana	1	4	Leaf	DQ158182	Wu *et al.* 2006
	cavicola	1	2	Leaf	DQ158181	Wu *et al.* 2006
	glutinosa	3	4, 6, 8	Leaf	AF205852	Choi *et al.* 2000
					AF205851	
					AF208020	Park *et al.* 2000
Solanum	*americanum*	2	2, 5	Leaf	AF174381	Xu *et al.* 2001
					AF209709	
	phureja	2	1	Leaf	AY517498	Bu *et al.* 2006
					AY247794	
	nigrum	2	1, 2	Leaf	AY422686	Schmidt *et al.* 2004
					GU133372	Hartl *et al.* 2010
	tuberosum	12	1, 2, 3	Leaf	U45450	Park and Thornburg 1996
					L37519	Jongsma *et al.* 1995
					DQ168323	Jørgensen *et al.* 2011
					DQ168321	
					DQ168313	
					EF469204	Li *et al.* 2011
					Z13992	Choi *et al.* 1992
					Z12753	Choi *et al.* 1990
					X03779	Sánchez-Serrano *et al.* 1986
					X03778	
					X04118	Keil *et al.* 1986
					AB110700	Shinogi *et al.* 2005
	lycopersicum	1	1	Leaf	L21194	Taylor *et al*. 1993
Lycopersicum	*esculentum*	3	1, 2	Leaf	AY007240	Kong and Ranganathan 2008
					X94946	Gadea *et al.* 1996
Capsicum	*annuum*	22	1, 2, 3, 4	Leaf	AF039398	Kim *et al.* 2001
				Stem	AF221097	Shin *et al.* 2001
				Fruit	AY986465-66	Tamhane *et al*. 2009
					DQ005912-16	
					DQ008950-51	
					EF136381-89	
					EF125182	
					EF144129	

repeat regions at the N- and C-terminal of the precursor (Scanlon *et al*. 1999). 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. 3B**).

The sequence of linker regions of 5 aa residues between IRDs is almost conserved in *Nicotiana* sp. (EEKKN), whereas in Pin-II PIs of other genera it is different though it functions similarly. For example, in *C. annuum* the linker sequences are QRNAK, EENAE, EASAE, EGNAE and EETQK. The linker region is very sensitive to proteases and is cleaved by endogenous proteinases *in planta* (Heath *et al.* 1995). Structurally linkers are not a part of IRD, but they link two IRDs on its either sides.

In all precursor Pin-II PI sequences, two 'types' of lin-

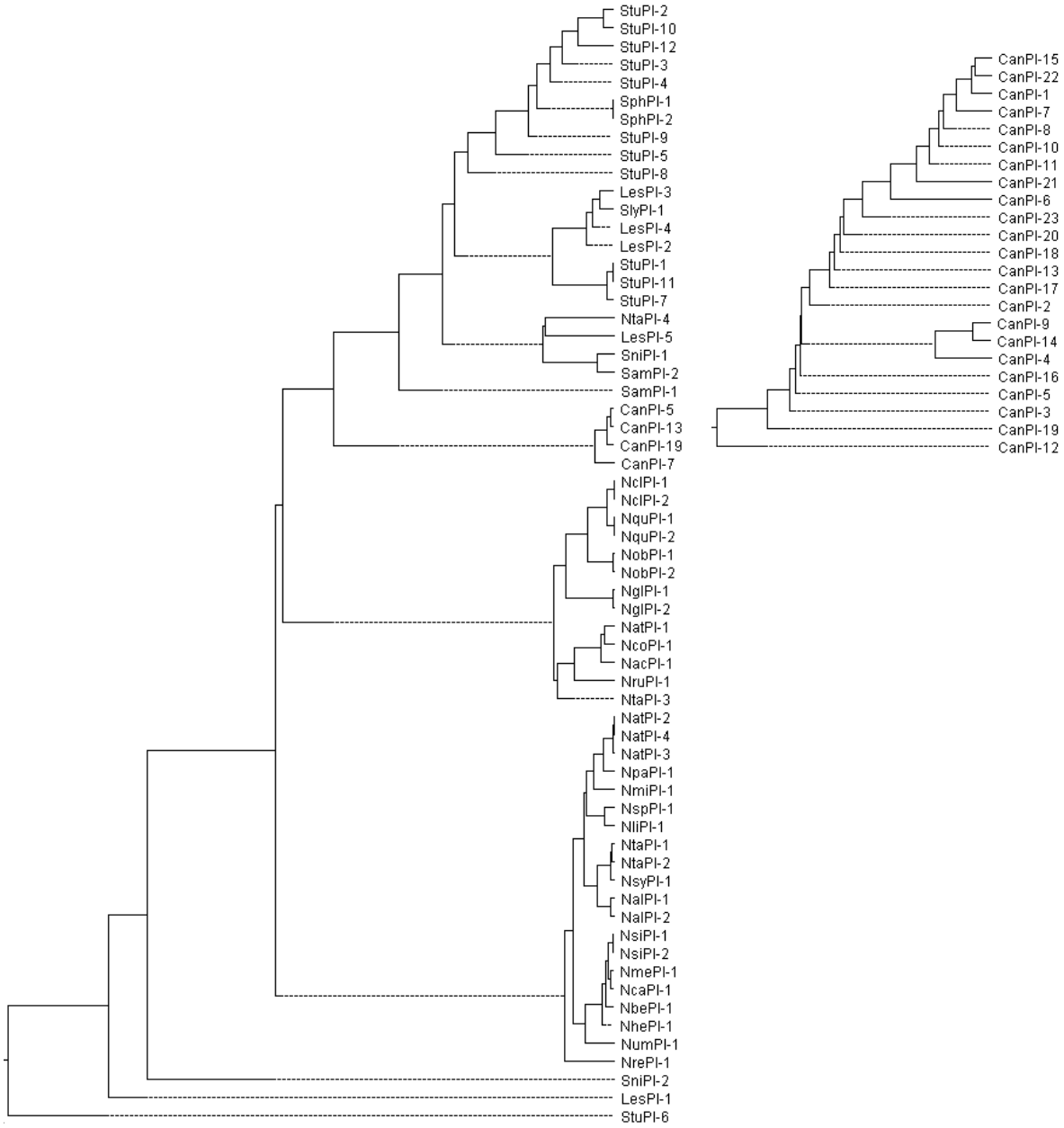

Fig. 1 Phylogenetic tree comprising the Pin-II PIs from Solanaceace genera *Nicotiana*, *Solanum*, *Capsicum* and *Lycopersicum*. A few representative PIs from *Capsicum annuum* have also been represented as an outgrown arm in the main Phylogenetic tree. This helps in giving an overview of the diversity and interrelationship of Pin-II PIs. The sequences were taken from NCBI as follows: *N. attenuata*: NatPI-1 (AY426751), NatPI-2 (DQ158200), NatPI-3 (AY297103), NatPI-4 (AF542547); *N. alata*: NalPI-1 (U08219), NalPI-2 (AF105340); *N. clevelandii*: NclPI-1 (DQ158203), NclPI-2 (DQ158203); *N. quadrivalvis*: NquPI-1 (DQ158202), NquPI-2 (DQ158198); *N. obtusifolia*: NobPI-1 (DQ158201), NobPI-2 (DQ158197); *N. rustica*: NruPI-1 (DQ158196); NcoPI-1 (*N. corymbosa*, DQ158195); NacPI-1 (*N. acuminata*, DQ158194); NpaPI-1 (*N. pauciflora*, DQ158193); NmiPI-1 (*N. miersii*, DQ158192); NspPI-1 (*N. spegazzinii*, DQ158191); NliPI-1 (*N. linearis*, DQ158190); *N. tabacum*: NtaPI-1 (Q158189), NtaPI-2 (DQ071272), NtaPI-3 (EF408803), NtaPI-4 (Z29537); NsyPI-1 (*N. sylvestris*, DQ158188); NrePI-1 (*N. repanda*, DQ158187); NumPI-1 (*N. umbratica*, DQ158186); NsiPI-1 (*N. simulans*, DQ158185); NmePI-1 (*N. megalosiphon*, DQ158184); NhePI-1 (*N. hesperis*, DQ158183); NbePI-1 (*N. benthamiana*, DQ158182); NcaPI-1 (*N. cavicola*, DQ158181); NglPI-1 (*N. glutinosa*, AF205852); NglPI-2 (*N. glutinosa*, AF205851); NglPI-3 (*N. glutinosa*, AF208020); *L. esculentum*: LesPI-1 (L21194), LesPI-2 (K03291), LesPI-3 (AY129402), Le sPI-4 (AY007240), LesPI-5 (X94946); SphPI-1 (*S. phureja*, AY517498), SphPI-2 (*S. phureja*, AY247794); *S. nigrum*: SniPI-1 (AY422686); SniPI-2 (GU133372); *S. tuberosum*: StuPI-1 (U45450), StuPI-2 (L37519), StuPI-3 (DQ168323), StuPI-4 (DQ168321), StuPI-5 (DQ168313), StuPI-6 (EF469204), StuPI-7 (Z13992), StuPI-8 (Z12753), StuPI-9 (X03779), StuPI-10 (X03778), StuPI-11 (X04118); SlyPI-1 (*S. lycopersicum*, AB110700); SamPI-1 (*S. americanum*, AF174381), SamPI-2 (*S. americanum*, AF209709); *C. annuum*: CanPI-7 (DQ005913), CanPI-1 (AF039398), CanPI-2 (AF221097), CanPI-3 (AY986465), CanPI-4 (AY986466), CanPI-5 (DQ005912), CanPI-8 (DQ005914), CanPI-9 (DQ005915), CanPI-10 (DQ005916), CanPI-11 (DQ008950), CanPI-13 (EF136387), CanPI-14 (EF136388), CanPI-15 (EF136389), CanPI-16 (EF125182), CanPI-17 (EF136381), CanPI-18 (EF136382), CanPI-19 (EF136383), CanPI-20 (EF136384), CanPI-21 (EF136385), CanPI-22 (EF136386), CanPI-23 (EF144129).

kers are present, (i) DPRNP like and (ii) EEKKN like. 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 IRD to an extended conformation (Schirra and Craik 2005). Kong and Ranaganathan (2008), after an alignment of sequences of IRDs from Pin-II family have suggested that each IRD is formed by a combination of two fragments namely 'Heavy' (H) and 'Light' (L). The H and L fragments may or may not be connected by a linker. Thus, they present a classification of domains into three types on the basis of the existence of linker sequences: 1) H-L type; here the H and L fragments are connected by Linker-1 i.e. DPRNP; 2) L-H type; here the H and L fragments are connected by Linker-2 i.e. EEKKN; 3) H+L type; here there is

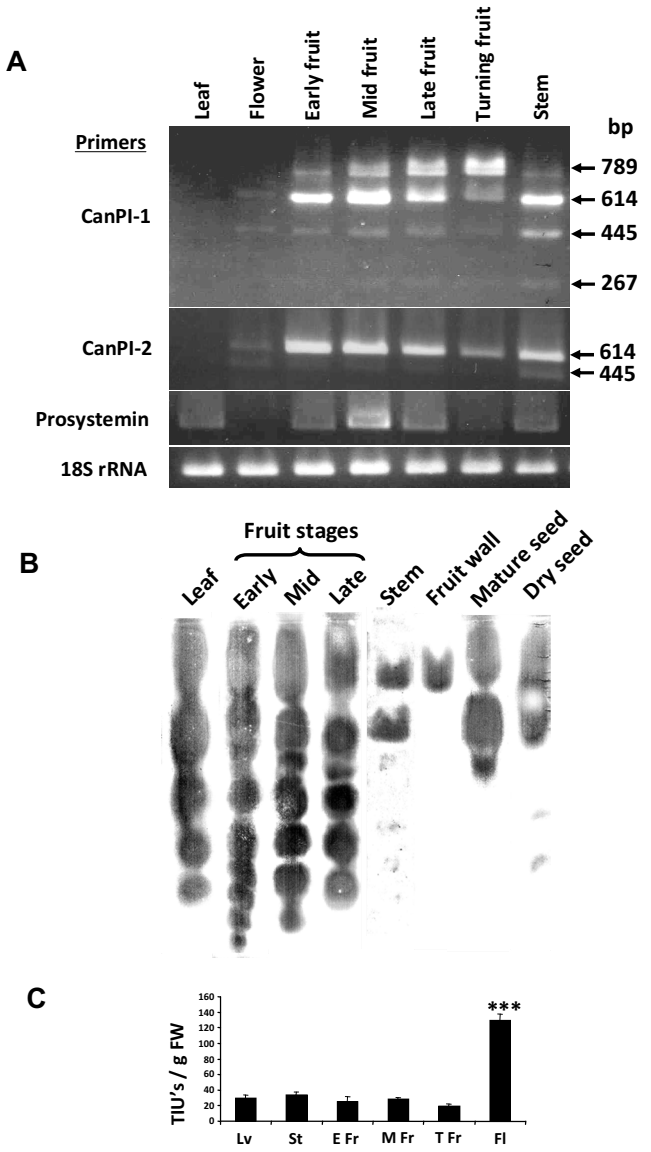

Fig. 2 Tissue-specific CanPI and prosystemin expression patterns. (**A**) RT-PCR analysis of CanPI and prosystemin expression in various tissues of mature *C. annuum* plants was performed. cDNA was equalized by comparing 18S rRNA amplification (row 4). (**B**) PI activity visualization. Trypsin inhibitor activity profiles from partially purified extracts of various tissues of *C. annuum*. Multiple TI activity bands were observed from extracts of fruit and leaf tissues. (**C**) Trypsin inhibitory activity from different *C. annuum* tissues. (**A-C**) Reproduced with kind permission from **Tamhane VA, Giri AP, Kumar P, Gupta VS** (2009) Spatial and temporal expression patterns of diverse Pin-II proteinase inhibitor genes in *Capsicum annuum* Linn. *Gene* **442**, 88-98, © Elsevier Science Ltd., Amsterdam.

no linker between the H and L fragments (**Fig. 4A**). They propose that H-L topology is favourable thermodynamically in comparison to L-H type of topology.

Single repeat Pin-II 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). Kong and Ranganathan (2008) also support the gene duplication hypothesis by Barta *et al*. (2002) for Pin-II family gene evolution. Unequal crossing over is presumed to be responsible for the expansion of the repeated domains (**Fig. 4B**). Domain replication i.e. duplication of the inhibitory domain sequence with the domains remaining fused may also play a role in generating such diversity (Christeller 2005).

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 (L-H type). This ancestral IRD, containing EEKKN linker would have led to the random incorporation of aa between the IRD fragments L and H. The other linker having sequence DPRNP seems to have got incorporated *via* this mechanism (Kong and Ranganathan 2008). Its integration into the PI structure results the two-repeat protein, to domain swap from the ancestral domain structure, attaining a thermodynamically more stable conformation (H-L type). The linker sequences are assumed to play important role in stabilizing the cross-repeat folding pattern of multi-domain Pin-II PIs. 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).

Phylogenetic analysis of Pin-II family by Kong and Ranganathan (2008) clubbed IRDs into seven clades on the basis of repeat number and species. In spite of high similarity among the PIs of *Solanaceae* species, clade 5 which includes IRD sequences from *C. annum* PIs, stands out from all others (**Fig. 5**). Unlike all the other clades, the *Capsicum* IRDs although being identical to the structural repeats observed in potato, tomato and in *Nicotiana* lack N- and C-terminal partial repeats, which form the "bracelet" link domain in other multi-IRD PIs.

PIN-II PI PROTEIN STRUCTURES

The three-dimensional structure of several Pin-II PIs, single- as well as multi-domain, have been determined either by X-ray crystallography or NMR and they give a good outline of the structure and dynamics of this class. The structures of single domain Pin-II PIs of *N. alata* (Nielsen *et al.* 1994), two domain precursor PIs from tomato individually and in ternary complex with two molecules of subtilisin Carlsberg (Barrette-Ng *et al*. 2003) and 6-IRD PIs from *N. alata* (Schirra and Craik 2005) have been studied (**Table 2**). The structure of chymotrypsin-binding domain (PCI-1) from potato PI-II, in complex with *Streptomyces griseus* proteinase B was solved to 2.1Å by X-ray crystallography (Greenblatt *et al*. 1989).

The sequence of IRDs is highly variable; however, presence of eight cysteines, a single proline residue and an active site either for trypsin or chymotrypsin inhibition is conserved throughout IRDs. The cysteines are involved in formation of four disulphide bonds, which stabilize the repeat structure (**Fig. 6A**). Single domain PIs having either trypsin inhibitory (TI) or chymotrypsin inhibitory (CI) active sites are formed by proteolytic cleavage at the linker regions of the multi-domain precursor (Heath *et al.* 1995; Lee *et al.* 1999). The single IRD CI protein contains a triple stranded β-sheet as the dominant secondary structural element, with several turns and a short region of helix. The putative CI 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 (Greenblatt *et al.* 1989; Nielsen *et al.* 1994, 1995). Due to the high sequence identity between TI and CI domains it has been anticipated that the TI domain also adopt 3D structures similar to CI.

Further structural refinement of the single domain TI or CI from *N. alata* by NMR (nulcear magnetic resonance) structure calculations (Schirra *et al*. 2008) has led to detailed information on Pin-II PIs, particularly single IRDs (**Fig. 6B**). The reactive site of the inhibitors containing the scissile peptide bond is positioned as a flexible loop and remains anchored to the core of the inhibitor by two disulfide bonds, C8-C37 and C4-C41. Further stabilization of the reactive site loop (RSL) is accomplished by: two additional disulfide bonds, C7-C25 and C14-C50; a prevalent network of hydrogen bonds; and presence of a proline residue at position P2 (Schirra and Craik 2005). The important functional role of the two disulfide bonds anchoring the RSL is reflected by their conserved presence among all

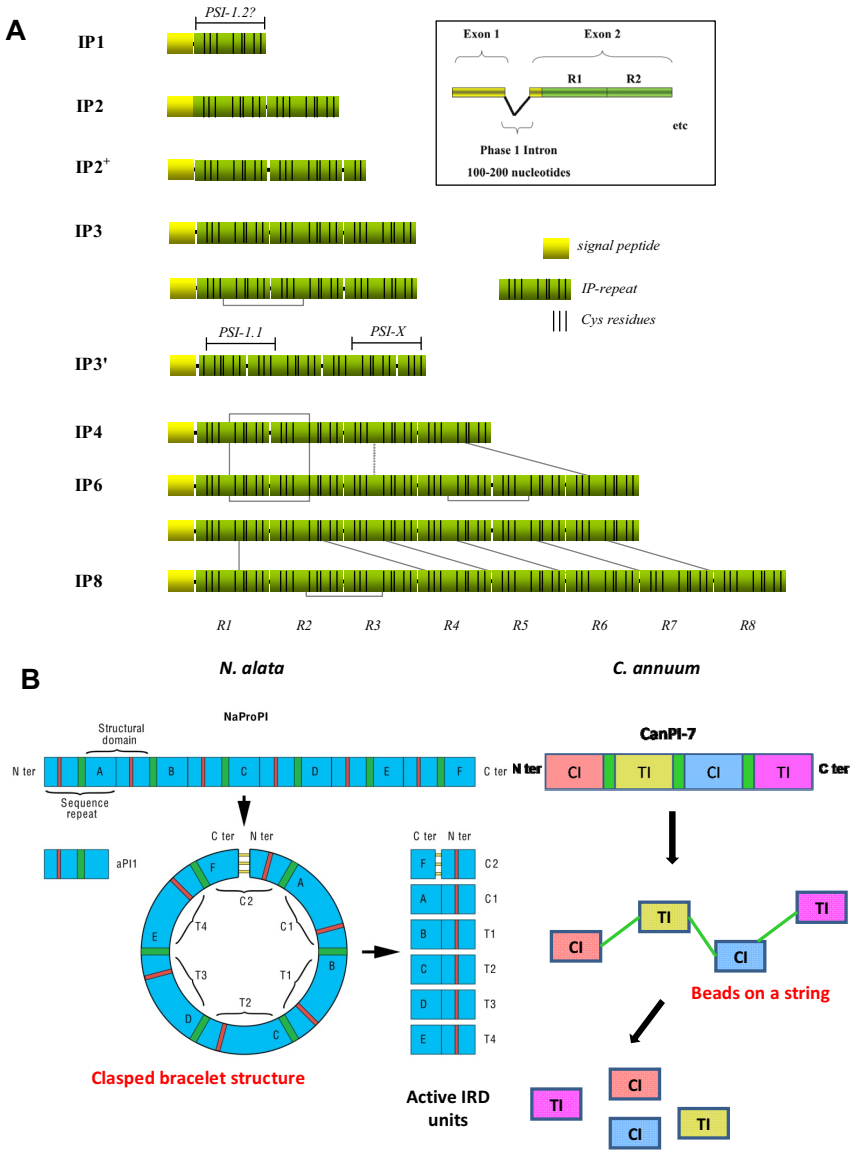

Fig. 3 (**A**) The domain structure of the potato type II proteinase inhibitor (Pin-II) family of precursors. The inset shows the consensus PI structure. IP1...IP8 designate the total number of IP repeats (green boxes) within each precursor. Yellow box, signal peptide; black vertical lines, Cys residues; 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. (**B**) Diagrammatic representation of the domain organisation of NaProPI and CanPI-7. The precursor protein NaProPI, shown as a linear gene product, forms a circular 'bracelet' structure that is 'clasped' by three disulphide bonds (yellow) between the N- and C-terminal repeats. Each repeat (labelled A–F) contains a protease-reactive site (red), which is specific for either chymotrypsin (C1 and C2) or trypsin (T1–4). Cleavage in each of the six linker regions (green) releases six active domains which are the native inhibitors. On the other hand, the precursor protein CanPI-7 is expected to form a "beads on a string" structure because of the absence of N- and C- terminal partial repeats. Proteolytic cleavage at the exposed linker regions (green) would generate 4 individual active IRD units. (**A**) Reproduced with kind permission from **Barta E, Pintar A, Pongor S** (2002) Repeats with variations: Accelerated evolution of the Pin2 family of proteinase inhibitors. *Trends in Genetics* **18**, 600-603, © Elsevier Science Ltd., Amsterdam. (**B**) Modified and reproduced with kind permission from **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* **7**, 793-802, © Elsevier Science Ltd., Amsterdam.

known Pin-II inhibitors. Other than these, there are few more highly conserved residues which are structurally important, such as Pro-18, Gly-38 and Gly-46 as they belong to the three β-turns, respectively (Kong and Ranganathan 2008). Flexibility of the RSL is remarkable in order to allow the binding of Pin-II PIs to wide range of proteinases (**Fig. 6C**).

In wild type (WT) IRD, the RSL is constrained by both C8-C37 and C4-C41 disulfide bonds thereby limiting its flexibility. NMR spectroscopy studies on the disulfide bond variant of IRD (T1) from *N. alata*, (C4A/C41A-T1) shows the similar conformation as the WT TI and only moderate decrease in its inhibitory potential (**Fig. 7A**). In contrast, the C8A/C37A-T1 variant shows a major disorder in the region of scissile bond making it's binding to proteinases more difficult and therefore, suppressing its ability as an inhibitor (Schirra *et al*. 2010). NMR relaxation experiments confirmed the much increased flexibility of binding loop for C8A/C37A-T1 variant. Thus, C8-C37 disulfide bond is indispensable for the stability and function of the IRD whereas the C4-C41 is not much critical as being supplemented by other stabilizing interactions (**Fig. 7B**).

The crystal structure of unbound form of two IRD PI of tomato inhibitor II by Barrette-Ng *et al*. (2003a, 2003b) 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, provides a unique opportunity to examine significant range of conformational flexibility present in the global structures of the inhibitor and flexibility

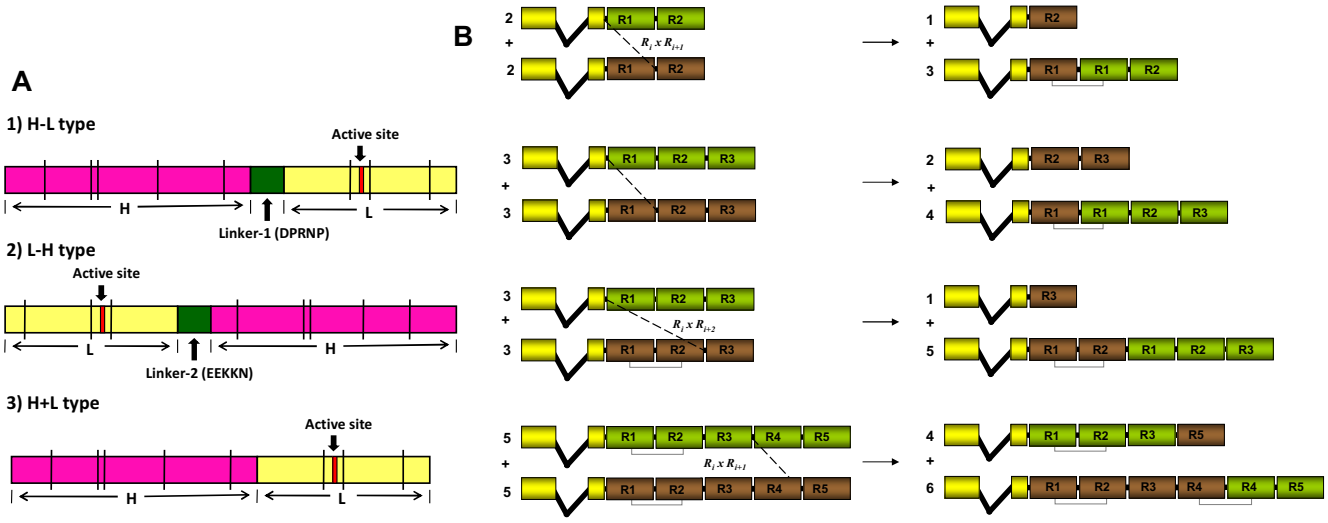

Fig. 4 (**A**) A schematic representation of the domain organization with respect to the existence of linker sequences and the heavy and light fragments. '|' indicates Cys residues. 1) H-L type; the H and L fragments are connected by Linker-1 i.e. DPRNP; 2) L-H type; the H and L fragments are connected by Linker-2 i.e. EEKKN; 3) H+L type; there is no linker between the H and L fragments. (**B**) Some of the potential unequal crossover (UECO) events that explain the emergence of sequence identity patterns of the potato II family precursors. The two partners are colored green and brown. Two types of UECO event involving either adjacent (Ri ×Ri+1) or nonadjacent (Ri ×Ri+2) repeats are shown by dashed lines. Gray lines indicate sequence identity (>98%). The IP1 type structure that corresponds to the homologs of the putative ancestral gene can arise as a result of various UECO events. For example, as the PSI-1.2 protein is similar to the R3 repeats it could have emerged from the Ri ×Ri+2 type recombination of two IP3 genes. IP5 type products have not been observed, but their putative Ri ×Ri+1 type recombination products are found in *N. alata* (bottom). In similar manner, the IP6 and IP8 type products found in *N. glutinosa* (Q9SDW7 andQ9SDW8, respectively; could have emerged from a putative IP7 protein as a result of an UECO type event. (**A**) Modified and reproduced with kind permission from **Kong L, Ranganathan S** (2008) Tandem duplication, circular permutation, molecular adaptation: How Solanaceae resist pests via inhibitors. *BMC Bioinformatics* **9 (Suppl 1)**, S22, © authors. (**B**) Reproduced with kind permission from **Barta E, Pintar A, Pongor S** (2002) Repeats with variations: Accelerated evolution of the Pin2 family of proteinase inhibitors. *Trends in Genetics* **18**, 600-603, © Elsevier Science Ltd., Amsterdam.

within RSLs. Conformational flexibility seen in the RSLs of unbound TI-II suggests a mechanism by which the inhibitor can balance the need for tight binding required for broad inhibitory function (Barrette-Ng *et al.* 2003a). The crystal structures of TI-II show dramatic change in conformational flexibility in the bound and unbound forms (Barrette-Ng *et al*. 2003b).

Studies have been conducted to find the structure and possible folding mechanism of single domains corresponding to aa sequence repeat and two contiguous structural domains, CI and TI, of the same precursor (Scanlon *et al.* 1999; Schirra *et al.* 2001). PI1 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 WT IRDs (Scanlon *et al.* 1999). 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 cross-repeat folding in combination with intra molecular domain swapping and circular permutation in the multi-domain proteins (Lee *et al.* 1999). Domain swapping has been commonly referred to as a mechanism for oligomerization in certain proteins like Diphtheria toxin (Bennet *et al*. 1994), RNase, Interferon, Interleukin, Cyt C (Hirota *et al*. 2010).

The first two single chains IRDs (CI-TI) of *N. alata* 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 six-domain precursor (Lee *et al.* 1999). 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. Both RSLs are positioned at the opposite ends, allowing the binding of two proteinases simultaneously without any steric interference. 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, especially if the number of domains is more than two in the precursor.

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 endogenous and exogenous PIs are also abundant in nature and they interact with the proteinases to modulate their activity for specific metabolic function (Fritz 2000; Gomes *et al.* 2011). Initially plant serine PIs were thought to have inhibition specificity for animal or microbial enzymes alone and not against plant proteinases. This led to demonstration of anti-metabolic effects of plant serine PIs on insects by inhibiting the gut proteases (Hilder *et al.* 1993; Gatehouse *et al.* 1999). Trypsin PIs are prime component of inducible plant system contributing to reduce the performance of folivores by targeting their main proteolytic digestive enzymes (Van Dam *et al.* 2001; Glawe *et al*. 2003; Zavala *et al*. 2004b; Horn *et al*. 2005; Zavala *et al*. 2008). Subsequently, evidences towards developmental regulation and tissue specific accumulation of PIs assigned endogenous functions to them. Plant organs that express Pin-II protein include flowers (Peña Cortés *et al.* 1991; Atkinson *et al.* 1993; Pearce *et al.* 1993; Sin and Chye 2004; Damle *et al.* 2005), fruits (Damle *et al*. 2005; Tamhane *et al*. 2009), stem (Xu *et al.* 2001), tubers (Sánchez-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 (Horn *et al*. 2005).

Dissecting out the defense and/or plant development specific role of PIs remains challenging and therefore, only

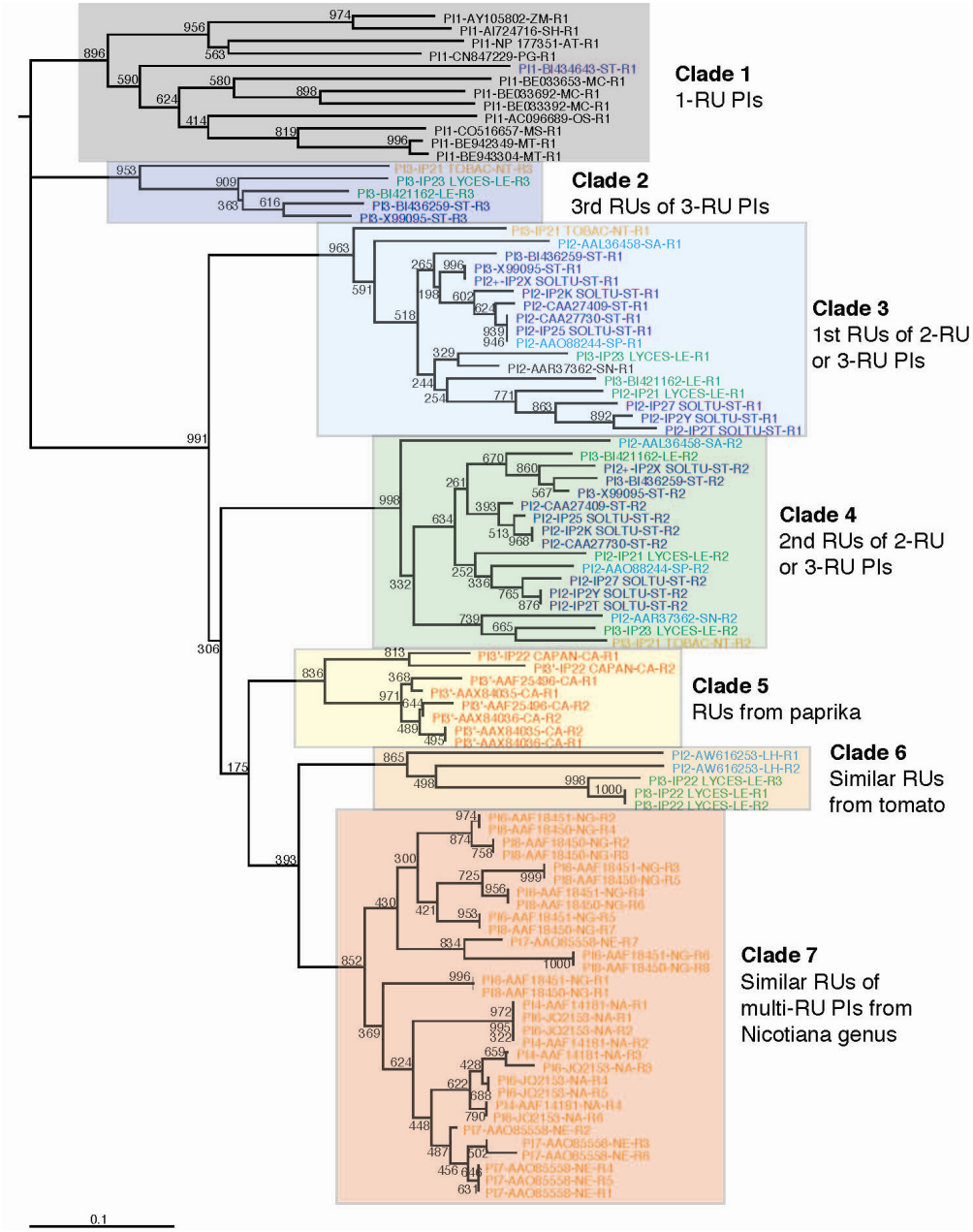

Fig. 5 Phylogenetic tree of Pot II PIs repeat units using NJ method. PIs from different species were colored into different colors. Green, tomato; dark blue, potato; red, paprika; orange, *Nicotiana* genus; blue, *Solanum* genus (except potato and tomato); black, non-*solanaceous* plants. Modified and reproduced with kind permission from **Kong L, Ranganathan S** (2008) Tandem duplication, circular permutation, molecular adaptation: How Solanaceae resist pests via inhibitors. *BMC Bioinformatics* **9 (Suppl 1)**, S22, © authors.

a few reports elucidating the endogenous functions of Pin-II PIs (Chye *et al*. 2006; Hartl *et al*. 2010).

Solanum americanum has two well characterized PIs SaPin-IIa and SaPin-IIb. It was observed that SaPin-IIa is abundantly expressed in stems especially in companion cells (CC) and sieve elements (SE) of phloem (Xu *et al*. 2001). The CC and SE 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. In stem SaPin-IIa is probably involved in regulating proteolysis. Transfer of SaPin-IIa gene to lettuce plants, which lack their own PI activity showed constitutive expression and the complete inhibition of endogenous protease activity thereby suggesting endogenous regulation of proteolysis by SaPin-IIa (Xu *et al*. 2004). SaPin-IIa and b both are strongly expressed in the floral tissue that are destined to undergo developmental programmed cell death (PCD) including stigma, stylar transmitting tissue, vascular bundles, nuclear cells of the ovule and the outermost cell layer of the placenta (Sin and Chye 2004). Expression profiles of SaPin-IIa and b, suggest their differential regulation and probably overlapping and complementary roles in floral development. They probably function by confining the PCD to the specific tissues, thereby protecting the adjacent tissues (Peña Cortés *et al*. 1991; Sin and Chye 2004). 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 not detected in these lines. Majority of seeds in the silenced lines were aborted due to defective seed coat that led to abnormal endosperm development (Sin *et al*. 2006). The seed coat needs to transport metabolites to the developing embryo. 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-

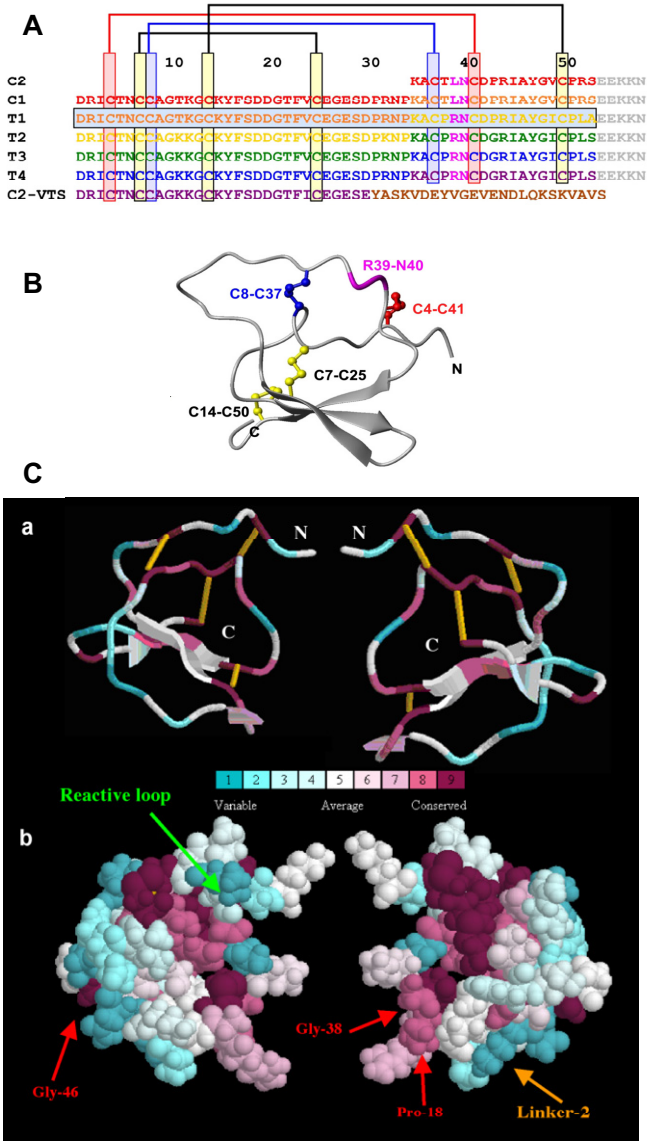

Fig. 6 (**A**) Amino acid sequence of NaProPI in single letter code. The sequence is arranged so that the sequence for each PI domain is shown on an individual line with the name of the respective proteinase inhibitor (PI) denoted at the beginning of the line. The repeats are highly similar with only small amino acid variations between them. Sequence numbers and disulphide bridging pattern are indicated on top of the sequence with cysteine residues highlighted by boxes. (**B**) Ribbon representation of the structure of T1. The disulfide bonds are shown explicitly as ball-and-stick models colored yellow, with the two disulfide bridges anchoring the reactive site loop to the core of the molecule (C4-C41 and C8-C37) colored red and blue, respectively. The reactive site is depicted in magenta. Created with MOLMOL. (**C**) Residue conservation analysis for the Pin-II family repeat units by ConSurf, mapped on to the structure ICE3. Different views of the same structure were shown, rotated by 180°, in (a) ribbon and (b) CPK representations. Residues are shaded from cyan (highly variable) through white (moderate conservation) to purple (highly conserved). (**A, B**) Reproduced with kind permission from **Schirra HJ, Guarino RF, Anderson MA, Craik DJ** (2010) Selective removal of individual disulfide bonds within a potato type II serine proteinase inhibitor from *Nicotiana alata* reveals differential stabilization of the reactive-site loop. *Journal of Molecular Biology* **395**, 609-626, © Elsevier Science Ltd., Amsterdam. (**C**) Modified and reproduced with kind permission from **Kong L, Ranganathan S** (2008) Tandem duplication, circular permutation, molecular adaptation: How Solanaceae resist pests via inhibitors. *BMC Bioinformatics* **9 (Suppl 1)**, S22, © authors.

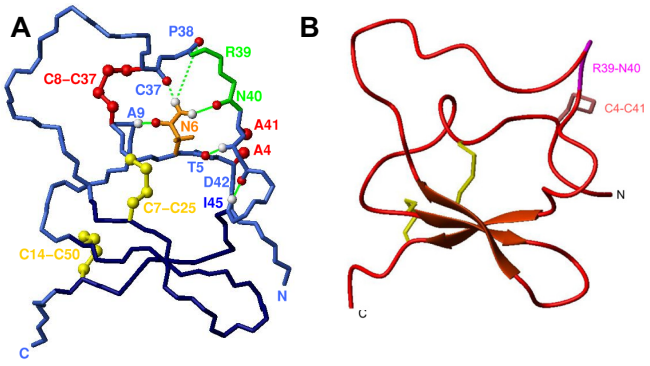

Fig. 7 (**A**) Conformational variations in the IRDs. Detailed view of C4A/C41A-T1 showing the stabilization of the reactive-site loop. The C8–C37 disulfide bond is indicated in light blue, and the side chains of A4 and A41 are indicated in light red. The reactive site, containing the P1–P1′ residues L38 and N39, is colored magenta. Hydrogen bonds stabilizing the reactive-site loop are indicated by continuous green lines. A hydrogen bond between the side chain of N6 (orange) and either C37 or P38 is indicated by dashed green lines. This figure was produced with MOLMOL. (**B**) Ribbon drawing of a representative model of the solution structure of C8A/C37A-T1, showing the regular secondary structure and global fold of the protein. C8–C37 disulfide bond is colored light blue. The reactive site R39–N40 is shown in magenta. C4–C41 disulfide bond colored light red. This figure was produced with MOLMOL. (**A, B**) Reproduced with kind permission from **Schirra HJ, Guarino RF, Anderson MA, Craik DJ** (2010) Selective removal of individual disulfide bonds within a potato type II serine proteinase inhibitor from *Nicotiana alata* reveals differential stabilization of the reactive-site loop. *Journal of Molecular Biology* **395**, 609-626, © Elsevier Science Ltd., Amsterdam.

associated proteinases generated in the seed.

The applications of Pin-II PIs in regulating endogenous proteinases 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 and regulation of Pin-II PIs (Chye *et al.* 2006). Liu *et al.* (2006) have shown that SaPin-IIb is also constitutively expressed in glandular trichomes. 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. Interestingly, over expression of SaPIN2a in tobacco plants resulted in a significant increase in glandular trichome density and a promotion of trichome branching, which provided an additional resistance against insect pests (Luo M *et al*. 2009).

SaPIN2a-overexpressing transgenic nightshade plants showed significantly lower height than wild-type plants. Transmission electron microscopy analysis showed that chloroplast-like organelles with thylakoids, which are not present in enucleate SEs of wild-type plants, were present in the SaPIN2a-overexpressing transgenic plants. The occurrence of these chloroplast-like organelles in the SEs of the SaPIN2a overexpressing transgenic plants might have resulted from inhibition of proteinase activities involved in plastid development and conversion. The ectopic presence of chloroplast-like organelles in the SEs of transgenic plants might impair the transport of various molecules through the phloem (Xie *et al.* 2007).

Four Pin-II PIs corresponding to their respective activity isoforms namely *SnSPI1*, *SnSPI2a*, *SnSPI2b* and *SnSPI2c* were identified in *S. nigrum*. The *SnSPI2a*, *SnSPI2b* and *SnSPI1* were found to be strong subtilisin-inhibitors, where as *SnSPI2c* was identified as a strong inhibitor of trypsin and chymotrypsin (Hartl *et al*. 2010). Contrary to the observations on the endogenous role of PIs in *S. americanum*, recent reports on the PIs from *S. nigrum* suggest not exactly similar roles (Hartl *et al*. 2010). Sin *et al*. (2006) have reported an increase in flower size and 80% seed abortion after silencing homologs of *SnSPI2a* and *SnSPI2b* in *S. americanum*. However, in *S. nigrum* upon silencing SPI2a and SPI2b, no effect on flower size was detected and only 0.7 to 2.8% of the seeds were aborted or

Table 2 Determined three-dimensional structures of Pin-II PIs.

Organism/ Source	Inhibitor	Remark	Technique	Resolution/ rmsd	Pdb ID	No. of IRDs	Inhibitory activity	Reference
Solanum tuberosum (potato)	PCI-1	In complex with *Streptomyces griseus* proteinase B	X- ray	2.1 Å	4sgb	1	Chymotrypsin	Greenblatt *et al*. 1989
N. alata	T1	6 kDa TIs isolated from 40.3 kDa precursor	NMR	1.79 Å	1tih	1	Tryspin and Chymotrypsin	Nielsen *et al*. 1995
N. alata (ornamental tobacco)	aPI1	Artificial construct – first IP repeat of NaProPI, equivalent to a putative ancestral precursor	NMR	2.19 Å	1ce3	1	Chymotrypsin	Scanlon *et al*. 1999
N. alata	C2	Two-chain inhibitor	NMR	2.60 Å	1qh2	1	Chymotrypsin	Lee *et al*. 1999
N. alata	C1-T1	Artificial construct – first two PI domains of NaProPI	NMR	2.05 Å (C1) 1.65 Å (T1)	1fyb	2	Chymotrypsin and Trypsin	Schirra *et al*. 2001
Lycopersicon esculentum	TI-II	Free form	X- ray	2.15 Å	1pju	2	Tryspin	Barette Ng *et al*. 2003
Lycopersicon esculentum (tomato)	TI-II	In complex with subtilisin Carlsberg	X- ray	2.5 Å	1oyv	2	Tryspin	Barette Ng *et al*. 2003
N. alata	C1	Free form	NMR	1.97 Å (backbone)	2jzm	1	Chymotrypsin	Schirra *et al.* 2008
N. alata	C4A/ C41A-T1	Artificial construct- disulfide variant of T1	NMR	2.26 Å	n.d.	1	Trypsin	Schirra *et al*. 2010
N. alata	C8A/ C37A-T1	Artificial construct- disulfide variant of T1	NMR	2.67 Å	n.d.	1	Weak trypsin inhibitor	Schirra *et al*. 2010

n.d. = not deposited

defective. Xie *et al*. (2007) have reported that the ectopic over expression of PI genes can affect plant growth. In comparative growth experiments in *S. nigrum* no differences in plant height were detected. Hartl *et al*. (2010) have shown that the PIs from of *S. nigrum* exhibit a certain degree of functional differentiation but also considerable functional overlap. The highly abundant PI (*SnSPI2c)* displays typical characteristics of a defense-related gene where as the other two, *SnSPI2a* and *SnSPI2b*, show an overlap of defensive and developmental properties. Both are very similar to each other and perhaps they represent an early stage in the differentiation of a developmental function for *SnSPI2a* and a defensive function for *SnSPI2b*. The specificity of most SnPIs for subtilisin and their involvement in seed development suggests an interaction with plant endogenous subtilases. The identification of these target proteinases will be an interesting task for future research (Hartl *et al*. 2010).

In *N. alata*, NaPIs account for up to 30% of soluble protein in stigma cells (Atkinson *et al.* 1993) and are present in its vacuoles. It has been observed by Johnson *et al*. (2007) that the high levels of NaPI synthesized in maturing stigmas produces two populations of PIs; 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 endoplasmic reticulum (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.

PI biosynthesis for defense of the plants against insect pests is an energy intensive process though it pays off by offering protection when the plants are attacked by herbivores (Zavala *et al*. 2004a, b). Silencing the PI gene in *N. attenuata* abolishes the plant's capacity to produce PIs and allows it to grow faster, flower earlier, and produce more seed capsules compared with PI-producing genotypes (Zavala *et al.* 2004a). Similarly, restoring PI production by transforming an ecotype of *N. attenuata* naturally deficient in PI production (Wu *et al.* 2007) reduces lifetime seed production (Zavala *et al.* 2004a). Studies highlight that PIs occur at high levels in reproductive organs, although their role in floral function has not been thoroughly explored. Bezzi *et al*. (2010) provide evidence for a role of PIs in the processing and secretion of nectar proteins, which, in turn, results in higher levels of nectar H_2O_2. Native flower visitors removed less nectar from trypsin-PI-silenced-*N. attenuata* plants (ir-pi) than from wild-type plants. The nectar from PI-silenced-flowers contained 3.6-fold more total protein than the nectar of wild-type flowers. Changed-nectar properties of PI-silenced-*N. attenuata*-lines repelled native flower visitors even in the absence of nicotine. The effect of silencing PIs on nectar protein accumulation suggests an endogenous regulatory function for PIs in *N. attenuata* flowers.

A qualitative as well as quantitative analysis of endogenous PI activity in different tissues of field grown tomato plants was undertaken by Damle *et al*. (2005). The TI activity, in flower, was reported to be hundred times higher than leaves and developing fruit stages. The estimation of insect protease inhibitory activity, from these tissues also yielded similar results with a fold difference in flower as compared to leaves and developing fruit stages. But interestingly, the PI activity profiles of these tissues yielded identical patterns. This observation can be viewed as a survival strategy by the plant, partitioning and diverting its metabolic resources to the reproductive organ i.e. the flower.

C. annuum Pin-II PIs (CanPIs) displayed high isoform diversity with PIs of 1- to 4-IRD expressing simultaneously (Tamhane *et al*. 2009). Expression patterns of *CanPI*s in the fruit and stem tissues of mature *C. annuum* plants varied qualitatively and quantitatively (Tamhane *et al*. 2009). In the fruit tissue, *CanPI*s with different IRDs (from 1 to 4) were expressed simultaneously. In stem tissue, 1- and 2-IRD *CanPI*s were strongly expressed along with moderate expression of 3- and 4-IRD CanPIs. Analysis of CanPI protein activity showed a range of active forms across tissues. *CanPI* expression was differentially up-regulated upon wounding and insect attack. Although infestation by aphids (*Myzus persicae*) and Lepidopteran pests (*Spodoptera litura*) specifically induced 4-IRD *CanPI*s, virus-infected leaves did not affect *CanPI* expression. Analysis of CanPI protein activity indicated that the up-regulation in *CanPI* expression did not always correlate with increase in PI activity indicating involvement of PI in plants endogenous function(s) (**Fig. 2**). *CanPI* expression is regulated spatially, temporally as well as qualitatively and quantitatively. Several studies have been directed to identify the nature and extent of involvement of Pin-II PIs in plants endogenous function. However new and not exactly similar findings emerge when different species are investigated, indicating a species dependant functional modification of the PIs. Conversely, these species have not been investigated simultaneously/together to identify common trends of Pin-II PI function. Further, the high degree of sequence similarity in the IRDs of Pin-II PIs at mRNA and protein level complicate the process of identifying/assigning a particular endogenous or defense function to specific PI variant(s).

POTENTIAL OF PIN-II PIS TO INHIBIT PROTEINASES

Each inhibitory repeat of the Pin-II precursor contains a single reactive site. Single IRD PI of Pin-II family can bind to a single protease, while two domain PIs of tomato and potato can simultaneously inhibit two protease molecules. The P1 residue of the reactive site, which reacts with protease active site, determines its specificity. Presence of lysine 'K', arginine 'R' or Leucine 'L' in the P1 position confers the inhibitor with either TI or CI potential. P3 to P2' a stretch of 5 aa close to the reactive site is important in determining enzyme specificity of the inhibitor. The RSL P4 to P3' of the inhibitor domain interacts with S6 to S2' of protease pocket to bring about its inhibition by mimicking a substrate. The core RSL i.e. (P_3-P_2) does not show very high sequence variability. It is bound by two disulfide bonds 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 to provide broad inhibitory potential. The RSL must retain a certain degree of flexibility in order to allow binding of the PI to the binding sites of a variety of different proteases (Barrette-Ng *et al.* 2003a).

The P1 residue contributes the largest number of contacts with the protease. The deep docking of P1 side chain in S1 binding pocket of the protease plays an extensive role in the energetics of the specificity of PI-protease interaction. The substitution of P1 residue with aa other than K/R showed a weaker side chain interaction of the P1 residue in the S1 binding pocket of trypsin (Otlewski *et al*. 2001). Replacement of P1 residue with aa A/G/L/V showed reduced association energy leading to many fold decrease in the association constants.

Mutational studies on PI-II from potato have highlighted the importance of secondary contacts not involving RSL as well, in determining the specificity of protease inhibition. The inhibition capacity of a TI domain could not be transferred to the other domain by mutating the P1 residue or the residues within the RSL (Schirra and Craik 2005). Interchange of reactive site of domain I (L) to domain II (R) in a two domain PI, did not result in exact inhibitory specificity transfer (Beekwilder *et al.* 2000).

The structural basis of inhibition of a multi-domain Pin-II inhibitor 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.* 2003b). The inhibitory RSL in each IRD is positioned at opposite ends of the elongated molecule facilitating inhibition of two protease molecules. There is a considerable reduction in flexibility of the loop on binding to proteases 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.

The diversity in the number of repeats and diversity within IRD sequences is predominantly observed in Solanaceae PIs. The different specificities within a multi repeat protein contribute to a PI cocktail to fight against varied pest/pathogenic attacks. The PIs from *C. annuum* have been found to diversify to an extent that there are 54 unique IRDs constituting 67 novel *Can*PI genes (Mishra *et al*. unpublished data). On the other hand *N. attenuata* express 7-IRD NaTPI gene consisting 7 IRDs with sequence diversity (Zavala *et al*. 2004b).

Study by Bryant *et al*. (1976) was one of the pioneering reports on the purification and characterization of Pin-II PIs from potato. PI with CI and TI properties, was found to be a heat-stable protein, with molecular weight of 21,000 Da. Reconstituted dimers 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, immuno-electrophoretic mobilities, ion-exchange properties and their inhibitory activities against trypsin. The properties of dimeric species are similar but not identical to inhibitors IIa and IIb reported from Japanese potatoes indicating the existence of intervarietal, as well as intravarietal, differences among potato tuber inhibitor II isoforms.

In various members of Pin-II precursors studied there is a combination of TI/ CI domains. For example the six domain *N. alata* PI (NaProPI) possesses four TI domains and two CI domains. In potato PI which has two IRDs, one is TI specific and the other is CI specific. However, the NaProPI cannot bind to six proteases simultaneously because of steric interference. This 6-IRD PI of *N. alata* could inhibit maximum of four chymotrypsin or 2.6 trypsin molecules. In order to realize total inhibition potential, individual IRDs must be released from the precursor. It is thus important to have a proteolytic processing of precursor for maximum protease inhibition (Heath *et al.* 1995). This also highlights the reason behind the absence of inter domain interactions in Pin-II PIs.

Two *C. annuum* seed PIs of around 50 aa, PSI1.1, PSI1.2, related to each other by circular permutation have been found to inhibit trypsin, chymotrypsin, thrombin and factor Xa with different specificities. The PSI1.2 shows the circularly permuted topology and also corresponds to a complete repeat representing a putative ancestral protein (L-H type, Kong and Ranganathan 2008) of Pin-II family (Antcheva *et al.* 2001). Two IRD PIs from *S. tuberosum* (Eddy *et al.* 1980) and *L. esculantum* (Plunkett *et al.* 1982) have been shown to inhibit chymotrypsin, subtilisin and trypsin. A 6 kDa protein with inhibitory activity against trypsin and chymotrypsin was purified from stigmas of *N. alata* rather than the precursor protein of 41.6 kDa because of the processing at the linkers to generate active inhibitory units (Atkinson *et al*. 1993).

Two proteinase inhibitors from *C. annuum* leaves, CapA1 and CapA2 exhibiting the molecular mass of 12 kDa inhibited bovine trypsin and chymotrypsin suggesting the presence of two inhibitory sites and two IRDs (Tamhane *et al*. 2005). PIs from *C. annuum* with variable number of IRDs and sequence variations within the IRDs, showed significant changes in their specificity and inhibitory potential towards proteases like trypsin, chymotrypsin and complex mixtures of insect gut proteases (Tamhane *et al*. 2007; Mishra *et al*. 2010) (**Fig. 8**). The detailed exploration for stability, activity and processing of multi-domain CanPIs having varied combinations of TI/CI IRDs has revealed the release of IRD units as a result of processing at the linker regions by proteases other than bovine trypsin and chymotrypsin (Mishra *et al*. 2010). Several fold processing of multi-IRD PIs, leads to release of IRD cocktail with various specificities and less steric hindrance thus facilitating the interaction with proteases.

The effect of mutation/variation in active site or conserved residues is reflected in the structure and reactivity of Pin-II proteins. In a recent study by Schirra *et al.* (2010), the WT and cysteine variants of T1 (TI domain) were assessed for their ability to inhibit bovine β-trypsin, resulting change in their 3D structure and dynamic behavior. The selective replacement of relevant cysteine residues responsible for disulfide bond formation flanking the reactive site resulted in poor inhibitory activity (Ki ~ 1.8 μM) by C8A/C37A-T1 variant (aa Cys-C at positions C8 and C37 are replaced by aa Ala-A). On the other hand, substantial retention of TI activity of C4A/C41A-T1 variant (Ki ~ 350 nM) as compared to the wild-type TI (Ki < 5 nM) affirmed the indispensability of C8-C37 bond in Pin-II proteins. The removal of the disulfide bonds had the profound effect on the flexibility of the RSL itself making its binding to the proteases more difficult thus decreasing its binding affinity. The findings complement the vitality of conserved residues in Pin-II proteins for their structure and thus their function (Schirra *et al.* 2010).

IN VITRO AND *IN VIVO* EFFECTS OF PIN-II PIS ON INSECT PROTEASES

Effect of plant's endogenous Pin-II PIs on insects

Wound induction of Pin-IIs 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).

In order to investigate the *in vivo* activity of tomato PIs, Damle *et al*. (2005) tested the inhibitory potential of these PIs against gut proteinases from chickpea-fed and tomato-fed *H. armigera* insects. In both the cases, the gut protease activity was inhibited to 50–60% and 90% and in feeding experiments, these PIs showed adverse effects on *H. armigera* development in a dose-dependent manner. This demonstrated that the host plant PIs are akin to other non-host PIs which, so far, have been claimed to be an emerging aid in plant defense.

The addition of soybean TI (SBTI) 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 PIs in the artificial diets (Broadway and Duffy 1986).

Three steps (namely PI *in vitro* assays, PI incorporation in artificial diet and PI transgenics) have been used to assess the potential of ornamental tobacco (*N. alata*) PIs in insect control (Heath *et al.* 1997). In an *in vitro* approach all five inhibitors (one 6-kDa CI and four 6-kDa TIs from a single 40.3-kDa precursor protein) were tested for their ability to inhibit gut protease activity in insects representing four orders. In most cases the pooled inhibitors inhibited the gut protease activity ranging from 37 to 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 (*H. punctigera*) and the black field cricket (*Teteogryllus commodus*) revealed a significant ($P < 0.01$) reduction in growth and were more lethargic and failed to complete molting than insects on the control diet. 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. The efficacy of *C. annuum* PIs against *H. armigera* gut proteases as well as larval growth and development was demonstrated by Tamhane *et al*. (2005). *In vitro* assays showed that the 68-91% trypsin activity of *H. armigera* gut protease was sensitive to PI while 39-85% chymotrypsin-like activity of gut proteases was insensitive to the CanPIs. When fed to *H. armigera*, CanPIs brought about retardation in growth of the insect as well as reduction in fertility and fecundity for two consecutive generations.

A novel exploitation of plant TPI (trypsin PI) activities and 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*, *N. obtusifolia*) and 2 allotetraploid (*N. clevelandii*, *N. quadrivalvis*) species, which are the descendents of the former. *N. attenuata*, *N. quadrivalvis* and *N. clevelandii* elicited higher TPI activity while *N. obtusifolia* elicited suppressed activity in response to application of insect oral secretion. It has been shown that a network composed of an upstream signaling system, downstream interactions between *cis*- and *trans*-elements and post-transcriptional regulators, probably regulates the PI expression. It was suggested that both the tetraploids probably retained the upstream signaling network from the diploid *N. attenuata* but abandoned those from *N. obtusifolia* and although both the systems might co-exist, tetraploids still possess the ability to recognize attack from *Manduca sexta* (Solanaceous specialist herbivore) larvae.

Hartl *et al*. (2010) examined *S. nigrum*'s complete serine-protease-inhibitor (SPI) profile and identified four PI genes *SnSPI2c*, *SnSPI2a*, *SnSPI2c-R2* and *SnSPI2c-R3*. In leaf tissue, especially *SPI2c* was most abundant and highly upregulated on application of MeJA, regurgitant from *M. sexta* and mechanical wounding. Comparatively lower transcripts of *SPI2a* and *SPI2b*, give indication of functional diversification of *SPI2c* towards plant defense. On conducting field experiments with transgenic plants silenced for SPIs, it was observed that the PIs of *S. nigrum* have no effect on the *M. sexta* performance. By contrast, generalist herbivore like *S. exigua* dwelled better on SPI-silenced plants. Moreover it, exhibited a compensatory feeding response to the expression of *SnSPI2c* along with a resultant increase in larval growth. However, the varied responses also suggest the involvement of several other factors like co-occurrence of pathogens, herbivores and intraspecific variations within the host species.

Heterogenous Pin-II PI activity by transgenic approach

Diverse CanPIs with 1- to 4-IRDs were expressed heterologously in *Pichia pastoris* and the recombinant proteins were characterized for their insect inhibitory potential. *H. armigera* larvae fed on rCanPI diet showed 30% mortality and 40% lower mass among the survivors, in the early instars (Tamhane *et al*. 2007). Pupal mass reduction of 12-25% was recorded, leading to decreased fecundity. The 4-IRD PI; CanPI-7 with two CI sites and two TI sites showed the strongest anti-metabolic effect on *H. armigera*. Further exploiting the interaction(s) of rCanPIs with *H. armigera* gut proteases by IF-MALDI-TOF analysis, Mishra *et al*. (2010) revealed PI processing patterns and the stability of these rPIs in presence of gut proteases of *H. armigera* (**Fig. 8**). The insect gut proteases act on the linkers in CanPI-7; processing the multi-IRD form to lower/single IRD forms. Terminal processing of precursor PIs/IRDs endogenously in plants or in insect gut upon ingestion, leads to increase in its IRD diversity; which mostly has a functional significance (Horn *et al.* 2005).

Dunse *et al*. (2010) explored the consequences of feeding *H. punctigera* and *H. armigera* with Pin-I and -II inhibitor proteins. *H. punctigera* larvae were fed with a cotton leaf-based artificial diet, composed of 0.26% (w/v) of *N. alata* proteinase inhibitor (NaPI) which is a Pin-II. They detected a higher mortality (80%) as well as lower larval mass (30 mg) in NaPI-fed larvae as compared to those fed with the control diet (i.e., 40% mortality and 100 mg larval mass). Interestingly, the consumption of NaPI by the larvae, led to the induction of a chymotrypsin which was found to be resistant to inhibition by NaPI. On the contrary, the activity of this chymotrypsin was found to be inhibited by a Pin-I inhibitor (StPin1A) isolated from wound-induced leaves of *S. tuberosum*. *H. armigera* larvae fed with diets containing NaPI and StPin1A were reported to weigh less than the larvae fed with control diet by 50 and 40%, respectively; 90% smaller larvae were observed when fed with an artificial diet composed of both the types of inhibitor proteins, namely, NaPI and StPin1A. Taking this outcome forward, they also conducted field trials with transgenic cotton plants, expressing the individual inhibitors and combination of inhibitors (NaPI-StPin1A), and subjected these artificially to *H. armigera* infestation along with the natural prevalence of *H. punctigera* at the field site. They recorded an increase in number of cotton bolls in the transgenic line expressing both NaPI and StPin1A than the parental untransformed line and also a boost in lint weight per plant [27.8 ± 0.59 (SE) g for these transgenic lines, when evaluated against the control line 22.9 ± 2.1 (SE)] (Dunse *et al*. 2010).

In the case of another pest, *M. sexta* larvae grown on

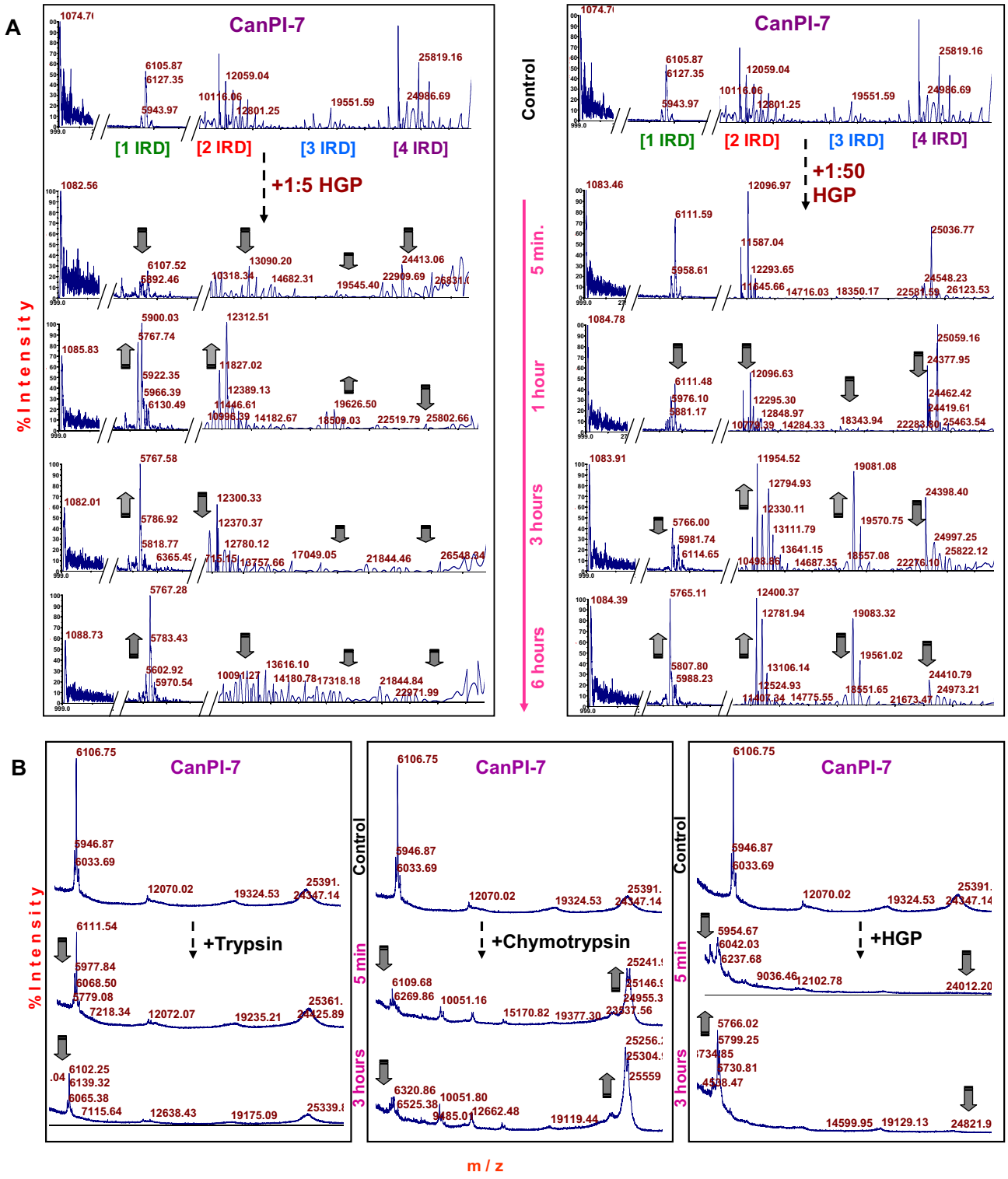

Fig. 8 IF-MALDI-TOF-MS analysis of rCanPI-7–proteinase interaction. (**A**) Different concentrations of HGP (0.5, 0.1, 0.01U) were incubated with rCanPI-7 (0.05 HGPI unit) for 5 min, 1, 3 and 6 h at 24°C. Due to the interactions between the PI and HGP, change in the intensity and diversity of the CanPI peaks was detected and is indicated by arrows. (**B**) IF-MALDI-TOF-MS analysis of the interactions between rCanPI-7 and bovine trypsin (0.5 U) (a), chymotrypsin (0.5 U) (b) and HGP (0.1 U) (c). Bovine trypsin and chymotrypsin do not act on the linker regions in the rCanPI-7, whereas HGP cleaves on the linkers in turn processing the multi-IRD forms to lower IRD forms. Reproduced with permission from **Mishra M, Tamhane VA, Khandelwal N, Kulkarni MJ, Gupta VS, Giri AP** (2010) Interaction of recombinant CanPIs with *Helicoverpa armigera* gut proteases reveals their processing patterns, stability and efficiency. *Proteomics* **10**, 2845-2857, © Interscience Wiley, Singapore.

transgenic tobacco plants expressing Pin-II proteins from tomato and potato showed severe inhibition at 50 µg inhibitor/g of tissue while still more inhibition and mortality at 100 µg/g tissue. Comparisons with inhibitor I and II showed that the TI activity of Pin-II PI was largely responsible for inhibition of growth (Johnston *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).

SaPIN2a, from the *S. americanum* was expressed under the control of the CaMV 35S promoter by Luo *et al*. (2009). Bioassays for insect resistance showed that transgenic

tobacco plants over expressing SaPIN2a were more resistant to *H. armigera* and *S. litura* larvae than the control plants. They also reported an increase in the glandular trichome density along with the promotion of trichome branching in transgenic tobacco plants.

Pin-II PIs of potato have also been used in transgenic rice and wheat plants to control biotic infestations by *Sesmia inferens* in 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 modes of action rather than combination of inhibitors might be more effective for insect control and stable resistance against pests (Abdeen *et al.* 2005).

The cost benefit studies of PI strategy for insect tolerance performed by Zavala *et al*. (2004a, 2004b) 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*. Behavior of different insects associated with *N. attenuata* in its natural ecosystem is influenced by presence/absence of PIs in the plant tissue (Bezzi *et al.* 2010; Diezel *et al.* 2011).

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 WT plants. Similarly, the polyphagous insect pest beet armyworm showed a 57% higher mass when reared on these anti-sense lines (Royo *et al.* 1999). Evidence is now gathering to say that many different types of insects and other non insect pest's growth is also influenced by PIs. Root knot nematode infestation upregulated PI genes and it negatively correlated with PI expression levels in tomato (Fujimoto *et al.* 2011).

Pin-II PIs span the gap between basic and applied sciences with their endogenous functions in plants and promising potential applications in pest control. The successful implementation of inhibitor-expressing transgenic plant lines in agricultural fields has paved the way towards development of superior crop varieties with insect resistance and high productivity. Though the biochemistry of this class of proteins continues to fascinate biologists, their worth in the light of future research and applications holds a real boon to plant biotechnology.

CONCLUSION AND FURTHER RESEARCH DIRECTIONS

The structural and functional diversity displayed by Pin-II PIs and their component IRDs has made this PI family extremely interesting in terms of exploring basic and applied research avenues. The Pin-II PIs have been analyzed to detect various phylogenetic trends responsible for the present day diversity of the family members. Pin-II genes in Solanaceae are important due to their evolution into multiple inhibitory repeat types (2 to 8 IRDs) from the ancestral single repeat Pin-II PI precursor (Barta *et al.* 2002). Significantly high diversity in precursor Pin-II PIs, giving rise to many variants of the precursor is particularly found in *C. annuum*, *S. americanum* and recently in *S. nigrum*. Further studies on co-relation of phylogeny of various genera in Solanaceae with their diversity of Pin-II PIs may provide better insights into evolutionary mechanisms acting in the family.

To date more than 60 different Pin-II PI transcripts have been detected from various tissues of *C. annuum*. This extraordinarily high transcript diversity has raised questions; to list a few; why this high diversity is found only in *C. annuum*? 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. The expression variability in these PIs and their correlation with the tissue types, inducibility provide an excellent example of temporal, spatial, qualitative and quantitative gene regulatory mechanism(s) operating in plants. Recent investigations on the Pin-II PI gene expression (Tamhane *et al*. 2009; Hartl *et al*. 2010) has shed light on some novel aspects and raised several questions about the PI regulatory mechanisms operating in plants, especially those that come into significance during biotic and abiotic stresses. The precise expression of a particular variant PI of the Pin-II family in response to specific environmental stimuli probably represents the ultimate result of the multi-faceted cross talk between various plant hormones and proteins/transcription factors. Several investigations are pointing towards the endogenous role of Pin-II PIs and the precision/regulation through which different Pin-II PIs are rendered for the two different roles *viz.* plant defense and plant endogenous function(s) (Hartl *et al*. 2010). The detailed study by Hartl *et al*. (2010) on *S. nigrum* PIs led to the identification of four PI variants with different substrate specificities, expression patterns and importantly varied influence on diverse natural herbivores. The accumulation pattern(s) of these PIs in the *S. nigrum* reproductive structures also suggest a probable developmental role of specific PIs.

Biotic stressed *C. annuum* showed induction of CanPIs with more IRDs per precursor, which at steady state exhibit low level expression. Observations indicate the involvement of different CanPIs in either tissue-specific endogenous physiological function and/or in induced defense against insects (Tamhane *et al*. 2009). This study surfaces many questions related to the physiological role of PIs, 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 as well? (ii) Why are one and two IRD PIs diverse in the stem tissues? (iii) Why do the three and four IRD PIs show high subtype diversity and variability in expression during stages of fruit development? And perhaps most importantly (iv) What is the mechanism through which plants bring about the coordinated regulation of Pin-II expression for endogenous and induced/defense functions?

It will be interesting to find out how much of the diversity in Pin-II PI mRNAs actually translates into PI proteins *in situ*. But it is certain that in the translated Pin-II PI 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 PI fragments with variable number of IRDs. The variation(s) in IRDs, linker sequences and structures of individual Pin-II fragments cumulatively contribute to modify their PI specificities. It has been reported that the expression levels of the precursor PIs as well as the plant proteinases increase due to insect attack (Horn *et al*. 2005) Pin-II PI proteins expressed and the post-translational variations they undergo are responsible to generate a PI cocktail, best suited for an endogenous and/or defense purpose in the plant. Several studies on the Pin-II PI proteins have identified important aa in the polypeptide chain of the IRD responsible for modulating the PI specificity and potential (Dunse *et al.* 2010b). PI with diverse combination of IRDs was found to be better in retarding the growth and development of a phytophagous pest as compared to PIs with similar IRDs (Tamhane *et al*. 2007). *In silico* followed by wet lab analysis of variant Pin-II PI/IRD structures and their interaction studies with protease will enable the identifica-

tion of IRD(s) combination best suited to target a gut protease cocktail eventually to combat a particular pest.

The plant–insect interactions result in complicated co-evolutionary phenomena, as the adaptations are mutual, leading to speciation and spread of each other. The interactions have 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. 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 in multiple plant systems, 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. Dunse *et al.* (2010) reported success in combating phytophagous insects by using PI transgenic cotton. It is an important study demonstrating the 'on field' potential of using combinations of plant PIs (NaPI-Pin-II and StPin1A-Pin-I) to prevent crop damage caused by insects.

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 reports of using combination of different defense molecules simultaneously in transgenic plants (as summarized in Christou *et al.* 2006). So in a long lasting co-evolutionary interaction between the two, plants appear to 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 (Harsulkar *et al*. 1999), if proper expression is ensured. Due to a high diversity in Pin-II PI IRDs it is possible to identify combinations of IRDs, which are best suited for a particular insect infestation.

The naturally occurring gene diversity in Pin-II PIs provides a very effective/elaborate starting material to select the best insect defensive combination 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 the goal of crop protection through productive, sustainable, yet environmentally friendly insect resistant strategies.

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REFERENCES

Abdeen A, Virgos A, Olivella E, Villanueva J, Aviles 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

Antcheva N, Pintar A, Patthy 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 annuum* seeds. *Protein Science* **10**, 2280-2290

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

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, Ng KK, Cherney MM, Pearce G, Ghani U, Ryan CA, James MN (2003) Unbound form of tomato inhibitor-II reveals interdomain flexibility and conformational variability in the reactive site loops. *The Journal of Biological Chemistry* **278**, 31391-31400

Barrette-Ng IH, Ng KK, Cherney MM, Pearce G, Ryan CA, James MN (2003) Structural basis of inhibition revealed by a 1:2 complex of the two-headed tomato inhibitor-II and subtilisin Carlsberg. *The 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 in Genetics* **18**, 600-603

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

Bennett MJ (1994) Domain swapping: Entangling alliances between proteins. *Proceedings of the National Academy of Sciences USA* **91**, 3127-3131

Bezzi S, Kessler D, Diezel C, Muck A, Anssour S, Baldwin IT (2010) Silencing NaTPI expression increases nectar germin, nectarins, and hydrogen peroxide levels and inhibits nectar removal from plants in nature. *Plant Physiology* **152**, 2232-2242

Broadway RM, Duffey SS (1986) 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

Bryant J, Gurusaddaiah T, Ryan CA (1976) Proteinase Inhibitor I1 from potatoes: Isolation and characterization of its promoter components. *Biochemistry* **15**, 3418- 3423

Bu QY, Wu L, Yang SH, Wan JM (2006) Cloning of a potato proteinase inhibitor gene PINII-2x from diploid potato (*Solanum phurejia* L.) and transgenic investigation of its potential to confer insect resistance in rice. *Journal of Integrative Plant Biology* **48**, 732-739

Chen H, Gonzales-Vigil E, Wilkerson CG, Howe GA (2007) Stability of plant defense proteins in the gut of insect herbivores. *Plant Physiology* **143**, 1954-1967

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

Christeller JT (2005) Review article: Evolutionary mechanisms acting on proteinase inhibitor variability. *FEBS Journal* **272**, 5710-5722

Chye ML, Sin SF, Xu ZF, Yeung EC (2006) Serine proteinase inhibitor proteins: Exogenous and endogenous functions. *In Vitro Cellular and Developmental Biology - Plant* **42**, 100-108

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

Diezel C, Kessler D, Baldwin IT (2011) Pithy protection: *Nicotiana attenuata*'s jasmonic acid-mediated defenses are required to resist stem-boring weevil larvae. *Plant Physiology* **155**, 1936-1946

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

Dunse KM, Stevens JA, Lay FT, Gaspar YM, Heath RL, Anderson MA (2010) Coexpression of potato type I and II proteinase inhibitors gives cotton plants protection against insect damage in the field. *Proceedings of the National Academy of Sciences USA* **107**, 15011-15015

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

Fritz H (2000) Foreword. In: von der Helm K, Korant BD, Cheronis JC (Eds) *Proteases as Targets for Therapy*, Springer-Verlag, pp 5-6

Fujimoto T, Tomitaka Y, Abe H, Tsuda S, Futai K, Mizukubo T (2011) Expression profile of jasmonic acid-induced genes and the induced resistance against the root-knot nematode (*Meloidogyne incognita*) in tomato plants (*Solanum lycopersicum*) after foliar treatment with methyl jasmonate. *Journal of Plant Physiology* **168**, 1084-1097

Gadea J, Mayda ME, Conejero V, Vera P (1996) Characterization of defense-related genes ectopically expressed in viroid-infected tomato plants. *Molecular Plant-Microbe Interactions* **9**, 409-415

Garcia-Olmedo F, Salcedo G, Sánchez -Monge RF, Gomez L, Royo J, Carbonero P (1987) Plant proteinaceous inhibitors of proteinases and α-amylases. *Oxford Surveys of Plant Molecular and Cell Biology* **4**, 275-334

Gatehouse AM, 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 (1998) 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. *Recent Research Developments in Phytochemistry* **8**, 117-137

Glawe GA, 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

Gomes MT, Oliva ML, Lopes MT, Salas CE (2011) Plant proteinases and inhibitors: An overview of biological function and pharmacological activity. *Current Protein and Peptide Science* **12**, 417-436

Graham JS, Pearce G, Merryweather J, Titani K, Ericsson LH, Ryan CA (1985) 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 defense mechanism against insects. *Science* **175**, 776-777

Greenblatt HM, 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

Hansen D, Macedo-Ribeiro S, Verissimo P, Im SY, Sampaio MU, Oliva MLV (2007) Crystal structure of a novel cysteinless plant Kunitz-type protease inhibitor. *Biochemical and Biophysical Research Communications* **360**, 735-740

Hartl M, Giri AP, Kaur H, Baldwin IT (2010) Serine protease inhibitors specifically defend *Solanum nigrum* against generalist herbivores but do not influence plant growth and development. *Plant Cell* **22**, 4158- 4175

Hartl M, Giri AP, Kaur H, Baldwin IT (2011) The multiple functions of plant serine protease inhibitors: Defense against herbivores and beyond. *Plant Signalling and Behaviour* **6**, 1-3

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, Van Heeswijck R, Anderson MA (1997) Proteinase inhibitors from *Nicotiana alata* enhance plant resistance to insect pests. *Journal of Insect Physiology* **43**, 833-842

Hilder VA, Gatehouse AMR, Boulter D (1993) Transgenic plants conferring insect tolerance: Proteinase inhibitor approach. *Transgenic Plants* **1**, 317-338

Hirota SYH, Nagao S, Taketa M, Komori H, Kamikubo H, Wanga Z, Takahashi I, Negi S, Sugiura Y, Kataoka M, Higuchi Y (2010) Cytochrome c polymerization by successive domain swapping at the C-terminal helix. *Proceedings of the National Academy of Sciences USA* **107**, 12854-12859

Horn M, Patankar AG, Zavala JA, Wu J, Doleckova-Maresova L, Vujtechova M, Mares M, Baldwin IT (2005) Differential elicitation of two processing proteases controls the processing pattern of the trypsin proteinase inhibitor precursor in *Nicotiana attenuata*. *Plant Physiology* **139**, 375-388

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

Johnson ED, Miller EA, Anderson MA (2007) Dual location of a family of proteinase inhibitors within the stigmas of *Nicotiana alata. Planta* **225**, 1265-1276

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

Jørgensen M, Stensballe A, Welinder KG (2011) Extensive post-translational processing of potato tuber storage proteins and vacuolar targeting. *The Federation of European Biochemical Societies Journal* **278**, 4070-4087

Keil M, Sanchez-Serrano J, Schell J, Willmitzer L (1986) Primary structure of a proteinase inhibitor II gene from potato (*Solanum tuberosum*). *Nucleic Acids Research* **14**, 5641-5650

Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: The emerging molecular analysis. *Annual Review of Plant Biology* **53**, 299-328

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

Kong L, Ranganathan S (2008) Tandem duplication, circular permutation, molecular adaptation: How Solanaceae resist pests via inhibitors. *BMC Bioinformatics* **9 (Suppl 1)**, S22

Lawrence PK, Koundal KR (2002) Plant protease inhibitors in control of phytophagous insects. *Electronic Journal of Biotechnology* **5**, 5-6

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. *Plant Journal* **33**, 567-576

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

Li L, Li C, Lee GI, Howe GA (2002) 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

Li X-Q, Zhang T, Donnelly D (2011) Selective loss of cysteine residues and disulphide bonds in a potato proteinase inhibitor II family. *PLoS ONE* **6**, e18615

Liu J, Xia KF, Zhu JC, Deng YG, Huang XL, Hu BL, Xu X, Xu ZF (2006) The nightshade proteinase inhibitor IIb gene is constitutively expressed in glandular trichomes. *Plant and Cell Physiology* **47**, 1274-1284

Lou Y, Baldwin IT (2004) Nitrogen supply influences herbivore-induced direct and indirect defenses and transcriptional responses in *Nicotiana attenuata*. *Plant Physiology* **135**, 496-506

Luo M, Wang Z, Li H, Xia KF, Cai Y, Xu ZF (2009) Overexpression of a weed (*Solanum americanum*) proteinase inhibitor in transgenic tobacco results in increased glandular trichome density and enhanced resistance to *Helicoverpa armigera* and *Spodoptera litura*. *International Journal of Molecular Sciences* **10**, 1896-1910

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

Mishra M, Tamhane VA, Khandelwal N, Kulkarni MJ, Gupta VS, Giri AP (2010) Interaction of recombinant CanPIs with *Helicoverpa armigera* gut proteases reveals their processing patterns, stability and efficiency. *Proteomics* **10**, 2845-2857

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

Narvaez-Vasquez J, Ryan C (2004) The cellular localization of prosystemin: A functional role for phloem parenchyma in systemic wound signaling. *Planta* **218**, 360-369

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

Otlewski J, Jaskolski M, Buczek O, Cicerpicki T, Czapinska H, Krowarsch D, Smalas AO, Stachowiak D, Szpineta A, Dadlez M (2001) Structure-function relationship of serine protease-protein inhibitor interaction. *Acta Biochimica Polonica* **48**, 419-428

Park KS, Cheong JJ, Lee SJ, Suh MC, Choi D (2000) A novel proteinase inhibitor gene transiently induced by tobacco mosaic virus infection. *Biochimica et Biophysica Acta* **1492**, 509-512

Pearce G, Strydom D, Johnson S, Ryan CA (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* **253**, 895-897

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

Peña Cortés H, Sanchez-serrano JJ, Mertens R, Willmitzer L, Prat S (1989) Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. *Proceedings of the National Academy of Sciences USA* **86**, 9851- 9855

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

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

Rawlings ND, Tolle DP, Barrett AJ (2004) Evolutionary families of peptidase inhibitors. *Biochemical Journal* **378**, 705-716

Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ (2008) MEROPS: the peptidase database. *Nucleic Acids Research* **34**, D320-D325

Royo JN, Leon J, Vancanneyt G, Albar JP, Rosahl S, Ortego Fl, Castanera P, Sánchez-Serrano JJ (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

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

Ryan CA (1990) Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. *Annual Review of Phytopathology* **28**, 425-449

Ryan CA (2000) The systemin signaling pathway: Differential activation of plant defensive genes. *Biochimica et Biophysica 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

Sánchez-Serrano JJ, 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

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* **7**, 793-802

Schirra HJ, Scanlon MJ, Lee MC, 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

Schirra HJ, Anderson MA, Craik DJ (2008) Structural refinement of insecticidal plant proteinase inhibitors from *Nicotiana alata*. *Protein and Peptide Letters* **15**, 903-909

Schirra HJ, Guarino RF, Anderson MA, Craik DJ (2010) Selective removal of individual disulfide bonds within a potato type II serine proteinase inhibitor from *Nicotiana alata* reveals differential stabilization of the reactive-site loop. *Journal of Molecular Biology* **395**, 609-626

Schmidt S, Baldwin IT (2006) Systemin in *Solanum nigrum*. The tomato-homologous polypeptide does not mediate direct defense responses. *Plant Physiology* **142**, 1751-1758

Shin R, Lee GJ, Park CJ, Kim TY, You JS, Nam YW, Paek KH (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

Shinogi T, Hamanishi Y, Otsu Y, Wang YQ, Nonomura T, Matsuda Y, Toyoda H, Narusaka Y, Tosa Y, Mayama S (2005) Role of induced resistance in interactions of *Epilachna vigintioctopunctata* with host and non-host plant species. *Plant Science* **168**, 1477-1485

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

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

Srinivasan T, Kumar KR, Kirti PB (2009) Constitutive expression of a trypsin protease inhibitor confers multiple stress tolerance in transgenic tobacco. *Plant and Cell Physiology* **50**, 541-553

Sun JQ, Jiang HL, Li CY (2011) Systemin/jasmonate-mediated systemic defense signaling in tomato. *Molecular Plant* **4**, 607-615

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 – General Subjects* **1722**, 156-167

Tamhane VA, Giri AP, Sainani MN, Gupta VS (2007) Diverse forms of Pin-II family proteinase inhibitors from *Capsicum annuum* adversely affect the growth and development of *Helicoverpa armigera*. *Gene* **403**, 29-38

Tamhane VA, Giri AP, Kumar P, Gupta VS (2009) Spatial and temporal expression patterns of diverse Pin-II proteinase inhibitor genes in *Capsicum annuum* Linn. *Gene* **442**, 88-98

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

Van Dam NM, Horn M, Mares M, Baldwin IT (2001) Ontogeny constrains systemic protease inhibitor response in *Nicotiana attenuata*. *Journal of Chemical Ecology* **27**, 547-568

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

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

Wu J, Kang JH, Hettenhausen C, Baldwin IT (2007) Nonsense-mediated mRNA decay (NMD) silences the accumulation of aberrant trypsin proteinase inhibitor mRNA in *Nicotiana attenuata*. *Plant Journal* **51**, 693-706

Xie J, Ouyang XZ, Xia KF, Huang YF, Pan WB, Cai YP, Xu X, Li B, Xu ZF (2007) Chloroplast-like organelles were found in enucleate sieve elements of transgenic plants overexpressing a proteinase inhibitor. *Bioscience*, *Biotechnology and Biochemistry* **71**, 2759-2765

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

Yang DH, Hettenhausen C, Baldwin IT, Wu J (2011) BAK1 regulates the accumulation of jasmonic acid and the levels of trypsin proteinase inhibitors in *Nicotiana attenuata*'s responses to herbivory. *Journal of Experimental Botany* **62**, 641-652

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

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

Zavala JA, Giri AP, Jongsma MA, Baldwin IT (2008) Digestive duet: Midgut digestive proteinases of *Manduca sexta* ingesting *Nicotiana attenuata* with manipulated trypsin proteinase inhibitor expression. *PLoS ONE* **3 (4)**, e2008

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