Interactions between diverse proteinase inhibitors from *Capsicum annuum* and insect pests: A biochemical and molecular approach

Lepidopteran insect pests are responsible for causing severe losses of several crop plants. Proteinase inhibitor (PI) based approach is extensively in focus, for environment friendly pest control approaches and detailed study of plant-insect interactions. I have carried out my thesis work on Pin-II type PIs from a non-preferred host of *H. armigera, Capsicum annuum*. The interaction between these *C. annuum* PIs and insect pests have been studied using various biochemical, molecular and proteomic approaches. The major objectives and the results obtained are summarized below:

Implications of induced proteinase inhibitor diversity in *Capsicum annuum*

*Capsicum annuum*, has an array of Pin-II type PI genes (*CanPIs*) displaying regulated expression in steady state and induced conditions. Induction experiments were performed on *C. annuum* leaves viz. mechanical wounding followed by treatment with oral secretions of *H. armigera* (*W+OS*), with water (*W+W*) and aphid infestation (*AI*). We cloned and sequenced the treatment specific *CanPI* transcripts to identify distinct *CanPI* genes for each treatment, which to our surprise yielded 44 new diverse Pin-II genes. Sixty seven *CanPIs* comprising 55 unique sequence variants of IRDs, illustrating varied distribution across treatments were identified. The overall abundance of 3-IRD PIs and TI domains were prevalent across these treatments whereas distinct equal affluence of 4-IRD PIs was credible in wounding with OS. The *CanPI* expression pattern in response to OS (elicitors) was markedly different from the other two treatments viz. wounding with water and aphid infestation, signifying the defense related role of CanPIs against lepidopteran pests. A differential pattern of induced PI activity accumulation was observed in *C. annuum* leaves upon various inductions. Further characterization of the induced PIs by protease inhibitory activity assays, 1D and 2-dimensional gel electrophoresis followed by TI visualization and MALDI-TOF-MS, corroborated their structural and functional diversity. Across various plant
parts of *C. annuum* also, the qualitative and quantitative variations in PI activity were evident with flower showing the highest accumulation of PI activity.

**Interaction of recombinant CanPIs with *Helicoverpa armigera* gut proteases reveals their processing patterns, stability and efficiency**

Six diverse representative *C. annuum* genes (*CanPI*-13, -15, -19, -22, -5 and -7) comprising one, two, three or four IRDs, were selected for cloning and expression in *P. pastoris*. Recombinant proteins were characterized with specific reference to their (i) processing by *H. armigera* gut proteinases (HGP) (ii) stability in proteolytic environment (iii) inhibitory activity against HGP. PAGE and MALDI-TOF-MS techniques revealed presence of multiple, processed repeats in the purified rCanPI proteins. rCanPIs were resolved on native and SDS-PAGE and visualized for TI profiles. Multiple TI activity isoforms were detected for the rCanPIs and indicated presence of heterogeneity at the activity level. rCanPIs were used for inhibition studies against trypsin, chymotrypsin and *H. armigera* gut proteinases of the 4th instar larvae fed on artificial diet (AD-HGP). The interactions of rCanPIs with various proteinases were studied by IF-MALDI-TOF-MS and revealed the processing of multi-IRD proteins at the linker regions by the HGPs. *In vitro* and *in vivo* stability of rCanPIs was analyzed by native in-gel TI activity visualization. The stability of individual rCanPI varied in the proteolytic environment. rCanPI-5 and rCanPI-7 showed maximum inhibition of HGP isoforms and their processed units were also found to be stable in presence of HGP. Even single aa variations in IRDs were found to significantly change the HGP inhibition specificity. Results demonstrated the low efficiency of single IRD CanPIs against HGPs; indicating importance of presence of multiple IRD genes *in planta* for defense.

**Structural-functional insights of single and multi-domain *Capsicum annuum* proteinase inhibitors**

Owing to the inducibility of 4-IRD CanPIs under insect infestation, higher stability and efficiency against HGPs, CanPI-7 was used for further biochemical and biophysical characterization under various conditions of temperature, pH and against diverse proteases. CanPI-15 and CanPI-7 were cloned in bacterial
expression system for the requirement of more purified protein for structural studies. Recombinant proteins were expressed and purified by Nickel affinity chromatography followed by size-exclusion. The purified protein preparations were analyzed for their interaction with broad range of proteases like trypsin, chymotrypsin, elastase, HGP, *Spodoptera exigua* gut proteases and showed 60-95% inhibition. The inhibition kinetic studies of CanPIs with trypsin revealed higher binding efficiency and potency of CanPI-7 as indicated by IC\textsubscript{50} and K\textsubscript{i}. The proteins were characterized for their optimum activity under varied conditions of temperature and pH and respective secondary structural changes in the proteins were monitored using the fluorescence spectroscopy, ANS binding assays and CD spectroscopy. CanPI proteins exhibited stability over a wide range of conditions. Secondary structure analysis by CD and *in silico* structure prediction for CanPI-7 gave interesting insights *viz.*, adaptability of CanPIs to attain polyproline fold and spatial arrangement of multiple domains in precursor molecules. The generated models were validated and assessed using bioinformatics tools. Molecular docking studies on CanPI-7 and the target proteases revealed the probable binding mechanism of CanPI precursors with multiple protease molecules at the same time thus, contributing its higher potency and inhibition efficiency.

The present study on *C. annuum* has brought in to light a diverse array of Pin-II PIs expressed in various tissues and under various conditions in the plant, with emphasis on their defense role. The characterization of representative CanPIs have indicated their high efficiency and promising potential in inhibiting lepidopteran insect gut proteases. Thus, the naturally occurring gene diversity in CanPIs provides an effective starting material to reach the goal of crop protection.