Functional characterization of proteinase inhibitors from *Capsicum annuum* using transgenic approach

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By

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Dedicated to.....

Aai, Anna, Rohini, Anvi and family

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CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled **"Functional characterization of proteinase inhibitors from *Capsicum annuum* using transgenic approach"** submitted by **Mr. Rahul Sadashiv Tanpure** to Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in Biological Sciences, embodies original research work under our supervision. We further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

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DECLARATION BY RESEARCH SCHOLAR

I hereby declare that thesis entitled **"Functional characterization of proteinase inhibitors from *Capsicum annuum* using transgenic approach"** submitted by me for the Degree of Doctor of Philosophy to Academy of Scientific and Innovative Research (AcSIR) is the record of work carried out by me at Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune-411008, India, under the supervision of Dr. Vidya S. Gupta (research supervisor) and Dr. Ashok P. Giri (research co-supervisor). The work is original and has not formed the basis for the award of any degree, diploma, associateship and/or fellowship titles in this or any other University or Institution. I further declare that the material from other sources has been duly acknowledged in the thesis.

Rahul Sadashiv Tanpure
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Rahul Sadashiv Tanpure

Contents

List of Abbreviations

aa	Amino acid
AI	Amylase inhibitor
BSA	Bovine Serum Albumin
EIC	Extracted Ion Chromatogram
PCR	Polymerase Chain Reaction
ESI-	Electrospray Ionization-Negative mode
ESI+	Electrospray Ionization-Positive mode
Fmol, mmole	Femtomole, millimole
g, mg, µg, ng	Gram, milligram, microgram, nanogram
HDMS	High Defination Mass Spectroscopy
GM	Genetically modified
Orbitrap	Ion Trap Quadrupole-Orbitrap
mRNA	Messenger RNA
OPLS-DA	Orthogonal Partial Least Squares-Discriminant Analysis
PCA	Principle Component Analysis
PLGS	ProteinLynxGlobal server
ppm	Parts per million
RNA	Ribonucleic acid
rpm	Revolutions per minute
PCR	Polymerase chain reaction
TCA	Trichloroacetic acid
Bt	*Bacillus thuringiensis*
CanPI	*Capsicum annuum* Proteinase Inhibitor
cDNA	Complementary deoxyribonucleic acid
CI	Chymotrypsin inhibitor
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetate
HaTry	*Helicoverpa armigera* trypsin
HaChy	*Helicoverpa armigera* chymotrypsin
HGPIUs	*Helicoverpa armigera* Gut Proteinase Inhibitor Units
HGPs	*Helicoverpa armigera* Gut Proteinases
IRD	Inhibitory Repeat Domain
kDa/kD	Kilo Dalton
L, mL, µL	Liter, milliliter, microliter
M, mM, µM	Molar, millimolar, micromolar
Fmol, µmole	Femtomole, micromole
CTAB	Cetyltrimethylammonium bromide
OD	Optical Density
PDB	Protein data bank
PI	Proteinase Inhibitor
Pin-I/II	Potato Proteinase Inhibitor I/II
rCanPI	Recombinant *C. annuum* Proteinase Inhibitor
RNA	Ribonucleic Acid
RSL	Reactive site loop
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction

TI	Trypsin inhibitor
TRIS	Tris-Hydroxymethyl Aminomethane
BA*p*NA	Benzoyl-DL-Arginyl-*p*-Nitroanilide
SAA*p*LNA	N-succinyl-L-ala-L-ala-L-pro-L-leu-p-nitroanilide
YEPD	yeast extract peptone dextrose
RFUs	relative fluorescent units
qRT-PCR	quantitative Real-Time PCR
Yca1	yeast metacaspase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
ZA	Zeatin
BAP	6-Benzylaminopurine
IAA	Indole-3-acetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
AC	Acetosyringone
WT	Wild type plants
SP	serine protease
CP	cysteine protease
EV	Empty vector yeast strain
NAA	Naphthaleneacetic acid

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Research articles published from this thesis work:

1. Joshi, R. S., **Tanpure R. S**., Singh, R. K., Gupta V. S. and Giri, A. P. (2014) Resistance through inhibition: Ectopic expression of serine protease inhibitor offers stress tolerance via delayed senescence in yeast cell. **Biochemical and Biophysical Research Communications** 452: 361-368

2. **Tanpure R. S.**, Barbole R. S., Dawkar V. V., Waichal Y. A., Giri A. P. and Gupta V. S. (2017) Improved tolerance against *Helicoverpa armigera* in transgenic tomato over-expressing multi-domain proteinase inhibitor gene from *Capsicum annuum*. **Physiology and Molecular Biology of Plants** 23 (3): 597-604

3. **Tanpure, R. S**., Lomate, P. R., Dawkar, V. V., Gupta, V. S. and Giri, A. P. (2013) Transgenic plants for insect tolerance: current status and future prospects. **Biotechnology: Beyond Borders** (Eds.) M.V. Deshpande, J. Ruiz-Herrera. 172- 187

Chapter 1

Review of Literature

1.1 Influence of insect pests on agriculture

Biotic stresses imposed by other organisms adversely affect the growth, development and productivity of plants. Mainly biotic stresses are caused by pests, pathogens, weeds and herbivores. Insect pests are the major constraints limiting the potential agricultural production. For example, *Helicoverpa armigera* causes damage to crops estimated at greater than US$ 2 billion annually, excluding social, economic and environmental costs associated with its control. The annual control costs and production losses due to *H. armigera* worldwide together is predicted to be about US$ 5 billion (Lammers and McLeod, 2007). It is estimated that herbivorous insects attack alone leads to about 26% crop yield loss all over the world while in India it is ~18% (Rs. 90,000 crore) (http://www.livemint. com/2009/02/25181538/India-loses-Rs90000 -cr-crop-y.html). India loses 15-25% potential crop output due to pests, weeds and pathogens. (http://www.financialexpress.com/india news/india loses 15-25-per-cent-potential-crop-output-due-pests-weeds-diseases). Reports indicate that in India, insect pest cause about 50% loss in cotton, 25% in rice, 20% in pulses and sugarcane and 5% in wheat (Dhaliwal and Arora, 1996; Singh, 2007). In terms of monetary value, Indian agriculture suffers an annual loss of about Rs. 276 crore due to insect pests out of which Rs. ~118 crore is caused by *H. armigera* alone. In India, the losses due to *H. armigera* infestation are estimated to be 15-46% in tomato, 4.2-39.7% in pulses, chickpea alone being 29.2%; 25-79% in cotton, 18-26% in sorghum, 50% in sunflower and over 40% in okra (Singh et al., 2014; Reddy and Zehr, 2004).

1.2 *Helicoverpa armigera* (Hübner): A global pest

Lepidoptera is one of the largest orders in the class Insecta which includes butterflies and moths. The larvae of various Lepidopteran species are herbivores and are major pests in agriculture. Some of the major pests belong to the families Noctuidae, Pyralidae and Tortricidae. The larvae of the *Helicoverpa* (corn earworm) and Noctuidae genus *Spodoptera* (armyworms) can cause wide damage to various crops. Members of genera *Heliothis* (Family: Noctuidae) are major agricultural pests of global significance. The host plant range for Lepidopteran insects may either be diverse (polyphagous or generalist), e.g., *H. armigera*, which feeds on various legumes, vegetables and fruits, or narrow (monophagous or specialist), e.g., *Manduca*

sexta, which shows preference for solanaceous plants (Ehrlich and Raven, 1964; Tamhane et al., 2007a; Wu and Baldwin, 2010).

H. armigera Hübner (Lepidoptera: Noctuidae) is a notorious and impactful insect pest of agriculture worldwide. It inhabits many countries of Asia, Europe, Africa and Australia; and has wide geographic presence. The major characteristic features which make it a successful pest is its polyphagy, high reproductive rate, mobility and facultative diapause (Fitt, 1989). *H. armigera* infests on an extensively wide range of plants (>180 plant hosts from >45 families) and the most important host crops include cotton, legumes, tomato, tobacco, okra, potato, sunflower, safflower, maize, groundnut, etc. (Fitt, 1989; Rajapakse and Walter, 2007). *H. armigera, H. zea, H. virescens* and *H. punctigera* are highly polyphagous and damage a large number of plant species, including food, fiber, oil, fodder and also horticultural and ornamental crops (Fitt, 1989). *H. armigera* shows higher tendency than similarly polyphagous *H. zea* for infestation and resistance development. *H. zea* was thought to be derived from *H. armigera* approximately 1.5-2 million years ago. Preferentially, larvae of *H. armigera* infests on reproductive structures and economically important plant parts like fruits, seeds, pods which eventually leads to major loss in crop yield.

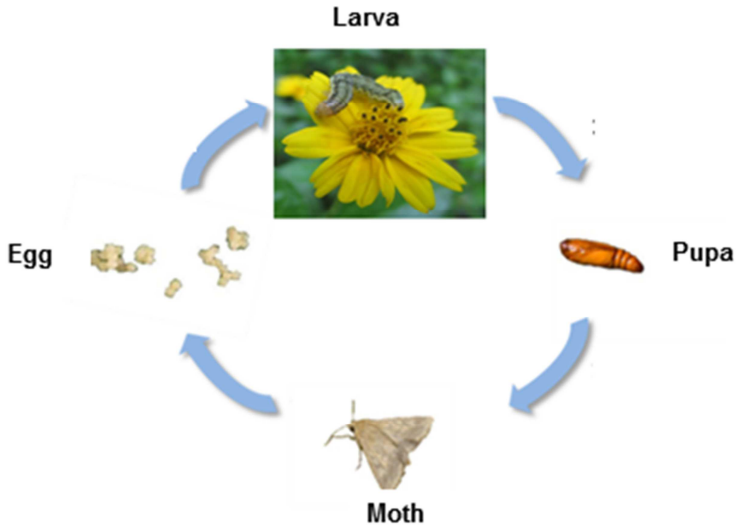


Figure 1.1: Stages in the life- cycle of *H. armigera*.

H. armigera has a life expectancy ranging between 25 to 35 days (**Figure 1.1**). Each female can lay several hundred eggs on lower surface of flowers, leaves, shoot tips and young pods. The eggs hatch after 3-4 days and larval stage persists for 12-16 days. The larval period is categorized into six instars in which the fourth and fifth are

extremely voracious and most damaging ones (Tamhane et al., 2007a). The caterpillars are aggressive, occasionally carnivorous and when opportunity arises, they become cannibalistic. Pupa is a non-feeding stage which lasts normally for 6-10 days. The moth remains alive for 4-5 days and feeds on nectar (Gowda and Sharma, 2005; Tamhane et al., 2007a). In case of adverse conditions, the larvae undergo facultative diapause i.e. a state of suppressed metabolism as pupae so as to survive. There is a difference in colour of the larval instars and the moth depending on the food on which they feed and the environment.

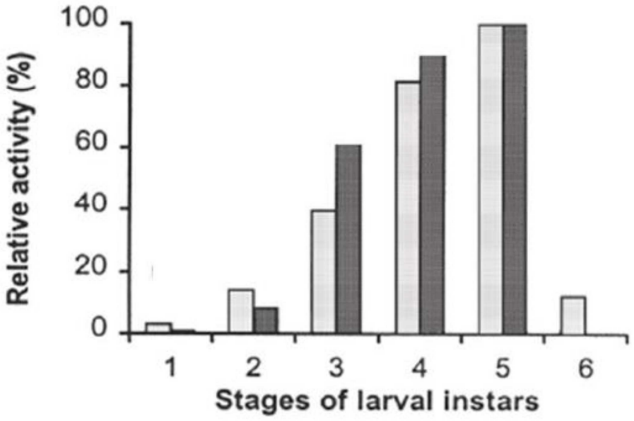


Figure 1.2: Relative gut proteinase activity of *H. armigera* during the stages of larval development [Reproduced and modified from Patankar et al., 2001].

Lepidopteran insects mostly rely on proteases for their digestive processes (Telang et al., 2001). The alkaline gut pH and presence of free glycine are other distinct characteristics of insect guts (Johnston et al., 1991). The major digestive enzymes of *H. armigera* midgut consist of endopeptidases like serine, cystine, cathepsin B like proteinases, metallo- and exopeptidases. Enzymes such as trypsin and chymotrypsin (serine proteinases), form the major mechanistic class (>95%) in the gut environment (Johnston et al., 1991; Purcell et al., 1992; Harsulkar et al., 1999; Patankar et al., 2001). Many of these proteinases have been isolated and the coding DNA/ cDNA have been characterized (Gatehouse et al., 1997; Bown et al., 1997, 1998; Mazumdar-Leighton et al., 2000; Patankar et al., 2001; Bayes et al., 2003; Chougule et al., 2005; Telang et al., 2003). The protein from complex food material is broken down into smaller oligopeptides by these endopeptidases mainly by trypsins and chymotrypsins. These oligo-peptides are further digested by exopeptidases releasing free amino acids. Thus, the digestion in the larval gut follows a rational

trend as a result of streamline digestive process. During larval development gut protease activity increases with the highest activity seen in the fifth instar larvae, followed by a sharp decline in the sixth instar (**Figure 1.2**). Polyphagy needs midgut proteases changes in insects in order to maximize the benefits from protein-rich plant reproductive structures, carbohydrate-rich leaves and even diverse unbalanced diets (Sarate et al., 2012). The total amount of carbohydrate, protein and lipid in the diet directly affect the insect growth and development. The gut protease composition varies according to the developmental stage of the larvae and the dietary content (Patankar et al., 2001; Chougule et al., 2005; Sarate et al., 2012). Significant relationships between nutritional qualities of the diet and larval and pupal mass have

Figure 1.3: *H. armigera* is a polyphagous pest which infests on various food crops *viz.* (A) marigold flower, (B) tobacco, (C) chickpea and (D) tomato.

been noticed on feeding *H. armigera* larvae on various host plants *viz.* vegetables (tomato and okra), legumes (chickpea and pigeon pea), cereals (sorghum and maize) and flowers (rose and marigold) (Fefelova and Frolov, 2008; Kotkar et al., 2009; Sarate et al., 2012) (**Figure 1.3**). Multigene families encoding variants of serine proteases in response to the dietary content have been recognized in *H. armigera* (Bown et al., 1997). The relative levels of amylase genes also vary among larvae feeding on various host plants (Kotkar et al., 2012). Variations in the digestive complement related to larval stage and diet explains the polyphagous nature of *H. armigera* which enables it to infest a various variety of agriculturally important crops simultaneously (Patankar et al., 2001).

1.3 Management of *H. armigera*

Owing to the huge loss in crop production due to *H. armigera*, various strategies used for its management include: cultural practices, chemical insecticides, biological control, host resistance, biotechnological approaches and integrated pest management. Cultural practices comprise hand picking of large sized larvae, deep ploughing of soil, weeding, shaking of plants, intercropping, use of trap crops, following a good time of sowing and fertilizer application (Dahiya et al., 1999). It also involves use of clean planting material, systematic trapping of adult insects (to control population build-up) and field sanitation (whereby residues that may form breeding grounds are removed). These cultural practices are economical and eco-friendly, however, they are usually very strenuous.

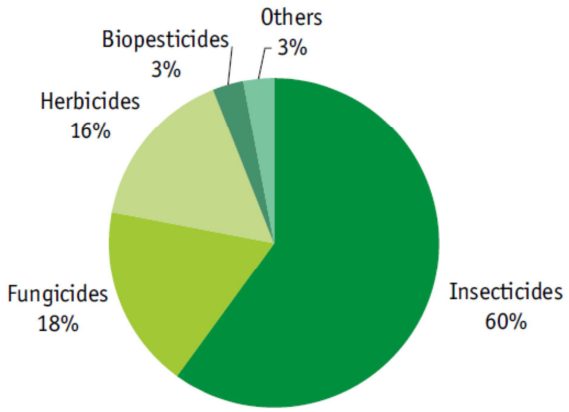


Figure 1.4: Indian crop protection market (2015) (Source: Industry reports, Analysis by Tata Strategic, Link: http://indiainbusiness.nic.in/newdesign/upload/Agrochemicals-Knowledge-report-2016.pdf.).

Since the decades, use of chemical pesticides is a major strategy used for controlling insect pests. The enormous economic success in agriculture is due to the application of pesticides because it helps in controlling biotic stresses. The Indian crop protection market is dominated by insecticides and almost 60% is covered by it (**Figure 1.4**). The major advantage of chemical pesticides is that they are effective even when used in an advanced stage of infestation but their continuous usage has resulted in the development of resistance against many chemical insecticides including organophosphates, organochlorides, carbamates, pyrethroids etc. (Mccaffery, 1998; Gunning et al., 1998; Dawkar et al., 2013). Moreover, chemical pesticides cause a serious threat to the atmosphere by contaminating soil, water,

vegetation and ecosystems and cause toxic effects on the biome including human beings and non-target organisms.

Biopesticides are new age crop protection products manufactured from natural sources like plants, animals, bacteria and fungi. They are eco-friendly, easy to use; require lower dosage amounts for the same performance as compared to chemical pesticides. Biopesticides involve utilizing the natural enemies (predators and parasitoids) of the pest such as live nematodes, fungi, bacteria, viruses or products derived from them and other plant products (Gurjar et al., 2011). Currently bio-pesticides constitute only 3% of Indian crop protection market (**Figure 1.4**). Being environment friendly, they are recently becoming very popular. For example, entomopathogenic fungi (*Beauveria bassiana* and *Metarhizium anisopliae*) and wasps (Trichogramma spp.) are treated as natural enemies of *H. armigera.* Among all the bio-pesticides, *Bacillus thuringiensis* (Bt) and Nuclear Polyhedrosis Virus (NPV) have been very popular. Plant products such as neem products, crude plant oils and vegetable oils are also used for pest control with limited success. However, major disadvantage of biopesticides is slow speed of action and requires specific condition for their survival.

One of important strategy adopted by breeders to control insect infestation is to use resistant variety. Host plant resistance to insect pests depends on the availability of resistance genes in the genome and their transfer to high yielding cultivars through conventional breeding process or modern biotechnological approaches. For example, in case of chickpea, few wild species (*Cicer bijugum*, *Cicer judaicum* and *Cicer reticulatum*) have been reported to be potential sources of resistance/ tolerance to *H. armigera* which can be utilised to increase the level of insect resistance in chickpea (Sharma et al., 2007).

One of the important approaches is to employ recombinant DNA technology to grow genetically modified (GM) crops with enhanced insect resistance by transfer of heterologous genes from various other sources. This technology has become popular worldwide, where in a total of 26 countries (19 developing and 7 industrial countries), planted such transgenic crops in the year 2016 (**Figure 1.5**). Moreover, GM crops grown area surged to a record of 2.1 billion hectares in 2016. In last 20 years, out of 2 billion hectares of GM crops grown commercially comprised 1.0 billion hectares of GM soybean, 0.6 billion hectares of GM maize, 0.3 billion hectares

of GM cotton, and 0.1 billion hectares of GM canola (ISAAA report 2016). In India, transgenic Bt cotton with enhanced resistance to *H. armigera* is the first biotech crop grown commercially since 2002 (Herring, 2015) and India has retained its number one position in transgenic cotton producing country in the world (ISAAA report, 2016). Very recently in the year 2016, Genetic Engineering Appraisal Committee (GEAC) of India has completed the biosafety assessment of transgenic mustard hybrid DMHII and parental lines containing Barnase/ Barstar gene and if permitted by

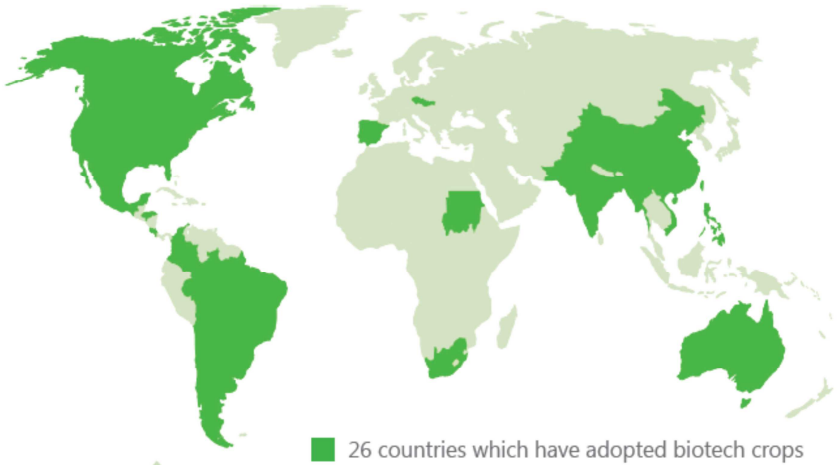


Figure 1.5: Global area of GM crops (Source: ISAAA report 2016).

the Government of India, GM mustard would be the first GM food crop developed by the Centre for Genetic Manipulation of Crop Plants (CGMCP), University of Delhi, New Delhi, India.

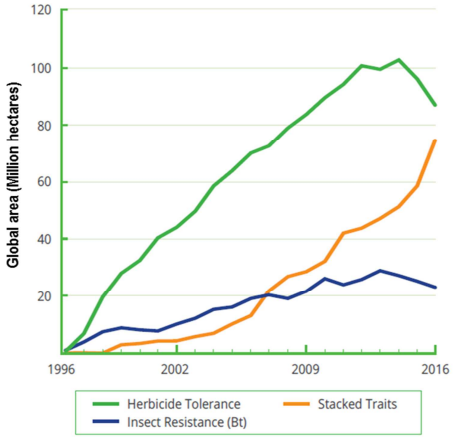


Figure 1.6: Global area of GM crops from 1996 to 2016 by trait (Million Hectares) (Source: ISAAA report, 2016).

Not only a single trait, but recently, there is also increase in stacked traits in GM crops all over the world (ISAAA report 2016) (**Figure 1.6**). Significance and utilization of the GM plants for insect tolerance has been further discussed in detail below.

1.4 Insect control through transgenic approach

Genetic engineering technique offers myriads of applications in improvement of crops for insect tolerance and for enhancement of quality. The most widely used traits in plant genetic engineering are herbicide and pest resistance (ISAAA report, 2016). During the last two decades, a large number of GM crops resistant to insect pest have been developed and more are underway. For providing protection against insect, various genes like *Cry* toxin, proteinase inhibitor, amylase inhibitor, chitinase, lectin, defensin and pathogenesis-related genes are being transferred to many crops world over.

Bacillus thuringiensis (Bt) Cry toxins as insecticidal proteins

Bacillus thuringiensis (Bt) is an aerobic, spore-forming, gram-positive and entomopathogenic bacterium that produces crystal proteins or δ-endotoxins (Cry). In the German province of Thuringia, Ernst Berliner isolated *B. thuringiensis* from a diseased Mediterranean flour moth (*Ephestia kuehniella*) around at 1911 (Dawkar et al., 2013). The insecticidal property of Bt is due to proteinaceous crystals that are generated during sporulation (Bravo et al., 2004). In 1938, first commercial Bt insecticide Sporeine was produced and later other Bt products such as Thuricides were developed. The mode of action of Bt toxins in insects involves several steps which occur in digestive track of insects, including solubilization of Bt-crystal, proteolytic processing of Bt pro-toxin by insect proteinases, binding of activated toxin to midgut receptors, and insertion of the toxin molecule into the gut epithelial cell membrane to create pores (Bravo et al., 2004, 2007). Alternatively, after being activated in the midgut, one of the domains of the toxin, domain II combines with receptor proteins, leading to a change in conformation of toxin (Bravo et al., 2007). The activated toxin binds to brush border membrane vesicles. Then the intestinal epidermal cells of insect are ruptured, causing the death of the insect (Bravo et al., 2004) (**Figure 1.7**). In Lepidopteran insects, cadherin like proteins, glycosylphophatidylinositol (GPI)-anchored aminopeptidase-N (APN), GPI-anchored alkaline phosphatase (ALP), gylcolipids and P252 have been identified as Cry toxin receptors (Pigott and Ellar, 2007). Cry1A toxins were reported to bind cadherin protein in

several insects such as *Manduca sexta, Bombyx mori, Heliothis virescens, H. armigera, Pectinophora gossypiella, Ostrinia nubilalis* etc. Cry1 toxins also bind to APN proteins families from *B. mori, H. armigera. H. virescens, Lymantria dispar, M. sexta* and *Plutella xylostella* (Pigott and Ellar, 2007). A cadherin protein was identified as Cry3Aa receptor in Coleopteran insects such as *Diabrotica virgifera* and *Tenebrio molitor* (Park et al., 2009; Fabrick et al., 2009).

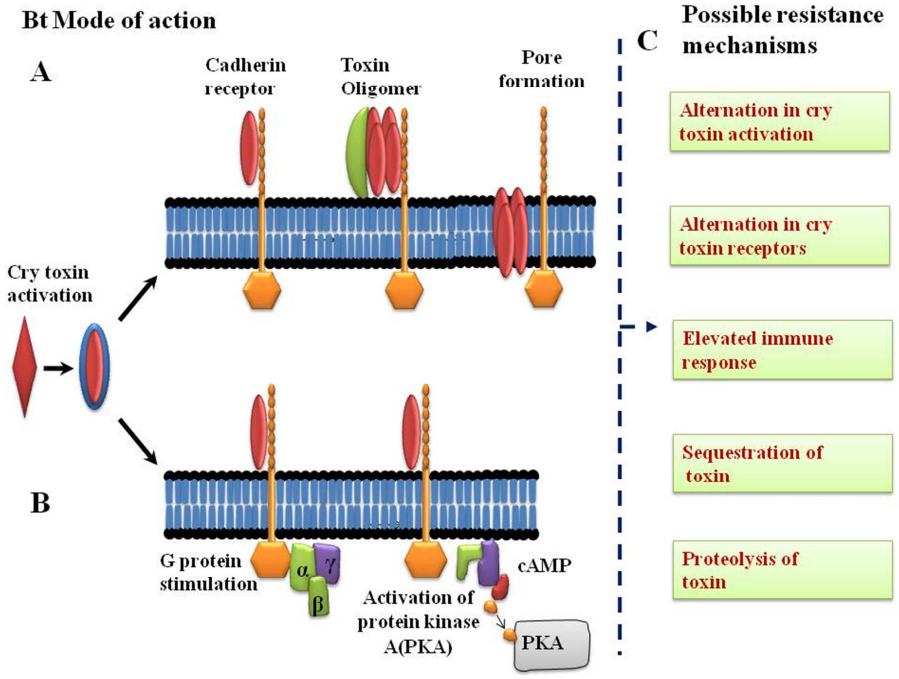


Figure 1.7: Mechanism of action of Bt (cry) toxins and possible resistance mechanism (A, B) Schematic representation of the mechanism of action of Bt-Cry toxins on intestinal epidermal cells of insect (C) Possible resistance mechanisms that are described in the literature (Adopted from Tanpure et al., 2013)

More than 500 different Cry gene sequences have been classified into 70 groups (*Cry1-Cry67*) based on their primary amino acid sequence (Crickmore et al., 2011). These groups may have different modes of action and may be grouped in four different families (i) three-domain Cry toxins (3D), mosquitocidal Cry toxins (Mtx), binary-like (Bin) and the Cyt toxins (Bravo et al., 2011). Insect specificity is largely determined by the specific binding of Cry toxins to surface proteins located in the microvilli of larvae midgut cells. Separate strains of Bt produce a variety of crystal toxins with distinct host ranges. Transgenic expression of Bt insecticidal toxins is presently the most significant and commonly used strategy for crop plants against the insect pests. To the best our knowledge following listed genes encoding different Bt toxins have been engineered into plants:

cry1Aa, cry1Ab, cry1Ac, cry1Ba, cry1Ca, cry1H, cry2Aa, cry3A, cry6A and *cry9C* (**Table 1.1**).

Table 1.1: Commercial transgenic plants expressing different toxins for protection against insects

Host Plant	Trade name	Toxins/ genes	Target pest and traits
Tomato	5345 tomato	Cry1Ac	Lepidopteran
Soybean	Intacta Roundup Ready 2 Pro	Cry1Ac, cp4 epsps	Lepidopteran and Herbicide tolerance
Rice	BT Shanyou 63	Cry1Ab, Cry1Ac	Lepidopteran
Potato	Atlantic New Leaf	Cry3A	Coleopteran
	New Leaf Russet Burbank	Cry3A, plrv_orf1, plrv_orf2	Coleopteran and Potato leaf roll virus
	Shepody NewLeaf Y	Cry3A, pvy_cp	Coleopteran/ Potato virus Y (PVY)
Maize	YieldGard	Cry1Ab	Lepidopteran
	Optimum Intrasect Xtreme	Cry1Fa2, cp4 epsps,Cry34Ab1, Cry35Ab1, Cry1Ab, mCry3A	Lepidopteran, Coleopteran and Herbicide tolerance
	Genuity SmartStax	cp4 epsps , Cry1Fa2, Cry2Ab2, Cry35Ab1, Cry34Ab1, Cry3Bb1, Cry1A.105	Lepidopteran, Coleopteran and Herbicide tolerance
	Agrisure Viptera 3110	Cry1Ab, Vip3Aa20	Lepidopteran
	Agrisure Viptera 3111	Cry1Ab, Cry3Aa, Vip3Aa20	Lepidopteran
Cotton	Roundup Ready Bollgard	cp4 epsps, Cry1Ac	Lepidopteran and Herbicide tolerance
	Bollgard	Cry1Ac	Lepidopteran
	VipCot	Vip3Aa19,Cry1Ab	Lepidopteran

In general, Bt toxins have been transgenically expressed in more than 26 different plant species. Usually codon-optimized genes are being transferred into crops, including cotton (*Gossypium hirsutum*), maize (*Zea mays*), potato (*Solanum tuberosum*), cabbage (*Brassica oleracea*) and alfalfa (*Medicago sativa*) (Schuler et al., 1998). The level of expression of

Bt toxins needs to be sufficient to cause high mortality of target pests in the field. Bt cotton has been released commercially in India, China, USA, Australia, South Africa and several other countries. This approach has resulted in the significant retardation of growth of many insect species in the field. However, its effectiveness is threatened by the development of resistance in some species. This may be due to disruption of any of the steps in the mode of action (Heckel et al., 2007) (**Figure 1.7**). Generally, Bt resistance mechanism is due to the variation of midgut receptor binding for Bt toxins (Ferré and Van Rie, 2002). Furthermore, several reports have shown the relationship between Bt resistance with reduced activity of digestive enzymes involved in the solubilization and activation of Bt pro-proteins (Forcada et al., 1996; Oppert et al., 1997; Li et al., 2004; Karumbaiah et al., 2007). The other mechanism reported is degradation of the Cry toxins (Forcada et al., 1996) and elevated immune status (Ma et al., 2005).

Vegetative insecticidal proteins (Vips) from *B. thuringiensis*

Many strains of Bt are known to produce insecticidal proteins during their vegetative growth. These proteins are called vegetative insecticidal proteins (Vips). *Vip* gene family does not show similarity with Bt toxins (Lee et al., 2003; Yu et al., 1997). At present, hundreds of Vip genes have been identified, cloned and characterized. These genes can be classified into three groups, eight subgroups and 25 classes according to the encoded amino acid sequence similarity. Vip has toxicity of the same degree as that of Bt toxin, however, does not need to be solubilized in the insect gut before it can act. Vip1 and Vip2 are binary toxins that have specificity to Coleopteran, whereas Vip3 toxins have specificity to Lepidopteran insects. Vip proteins have different mechanisms than Cry proteins for toxicity and distinctive receptors in the midguts of insects. Upon activation, the Vip protein can bind to midgut epithelial cells in the insect and can activate programmed cell death in those cells (Yu et al., 1997).

Syngenta Company Pvt. Ltd. developed Vip based transgenic cotton and corn with pyramided traits that expressed two insecticidal proteins derived from *B. thuringiensis viz.* Vip3A and Cry1Ab (**Table 1.1**). Together these proteins were highly effective against wide range of Lepidopteran pests (Estruch et al., 1996). VipCot cotton plants lack cross-resistance between Vip3A and Cry proteins (Kurtz et al., 2007). This would also help in delaying the development of resistance in pests to transgenic Bt traits. Recently, chimeric *Vip3A* and *Cry1Ac* gene was transformed into cotton, which exhibited

a broad insecticidal spectrum against lepidopteran pests (Chen et al., 2017). Maize plants containing *Cry1B* and *Vip3Aa1* gene showed toxicity against the fall armyworm and European corn borer (Estruch, 2000). Field trials of various Vip based transgenic crops are currently at advance stages. The cultivation of Vip based transgenic crops is likely to increase the production and further reduce global chemical pesticide usage.

Other defense proteins

Protease inhibitors (PIs): Plant defensive molecules against insect herbivory

Protease inhibitors (PIs) play important role in plant protection. There have been many studies demonstrating that growth and development of insect retard upon exposure to PIs. Antibiosis nature of PI leads the concept of developing PI-based transgenic for insect tolerance (Ryan, 1990; Gatehouse 2011) (**Table 1.2**). Significance and utilization of PIs has been further discussed in section 1.5 and further sections.

α-Amylase inhibitors (AIs)

In plants, proteinaceous AIs as a part of the natural defense mechanisms are present in cereals such as wheat, barley, sorghum, rye and rice and in legumes such as pigeon pea, cowpea and common bean (Franco et al., 2002). AIs exhibit different specificities against α-amylases from diverse sources (**Table 1.2**). Thus, AIs are attractive candidates for the control of seed weevils as these insects are highly dependent on starch as an energy source. The AIs have long been proposed as possibly important weapons against insect pests whose diets make them highly dependent on the α-amylase activity. For example, the expression of α-AI from common bean (*Phaseolus vulgaris*) seeds is lethal to several insects, including pea weevil and some species of bruchids in transgenic plants (Ishimoto et al., 1996). Transgenic pea expressing *α-AI-1* gene showed larval mortality in pea weevil while *α-AI-2* delayed larval maturation (Schroeder et al., 1995). Further, *in vitro* and *in vivo* trials, including those made under field conditions, have now fully confirmed this potential, raising the possibility of significantly increased yields. Further research to identify potent AIs needs to be performed (Franco et al., 2002). However some AIs from wheat and barley are identified as an allergen for human body (Fränken et al 1994; Barber et al., 1989).

Lectins

Lectins are carbohydrate-binding proteins that are highly specific for sugar moieties. The insecticidal properties of some lectins for species from various insect orders have been known (**Table 1.2**). Lectins disrupt the gut cell wall of insects by binding to glycoproteins of brush border cells and thus, the nutrient uptake is hampered. The first transgenic plant expressing lectin showing insecticidal activity was produced in 1990 (Boulter et al., 1990). Lectins have attracted substantial research attention as some of them showed toxicity to various pest insects, while showing no/ low mammalian toxicity (Murdock and Shade, 2002).Though mode of action is not yet fully known, few lectins showed to interact with insect gut brush-border membranes and others to the peritrophic membrane. Initial reports to explore the role of lectins for insect control indicated that, wheat germ agglutinin exhibited insecticidal potential but exhibited toxicity toward mammal. *Galanthus nivalis* agglutinin (GNA), have reached commercialization stage, since it did not showed mammalian toxicity. In terms of commercialization, plants with GNA are the most advanced and considerable efforts are focused on finding similar lectin genes (Murdock and Shade, 2002).

Chitinases

Chitinases are potential candidates for developing insect resistant plants, seeing that chitin occurs specifically in nematodes, arthropods, fungi and some algae and thus, adverse effects on vertebrates are unlikely. Chitinases derived from plants, microbes and animals have raised interest for producing transgenic plants for increase resistance against insect pests or fungal pathogens (**Table 1.2**). Chitinase derived from plants are being used for engineering transgenic plants against fungal diseases. However, recent studies on chitinases for insect resistance have been more attention on insect-derived chitinases. A chitinase gene derived from *M. sexta*, have been expressed in papaya plants showing resistance to *Tetranychus cinnabarinus* (McCafferty et al., 2006). *M. sexta* chitinase have been expressed in cotton plants, however their efficacy in controlling insect pests have not reported so far (Hao et al., 2005). Apparently, every arthropod might be vulnerable to a chitinase, however it would require to be adequately exposed to it at suitable stage of life for effectiveness.

Defensins

Defensins consist of peptides derived from several plant species exhibiting antimicrobial porperties towards various types of microorganisms. Some of these have also been shown to have insecticidal activity (Lay et al., 2003; Liu et al., 2006b). Few defensins from different plant species have been characterized and probably will be exploited for their antimicrobial and insecticidal activities (Lay and Anderson, 2005).

Lipid acyl hydrolase

The first evidence that lipid acyl hydrolase exhibit insecticidal properties came in 1995, when Strickland et al. (1995) reported that the growth of western and southern corn rootworms was inhibited by patatin. Some transgenic plants are produced demonstrating insecticidal properties of these glycoproteins (Alibhai and Rydel, 2003). However, transgenic plants expressing lipid acyl hydrolases is not currently available for commercial development.

Table 1.2: Defense related proteins having potential to develop insect-resistance transgenic plants.

Protein	Mode of action	Target insects	References
Protease inhibitors	Inhibit digestive proteases	Lepidopteran, Coleopteran, Dipteran	(Dunse et al., 2010) (Calderon et al., 2005) (Cruz et al., 2013)
Amylase inhibitors	Inhibit amylases	Coleopteran Lepidopteran	(Franco et al. 2005) (Pytelkova et al., 2009)
Lectins	Agglutination of insect gut brush-border and peritrophic membrane	Lepidopteran, Dipteran, Hemipetran, Coleopteran	(Boulter et al., 1990) (Murdock and Shade, 2002)
Chitinases	Degradation of insect chitin	Lepidopteran, Dipteran, Hemipetran, Coleopteran	(McCafferty et al., 2006)
Defensin	Bind to the cell membrane and form pores and leads to efflux of essential ions and nutrients	Lepidopteran, Dipteran, Hemipetran, Coleopteran	(Lay et al., 2003; Liu et al., 2006)
Lipid acyl hydrolases	Cleave fatty acids from membrane lipids	Lepidopteran, Dipteran, Hemipetran, Coleopteran	(Strickland et al., 1995)

Metabolic engineering in plants for improving insect resistance

Metabolic engineering offers enormous potential for plant improvement because of the great contribution of volatile secondary metabolites to reproduction, defense and food quality. Metabolic pathway engineering in plants requires a systematic study of the molecular mechanisms underlying plant resistance to an insect. To cope up with herbivores attack, it requires built-in and inducible systems that can help plants to survive and overcome the attack of insects. Thorough understanding of protective pathways is necessary to operate in the form of cascades and show crosstalk, among them for metabolic, developmental and defense processes of the cell. Such studies will help to identify the potential intervention points which can be manipulated in order to strengthen endogenous defenses and will finally lead to the modification of endogenous pathways to increase particular desirable molecules (Kappers et al., 2005). This strategy has relied on expression of genes involved in signaling and regulatory pathways or enzymes involved in detoxification pathways or in the synthesis of protective metabolites (Kos et al., 2013). However, enhanced production of defense molecules in plants in a predictable and useful manner is a need of time. In combination with emerging biotechnological methods, attention has, therefore, shifted to metabolic pathway engineering. For example, transcription factor, *AtMYB12* has been heterologously expressed in plants, like tobacco and tomato leading to high-level accumulation of polyphenolic compounds (Misra et al., 2010; Pandey et al., 2014; Luo et al., 2008). This has been strategically used for developing safer insect pest-resistant transgenic plants. By switching the subcellular localization of the introduced sesquiterpene synthase to the mitochondria of *Arabidopsis thaliana*, two new isoprenoids were emitted that aided the defense mechanisms of plants (Kappers et al., 2005).

Although, metabolic engineering approach holds considerable promise for improving crop protection through a transgenic approach, there are some constraints which need to be kept in mind to develop resistant plants against insect pests. However, using transgenic plants with modified emission of metabolites might attract other herbivores which may lead to environmental risks (Halitschke et al., 2008; Carroll et al., 2006). The wider applications of available metabolite profiling technologies are likely to increase our understanding of metabolic networks. Identifying correlations and links between different metabolites facilitate the process of hypothesis generation. Despite the modern prevalence

of claims, there is no simple technology that allows the quantification and identification of all the metabolites in a tissue. Based on purely global metabolite profiling, modeling can predict and make it possible to engineer plants that can produce required components and attain insect resistance in target plants.

RNA interference (RNAi) approach for insect control

RNAi is a post-transcriptional gene silencing mechanism that is initiated by the introduction of double-stranded RNA (dsRNA) into a cell (Fire et al., 1998) and can offer pest protection as demonstrated by several exploratory studies (Baum et al., 2007; Mao et al., 2007). Several examples in recent literature suggest that silencing of respective gene(s) can target particular metabolic and vital processes of insects. A molt-regulating transcription factor, *HaHR3* gene of *H. armigera* was silenced by feeding recombinant bacteria and transgenic plants to disrupt *H. armigera* development (Xiong et al., 2013). Hemipteran insect *Nilaparvata lugens* midgut genes such as hexose transporter (*NlHT1*), carboxypeptidase (*Nlcar*) and trypsin-like serine protease (*Nltry*) were silenced using transgenic rice plants (Zha et al, 2011). Dramatic decrease in larval growth of *H. armigera* was observed upon silencing of cytochrome P450 (*CYP6AE14*) and glutathione S-transferase (*GST1*) genes (Mao et al., 2007). Transgenic corn plants expressing dsRNA of V-ATPase subunits and α-tubulin gene from *D. virgifera* showed a significant reduction in larval feeding damage using growth chamber assay (Baum et al., 2007). However, biosafety of RNAi crops is still the major issue which needs to be carefully considered and more research is required to determine potential exposure pathways and hazards, including off-target effects, non-target effects and impacts from genetic mutations.

Multiple genes engineering in plants for improving insect resistance

Use of multiple genes in transgenic will have advantage to bring more than one functional proteins in target plant with desired traits. Successive expression of target gene(s) of similar or different function in plants allows fine-tuning of trait manipulation. “Second” and “Third” generation insect tolerant transgenic plant varieties pyramided with multiple *Bt* genes demonstrated their benefits. For example, pyramided Bt toxins expressing transgenic plants greatly reduce the probability of resistance evolution, as target insects would need to develop simultaneous mutations in diverse toxin receptors to acquire resistance (Bravo and Soberón, 2008). Combination of *cry1Ac* and *cry2Ab* or *cry*

and *Vip* toxin genes are best-known examples (Chitkowski et al., 2003; Gatehouse 2008). Transgenic plants are in pipeline with multiple genes that can impart resistance to insect, fungi, bacteria and virus.

To provide more durable and cleaner transgenic technologies for the future, refinement of strategies for multigene expression is necessary. Such technological advancement has yet to be realized since current constraints are (i) prevalence of gene silencing, (ii) multigene transformation without antibiotic resistance gene and (iii) well-characterized promoters and their functioning in heterologous system for regulated expression. Thus, there are significant limitations for introducing multiple genes in a transgenic plant and ensuring that they all will be expressed in an anticipated format. Resolutions to these challenges are essential for revolutionary change in agriculture field.

1.5 Proteinase inhibitors (PIs) against insect herbivory

PIs act as anti-metabolic proteins by blocking the digestive proteinases of insect gut (Hilder and Boulter, 1999). PIs are expressed in response to various biotic and abiotic stress conditions, e.g. insect attack, pathogen invasion, wounding and environmental stress. A wide range of PI genes with distinct modes of action have been isolated from a broad range of plant species. The earliest investigation on possible role of PIs in plant protection dates back to 1947 when, Mickel and Standish observed abnormal development of insect larvae on soybean-based products (Mickel and Standish, 1947). Later, the antibiosis effects of soybean trypsin inhibitors were shown on larvae of flour beetle, *Tribolium confusum* (Lipke et al., 1954). PIs are naturally encountered in many plant species; their expression varies between various plant tissues including leaves, flowers and fruits/ seeds (Damle et al., 2005; Tamhane et al., 2005). PIs are abundantly present in the storage tissues of plants and can represent up to 10% of total protein (Ussuf et al., 2001). The accumulation of trypsin and chymotrypsin-like PIs throughout the aerial tissues of tomato and potato plants was demonstrated to be a direct consequence of insect-mediated damage or mechanical wounding (Green and Ryan, 1972; Koiwa 1997). PIs act as substrate mimic and block the digestive proteinases in the larval gut, thereby, limiting the release of amino acids from food proteins (Broadway and Duffey, 1986; Hilder and Boulter, 1999). As a result, the depletion of amino acids exerts a profoundly detrimental effect on larval physiology and thereby retards the growth and development of the larvae (Broadway and Duffy, 1986; De Leo et al., 2001;

Telang et al., 2003, 2009; Damle et al., 2005; Tamhane et al., 2007b; Hartl et al., 2010; Dawkar et al., 2011). Further, the decreased fertility and fecundity of the adult moths was observed in various insect pests reducing the overall fitness of the insect populations. PIs may also adversely affect the proteinases of phytopathogenic fungi, nematode and microorganisms (Mosolov et al., 1976; Valueva et al., 2004; Urwin et al., 1998). Additionally, antagonistic effects of PIs act synergistically with other components of the plant defense mechanism *viz*. retarded insects become easy targets for their parasites (Lewis et al., 1997). Many PI proteins are rich in cysteine and lysine, contributing to better and enhanced nutritional quality (Ryan, 1990). These advantages make PIs an ideal choice to use in development of transgenic crops resistant to insect pests.

1.6 Proteinase inhibitor families in plants

PIs have been grouped based on their specificity in four mechanistic classes as serine, cysteine, aspartic and metallo-protease inhibitors (**Figure 1.8**) (Ryan, 1990). Furthermore, each type comprises various inhibitor families which have been classified based on their sequence homology, structural characteristics, molecular mass and expression patterns (Laskowski and Kato, 1980; Ryan, 1990). Generally, PIs shows molecular mass from 5 to 25 kDa. Most PIs are products of multigene families and various isoinhibitors have been identified in a single species displaying different specificities towards proteases (Wu et al., 2006; Tamhane et al., 2009). Serine proteinase inhibitors are further classified into various families such as soyabean trysin inhibitor (Kunitz), Bowman-Birk, barley trypsin inhibitor, wound-inducible potato proteinase inhibitor (PIN) type I and II, Squash inhibitor and mustard seed trypsin inhibitor.

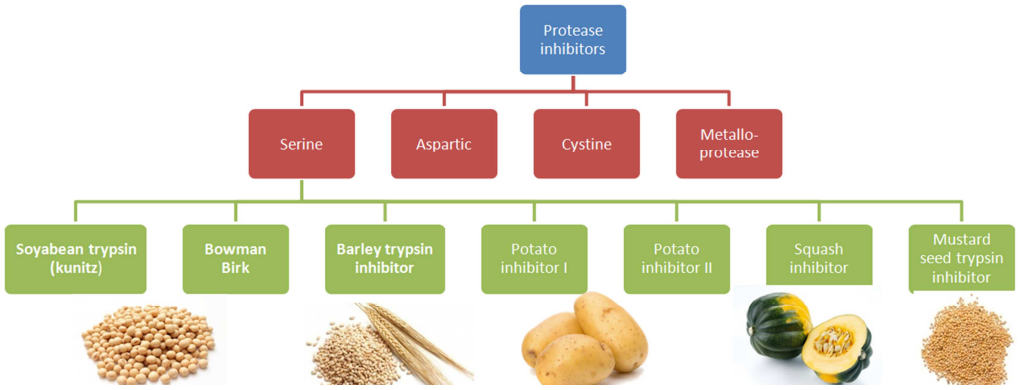


Figure 1.8: Classification of proteinase inhibitors.

Members of serine and cysteine PI families have been more important to the area of plant defense due to their wide occurrence across various plants whereas metallo- and aspartic PIs have limited presence (Ryan, 1990).

1.7 Serine PIs and their standard mechanism of inhibition

Serine PIs are the most extensively distributed family of PIs and they all use the competitive mechanism of inhibition. Protease-serine PI interaction is an entropy driven process and is further affected by non-contact residues of the inhibitor by means of Van der Waal's interaction and hydrogen (H) bonding (Otlewski et al., 2000). Serine PIs possess a rigid reactive-site loop that is complementary to the substrate binding site of serine proteinases and hence, they bind in a substrate like manner (Laskowski et al., 1980; Bode and Huber, 1992; Krowarsch et al., 2003). Tight binding to the proteinase is achieved by retaining a conformational stability of reactive-site loop (RSL) which extends from the protein scaffold and functions as a recognition motif. The P1 residue of the RSL makes contacts with the proteinase at S1 pocket and is the key determinant of the inhibitory specificity (Laskowski et al., 1987). Phe, Leu or Tyr for chymotrypsin enzyme, Arg or Lys for trypsin-like enzymes and Ala for elastase are usually present at the P1 position at RSL of the PI. The protease recognizes the specific peptide bond P1-P1'in the RSL and the two proteins are frozen into a stable complex in which the protease is unable to complete the hydrolysis of the peptide bond, nor can the complex easily dissociate. The strength of the protease-PI interaction is determined by the compatibility of all the amino acid residues (P4-P4') which also aid to direct the inhibitor towards the active site cleft of the PI (Bateman et al., 2011). Tight binding and slow hydrolysis are the characteristic features which are result of stable hydrogen bonding that surround the scissile peptide bond and the Van der Waals contacts at the interface. Disulfide bonds are present extensively in many of the proteinase inhibitor families and helps to provide stability to the exposed RSL by holding it through covalent attachments. These interactions restrict any distortion of the P1-P1' peptide bond and increase the activation-energy barrier for hydrolysis. The inhibitory loops of serine PIs have a typical conformation, defined by the torsion angles of the P3-P3' segment, regardless of the family they belong to, while scaffold has widely different folds in different families of inhibitors (Krowarsch et al., 2003). Recent studies have led to the emergence of a hypothesis

that functional specifications of the serine PIs are closely related with their sequence and structural variations (Schirra et al., 2010; Li et al., 2011b).

1.8 Effect of PIs on insects

Key discovery by Green and Ryan (1972) on wound inducible nature of PI leads the concept of developing PI-based transgenic for insect tolerance. The direct evidence for inhibitory effect of PIs in plant leaves against insects was first demonstrated by Hilder et al. (1987) by expressing a cowpea trypsin inhibitor gene in transgenic tobacco plants. There have been many examples demonstrating PI activity against certain insect species, both by *in vitro* and *in vivo* bioassays (Giri et al., 2003, 2006; Broadway and Duffy, 1986; Johnston et al., 1993; Harsulkar et al., 1999; Bown et al., 2004; Giri et al., 2005; Tamhane et al., 2005, 2007b). Firm establishment of PIs as antagonists have led the way to transgenic expression of PIs in various crop plants rendering them with higher resistance to pests (**Table 1.3**). Various PIs have been isolated and used with divergent modes of action against different insect pest species to create transgenic crop plants.

Recent studies have indicated that the combined expression of defense genes with different modes of action and combination of inhibitors might be more effective for insect control and stable resistance against pests. For example, combined inhibitory effect of two PIs on *H. armigera* larval growth was reflected by an increased yield of cotton bolls in field trials of transgenic plants expressing both the inhibitor genes (Dunse et al., 2010). Thus, stability of PIs to proteolytic degradation and the synergistic interaction of different PIs can drastically influence the efficacy of PIs. Making use of combinations of a variety of defense molecules has risen as a futuristic approach to improve insect resistance in crop plants and the enormous potential of the PIs in agriculture awaiting full-scale exploration.

1.9 Potato inhibitor type II (Pin-II): Role in endogenous and defense functions

Potato inhibitor type II (Pin-II or Pot-II) family of serine PIs has been studied at molecular level such as gene and protein level. The Pin-II PIs, mainly in Solanaceae, have a distinctive single or multiple inhibitory repeat domains (IRDs) with sequence and structure variations. Pin-II show wound induced up-regulation and expression, post-translational interactions with proteases leading to modification in PI protein

Table 1.3: Transgenic plants harbouring PI genes with increased resistance against various classes of pests

Plant source of PI gene	Transformed plant	Target organisms	References
Beta vulgaris	Tobacco	*Spodoptera frugiperda, S. exigua, Manduca sexta*	(Smigocki et al., 2013)
Zea mays, Solanum tuberosum	Rice	*Chilo suppressalis, Magnaporthe oryzae*	(Quilis et al., 2014)
Ipomoea batatas Colocasia esculenta	Tobacco	*Helicoverpa armigera, Erwinia carotovora, Pythium aphanidermatum*	(Senthilkumar et al., 2010)
Solanum tuberosum	Chinese cabbage	*Pieris rapae, Plutella xylostella,*	(Zhang et al., 2012b)
Solanum tuberosum	Tobacco	*Rhizoctonia, Helicoverpa armigera*	(Majeed et al., 2011)
Solanum tuberosum	Tomato	*Liriomyza trifolii, Heliothis obsolete*	(Abdeen et al., 2005)
Nicotiana alata, Solanum tuberosum	Cotton	*Helicoverpa punctigera*	(Dunse et al., 2010)
Solanum lycopersicum	Tobacco	*Manduca sexta*	(Johnson et al.,1989)
Nicotiana tabacum	Tobacco	*Spodoptera litura, Helicoverpa armigera*	(Srinivasan et al., 2009)
Nicotiana alata	Tobacco	*Helicoverpa punctigera; Teleogryllus commodus*	(Heath et al., 1997)
Nicotiana attenuata	Tobacco	*M. sexta , Tupiocoris notatus*	(Zavala et al., 2004b)
Solanum americanum	Tobacco	*Helicoverpa armigera, Spodoptera litura*	(Luo et al., 2009)
Ipomoea batatas, Colocasia esculenta	Tobacco	*Spodoptera litura, Spodoptera exigua*	(Chen et al., 2014)
Arabidopsis thaliana	White polar	*Chrysomela populi*	(Delledonne et al., 2001)
Solanum tuberosum	Rice	*Sesamia inferens*	(Duan et al. 1996)
Vigna unguiculata	Strawberry	*Otiorhynchus sulcatus*	(Graham et al., 1997)
Nicotiana alata	Apple	*Epiphyas postvittana*	(Maheswaran et al., 2007)
Oryza sativa	Alfalfa	*Pratylenchus penetans*	(Samac and Smigocki, 2003)

structure, activity and function. Together all these characteristics make them an interesting subject area for plant and insect biologists. Plant organs that express Pin-II protein include leaves, flowers, fruits, stem, tubers and roots (Tamhane et al., 2012).

PIs are involved in various other physiological and developmental responses of plants (Chye et al., 2006; Hartl et al., 2011). For instance, potential roles of PI have been suggested in seed germination, tapetum degeneration, programmed cell death etc. Reports on proteinase inhibitor II (Pin-II), showed that it could play endogenous roles in development and responses (Hendriks et al. 1991; Pena-Cortes et al. 1995; Sin and Chye 2004; Sin et al. 2006). Sin et al. (2006) showed that an increase in flower size and 80% seed abortion after silencing homologs of *SnSPI2a* and *SnSPI2b* in *S. americanum.* However, there was no effect on flower size and only 0.7-2.8% of the seeds were aborted or defective upon silencing of *S. nigrum* PIs (*SPI2a* and *SPI2b*) (Hartl et al., 2010). The PIs in developing seeds of *S. americanum* helps in protection of the embryo and endosperm by controlling proteinases generated within the seed (Sin et al., 2006). *Solanum americanum* PI (*SaPin-IIb*) play an important role in trichome-based defense by functioning as a constitutive component of trichome chemical defense and/or by regulating the development of glandular trichomes (Liu et al., 2006a; Luo et al., 2009). There are some reports that Pin-II PIs can control cell proteolysis by their action on endogenous proteinases, thus controlling protein turnover and metabolism (Horn et al., 2005; Sin and Chye, 2004). PI might play important role in several endogenous processes in cells. Abundant expression of *Solanum americanum* PI (*SaPin-IIa*) in stem especially in companion cells (CC) and sieve elements (SE) of phloem indicated their role in regulating proteolysis in SE and phloem development (Xu et al., 2001, 2004).

1.10 *Capsicum annuum*: Occurrence and diversity in Pin-II genes

Capsicum annuum (Chili pepper) is a domesticated species of genus Capsicum from Solanaceae family native to northern South America and southern North America. Amongst different members of Solanaceae *C. annuum* has the lowest number of chromosomes i.e. (2n = 24). *C. annuum* is one of the non-preferred hosts of *H. armigera*. Seventy nine *C. annuum* PI (*CanPIs*) genes have been identified which show homology to Pin-II family and constitute 1, 2, 3, or 4-IRD PIs (Shin et al., 2001; Kim et al., 2001; Tamhane et al., 2007b, 2009; Mahajan, 2015). Expression patterns

of *CanPIs* in different tissues differ qualitatively, quantitatively as well as spatially and temporally. The flower tissue has significantly higher level of PI activity compared to the leaf, stem and fruit tissues (Tamhane et al., 2009). Precursor Pin-II PIs comprise of 1- to 8- inhibitory repeat domains (IRDs) linked by linker peptides, which release IRDs upon proteolysis. Several novel and diverse Pin-II PIs having 1- to 4-IRDs were isolated from developing fruit and stem tissues of *C. annuum*. Precursor CanPIs interact with the *H. armigera* gut proteases and get processed into their constituent IRDs (Mishra et al., 2010). In precursor Pin-II, the IRDs have ability of simultaneously inhibiting one or more protease molecules (Barrette et al., 2003a, 2003b). Each IRD is a peptide of around 50 amino acid (aa) length with a molecular mass of ~6 KDa. The sequence of IRDs shows variations and at the same time has conserved 8 cysteine residues that form disulfide bridge (Nielsen et al., 1995; Scanlon et al., 1999; Lee et al., 1999; Schirra et al., 2010). One structural feature of Pin-II IRD

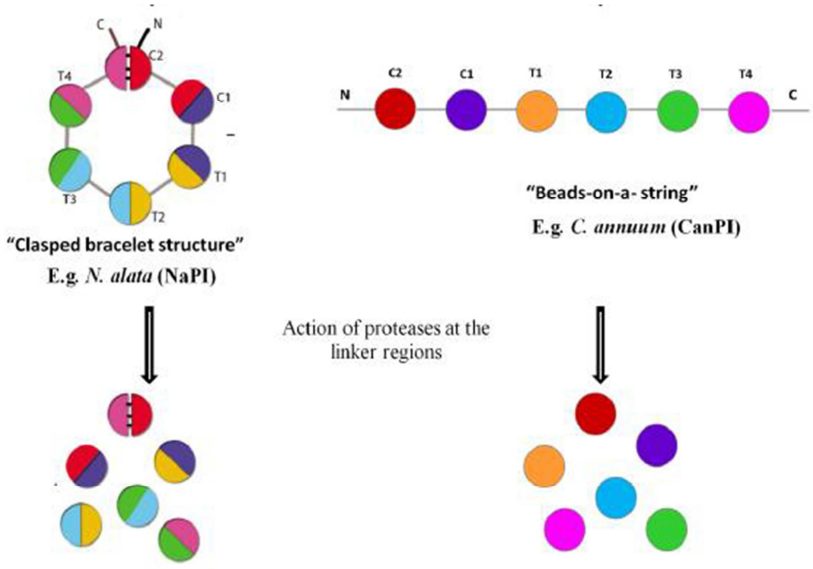


Figure 1.9: Models of Pin-II PI precursors. Structure and processing of the *Nicotiana* and *Capsicum* PIs. Clasped bracelet and beads-on-a-string and like structure is expected to be assumed by the precursor *CanPIs*. T and C indicate trypsin and chymotrypsin IRDs, respectively (Modified from Dunse and Anderson, ISB news report, June 2011).

is a triple stranded β sheet scaffold present in disordered loop. Each IRD possesses a single reactive site, which inhibits trypsin (TI) or chymotrypsin (CI) depending on whether a Lys/Arg or a Leu residue is present at the P1 position. The diversity in *CanPIs* can be assigned to individual IRDs, which show a sequence variation ranging from 2-25% within the vicinity of the reactive site loops and C-terminal region.

CanPIs are expected to assume Beads-on-string (CanPI7) and Bracelet like (CanPI69) structure (Mahajan et al., 2015) (**Figure 1.9**). Active site variants of TI domains , 'CPRDC', 'CPKNC', 'CPRYC' and 'CPRNC'; and two types of CI domains 'CTPNC' and 'CTLNC' have been reported to be present among the identified IRDs. In earlier studies, fruit tissue showed higher expression of 3- and 4-IRD *CanPIs* while stem tissue showed higher proportion of expression of 1- and 2-IRD *CanPIs* (Tamhane et al., 2009). Significantly high expression levels of *CanPIs* were reported upon natural infestation by Lepidopteran insects and aphids, demonstrated the involvement of *CanPIs* in plant defense (Tamhane et al., 2009). Biotic (virus, aphid and lepidopteran insect) and wounding stress to the plant tissues induced deferential CanPI profiles (Mishra et al., 2012). Recent studies on the defensive roles of PIs and/or endogenous roles from various solanaceous species and simultaneous expression of multiple *CanPIs* emphasize their prospective involvement in many of the plant's biological processes (Sin and Chye, 2004; Xu et al., 2004; Wu et al., 2006; Johnson et al., 2007; Tamhane et al., 2009; Hartl et al., 2011).

1.11 Antibiosis of *CanPIs* towards *H. armigera*

The efficacy of various *CanPIs* against *H. armigera* gut proteases and larval growth and development was demonstrated by Tamhane et al. (2005) (**Figure 1.10**). *In vitro* assays showed that most of the trypsin-like activity of the *H. armigera* gut protease isoforms was inhibited (upto 68-91%) by CanPIs. Futher, CanPIs inhibited more than

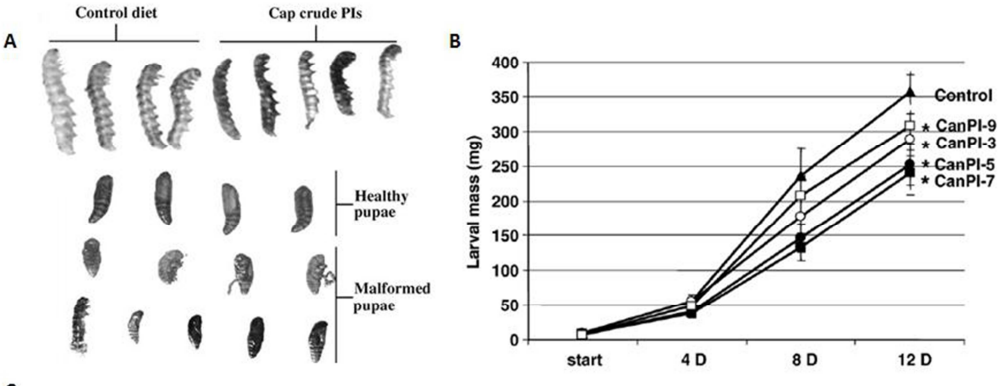


Figure 1.10: The effect of *C. annuum* PIs on the growth and development of *H. armigera* larvae and pupae (A) Reduction in larval weight of CanPI fed compared to control diet and malformed pupae of larvae fed on CanPI diet as compared to healthy pupae fed on control diet. (Tamhane et al., 2005). (B) Reduced larval weight gain in the larvae fed on recombinant CanPIs (Tamhane et al., 2007b).

60% of total proteolytic activity of larvae fed on cotton and chickpea plants. Feeding of *C. annuum* leaf extracts and purified PIs in varied doses to *H. armigera* larvae for two successive generations demonstrated retarded larval growth and development. Delayed pupation and reduction in fertility and fecundity in *H. armigera* were also noticed. Various *CanPIs* with 1- to 4-IRDs were expressed heterologously in *Pichia pastoris* and the recombinant proteins were characterized for their insect inhibitory potential (Tamhane et al., 2007b). *H. armigera* larvae fed on rCanPI diet showed 30% mortality and 40% reduced weight among the survivors, in the early instars. Pupal mass reduction of 12- 25% was recorded, leading to decreased fecundity. The 4-IRD rCanPI- 7 with two chymotrypsin inhibitory sites and two trypsin inhibitory sites showed the strongest anti-metabolic effect on *H. armigera* among the selectively studied rCanPIs. Further exploiting the interaction(s) of recombinant CanPIs with *H. armigera* gut proteases by Intensity Fading Matrix Assisted Lased Desorption/ Ionization Time of Flight (IFMALDI- TOF) analysis. Mishra et al. (2010) revealed PI processing patterns and the stability of rCanPIs in presence of gut proteases of *H.*

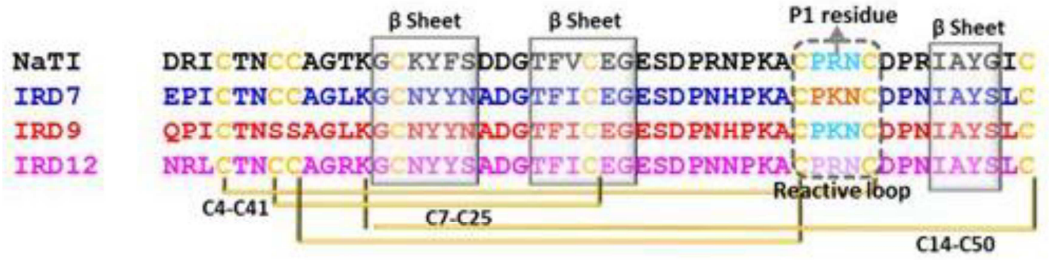


Figure 1.11: Sequence and structural diversity of IRDs. Multiple sequence alignment of IRD-7, -9, -12 and *Nicotiana alata* trypsin inhibitor (NaTI) using DNASTAR and ClustalX2 software. (Adopted from Joshi et al., 2014a)

armigera. The stoichiometry of rCanPI7 with protease molecules was analyzed by molecular docking. The static complex of CanPI7 representing at least three protease molecules in close proximity to the RSLs also indicate its higher potency (Mishra et al., 2013). Futhermore, *in silico*, *in vitro* and *in vivo* studies with individual IRDs (IRD-7, -9 and -12) of *C. annuum* (**Figure 1.11**) demonstrated their efficacy in inhibition of insect proteases and thus, the effect of sequence variation on inhibition potential was evident (Joshi et al., 2014a, 2014b).

1.12 Importance of *Arabidopsis* and tomato

Arabidopsis thaliana (2n=5) is a small annual flowering plant in the mustard family (Brassicaceae) and about 750 natural accessions have been collected from around the world. *Arabidopsis* was first discovered by Johannes Thal (hence, thaliana) in the Harz Mountains (Gemany) in the sixteenth century. *Arabidopsis* is not of agronomic significance, but it offers major advantages for basic research in genetics and molecular biology of plants. The success of *Arabidopsis* as a model organism for the study of plant genetics, physiology, biochemistry and development is mainly due to relatively small nuclear genome, rapid life cycle, large number of offspring, ease of genetic transformation and ability to grow in the laboratory. In addition, *Arabidopsis* genome contains fewer repetitive sequences than any known higher plant, greatly facilitating molecular studies and map-based cloning. Mutants available for functional studies have become a great tool in the era of plant molecular biology. Two principal methods are used successfully as biological mutagenesis in *Arabidopsis*: *Agrobacterium*-mediated T-DNA (transferred DNA) transformation and two transposable element systems that originate from maize (Page and Grossniklaus, 2002). Mostly foral dip method is used for *Agrobacterium* mediated T-DNA transformation for *Arabidopsis*. The primary reasons for the popularity of the floral dip method have been its reliability and simplicity. The elimination of tissue culture technique of regeneration largely reduces hands-on time, and success can be achieved even by non-experts (Clough and Bent, 1998). The advantages of using *Arabidopsis* in molecular genetic experiments have been reviewed extensively. Recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) technique has been applied for targeted genome engineering in *Arabidopsis* (Schiml et al., 2014; Feng et al., 2014). The implications of these discoveries are not only relevant for plant biologists, but may also affect evolutionary biology, functional and comparative genomics and agricultural science.

Tomato (2n=12) is one of the most important crop plants worldwide, because it propagates easily and has a short lifecycle. Tomato has more than 3,000 species that achieved tremendous popularity over the last century and is grown in almost every country of the world. Tomato is one of the major horticulture crops of India. India is one of the largest producers of tomatoes in the world with around 11% of the total global production. Andhra Pradesh is the leading state in India for tomato production,

even after creation of Telangana (https://www.mapsofindia.com/answers/india/state-largest-producer-tomatoes-india/). Tomato plants are vulnerable to attack of a broad spectrum of pathogens such as insect, virus, fungus and bacteria. Numerous transgenic approaches have been tried in tomato to improve its tolerance to biotic stresses and other traits.

Flavr Savr tomato was the first grown GM food crop permitted for human consumption. Tomato varieties permitted commercially include: 351N (Portland, USA), 8338 and 5345 (St. Louis, USA) and 1345-4 (Oakland, USA) (Gerszberg et al., 2015). Tomato having approximately 900 megabases (Mb) genome size, is considered as a model plant for physiological, biochemical and molecular-genetic investigations (Kalloo, 1991; Gerszberg et al., 2015). In addition, tomato has been used extensively as a model system for *Agrobacterium*-mediated transformation. Recently, CRISPR/Cas9 technique has been applied for targeted genetic engineering in tomato and rice (Shimatani et al., 2017).

1.13 Genesis of thesis

PIs are important proteins used to control Lepidopteran insect pests. In the past studies in our laboratory, PIs from Chickpea, Bitter gourd, Winged bean and Capsicum have been well characterized and their interaction with insects was studied by *in vitro* and *in vivo* assays. Hence, with this background development of transgenic tomato with *C. annuum* PIs was further planned and undertaken. The plant and insect systems considered in this study are *C. annuum* and *H. armigera*, respectively. *C. annuum*, a solanaceous plant, is a non-preferred host for *H. armigera*. Early observations showing reduced fitness of *H. armigera* larvae upon ingestion of *C. annuum* PIs (*CanPIs*) inspired us to further investigate this interaction. Study of the induced PI diversity, in *C. annuum*, revealed an array of different PI genes formed as a result of combinations of various IRDs. The effects of PI proteins from *C. annuum* were pronounced on *H. armigera* and showed not only reduction in larval and pupal weights but also dramatic reduction in the fertility and fecundity which was carried on up to the second generation (Tamhane et al., 2005). Recombinant CanPI proteins (3- and 4-IRD type) inhibited *H. armigera* gut proteinases and exerted anti-metabolic effects on the larval growth and development (Tamhane et al., 2007b). The interesting leads obtained in *CanPIs* with respect to their diversity and efficiency against *H.*

armigera growth retardation, were thus, thought to be pursued further and became the rationale behind my thesis work. Hence, generation and characterization of transgenic plants expressing *CanPIs* was undertaken. On the other hand, PIs are also known for increasing tolerance to abiotic stress. The study of *CanPIs* for abiotic stress in yeast model might give insights on stress tolerance mechanisms and would help us in improving our approach for development of CanPI expressing transgenic plants against abiotic stress. Altogether, functional assessment of *CanPIs* in biotic and abiotic stress tolerance by using transgenic approaches is addressed in the present thesis. Understanding these aspects will provide valuable knowledge for the development of *CanPIs* as a viable transgenic plant protection technology against different stresses. Keeping this in view following objectives were formulated.

1.14 Objectives:

- To study efficacy of *CanPI7* and *IRD7* for insect tolerance through *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*.
- To develop *CanPI7* expressing transgenic tomato using *Agrobacterium*-mediated transformation.
- To study and characterize single domain *CanPIs* (IRDs) in yeast strain against abiotic stresses.

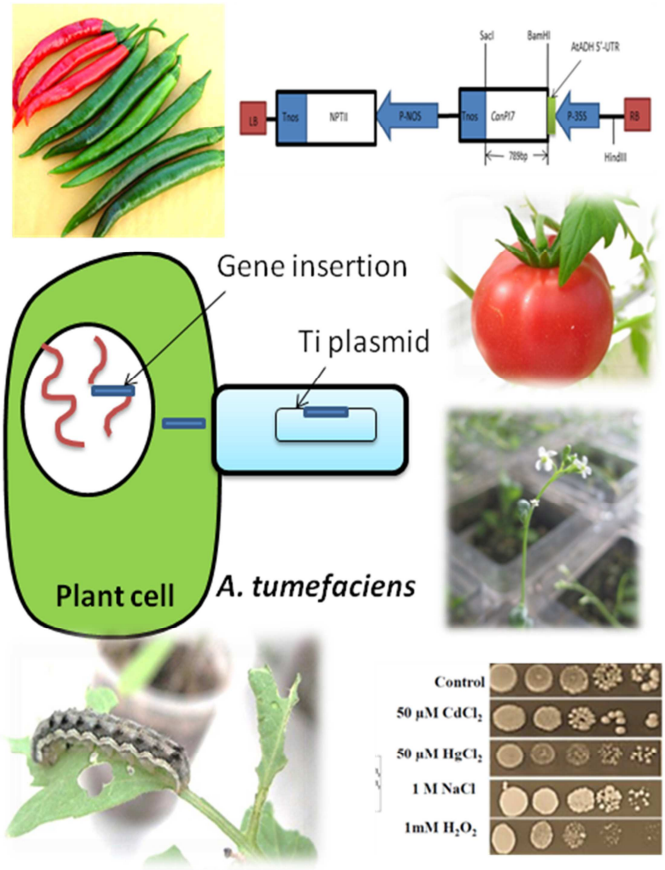
1.15 Organization of the thesis

The thesis is organized into five chapters; the first being the introduction to plant and insect interactions and detailed review of techniques and relevant information currently available to study their interaction form the basis of this study. The leads coming out of the earlier studies on *CanPIs* were taken up as a foundation to further investigate the functional role of *CanPIs* using transgenic approaches. Second chapter describes the materials and methods used for all the experimental work. The third chapter includes the results of transgenic *Arabidopsis* and tomato plants expressing *CanPIs* characterized for insect tolerance. Characterization of the transgenic tomato plants by label free quantitative proteomics, LC-MS^E and UHPLC-Orbitrap based metabolomics approaches has also been reported. Also yeast strain expressing single domain *CanPIs* (IRDs) have been characterized for abiotic stress tolerance. The fourth chapter includes the discussion on the results obtained and the significance of

this work. Several aspects of transgenic *Arabidopsis* and tomato plants expressing *CanPIs* have been discussed. The outcome of present work throws light on the significance and advantage of using *CanPIs* for developing insect tolerant transgenic plants. Also, yeast expressing recombinant *CanPIs* has been discussed for abiotic stress tolerance. In addition to this, possible molecular mechanism of PI-based multiple abiotic stress tolerance in *CanPI* expressing yeast strains is described. In the fifth and the final chapter outcome of the present work has been summarized and some future directions emerging from the present work have also been discussed which can aid in further understanding CanPI for better implementation in crop development against stress tolerance. Finally, all the literature referred, to develop protocols and to infer our results in relation to other systems has been listed as bibliography chapter at the end of the thesis.

Chapter 2

Materials and Methods



Section 2.1 *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*

2.1.1 Bioinformatics analysis

To investigate the interrelationships and groupings within PIs of various plant species and CanPI7, phylogenetic analysis was carried out. The amino acid (aa) sequences of 11 different PIs were taken from Uniprot database (http://www.uniprot.org/). Phylogenetic analysis of aa sequences and CanPI7 sequence was carried out using MEGA5 software (http://www.megasoftware.net/). Multiple sequence alignment of deduced aa sequences among all individual IRDs of CanPI7 was also performed using ClustalW program (http://www.ebi.ac.uk/clustalw).

Furthermore, three dimensional structures for these individual IRDs of CanPI7 were predicted using homology modeling. It was also performed to predict the structures of *H. armigera* trypsins, chymotrypsins and cathepsins. These proteases were selected on the basis of sequence divergence obtained from phylogenetic and sequence analysis. The sequences for trypsins, chymotrypsins and cathepsins were taken from Uniprot database. Three-dimensional models for all the *H. armigera* proteases were generated using a protein structure prediction server (http://ps2.life.nctu.edu.tw/), which implements an approach to comparative modeling by satisfying spatial restraints (Chen et al., 2006). Sequence similarity search was performed for the selected model using PSI-BLAST against a database of known protein structures with default parameters for validation. Taking into consideration the maximum query coverage, the desired model was preferred and the predicted models were validated by MOLPROBITY (Chen et al., 2010). A docking study was performed to determine the binding energy and interaction of IRDs with *H. armigera* proteases. Predicted structures of proteases were refined by energy minimization and restraint relaxation using Swiss PDB-Viewer (v4.1.0) (Guex and Peitsch, 1997). In order to perform molecular docking, models of *H. armigera* proteases and inhibitors were submitted to Patchdock online server (http://bioinfo3d.cs.tau.ac.il/ PatchDock/) following the standard package protocols and further refined by FireDock online server (Schneidman-Duhovny et al., 2005). Binding energy obtained for each complex was normalized by mean values and represented in heat map format using MeV software packages (http://www.tm4.org/mev/). The gradient ruler from -5 to 5 is an indicator of interaction strength. Data were clustered using hierarchical clustering method (Saeed et al., 2006).

2.1.2 Construction of plant expression vector

C. annuum variety Phule Jyoti, mid-mature green fruits (**Figure 2.1.1**), were obtained from Agriculture College, Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, MS,

Figure 2.1.1: Green fruits of *C. annuum* (cultivar: Phule Jyoti)

India. Amplified cDNAs of *CanPIs* were isolated from mature green fruits of *C. annuum* and cloned in pGEM-T-Easy vector (Tamhane et al. 2007b). For expression and transformation, *CanPI7* gene ORF (Accession No.: DQ005913.1) was amplified with gene specific primers with *BamH*I and *Sac*I restriction sites (Appendix I) using full-length gene cloned in the pGEMT-T-Easy vector (Promega, Madison, WI, USA) as template in insert PCR. The amplicon was digested with *BamH*I and *Sac*I restriction enzymes (Promega) and cloned into pRI101-AN vector (**Figure 2.1.2**) (Takara, Shiga, Japan). Similarly, *IRD7* gene was cloned in pRI101-AN vector.

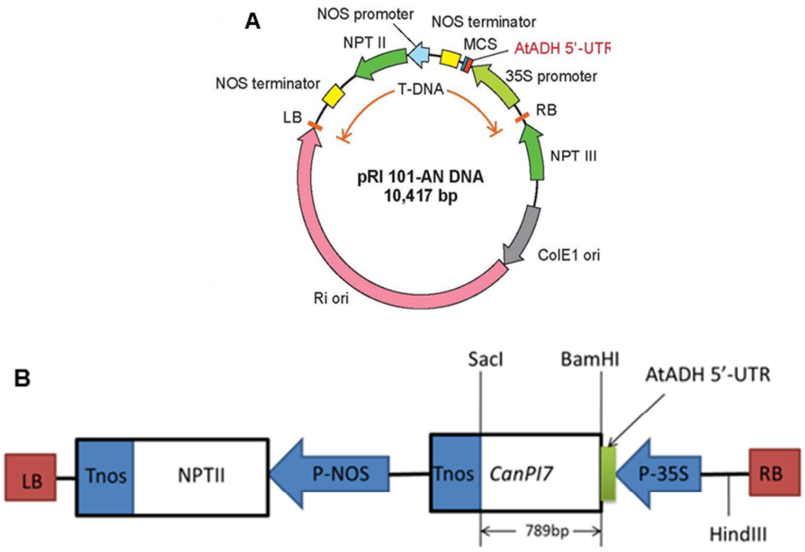


Figure 2.1.2: (A) Vector map of pRI101-AN vector (Source: Takara, Clontech) (B) Representative image of cloned region of recombinant pRI101-AN vector.

2.1.3 Confirmation and transformation of *Agrobacterium* GV3101 strain

Confirmation of *Agrobacterium* strain GV3101 was done using Benedict's reagent following the protocol described by Bernaerts and De (1963). *E. coli* and *Agrobacterium* strain GV3101- pRESC were used as negative and positive control, respectively. Additionally, *Agrobacterium* strain GV3101 were subjected to antibiotic (Rifampicin= 50 mg/l) sensitivity for further confirmation. Recombinant plasmid was introduced into this *Agrobacterium* strain GV3101 by using freeze thaw method (Weigel and Glazebrook, 2006).

2.1.4 Germination and growth of *A. thaliana*

A. thaliana seeds (~1000) were transferred in microcentrifuge tube for surface sterilization. Then seeds were treated with ethanol (75%) for 30 s. After removing ethanol from microcentrifuge tube, seeds were treated with sodium hypochlorite (3%) containing Tween20 (0.01%) for 5 min. To remove sodium hypochlorite and Tween20, seeds were thoroughly washed with sterile distilled water. After surface sterilization, seeds were platted on Murashige and Skoog (MS) medium (HiMedia Laboratories, Mumbai, India), at pH 5.7 (Murashige and Skoog, 1962) (150 seeds/plate). Seeds were germinated and grown in long day condition (16 h light/ 8 h dark, 22°C) for 2 weeks. Then these grown plants were transferred on to soilrite (**Figure 2.1.3)**.

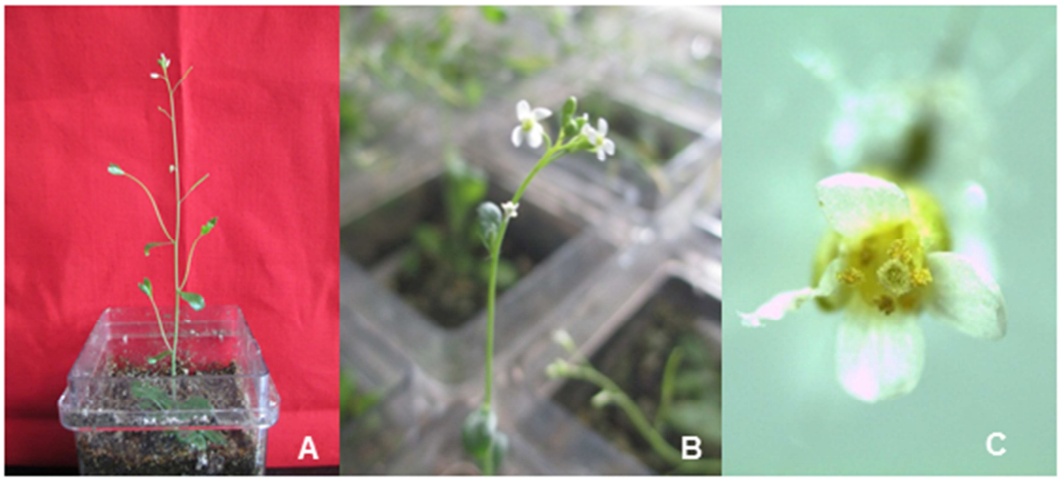


Figure 2.1.3: (A) Mature *A. thaliana* plant, variety Columbia (Col 0) (B) Inflorescence of *A. thaliana* (C) Flower of *A. thaliana*.

2.1.5 *Agrobacterium*-mediated transformation of *A. thaliana*

Agrobacterium strain GV3101 which contained the gene of interest was inoculated in Luria-Bertani media (5 mL) containing appropriate antibiotics (100 mg/L kanamycin and 50 mg/L rifampicin) and culture was incubated at 28°C for 1 day. *Agrobacterium* cells were collected by centrifugation at 4000 rpm for 10 min at room temperature. Cell pellet was dissolved in equal volume of freshly prepared 5% (W/V) sucrose, 0.01% of Tween 20 and 100 µM acetosyringone, then incubated on shaker at 24°C for 2 h. *A. thaliana* flowers (unopened flowers) were infected with *Agrobacterium* suspension using micropipette (Clough and Bent, 1998). *Agrobacterium* infected plants were covered with plastic bags to maintain high humidity for 1 h. Then plastic bags were removed and plants were kept in growth chamber till maturation of seeds. Seeds were collected and stored at room temperature for 10 d (for drying).

2.1.6 Screening of primary putative transgenic *A. thaliana*

Seeds from *Agrobacterium* infected plants were collected and stored at room temperature for 10 days (for drying) followed by cold treatment for 2 days. Then seeds of putative transgenic *Arabidopsis* and control untransformed wild type (WT) were treated with ethanol (75%) for 30 s. Subsequently seeds were treated with sodium hypochlorite (3%) containing Tween20 (0.01%) for 5 min. To remove sodium hypochlorite and Tween20, seeds were thoroughly washed with sterile distilled water. After the surface sterilization, seeds were placed on MS media plates (150/plate), containing 50 mg/L of kanamycin. These plates were then kept in growth chamber and daily observed till germination. This procedure was allowed to differentiate transgenic and non-transgenic plants.

2.1.7 Molecular characterization of putative transgenic *A. thaliana*

Genomic DNA of transgenic and WT plants was isolated using standard cetyltri-methylammonium bromide (CTAB) based extraction procedure (Doyle, 1990). Plant tissue (~100-200 mg) was grinded by using mortar and pestle in liquid nitrogen. Then CTAB (500 µl) buffer (Appendix II) was added and incubated at 65°C in the water bath for 2 h. Chloroform (300 µL) was added in samples and mixed manually. Samples were centrifuged at 13,000 rpm at 25°C for 20 min. Then aqueous layer was transferred into fresh micro centrifuge tube without disturbing the other layers. After

separation, ice-cold isopropanol (200 µL) was added in samples and mixed well for 15 min. Then samples were incubated at -20°C for 1 h. Next to this, these samples were centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was slowly removed and pellet was washed by adding 200 µl of 75% ethanol and centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was discarded after centrifugation and pellet was air dried at 28°C. Dried pellet was dissolved in 50 µL of sterile water. DNA was quantified using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and was stored at 4°C.

2.1.8 Proteinase inhibitor and protease activity assays

Total soluble protein extracted from leaves of 5 weeks old WT and T2 transgenic plants was assayed for proteinase inhibitory activity. A leaf tissue (200 mg) was grinded to a fine powder and extracted in 500 µL of Tris-HCl buffer (0.1 M; pH 7.8) at 4°C for period of 2 days. The extract was then centrifuged at 12,000 rpm (4°C; 20 min) and clear supernatant was used for enzymatic assay. Total protein of clear supernatant was quantified following method described by Bradford (1976). For the enzyme inhibition assay, a suitable volume of enzyme equivalent to 0.4 U *H. armigera* gut proteinases (HGP), was mixed with leaf crude protein (2 µg), incubated at 37°C for 10 min and residual proteinase activity was estimated using enzyme specific chromogenic substrates, Benzoyl-L-Arginyl-p-Nitroanilide (BA*p*NA) or N-succinyl-L-ala-L-ala-L-pro-L-leu-p-nitroanilide (SAA*p*LNA) (Sigma, St. Louis, MO, USA), as reported earlier (Damle et al., 2005; Tamhane et al., 2005). One proteinase unit was defined as the amount of enzyme that increased the absorbance by 1.0 OD under the given assay conditions.

Protease assays using azocasein and BA*p*NA as substrates were performed in order to estimate total protease-like and trypsin-like activities, respectively. An azocasein assay was carried out using 1% (w/v) solution of the substrate in the assay buffer. HGPs were added to 200 µL substrate and incubated for 30 min at 37°C and the reaction was terminated by adding 300 µL of 5% trichloro-acetic acid. The precipitated proteins were centrifuged at 10,000 rpm for 10 min and 0.5 µL of supernatant was added to 0.5 mL of 1 M NaOH. The absorbance of this solution was measured at 450 nm. Protease inhibitors were prepared as the following stock solutions: N-p-Tosyl-L-Lysine Chloromethyl Ketone (1 mM), N-ethylmaleimide (1

mM), in water. Inhibitors were first pre-incubated for 15 min at ambient temperature with enzyme extracts prior to azocasein addition and activities were measured as described above. Control assays were performed with the corresponding solvents.

2.1.9 Insect bioassay of transgenic *A. thaliana*

The efficacy of *C. annum* inhibitors was studied by feeding assays using laboratory-established culture of *H. armigera*. *H. armigera* larvae were obtained from Dr. Alok Sen (Laboratory of Entomology, Division of Organic Chemistry, CSIR-National Chemical Laboratory, Pune). The culture was maintained under controlled conditions at 25°C, 65% relative humidity with 12 h dark/ 12 h light cycles in growth chamber. *H. armigera* larvae were maintained on an artificial diet composed of **(A)** chick pea flour; 50 g, yeast extract; 12 g, wheat germ; 5 g, casein; 3.5 g, sorbic acid; 0.5 g, methyl paraben; 1 g in 150 ml distilled water, **(B)** choline chloride; 0.35 g, streptomycin sulphate; 0.02 g, ascorbic acid; 2 g, cholesterol; 0.15 g, multivitamin multi-mineral capsule (Becadexamin, GlaxoSmithKline Pharmaceuticals Limited, Bangalore, India), Vitamin E; 200 mg, formaldehyde; 1 mL, bavistin; 0.3 g, distilled water; 30 mL and **(C)** agar; 6.5 g in 180 mL distilled water (Nagarkatti and Prakash, 1974). A and B were mixed together and molten agar **(C)** was added. Individual insects were fed on 1 cm ×1 cm ×1 cm cubes of the artificial diet daily and subsequent generations were used for feeding assays.

The total gut proteolytic activity of a single gut of the fourth instar larva was estimated. The minimum inhibitor amount (X) of *A.thaliana* leaf extract of WT plant required to inhibit maximum total proteolytic activity present in single gut was calculated and identified inhibitor amount (3X) was incorporated per gram of the artificial diet. Fifteen early first instar larvae were analyzed for each diet containing crude protein from transgenic plants and WT group, also artificial diet without crude protein was used. Larval weights were taken every day and percent weight reduction in the larvae fed on PI conatining diet was compared to that of larvae fed on diet containing WT crude protein. Also mortality of *H. armigera* larvae was recorded for a period of 7 days. The experiment was repeated thrice.

2.1.10 Statistical analysis

Data was analyzed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test. Data points were considered significant at $p \leq 0.01$ (c), $p \leq 0.001$ (b) and $p \leq 0.0001$ (a).

Section 2.2 *Agrobacterium*-mediated transformation of tomato

2.2.1 Regeneration and co-cultivation studies of tomato (cv Pusa Ruby)

Pusa Ruby variety of tomato (*Solanum lycopersicum* Mill.), was used in this study. Seeds of Pusa Ruby variety released by Indian Agricultural Research Institute (IARI, New Delhi) were collected from Sumeet Seeds Ltd (Maharashtra, India). It is an early growing determinate cultivar and fruits are lightly furrowed, medium-sized, lobed and uniformly red when ripen (**Figure 2.2.1**). Variety is suitable for sowing in spring-summer as well as autumn-winter seasons and has average yield of 32.5 t/ha. It is used for table as well as processing purpose.

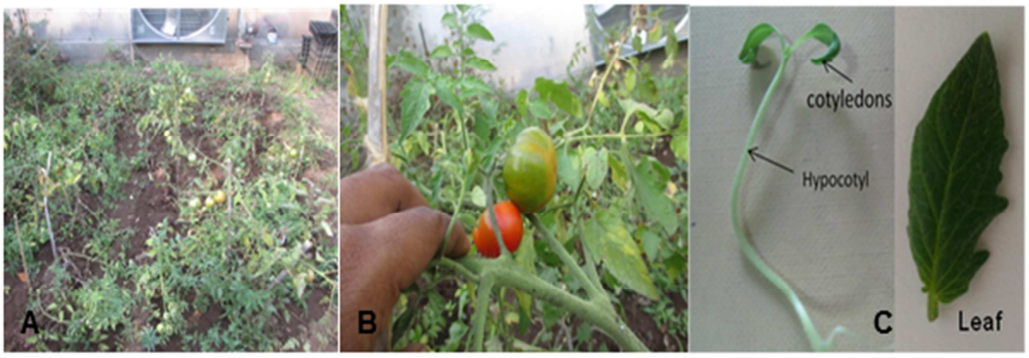


Figure 2.2.1 Pusa Ruby tomato: (A) Pusa Ruby tomato variety grown in field (B) fruits (C) Explants used for regeneration studies

For the preparation of explants the seeds were washed with 70% (v/v) ethanol for 1 min and washed twice with sterile distilled water. Then seeds were surface sterilized in the laminar flow with 4% (v/v) sodium hypochlorite solution containing 0.1% Tween20 for 12 min. The seeds were then washed five times with sterilie distilled water. To facilitate germination, seeds were then cultured on MS media. Incubation conditions for *in vitro* culture, unless stated otherwise, were 25±1°C and 16 h photoperiod of light intensity 2500 to 3000 lux. All media combinations contained 3% sucrose solidified with a 0.8% agar and with a pH of 5.7, adjusted before autoclaving.

Leaf, hypocotyls, and cotyledonary leaf were collected from *in vitro* grown seedlings (**Figure 2.2.1 c**). These explants were plated individually, on regeneration media, i.e., MS supplemented with zeatin (2 mg/L) for the determination of best type of explant producing maximum number of calli and shoots.

For induction of callus and production of multiple shoot buds from cotyledonary leaf explants, MS media supplemented with different concentrations and combinations of BAP, Kinetin, Zeatin and 2,4-D were used. Cotyledonary leaves were collected from 10 days old *in vitro* grown seedlings. Each cotyledonary leaf was transversely cut at tips. For shoot formation from the regenerated callus, MS media with different concentrations of BAP, Zeatin and TDZ were used. For root formation from the cut ends of regenerated excised shoots, MS media with different hormones such as IBA, IAA and NAA were used. Following the development of sufficient roots, plantlets were transferred to small plastic pots containing soil containing 50% soil, 25% Soilrite (Kelperilite, Banglore, India) and 25% cocopeat for hardening. These plantlets were acclimated in pots covered in plastic bags initially for 4-5 days in the greenhouse and plants were maintained further in greenhouse till flowering and fruiting.

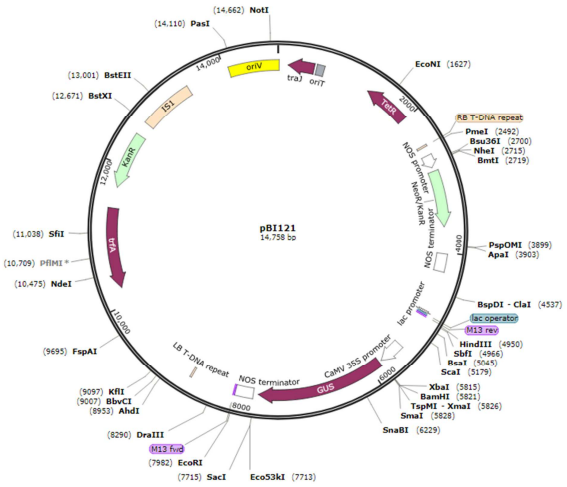


Figure 2.2.2 Vector map of pBI121 vector
(Adopted from http://www.snapgene.com/resources/plasmid_files/plant_vectors/pBI121/)

Agrobacterium strain GV3101 with the binary plasmid pBI121 was used for co-cultivation studies (**Figure 2.2.2**). It contains a reporter GUS gene and a selectable marker gene *nptII* encoding the enzyme neomycin phosphotransferase conferring

kanamycin resistance. 10 mL of liquid LB containing 50 mg/L kanamycin were inoculated with *Agrobacterium* culture. *Agrobacterium* culture was grown for 48 h at 28°C and 200 rpm. Cells were pellet down at 25°C and 5,000 rpm, followed by washing with sterile liquid MS media. The pellet was resuspended in 10 mL of sterile liquid MS media used for co-cultivation experiments. Effect of various bacterial densities and acetosyringone concentrations on transformation efficiency was studied.

Histochemical assay was performed to visualize GUS activity as described by Chaudhury et al. (1995). Briefly, co-cultivated cotyledons were incubated in GUS histochemical buffer [50 mM sodium phosphate, pH 7.0; 50 mM EDTA, pH 8.0; 0.5 mM $K_3Fe(CN)_6$; 0.5 mM $K_4Fe(CN)_6$; 0.1% Triton X-100; 1 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide)] at 37°C for up to 24 h. Chlorophyll in cotyledons was subsequently extracted by incubation in acetone: ethanol (1:3) before assessment for GUS activity.

2.2.2 Generation of transgenic tomato plants (T0)

Optimized conditions from regeneration and co-cultivation studies combined with standard optimized tomato transformation protocol for Pusa Ruby variety as described by Sharma et al. (2009) were used for generation of transgenic tomato plants.

In brief, seeds were surface-sterilized with 4% sodium hypochlorite solution for 12 min with intermittent shaking, followed by 4-6 washes with sterile distilled water. Finally, seeds were germinated on MS medium in plastic square vessels. After 10 days of growth, cotyledons were cut at the tip as well as at the base and middle segments were placed on preculture medium with their adaxial surface in contact with the medium for 48 h. *Agrobacterium* culture containing binary vector were grown in shaking culture for 48 h at 28°C and 200 rpm. Cells were pellet down at 25°C and 5,000 rpm, followed by washing with sterile liquid MS media. The pellet was resuspended in 10 mL of sterile liquid MS media containing 200 µM acetosyringone. Explants were incubated in the bacterial suspension for 20 min and inverted at 10 min interval during incubation. For every individual transformation experiment, there were also positive and negative controls. Positive control means non-transformed leaf discs, they were not treated with *Agrobacterium* cells and they were placed on nonselective MS media. On the other hand, negative control means non-transformed leaf discs, again they were not treated with *Agrobacterium* cells but they were placed

on selective MS media. The explants were then blotted on sterile tissue paper and co-cultured on the same pre-culture medium for 72 h at 28°C. Co-cultured explants were transferred to a selection medium containing 1 mg/l trans-zeatin for regeneration. Explants that showed regeneration were sub-cultured onto fresh selection medium every 2 weeks. Regenerated shoots were excised from the callus and transferred to a rooting medium containing 1 mg/l Indole-3-butyric acid (IBA). Those plantlets which attained good shoot development (~7 cm in height) and produced roots were transferred to pots containing 50% soil, 25% soilrite (Kelperilite, Banglore, India), and 25% cocopeat for hardening. Initially pots were kept covered in plastic bags for 4-5 days in the greenhouse. After acclimatization, plastic bags were removed and plants were further grown till fruiting for seed collection. The transformation efficiency was calculated as the percent co-cultivated explants producing independent transformation events, leading to regeneration of a complete plant on the rooting medium.

2.2.3 Molecular characterization of transgenic tomato plants

Initial T0 rooted kanamycin resistant plantlets were confirmed by genomic PCR and transferred to a greenhouse for fruiting. The seeds collected from potentially positive transgenic plants were selected on MS plates containing 200 mg/L kanamycin for segregation and selection of transgenic progeny. Germinated T1 plants were transferred to greenhouse for further investigation. Putative T1 transgenic plants were examined by genomic PCR, Southern blot and reverse transcriptase PCR (RT-PCR) analysis. PCR amplification of genomic DNA for the presence of *CanPI7* gene was performed using genomic DNA as template and gene-specific primers (Appendix I).

Total RNA was isolated from Wild type (WT) and six transgenic tomato (T1) plants under normal growth condition using Trizol reagent (Invitrogen, Carlsbad, CA, USA). For total RNA isolation, plant tissue (100 mg) was grinded by using mortar and pestle in liquid nitrogen. Then 1 mL of Trizol reagent was added, samples were again grinded by pestle, transferred in micro centrifuge tube and vortexed for 5 min at 25°C. Then 300 µL of chloroform was added in samples and mixed till emulsion form by inverting tubes manually. Sample was centrifuged at 13,000 rpm at 4°C for 15 min. After centrifugation, supernatant was transferred in fresh micro centrifuge tube without disturbing separated layers. Samples were again washed with 300 µL of

chloroform. The aqueous layer was transferred in new micro centrifuge tube after centrifugation of sample at 13,000 rpm at 4°C for 15 min. After separation, 500 µL of isopropanol was added to samples and mixed by inverting tube for 15 min. Sample was then kept at 25°C for 5-10 min and centrifuged at 13,000 rpm for 10 min at 4°C. Then pellet was washed with 75% ethanol prepared in DEPC treated water. After mixing, sample was centrifuged at 13,000 rpm for 10 min at 4°C. Supernatant was discarded after centrifugation and pellet was again washed with ethanol. Dried pellet was suspended in 50 µL of DEPC treated water. Cocktail of 1 µL of DNase in 3.5 µL of reaction enzyme buffer was added in 50µL of isolated RNA. This mixture was incubated at 37°C for 30 min. Then 2 µL of stopping solution was added and incubated at 65°C for 15 min. Total RNA was quantified using Nanodrop and was stored at -20°C. cDNA was synthesized using a reverse transcriptase (RT) kit with ~2 µg purified total RNA, according to the manufacturer's protocol (Applied Biosystems, Foster, CA, USA). PCR amplification of cDNA was performed using specific primers of *CanPI7* gene, with tomato *PIN-II* and *elongation factor 1α* (EF) gene as an internal control (Appendix I).

For Southern blotting, DNA (10 µg) was digested by restriction enzymes (*BamH*I and *Sac*I) (Promega), resolved on 0.8% agarose gel and transferred onto nylon membrane (Hybond-N^+, Roche, Switzerland) using standard protocol according to Sambrook et al. (1989). The gene-specific probe (789 bp) was used to hybridize blotted membranes. Digoxigenin-labeled gene-specific probe generation, hybridization and detection was performed with DIG High Prime DNA Labeling and Detection Starter Kit I according to the manufacturers' instructions (Roche, Switzerland).

2.2.4 Segregation analysis of the progeny (T1) plants of transgenic tomato

The segregation ratios of kanamycin-resistant (Km^R) to kanamycin-sensitive (Km^S) plants in the progeny (T1) of self-fertilized primary (T0) transgenic lines were determined by germinating surface sterilized seeds from two T0 lines on MS medium containing kanamycin sulphate (200 mg/L). After incubation for 2-3 weeks in a tissue culture chamber (25-27 º C, 16 h light/ 8 h dark), the seedlings were scored for kanamycin resistance. The segregation ratios were assessed by Chi-square analysis.

2.2.5 Proteinase inhibitor activity assays of transgenic tomato

Total soluble protein extracted from leaves of 2 month old WT and transgenic plants (T1, T2 generation) was assayed for proteinase inhibitory activity. Leaf tissues (200 mg) were grinded to a fine powder and extracted in 500 µL of Tris-HCl buffer (0.1 M; pH 7.8) at 4°C for a period of 2 days. The extract was then centrifuged at 12,000 rpm (4°C; 20 min) and clear supernatant was used for enzymatic assays. Total protein of clear supernatant was quantified following method described by Bradford (1976). For the enzyme inhibition assay, a suitable volume of enzyme equivalent to 0.4 U *H. armigera* gut proteinases (HGP), was mixed with crude leaf protein (50 µg), incubated at 37°C for 10 min and residual proteinase activity was estimated using enzyme specific chromogenic substrates, Benzoyl-DL-Arginyl-p-Nitroanilide (BA*p*NA) or N-succinyl-L-ala-L-ala-L-pro-L-leu-p-nitroanilide (SAA*p*LNA) (Sigma, St. Louis, MO, USA), as reported earlier (Damle et al., 2005; Tamhane et al., 2005). One proteinase unit was defined as the amount of enzyme that increased the absorbance by 1.0 OD under the given assay conditions.

2.2.6 Feeding-choice assay of transgenic plants

Two leaves each of WT and two transgenic tomato line 1 and line 2 (T2 generation) were arranged in plastic petri plates (15 cm diameter) opposite to each other on moist filter paper. First-instar *H. armigera* larvae were randomly transferred to the petri plates (6 larvae/plate; n=3). The amount of tissue remaining was noted each day at the same time for four days. The insects preference for a particular tissue type was proportional to the amount of tissue consumed. Greater consumption indicated greater preference in the choice assay.

2.2.7 Insect bioassay of transgenic plants

Insect resistance of transgenic plants (T1, T2 generation) was assayed by using detached leaves of WT and transgenic tomato plants using first instar larvae of *H. armigera* following the method of Senthilkumar et al. (2010). For leaf bioassay, fully expanded leaves of similar size and approximately 2 month old greenhouse grown transgenic tomato plants were placed in 9 cm petri dishes containing 2% (w⁄ v) agar and covered with filter paper to retain proper moisture. To carry out this assay, 15 leaves from WT and 15 leaves from each of three transgenic tomato lines were taken

and one leaf per larva of *H. armigera* (60/treatment) was placed in Petri dish. Fresh leaves were supplied after every 48 h. Individual larval body mass was measured after every 24 h period. Also mortality of *H. armigera* larvae was recorded for a period of 7 days. The experiment was repeated thrice.

2.2.8 Protein extraction and mass spectrometry analysis

Total proteome of leaf tissue of 2 month old three T1 transgenic lines and two WT plants was extracted as described by Kumar et al. (2016) (**Figure 2.2.3**). In brief, this comprises, removal of phenolic compounds by protein precipitation using 10% TCA/

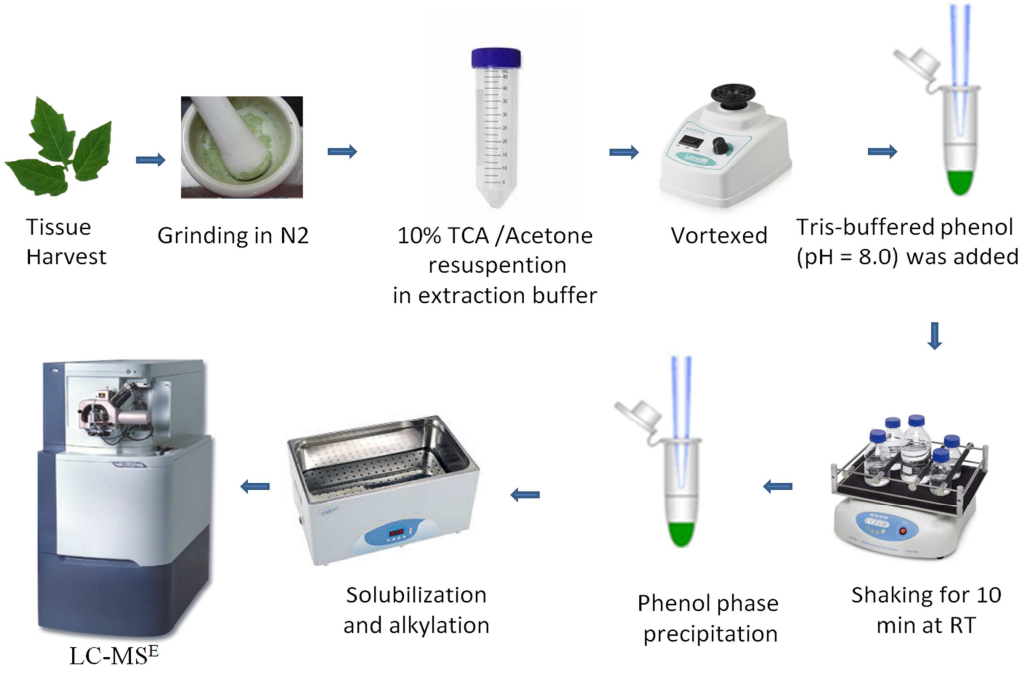


Figure 2.2.3: Schematic presentation of plant proteome extraction and mass spectrometry analysis

Acetone resuspension of pellet in 10 mL of extraction buffer containing 0.7 M Sucrose; 0.1 M KCl; 50 mM EDTA, 0.5 M Tris-HCl and pH 7.5. The reducing agent β-mercaptoethanol was added to a final concentration of 2% (vol/vol). Samples were vortexed and incubated by shaking on ice for 10 min. Subsequently, an equal volume of Tris-buffered phenol (pH = 8.0) was added, and solutions were incubated on a shaker at room temperature for 10 min. The phenol phase was transferred to another tube and four volumes of precipitation buffer, containing ammonium acetate in ice-

cold methanol and samples were incubated overnight at -20°C. After centrifugation, the pellets were washed thrice with ice-cold precipitation buffer and finally, pellets were dried at 25°C. Furthermore, protein pellets were solubilized in 50 mM ammonium bicarbonate buffer containing 0.1% Rapigest (Waters, USA). The dissolved proteins were reduced and alkylated by DTT and iodoacetamide, respectively followed by overnight tryptic hydrolysis at 37°C utilizing sequencing grade trypsin (Promega). The digested peptides were examined with LC-MS^E workflow using nano-ACQUITY online coupled to a SYNAPT-HDMS system (Waters, USA). Nano-LC separation was carried out with symmetry C18 trapping column (180 µm x 20 mm, 5 µm) and bridged-ethyl hybrid (BEH) C18 analytical column (75 µm x 250 mm, 1.7 µm). The binary solvent system comprised solvent A (0.1% formic acid in water), and solvent B (0.1% formic acid in acetonitrile). Each sample (500 ng) was initially transfered to the trapping column and desalted by flushing with 1% solvent B for 1 min at a flow rate of 15 µL/min. Elution of the tryptic digested sample was performed at a flow rate of 300 nL/min by increasing the solvent B concentration from 3% to 40% over 90 min. Ahead of data acquisition, the mass analyzer was calibrated using Glu-fibrinopeptide B (St. Louis, MO, USA) from m/z 50 to 1990. The Glu-fibrinopeptide B (GFP-B) was supplied at 500 fmole/µL to the mass spectrometer via a NanoLockSpray interface using the auxiliary pump of the nano-ACQUITY system at every 30 s interval for lock mass correction during data acquisition. Data independent acquisition was carried out (LC-MS^E) as described by Patel et al. (2009). Further, the percent coefficient of variance of retention time was calculated to assess the separation stability and coefficient of variance of 0.3 min, which also suggested stability in chromatographic separation. LC-MS^E data were processed and protein identification was carried out using ProteinLynx Global Server 2.4 (PLGS) software (Waters).

2.2.9. Extraction of metabolites and UHPLC profiling

Metabolites of each leaf tissue (100 mg) sample from two months old six transgenic lines and five WT plants were extracted with 1 mL of 60% ice cold methanol and 0.1% formic acid followed by sonication for 20 min and centrifugation at 4°C at 15,000 rpm for 30 min (Kumar et., 2015) (**Figure 2.2.4**). Supernatant was filtered with 0.2 micron filter (Millipore, Hessen, Germany). An Accela™ ultra high

performance liquid chromatography (UHPLC) system (ThermoFisher, Waltham, USA), coupled online via heated electrospray ionization source (HESI) with a Q-Exactive-Orbitrap mass spectrometer (ThermoFisher), was used for non-targeted metabolomics profiling with 3 µL sample injection volume. The metabolites were analysed using a C18 Hypersil Gold column (1.9 µm, 2.1 mmX150, ThermoFisher). The temperature of the column oven and sample manager was set at 40°C and 4°C,

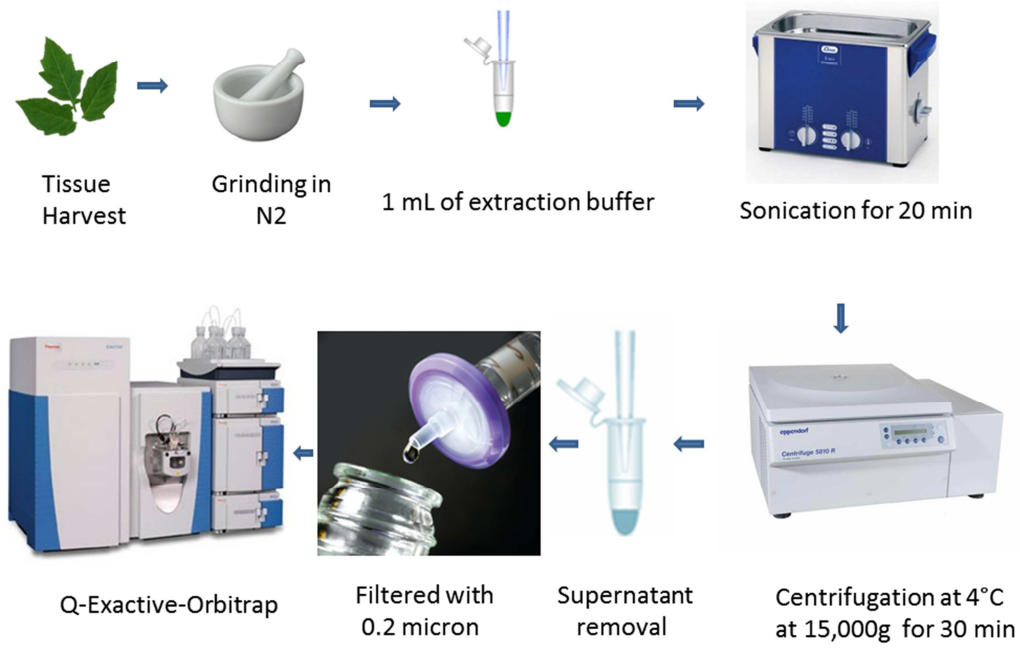


Figure 2.2.4: Metabolite extraction protocol and data acquisition methodology

respectively. The eluents A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid) were used in the electrospray ionization-negative (ESI-) mode and electrospray ionization-positive (ESI+) mode. The flow rate was adjusted at 0.6 mL/min with a linear gradient elution for 15 min. In the ESI- mode, the MS spray voltage was 2.8 KV while it was 3.7 KV in the ESI+ mode. The mass scan range was set from 100 to 1000 m/z and the tube lens was set to 45V. The capillary temperature was set at 300°C with the aux gas at 5 arbitrary units and the sheath gas at 45 arbitrary units. The resolution of the Orbitrap was fixed at 70,000. The tandem mass spectrometry (MS/MS) data were collected with the collision energy range between 10 and 35 eV. Pooled quality control (QC) samples were made by mixing all the samples to check the quality of metabolic profiling data. Five QC samples were run

before analyzing the sample sequence. The stability of the system during the analysis of sample sequence was monitored by running one QC sample every 10 sample injections. Extracted ion chromatograms (EICs) of isoleucine (Ile) and leucine (Leu) were choosed to verify the resolution of the mass spectrometer, intensity deviation, retention time shift and ppm error. Moreover, the retention times, mass accuracies and peak areas of these two selected EICs in the QC samples were also verified to validate the system stability. The raw data alignment and peak picking were carried out using the Progenesis QI software (Waters and Nonlinear Dynamics) for positive (ESI+) and negative (ESI−) ionization modes individually. All the detected ions in each sample with ANNOVA $p<0.005$, FDR <1% and minimum fold change of 1.5 between control and their respective inoculated samples, were normalized using total intensity before importing into SIMCA-P v. 13.0 software (Umetrics) for multivariate data analysis. The overall pattern and trend in data was analyzed by PCA performed with mean centered data to check.

Further, OPLS-DA was performed on pareto scaling data to identify the discriminating metabolites between control and inoculated samples. The parameters of the models, such as the R^2X, R^2Y, Q^2Y and the R^2Y-, Q^2Y-intercepts, were analyzed to ensure the quality of the multivariate models and to avoid the risk of over-fitting using 200 iteractions. The VIP values of all the peaks from the 7-fold cross-validated OPLS-DA model were considered as a coefficient for peak selection. Discriminating variables were selected according to their highest influence on loading, VIP values (VIP>1.0), S-plot and jack-knifed-based confidence intervals of OPLS-DA model. Identification of metabolites was carried out by searching the available databases such as KEGG (http://www.kegg.com), Massbank (http://massbank.imm.ac.cn/MassBank), KNApSAcK (http://kanaya.naist.jp/KNApSAcK) and METLIN (http://metlin.scripps.edu) using exact mass and MS/MS fragmentation patterns. *In silico* prediction of the mass fragmentation of the candidate structures was also performed using Mass Frontier™ software (ThermoFisher) and compared with MS/MS fragmentation pattern of identified metabolites.

Section 2.3 Characterization of single domain *CanPIs* (IRDs) in recombinant yeast against abiotic stress

2.3.1 Cloning of *CanPI* (IRDs) genes in *Pichia pastoris*

The mature peptide regions of single domain *CanPIs* (IRDs) were cloned in expression vector pPIC9 (Invitrogen, Carlsbad, CA, USA) in *P. pastoris* GS115 (Joshi et al., 2014b). The primers containing *Xho*I and *Not*I sites were used for amplification of the mature peptide regions from the full-length gene cloned in the pGEMT vector using Accuprime Pfx polymerase (Promega). The amplified product was digested with *Xho*I (Promega) and *Not*I (Promega) and purified using gel elution kit (Sigma, USA). Yeast expression vector pPIC9 (**Figure 2.3.1**) was prepared by digesting it with the same restriction enzymes (*Xho*I and *Not*I). The digested insert and vector were quantified and ligated in 3:1 ratio using T4 ligase (Promega). The ligated product was used for transformation of competent cells of *E. coli* strain (Top10). Positive colonies were identified by colony PCR. Plasmids were isolated from them and checked by restriction digestion with *Xho*I and *Not*I enzymes. The plasmids were digested with *Sal*I (Promega) for linearization and were utilized for *P. pastoris* (GS115) transformation.

2.3.2 Viability and growth studies of EV and PpIRD^{+} strains

Transformed *P. pastoris* have three different IRDs cloned independently i.e. IRD-7, -9 and -12, with differential protease inhibition potential. These four different *in vitro*

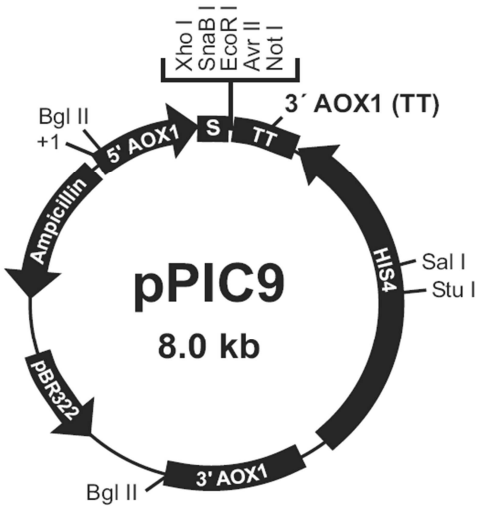


Figure 2.3.1: Vector map of pPIC9 vector (Source: Invitrogen).

phenotypes, containing empty and IRD containing vector, were assessed on various conditions by spotting 10 µl aliquots of diluted stationary phase cultures on plates containing high salt (1 M NaCl), metal (50 µM $CdCl_2$ and 50 µM $HgCl_2$) and 1 mM H_2O_2. These phenotypic tests were performed by serial dilutions of cultures onto solid agar yeast extract peptone dextrose (YEPD)-based plates. Yeast cultures were grown overnight in liquid YEPD and diluted to a density of 1.5×10^5 cells/mL. Four serial 10-fold dilutions were performed at a final dilution containing 1.5×10^1 cells/mL. 4 µL of each dilution were spotted onto YEPD-based plates and incubated at 28°C for 48 h. To assay the viability of yeast containing empty and inhibitor containing vector, the overnight cultures were adjusted to OD_{600} of 0.1, and the cultures were grown for 14 h at 28°C with shaking (220 rpm), and the OD_{600} was monitored after every 2 h. Time course growth was plotted and further analyzed (Grant et al., 1997).

2.3.3 Trypsin inhibitor activity and protease assays

Different yeast strains under control and stress conditions were grown and pelleted down. Cell mass was suspended in lysis buffer without any protease inhibitor. Protein lysate of yeast was incubated with trypsin for 15 min at 37°C and residual protease activity was estimated by BA*p*NA assay. The details of the assay were described previously (Joshi et al., 2014a). Protease assays using azocasein and BA*p*NA as substrates were performed as described previously in section No. 2.1.8

2.3.4 Metacaspase activity assay

Metacaspase activity in yeast cells were estimated as described by Vercammen et al. (2004). Intracellular metacaspase activity was determined using a commercially available fluorogenic system that uses the peptide Ac-Val-Arg-Pro-Arg-7-amido-4-methylcoumarin (Ac-VRPR-amc) as substrate. The assay was performed using 50 µM substrate and cell extract containing 100 µg of protein made up to total reaction volume of 100 µL. After 2 h incubation of this mixture in the dark at 37°C, the release of fluorescent amc was measured at λex of 380 nm and λem of 460 nm with a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany). The activity is presented as relative fluorescent units (RFUs), calculated by subtracting

background fluorescence in the absence of cell extract from the fluorescence obtained in the presence of cell extract.

2.3.5 Quantitative real-time PCR

Total RNA was isolated from the yeast using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and synthesis of the first strand cDNA was carried out with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA) using random primers based on the manufacturer's protocol. Relative transcript abundance of subtilisin-like protease 3, calpain-like protease 1 and metacaspases was determined by quantitative Real-Time PCR (qRT-PCR) using 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster, CA, USA) and Faststart Universal SYBR Green Master (Rox) 2× concentrate (Roche Diagnostics, GmbH, Germany). The relative expression of serine and cysteine protease genes (subtilisin-like protease 3, Calpain-like protease 1 and metacaspases) was assessed (**Appendix III**). For each gene, amplification efficiency was assessed by constructing a standard plot utilizing 5 serial dilutions of cDNA pool which was prepared by combining aliquots from all the cDNA samples under study. Actin (ACT1; Accession no.: CAA24598) was invoked as a reference gene for normalization. Reaction mix for qRT-PCR and thermal cycler conditions were followed as described by Chikate et al. (2013). For each treatment/time point, two biological replicates (each constituting three technical replicates), the average transcript abundance and subsequent fold difference with respect to the control were calculated.

2.3.6 Molecular docking study

In order to analyze the interaction between yeast metacaspase and IRDs, protein-protein docking was carried out. Structure of yeast metacaspase (Yca1) was retrieved from PDB (PDB ID: 4F6O) and structures of IRDs were predicted using homology modeling (Grant et al., 1997; Wong et al., 2012). ZDOCK 3.0.1 rigid-body docking program was utilized for generating Yca1-IRD complex structure. The catalytic residues of Yca1 (CYS220, CYS276) were allowed to interact with the reactive loop of the IRDs (37CPxNC41). The best composite structure was chosen based on the ZDOCK scores (Chen et al., 2003). Top score structure obtained for complex was energy minimized with the GROMOS96 force field using Swiss PDB viewer

(http://spdbv.vital-it.ch/) and binding energy was estimated using PDBePISA server (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver) (Krissinel et al., 2007). Occurrence and distribution of different polar interactions in the active site of Yca1 and reactive loop of IRDs were analyzed using the PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.

2.3.7 Viability and growth studies of metacaspase knockout *S. cerevisiae* strain (ΔYca1)

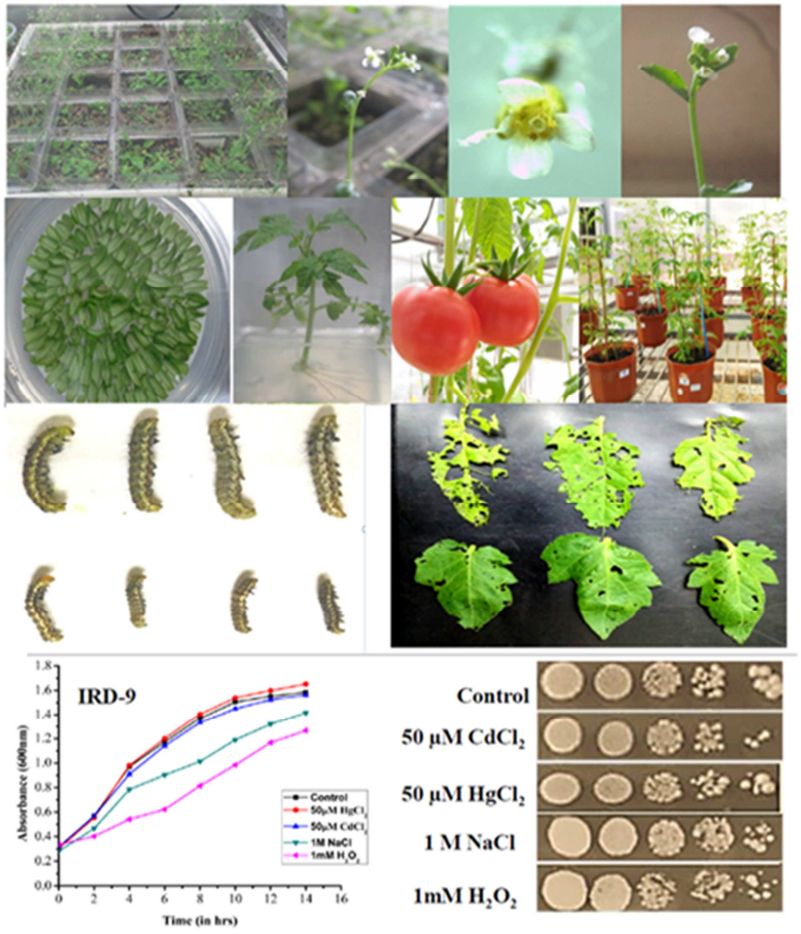
S. cerevisiae metacaspase (Yca1) knockout yeast strain was provided by Prof. Lynn Megeney (Ottawa Hospital Research Institute, Ottawa, Canada) as a kind gift. Viability and growth curve assay of homozygous diploid *S. cerevisiae* metacaspase knockout (ΔYca1) was performed to confirm the role of metacaspase in apoptosis and its correlation with delayed senescence in the presence of PIs. Overnight cultures of ΔYca1 strain was adjusted to OD_{600} of 0.1, and the cultures were grown for 14 h at 28°C with shaking (220 rpm), and the OD_{600} was monitored after every 2 h.

2.3.8 GAPDH activity assay

Silva et al. (2011) had shown that yeast metacaspase specific substrate and primary target in the cell is Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hence its relevance to yeast apoptosis. The assay mixture (1 mL) contained 10 mM sodium pyrophosphate (pH 8.5), 20 mM sodium phosphate, 0.25 mM NAD, 3 µM dithiothreitol, and either 1 µg of purified GAPDH or enzyme from yeast extract. After incubating in a spectrophotometer at 25°C for 5 min to achieve temperature equilibrium and to establish blank values, the reaction was initiated with the addition of glyceraldehyde 3-phosphate (0.4 µM). Absorbance at 340 nm was recorded from 0 to 5 min.

Chapter 3

Results



Section 3.1 *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*

3.1.1 IRDs of CanPI7 exhibit strong binding efficiency with various proteases

Multiple sequence alignment of amino acid sequences of IRD variants of CanPI7 with other reported gene sequences in the NCBI database indicated that domain 1 and 3

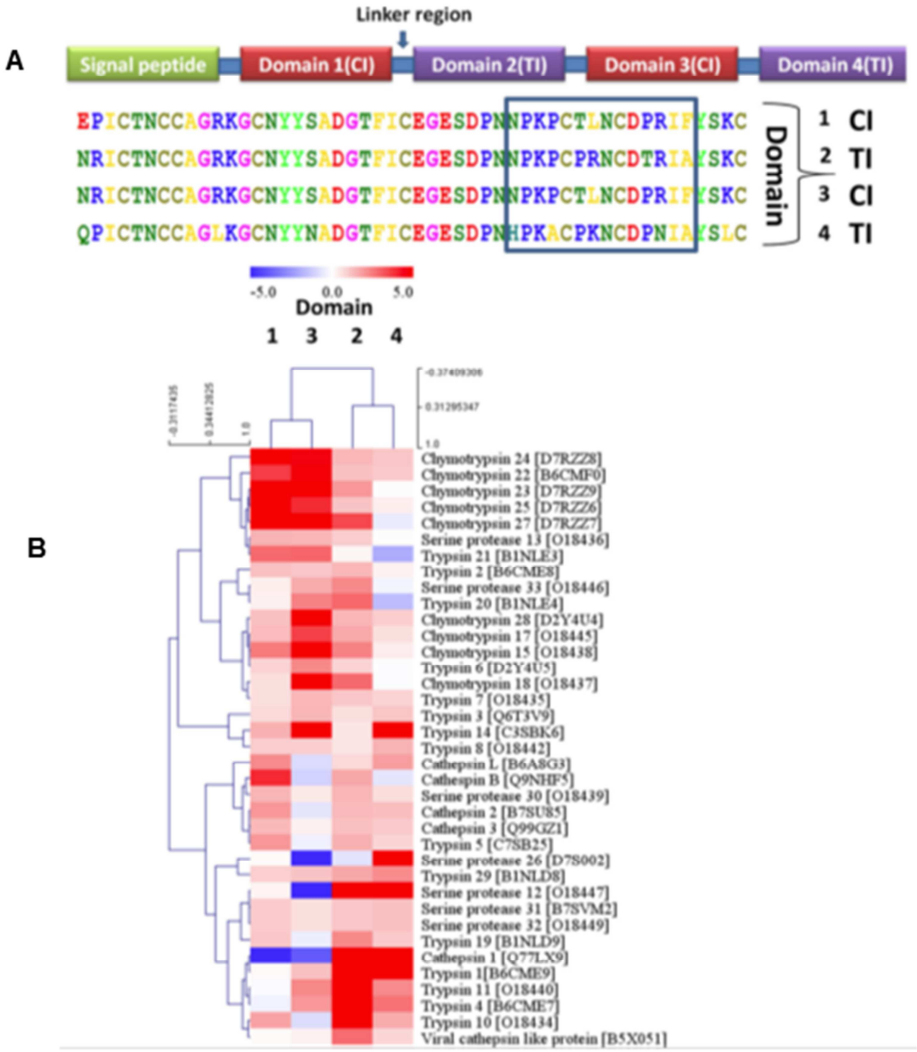


Figure 3.1.1: (A) Multiple sequence alignment of AA sequences of IRD variants of CanPI7. The region close to the active site showing major variation is marked by box (B) Interaction energy of binding of all four IRDs with *H. armigera* proteases compared by using Heatmap analysis. Heatmap with hierarchical clustering of relative free binding energy (obtained from docking study) was normalized by mean values of cumulative free energy obtained from *H. armigera* trypsins, chymotrypsin, cathepsins and other serine proteases binding with IRDs of CanPI7. The gradient ruler from -5 (blue) to 5 (red) is an indicator of interaction strength, where blue colour indicates weak binding and red indicates strong binding. Accession number of each protease sequence (Uniprot IDs) is given in the bracket. The scale values (-0.37 to 1.0) on the cluster branches indicate the degree of correlation in different interaction and hence, further used for the clustering.

were typical trypsin inhibitory domain (TI) while domain 2 and 4 represented a chymotrypsin inhibitory domain (CI) (**Figure 3.1.1A**). These IRDs showed variation in active site residues. Trypsin and chymotrypsin inhibitory sites had Lys (K) or Arg (R) and Leu (L),respectively at P1 site of reactive core. Docking studies revealed significant differences in binding energies of IRDs with those of HGPases suggesting that inhibitory domains had variable interaction with *H. armigera* serine proteinases. Among these domains, 1 and 3 showed strong interaction with the lowest binding energy with various *H. armigera* chymotrypsins (**Figure 3.1.1B**). Domain 2 and 4 showed interaction with most of the serine proteinases, but they exhibited remarkable binding with *H. armigera* trypsins. Interaction patterns for domain 1 and 3 with most of the serine proteinases were found to be similar. Domain 1 and 3 were clustered together for all the analyzed serine proteinases, while domain 2 and 4 were clustered in separate clan. This suggested difference in overall binding patterns of most of the gut serine proteases with domain 1 and 3 as compared to domain 2 and 4.

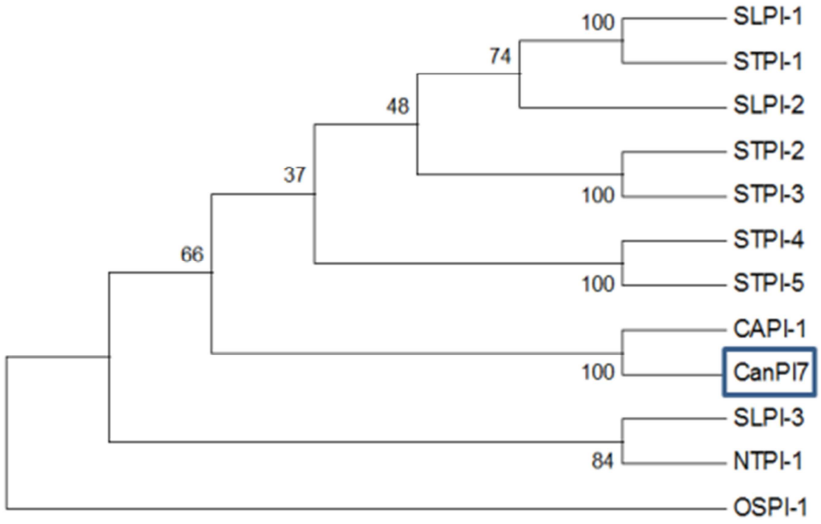


Figure 3.1.2: Phylogenetic analysis of CanPI7 protein with PIs of Solaneceous species. SLPI-1 (*S. lycopersicum*, Q43502), SLPI-2 (*S. lycopersicum*, Q43710), SLPI-3 (*S. lycopersicum*, P05119), STPI-1 (*S. tuberosum*, Q41488), STPI-2 (*S. tuberosum*, P01080), STPI-3 (*S. tuberosum*, Q00782), STPI-4 (*S. tuberosum*, Q41435), STPI-5 (*S. tuberosum*, Q43652), CAPI-1 (*C. annuum*, O49146), NTPI-1 (*Nicotiana tabacum*, Q40561), CanPI7 (*C. annuum* Q4ZIQ5), OSPI-1 (*Oryza sativa*, Q0JR25) used as outgroup.

For evaluating the phylogenetic relationship, an Neighbor-joining (NJ) phylogram was generated based on deduced amino acid residues of total 10 PI sequences from solaneceous plants (*Oryza sativa* PI as out group) with 1000

bootstrap replicates using MEGA (Molecular Evolutionary Genetic Analysis) 6 programs. Within phylogenetic tree, it was seen that CanPI7 protein is closely related to PI of *Solanum tuberosum*, which indicated that they are evolutionary closely related than with other PIs (**Figure 3.1.2**).

3.1.2 *Agrobacterium* GV3101 strain confirmation by 3-ketolactose and antibiotic resistance test

The confirmation of empty strain of *A. tumefaciens* GV3101 was conducted based on specific tests including antibiotics resistance test and production of 3-ketolactose test along with positive control of *Agrobacterium* strain GV3101-pRESC and negative control of *E. coli*. Empty *Agrobacterium* strain GV3101 and GV3101-pRESC showed ability for oxidation of lactose to 3-ketolase (yellow colouration) using the Benedict's reagent (**Figure 3.1.3A** and **B,** respectively), whereas negative control strain of *E. coli* (Top 10) showed no colouration. Antibiotics resistance test showed that empty *A. tumefaciens* GV3101 and GV3101-pRESC (positive control) were resistant towards rifampicin (50mg/L) in liquid LB medium, whereas negative control strain of *E. coli* (Top 10) was sensitive towards rifampicin.

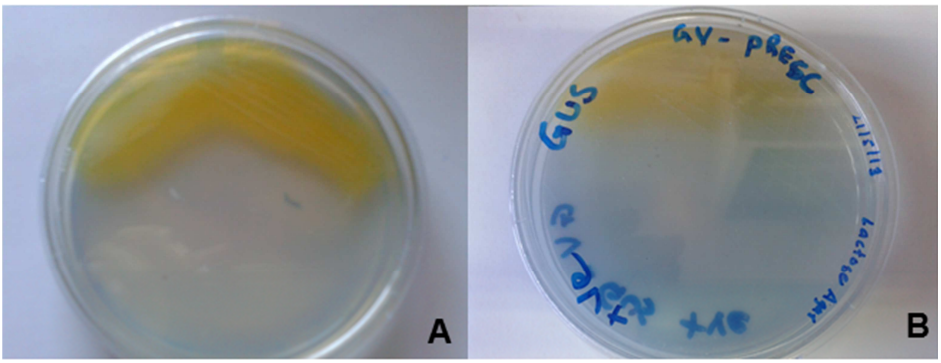


Figure 3.1.3: Identification of *Agrobacterium* culture through 3-ketolactose test (A) *Agrobacterium* strain GV3101 (empty strain) (B) positive control strain (GV3101-pRESC).

3.1.3 Cloning of *CanPI* genes

The open reading frame of *CanPI7* gene was amplified with gene specific primers modified to include *BamH*I and *Sac*I restriction sites (**Figure 3.1.4**) and the*BamH*I and *Sac*I digested amplicon was cloned in plant expression vector pRI101-AN digested with the same restriction enzymes. The digested insert and vector were

quantified and ligated in 10:1 ratio using T4 ligase (Promega). The resulting ligated construct was then transfered into empty *A. tumefaciens* GV3101 strain by freeze/thawing method (**Figure 3.1.4**). Similarly, *IRD7* gene was also cloned into *A. tumefaciens* GV3101 strain.

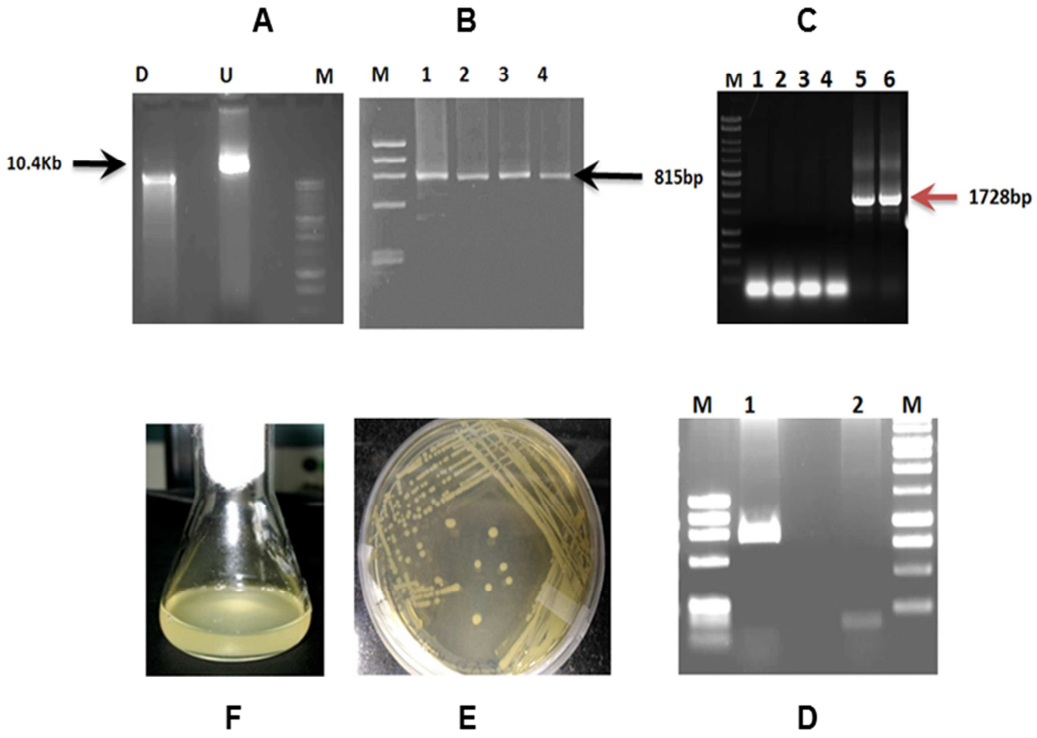


Figure 3.1.4: A schematic representation of sequential steps of cloning of *CanPI7* gene in pRI101-AN vector and transformation in *Agrobacterium* GV3101 strain. (A) pRI101-AN vector digestion with *BamH*I and *Sac*I restriction enzyme (Lane D: digested vector, Lane U: undigested vector, Lane M: 1Kb Marker) (B) Insert PCR of *CanPI7* gene (Lane 1-4: amplification of *CanPI7* fragment, Lane M: ΦX marker) (C) Confirmation of the presence of *CanPI7* (Lane 5 and 6) gene in pRI101-AN vector by the colony PCR technique (D) Confirmation of the presence of *CanPI7* gene (Lane 1) and *IRD7* gene (Lane 2) with restriction digestion of recombinant plasmid (*BamH*I and *Sac*I). (Lane M: marker lane) (E) Plate culture of transformed colony of *Agrobacterium* strain GV3101 (F) Overnight grown *Agrobacterium* GV3101 culture containing recombinant plasmid.

3.1.4 Generation of transgenic *A. thaliana* using floral dip method

Clough and Bent (1998), screened various *Agrobacterium* infection methods and found floral dip or floral spray method effective over conventional co-cultivation and agro-infiltration methods for transformation of *Arabidopsis*.

We, therefore, implemented floral dip method for *A. thaliana* transformation. Flowers from 35 days old *A. thaliana* ecotype Columbia-0 were infected with two constructs (*CanPI7* and *IRD7*) (**Figure 3.1.5**). Unopened flower, an ideal flowering tissue of

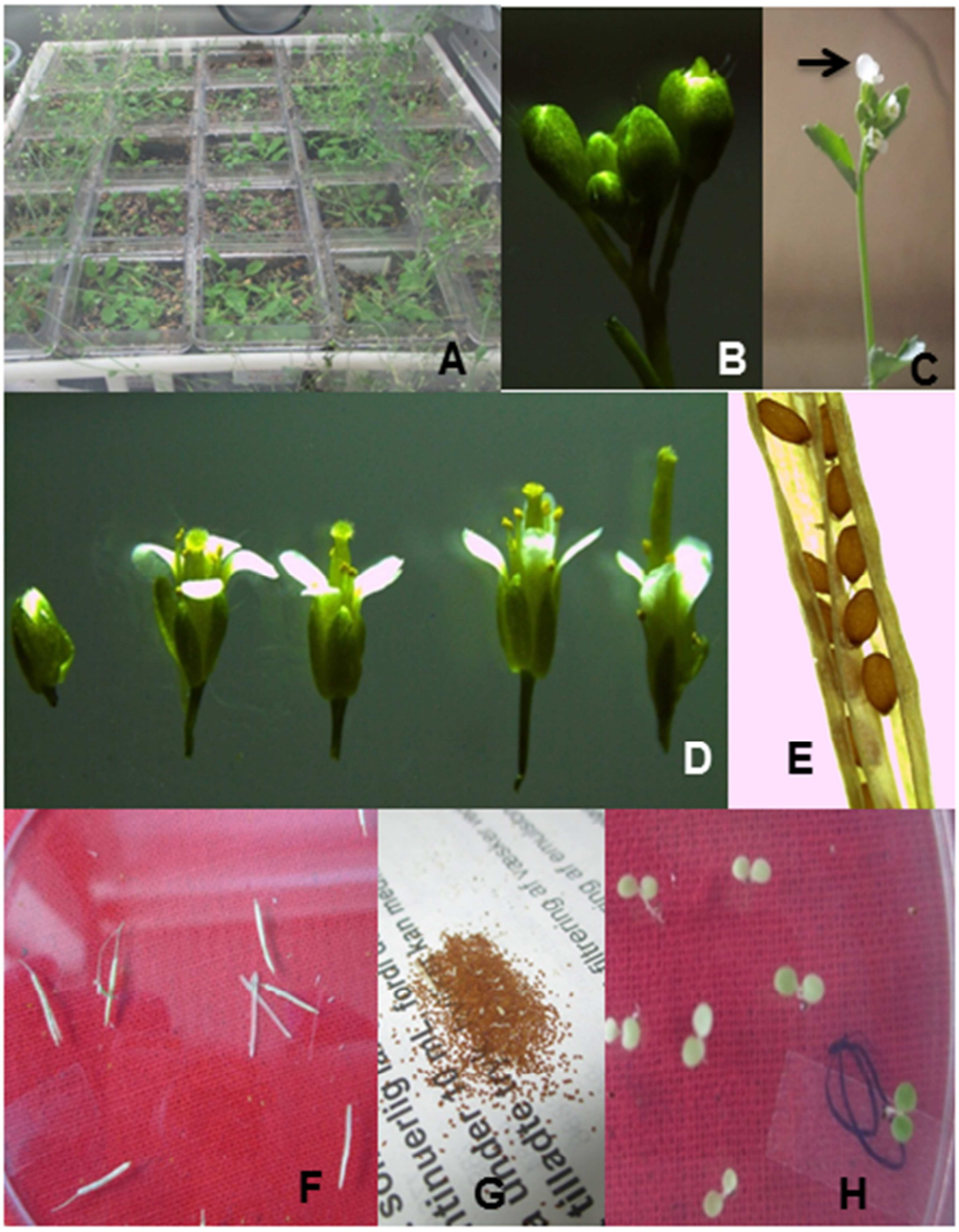


Figure 3.1.5: A schematic representation of the sequential stages of *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* plants. (A) Growth of *Arabidopsis* plants (35 days old) (B) Inflorescence (unopened flowers) (C) *Agrobacterium* infection to flowers (D) Silique development stages (E) Formation of mature seeds (F) Silique collection (G) Seed isolation (H) Seed germination.

Arabidopsis for transformation (Clough and Bent 1998) was infected to get high rate of transformation. Two *Agrobacterium* strains of GV3101 containing plasmid pRI101-*CanPI7* and pRI101-*IRD7* were used to transform *A. thaliana*. Different developmental stages of flowers of transformed *A. thaliana* plants were observed under stereo microscope (Laica S8APO). Siliques were collected from infected plants, seeds were removed and dried. Further, these seeds were germinated on selection MS medium containing kanamycin (50 mg/L).

Table 3.1.1 Transformation efficiency of *CanPI7* and *IRD7* in *A. thaliana*

Construct	Total seeds germinated	Plants survived on selection medium	Transformation efficiency (%)
CanPI7	2250	14	0.62
IRD7	3800	31	0.82

3.1.5 Selection and molecular characterization of transgenic *A. thaliana*

The selection method was applied to germinated seedlings of T1 seeds of *Arabidopsis* to obtain kanamycin-resistant plants. Following *Arabidopsis* transformation protocol with pRI101 constructs containing *CanPIs* (*CanPI7* and *IRD7*) putative transformed plants were obtained, which conferred kanamycin resistance via *nptII* gene. In floral dip method, higher frequency of transformation was observed for *IRD7* (0.82%) as compared to that with *CanPI7* (0.62%), which may be due to the difference in size of the genes used for construct development. Floral dip method was found to be suitable and easy for *A. thaliana* transformation using *CanPI* genes. Approximately, 6050 seeds were screened on selection media using these two constructs (**Table 3.1.1**). Putative transgenic lines were differentiated from non-transformed plants by their good growth and dark green foliage on selection media. Primary transformants were selected based on their resistance to kanamycin (50 mg/L) (**Figure 3.1.6**). Kanamycin-resistant seedlings were clearly distinguished from non-resistant seedlings by green expanded cotyledons whereas non-resistant seedlings had pale unexpanded cotyledons. Transformants had long hypocotyls, whereas non-resistant seedlings had short hypocotyls. Transformants were also identified by well-established roots within the selective medium. The transformed and non-transformed plants were differentiated in 10 days. After 10 days, transformants continued to grow and

remained green, while untransformed plants remained small, turned white and died in two weeks on selection media (**Figure 3.1.6**). Seeds of T2 plants roduced after self-pollination of T1 plants were germinated on selection media. These plants were used for assessment of insect tolerance by activity assay and insect bioassay.

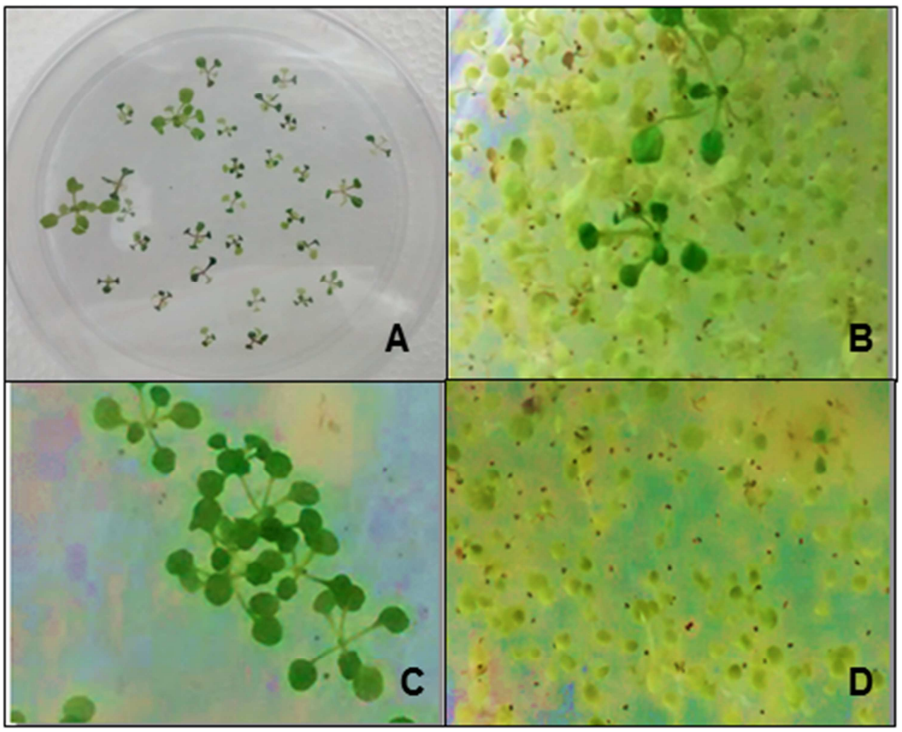


Figure 3.1.6: Screening for transgenic *Arabidopsis* plants. (A) Transgenic seedlings that have stably integrated kanamycin phosphotransferase (*nptII*) transgenes showing healthy growth are selected for kanamycin-resistance after 14 days of selection on 50 mg/L kanamycin (B) Magnified view of transgenic plants with green colour and healthy growth on selection medium (C) Positive control consisting of WT plants grown on MS media (D) Negative control consisting of WT plants grown on MS media containing kanamycin. None of the plants show green colour and good growth.

Homozygous T2 plants were selected based on segregation analysis of their progeny resistant to kanamycin selection media. Further, these transgenic *Arabidopsis* plants were examined by genomic PCR. Germinated kanamycin resistant T2 transgenic plants are shown in (**Figure 3.1.7**). The presence of *CanPI7* transgene in developed T2 transgenic plants was confirmed by PCR analysis using transgene specific primers. Genomic DNA was isolated from putative transgenic lines of *CanPI7* and *IRD7* using CTAB method (**Figure 3.1.7**). This DNA was used as template for PCR with 35S forward and gene specific reverse primers.

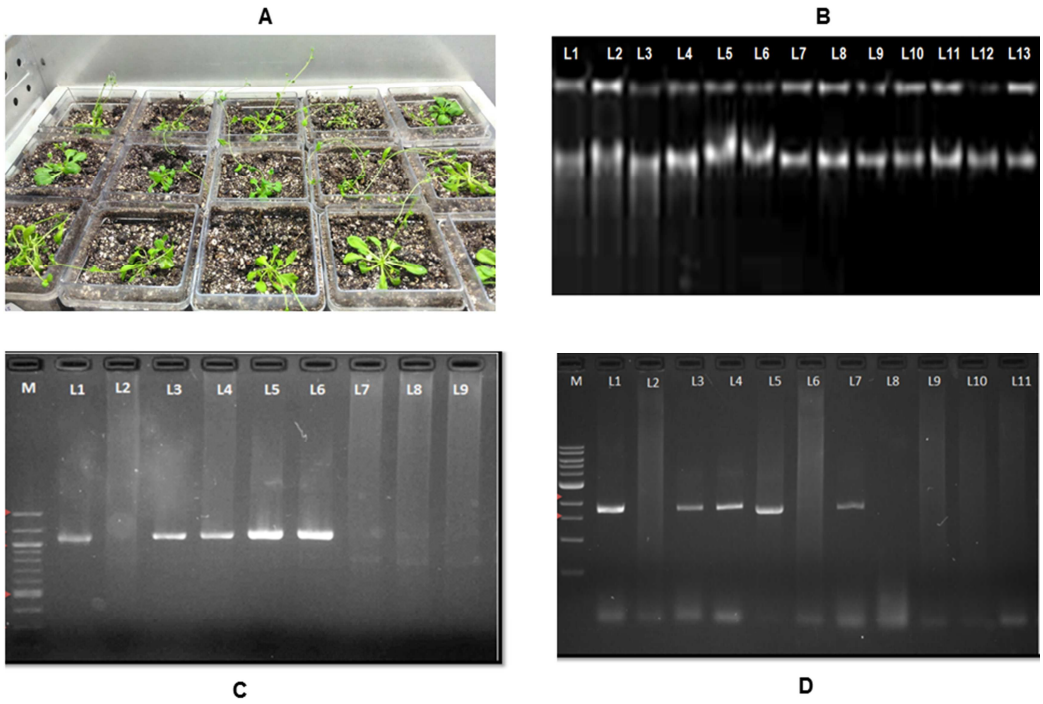


Figure 3.1.7: Growth and characterization of T2 transgenic lines (A) Growth of T2 transgenic lines in soilrite (B) DNA isolation of transgenic and WT lines, Lane 1-10: DNA of transgenic lines, Lane 11-13: DNA of WT plants (C) Genomic PCR analyses to detect the presence of *IRD7* in putative transgenic plants. M- 100bp ladder, L1- positive control, L2- negative control, L3 to L6 test of putative transformed plants of *IRD7* and L7 to L9 wild types plants **(**D**)** Genomic PCR analyses to detect the presence of *CanPI7* in putative transformed *A. thaliana* plants. M-1kb ladder, L1 - positive control of *CanPI7*, L2 - negative control, L3to L7 - test samples of putative transgenic plants for *CanPI7*, L8-L11 -test samples of WT plants.

A product of 1728 bp and 1184 bp was amplified from genomic DNA of randomly selected transgenic lines with *CanPI7* and *IRD7* transgenes. PCR analysis confirmed the presence of *CanPI7* and *IRD7* transgenes in individual transgenic lines and PCR positive transgenic lines were randomly selected for screening by insect bioassay. No gene specific amplicon was detected in the WT control plants.

3.1.6 Proteinase inhibitor activity assay indicates variable inhibition of HGP activity

The inhibitory activity of protein extracts of transgenic and WT *Arabidopsis* leaf tissue was assayed against HGP. Crude extract of transgenic lines (Line1 and Line2) of *CanPI7* and *IRD7* exhibited significantly higher HGP inhibition as compared to

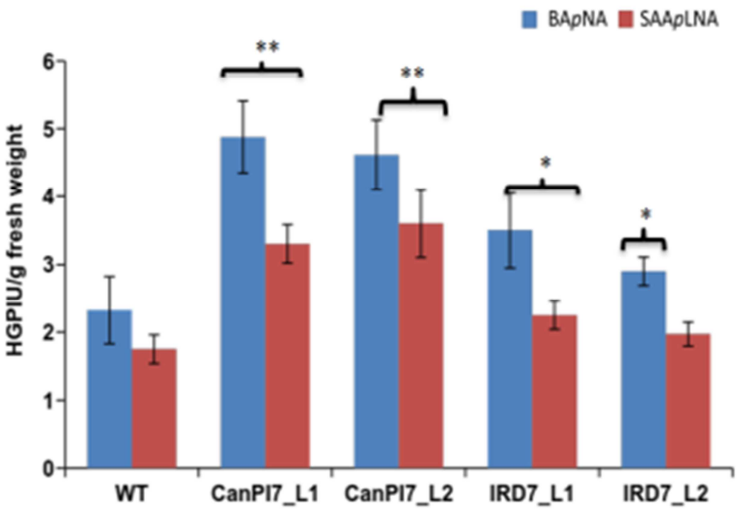


Figure 3.1.8: HGP inhibitory activity of WT and transgenic plant. Graph indicates the concentration in terms of HGP inhibitory units (HGPIUs) in WT and transgenic leaves.

crude extract of WT (**Figure 3.1.8**). HGP inhibition was observed higher in transgenic lines of *CanPI7* as compared with transgenic lines of *IRD7*. Qualitative analysis of crude protein of WT and transgenic leaves revealed that inhibition of trypsin-like activity was higher than inhibition of chymotrypsin-like activity of HGPs. Quantitatively in transgenic *CanPI7* lines, the amount of HGPIs was 1.98 to 2.09 times higher in case of trypsin like activity while 1.88 to 2.05 times higher for chymotrypsin like activity in transgenic leaves as compared to those in WT leaves. Similarly, in transgenic *IRD7* lines, the amount of HGPIs was 1.24 to 1.5 times higher in case of trypsin like activity while 1.12 to 1.28 times higher for chymotrypsin like activity in transgenic leaves as compared to those in WT leaves.

3.1.7 *CanPI7* transgenic *Arabidopsis* retards the growth and development of *H. armigera* larvae

After feeding for 7 days, average mass of larvae fed on AD and WT were 92.26 mg and 81.85 mg respectively (**Figure 3.1.9**). Most of larvae recovered from AD and WT diet reached to higher larval mass (>75 mg). Larvae recovered from *CanPI7* transgenic lines remained in larval mass range of 52.4 to 71.7 mg. Larvae feeding on

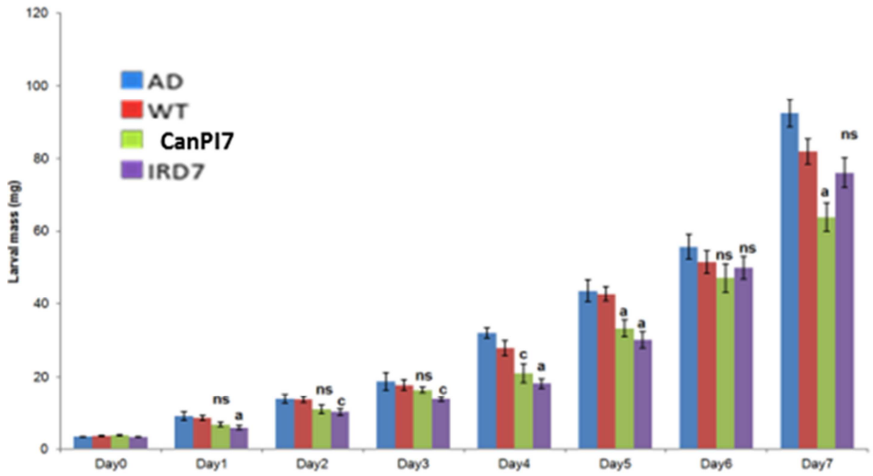


Figure 3.1.9: Larval mass of first instar *H. armigera* larvae fed on diet containing leaf crude protein of WT and transgenic *Arabidopsis* plant. Larval mass was recorded after every 24 h for 7 days. Data was obtained from the average weight (±SE) of 60 larvae per treatment from independent experiments performed in triplicate. Standard mean errors are indicated. a***, b** and c* indicate that values are significantly different from each other at $p < 0.0001$, $p < 0.001$ and $p < 0.01$ respectively, 'ns' denotes non-significant.

diet containing crude protein of *CanPI7* transformed plants had significantly reduced mean larval mass at 4, 5 and 7 days as compared to WT. Moreover, larvae recovered from *IRD7* transgenic lines showed larval mass range of 72.4 to 89.7 mg. Highest reduction in mass of larvae was upto 22.12% in *CanPI7* transgenic line diet as compared to that on diet containing WT crude protein on 7th day.

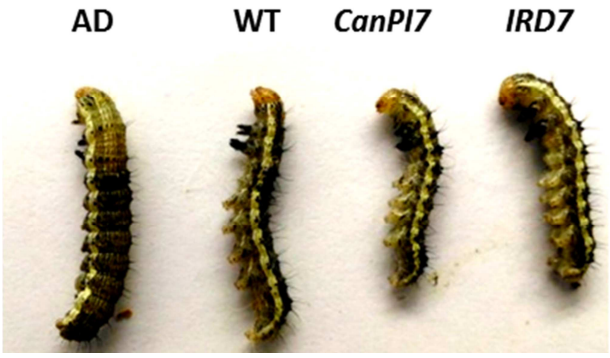


Figure 3.1.10: Representative *H. armigera* first instar larvae fed on diet containing leaf crude protein of WT and transgenic *Arabidopsis* plant for 7 days.

Representative image of larvae recovered at the end of 7th day experimental period is shown in **Figure 3.1.10.** No significant differences in larval mortality rate on transgenic and WT diet were noted. Together, feeding experiment established that larvae fed on AD containing crude protein of transgenic *Arabidopsis* plants expressing a multidomain *CanPI7* gene showed a higher reduction in their mass compared to that of larvae fed on diet containing crude protein of transgenic *IRD7* plants.

Section 3.2 *Agrobacterium*-mediated transformation of tomato

3.2.1 Variable regeneration response of tomato cultivar 'Pusa Ruby'

The development of an improved protocol for simple and efficient regeneration system was carried out for tomato indeterminate cultivar Pusa Ruby. The regeneration capacity of three types of explants (segments from hypocotyl, cotyledonary leaf and leaf) were compared in tomato. Leaf, hypocotyls and cotyledonary leaf were grown on MS medium supplemented with zeatin (ZA) (1.0 mg/L)(**Figure 3.2.1**). The highest regeneration capacity was found in cotyledonary leaf explants, i.e., 74% of the cotyledonary cultured, produced callus (**Figure 3.2.2**). The second highest regeneration response was observed in hypocotyls, i.e., 64.3% produced. The minimum regeneration response obtained was for leaf explants (56.3%).

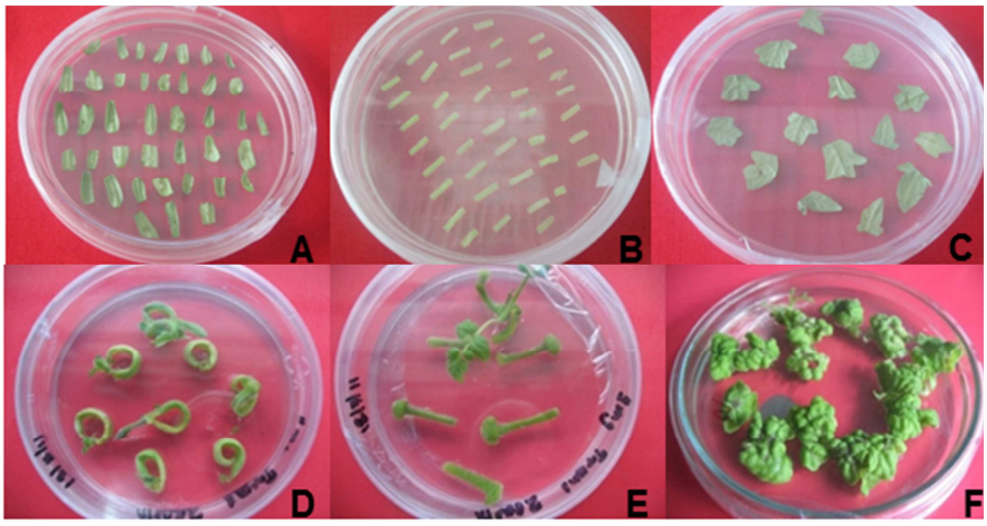


Figure 3.2.1: Regeneration response of different tomato explants (A) Cotyledonary leaf (B) Hypocotyls and (C) Leaf placed on regeneration medium. Corresponding response of tomato explants (D) cotyledonary leaf (E) hypocotyls and (F) leaf on regeneration medium.

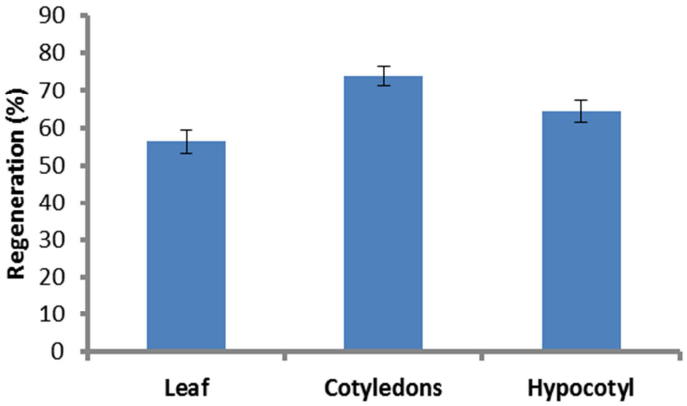


Figure 3.2.2: Graph showing regeneration response of different tomato explants.

Different sets of experiments were carried out to find best medium for tomato regeneration. In a preliminary experiment, we compared the effect of different media, differing only in their growth hormone compositions, on the regeneration of cultivar Pusa Ruby. In the first set of experiments four hormones such as zeatin (ZA), 6-Benzylaminopurine (BAP), Kinetin + Indole-3-acetic acid (IAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) were added in MS medium to find the best medium for callus regeneration. Medium which contained zeatin at 1 mg/l gave the best results in terms of regeneration percentage and morphological quality of the regenerated plants compared to the medium containing BAP, Kinetin + IAA, and 2,4-D (**Figure 3.2.3**). BAP and Kinetin produced reduced percentage of regeneration (73 and 67.6%, respectively). Response of all the different combinations and concentrations of growth regulators supplemented with MS for the induction of callus from cotyledonary leaf explants are presented in (**Table 3.2.1**) It was observed that Kinetin+ IAA, ZA and BAP had positive effect towards shoot regeneration. However, 2,4-D supplemented MS did not show any response towards shoot bud formation.

In second set of experiment, 4 different hormone concentrations were suplement to the MS medium and regeneration of explants was done on these media to examine their effect on shoot induction and their development. The maximum callus regeneration, i.e., ~78% was observed in MS medium supplemented with zeatin 1 mg/L (**Figure 3.2.3A**) (**Table 3.2.1**). Transfer of cotyledons to regeneration medium stimulated shoot buds in callus within 3 weeks. Shoot buds emerged mostly from the proximal

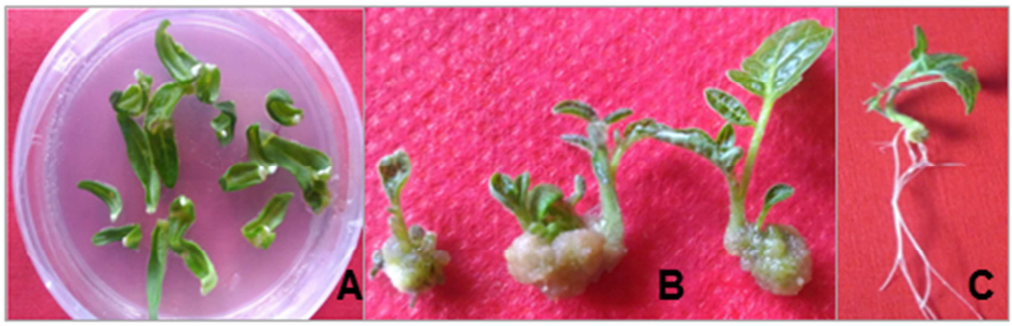


Figure 3.2.3: Regeneration response of cotyledon explants on callus, shoot and root induction media **(**A) Callus regeneration in cotyledon explants of tomato cultivar "Pusa Ruby" placed on callus induction medium. (B) Regeneration of shoot buds in callus of tomato explants on MS medium supplemented with ZA. The data were taken after 5 weeks of culture. (C) Rooting of excised shoots in root induction medium (IBA= 1mg/L).

Table 3.2.1 Regeneration response from cotyledon explants in different callus, shoot and root induction media.

Media	**Media composition (mg/L)**	**% Regeneration**
Callus induction		
C1	Kinetin (1)+IAA (0.1)	67.6 ± 3
C2	BAP (1)	73 ± 2.7
C3	Zeatin (1)	78.6 ± 3.5
C4	2,4-D (1)	74.6 ± 2.9
Shoot induction		
S1	BAP (1)	32 ± 2
S2	Zeatin (1)	44.3 ±3
S3	TDZ (1)	22 ± 2.6
S4	BAP (0.5) + zeatin (0.5)	36 ± 4.5
Root induction		
R1	NAA (1)	62.2 ± 3.5
R2	IAA (1)	85.3 ± 4.5
R3	IBA (1)	92.7 ± 3.1

[±]Values are mean of three experiments (SD).

end of the cotyledons. Shoots were also initiated from the remaining wounded parts of the cotyledons. The development of shoot buds was fast in MS containing ZA 1 mg/L compared to other media (**Figure 3.2.3B**) (**Table 3.2.1**). Successful induction of roots in plantlets is an integral part of regeneration studies. In the third set of experiments excised shoots were transferred on MS medium supplemented with various auxins, *viz.* IAA, IBA and NAA. Induction of root was observed within 15 days (**Figure 3.2.3C**). MS medium containing 1 mg/L IBA was found to be the most effective in producing maximum number of strong and healthy roots (**Table 3.2.1**). For acclimatization, *in vitro* rooted shoots were removed from rooting medium and washed to remove adhering gel and transplanted to plastic pots containing autoclaved soilrite. Plants were kept under culture room conditions for 7 days, then transferred to greenhouse and placed under shade until growth was observed. Rooted plantlets were transferred to soil mixture containing soilrite and cocopeat (2:1:1) where they acclimatized successfully.

3.2.2 Co-cultivation studies of *Agrobacterium* strain GV3101

Tomato is a natural host for *Agrobacterium*, valuable to present plant transformation techniques. To monitor early events of plant transformation 35S-GUS construct was used, since it ensures that the GUS coloration arises from transformed plant tissue. Interaction between *A. tumefaciens* strain GV3101 with the cotyledonary leaf explants of tomato variety (cv Pusa Ruby) exhibited the explants to be susceptible towards infection. GUS positive regions were detected predominantly along the cut ends.

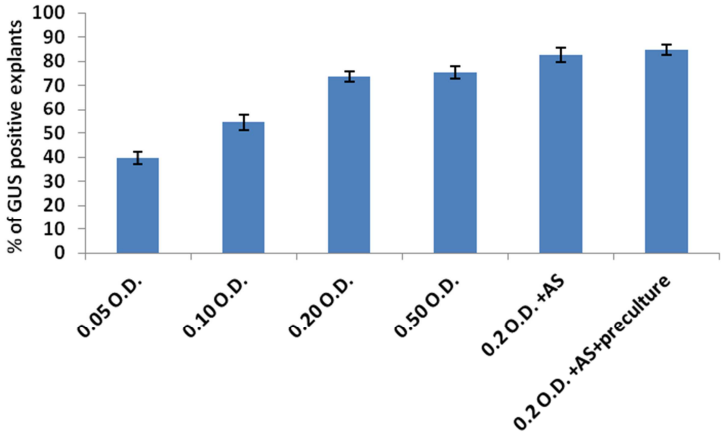


Figure 3.2.4: Effect of various concentrations of bacterial density on transient GUS expression of explants. Standard mean errors are indicated.

Parameters affecting *Agrobacterium*-mediated genetic transformation such as optical density of bacterial culture and acetosyringone (AS) were optimized in present study. Transient transformation efficiency was compared according to the percentage of GUS-positive explants after co-cultivation. Bacterial suspensions with optical densities of 0.01, 0.05, 0.1, 0.2 and 0.5 were used. Among the various optical densities (OD_{600}) tested, it was observed that maximum transformation (75.3%) was observed at OD_{600} of 0.5 while minimum (39.6%) was at OD_{600} of 0.05 (**Figure 3.2.4.**). Transformation frequency of transient GUS expression in explants were found to be increasing with increase of the optical density of the *Agrobacterium* suspension. Increasing bacterial suspension concentrations markedly increased the contamination, and higher necrosis rate. In the present study, addition of 200 µmol AS in the medium could significantly increase the frequency of transient GUS expression in tomato explants (**Figure 3.2.5**). Also use of AS increased the area of infection of transformed explants. Our results indicated that AS might be valuable to improve the transformation efficiency for tomato explants. The results showed that *Agrobacterium* strain GV3101 was active in cut surfaces of leaf section.

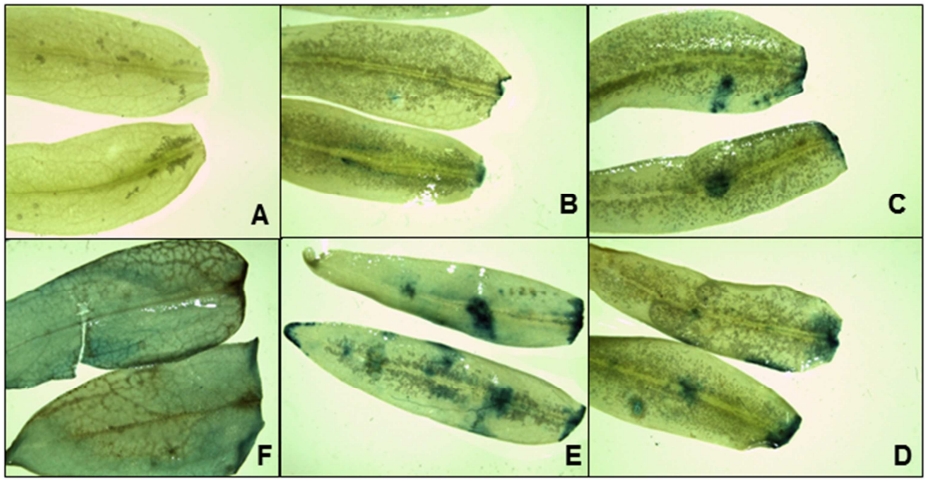


Figure 3.2.5: Magnified view showing histochemical localization of GUS activity (blue colour) at the cut ends of cotyledonary leaf explants of Pusa Ruby. (A) Control explants (without infection) (B) Infected explants at O.D. = 0.1 (C) Infected explants at O.D.= 0.2 (D) Infected explants at O.D.= 0.5 (E) Infected explants at O.D.= 0.2 supplemented with 200µM AS in infection medium (F) Infected precultured explants at O.D.= 0.2 supplemented with 200µM AS in infection medium.

No histochemical staining was detected in the explants of control plants as shown in **Figure 3.2.5**. Using this optimised regeneration and co-cultivation parameters, future

studies were conducted to transfer *CanPI7* candidate gene in the tomato plant for conferring insect resistance.

3.2.3 Regeneration and molecular characterization of transgenic T0 plants

For tomato, different transformation methods are available, but *Agrobacterium*-mediated method was preferred for cultivar Pusa Ruby, since it is a well established (Sharma et al, 2009) and highly efficient procedure. To study the role *CanPI7* gene in insect resistance, *A. tumefaciens* strain GV3101 having plasmid pRI101-*CanPI7* was used to generate transgenic tomato plants (**Figure 3.2.6**).

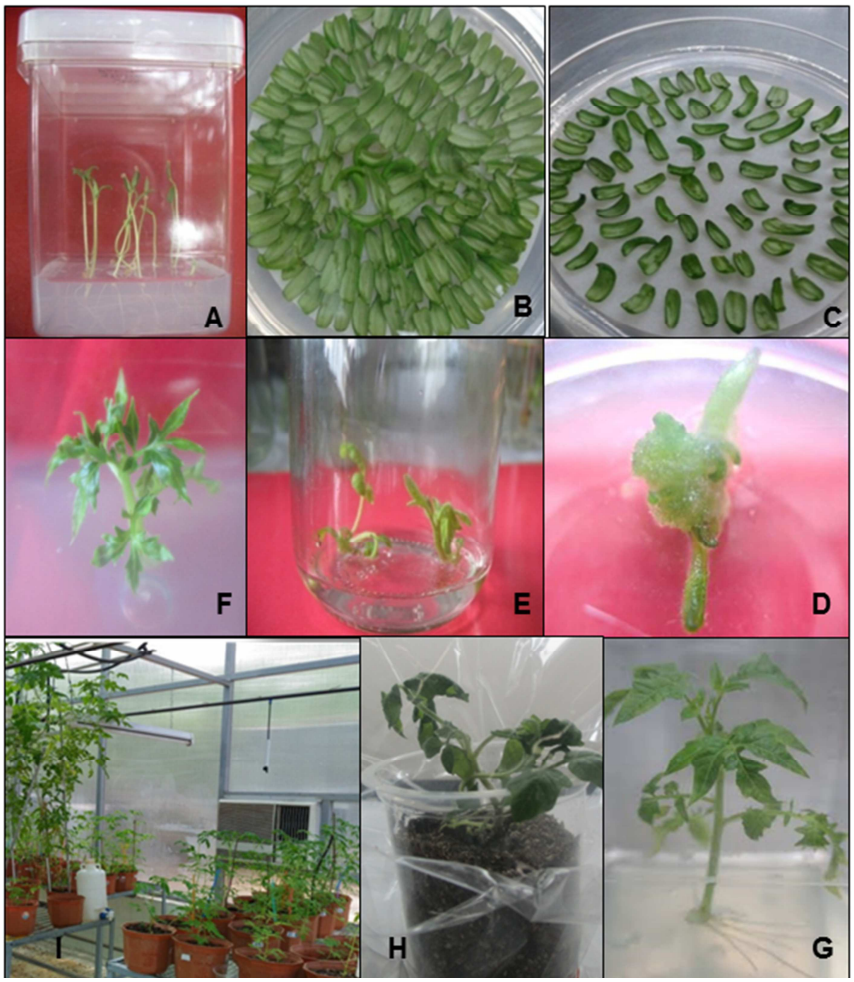


Figure 3.2.6: Various stages of *Solanum lycopersicum* var. Pusa Ruby transformation (A) Germination of seeds (B) Preculture of cotyledonary leaf explants (C) Co-cultivation of explants with *A. tumefaciens* (D) Formation of shoots on callus of transformed explants after 5 weeks (E) Surviving shoots of selection medium in presence of 100 mg/L kanamycin (F) Elongation of healthy shoots following kanamycin selection. (G) Rooting of elongated shoots (H) Hardening of plantlets in soilrite in growth chamber (I) Putatively transformed plant (T0) transferred to plastic pots containing soil mixture in greenhouse.

Callus formation was seen in positive control explants on medium without kanamycin. Negative control explants, which were not infected with *Agrobacterium* placed on selection medium, did not showed callus formation and necrosis was seen because of kanamycin. Response of positive and negative control explants indicated that regeneration and selection system was appropriate, respectively. After 2-3 weeks, formation of calli was observed due to *npt* gene which deactivates the antibiotic (kanamycin) selection. In addition to this, it was observed that most of *Agrobacterium* treated explants showed growth on selective medium containing 100 mg/l kanamycin and this indicated the first sign that *CanPI7* was transformed into explants (**Figure 3.2.6**). After 3-4 weeks, shoot buds started to emerge from the calli. The root formation ability shown by the transformed shoots was another evidence that transgenes (*CanPI7* and *npt*) were transformed. Root development was seen in shoots within 15 days. Around 4 weeks later, the regenerated plantlets were transferred from glass bottles to soilrite. Before taking to the greenhouse, the plantlets in pots containing soilrite were acclimatised in plant growth chamber at 25 ± 1 °C with high relative humidity (70%) at a photoperiod of 16 h for 7 days. Regenerated plantlets were transferred to greenhouse and were covered with plastic bags for first 4 days. The independently derived transgenic lines exhibited phenotypes that were indistinguishable from the untransformed control plants (WT) except for line 2, which showed change in leaf morphology (**Figure 3.2.7**). Transgenic line 2 exhibited oval type leaves compared to pinnate type leaves of WT (**Figure 3.2.8**). Overall, 7.5% efficiency of tomato transformation was achieved. Tomato transformation events gave rise to 9 kanamycin resistant T0 plants, which were confirmed as transgenic origin by genomic PCR analysis. DNA isolation of WT and transgenic plants was done using CTAB (**Figure 3.2.9A**) and treated with RNAse to remove RNA contamination (**Figure 3.2.9B**). Further, these transgenic tomato plants were examined by genomic PCR (**Figure 3.2.9C**). A band of the expected size (1728 bp) was detected in putative first generation transgenic plants. This indicated successful integration of the *CanPI7* transgene into the tomato genome. The PCR product was not detected in non-transgenic WT, the negative control.

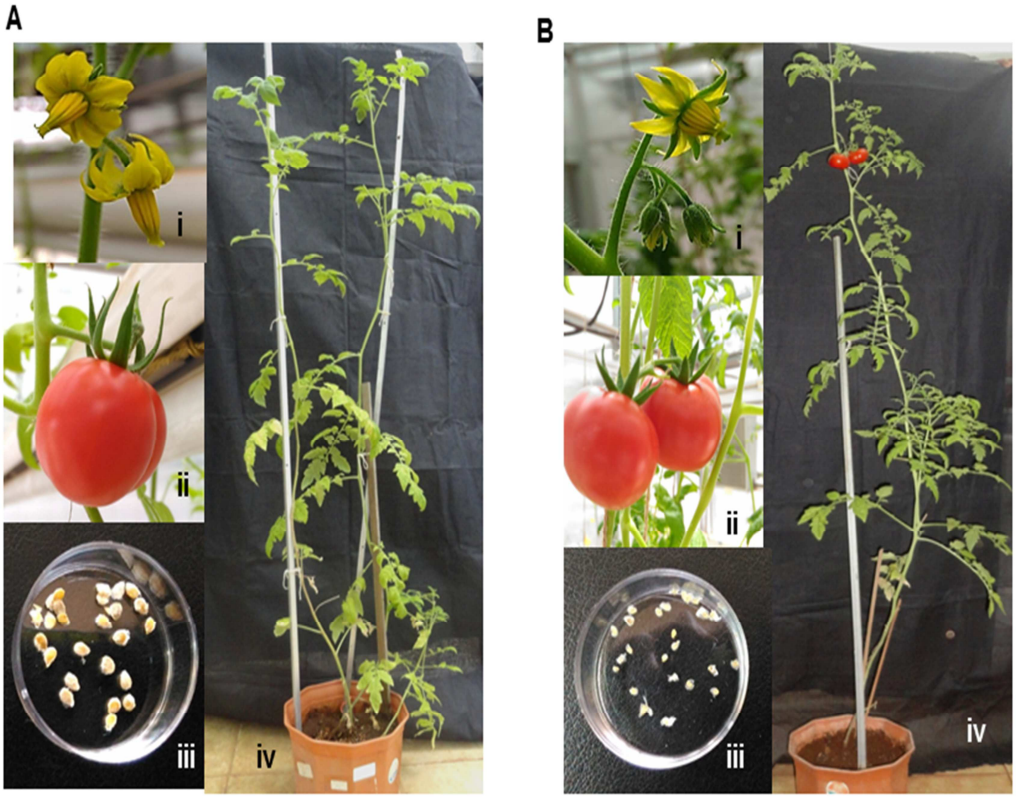


Figure 3.2.7: Different stages of WT and transgenic tomato plants.
(A) (i) Flowers (ii) fruit (iii) seeds of (iv) WT plant
(B) (i) Flowers (ii) fruits (iii) seeds of (iv) Transgenic (T0) plant.

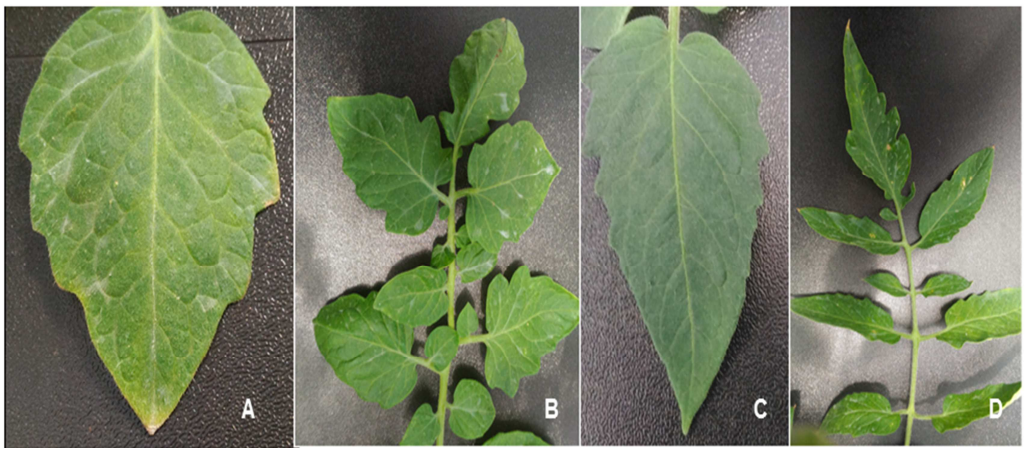


Figure 3.2.8: Representative picture of transgenic and WT leaf. (A) Leaflet and (B) compound leaf of transgenic line 2 (C) Leaflet and (D) compound leaf of control WT plant.

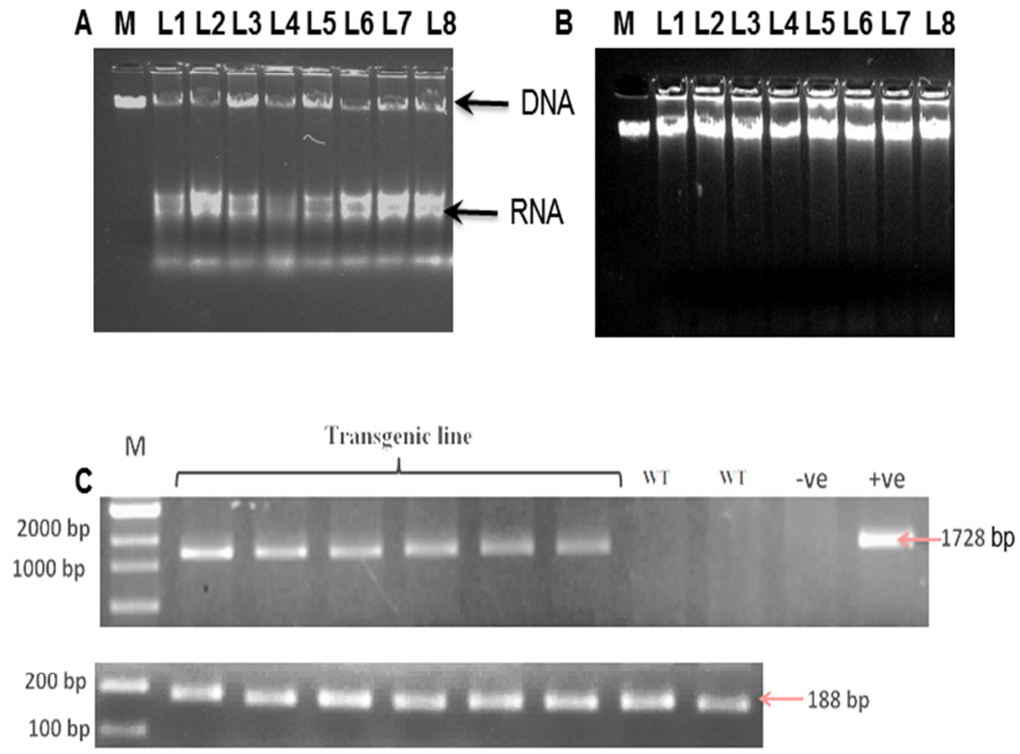


Figure 3.2.9: Molecular analysis of T0 transgenic plants (A) DNA isolation of WT and putative transgenic plants (B) RNase treatment to remove RNA contamination Lane M: marker; Lanes 1-6: DNA from transformed plants; Lane 7-8: DNA from WT (C) PCR amplification of the 1728 bp and 188bp fragments of the transgene (35S + *CanPI7*) and elongation factor gene, respectively. Lane M: marker; Lanes 1-6: DNA from transformed plants; Lane 7-8: DNA from WT; Lane 9: negative template control.

3.2.4 Molecular characterization of transgenic T1 tomato plants

Seeds of T1 plants that were produced after self-pollination were germinated on selection medium (**Figure 3.2.10A**). Of the 55 seeds tested from two separate T0 plants, 34 turned out positive for the *CanPI7* transgene following kanamycin selection screening (**Table 3.2.2**). WT plants were unable to grow on selection medium (**Figure 3.2.10B**). Though the untransformed seeds could germinate, they did not develop leaves and roots on selective medium. Further, these transgenic tomato plants were examined by genomic PCR, Southern blot and RT-PCR. A total of 6 healthy and best grown transgenic lines (L1, L2, L3, L4, L5 and L6) were randomly selected for further investigation.

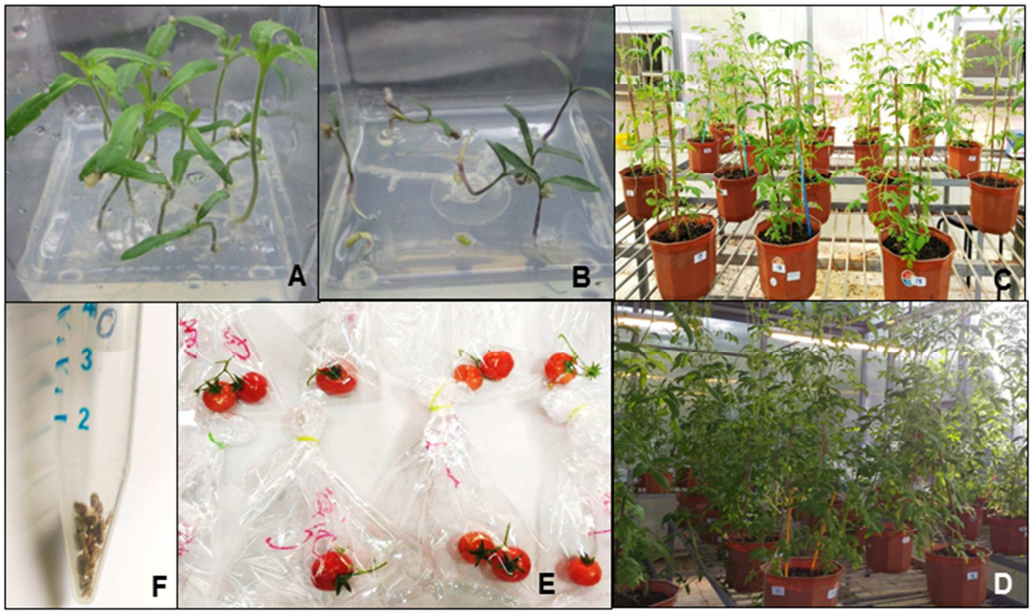


Figure 3.2.10: Growth and selection of T1 plants (A) Germination of T1 seeds on selection medium (B) WT seeds on selection medium (negative control) (C) 2 month old T1 transgenic plants in greenhouse. (D) 4 months old T1 transgenic plants in greenhouse (E) Transgenic fruit collection (F) Transgenic seeds (T2) collection.

3.2.4.1 Mendelian inheritance pattern of T1 seeds

Seeds from the T0 lines were germinated on selection medium supplemented with 200 mg/L kanamycin. For each transgenic line, 20-25 seeds were used and the numbers of resistant plants on selection medium were calculated. The result is as presented in **Table 3.2.2**. Antibiotic screening of T1 seeds revealed segregation according

Table 3.2.2 Segregation of *CanPI7* gene in T1 progeny of transgenic tomato plants

T0 plants	**Response of T1 seeds on kanamycin selection medium**			**χ^2 value**
	Total	Kan^r	Kan^s	
WT	20	0	20	60
Line 1	25	18	6	0.04
Line 2	20	14	6	0.26

Kan^r = kanamycin resistant; Kan^s = kanamycin susceptible

to Mendelian ratio 3:1 (resistant: susceptible, $p \leq 0.05$, $\chi^2 = 3.841$) for kanamycin tolerance as expected for typical single dominant gene inheritance. The appearance of

transgenic seed on selection medium is shown in (**Figure 3.2.10a**). As can be seen from the photographs, transgenic seeds grew faster than the WT plants on selection medium. The χ^2 test indicated non-significant difference between expected 3:1 ratio and observed values. These results showed that the T-DNA insert was transmitted to the next generation and expressed in the ratio expected for a single dominant gene.

The presence of *CanPI7* transgene in putative T1 transgenic plants was confirmed by PCR analysis using transgene specific primers (**Figure 3.2.11 A and B**). A product of 323 bp was amplified from genomic DNA of randomly selected

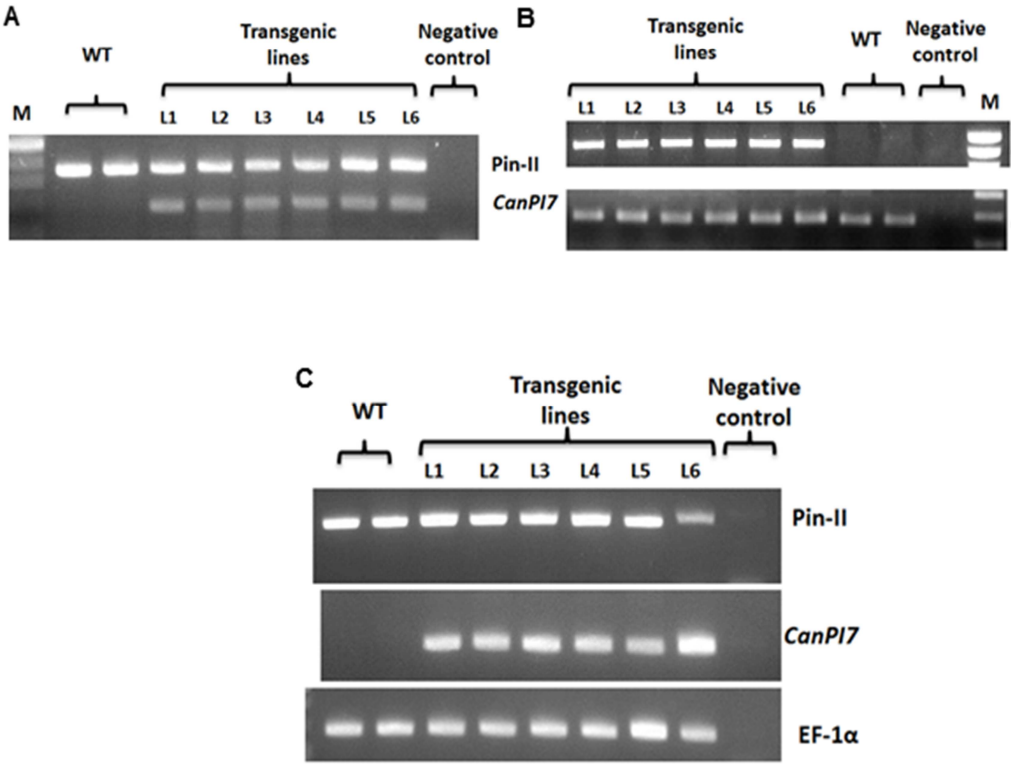


Figure 3.2.11: Molecular analysis of T1 transgenic plants (A) PCR amplification of the 342 bp and 173 bp fragment of the tomato PINII and *CanPI7* genes, respectively. Lane M: marker; Lane 1-2: DNA from WT; Lanes 3-8: DNA from transformed plants; Lane 9: negative template control (B) PCR amplification of the 1728 bp and 188bp fragments of the transgene (35S + *CanPI7*) and elongation factor (EF-1α) gene, respectively. (C) RT-PCR analysis of *CanPI7* expression from transgenic tomato and WT. Lane 1-2: cDNA from WT; Lanes 3-8: cDNA from transformed plants, EF-1α and Pin-II gene were analysed as internal control; Lane 9: negative template control.

CanPI7 transgenic lines. No amplicon was detected in the WT control plants. In addition, an amplicon of 188 bp for housekeeping gene (Actin) was generated from genomic DNA of the WT and the transgenic plants to check the quality of DNA. PCR

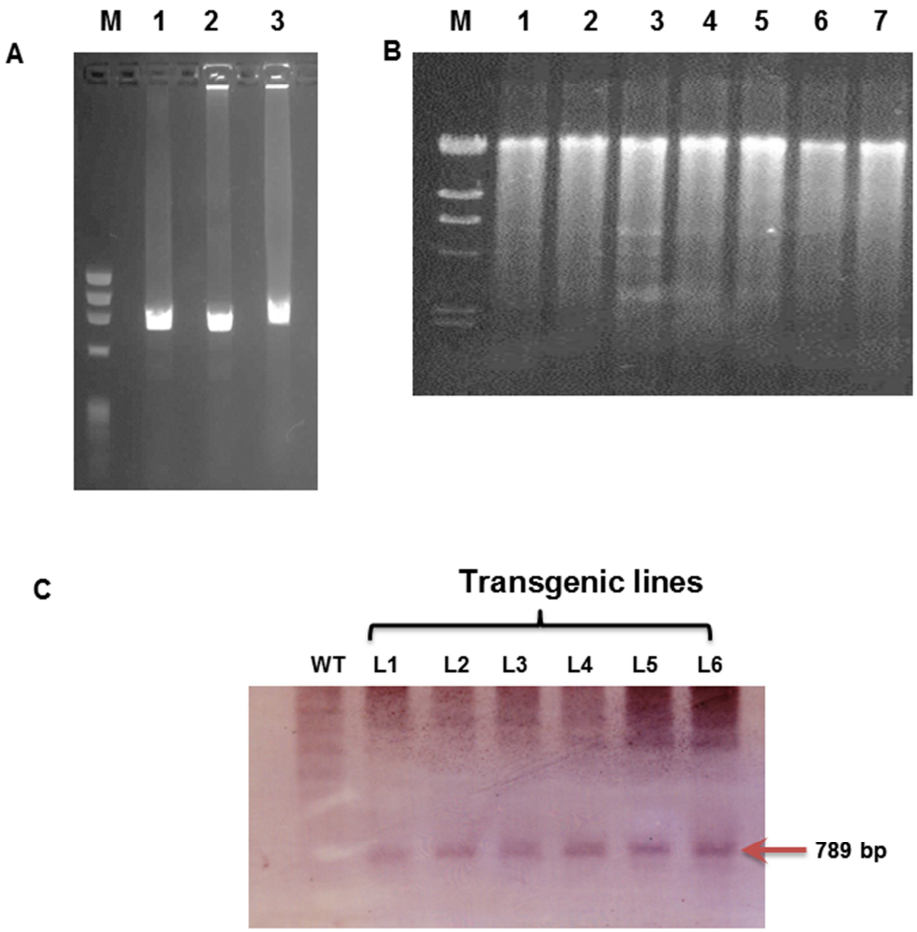


Figure 3.2.12: Southern blotting of T1 plants (A) Probe preparation M: marker L1-L2: Template for probe preparation, L3: probe (B) DNA digestion with *BamHI* and *SacI* restriction enzymes (C) Southern blot hybridization analysis of six transgenic PCR positive T1 lines. The genomic DNA (digested with *BamHI* and *SacI*) hybridized with *CanPI7* probe. Lane 1: genomic DNA from WT; Lanes 2-7: genomic DNA from six T1 lines.

analysis confirmed the presence of *CanPI7* transgene in individual transgenic lines. Then PCR positive transgenic lines were randomly selected for later screening of insect resistance. Semi-quantitative RT-PCR was performed to analyze the expression of *CanPI7* gene and tomato PIN II gene using total RNA prepared from WT and transgenic leaves. Accumulation of mRNAs of transgene was detected only in T1 plants (**Figure 3.2.11C**). Tomato PIN II (*Sly Pin-2*) and elongation factor-α (*SylEF-1*) mRNA transcripts used as reference genes, were detected in all the T1 and non-transgenic plants. The same 6 transgenic lines with *CanPI7* gene representing two independent events as explained above were selected for Southern blot analysis using genomic DNA of transgenic plants with *CanPI7* probe to determine the transgene

integration in tomato genome, which confirmed the presence of *CanPI7* gene (**Figure 3.2.12**). Detected bands were very faint in line 1 and 3. No such bands were detected in the non-transgenic WT plants.

3.2.6 Proteinase inhibitor activity assay of T1 transgenic plants shows variable inhibition of HGP activity

The inhibitory activity of protein extracts of transgenic and WT tomato leaf tissue was assayed against HGP. Transgenic plants exhibited significantly higher HGP inhibition as compared to WT (**Figure 3.2.13**). Qualitative and quantitative analysis of WT and transgenic leaves revealed that inhibition of trypsin-like activity was about 4 to 6 times higher while that of chymotrypsin like activity of HGPs was 4 to 7 times higher in transgenic leaves as compared to those in WT leaves. Line 1 exhibited maximum inhibition of chymotrypsin like activity while Line 3 indicated more of trypsin inhibitory activity. Overall, HGP inhibition was observed to be better in transgenic line 1 as compared to that of Line 3.

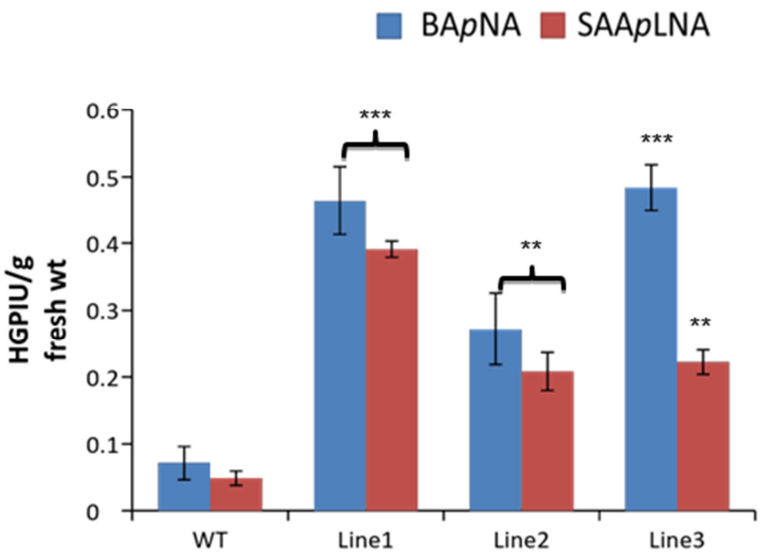


Figure 3.2.13: HGP inhibitory activity of WT and transgenic leaves (T1). Graph indicates the concentration in terms of HGP inhibitory units (HGPIUs) in WT and transgenic leaves (T1). Standard mean errors are indicated. ** and *** indicate that values are significantly different from each other at $p < 0.01$ and $p < 0.001$, respectively.

3.2.7 *CanPI7* transgenic (T1) tomato plants retard the growth and development of *H. armigera* larvae

H. armigera larvae of the first instar were fed on transgenic leaves of all the three lines separately with WT leaves as control. Representative image of larvae recovered at the end of 7th day experimental period is shown in (**Figure 3.2.14**). Insects made severe damage to WT leaves compared to the transgenic plants (**Figure 3.2.14**). After feeding for 7 days, average mass of fifteen larvae per line fed on transgenic leaves ranged from 4.4 to 9.5 mg per larva depending on line while on WT leaves it was 11 mg per larva (**Figure 3.2.15**). Larvae feeding on leaves from *CanPI7* transformed plants had significantly reduced mean larval mass at 2, 3, 4 and 6 days as compared to larvae fed on WT. Based on mass of larvae recovered in this insect bioassay experiment, larval population was distributed into various groups: 3.1-4.9 mg, 5.0-9.9 mg and 10.0-20.0 mg. Larvae from transgenic lines remained in the lowest larval mass range (3.1-4.9 mg) upto almost 6 days of feeding while entered into next range group of 5.0- 9.9 mg) on the 7th day.

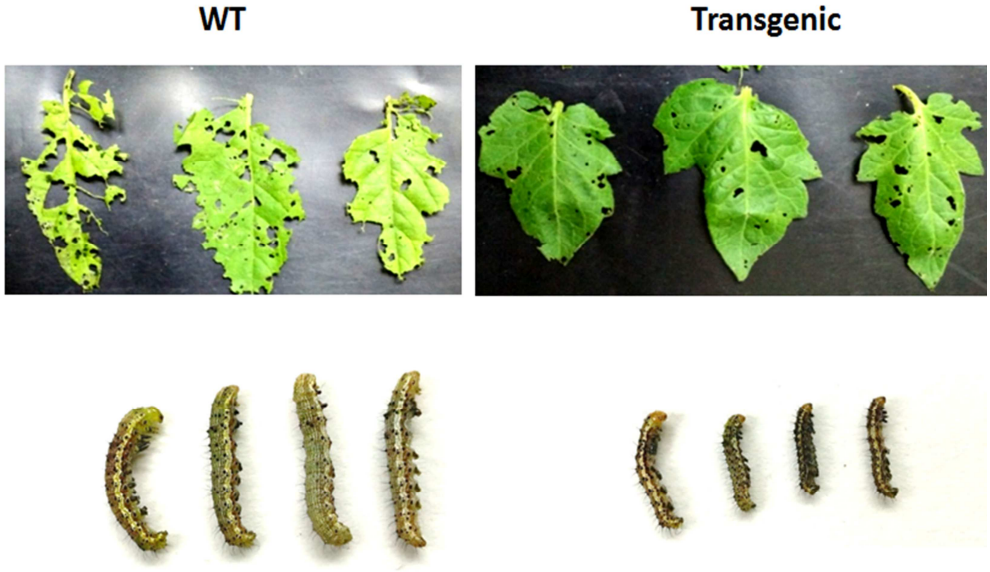


Figure 3.2.14: Representative *H. armigera* first instar larvae fed on WT and transgenic tomato plant leafs. Three independent plants were used per assay and data were obtained from the average weight (±SE) of 60 larvae per treatment from independent experiments performed in triplicate. Representative WT and transgenic tomato leaves showing damage after infestation by *H. armigera* larvae.

Most of larvae recovered from WT leaves reached to higher larval mass (>10 mg). Larval mass reduction was observed in all the three transgenic lines, however, it was

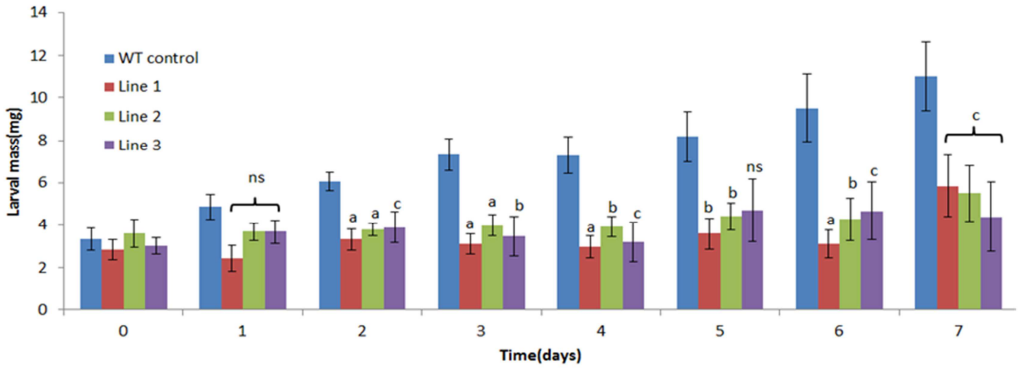


Figure 3.2.15: Larval mass of first instar *H. armigera* larvae fed on transgenic lines and WT plant leaves. Larval mass was recorded after every 24 h for 7 days. Standard mean errors are indicated. a***, b** and c* indicate that values are significantly different from each other at $p < 0.0001$, $p < 0.001$ and $p < 0.01$ respectively, 'ns' denotes non-significant.

much higher in Line 1compared to Lines 2 and 3 upto six days of feeding. On the seventh day highest reduction in mass of larvae (60%) was observed in transgenic line 3 as compared to larvae fed on WT leaves and was also more compared to line 1 and 2. However, no significant differences in larval mortality rate on transgenic and WT leaves were noted. Together, feeding experiment established that larvae fed on tomato plants expressing *CanPI7* gene under a constitutive promoter showed a significant reduction in their mass when compared with that of larvae fed on WT leaves.

3.2.8 Molecular characterization of transgenic T2 tomato plants

Seeds of T2 plants that were produced after self-pollination were germinated on selection medium. Homozygous T2 plants were selected based on analysis of their progeny resistant to kanamycin. Further, these transgenic tomato plants were examined by genomic PCR (**Figure 3.2.16**).

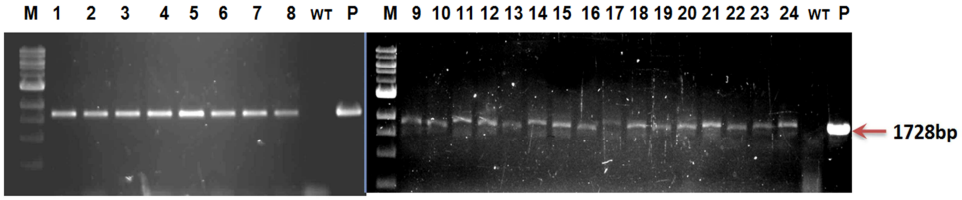


Figure 3.2.16: Molecular analysis of T2 transgenic tomato plants. PCR amplification of *CanPI7* gene (1728 bp) was carried out by using 35S forward primer and gene specific reverse primer. Lane M: 1Kb marker; Lane WT: DNA from WT; Lanes 1-24: DNA from transformed plants; Lane P: Positive control (plasmid template).

The presence of *CanPI7* transgene in putative T2 transgenic plants was confirmed by PCR analysis using 35S forward primer and gene specific reverse primer. A product of 1728 bp was amplified from genomic DNA of randomly selected transgenic lines. In addition, fragment of 188 bp for EF-1α was amplified from genomic DNA of WT and transgenic plants to check the quality of DNA. PCR analysis confirmed the presence of *CanPI7* transgene in individual transgenic lines and PCR positive transgenic lines were then randomly selected to screen for inhibition activity assay and insect resistance. No amplicon was detected in the WT control plants.

3.2.9 Proteinase inhibitor activity assay of T2 transgenic plants shows variable inhibition of HGP activity

The inhibitory activity of protein extracts of transgenic (T2) and WT tomato leaf tissue was assayed against HGP (**Figure 3.2.17**). Transgenic plants (T2) exhibited significantly higher HGP inhibition as compared to WT. Qualitative and quantitative analysis of WT and transgenic leaves revealed that inhibition of trypsin-like activity was about 5.7 to 6.1 times higher while that of chymotrypsin like activity of HGPs was 8.6 to 11 times higher in transgenic leaves as compared to those in WT leaves. Overall, HGP inhibition was observed to be better in transgenic Line 1 as compared to that of Line 2 and Line 3.

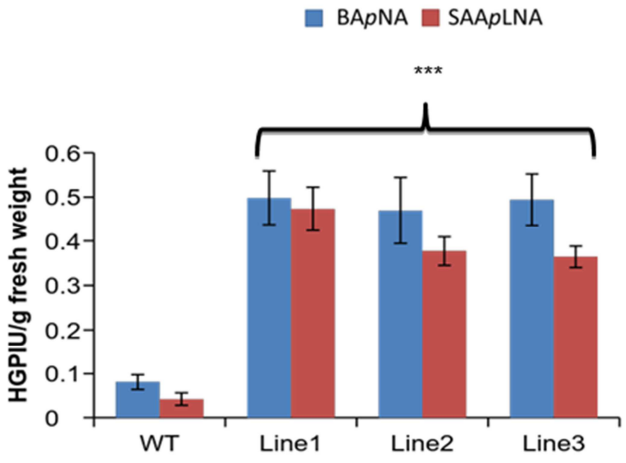


Figure 3.2.17: HGP inhibitory activity of WT and transgenic plants (T2). Graph indicates the concentration in terms of HGP inhibitory units (HGPIUs) in WT and transgenic leaves.

3.2.10 *CanPI7* transgenic (T2) tomato plants retard the growth and development of *H. armigera* larvae

H. armigera larvae of the first instar were fed on transgenic leaves of all the three lines (T2) separately with WT leaves as control. Representative image of larvae recovered at the end of 7th day experimental period is shown in (**Figure 3.2.18**). Insects made severe damage to WT leaves compared to the transgenic plants (**Figure 3.2.18**). After feeding for 7 days, average mass of fifteen larvae per line fed on transgenic leaves ranged from 9.3 to 11.7 mg per larva depending on line while on WT leaves it was 17.22 mg per larva (**Figure 3.2.19**). Larvae feeding on leaves from

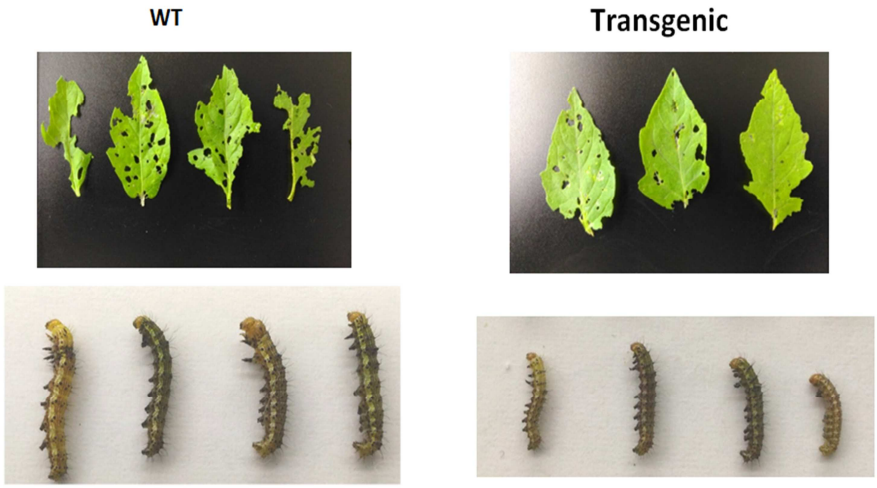


Figure 3.2.18: Representative WT and transgenic leaves (T2) showing damage after infestation by *H. armigera* larvae. Representative *H. armigera* first instar larvae fed on WT and transgenic tomato plant leafs (T2). Three independent plants were used per assay and data were obtained from the average weight (±SE) of 60 larvae per treatment from independent experiments performed in triplicate.

CanPI7 transformed plants had significantly reduced mean larval mass at 2, 3, 4, 5, 6 and 7 days as compared to larvae fed on WT. Based on mass of larvae recovered in this insect bioassay experiment, larval population was distributed into various groups: 3.1-4.9 mg, 5.0-9.9 mg and 10.0-20.0 mg in this experiment also. Larvae from transgenic lines remained in the lowest larval mass range (9.7-13.6 mg) on the 7th day. While, most of larvae recovered from WT leaves reached to higher larval mass (>15 mg). On the 7th day the highest reduction in mass of larvae was observed in transgenic Line 2 as compared to larvae fed on WT leaves and was also higher compared to Line 1 and 3. However, no significant difference in larval mortality rate on transgenic and WT leaves was noted. Together, feeding experiment established that larvae fed on

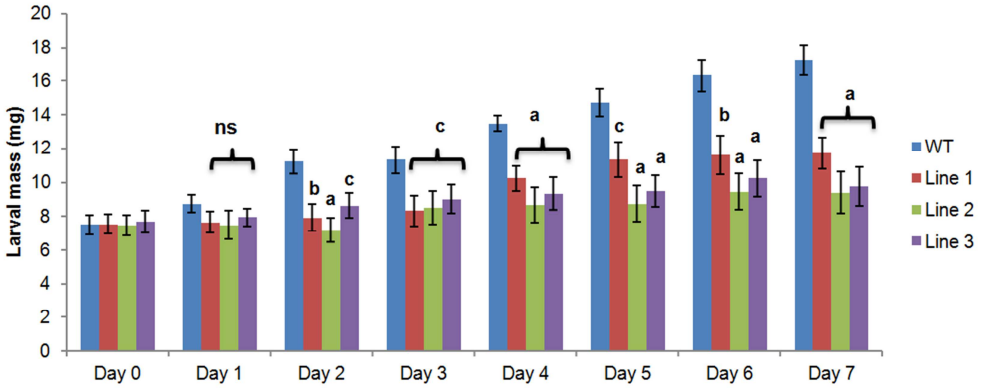


Figure 3.2.19: Larval mass of first instar *H. armigera* larvae fed on transgenic lines (T2) and WT plant leaves. Larval mass was recorded after every 24 h for 7 days. Standard mean errors are indicated. a***, b** and c* indicate that values are significantly different from each other at $p < 0.0001$, $p < 0.001$ and $p < 0.01$ respectively, 'ns' denotes non-significant.

transgenic tomato plants (T2)expressing *CanPI7* gene under a constitutive promoter showed a significant reduction in their mass when compared with that of larvae fed on WT leaves.

3.2.11 Choice assay of T2 transgenic tomato plants

Feeding-choice assays showed that *H. armigera* larvae consumed less transgenic leaf tissue than that of WT tomato (**Figure 3.2.20**). By the end of the 4th day, larvae consumed all WT tomato leaves and showed less preference for *CanPI7* transgenic leaves. Such avoidance to feeding on transgenic leaves by *H. armigera* larvae clearly indicated the increased insect resistance in transgenic plants, which deter larval feeding.

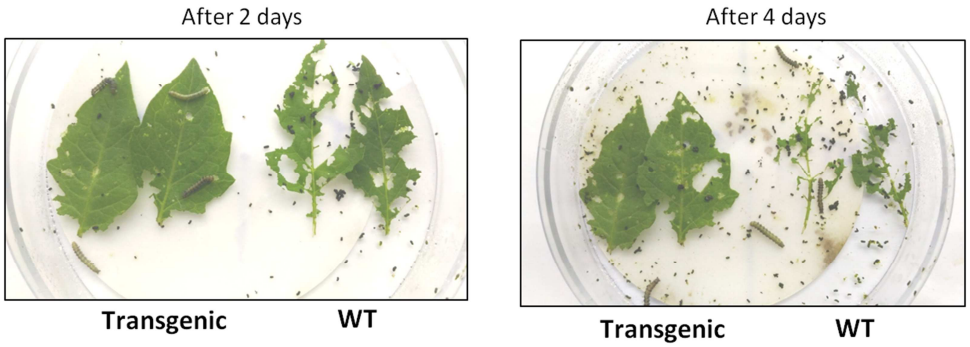


Figure 3.2.20: Performance of *H. armigera* feeding on WT tomato leaves and transgenic leaves in choice assay. Feeding assay indicated preference of *H. armigera* first-instar larvae for WT leaves over transgenic leaves for their consumption.

3.2.12 Proteome profiling of transgenic leaves

Proteome analysis of three transgenic lines (T1) and WT lines was performed as detailed in materials and methods chapter. Out of total 272 proteins identified, 25 showed differential accumulation (**Table 3.2.3**). The ratio of normalized intensity of proteins from WT and transgenic plants revealed increased or decreased expression in the leaf tissue. CanPI7 protein was detected uniquely in the range of 51 to 1435.51 fmole in the leaves of three T1 transgenic lines, while it was completely absent in WT. Enzymes related to photosynthesis such as carboxylase, aldolase, reductase and phosphopentokinase were up accumulated (1.53-6.55 fold). Similarly, enzymes involved in biosynthesis of secondary metabolites *viz*. carboxylase ammonia-lyase, transferase and aldolase and other enzymes such as, dehydrogenase (ascorbate), transferase, peroxidase, carboxylase, phosphatase adenylpyro-phosphatase, phosphatise, lactoperoxidase, pentose phosphate pathway and fructose/mannose metabolism enzymes were accumulated (1.53-5.8 fold and 1.5 to 2 fold, respectively) as compared to WT plants. Ammonia-lyase involved in amino acids, glycine, serine, threonine, valine, leucine and isoleucine metabolism was also accumulated up to 5.8 fold. Other proteins such as glutathione peroxidase and heat shock protein (Hsp70) were highly upregulated in transgenic plants. However, superoxide dismutase enzyme of phenylpropanoid biosynthesis was down regulated by 3.7 fold. Other proteins such as cysteine proteinase, eukaryotic translation initiation factor, profilin and plastocyanin were also lower in transgenic plants as compared to WT. Moreover, defensin protein was uniquely detected in only transgenic plants. All other proteins in transgenic and WT plants did not reveal any change.

3.2.13 Metabolome profiling of transgenic leaves

UPHPLC-based global metabolic profiling of leaf WT and six transgenic tomato plants (T1) were performed. Reproducibility and stability of system were validated using various parameters before the samples were measured. Stability of the system was based on the retention times (RT), peak areas and intensity coefficient variation of Leu (0.148) and Ile (0.145) in all the QC samples. Mass accuracy was ~2 ppm during data acquisition. These results indicated that stability and reliability of system were qualified for analysing the samples for data acquisition. Principal component analysis (PCA) which is was employed on whole metabolic data set to observe a

Table 3.2.3: LC-MS^E identification of differentially expressed proteins in T1 transgenic tomato plants compared to WT plants.

Accession No.	Description	MW (Da)	p*I* (pH)	PLGS Score	Pepti-des	Theoreti-cal Peptides	Cover-age (%)	Digest Peptides	Fold change
Up-regulated proteins									
K4BE01	Chlorophyll a-b binding protein	28287	5.33	8525.88	9	13	61.79	9	6.55
P25306	Threonine dehydratase	64896	5.07	17945.4	32	41	60.67	27	5.81
K4BE00	Chlorophyll a-b binding protein	28301	5.33	7578.81	7	13	56.17	7	5.1
P07370	Chlorophyll a b binding protein 1B	28054	4.97	5282	6	13	54.7	6	5
K4B876	Chlorophyll a-b binding protein	28024	4.97	29997.76	7	13	52.45	6	3.89
K4B6A3	Ferredoxin NADP reductase	40553	8.63	3418.19	8	33	29.55	8	2.27
Q40151	Hsc70	71470	4.98	1531.59	13	52	27.18	13	2.24
H1ZXA9	Heat shock protein 70	71345	4.94	2431.8	14	52	31.95	14	2.24
K4CVX2	Proteinase inhibitor i	12572	8.24	8262.21	5	12	72.97	5	2.11
K4D9L5	Heat shock cognate 70 kda protein 2-like	77093	5.35	2408.16	20	55	34.24	17	2.054
K4CR90	Heat shock cognate 70 kda protein 2-like	71180	4.94	2035.17	13	52	36.05	13	2.01
H1ZXA8	Heat shock protein 70 isoform 2	70742	4.88	1801.31	15	51	31.21	14	1.97
K4B6C3	Fructose bisphosphate aldolase	42643	6	2008.93	9	28	37.46	9	1.93
Q5NE20	Carbonic anhydrase	34445	6.73	8582.78	12	32	47.04	12	1.89
K4CVX5	Wound-induced proteinase inhibitor	12358	7.02	3745.2	4	10	56.36	4	1.87
K4D489	Rubisco	49206	8.68	25990.89	27	38	63.37	22	1.78
K4CMY9	Phosphoribulokinase	44393	5.92	12420.6	18	31	56.5	15	1.71
K4BLT6	Rubisco	49883	5.64	1333.84	6	42	15.61	6	1.68
K4DFV4	Glutathione peroxidase	19271	4.94	6838.47	8	17	52.94	8	1.61
Down- regulated proteins									

Q40143	Cysteine proteinase 3	38919	8.33	13706.1	8	21	9.83	3	3.12
Q7XAV2	Superoxide dismutase Cu Zn	22268	6.03	28625.86	6	11	38.70	4	3.70
K4DBU7	Profilin	10806	5.81	1549.28	2	6	50	2	2.27
P17340	Plastocyanin chloroplastic	16981	4.87	90513.91	8	10	40.58	5	2.27
Uniquely detected in transgenic plants									
B1N678	Defensin	8439	8.5327	6477.4	2	5	22.368	2	
Q4ZIQ5	CanPI7	28501	6.1011	12245	9	17	53.435	8	

potential clustering behaviour and pattern in metabolite data set. Unit variance scaled PCA score plots showed fairly clear differences between WT and transgenic leaf tissue, indicating significant changes in their metabolite profiles. PCA trajectory plots

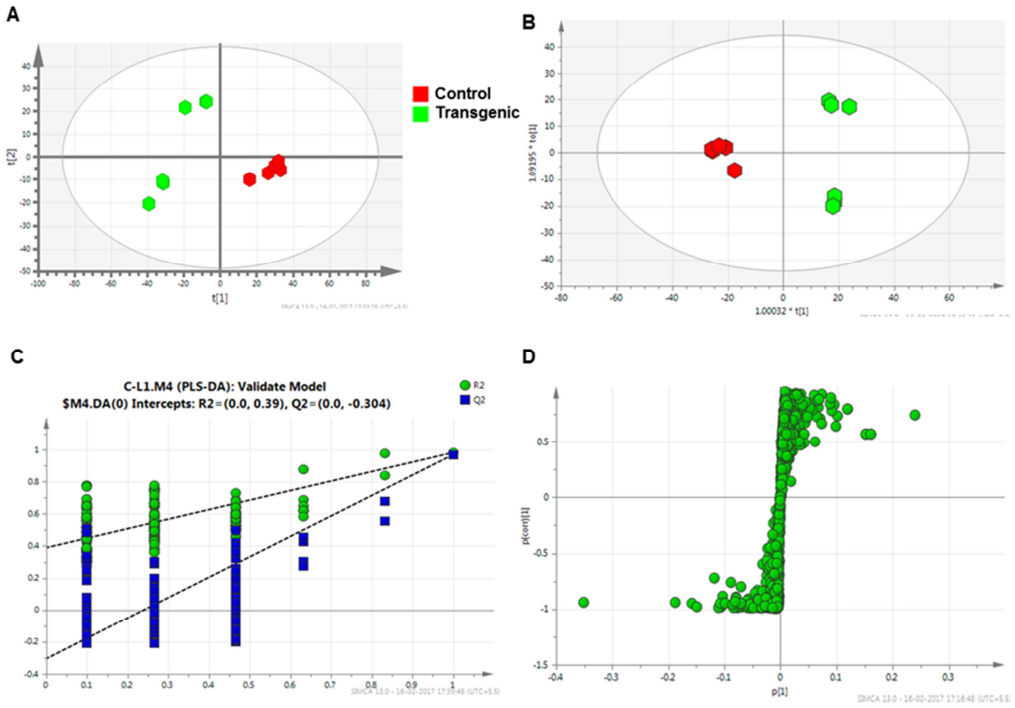


Figure 3.2.21: (A) Comparison via PCA considering all samples of WT and transgenic plants (B) Comparison via OPLS-DA considering all samples of WT and transgenic plants (C) Permutation test results for OPLS-DA models with two components and 200 permutations. (D) S-plot obtained from metabolic profiles considering all samples.

Table 3.2.4: Differentially accumulated metabolites identified from UPHPLC in transgenic tomato plants.

Metabolites	Formula	Mass	Fold change	Pathway
Up- accumulated				
L-Malate	$C_4H_6O_5$	134.02	2.70	TCA cycle
Isocitrate	$C_6H_8O_7$	192.02	6.14	TCA cycle
L-Glutamate	$C_5H_9NO_4$	147.05	2.43	Amino acid
D-Glucarate	$C_6H_{10}O_8$	210.03	1.98	Pentose and glucuronate
3-(3'-Methylthio)propylmalic acid	$C_8H_{14}O_5S$	222.05	3.71	Glucosinolate
D-Glucuronate	$C_6H_{10}O_7$	194.04	2.74	Ascorbate and aldarate
2,5-Dioxopentanoate	$C_5H_6O_4$	130.02	6.62	Pentose and glucuronate
4-Aminobutanoate	$C_4H_9NO_2$	103.06	3.38	Alanine, aspartate and glutamate
3-Phosphonooxypyruvate	$C_3H_5O_7P$	183.97	8.38	Calvin cycle
Down- accumulated				
Tomatidine	$C_{27}H_{45}NO_2$	415.34	0.25	Secondary metabolism
Solasodine	$C_{27}H_{43}NO_2$	413.32	0.18	Secondary metabolism
1,2-Dihydroxy-5-(methylthio)pent-1-en-3-one	$C_6H_{10}O_3S$	162.03	0.04	Cysteine and methionine
2-Aminobenzoate	$C_7H_7NO_2$	137.0	0.44	Tryptophan
2-Hydroxy-2,4-pentadienoate	$C_5H_6O_3$	114.03	0.23	Phenylalanine
Glutathione	$C_{10}H_{17}N_3O_6S$	307.0	0.11	Cysteine and methionine
L-Phenylalanine	$C_9H_{11}NO_2$	165.07	0.22	Amino acid
Indole-3-acetaldehyde	$C_{10}H_9NO$	159.06	0.20	Tryptophan
Serotonin	$C_{10}H_{12}N_2O$	176.09	0.20	Tryptophan
2-Hydroxy-2,4-pentadienoate	$C_5H_6O_3$	114.03	0.30	Phenylalanine
Chlorogenic acid	$C_{16}H_{18}O_9$	354.09	0.05	Phenylpropanoid, Flavonoid
5-O-Caffeoylshikimic acid	$C_{16}H_{16}O_8$	336.08	0.05	Phenylpropanoid, Flavonoid
Rutin	$C_{27}H_{30}O_{16}$	610.15	0.10	Flavone and flavonol
Quercetin	$C_{15}H_{10}O_7$	302.04	0.10	Flavone and flavonol

clearly illustrated differential pattern with significant metabolic deviations (**Figure 3.2.21**). Orthogonal partial least squares-discriminant analysis (OPLS-DA) model was

generated for WT and transgenic plants. OPLS-DA score plot revealed complete separation between WT and transgenic plants with two distinguished clusters. Moreover, permutation tests with 200 iterations further confirmed that OPLS-DA model was robust. Metabolic variables with high variable importance in projection (VIP >1) and influence on loading were used for further analysis. Jack-knifed-based confidence interval was used to remove variables with low reliability. S-plot from above generated model between WT and transgenic genotypes identified key intermediate metabolites of various biosynthesis pathways such as pentose and glucuronate, ascorbate and alderate, glucosinolate, secondary metabolism, flavonoid, flavone, flavonol, TCA cycle, amino acid biosynthesis and Calvin cycle (**Table 3.2.4**). A marked increase in level of L- malate and isocitrate of TCA cycle was apparent in transgenic plants compared to WT. Additionally, it was observed that L-glutamate was upregulated in transgenic plants. Various metabolites from glucosinolate, ascorbate and aldarate pathways were also upregulated in transgenic plants. Increase in the level of D-glucarate and 2,5-dioxopentanoate of pentose and glucuronate pathway in transgenic compared to WT was apparent. Secondary metabolites such as tomatidine and solasodine decreased in transgenic plants, compared to WT plants. Further, transgenic plants showed reduction in the levels of metabolites related to amino acids metabolism such as 1,2-Dihydroxy-5-(methylthio) pent-1-en-3-one, 2-aminobenzoate, 2-Hydroxy-2,4-pentadienoate, glutathione, L-phenylalanine, Indole-3-acetaldehyde and serotonin. Chlorogenic acid and 5-O-Caffeoylshikimic acid of phenypropanoid and flavonoid pathway were downregulated. Similarly, two of the metabolites from flavone and flavonol metabolism, such as rutin and quercetin were down regulated.

Section 3.3 Characterization of single domain *CanPIs* (IRDs) in recombinant yeast against abiotic stress

3.3.1. Ectopic expression of IRDs confers tolerance towards different stresses

Stress tolerance of $PpIRD^{+}$ strains was evaluated in serial dilution tests, as showed in **Figure 3.3.1**. Yeast cell with an empty vector (EV) showed susceptibility to elevated salt, heavy metal and oxidative stress as significant growth retardation was marked (**Figure 3.3.1A**). $PpIRD^{+}$ strains containing either IRD 7 or 9 or 12 of CanPIs appeared to be highly tolerant to various abiotic stress inducing agents. Moreover,

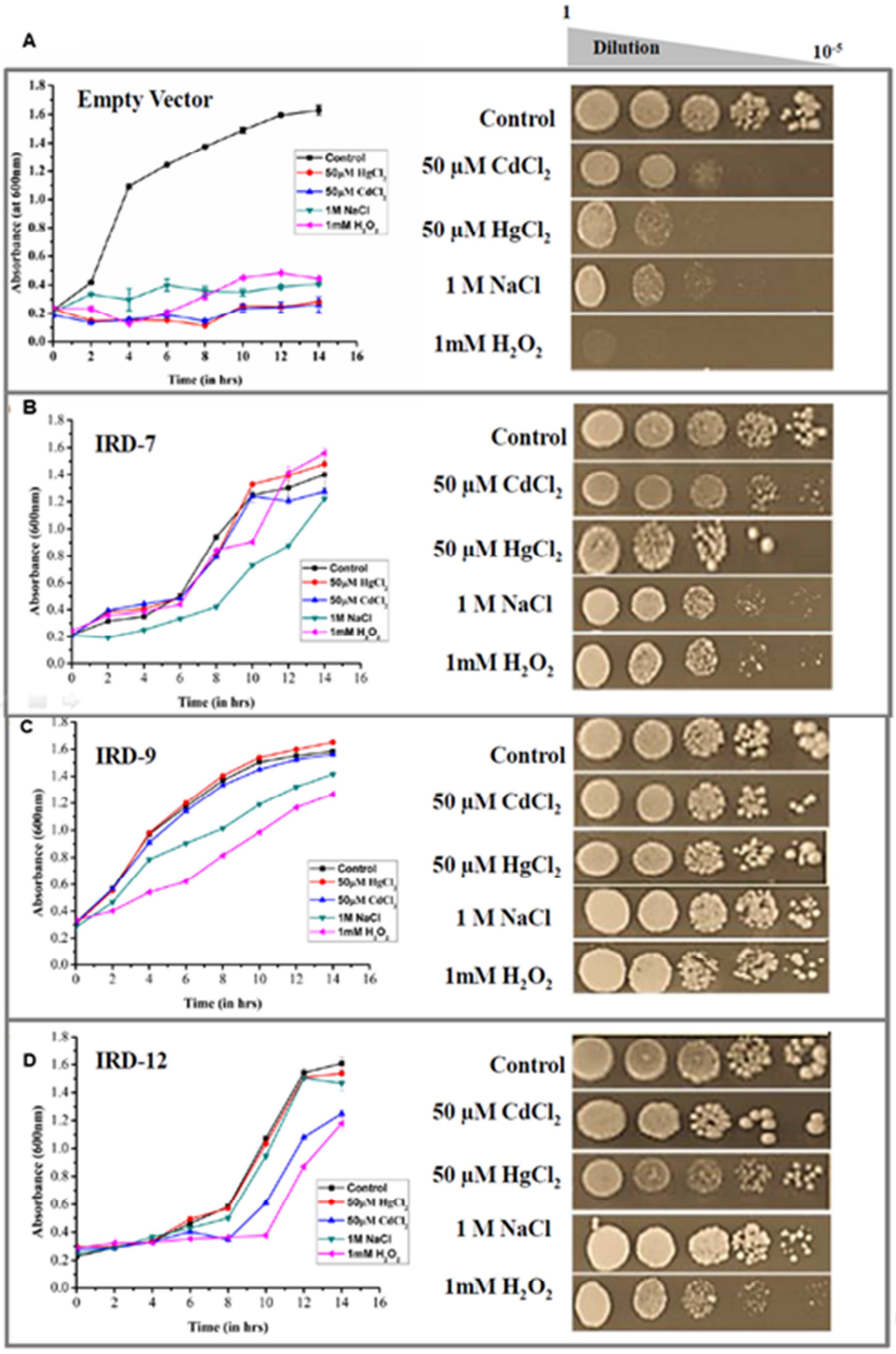


Figure 3.3.1: Growth of yeast cells expressing (A) empty pPIC9 vector (EV) and IRDs namely (B) IRD-7, (C) -9, (D) -12 in yeast extract peptone dextrose (YPD) broth and plate supplemented with 50 µM $CdCl_2$, 50 µM $HgCl_2$, 1 M NaCl and 1 mM H_2O_2 stress.

PpIRD-9^{+} strain (**Figure 3.3.1C**) containing IRD 9 showed higher tolerance to all the stresses than those in IRD-7 (**Figure 3.3.1B**) and -12 (**Figure 3.3.1D**). Previous study from our laboratory has also shown that IRD-9 had higher protease inhibition potential as compared to IRD-7 and -12. In growth curve analysis (14 h), IRD-9 showed faster rescue of the phenotype analogous to that of the control condition under all the stress conditions. In case of PpIRD-7^{+} and -12^{+} strains, initially growth was retarded up to 4 h, indicating as extension of the lag phase under stress condition; later the cells were adapted to stress condition. Stress adaptation resulted in the normal growth of yeast cells in the exponential phase. These results clearly demonstrated that expression of PIs provided endurance against various abiotic stresses.

3.3.2. Enhanced expression and trypsin protease inhibition (TPI) activity in PpIRD^{+} strains

Gene expression analysis of CanPI-IRDs in PpIRD^{+} showed that all the three IRDs (IRD-7, -9 and -12) overexpressed as compared to EV strain under stress inducing

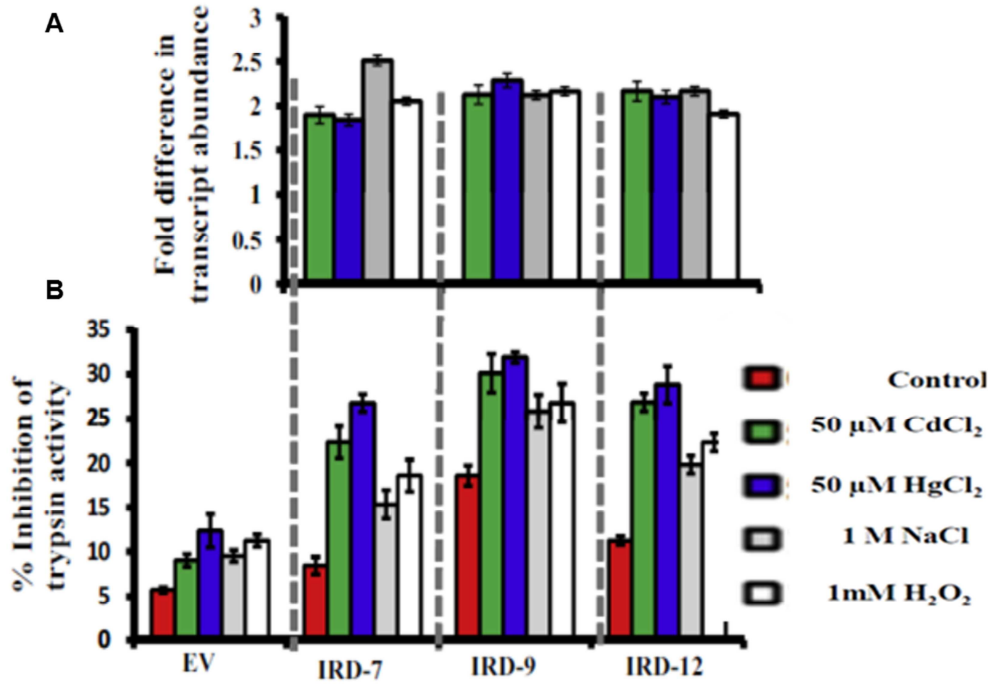


Figure 3.3.2: Protease inhibitor and protease gene expression and activity of *CanPI* expressing yeast cells under stress. (A) Fold difference in transcript abundance of IRDs; (B) trypsin inhibitory activity.

conditions (**Figure 3.3.2A**). Parallel to this transcriptional activation, intracellular TPI activity was also observed to be significantly higher in PpIRD^{+} strains after stress inducing agents' treatment as compared to EV strain (**Figure 3.3.2B**). Cell lysate of IRD-9 expressing strain showed maximum protease inhibitor activity as compared to IRD-7 and -12 expressing *P. pastoris* strains under all the stress conditions. Further as pPIC9 has alpha secretion tag before IRD sequence, extracellular TPI activity of one of the three IRDs, i.e. IRD-9 was tested and found to be higher as compared to its intracellular activity (**Figure 3.3.3**).

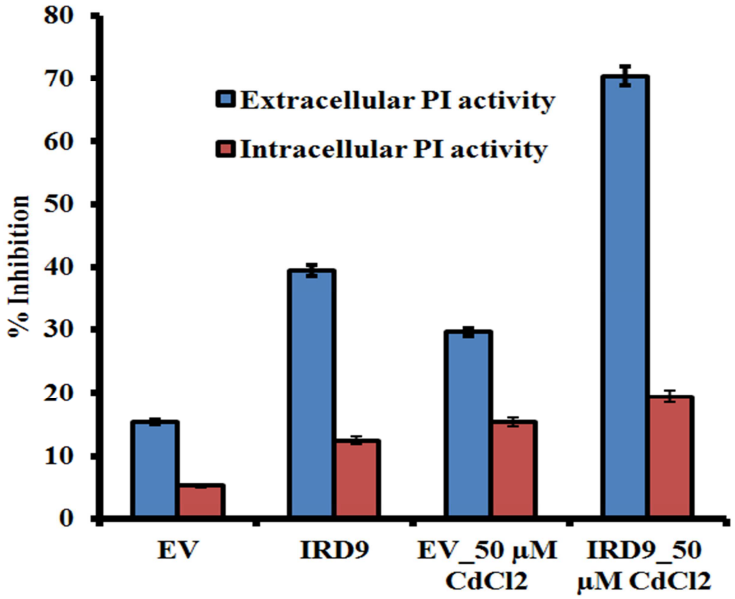


Figure 3.3.3: Extracellular and intracellular trypsin PI activity of EV and IRD9_PpIRD^{+} yeast cells under 50 µM $CdCl_2$ stress. Significant inhibition of trypsin activity was observed in extracellular media (50 µg of protein) of IRD9_PpIRD^{+}, which was indication of secretion of overexpressed PIs. While, before reaching to level of extracellular secretion considerable amount of PI is expressed inside cell and thus gives residual inhibitory activity of cell lysate (50 µg of protein). Values are the means of three measurements with standard error.

Before reaching to level of extracellular secretion, considerable amount of PI probably expressed inside cell that may gave residual intracellular inhibitory activity of cell lysate (50 µg of protein). Similarly, PpIRD^{+} strains showed enhancement of PI activity on stress stimuli, which might inhibit the cellular proteases which are involved in protein degradation under stress.

3.3.3. Inhibition of serine (SP) and cysteine protease (CP) activities in PpIRD$^+$ strains

In stress conditions, the gene expression and ultimately activity of SP and CP increased rapidly in EV cells as compared to the control condition (**Figure 3.3.4A** and **B**). Overexpression of subtilisin-like protease 3 and calpain-like protease transcripts was observed in PpIRD$^+$ strains (**Figure 3.3.4A**). However, at activity level no significant difference in SP and CP activities was observed in stress inducing agent treated cells as compared to the control cells. EV strains showed elevated level of SP and CP activity, while PpIRD$^+$ cells showed optimal level of protease. This inhibition of SP activity might be due to SP inhibitor. However, the cross reactivity of IRDs toward inhibition of CP might have influence on regulation of CP expression and activity in PpIRD$^+$ strains under stress (**Figure 3.3.4B**).

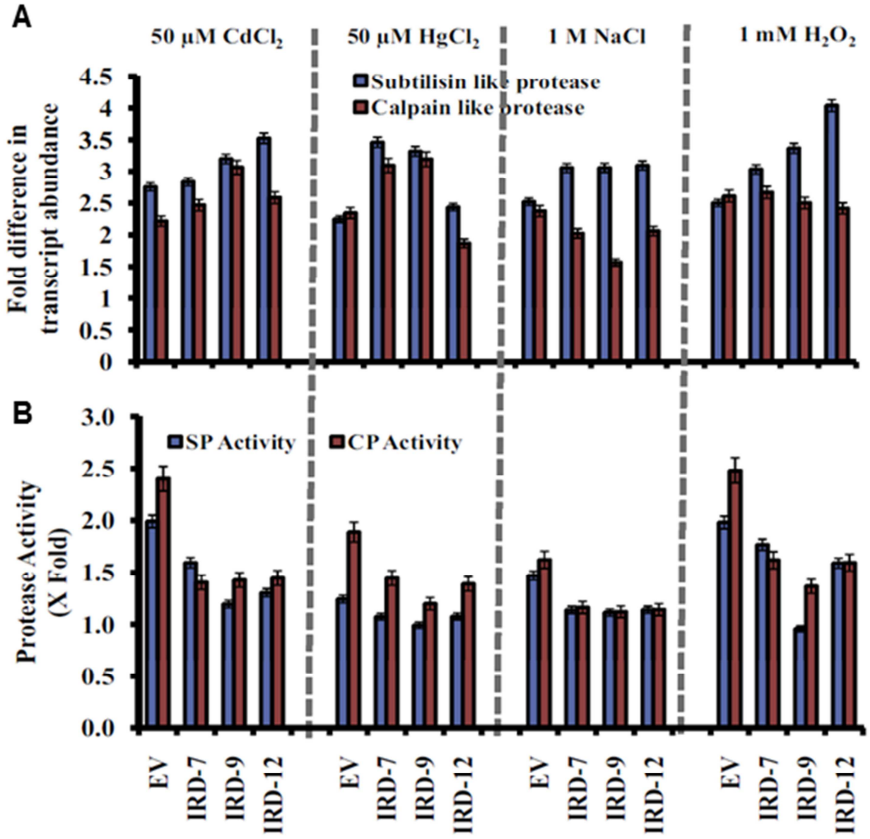


Figure 3.3.4: (A) Fold difference in transcript abundance of subtilisin like and calpain like protease and (B) serine and cysteine protease activities in yeast cells expressing IRD-7, -9 and -12 under stress inducing agents like 50 µM $CdCl_2$, 50 µM $HgCl_2$, 1 M NaCl and 1 mM H_2O_2 compared to control condition. Values are the means of three measurements with standard error.

3.3.4. PpIRD$^+$ exhibits reduced metacaspase activity under stress conditions

The metacaspase mRNA level was markedly elevated in EV and PpIRD$^+$ strains under controlled condition. Similarly, the metacaspase expression level appeared to be stimulated also by stress-inducing agents (**Figure 3.3.5A**). Metacaspase specific substrate was further used to assess the level of metacaspase activity in EV and PpIRD$^+$ under various stress conditions. PpIRD$^+$ stains showed 50–60% reduction in metacaspase activity as compared to EV strain (**Figure 3.3.5B**). This indicated that under stress condition the metacaspase activity was inhibited by the induced expression of IRDs.

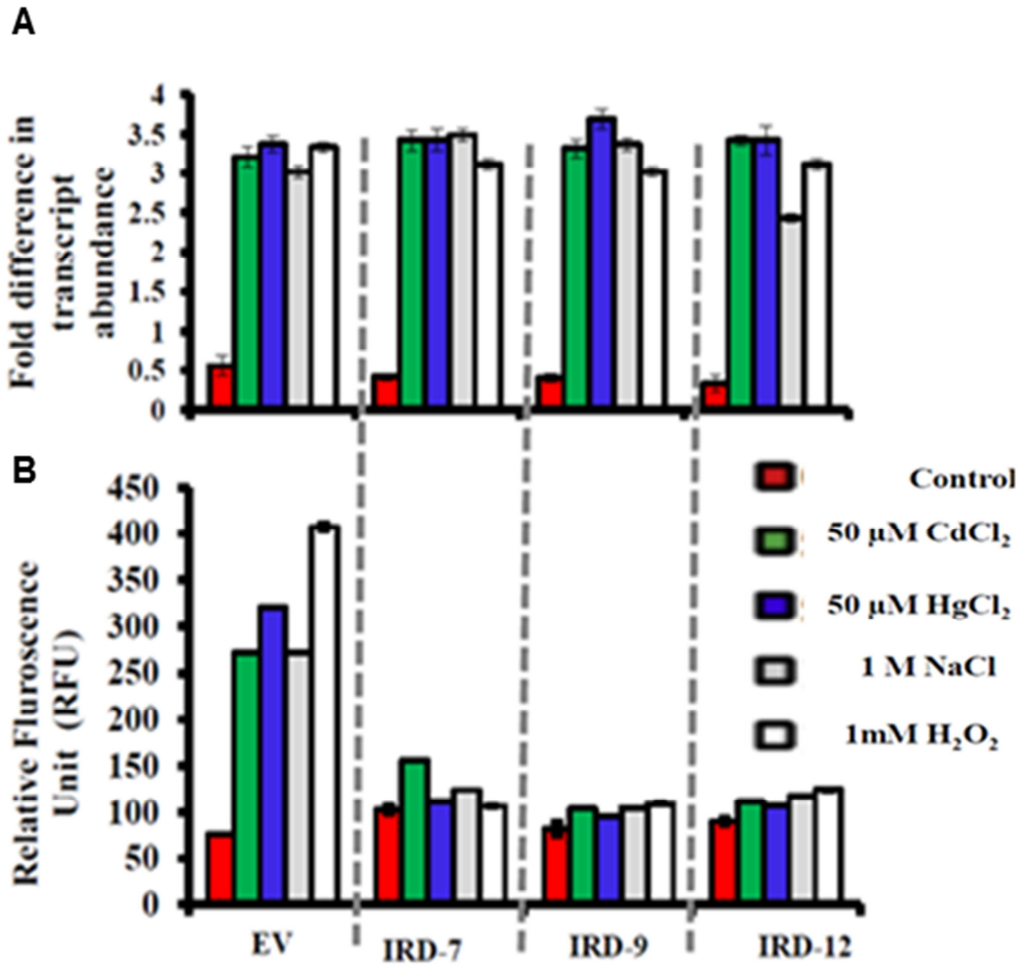


Figure 3.3.5: (A) Metacaspase gene expression and (B) intracellular metacaspase activity of *CanPI* expressing yeast cells under stress inducing agents like 50 µM $CdCl_2$, 50 µM $HgCl_2$, 1 M NaCl and 1 mM H_2O_2. Values are the means of three measurements with standard error.

3.3.5. Reactive loop of IRDs forms multiple contacts with the active site of metacaspase (Yca1)

Comprehensive view of IRD-metacaspase complex showed that IRD was bound at the active site region of metacaspase and blocked the access of the substrate to the active site (**Figure 3.3.6A-C**). Close view of this interaction depicted the formation of a hydrogen bond in the reactive loop of IRDs (CPXXC) and the active site of metacaspase (His220 and Cys276). In IRD-7 and -9, carbonyl oxygen atoms of Asn40 of IRD reactive loop formed hydrogen bond with metacaspase active site residue i.e. His220 (**Figure 3.3.6 A** and **B**). In case of IRD-12 and metacaspase complex, there

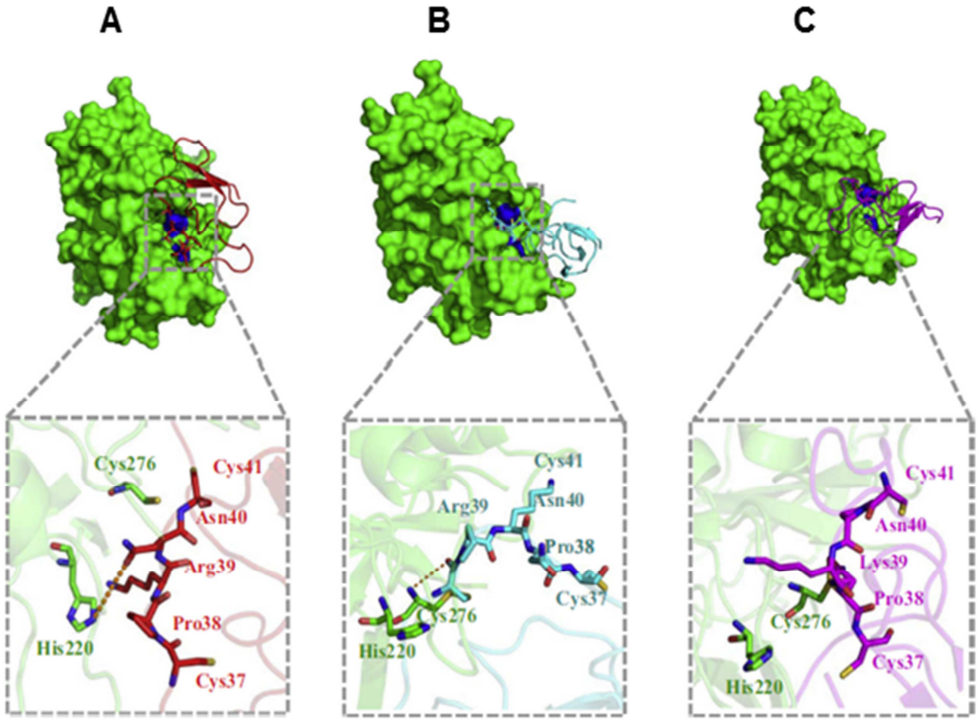


Figure 3.3.6: Interaction between the active site of yeast metacaspase (Yca1) and reactive loop of IRD evaluated by docking analysis. Reactive loop of (A) IRD-7, (B) IRD-9 and (C) IRD-12, showed close interaction with metacaspase active site residues (His220 and Cys276). In case of IRD-7 and IRD-9, Arg39 of reactive loop forms hydrogen bond with His220 and Cys276, respectively.

was absence of polar contact in interacting regions (**Figure 3.3.6C**). IRD-9 showed stronger binding to metacaspase with energy of 32 kcal/mol as compared to IRD-7 (18 kcal/mol) and IRD-12 (19 kcal/mol). Results derived from energy calculations were in well accordance with our earlier reports (Joshi et al., 2014b). This indicated that interaction of IRD with metacaspase was similar to IRD-trypsin interaction.

Substrates with arginine/lysine residue at the P1 position could act as superior and specific substrates for metacaspase. The S1-S3 position of binding pocket of Yca1 constituted negative environment and showed enhanced binding affinity towards positively charged amino acids. The reactive loop of IRDs contained arginine/ lysine (positively charged) residue at the P1 position, thus increasing the reactivity and specificity of IRDs toward Yca1. Yca1-IRDs complex showed enzyme-substrate like binding with relatively higher binding energy. Docking studies provided supportive indication of the interaction between two heterogeneous partners, Yca1 and IRDs.

3.3.6. Metacaspase knockout *S. cerevisiae* strain (ΔYca1) shows PpIRD$^+$ like growth characteristics and maintenance of GAPDH activity in PpIRD$^+$ strains

To examine the effect of various stresses on the growth of ΔYca1 cells, the time-dependent changes in optical density (OD_{600}) of culture were measured subjected to various stresses. As shown in **Figure 3.3.7A**, growth of ΔYca1 strain was delayed

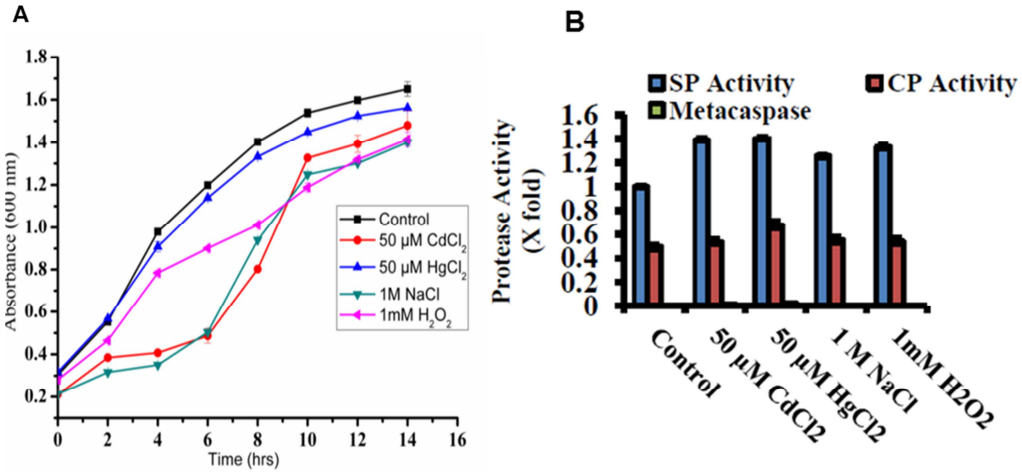


Figure 3.3.7: (A) Growth of metacaspase knockout yeast cell (ΔYca1) in the YPD medium supplemented with 50 µM $CdCl_2$, 50 µM $HgCl_2$, 1 M NaCl and 1 mM H_2O_2. (B) Intracellular serine protease and metacaspase activity in ΔYca1 cell under stress.

during the first 2 h of exposure to stress inducing agents, but it started to increase after 3 h, the density of the ΔYca1 culture was higher and almost alike of those observed in the absence of stress. Growth pattern of the ΔYca1 strain was comparable like PpIRD$^+$ strains, specifically more similar with growth of IRD-9 expressing yeast cell

under stress. Assessment of various protease activities showed that SP activity was significantly increased after stress treatment, while CP activity was marginally increased. Metacaspase activity was almost absent in all the different stress conditions (**Figure 3.3.7B**).

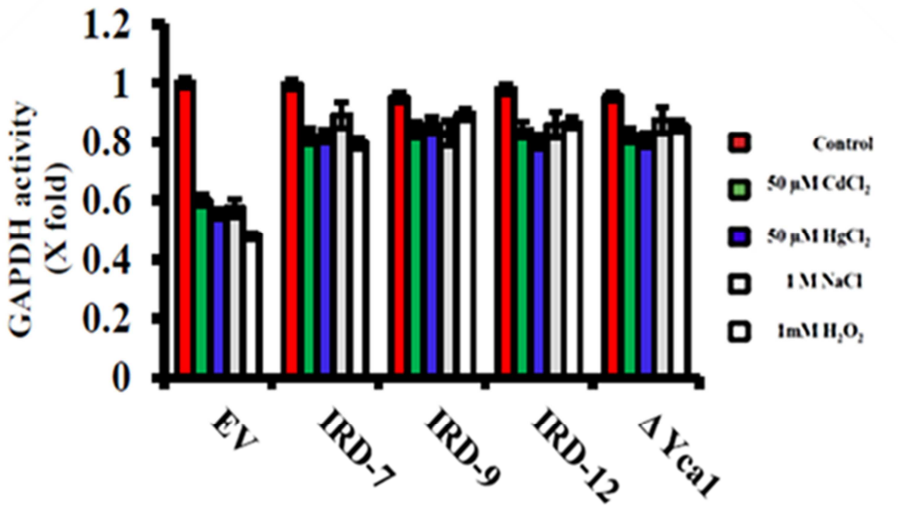
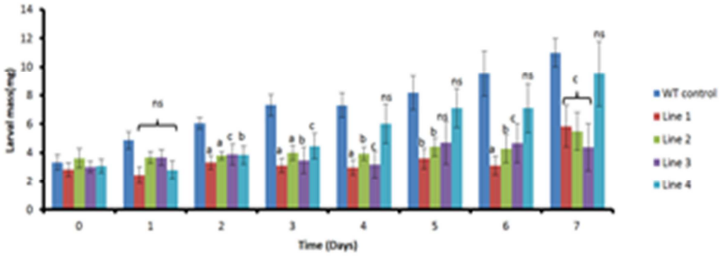


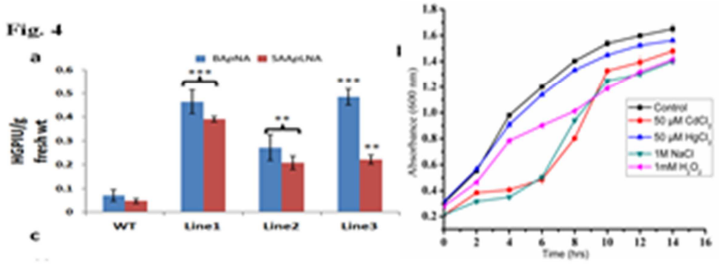
Figure 3.3.8: GAPDH activity of EV, PpIRD^{+} (IRD-7, -9 and -12) and ΔYca1 strain under multiple stresses. Values are the means of three measurements with standard error.

It was observed that ΔYca1 and PpIRD^{+} strains showed maintenance of GAPDH activity under various stresses, while there was considerable reduction (50%) in this activity in the case of EV strains (**Figure 3.3.8**).

Chapter 4

Discussion





Rapid and broad spectrum development of molecular techniques for agricultural exploitation has been mainly observed from the last three decades. The database built using these techniques is being employed to improve the quality and yield of various crop plants. Molecular studies on plant resistance mechanisms allowed the identification of genes whose manipulation could improve plant resistance to insect pests. Analysis of natural variation at all levels of biological organization, i.e. genetic, transcriptomic, proteomic and metabolomic, has become possible due to recently developed technologies. Utilizing this information, genetic engineering has further become a reasonable solution to increase plant resistance against many biotic and abiotic stresses as well as to improve food quality and yield. New transformation technologies using targeted/ untargeted approaches may also aid to unravelling the mechanism(s) behind resistance mediated by new genes. Furthermore, an examination of plants resistant to different insects allow the identification of genes, which are crucial for plant resistance to pests and can be potential targets for developing transgenic plants. Thus, plant transformation technologies have radically changed biological research and have a major impact on crop improvement towards biotic and abiotic stress resistance.

In the present work, an attempt has been made to highlight functional analysis of proteinase inhibitor from *C. annuum* (*CanPI*) using transgenic approach. For this, *Agrobacterium*-mediated transformation was carried to produce transgenic *Arabidopsis* and tomato plants. Furthermore, model yeast system was used to functionally characterize single domain *CanPIs* (IRDs) for abiotic stress tolerance. The inferences drawn in our studies can be correlated, validated or extrapolated for other transgenic studies prevalent in rest of the world. The results are discussed in this chapter in the context of the available literature and their potential utility in crop improvement through transgenic approach.

Section 4.1: Transgenic *Arabidopsis thaliana* with multidomain *CanPI7* shows better insect tolerance than that with single domain *IRD7*

Many plant species have a long life cycle and are difficult for genetic transformation, which makes it time-consuming to produce transgenic lines for functional characterization of genes. *A. thaliana* have being used as model plant for most of molecular studies focussing on plant responses to herbivorous insects. Results derived from *A. thaliana* might be helpful for understanding plant resistance mechanism against insects in other plants considering assumption that genes involved and the resistance mechanisms are conserved among species (Salentijn et al., 2007). *Arabidopsis* is not of agricultural important crop but have been extensively used as a model plant due to its user-friendly features, such as relative small genome size and short generation time (Van Poecke and Dicke, 2004). Furthermore, many important tools, such as single-nucleotide polymorphism arrays, full-genome microarrays and wide mutant collections, are available to enable molecular studies (Meinke et al., 2003; Clark et al., 2007). Columbia -0 is the most commonly used *A. thaliana* variety in plant-insect interaction studies and serves as an appropriate host to several herbivore species (Van Poecke et al., 2007). Till date, so far no strong sources of resistance have been observed in *A. thaliana*, however variation in resistance to insect herbivores has been reported among different *Arabidopsis* accessions (Snoeren et al., 2010; Todesco et al., 2010).

Pin-II proteinase inhibitors (PIs) are the focus of this research because of their large structural-functional diversity and relevance in plant defense. Heterologous PI genes expressed in some other plants has led increased resistance to a varied range of insect pests (Abdeen et al. 2005; Quilis et al. 2014; Schneider et al. 2017). The effectiveness of PIs against other pathogens such as bacteria, fungi and nematodes has also been reported. Up-regulated yet specialized PI expression upon wounding and insect infestation provides insights into the evolution of PI based plant defence mechanisms against insects. PIs, being an innate part of the plant defense system for protecting them from insects, fungi and bacteria, exhibit enough variability to interact with a wide range of proteases they come across. However, insects have also been shown to be adequately adaptable to the existence of either endogenous PIs of their host plants or transgenic PIs. Thus, discovery and characterization of novel PIs to address induced and inherent complexity of insect gut proteinases is obligatory.

Keeping this aspect in view, PIs derived from non-host plants containing multidomains of inhibitors active against variants of proteinases could be a more effective approach to provide sustainable crop protection against insect pests.

In earlier reports from our lab it was shown that *C. annuum* possesses an array of Pin-II *PI* (*CanPIs*) genes ranging from 1- to 4- IRD. *CanPIs* are known for their differential patterns of expression in various tissues. *CanPIs* displaying high isoforms diversity with PIs of 1- to 4-IRDs, have been isolated and characterized to assess their defense potential against Lepidopteran proteases. Since *H. armigera* has limited exposure to *C. annuum*, this insect is less likely to have developed resistance to *C. annuum* proteinase inhibitor. Further studies have also revealed the induced accumulation of multi-IRD *CanPIs* in leaves in response to mechanical wounding and insect infestations (Tamhane et al., 2009). Varying inhibition of trypsin, chymotrypsin, elastase and total gut proteinase activities of Lepidopteran insects using single domain (IRD7) and multi-domain CanPI7 (4-IRD) was previously reported from our laboratory (Tamhane et al. 2007b; Joshi et al., 2014a, b). Moreover, structure prediction and docking studies of IRD7 alone with proteases were also performed to exhibit its interaction with target protease (Joshi et al., 2014a).

In the present study, structure prediction and docking studies of CanPI7 with proteases exhibited that all the four reactive sites were exposed on unordered loops which facilitated its interaction with multiple target protease molecules, affirming its higher inhibition potency. Initial binding energy comparison and hierarchical clustering analysis provided overview of specific interaction of *CanPIs* with various proteases. It may also give speculation about mode of action and effect of various inhibitory domains on *H. armigera* digestive proteases. Moreover, insights into underlying mechanism of differential inhibition between proteases by IRDs could potentially be useful in designing improved strategies for insect control. Furthermore, single domain (*IRD7*) and multidomain *CanPI7* (4-IRD), were selected for *in planta* characterization through transgenic *Arabidopsis*. *Agrobacterium*-mediated transformation of *Arabidopsis* using floral dip method obtained transformation efficiency of 0.62 % (*CanPI7*) and 0.82% (*IRD7*), respectively. Similar transformation efficiency range was reported by protocol followed by Clough and Bent (1998). Evaluation of biological activity of *CanPIs* (*IRD7*/ *CanPI7*) expressed in transgenic *Arabidopsis* leaves was performed using HGP inhibition assay. Crude

protein of CanPI7 and IRD7 from respective transgenic *Arabidopsis* showed increased inhibition of HGP activity as compared to WT plants. However, compared to *IRD7* transgenic, *CanPI7* transgenic lines showed higher inhibition in HGPs when expressed in *Arabidopsis*. The present findings corroborate with our earlier data on PI activity of *H. armigera* feeding on artificial diet (AD) containing recombinant PI (Tamhane et al., 2007b). The clear decrease on both trypsin and chymotrypsin like specific activities confirmed the potential for direct interference of *CanPIs* on *H. armigera* proteases and eventually its digestion. Cross reactivity in binding of trypsin or chymotrypsin molecules to either TI or CI sites of CanPI7 was also earlier reported which suggested that the reactive site loops retained adequate conformational flexibility to allow recognition by a variety of proteinase molecules. This might be the key reason behind the higher inhibition of HGP by crude protein from transgenic *CanPI7 Arabidopsis*. Similar inhibition pattern was observed in other studies, using protein extract of transgenic *Arabidopsis* expressing PIs against various proteases. Co-expressing two barley proteases inhibitors, cystatin and trypsin inhibitor genes in *Arabidopsis* showed significant inhibitory activity against commercial proteases such as papain / trypsin and also inhibited cathepsin B- and L-like activities of spider mite (*Tetranychus urticae*) (Santamaria et al., 2012). Transgenic *Arabidopsis* expressing *HvCPI-6* had also shown *in vitro* inhibition of cathepsin L- and/or B-like proteinases activity of two aphids, *Myzus persicae* and *Acyrthosiphon pisum* (Carrillo et al., 2011). Plant extract from transgenic *Arabidopsis* expressing protease inhibitor from barley showed strong inhibition of chymotrypsin activity as compared to WT plants (Losvik et al., 2017). Overall, our results of inhibition assay imply that *CanPI7* could effectively inhibit the gut proteases of *H. armigera* which are complex mixtures of several isoforms of trypsin, chymotrypsin and elastases.

In order to test the efficacy of CanPI proteins expressed in plants against *H. armigera*, insect bioassay was also carried out. This study indicated significant reduction in larval mass of *H. armigera* after feeding on AD containing crude protein from *CanPI7* transgenic lines, which substantiated the impact of the CanPI7 protein on *H. armigera* target proteinases. Our findings were in well accordance with the earlier reports of insect feeding assay on AD containing recombinant CanPI7 protein (Tamhane et al., 2007b). Based on the previous reports of inhibitory properties of CanPI7, a broad spectrum of insect digestive proteinases might be affected by activity

of inhibitory domains. The inhibition of proteolysis through PIs may decrease access to essential amino acids and consequently protein functions can be impaired disrupting crucial physiological events of *H. armigera* such as nutrition, redox status and development. In our present studies, single and multidomain PIs varied in their biochemical properties like inhibition potential and binding efficiencies against target proteases, growth, etc. Similarly in various *in vivo* and *in vitro* studies, transgenic *Arabidopsis* expressing different PIs have shown to confer resistance against various insect species in their respective bioassays. Overexpressing Kunitz trypsin inhibitor of poplar in *Arabidopsis* caused improved resistance against *H. armigera* larvae (Hu et al., 2012). Barley cysteine PI had shown reduced performance of two aphid species in AD and transgenic *Arabidopsis* (Carrillo et al., 2011). Also, the protease inhibitor from barley had been reported to inhibit aphid fecundity in transgenic *Arabidopsis* (Loswik et al., 2017). Transgenic *Arabidopsis* expressing cowpea trypsin inhibitor and rice cysteine PI also conferred resistance to the nematode *Rotylenchulus reniformis* (Urwin et al., 2000). Gene stacking of two barley PIs in *Arabidopsis* enhanced plant resistance to *T. urticae* (Santamaria et al., 2012). Moreover, ectopic expression of a PI (*MtPiI4*) gene from *Medicago truncatula* in transgenic *Arabidopsis* conferred resistance against bacterial pathogen *Pseudomonas syringe* (Sun et al., 2015). However, in the present study AD containing IRD7 did not show significant reduction in larval mass compared to the control, although inhibition of proteases was observed as detailed above. The number and class of gut proteases of *H. armigera* (Trypsins and Chymotrypsins) might not be significantly inhibited by single TI domain of IRD7. This indicated that the level of *IRD7* when expressed in transgenic *Arabidopsis* might not be sufficiently high to inhibit *H. armigera* growth and development.

In conclusion, *CanPI7* protease inhibitor gene in *Arabidopsis* has resulted to be more effective and enhance resistance ability than a single domain *IRD7* transgenic *Arabidopsis*. These results demonstrate that, PIs with multiple and varying specificities are better approach for tackling a wide range of insect gut proteases. These results propose that *CanPI7* containing multiple domains of CI and TI, is a potential candidate to further elucidate the antibiosis properties of *CanPIs* in transgenic crop plants. Ultimately, the integration of *CanPI* genes into cultivated

plants may become an effective approach to develop crops with improved and durable resistance towards insect pests.

Section 4.2: Transgenic tomato with *CanPI7*: Sustainable approach for insect tolerance

Tomato, being one of the most important food crops in the world, is in constant need for improvement in agronomic yield and quality to meet the demands of the vegetable market and processing industry. Many insect resistant varieties of tomato have already been developed and many are in pipeline, which can be made available to farmers. Several attempts to generate transgenic tomato plants showing resistance to insect were and are being made (Abdeen et al. 2005; Delannay et al. 1989). In the present work, an attempt has been made to establish transgenic tomato line expressing multidomain proteinase inhibitor, *CanPI7* gene to improve insect tolerance. Proteome and metabolome alterations in transgenic tomato plant, if any, were also analysed using LC-MS^E and UHPLC-Orbitrap techniques, respectively.

The establishment of simple and efficient regeneration system is a fundamental prerequisite of taking advantage of cell and tissue culture for developing genetically transformed plants. The *in vitro* culture of tomato has been successfully used in different biotechnological applications such as the clonal propagation of high-value commercial cultivars and genetic transformation (Li et al., 2011a; Yarra et al. 2012; Namitha and Negi, 2013). Many independent reports have previously shown that tomato plant regeneration achieved through organogenesis was affected by several factors such as genotype, explant source, age of explants, media composition and environmental conditions (Mamidala and Nanna, 2011; Namitha and Negi, 2013; Sherkar and Chavan, 2014; Wayase and Shitole, 2014). For regeneration studies of tomato different types of explants have been used. Type of explants not only determines frequency of the explant organogenesis but also shoots induction efficiency (Bahurupe et al., 2013; Jehan and Hassanein, 2013). There are many reports regarding tomato transformation and *in vitro* plant regeneration from different explants (including seed-cut cotyledons, hypocotyls, leaves, stem sections, pedicels, petioles and inflorescences) *via* organogenesis (Khoudi et al. 2009; Yasmeen 2009; Goel et al. 2011; Rai et al. 2013; Namitha and Negi, 2013; Sherkar and Chavan, 2014; Wayase and Shitole, 2014). Most tissues of tomato were reported to have high totipotency; however, the choice of right explant might vary with the genotype. Our

results implied that overall regeneration ability was higher in cotyledons over hypocotyl and leaf explants. Our findings were in well accordance with the earlier regeneration studies reported by Mamidala and Nanna (2011). Zhang et al. (2012b) indicated that location of the cutting wound in explants significantly affected callus induction and adventitious bud formation. They demonstrated that the highest frequency of bud induction occurred at middle part of cotyledon segment. Similar result was obtained in our regeneration studies also.

Plant growth regulators (PGRs) in growth medium play an important role in regulating callus induction and tissue differentiation of explants in plant tissue culture technique. According to numerous reports IAA, NAA, 2,4-D, ZA and 6-BAP are the hormones commonly used in *in vitro* cultures of tomato to ameliorate callus induction and shoot regeneration (Kantor et al., 2013; Mamidala and Nanna, 2011; Ashakiran et al., 2011; Zhang et al., 2012b; Namitha and Negi, 2013). Kietin, 6- (c,c-dimethylallylamino) purine (2iP) and Thidiazuron (TDZ) are new plant PGRs used in some other studies (Ishag et al., 2009; Chaudhry et al., 2010;Wu et al., 2011; Ashakiran et al., 2011). From our regeneration studies, highest callus induction with simultaneous multiple shoots development was noted with MS media containing ZA (1 mg/L). ZA is a cytokinin reported to be used mostly in regeneration and transformation studies of tomato (Ichimura and Oda, 1995; Costa et al., 2000). Frary and Earle (1996) used ZA (2 mg/L) for regeneration of tomato cultivar Moneymaker, while McCormick (1991) used 1 mg/L of it in addition to Gamborgs B5 Vitamins for shoot regeneration.

Rooting is the final step of the regeneration protocol in plant tissue culture for generation of *in vitro* plantlets. There are several factors affecting the root induction in shoots such as the physiological status of plantlets, medium composition and growth regulators. Some reports suggested that for root induction, tomato does not require any exogenous PGRs (Mensuali- Sodi et al., 1995; Rashid and Bal, 2010; Bahurupe et al., 2013). However, in most cases, root formation would be achieved with auxins (IAA, NAA or IBA) alone with concentration ranging from 0.1 to 1 mg/L (Chaudhry et al., 2010; Ashakiran et al., 2011; Mamidala and Nanna, 2011; Zhang et al., 2012b; Namitha and Negi, 2013; Shitole and wayse, 2014; Sherkar and Chavan, 2014). In the present study, maximum root induction from the regenerated shoots was achieved on MS medium supplemented with 1 mg/L IBA. Izadpanah and Khosh-Khui

(1992) obtained the highest percentage of rooting with medium containing 0.5 mg/L IBA. This difference in concentration might be due to genotype difference.

For tomato transformation, *Agrobacterium* concentration in co-cultivation usually ranges between 0.01 and 1.0 at OD_{600} nm (João and Brown, 1993; Frary and Earle, 1996; Ling et al., 1998; Krasnyanski et al., 2001; Pozueta-Romero et al., 2001; Park et al., 2003; Dan et al., 2006; Wu et al., 2011; Qiu et al., 2007). We found maximum transformation efficiency at OD 0.5, whereas at OD 0.2 it was slightly less than that at 0.5 OD. However, at higher optical density (0.5OD) necrosis rate was higher, hence, use of bacterial suspension at this OD was not feasible for transformation event. Addition of phenolic compound, acetosyringone (AS) to the co-cultivation media has been shown to increase the transformation frequency in the present study. Similar findings were observed in other studies also (Park et al., 2003; Cortina and Culianez-Mica, 2004). Preculture of explants for 2 days increased the transformation frequency and area of infection. The 2 days preculturing treatment has also been suggested in many reports (McCormick et al., 1986; Hamza and Chupeau, 1993; Ellul et al., 2003). Thus, in the present study, for more transformation efficiency of precultured explants, bacterial suspension with optical density 0.2 at 600 nm supplemented with 200 µM AC and 2 days of co-cultivation period were used.

Transformation efficiencies by *Agrobacterium* method have been reported to range from 6 to 40% (Vidya et al., 2000; Park et al., 2003; Sun et al., 2006). In the present study, tomato transformation efficiency was 7.5%. There were no differences in plant morphology between WT and PI overexpressed transgenic tomato plants, except that the line 2 of T0 generation showed change in leaf morphology, which might be due to position effect of integration of transgene. Silencing or ectopic overexpression of PI genes in plants has been reported to affect plant growth in certain cases, such as tobacco, nightshade and lettuce (Zavala et al., 2004a; Xie et al., 2007). Furthermore, reduced number of viable seeds in fruits of transgenic plants was observed in the present study, which might be due to overexpression of *CanPI7* transgene. Similar observation on reduced seed set was reported by RNAi mediated silencing of *SaPIN2 PIs* (Sin et al., 2006).

Based on earlier preliminary studies in transgenic *Arabidopsis*, the *CanPI7* was considered as a potential candidate for developing and testing transgenic crop plants for improved resistance to *H. armigera*. Moreover, in earlier reports from our

lab, it was shown that neonates as compared to third instar larvae had more prominent retardation upon exposure to recombinant CanPI7 (Mahajan et al., 2013). Thus, to obtain maximum effect of CanPI7 protein on growth of neonates and first instar *H. armigera* larvae and to get insight into antibiosis effect of CanPI7 during initial stage of insect development from crop protection point of view, it was crucial to establish transgenic crop plant, such as tomato overexpressing *CanPI7* gene. Protein extracts from *CanPI7* plants exhibited inhibitory activity against HGP, which was in agreement with earlier reports of inhibition assays for recombinant CanPI7 protein from our lab (Tamhane et al., 2007b). Moreover, different transgenic events showed variation in inhibitory activities suggesting scope to produce more transgenic events to have maximum and specific protease inhibitory activity of this multidomain PI.

Insect feeding experiments further revealed that the ingestion of *CanPI7* containing transgenic tomato leaves had deleterious effect on metabolism and growth of the *H. armigera* insect. Mass of the larvae fed on transgenic plants was observed to be decreased as compared to larvae fed on WT plants. Based on these as well as previous reports of inhibitory properties of *CanPI7* (Tamhane et al., 2007b), a broad spectrum of insect digestive proteinases (serine proteinases) might be inhibited by activity of multiple domains of *CanPI7*. The possible reasons for reduced growth of *H. armigera* larvae could be the amino acid starvation due to inhibition of insect gut proteinases and increased stress on gut proteinase expression system to express more and higher amounts of proteinases (Broadway, 1995). Moreover, because of presence of multi inhibitory domains in CanPI7, insect might face problem in expressing various forms of proteases leading to detrimental effects on larvae. In insect bioassay of T1 transgenic plants, variation in reduction in larval mass was seen at the 6^{th} and 7^{th} day among the transgenic lines. Line 1 showed higher reduction in larval mass as compared to the other transgenic lines at 6^{th} day. However on 7^{th} day, Line 3 showed higher larval mass reduction as compared to transgenic Line 1 and 2. This variation might be due to change in overall inhibition shown by transgenic lines due to interplay between PIs of tomato (SlPinII) and CanPI7 in transgenic tomato and HGP proteases. In the present choice assay, functional analysis of CanPI7 was performed to check its potential in mitigating insect infestation. Choice assay of homozygous T2 transgenic plants confirmed the deterrent property of transgenic tomato expressing CanPI7 protein. Several groups have reported the enhanced protection of plants

towards insect attacks using *PI-II* genes (Ryan, 1990; Luo et al., 2009; Dunse et al., 2010; Majeed et al., 2011). Transgenic tobacco expressing Pin-II genes from tomato and potato increased tolerance against *Manduca sexta* (Johnson et al., 1989). Potato PI-II gene expressed in Chinese cabbage resulted in conferring resistance against *Pieris rapae* and *Plutella xylostella,* (Zhang et al., 2012a). The expression of PI-II (*SaPIN2*) in transgenic tobacco plants inhibited the growth of *H. armigera* and *Spodoptera litura* larvae (Luo et al., 2009). Transgenic tomato over-expressing carboxypeptidase inhibitors (PCI) and potato PI-II increased resistance against larvae of *Liriomyza trifolii* and *Heliothis obsolete* (Abdeen et al., 2005). Transgenic tobacco plants expressing the pyramided inhibitor genes from potato and taro exhibited broad-spectrum resistance to *H. armigera, Erwinia carotovora* and *Pythium aphanidermatum* (Senthilkumar et al., 2010). Also, in transgenic tomato over-expression of cysteine proteinase inhibitor from rice conferred protection against white potato cyst nematode (Urwin et al., 1995). Recently, transgenic sugarcane overexpressing cysteine peptidase inhibitor negatively affected the growth and development of *Sphenophorus levis* (Schneider et al. 2017).

Although plant PIs have been found effective against several insects, their effects are transient in some cases as insects can adapt to PIs by (i) over expressing PI-insensitive proteinases (Broadway, 1995, 1996, 1997; Jongsma et al., 1995, 1997), (ii) regulating the level of existing proteinases (Broadway, 1997; Gatehouse et al., 1997) and (iii) degrading the PIs (Michaud, 1997; Giri et al., 1998). After prolonged exposure and selection pressure mediated by PI, resistant biotypes of insects may evolve (Lawrence and Kuondal, 2002). Considering the high complexity of protease-PI interactions in host insect systems and the diversity of proteolytic enzymes used by insects to hydrolyze dietary proteins, the choice of appropriate PIs is important in deciding the success or failure of PI transgenic plants. *S. exigua* larvae fed on detached leaves of tobacco plants transformed with trypsin/ chymotrypsin-specific potato 2 inhibitor (PI2) under the control of constitutive promoter remained unaffected and showed 2.5 fold induction of new tryptic gut activity that was insensitive to inhibition by PI2 (Jongsma et al., 1995). This insect adaption to PIs can be reduced by using novel inhibitors and multiple genes stacking. Novel inhibitor containing artificial multidomain inhibitors with more potent inhibitor potential than its natural counterparts can be designed (Outchkourov et al., 2004). It has also been

shown that by stacking two insecticidal genes with different mechanisms of action or by using combinations of PI genes, the possibility of evolution of adaptation or resistance in the larvae can be significantly reduced (Dunse et al., 2010; Chen et al., 2014). Together, the results presented here suggest that *CanPI7* in combination with other PI or resistance transgenes may provide a valuable strategy for the protection of economically important crop species against *H. armigera* pest.

Variations in the efficacies of insect tolerance observed in the transgenic lines in the present study guided us to carry out in depth proteomic and metabolomics analyses of the plants. In order to identify differential abundance of proteins under overexpressed *CanPI7* condition, a quantitative comparative protein profiling was performed using LC-MS^E. A few reports of comparative proteomic analysis of the transgenic plant with WT are available (Gong et al., 2012; Ghosh et al., 2016; Zolla et al., 2008; Tan et al., 2016). However, the target protein could not be detected in some cases, possibly due to the low expression of the target gene and/ or limitations in the detection techniques (Zolla et al., 2008; Tan et al., 2016). On the other hand in the present study, CanPI7 protein was uniquely detected in transgenic lines probably due to overexpression of the gene under promoter containing enhancer element of *Arabidopsis* and high sensitivity of SYNAPT-HDMS system (Waters, USA) used in our case. Further, pathway analysis revealed that most of the differentially expressed proteins were implicated in photosynthesis, glutathione, secondary metabolites, glyoxylate and dicarboxylate, purine, thiamine, phenylpropanoid and nitrogen metabolism. These up-regulated effects of photosynthesis might be for generating extra energy in response to the insertion of exogenous genes (Gong et al., 2012). Similar studies were performed on transgenic leaves of cotton, maize, mint and tomato (Gu et al., 2013; Sinha et al., 2013; Wang et al., 2015; Tan et al., 2016). The transgenic plants also showed up-accumulation of proteins related to stress and anti-oxidation. The abundance of the threonine deaminase (TD) protein involved in isoleucine biosynthesis was increased in transgenic plant. TD is generally up regulated in leaves after herbivore attack and plays an important role in herbivore resistance by mediating JA-Ile signalling and also acts as an anti-nutritional protein by depleting threonine levels (Gonzales-Vigil et al., 2011). Likewise, ferredoxin NADP reductase was also identified as up-accumulated in transgenic plant. It has been demonstrated that transgenic plants overexpressing ferredoxin show high resistance to

many bacterial pathogens (Huang et al., 2004). Plant defensins are cationic peptides that are ubiquitous within the plant kingdom and belong to a large superfamily of antimicrobial peptides found in several plants species (De Samblanx et al., 1997; Thevissen et al., 2000; Aerts et al., 2007). Defensin protein was uniquely detected in transgenic plants as compared to their wild counterparts. These results suggested that CanPI7 and defensin cumulatively might strengthen defense mechanism against insects in transgenic tomato lines. Together, this data suggested that although some significant differences were detected, the proteomic patterns were not substantially different between leaves of the transgenic tomato and WT.

Based on the metabolomic results, metabolites involved in various pathways such as TCA cycle, amino acid (L- glutamate), glucosinolate, ascorbate and aldarate pathways were upregulated in the transgenic lines as compared to the WT control plants. Moreover, our analysis showed decreased accumulation of metabolites related to secondary metabolite pathways, amino acids, flavones, flavonols, phenypropanoids and flavonoids metabolism in the transgenic plants in comparison to their respective WT controls. Overexpression of *CanPI7* might modulate biological pathways in plant cell through inhibition of regulatory proteases/enzymes involved in several metabolisms; however, further studies would be required to confirm this mechanism. Le Gall et al. (2011a, b) investigated GM tomatoes with altered flavonol metabolism. The metabolite profiles of the GM tomatoes during ripening were compared with those of non-GM controls by means of 1H-NMR metabolomics. The assessment of GM tomatoes overexpressing the taste-modifying protein miraculin was conducted on a multiplatform approach using GC-MS, LC-MS and CE-MS (Kusano et al., 2011). Lastly, present metabolic data suggested interactions among various pathways but was unlikely to specifically contribute to defence of transgenic plant against *H. armigera*. Untargeted metabolomics-based profiling of transgenic crops are thus of importance for comprehensive and comparative assessment of transgenic crop plants. Overall, overexpression of CanPI7 along with induction of defensin like proteins with no adverse changes in proteome and metabolome of transgenic tomato as compared to its non-transgenic counterpart would lead to a sustainable strategy to develop insect tolerance in crop plants such as tomato.

Section 4.3: Single domain CanPIs (IRDs) in recombinant yeast indicate tolerance against abiotic stress

Abiotic stresses, such as extreme temperatures, drought, salinity, chemical toxicity and oxidative stress are important threats to plant growth and productivity. Protein synthesis and degradation, which depend on proteolytic enzymes, are important parts of the plant responses to abiotic stresses (Ingram and Bartels, 1996; El Maarouf et al., 1999). Proteolysis in plants is a complex process involving several enzymes in various pathways in many cellular compartments. Cysteine proteinases are a major group of enzymes in this process (Grudkowska and Zagdanska, 2004). Cysteine proteinases such as metacaspases accumulate when tissues are exposed to different abiotic stresses (Callis et al., 1995; Koizumi et al., 1993). Metacaspases in plants, fungi, and protozoa constitute important members of a conserved superfamily of caspase-related proteases. Yeast caspase-1 protein, which is the single metacaspase in *S. cerevisiae*, mediates apoptosis triggered by oxidative stress or aging in yeast. Two *Arabidopsis* metacaspases, AtMCP1b and AtMCP2b activated apoptosis-like cell death in yeast. On the other hand, cell damage also leads to synthesis of PIs, which regulate the activity of cysteine proteinases (Yang and Yeh, 2005). The expression of PI genes has been noted in salt and water deficit and temperature stress responses of plants (Dombrowski, 2003; Huang et al., 2007; Singh et al., 2009; Srinivasan et al., 2009). In peanut, the Bowmane Birk PI gene was upregulated by water deficit and the level of transcript accumulation was higher in tolerant as compared to susceptible cultivar (Dramé et al., 2013). In chestnut, a cysteine-PI gene was induced by low temperature, salt and heat stresses (Pernas et al., 2000). Similarly a Kunitz-type PI was induced by heat and drought stress in Brassica (Satoh et al., 2001). A soybean cysteine PI has been designated a novel role in modulating the programmed cell death (Solomon et al. 1999). Furthermore, Shitan et al. (2007) showed that expression Bowman Birk inhibitors award heavy metal and multiple drug tolerance in yeast. The overexpression of an *Arabidopsis* cysteine proteinase inhibitor gene (AtCYS1) blocked cell death activated by either avirulent pathogens or by oxidative and nitric oxide (NO) stress in transgenic tobacco plants (Belenghi et al., 2003). Thus, these reports suggested important role of PIs during abiotic stress management and prompted us to study role of CanPI in abiotic stress tolerance.

To examine whether yeast metacaspases are functionally related to PI, we carried out analyses of IRDs, utilizing recombinant yeast strains with wild-type and the disrupted YCA1 gene (yca1). We reported the response of yeast strains expressing *C. annuum* protease inhibitors (*CanPIs*) containing single inhibitory repeat units (IRD-7, IRD-9 and IRD-12) to various abiotic stresses. It was observed that ectopic expression of IRDs in yeast conferred tolerance towards different stresses such as salt (NaCl), heavy metals ($CdCl_2$, $HgCl_2$) and oxidative stress (H_2O_2). These results demonstrated that expression of PIs provides endurance against various abiotic stresses. There is a definite correlation in growth rate and inhibition potential of IRDs indicating the significance of protease inhibition on cell survival under stress. There was a significant increase in intracellular TPI activity in $PpIRD^+$ strain as compared to EV cells under stress conditions. Domash et al. (2008) has discussed about regulation of proteases expression through the modulation of PIs expression under stress conditions. Similarly, $PpIRD^+$ strains showed enhancement of PIs activity on stress stimuli, which might inhibit the cellular proteases which are involved in protein degradation under stress. The impact of abiotic stress on the activity of serine and cysteine proteases was investigated in yeast cells. It was observed that the activities of SP and CP increased rapidly in EV cells, while $PpIRD^+$ cells showed optimal level of protease. The inhibition of SP and CP activity might be due to elevated expression and activity level of PIs i.e. IRDs under stress. Induction of protease under stress condition could help the cells in clearance of misfolded protein load and thus, overcome the lethal effect of stress. Although IRDs are SP inhibitor, the cross reactivity of IRDs toward inhibition of CP might have influence on regulation of CP expression and activity in $PpIRD^+$ strains. According to various reports, increase in protease activity under stress conditions could be related to physiological processes such as senescence and programmed cell death (Palma et al., 2002). An increase in proteolytic activity under stress conditions can lead to a disturbance in the balance between protein synthesis and decay, which might cause premature senescence and cell death (Djebali et al., 2008). To prevent this, valid mechanisms are required to control the proteolytic activity at the transcriptional, translational and post-translational levels. Solomon et al. (1999) has shown that expression of cystatin, an endogenous cysteine PI, can cause inhibition of the programmed cell death related cysteine proteases, which are induced by biotic or oxidative stress (Solomon et al.,

1999). Quian et al. (2014) evidenced that expression of oryzacystatin-I inhibited cysteine proteases participate in the control of growth and stress tolerance through effects on strigolactones. These results suggested that, there is a definite correlation in growth rate and inhibition potential of IRDs indicating the significance of protease inhibition on cell survival under stress**.** Thus, these observations suggest that ectopic expression of PIs might lead to inhibition of proteases participating in the growth control and thus, might provide multiple stress tolerance in yeast cells.

Level of metacaspase activity is an indicator of survival status of the cell. Enhanced transcriptional and translation level of metacaspase was observed in the apoptosis or senescence cells (Lee et al., 2010; Tsiatsiani et al, 2011). It is known that expression of yeast metacaspase is regulated by apoptosis stimuli like oxidative stress, low pH, and high salinity stress. Numerous reports highlight the interlinking in ectopic expression of PI, its effect on cysteine proteases and abiotic stress tolerance (Solomon et al., 1999; Quain et al., 2014). In EV cells, higher metacaspase activity leads to activation of apoptosis pathway and that may result in reduced growth under stress condition. Inhibition of metacaspase was seen in $PpIRD^{+}$ strain which might result in delayed senescence in $PpIRD^{+}$ strains. Docking results between IRDs and yeast metacaspase, suggested that strong interaction between the reactive loops of IRD with the active site of metacaspase might be the key reason behind delayed apoptosis and multiple tolerance in yeast cells. Yeast metacaspases have arginine/ lysine-specific endopeptidase activity, thus various synthetic substrates containing an arginine/ lysine residue at the P1 position could be cleaved by the metacaspase examined in this study (Vercammen et al., 2004). The reactive loop of expressed IRD contains arginine/ lysine residue at the P1 position, thus enhancing the specificity of IRDs toward Yca1. These data, in turn, suggest that Yca1 interacts with IRDs in substrate specific manner, and these results in competitive inhibition of its activity. This indicated that under stress condition the metacaspase activity was inhibited by the induced expression of IRDs and thus resulted in delayed senescence in $PpIRD^{+}$ strains. Growth characteristics of yeast metacaspase knockout (ΔYca1) were also studied to confirm the role of metacaspase in survival of the cells. Growth and metacaspase activity analysis of this knockout indicated that ΔYca1 strain exhibited similar growth characteristics like $PpIRD^{+}$ strains and suggested that metacaspase

activity of yeast cell under stress might be inhibited by IRD expression and thus exhibited phenotype like ΔYca1 strain (Lee et al., 2010).

Assessment of metacaspase inhibition was performed indirectly by estimation of *in vivo* GAPDH activity, as it is the primary target of metacaspase during apoptosis process. Silva et al. (2014) showed that in yeast cells GAPDH is the primary target of metacaspase. Increased activity of metacaspase under stress enhances GAPDH degradation and thus it would inhibit the basic energy metabolism of the cell (Joshi et al., 2014b). Inhibition of metacaspase activity by PIs expression causes maintenance of GAPDH level; this in turn helps in survival and growth maintenance of the yeast cells under stress. Assessment of intracellular activity showed that inhibition of metacaspase activity causes the maintenance of cell's energy metabolism and thus its survival. This phenomenon of multiple stress tolerance via delayed senescence is mediated through maintenance of cells protein turnover and energy metabolism by inhibition of proteases. Together, the results strongly implicate the potential molecular mechanism of PI-based multiple abiotic stress tolerance in yeast strains due to inhibition of cellular metacaspase and proteases. Metacaspases and proteases from higher plants may serve similar functions.

Thus in conclusion, bioinformatics studies indicated that individual IRDs of *CanPI7* showed differential interaction with *H. armigera* proteases. Transgenic *CanPI7 Arabidopsis* plants showed better antibiosis activity against *H. armigera* larvae compared to transgenic *IRD7 Arabidopsis*. Transgenic tomato overexpressing *CanPI7* showed increased resistance against *H. armigera* larvae. Recombinant yeast strains expressing single domain *CanPIs* (IRDs) showed multiple abiotic stress tolerance via delayed senescence.

Chapter 5

Summary and Future Directions

Summary

Helicoverpa armigera is one of the most devastating insect pests of crop plants. Biotic stress caused by such pests and pathogens accompanies metabolic reprogramming in plants, which in turn leads to accumulation of defensive molecules. *Capsicum annuum* is one of the non-preferred host plants of *H. armigera*, a polyphagous pest. In *C. annuum* (Family: Solanaceae), elicitation of wound response leads to the systemic induction of significant amount of protease inhibitor (CanPI) proteins. In order to identify the antibiosis property of CanPI, the investigation of insect responses to transgenic plants expressing *CanPIs* needs to be performed. Keeping in view the dynamic nature of insect gut proteinases, carefully planned strategies will surely lead to development of sustainable solutions for *H. armigera* control. Furthermore, it is also beneficial to check activity of *CanPIs* (IRDs) for abiotic stress tolerance, thus, indicating dual advantage of *CanPIs* for crop improvement. The major objectives and the results obtained are summarized below.

5.1. Response of transgenic *A. thaliana* expressing *CanPI7* and *IRD7* genes towards the insect *H. armigera*

Docking study was performed to determine binding energy and interaction of inhibitory domains of CanPI7 with *H. armigera* proteinases. *CanPI7* and *IRD7* genes were cloned in plant expression vector pRI101-AN and transformed into *Agrobacterium* strain GV3101. Transformation of model plant *Arabidopsis* (cultivar: Col 0) was carried out using floral dip method, which avoids plant tissue culture technique. *A. thaliana* transformation efficiency of 0.62% and 0.82% was achieved for *CanPI7* and *IRD7*, respectively. Kanamycin resistant plants were confirmed as transgenic origin by genomic PCR analysis. Protein extract of transgenic *Arabidopsis* lines showed increased inhibitory activity against *H. armigera* gut proteinases, supporting those domains of CanPI protein to be effective and active. Transgenic *Arabidopsis* plants exhibited antibiosis effect against first instar larvae of *H. armigera*. Further, larvae fed on artificial diet containing crude protein of transgenic *Arabidopsis* showed delayed growth relative to larvae fed on control diet, but did not significantly change mortality rates. *H. armigera* larvae fed on *CanPI7* diet showed higher antibiosis effect than that on *IRD7* diet. Biochemical and molecular

information generated through above study indicated that multidomain *CanPI7* can be a candidate gene for improvement of resistance in crop plants such as tomato against *H. armigera*.

5.2. Antibiosis against *H. armigera* by transgenic tomato over-expressing multidomain proteinase inhibitor (*CanPI7*)

Regeneration studies of tomato cultivar Pusa Ruby were carried out for efficient *in vitro* propagation of tomato plants. Factors influencing *Agrobacterium*-mediated genetic transformation, namely, optical density of bacterial suspension and acetosyringone were optimized during present investigation. Using this optimised regeneration and co-cultivation parameters, future studies were conducted to transfer *CanPI7* in the tomato plant for conferring insect resistance. Stable integration and expression of the transgene in T0 transgenic generation, were confirmed by established molecular techniques. Overall, 7.5% efficiency of tomato transformation was achieved. Seeds of T0 plants that were produced after self-pollination were germinated on selection medium and further, these germinated plants were examined by genomic PCR, Southern blot and RT-PCR. Segregation results of T1 transgenic plants showed that the *CanP7* gene was transmitted to the next generation and segregated in the ratio of (3:1) as expected for a single dominant gene. Protein extract of transgenic tomato lines showed increased inhibitory activity against *H. armigera* gut proteinases, suggesting effective and active role of those domains of CanPI7 protein. When analyzed in T1 and T2 generation transgenic plants, they exhibited antibiosis effect against first instar larvae of *H. armigera*. Further, larvae fed on transgenic tomato leaves showed delayed growth relative to larvae fed on control plants, but did not change mortality rates significantly. Feeding-choice assays showed that *H. armigera* larvae consumed less transgenic leaf tissue than that of WT tomato. Moreover, proteomic and metabolomic studies showed that although some differences in proteins/ metabolites accumulation were detected, the overall proteomic and metabolomic patterns were not substantially different between the leaves of the transgenic and WT plants. Together, the results presented here suggest that *CanPI7* gene would be useful for protection of economically important crop species against *H. armigera* pest.

5.3. Yeast (PpIRD$^+$) strain expressing single domain *CanPIs* (IRD) responds against various abiotic stresses

PIs are involved in abiotic stress tolerance, though their exact role and mechanism are yet to be studied. To examine whether single domain *CanPIs* (IRDs) are involved in abiotic stress tolerance, we carried out primary analyses of IRDs, utilizing recombinant yeast strains with empty vector and the disrupted YCA1 gene (*yca1*). Growth studies of recombinant yeast strains (PpIRD$^+$) expressing *CanPIs* (IRD-7, IRD-9 and IRD-12) was assessed under heavy metal, high salt and oxidative stress. PpIRD$^+$ strains showed multiple abiotic stress tolerance and exhibited sustainable growth with reduced intracellular proteases (serine and cysteine protease) activities upon exposure to heavy metals, high salt and H_2O_2. PpIRD$^+$ strain displayed significant decrease in metacaspase (Yca1) activity, indicating the likelihood of cross reactivity of IRDs (serine protease inhibitor) with cysteine proteases. *Saccharomyces cerevisiae* knockout with Yca1 (ΔYca1) strain and PpIRD$^+$ exhibited similar growth characteristics under stress conditions, which demonstrated the delayed senescence because of cellular metacaspase inhibition. Docking study indicated a close proximity of IRDs reactive site and the active site of metacaspase in the complex that indicated their strong interactions. Maintenance of GAPDH activity in PpIRD$^+$ strain provided the evidence for the inhibition of metacaspase activity and survival of cells under stress conditions. Thus, these results strongly implicate the potential molecular mechanism of PI-based multiple abiotic stress tolerance in yeast strains through inhibition of cellular metacaspase and proteases.

The present study on *C. annuum* has brought in to light antibiosis property of *CanPIs* (Pin-II) expressed in transgenic plants, with emphasis on their defense role. The characterization of transgenic *Arabidopsis* and tomato expressing *CanPI7* have indicated high efficiency and promising potential in control of *H. armigera*. Also, recombinant yeast expressing single domain *CanPIs* have indicated their potential role in abiotic stress tolerance. Thus, the naturally occurring gene diversity in *C. annuum* (*CanPIs*) provides an effective dual strategy to reach the goal of crop protection against biotic stress by pests and abiotic stress.

Future directions

The information from transgenic studies revealed by the present analysis can be used in further studies for crop improvement. Pursuing of the following leads in future would be worth in order to effectively implement *CanPIs* in stress tolerance:

- Trials of transgenic plants in green house and field controlled conditions following biosafety regulations with whole plant for assessment of its insect controlling potential.
- Collecting seeds of elite transgenic lines through self-crossing and screening of plants for traits of resistance against insect pests and better yield.
- Stacking of *CanPI7* with other PIs or Bt genes which will offer enhanced insect tolerance to prevent or delay the emergence of resistance. As an extension of transgenic studies, focus on application of different promoter (replacing 35S prometer) and removal of selection marker gene (Kanamycin) need to be carried out.
- To prepare synthetic PIs with artificial domains using site-directed mutagenesis and protein engineering to increase efficacy against different proteases and further transformation of these inhibitor molecules into crop plants.
- Characterization of transgenic *Arabidopsis* and tomato expressing *CanPIs* against abiotic stress tolerance.
- To identify and study the endogenous roles of *CanPIs* such as programmed cell death, growth and development in plant cell.

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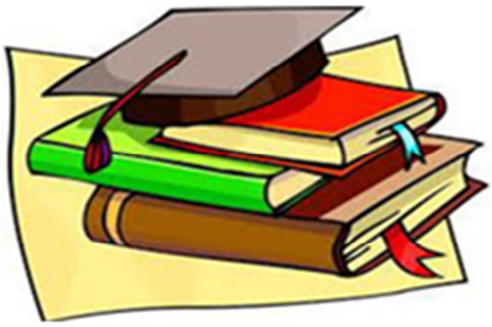

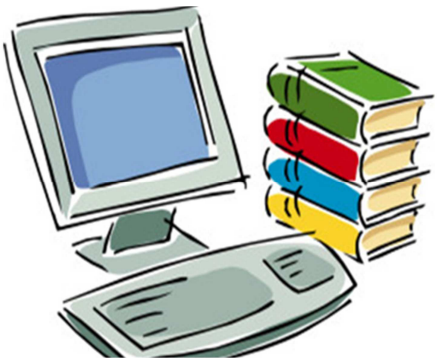

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Appendices

APPENDIX I

Primers used in present study

Gene	Primer	Sequence (5′–3′)
	For cloning	
CanPI_7	Forward	AAAAAAAGGATCCATGGCTGTTCCC
	Reverse	AAAAAAAGAGCTCCTGTTCATGCTTTTAC
	For Genomic PCR	
Tomato PI	Forward	CTTGGGTTCGGGATATGC
	Reverse	CATTACAGGGTACATATTTGCC
CanPI 7	Forward	GTTCCGAAGCAAGTGCAG
	Reverse	CATACAGACATAGTGAATAGGC
35S promoter	35S Forward	GCTCCTACAAATGCCATCA
CanPI7	Reverse primer	GAGCTCCTGTTCATGCTTTTAC
Actin	Forward	CCCAGAGGTACTCTTCCAACC

List of primers used for real-time PCR

Gene	Primers	Sequence (5′–3′)
Metacaspase-1	Yca1_FP	CATATGCCACAGGAAACAGG
	Yca1_RP	CACGCGTTCTCTATCCACAT
Subtilisin-like protease 3	Ysub_FP	TAATCTCCCAGGACCCAATC
	Ysub_RP	TCCCTGTGCGAAACTCTATG
Calpain-like protease 1	Cal_FP	CTTGGTGCTGGAACACCTTA
	Cal_RP	ATTCCCATCAGTGAGCTTCC
IRD-7	IRD7_FP	TGTTGTGCAGGCCTCAAG
	IRD7_RP	GGTCAGACTCTCCCTCACAAA
IRD-9	IRD9_FP	CAATAGTAGTGCAGGCCTCAAG
	IRD9_RP	GGTCAGACTCTCCCTCACAAA
IRD-12	IRD12_FP	TATGCACCAATTGCTGTGC
	IRD12_RP	CACAAATGAAAGTCCCGTCA
Tomato PI	Forward	CTTGGGTTCGGGATATGC
	Reverse	CATTACAGGGTACATATTTGCC
CanPI7	Forward	GTTCCGAAGCAAGTGCAG
	Reverse	CATACAGACATAGTGAATAGGC
Elongation factor	Forward	GGCCATCAGACAAACCACTC
	Reverse	TCCTGGAGAGCTTCGTGGTGC

APPENDIX II

MS composition used for tomato transformation

Component	Germination	Co-cultivation	Selection media	Rooting media
MS salt (g/ L)	4.4	4.4	4.4	4.4
Sucrose(g/ L)	30	30	30	30
Agar%	0.8	0.8	0.8	0.8
pH	5.7	5.7	5.7	5.7
Cefotaxime (mg/ L)	0	0	400	400
Kanamycin (mg/ L)	0	0	100	100
Hormone	0	BAP (2 mg/ L)	Zeatin (1 mg/ L)	IBA (1 mg/ L)

CTAB buffer

Reagent	Amount to add (for 10 mL)
Cetyltrimethyl ammonium bromide (CTAB) (10% in H2O)	3 mL
5 M NaCl	2.8 mL
0.5 M EDTA (pH 8.0)	0.4 mL
1 M Tris-Cl (pH 8.0)	1 mL
Polyvinylpyrrolidone	0.3 g
β-Mercaptoethanol	0.02 mL
H2O	2.48 mL

Luria-Bertani medium

Ingredients	g / Litre
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

APPENDIX III

Sequences used in the present study:

CanPI7 nucleotide sequence (GenBank: DQ005913.1) (789 bp)

ATGGCTGTTCCCAAAGAAGTTAGTTTCCTTGCTTCCCTACTTGTACTTGGAATATTGCTTCTACATGTTG
ATGCCAAGGCTTGTTCACAAAGAAACGCAAAAGAACCCATATGCACCAATTGTTGTGCAGGCCGTAAGGG
TTGCAACTATTACAGTGCTGATGGGACTTTCATTTGTGAAGGAGAGTCTGACCCCAACAACCCAAAACCT
TGTACTTTGAACTGTGATCCAAGAATTTTCTATTCAAAGTGTCCACGTTCCGAAGGAAACGCAGAAAATC
GCATATGCACCAATTGCTGTGCAGGCCGTAAGGGTTGCAACTATTACAGTGCTGATGGGACTTTCATTTG
TGAAGGAGAGTCTGACCCCAACAACCCAAAACCTTGCCCTCGGAATTGTGATACAAGAATTGCCTATTCA
AAATGTCCACGTTCCGAAGGAAACGCAGAAAATCGCATATGCACCAATTGCTGTGCAGGTCGTAAGGGTT
GCAACTATTACAGTGCTGACGGGACCTTCATTTGTGAAGGAGAGTCTGACCCCAACAACCCAAAACCTTG
CACTCTGAACTGTGATCCAAGAATTTTCTATTCAAAGTGTCCACGTTCCGAAGCAAGTGCAGAACAACCC
ATATGCACCAATTGTTGTGCAGGCCTCAAGGGTTGCAACTATTACAATGCTGACGGGACTTTCATTTGTG
AGGGAGAGTCTGACCCCAACCACCCAAAAGCTTGCCCCAAGAATTGTGATCCTAATATTGCCTATTCACT
ATGTCTGTATGAAAAGTAA

CanPI7 protein sequence (Uniprot ID: Q4ZIQ5)

MAVPKEVSFLASLLVLGILLLHVDAKACSQRNAKEPICTNCCAGRKGCNYYSADGTFICEGESDPNNPKP
CTLNCDPRIFYSKCPRSEGNAENRICTNCCAGRKGCNYYSADGTFICEGESDPNNPKPCPRNCDTRIAYS
KCPRSEGNAENRICTNCCAGRKGCNYYSADGTFICEGESDPNNPKPCTLNCDPRIFYSKCPRSEASAEQP
ICTNCCAGLKGCNYYNADGTFICEGESDPNHPKACPKNCDPNIAYSLCLYEK

IRD7

EPICTNCCAGLKGCNYYNADGTFICEGESDPNHPKACPKNCDPNIAYSLC

IRD9

QPICTNSSAGLKGCNYYNADGTFICEGESDPNHPKACPKNCDPNIAYSLC

IRD12

NRICTNCCAGRKGCNYYSADGTFICEGESDPNNPKACPRNCDTRIAYSKC

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2. **Tanpure R. S**. , Barbole R. S. , Dawkar V. V. , Waichal Y. A. , Giri A. P. and Gupta V. S. Improved tolerance against *Helicoverpa armigera* in transgenic tomato over-expressing multi-domain proteinase inhibitor gene from *Capsicum annuum*. ***Physiology and Molecular Biology of Plants***: 23, Issue 3, (2017) 597-604

3. **Tanpure, R. S**., Lomate, P. R., Dawkar, V. V., Gupta, V. S. and Giri, A. P. Transgenic plants for insect tolerance: current status and future prospects. ***Biotechnology: Beyond Borders***. M.V. Deshpande, J. Ruiz-Herrera. (2013) 172- 187.

4. Dawkar, V. V., Chougale, A. D., Barvkar, V. T., **Tanpure, R. S.**, Giri, A. P. Genetically Engineered Crops: Opportunities, Constraints, and Food Security at a Glance of Human Health, Environmental Impact, and Food Quality. ***Genetically Engineered Foods*** (Series: Handbook of Food Bioengineering), 6 (2017) 309-332.

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