# Development of efficient strategies for control of Lepidopteran insect pest

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In

**BIOTECHNOLOGY**

By

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*Dedicated to my beloved parents*

*And*

*The biotechnologists*

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# CERTIFICATE

Certified that the work in the Ph.D. thesis entitled **'Development of efficient strategies for control of Lepidopteran insect pest'** submitted by **Ms. Yojana R. Chikate** was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

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# DECLARATION

I hereby declare that the thesis entitled **'Development of efficient strategies for control of Lepidopteran insect pest'** submitted for Ph.D. degree to the Savitribai Phule Pune University has not been submitted by me for a degree at any other university.

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# Contents

## Abbreviations

| | |
|---|---|
| ACN | Acetonitrile |
| AD | Artificial diet |
| Ba*p*NA | N-a-benzoyl-DL-arginine *p*-nitroanilide |
| BSA | Bovine serum albumin |
| Bt | *Bacillus thuringiensis* |
| CanPI | *Capsicum annuum* proteinase inhibitor |
| CBB-R250 | Coomasssie brilliant blue-R250 |
| cDNA | Complementary deoxyribonucleic acid |
| CDNB | 1, 2-dichloro-1-dinitrobenzene |
| CHAPS | 3-[(3-cholamidopropyl) dimethylammonio]-1 propane sulfonate |
| CI | Chymotrypsin inhibitor |
| DDT | Dichlorodiphenyltrichloroethane |
| DIA | Data independent analysis |
| DNA | Deoxyribonucleic acid |
| DPPH | 1, 1-Diphenyl-2-picrylhydrazyl |
| dsRNA | Double stranded ribonucleic acid |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetra acetic acid |
| g, mg, µg, ng | gram, milligram, microgram, nanogram |
| GC-MS | Gas chromatography mass spectrometry |
| GFP | Green fluorescent protein |
| GXCT | Gel-X ray film contact-print technique |
| HaAce4 | *Helicoverpa armigera* acetylcholinesterase |
| HaCAHTL | *Helicoverpa armigera* cathepsin L |
| HaCAT | *Helicoverpa armigera* catalase |
| HaCda | *Helicoverpa armigera* chitin deacetylase |
| HaChy | *Helicoverpa armigera* chymotrypsin |
| HaCuZnSOD | *Helicoverpa armigera* super oxide dismutase |
| HaFabp | *Helicoverpa armigera* fatty acid binding protein |
| HaGAPDH | *Helicoverpa armigera* glyceraldehyde phosphate dehydrogenase |
| HaGST | *Helicoverpa armigera* glutathione S- transferase |
| HaJHE | *Helicoverpa armigera* juvenile hormone esterase |
| HaTry | *Helicoverpa armigera* trypsin |
| HCl | Hydrochloric acid |
| HDMS | High definition mass spectrometry |
| HGP | *H. armigera* gut proteases |
| IC50 | Inhibitor concentration at 50% inhibition |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| IRD | Inhibitory repeat domain |

| | |
|---|---|
| KCL | Potassium chloride |
| kDa/kD | kilo Dalton |
| L, mL, μL | liter, milliliter, microliter |
| LCMS | Liquid chromatography mass spectrometry |
| M, mM, μM | molar, millimolar, micromolar |
| MALDI-TOF | Matrix assisted laser desorption ionization time of flight |
| mmole, μmole | millimole, micromole |
| mRNA | messenger RNA |
| MSA | Multiple sequence alignment |
| MW | Molecular weight |
| MZ | Maize (*Zea mays*) |
| NaCl | Sodium chloride |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NaOH | Sodium hydroxide |
| NBS | National Bureau of Standards |
| $NH_4HCO_3$ | Ammonium bicarbonate |
| NIST | National institute of standards and technology (mass spectral library) |
| OK | Okra (*Abelmoschus esculentus*) |
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| PDB | Protein data bank |
| pI | Isoelectric point |
| PI | Proteinase inhibitor |
| PLGS | Protein lynx global server |
| PP | Pigeon pea (*Cajanus cajan*) |
| PSI-BLAST | Position specific iterated Basic local alignment search tool |
| rCanPI | recombinant *Capsicum annuum* proteinase inhibitor |
| RMSD | Root mean square deviation |
| RNA | Ribonucleic acid |
| RO | Rose (*Rosa rubiginosa*) |
| rpm | Revolutions per minute |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SAAPLpNA | N-Succinyl-Ala-Ala-Pro-Leu-nitroanilide |
| SDS | Sodium dodecyl sulphate |
| TCA | Trichloroacetic acid |
| TFA | Trifluoroacetic acid |
| TI | Trypsin inhibitor |
| TIU | Trypsin inhibitory units |
| TLCK | N-a-*p*-tosyl-l-lysine chloromethyl ketone |
| TPCK | N-tosyl-l-phenylalanine chloromethyl ketone |
| TRIS | Tris-hydroxymethyl aminomethane |
| UPLC | Ultra performance liquid chromatography |

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# THESIS SUMMARY

## Development of efficient strategies for control of Lepidopteran insect pest

*Helicoverpa armigera* is one of the major crop pests and is less amenable to current pest control methods. Sequence-specific mRNA degradation by RNA interference (RNAi) is emerging as a potent insect pest control strategy. This technology offers great potential in developing effective pest control strategy over current control methods and their resistance by pest. With this background, I have carried out my thesis work on polyphagy and adaptability in *H. armigera*. This involved identification of proteases and other enzymes/proteins responsible for polyphagy and its adaptation to non-host plant and synthetic pesticide, using comparative biochemical and molecular approaches. Following which the identified candidate proteins/enzymes were checked for gene silencing using dsRNA in *H. armigera* and their possible use as species specific pesticide.

## Differential protease activity augments polyphagy in *Helicoverpa armigera*

We studied the diversity of *H. armigera* gut trypsins and chymotrypsins through insect developmental stages (larvae, pupae and adult) on four nutritionally diverse host plants, namely, okra (OK; *Abelmoschus esculentus*), rose (RO; *Rosa rubiginosa*), pigeon pea (PP; *Cajanus cajan*) and maize (MZ; *Zea mays*), using real-time quantitative PCR and enzyme activity assays. The expression of *HaTry*(s) across developmental stages, when *H. armigera* were reared on OK, RO, PP and MZ was relatively high compared with that of *HaChy*(s). All HaTrys accumulated more at the fourth-instar larval stage, with *HaTry4* the highest, followed by *HaTry2*, *HaTry3* and *HaTry1*; in contrast, *HaTry5*, *HaTry7* and *HaTry8* showed weak expression. The pupal stage showed enormously high expression of *HaTry8* and *HaTry2*. The expression level of HaTrys at the adult stage was the lowest regardless of diet. *HaChy4* was expressed at the highest levels in larvae reared on MZ, followed by those reared on PP, OK and RO. The pupal stage showed higher expression for *HaChy1*, with maximum expression in pupae of the larvae reared on PP, followed by OK, MZ and RO. The expression of *HaChy3* was the highest in adults of the larvae reared on MZ followed by those reared on PP. We assessed protease activities in developmental stages and characterized those using synthetic inhibitors and diverse *Capsicum annuum* recombinant PIs (rCanPIs). A nano-Liquid Chromatography Mass

Spectrometry-elevated energy (nano-LCMSE) approach was used for the identification of differentially expressed proteases from larvae feeding on RO and PP and was confirmed by gene expression analysis. By superimposing predicted structures, we observed divergence in the catalytic and binding sites of differentially expressed trypsin isoforms. This data enhances the understanding of digestive physiology of *H. armigera* with emphasis on dynamics of gut protease expression and their interactions with different substrates and inhibitors.

**Molecular responses of *H. armigera* upon exposure to host and non-host plants, and synthetic pesticide**

To develop effective toxin resistance management strategies, a complete understanding of the physiological and genetic mechanisms by which insects become resistant to insecticidal proteins and/or phytochemicals is needed. The purpose of this study was to identify the key proteins and/or enzymes governing adaptation and/detoxification capability in *H. armigera* larvae. This involved comparative biochemical and molecular analyses to investigate the proteomic differences in gut, hemolymph and frass of *H. armigera* reared on normal host diet chickpea, *Cicer arietinum* (Cp), non host *Cassia tora* (Ct) and synthetic insecticide (chlorpyrifos-based) CH diet. There was a 2- to 3-fold reduction in larval mass in the larvae fed on the Ct diet. Food intake was dramatically reduced in the larvae grown on the Ct diet compared to the Cp diet resulting into stunted growth. Furthermore with proteomic analysis, overall 46 differentially expressed proteins were successfully identified, among which 25 proteins were from gut, 15 from hemolymph and 6 from frass. In gut proteome of *H. armigera*, 17 proteins related to digestion, immunity, energy production and apoptosis mechanism were up-regulated and 8 proteins involved in carbohydrate metabolism, lipid metabolism and energy transfer were down-regulated. Biochemical studies revealed that various enzymatic activities were reduced in the Ct-fed *H. armigera* larvae than in the control diet (Cp) fed larvae. The results of qRT-PCR indicate tissue specific expression profiles of selected isoforms of the identified candidate proteins. Decoding *Helicoverpa armigera* resistance mechanism against phytochemicals and synthetic insecticides is a challenge. Here, we investigated the response of *H. armigera* to synthetic organophosphate insecticide, chlorpyrifos (CH) by feeding the larvae on artificial diet supplemented with CH (CH-AD). The $LD_{50}$ concentration of CH was found to be 8 ppm and surviving larvae showed stunted growth, abnormal pupae and adult development. Proteomic analysis of *H. armigera*

gut tissue using nano-Liquid chromatography mass spectrometry revealed up regulation of 23-proteins (fold change from 1.5 to 8.4) upon CH-exposure. These proteins were identified with high confidence, number of peptides 3 to 28 and protein coverage from 12 to 66%. These up-regulated proteins belong to metabolic processes such as digestion, defense, energy metabolism, detoxification, etc. shown by gene ontology analysis. Biochemical data of candidate proteins such as proteases, CYP, Arg kinase indicated that insects struggle to obtain required nutrient and energy in presence of CH at the same time endeavoring to metabolize CH, which was also evident from gene expression profiles. Furthermore, we proposed a potential processing pathway of CH in *H. armigera* gut by examining the metabolites by gas chromatography mass spectrometry. Our results suggest that *H. armigera* can regulate the expression of proteins involved in digestion and detoxification mechanisms to adjust the nutritional requirements and neutralize the toxicity caused by insecticide molecules. This study showed that larva deals with plant antifeedant and a synthetic pesticide (toxin) by distinct pathways.

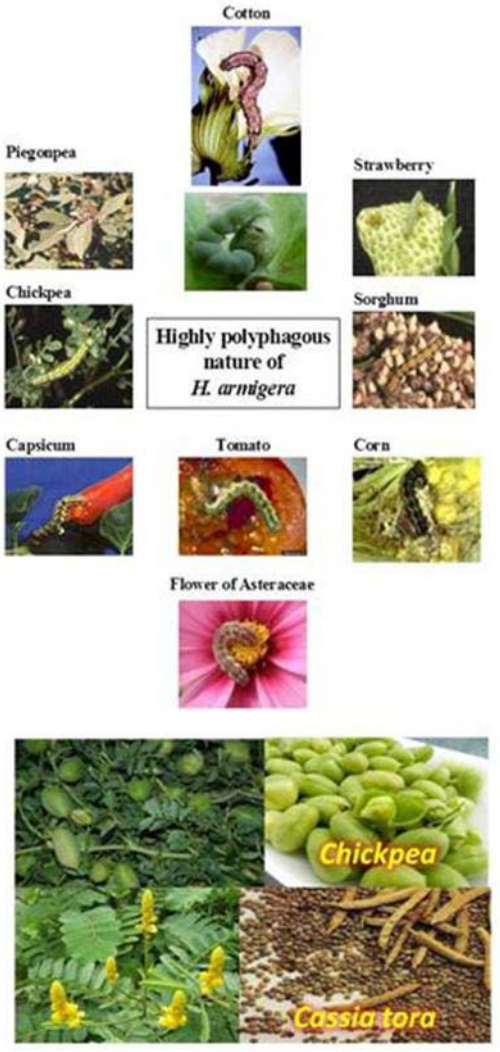
**Interruption of *Helicoverpa armigera* development upon silencing of diverse genes by dsRNA feeding**

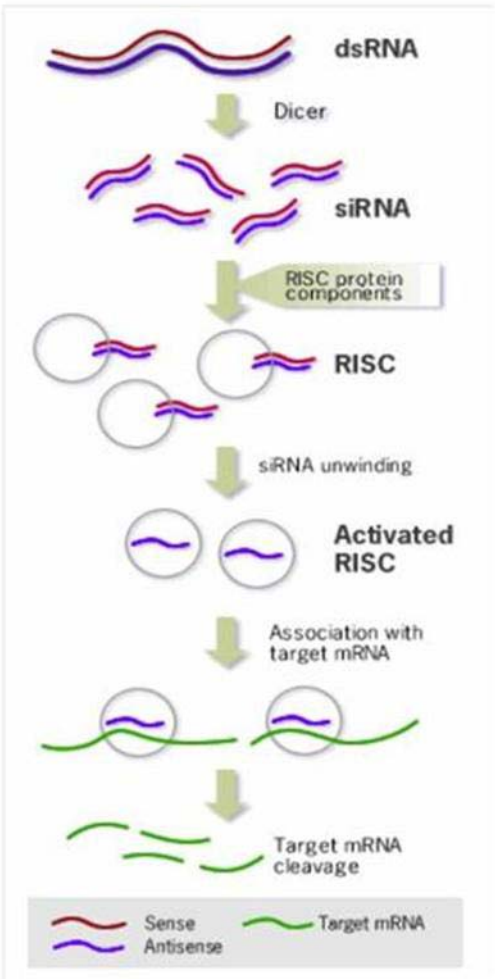
In this study we tested total of 15 dsRNAs' targeting major classes of enzyme/key proteins namely, proteases like trypsin (*HaTry2, 3, 4* and *6*), chymotrypsin (*HaChy4*) and cysteine proteases like Cathepsin (*HaCATHL*); glutathione-s-transferases (*HaGST1a, 6 and 8*); esterases (*HaAce4, HaJHE*); catalase (*HaCAT*); super-oxide-dismutase (*HaCu/ZnSOD*); fatty acid binding protein (*HaFabp*) and chitin deacetylase 5b (*HaCda5b* for gene silencing in *H. armigera* by supplying them with artificial diet (AD). Around 300 bp unique sequence region was selected from each mRNA for design and synthesis of dsRNA. The 300-400 bp fragments were amplified using gene-specific sequence adapted to T7 promoter sequence (in inverted orientation) as primer and cloned into $P^{GEMT}$ vector. Upon sequence confirmation of these plamids they were used as a template for *in vitro* dsRNA synthesis using Megascript RNAi kit. Initially 20 µg of dsRNA (coated on AD) was tested for trypsin and GST mRNA, which showed minimal effect on larvae and the mRNA levels were not reduced significantly. Therefore we tested 60 µg of dsRNA for all chosen 15 genes by delivery through AD, as surface applications (three applications at interval of 24 h.). *HaGAPDH* and *GFP* targeting dsRNA were used as positive and negative control respectively. Growth and development of *H. armigera*

larvae was observed for all treatments for a period of 10 days, in terms of phenotypic abnormalities, stunting and larval mass gain. Along with this, larval tissues from all treatment groups were harvested at interval of 2, 4 and 8 DPE (days post exposure) for further biochemical and molecular analyses. Highest mortality was observed in *HaAce4* dsRNA fed larvae (85%) followed by those fed with *HaJHE*; *HaCAT*; *HaCuZnSOD*; *HaFabp* and *HaTry3* whereas the rest dsRNAs' showed less mortality. Significant reduction of larval weight gain was observed at 48h., which later was consistent with only 6 dsRNAs' (*HaCATHL, HaAce4*, *HaJHE*, *HaCAT, HaCuZnSOD* and *HaCda5b*, 144h. post dsRNA feeding. Out of 15 dsRNAs, 7 dsRNAs' (*HaTry4*, *6*; *HaFabp*; *HaGSt1*, *8*; *HaAce4*; *HaCda5b*) resulted into malformed larvae at a dose of 60 µg (applied thrice at 24h. interval) when observed over a period of one week. At pupal stage *HaTry2*, *3*, *4*, *6*; *HaChy4*; *HaCATHL* and *HaCuZnSOD* exhibited the deformity in pupae. However the extent of malformation was more in case of moth as shown by 12 dsRNAs'. Significant reduction of cognate mRNA expression was observed 2 days post dsRNA feeding, but the mRNA level was found to be upregulated at 8 days post feeding. Interestingly trypsin and GST-like enzyme activities were found to be decreased at 2 and 8 days post dsRNA feeding. These findings might provide far scenario of designing potent dsRNA as species-specific pesticide.

# Chapter 1

## General introduction and review of literature

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# Chapter 1

## General introduction and review of literature

### 1.1 Lepidopteran insect pests

Insecta (comprising about 1 million species of insects) is one of the largest and most diverse groups of invertebrates evolved 400 million years ago. Insects being involved in diverse roles such as soil turning and aeration, dung burial, pest control, pollination and wildlife nutrition, form one of the important component of ecosystems. Insecta consists of around 30 orders out of which four namely, Coleoptera (beetles), Lepidoptera (butterflies and moths), Diptera (flies) and Hymenoptera (wasps, bees and ants) are dominant. Lepidoptera is the second largest order of insects that includes the butterflies, skippers and moths. The order has more than 1, 80, 000 species in 127 families and 46 superfamilies (Kristensen, 1999). The word "Lepidoptera" means "scaly-wing", from the ancient Greek words *lepidos* and *pteron* (Douglas, 2011). Lepidopterans are found throughout the world in all types of environment. Many of them have survived since the last ice age on isolated southern mountaintops (Scoble, 1995). This is primarily due to ultra adaptive nature of Lepidopterans. Dealing to different type of food is complex process which involves biochemical and chemical reactions that power life.

Understanding the biology and ecology of Lepidopteran pest is critical from an applied perspective, as several species target cultivated crops (Kozhanchikov, 1956). Major agricultural pests belong to Lepidoptera and can be oligophagous or ploy phagous. It seems that with different adaptive value linked with physiological situations in the environment, Lepidopterans have evolved distinct mechanisms to save themselves. Lepidopteran insect pests cause a significant amount of damage to important crops in the world affecting global crop yields and hence efficient control strategy is required to protect high value crops (Chatterjee and Mondal, 2012). The larval stage causes major damage to an array of economically valuable crops including chickpea (*Cicer arietinum*), maize (*Zea mays*), okra (*Abelmoschus esculentus*), sorghum (*Sorghum bicolor*), cotton (*Gossypium spp*), tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*),

potato (*Solamum tuberosum*), flax (*Linum usitatissimum*), soybean (*Glycine max*), other Leguminosae, and a number of fruits and forest trees (Giri *et al.,* 1998; Harshulkar *et al.,* 1999; Patankar *et al.,* 2001; Chougule *et al.,* 2005; Srinivasan *et al.,* 2006; Kotkar *et al.,* 2009; Sarate *et al.,* 2012; Chikate *et al.,* 2013); while moths and butterfly larvae are also serious pests in agriculture and forestry. Lepidopteran pests such as *Helicoverpa armigera*, *Plutella xylostella*, *Spodoptera litura*, *Leucinodes orbonalis* and *Earias fabia* are among the major pests of crops. Although they damage valuable plants, many species are important for their role in pollination and few species such as silkworm (*Bombyx mori*) are beneficial to humans. Silkworms are well-known for silk production, can be used as a bioreactor for proteinaceous drugs and as a source of biomaterials.

Each year billions of dollars are spent worldwide on pest control in agriculture (Krattiger, 1996; Fernandez-Cornejo *et al.,* 1998a; Grube *et al.,* 2007). Classically broad spectrum chemical insecticides have been the primary control agent for agricultural pests, with about 40% targeted to the control of lepidopteran insects (Brooke *et al.,* 1999). Despite this expenditure, 40% crop losses are due to insect damage, particularly in developing countries (**Fig. 1.1**). Widespread use of pesticides has resulted into pesticide resistant insects, a drop in beneficial insect populations and harmful effects to humans and the environment (Gunning *et al.,* 1991; Fitt, 1994; Gatehouse *et al.,* 1994; Haq *et al.,* 2004). These problems have putforth major challenges to develop novel and efficient pest control strategies using both synthetic and natural molecules that are more environmentally friendly.

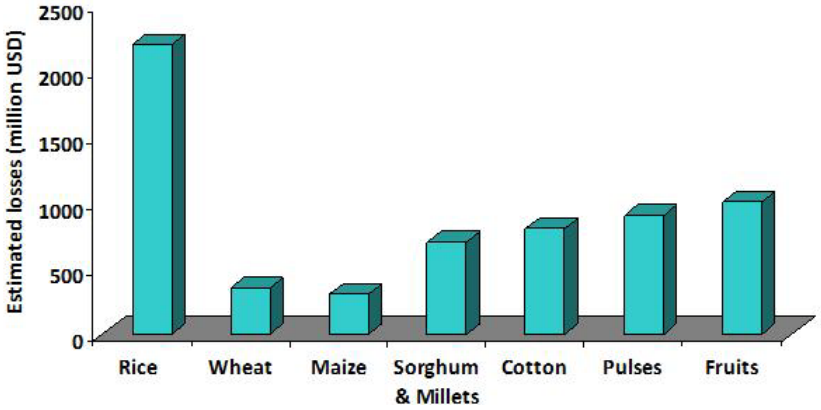



**Figure1.1** Crop losses due to insect pests (Modified from Reddy and Zehr, 2004)

## 1.2 Plant-pest interactions

Plants have evolved various dynamic defense strategies to improve their survival and reproduction. Plants can produce various secondary metabolites (phytochemicals), which affect the performance and endurance of herbivores (Scoble, 1995; Karban *et al.,* 1997). Plant defense mechanisms vary widely, ranging from mechanical to specific chemical effects that include digestibility reducers and toxins (Tollrian and Harvell, 1999; Sarmento *et al.,* 2011). One advantage of inducible defense mechanisms over constitutive ones is that they are initiated only when needed and are therefore potentially less costly (Karban *et al.,* 1997; Walling, 2000). Plant secondary metabolites include phenolics, terpenoids, alkaloids, and their derivatives. Co-evolution with herbivores has allowed plants to recognize key metabolic processes involved in insects physiology such as nervous (neurotransmitter synthesis, receptor activation, enzymes involved in signal transduction), digestive, and endocrine systems and produce specialized plant defense molecules targetting these processes (Rosenthal *et al.,* 1992; Wink, 2000; Wu and Baldwin, 2009). Essential oils and phenolics act by disrupting the endocrine system of insects and inhibiting important enzymes, respectively (Reynolds *et al.,* 1987, Balandrin *et al.,* 1988; Cheeke, 1989). For example, the well-known cotton phenolic pigment gossypol is a repellent for several insects due to its toxic effects (Maxwell *et al.,* 1965; Abou-Donia, 1989). Terpenoids are chemically the most diverse class of bioactive natural products (over 50,000 structures) produced by plants, which have key role against insects as antifeedants, repellents, and toxins (Aharoni *et al.,* 2005). For instance, phytoecdysones steroids from the common fern (*Polypodium vulgare*) disrupt insect molting by mimicking molting hormones (Canals *et al.,* 2005).

In the case of Lepidopteran larvae, plant drimane sesquiterpenes block stimulatory effects of glucose and inositol on chemosensory receptor cells located on insect mouthparts (Gershenzon and Dudareva, 2007). Alkaloids (over 12, 000 structures) can modulate insect enzymes or alter carbohydrate and fat storage by inhibiting the formation of phosphodiester bonds (Howe and Jander, 2008; Ziegler and Facchini, 2008). By disrupting nerve transmission in insects, alkaloids can also affect cell membrane and cytoskeletal structures, causing cells to weaken, collapse, or even leak (Petterson *et al.,* 1991; Fattorusso *et al.,* 2007). Interestingly, certain alkaloids such as pyrrolizidine occur

naturally in many plants as nontoxic N-oxides, which are converted into the toxic, uncharged, hydrophobic tertiary alkaloids in alkaline digestive tracts of insects (Hartmann, 1999). Upon ingestion by insects, plant cyanogenic glycosides are metabolized by β-glucosidases, resulting in the formation of the sugar and cyanohydrin moieties, and the latter spontaneously decomposes to toxic hydrogen cyanide (Vetter, 2000). Hydrogen cyanide inhibits the insect mitochondrial respiratory pathway enzyme cytochrome c oxidase (Brattsten *et al.,* 1983; Davis *et al.,* 1985; Zagrobelny *et al.,* 2009). Similar to cyanogenic glycosides, glucosinolates (GSLs) are protected in vacuoles from thioglucosidases called myrosinases (Grob and Matile, 1979) that hydrolyze GSL into toxins and feeding repellents such as isothiocyanates, nitriles, and thiocyanates.

Plants also produce several defense proteins that limit the rate of enzymatic conversion of ingested food in insects by altering either physical availability or chemical identity (Duffey and Stout, 1996). The major classes of such defense proteins are proteinase inhibitors (PIs), α-amylase inhibitors, lectins, polyphenol oxidases (PPOs), threonine deaminase, and arginase. PIs inhibit digestive proteases in insects, leading to reduced growth performance, weight loss, or even death of insects (Tamhane *et al.,* 2005, Dunse *et al.,* 2010; Lomate and Hivrale, 2012). Insect can overcome the adverse effects of plant proteinaceous molecules in myriad ways. Switching enzymes with altered substrate specificity and upregulation of chymotrypsin-like activity to counter trypsin inhibitor has been reported in *H. armigera* (Tamhane *et al.,* 2005; Mahajan *et al.,* 2013). Lectins are sugar-binding proteins found particularly in storage organs and protective structures of plants and inhibit nutrient absorption in insects (Chrispeels and Raikhel, 1991).

Plant PPOs are known to be induced upon wounding, and this is suggestive of their role in defense. For example, *S. litura* showed decreased growth with increased mortality upon feeding on transgenic tomato plants overexpressing a PPO (Mahanil *et al.,* 2008). Furthermore, PPO activity produces highly reactive O-quinones that might reduce the nutritive value of plant proteins by covalently modifying free amino groups (Baldwin and Preston, 1999; Constabel *et al.,* 2000). Threonine deaminase and arginase deplete the level of amino acids Thr and Arg, respectively, leading to adverse effect on insects (Chen *et al.,* 2005; Kang *et al.,* 2006).

## 1.3 *Helicoverpa armigera*: Major polyphagous pest of agricultural crop plants

*H. armigera* (Hübner) (Lepidoptera: Noctuidae) is a highly polyphagous agricultural pest. It is also known as pod borer, cotton bollworm, corn earworm, tobacco budworm and tomato fruit borer. The voracious caterpillars of *H. armigera* can feed on leaves and stems, but they exhibit a strong preference for reproductive organs such as buds, inflorescences, berries, pods, capsules etc. They bore into these parts, leaving large, round holes. *H. armigera* has been recorded feeding on around 300 cultivated and uncultivated plant species belonging to 45 families in India (Manjunath *et al.,* 1989; add recent reference here) with uncovering reports of new plant species as its host. This pest has received attention due to its wide geographical presence and heavy economic losses imparted to a variety of crops such as cereals, pulses, cotton, vegetables, chickpea, pigeon pea, sorghum, sunflower, soya bean and groundnuts fruit crops (Cunningham *et al.,* 1999). The global distribution of *H. armigera* is shown in **Fig. 1.2**. The pest is widely present in Asia, Africa and Oceania (EPPO, 2006). It is one of the most serious pests in cotton-producing countries like Australia, India and China, causing enormous economic problems (Estébanez-Perpiñá *et al.,* 2001; Downes *et al.,* 2007).

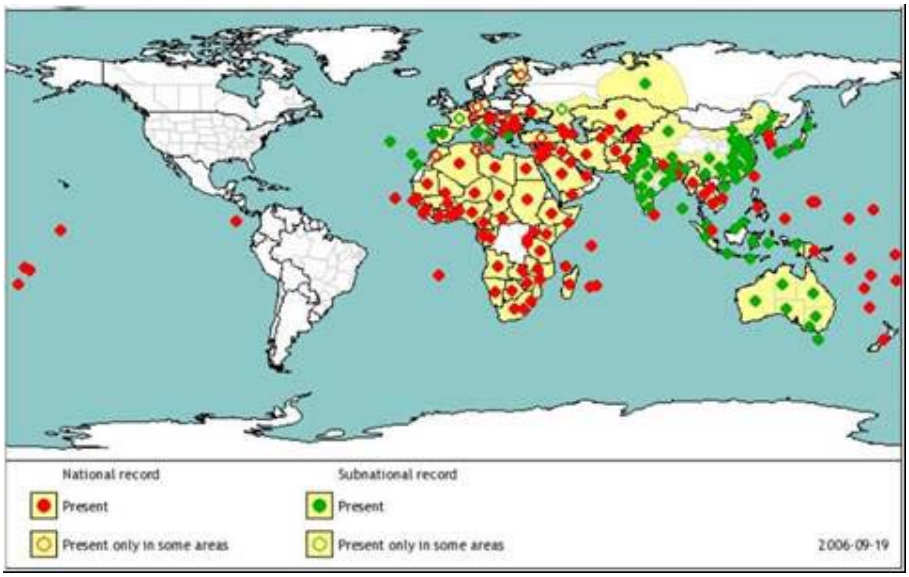



**Figure 1.2** Geographical distribution of *H. armigera* (Source: Plant protection service (NL) and Central science laboratory (UK) joint pest risk analysis for *H. armigera,* June 2007)

*H. armigera* infestation causes a worldwide loss of US$ 7.5 billion despite the use of insecticide worth US$ 2 billion (Gowda *et al.,* 2005). It has developed resistance to many widely used chemical insecticides such as organophosphates, carbamates and pyrethroids. The attributes of polyphagy, high adaptability, high fecundity, facultative diapauses, excellent migratory abilities (moths) and its cosmopolitan nature makes it a severe pest of agronomic importance. Currently widely accepted methods for *H. armigera* control are spraying of synthetic insecticides and use of transgenic *Bacillus thuringiensis* (Bt) toxin plants (Peferoen 1997; Schnepf *et al.,* 1998; Heckel, 2012). However, due to extensive but unplanned spraying of insecticides, this pest have become resistant to most of the insecticides by mutations in the target receptor or overexpression of detoxifying enzymes (Metcalf 1989; Fournier *et al.,* 1992; Park *et al.,* 1997; Enayati *et al.,* 2005; Dawkar *et al.,* 2011, 2013). Transgenic plants producing Bt toxins have proven to be successful in controlling few major insect pests but their long-term and large area of cultivations has lead to sporadic cases of development of resistance against Bt toxin (Edwards 1993; Tabashnik *et al.,* 2008, 2013).

### 1.3.1 Life Cycle of *Helicoverpa armigera*

*H. armigera* progresses through four stages of development; egg, larva (six instars), pupa and adult (moth) as shown in **Fig. 1.3**. Oviposition occurs at dawn, female moths lay around 500-3000 eggs per generation (Ramaswamy, 1990) on reproductive structures of plants such as on the lower surface of leaves, flower buds, flowers and young pods and on the shoot tips (Fitt, 1989). Eggs upon hatching develop into larvae through six larval instars, a period of 12 to 16 days. The pre-pupal stage lasts for 1-4 days. Sixth instar larvae undergo pupation where they become dormant, period of which is variable. The pupal stage lasts for 10 to 14 days if not in diapause, while in case of diapauses, the pupal period can take several months to complete (Reed, 1965). Pupae emerge into moths just after dark to midnight; they are active for 4 to 5 days and are further maintained in breeding chambers for next generation larvae. Moths feed on nectar (honey or sucrose in laboratory conditions), females release sex pheromones and mating occurs approximately 4 days after emergence. During winter, the pupal duration is longer and moths emerge out on the onset of warmer weather, which coincides with the podding

stage of the hosts like chickpea and pigeon pea, and therefore cause a severe damage to crops (Shanower and Romeis, 1999).

## Classification of *H. armigera:*

| | |
|---|---|
| Kingdom | *Animalia* |
| Phylum | *Arthropod* |
| Class | *Insecta* |
| Order | *Lepidoptera* |
| Suborder | *Ditrysia* |
| Superfamily | *Noctuoidea* |
| Family | *Noctuidae* |
| Genus | *Helicoverpa* |
| Species | *H. armigera* |

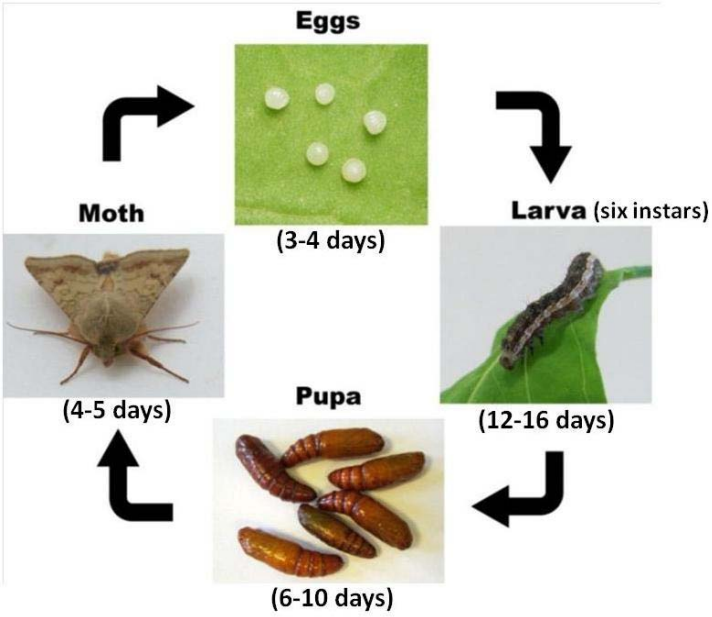



**Figure 1.3** Life cycle of *Helicoverpa armigera*

### 1.3.2 Biochemical and physiological strengths of *H. armigera*

The larvae of *H. armigera* is extremely damaging because they feed voraciously on the reproductive structures of crops which are rich in nitrogen (Fitt 1989) and these structures are often part of the crop that is harvested. The ability of larvae to feed on wide range of crops (i.e. it tackles variety of plant phytochemicals) indicate its highly adaptive biochemical and physiological strengths. Complex and diverse cocktail of digestive and

detoxifying enzymes facilitates polyphagy in *H. armigera*. These properties impart severe pest status to this insect.

In insects, proteases are a major group of hydrolytic enzymes categorized as endo- and exo-proteases (**Table 1**) and are predominantly involved in digestive processes, proenzyme activation, metamorphosis, release of physiologically active peptides and complement activation (Terra and Ferreira, 1994; Borovsky and Mahmood, 1995; Terra, 1988; Huang *et al.,* 2010). Serine proteases, namely, trypsin and chymotrypsin are abundant in the digestive tract of *H. armigera* (Srinivasan *et al.,* 2006). Twenty-one trypsin, 14 chymotrypsin, two elastase-like, and several aminopeptidase and carboxypeptidase genes were found in the gut tissue of *H. armigera* reared on a high-protein diet free of inhibitors (Bown *et al.,* 1997, 1998, 2004; Gatehouse *et al.,* 1999; Chougule *et al.,* 2005; Angelucci *et al.,* 2008). These enzymes are released extracellularly into the gut lumen and are active at an alkaline pH (Johnston *et al.,* 1991; Purcell *et al.,* 1992). The gut proteinase activity increases during larval development with the highest activity seen in the fifth instar larvae, followed by a sharp decline in the sixth instar. Over 90% of the gut proteinase activity of the fifth instar larvae is of serine proteinase type; however the second instar larvae show presence of other mechanistic classes like metalloproteases, aspartic and cysteine proteinases along with serine proteinase activity (Patankar *et al;* 2001). Trypsin and chymotrypsin like proteases from *H. armigera* gut have been purified and characterized (Johnston *et al,* 1991; Telang *et al,* 2005). The levels of gut protease genes and the composition vary according to diet and developmental stage of the larvae (Broadway *et al* 1997; Patankar *et al,* 2001; Chougule *et al,* 2005; Chikate *et al.,* 2013) (**Fig. 1.4**).

Serine endopeptidases belong to the S1 family (chymotrypsin family) of peptidases which contain a catalytic triad of histidine, aspartic acid and serine (http://merops.sanger.ac.uk/). In the case of trypsins, the Asp (189), Gly (216) and Gly (226) residues contribute to a negatively charged S1 (ratio of residues flanking to the catalytic site) site; thus, they are highly specific for the positively charged side chain of arginine or lysine in the substrate. Similarly, residues of S1 sites form a deep hydrophobic pocket of chymotrypsin and make it preferable for phenylalanine, tryptophan, and tyrosine at P1 on the substrate (Srinivasan *et al.,* 2006). Dynamic

expression and an ability to regulate gut proteases augment the insect's adaptability to diverse dietary protein/anti-nutritive/toxic constituents. The midgut enzyme profile of Lepidoptera larvae reared on an artificial diet (AD) or transgenic plants containing proteinase inhibitors (PIs) show that the insects are capable of up-regulating normal gut proteases (Christeller *et al.,* 1992). Moreover, they also induce several novel inhibitor-resistant/ degrading proteases (Jongsma *et al.,* 1995; Bown *et al.,* 1997, 2004; Broadway, 1997; Gatehouse *et al.,* 1997; Jongsma & Bolter, 1997; Wu *et al.,* 1997; Giri *et al.,* 1998; Mazumdar-Leighton & Broadway, 2001a, b; Zavala *et al.,* 2008; Dunse *et al.,* 2010a, b). Previous studies have indicated that the insects possess the strength to modify gut protease, amylase and lipase expression in accordance with both natural and PI-containing diets (**Fig. 1.5**) (Harsulkar *et al.,* 1999; Patankar *et al.,* 2001; Chougule *et al.,* 2005; Tamhane *et al.,* 2005; Kotkar *et al.,* 2009, 2012; Sarate *et al.,* 2012). The insect may overcome the effect of PIs in the diet by (i) over expressing native proteinases (Gatehouse *et al,* 1997) or (ii) by expressing a new set of proteinases that are insensitive to the inhibitors or (iii) proteolytic inactivation of PIs mediated by endogenous proteinases of the insect midgut or by combination of any of the above (Giri *et al,* 1998). Thus, continuous exposure to plant PIs usually results in insect adaptation by any or all of the available choices on variety of host plants

Adaptation of insects to dietary PIs has been observed with a diverse variety of PIs including serine PIs like soybean Kunitz trypsin inhibitor (Bown *et al,* 1997), potato PI-II (Broadway and Duffey, 1986; Jongsma *et al*, 1995), aprotinin (Gatehouse *et al*, 1997) as well as cysteine PIs. Legume seeds are rich in serine PIs exhibits strong inhibitory activity against trypsin, chymotrypsin or both, but weak or null activity against other proteolytic enzymes. *H. armigera* modifies its gut composition accordingly. While feeding on legume seeds may be the larvae produces more proteolytic enzymes in order to nullify the effect of PIs. The following reports have demonstrated that insect gut proteinases neutralize the effect of PIs by degrading them: multicystatin of potato tubers by *Diabrotica* larval proteinases (Orr *et al,* 1994); oryzacystatin by black vine weevil proteinases (Michaud *et al,* 1995); TIs of chickpea by gut proteinases of podborer (Giri *et al,* 1998); and oryzacystatin and soybean Bowman-Birk TI by beetle larvae (Girard *et al*, 1998).

**Table 1: Major *Helicoverpa* insect pests and their primary digestive proteinases**

| Insect Pest | Digestive proteases (%)* |
|---|---|
| *H. armigera*, *H. zea*, *H. virescens* | trypsin (90), chymotrypsin (5), elastase (1), carboxypeptidase (1), aminopeptidase (1), cathepsin B-like (1), metalloprotease (1) |

*Figures in parentheses represent approximate percentage contribution to total gut protease activity. Data modified from Giri *et.al*; 2006

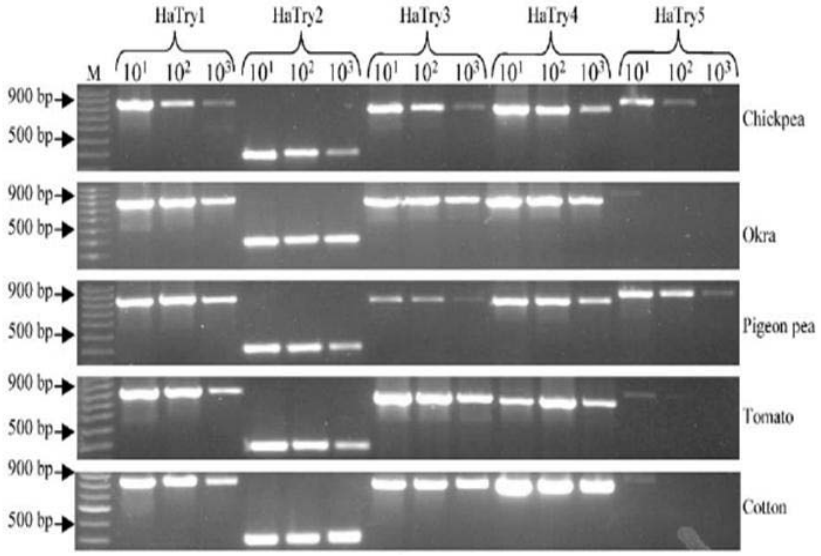



**Figure 1.4** Expression diversity of *H. armigera* trypsins in response to various diets (Adopted from Chougule *et al.*, 2005)

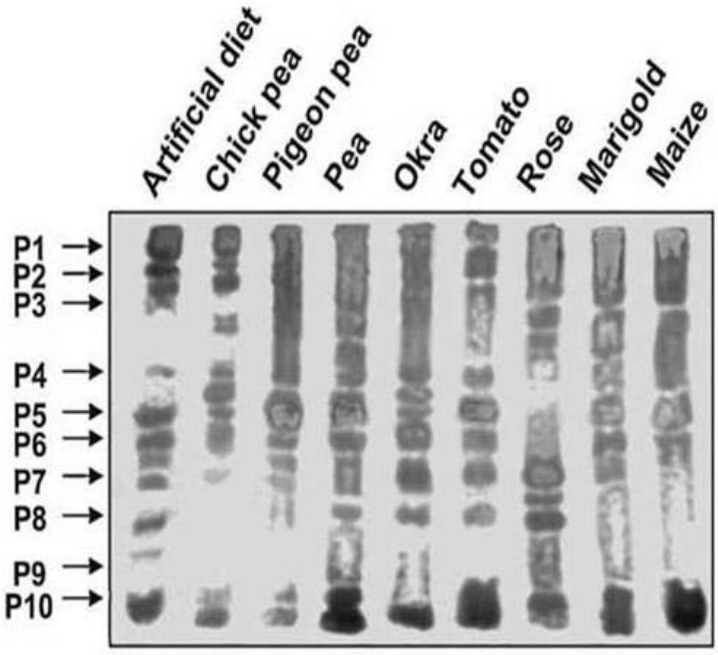



**Figure 1.5** *H. armigera* gut protease activity profile (on Native PAGE) when reared on various host plants (Adopted from Kotkar *et al.*, 2009)

## 1.4 Classical methods of *H. armigera* control

Classically farmers are practicing various methods for *H. armigera* control. These involve agricultural practices; use of bio-control agents such as pheromone traps, *H. armigera* nuclear polyhedrosis virus (HaNPV) sprays and Bt toxin sprays. Additionally there is paradigm shift in these practices and majority of control agents are chemical pesticides. Agricultural practices mainly involved manipulating the time of sowing, cropping season, spacing, and fertilizer application, deep ploughing and intercultural operations reduce the survival and build up of *H. armigera* population. Hand picking the large sized larvae, shaking the plants to make the larvae fall down, which are later, destroyed (Shanower *et al.*, 1998). Pheromones are sex hormones released by female moths to attract male moths. In fields different traps pre-applied with chemical pheromones are installed to trap the pests. NPV is *Helicoverpa* specific viral pathogen and has been used as sprays for pest control. However, these sprays have maintenance and application drawbacks and they are not cost-effective. Another well-known practice

was use of Bt toxin sprays; these proteins permeate insect gut epithelium and impair digestion thereby killing the pest. These toxins have been very effective for targeting most of the Lepidopteran pests. All these methods are environment friendly and target-specific but they are not commercially viable. These were therefore, largely replaced by chemical pesticides. Broad range of chemical pesticides has been developed and is being widely used for control of most of the crop pests.

## 1.5 Synthetic insecticides and their targeted actions

Use of chemical pesticides has become widely accepted and routine practice for crop pest control in fields. In 1939, Swiss chemist Paul Hermann Mu□ller developed the first synthetic pesticide dichlorodiphenyltrichloroethane (DDT). Furthermore, technological advancement led to the development of more efficient pest control strategies. Currently, several classes of synthetic insecticides are available in the market, *viz*. organochlorides, pyrethroids, neonicotinoids, ryanoids, organophosphates, carbamates, etc (**Table 2**). In general, these pesticides cause membrane disruption, inhibition of nutrient and ion transport, signal transduction processes, inhibition of metabolism, or the disruption in hormonal control of physiological processes in insect pests (Heckel, 2012). Most of these are broad spectrum insecticides and therefore are popular. Constantly newer derivatives of existing insecticide molecule are being developed to tackle most of the serious crop pests effectively. However, the success of these synthetic molecules is challenged increasingly by emergence of insecticide-resistant strains of pests. Another limitation for insecticide usage is their side-impact on non-pest (beneficial) insect species, disturbance of ecosystem and severe contamination of environment and human health. Yet these are quite oftenly used by farmers for pest control.

**Table 2: Mode of action of major insecticides, their target insects, and resistance mechanisms in insect pests**

| Class | Insecticide | Toxicity | Mode of action | Insect resistance status | Resistance mechanism |
|---|---|---|---|---|---|
| Organochlorides (Gunning *et al.*, 1996; Heckel, 2012) | DDT endosulfan aldrin methoxychlor dieldrin | broad spectrum | Modulate sodium channel of plasma membrane of the nervous cells | resistance | modification of para sodium channel, enzymatic detoxification, strong nerve insensitivity, penetration resistance |
| Organophosphates (Newcomb *et al.*, 1997) | chlorpyrifos acephate monocrotophos parathion piperonyl butoxide | broad spectrum | Acetylcholine esterase inhibitors | resistance | target site insensitivity mutations, knockdown resistance, enzymatic detoxification |
| Carbamates (Newcomb *et al.*, 1997) | aldicarb methomyl carbaryl carbofuran formetanate | broad spectrum | Acetylcholine esterase inhibitors | resistance | target site insensitivity mutations, knockdown resistance |
| Pyrethroids (Class and Kintrup, 1991) | cypermethrin deltamethrin fenvalerate tetramethrin etofenprox | broad spectrum | sodium channel modulators | resistance | modifications of the sodium channel protein, penetration resistance, strong nerve insensitivity |
| Neonicotinoids (Matsuda *et al.*, 2001; Nauen and Denholm, 2005 and Elbert *et al.*, 2008) | acetamiprid clothianidin imidacloprid nitenpyram nithiazine | broad spectrum | Nicotinic acetylcholine receptor agonists/ antagonists | resistance | Overexpression of microsomal oxidases, target site resistance, oxidative detoxification |
| Ryanoids (Lahm *et al.*, 2005, 2007 and Liu *et al.*, 2010) | rynaxypyr | Lepidoptera | activators of ryanodine receptor of $Ca^{2+}$ channels in cardiac and skeletal muscles | No resistance | resistance not reported yet |

## 1.6 Resistance mechanisms in insects: key to survival

Insects employ a variety of resistance mechanisms, including detoxification, target site modifications, and nerve insensitivity. Detoxification occurs when toxins are modified by reduction, oxidation, and conjugation reactions, resulting in the excretion of modified toxin molecules. The metabolic defense system in insects generally involves three main groups of enzymes acting in three phases against a number of insecticides and phytochemicals. Phase I involves reduction of toxicity of substrates by cytochrome P450 monooxygenases (CYPs). In phase II, hydrophobic toxic compounds are converted to hydrophilic products by action of the glutathione S-transferases (GSTs), uridinediphosphate (UDP)-glucuronosyltransferases (UGTs), and carboxylesterases (COEs) facilitating their excretion. Finally, phase III includes ATP binding cassette (ABC) and major membrane transporters that actively pump conjugated xenobiotics out of the cell. Usually, insects deal with toxic chemicals via avoidance (Chapman, 2003), sequestration , excretion, target site mutation, alteration of sensitivity, overexpression, and production of multiple isoforms of detoxifying enzymes (Price, 1997; Scott *et al.*, 2001; Silva *et al.,* 2001 and Nishida, 2002) (**Fig. 1.6**).

The predominant biochemical mechanism for metabolic detoxification of toxic chemicals involves CYP or COE mediated reactions, resulting in the reduction or oxidation of toxin. GSTs then convert the detoxified molecule into a more water-soluble form by glutathione conjugation, which facilitates their rapid removal from the cell (Enayati *et al.,* 2005). This can be achieved by either overexpression (Silva *et al.,* 2001) (**Fig. 1.6A**) or expressing duplicated isoforms of these enzymes (Heckel, 2012) (**Fig. 1.6B**). Alternatively, the modification of target site (mutation of amino acid residue) might result in insensitivity or adaptation of insects to toxic chemicals (Park *et al.,* 1997; Franck *et al.,* 2012) (**Fig. 1.6 C, D**). Physiological adaptations involve (i) sequestration, which refers to selective transport and storage of toxic chemicals preventing their interference in normal physiological processes of insects (stored toxin can be subsequently used for defense against predators by insect) (Bowers, 1991; Rimpler, 1991; Camara, 1997; Theodoratus and Bowers, 1999; Nishida, 2002; Kuhn *et al.,* 2004) (**Fig. 1.6 E**). Insects avoid potentially detrimental allelochemicals from sensitive sites by exoskeletal segregation (Nishida, 2002). Physiological adaptations also involve (ii) early

inactivation of the toxic chemical (e.g., by insect oral secretion) or avoidance of toxic chemicals (e.g., by vein cutting), most of which are usually detected by visual, olfactory, or contact mechanisms, (Glendinning *et al.*, 2002; Chapman, 2003) and (iii) excretion (the toxin is readily expelled by insect) (Zagrobelny *et al.*, 2004) (**Fig. 1.6 E**).

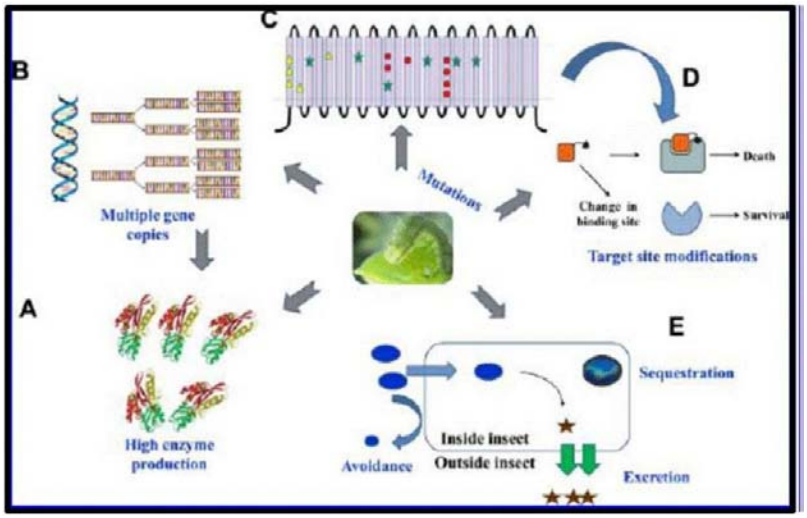



**Figure 1.6** General defense mechanisms in insects. Insect pests employ above mentioned mechanisms to overcome plant allelochemicals or insecticides. Increased resistance to insecticides can be due to overproduction of detoxifying enzymes such as GST, CYP, and COE (**A**), gene duplication (multiple isoforms of enzymes) (**B**), mutations (yellow triangle, insertion; green star, deletion; or red circle, substitution) in the target that result into insensitivity to insecticide (**C**), target site modification causing reduced insecticide binding (**D**), and sequestration (selective compartmentalization and storage of toxic chemicals), avoidance by visual, olfactory signals, or contact and excretion (ready removal of toxicants) (**E**).

Sequence-based transcript expression profiling studies have revealed that in *H. armigera* fed with various host plants differential expression was identified for genes involved in primary and secondary metabolism, environmental response, cellular processes, xenobiotic metabolism, and extracellular matrix-receptor pathways (Celorio-Mancera *et al.*, 2012). Similarly, in *Plutella xylostella*, whole genome sequencing revealed duplications in ABC transporter gene families along with CYPs, GSTs, and COEs involved in xenobiotic metabolic pathways (You *et al.*, 2013). Interestingly,

insects adapt to plant-derived toxic compounds, such as cardenolides, by making a single residue change in $Na^+/ K^+$ ATPases and high- level molecular convergence of these genes have helped insects to attain reduced sensitivity toward cardenolides (Dobler *et al.*, 2012). Another possible factor responsible for metabolizing such defensive compounds could be the alkaline pH of the herbivore gut that might inhibit plant β- glucosidases (Vetter, 2000). Resistance pathways against particular plant secondary metabolites [pyrrolizidinealkaloids (PA), GSL, furanocoumarin, etc.] governed by specific insect enzymes (CYPs, COEs, GSTs, nitrilase, etc.) are illustrated in **Fig. 1.7**.

Benzoxazinoids or hydroxamic acids are known to be highly toxic plant defensive compounds against the Lepidopteran insect species (Glauser *et al.*, 2011). However, *Mythimna separata* caterpillars can survive high levels of such benzoxazinoid compounds by possibly glucosylating them. UGTs are catalysts for the transfer of glycosyl group from UDP-glucose to a variety of acceptor molecules. For example, in *Manduca sexta* and *B. mori*, metabolism of phytochemicals such as flavonoids and coumarins and their removal has been shown to occur through UGTs (Zangerl, 1990). *Papilio polyxenes* has adapted to feeding on toxin containing host plants through diversification of the CYPs involved in detoxification and through its furanocoumarin responsive regulatory cascades (**Fig. 1.7A**) (Zangerl, 1990). Recent studies using plant-mediated RNAi have shown that silencing of *H. armigera* CYP6AE14 transcripts results in impaired tolerance to gossypol and reduced larval growth (Mao *et al.*, 2007, 2010). Lepidopterans sequester plant secondary metabolites such as terpenes, phenols, and many nitrogen-containing compounds and use them as toxic or unpalatable molecules for predators (Nishida, 2002). *M. sexta* accumulates nicotine synthesized by tobacco plants in its own body and uses it as a deterrent to parasitoids. Similarly poplar leaf beetle, *Chryosomela populi* uses an ATP-binding cassette transporter (CpMRP) as a pacemaker for sequestration of plant glucosides (Stauss *et al.*, 2013)

Some Lepidopteran insects such as *Utetheisea ornatrix* can detoxify alkaloids, store them in their bodies, and use them in defense against their own predators such as the lacewing *Ceraeochrysa cubana* (Hartmann, 1999). Similarly, metabolic pathway via N-oxygenase or flavin-dependent monooxygenases has been shown to detoxify PAs in *Estigmene acrea* and *Tyria jacobaeae* (**Fig. 1.7B**) (Eisner *et al.*, 2000; Despres *et al.*,

2007). Larvae of *Zygaena filipendulae* can sequester cyanogenic glucosides or synthesize them *de novo* and use them as defense molecules (Davis *et al.*, 1985; Jensen *et al.*, 2011). Some insects redirect the hydrolysis of GSL, which is usually catalyzed by myrosinase, toward the formation of less toxic nitriles, which eventually inhibit the production of toxic isothiocyanates production. This process is found in *Pieris rapae*, which utilizes the nitrile specifier protein to excrete nitriles in their frass (Wittstock *et al.*, 2004). Conversely, *P. xylostella* uses a GSL sulfatase enzyme instead of a nitrile specifier protein to convert isothiocyanates and nitriles into desulfoglucosinolates, which are less toxic and easily excretable (**Fig. 1.7C**) (Ratzka *et al.*, 2002; Furstenberg-Hagg *et al.*, 2013).

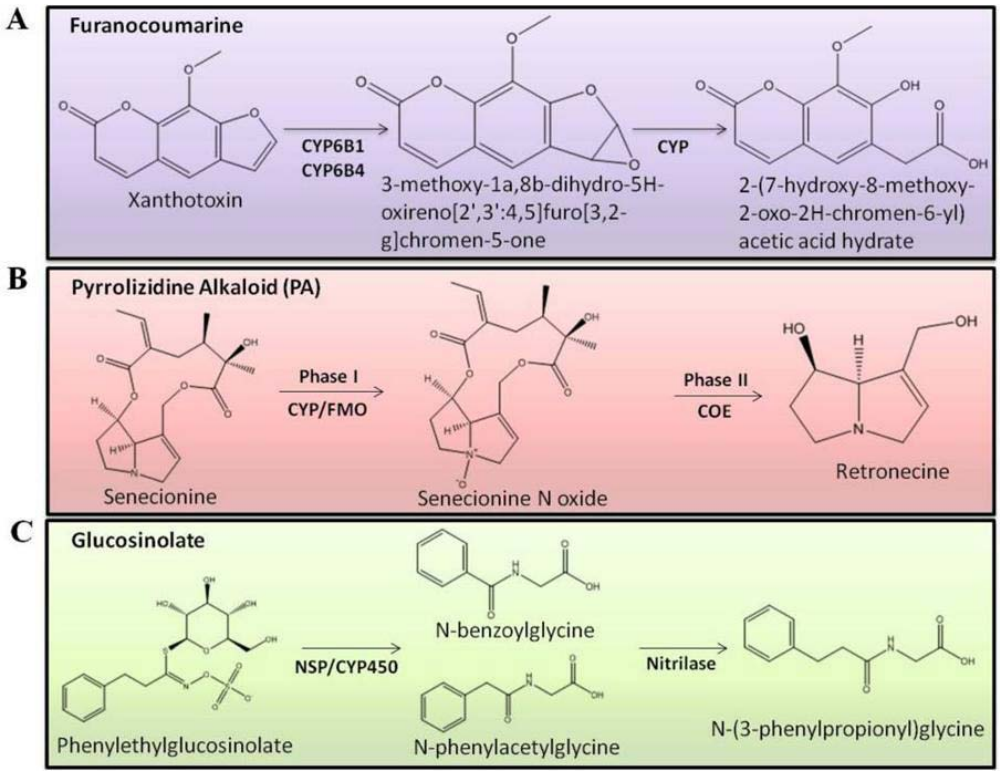



**Figure 1.7** Detoxification pathways for certain classes of plant allelochemicals. Furanocoumarin (**A**), pyrrolizidine alkaloid (**B**), and glucosinolate (**C**) are reproduced from reported studies. FMO: flavin-containing monooxygenase, NSP: nitrile specifier protein.

Chemical insecticides kill the pests, while the pests become resistant and eventually require increased doses of chemicals for their control. An overview of classes

of chemical insecticides, their representative compounds, and modes of action along with potential insect resistance mechanisms are presented in **Figs 1.8** and **1.9**. Different classes of insecticides, acting via different molecular pathways, can target similar or common insect physiological and biochemical mechanisms. For example, pyrethroids and organochlorides target sodium channels of nervous cells, whereas organophosphates and carbamates target acetyl cholinesterases (AChEs) in the insect nervous system (**Fig. 1.8**).

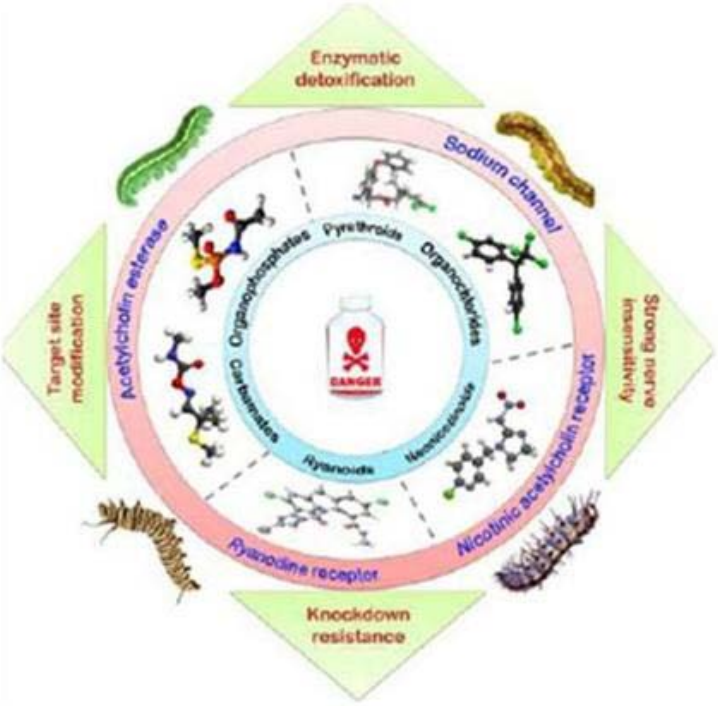



**Figure 1.8** Major classes of chemical insecticides, their mode of action, and resistance mechanism in insects against these insecticides. Internal blue-colored circle shows the class of insecticide and external pink circle illustrates the target of a corresponding insecticide in insect. The ring-and-ball structure of representative insecticide (e.g., acephate for organophosphates, cypermethrin for pyrethroids, DDT for organochlorides, imidacloprid for neonicotinoids, rynaxypyr for ryanoids, and aldicarb for carbamates) from each class has been placed in between two circles. External green triangles indicate the mechanism of detoxifications used by the insects to cope up with the adverse effects of insecticides.

Organophosphorus insecticides irreversibly inhibit carboxyl/cholin esterase family enzymes (Aldridge and Reiner, 1973; Cygler *et al.*, 1993). Resistance to

organophosphates is associated with decreased COEs activity in insect species (**Fig. 1.9A**) (Cygler *et al.,* 1993). Two classes of compounds, organophosphates and carbamates, are commonly used to inhibit AChE. However, target-site mutations in AChE are known to compensate for these insecticides, and sodium channel mutations confer pyrethroid resistance (**Fig. 1.9B**) (Oppenoorth, 1985; Bass *et al.,* 2011). Intensive insecticide use has resulted in the emergence of several resistant insect species possessing altered AChEs (Hemingway, 2000). Conventional insecticides are designed to target the ligand-gated ion channels, voltage-gated ion channels, and AChEs. DDT and pyrethroids are old and widely used pesticides known to inhibit voltage-gated sodium channels. Inherited resistance arises from acquired target-site mutations in the insects.

Genetic variation can also result in overproduction of detoxification enzymes, by either gene amplification or gene duplication events (Schmidt *et al.,* 2010). Several mutations in voltage-gated sodium channel locus have been reported for pyrethroid resistance in insects. For example, a single amino acid change (from Leu to Phe) is known to provide knockdown resistance (kdr) to pyrethroids, as first reported in house flies (Farnham, 1977). In the case of *Cydia pomonella*, a single amino acid substitution in an AChE target site was shown to counteract insecticides (Cassanelli *et al.,* 2006). Moreover, DNA sequence variation among 38 sequences of para sodium channel gene revealed a unique kdr mutation (L1014F) involved in pyrethroid resistance (Franck *et al.,* 2012). In *Heliothis virescens*, resistance is associated with mutations encoding Leu to His or Val to Met changes in voltage-gated sodium channel (Park *et al.,* 1997; Konus *et al.,* 2013) have reported that *H. armigera* can metabolize pyrethroids by modulating energy metabolism by CYPs, ATP synthase, and arginine kinase. DDT resistance has been associated with CYP6G, where the resistant alleles show insertion of partial accord transposable element at the ′5nd ( **Fig. 1.9C**) (Daborn *et al.,* 2002). Amplification or production of alternative truncated forms of esterases is also known to be involved in organophosphate resistance. A rich understanding of the relevant biology of insecticide resistance at molecular level will pave a way to design a better and more effective insect control strategy.

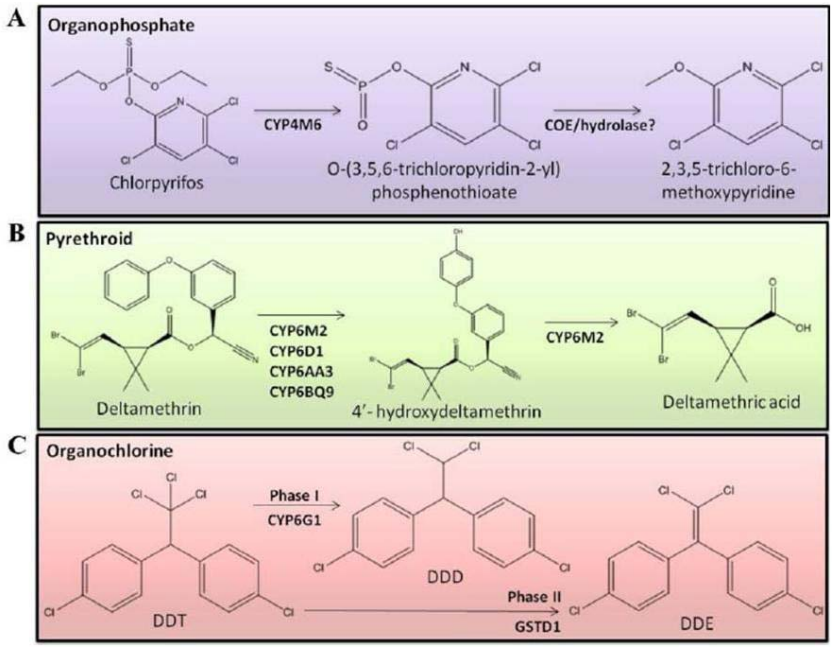



**Figure 1.9** Defense pathways of insect pests against particular synthetic insecticide. Phase I and II reactions for organophosphate (**A**), pyrethroid (**B**), and organochlorines (**C**) are reproduced from reported studies. DDT: dichlorodiphenyltrichloroethane, DDD: dichlorodiphenyldichloroethane, and DDE: dichlorodiphenyldichloroethylene.

## 1.7 Biotechnological methods of *H. armigera* control

Transgenic approaches have been widely practiced technology for pest control in modern time. However, field level success of transgenic plants remains only for Bt toxins and vegetiative insecticidal proteins. Transgenic plants expressing other variety of insect control agents have been produced; these majorly consisted of proteinase inhibitors (PIs) α-amylase inhibitors (α-AI) and lectins.

Numerous attempts are being done for successful transgenics expressing potent proteinase inhibitors that can specifically target insect's digestive proteases. Out of these few of the noticeable findings were (i) constitutive expression of tobacco trypsin protease inhibitor in transgenic tobacco showed resistance agaist *S. litura* and *H. armigera* (Srinivasan *et al.,* 2009) (ii) pyramiding sporamin + taro cystatin (PI) genes for dual broad-spectrum resistance against insect and phytopathogens in transgenic tobacco (Senthilkumar *et al.,* 2010) and (iii) Coexpression of potato type I and II proteinase

inhibitors exhibited protection against *H. armigera* damage in the field (Dunse *et al.*, 2010).

Certain successful examples of transgenic plants expressing α-AI include transgenic pea and azuki bean plants resistant to the *Bruchus pisorum* and *Callosobruchus chinensis* weevils (Morton *et al.*, 2000). Rye α-AI expressed in *Nicotiana tabacum* seeds, seed flour based artificial diet exhibited 74% mortality in *Anthonomus grandis* (Dias *et al.*, 2010). α-AI from Triticale seeds effectively inhibited *Eurygaster integriceps* salivary α-amylases (Mehrabadi *et al.*, 2010, 2012). Transgenic peas expressing bean α-AI inhibited development of pea weevil larvae (De Sousa-Majer *et al.*, 2007). Potent inhibition of α-amylases from the coffee berry borer pest by *Phaseolus vulgaris* α-AI-1 gene expressed in *Coffea arabica* plants (Barbosa *et al.*, 2010). Snowdrop lectin, *Galanthus nivalis* agglutinin (GNA) the first most plant lectin was found to be stable, active and resistant to proteolytic activity. Transgenic plants expressing GNA showed reduced feeding and delayed larval development of insect pests (Nagadhara *et al.*, 2004; Mehlo *et al.*, 2005).

Vegetative insecticidal proteins (Vips), are secreted during the growth stages and are toxic to a taxonomically diverse group of Lepidoptera (Estruch *et al.*, 1996; Llewellyn *et al.*, 2007). They show no sequence or structural homology with the δ-endotoxins. Thus, Vip toxins could be useful against insects that are resistant to Cry toxins. Exceptionally robust pyramids can be produced by stacking two or more toxins against specific insect pests (Roush *et al.*, 1998; Zhao *et al.*, 2003). Phenotypically resistant insects to more than one toxin are rare and therefore pyramiding of genes become valuable. However the resilience of a pyramid is determined by levels of pre-existing resistance to each toxin.

The examples of each strategy are mentioned in **Table 3.** Each strategy developed so far has faced certain limitations in pest control and insect pests are becoming resistant. In this scenario RNAi is emerging as a potent pest control strategy wherein, specific insect genes can be targeted by dsRNA.

**Table 3: Biotechnological approaches for the control of Lepidopteran pests using transgenics**

| Transgene | Source/mode of action | Example of use |
|---|---|---|
| *Bacillus thuringiensis* (Bt) endotoxin | Ingard ® - Cry1Ac<br>Bollgard II ® - Cry1Ac, Cry2Ab | Bt Cotton (*H. armigera*)<br>Bt Maize |
| Vegetative insecticidal protein (VIP) | Vip3 is toxic to Lepidopteran insects | *Agrotis ipsilon* and *Spodoptera frugiperda* |
| Protease inhibitors (PI) | [NaPI], [SBTI, SKTI]: blocks digestion of protein | Cotton, tobacco plants resistant to *H. armigera, S. litura, S. littoralis* |
| Alpha-amylase inhibitors | Alpha-amylase inhibitors (*P. vulgaris*) block starch digestion | *L. oleracea* (Lepidoptera) |
| Lectins | Breakdown of nutrient transport | (WGA), snowdrop lectin (GNA) *O. nubilalis* |
| RNAi constructs:<br>1) Vacuolar ATPase<br>2) Cytochrome P450 monooxygenase | K+ pump regulates midgut lumen pH; Cytochrome P450 monooxygenase permits insects to tolerate gossypol | Corn: dsRNA of a V-ATPase from *D. virgifera*<br>Cotton: cytochrome P450 dsRNA from *H. armigera* |

(Data modified from Stevens *et al.,* 2012)

## 1.7.1 *Bacillus thuringiensis* (bt) cry toxins: an effective bioinsecticide

Transgenic crops expressing Bt Cry proteins are presently the most significant and commonly used strategy against insect pests. The Bt toxin mode of action has been explained by two models: The pore formation model and the signal transduction model (**Fig. 1.10**).

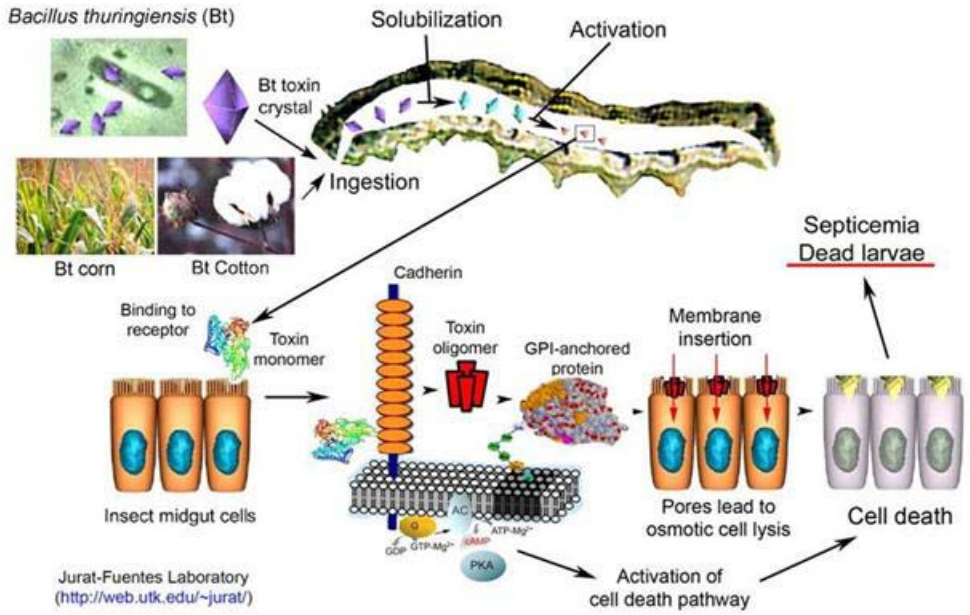



**Figure 1.10** Mode of action of Bt Toxin. Proposed two models: pore formation and signal transduction pathways are represented in figure. (Source: http://web.utk.edu/~jurat/)

The initial steps of both models are the same. Upon ingestion by insects the crystalline protein is solubilized in the midgut (Gill *et al.*, 1992). The 130 kDa protoxins are activated by insect gut proteases, forming a 43to 65 kDa protease-resistant active core (Tojo and Aizawa, 1983; Nagamatsu *et al.*, 1984; Rukmini *et al.*, 2000; Diaz-Mendoza *et al.*, 2007). Activated toxins bind to the primary receptors in the brush border membrane of the midgut epithelium columnar cells i.e cadherin-like proteins (Vadlamudi *et al.*, 1993 and 1995; Nagamatsu *et al.*, 1998; Jurat-Fuentes and Adang, 2004; Jurat-Fuentes *et al.*, 2004). Binding to cadherin facilitates further proteolytic cleavage of the toxin and promotes the formation of oligomers (Gómez *et al.*, 2002; Bravo *et al.*, 2004). Followed by this toxins interact with secondary receptors in the midgut larval membrane. These secondary receptors are GPI-anchored proteins; either aminopeptidases or alkaline phosphatases (Gill *et al.*, 1992; Knight *et al.*, 1994; Bravo *et al.*, 2004; Jurat-Fuentes and Adang, 2004). Following secondary receptor binding, the toxin inserts into the membrane and creates pores (Bravo *et al.*, 2004). This leads to the disruption of membrane integrity and cause an electrolyte imbalance resulting into death by starvation or septicaemia

(Knowles, 1994; Jiménez-Juárez *et al.*, 2007). Another model for the Bt toxin mechanism of action proposes that Cry toxins trigger a signalling cascade pathway (Zhang *et al.*, 2006; 2008). In this model, binding of toxin to the cadherin receptor initiates $Mg^{2+}$ dependent signal cascade pathway that includes a guanine nucleotide-binding protein, adenylyl cyclase, and protein kinase A which ultimately results in cell death.

The most commonly documented Bt resistance mechanism in insects is the alteration of midgut receptor binding for Bt toxins (Gahan *et al.*, 2010; Atsumi *et al.*, 2012). However, several studies have reported association of Bt resistance with reduced activity of digestive enzymes involved in the solubilization and activation of Bt pro-proteins. Resistance to Cry1Ac and Cry1Ab in insects falls into this category. Another mechanism reported is overexpression of certain proteases involved in degradation of the Cry toxins (Li *et al.*, 2004).

## 1.8 Gene silencing: Budding pest management strategy

RNA interference (RNAi) is an evolutionarily conserved mechanism of gene regulation across all eukaryotes; essentially it functions in maintaining genome integrity by blocking transposons and immunity in plants against invading viruses. Pioneering observations were found in plants (Napoli *et al.*, 1990) after which RNAi events were described in almost all eukaryotes including protozoa, flies, nematodes, insects, parasites, and mouse and human cell lines. The underlying mechanism still remained unkown. The mechanism of RNAi was discovered in nematode *Caenorhabditis elegans*, in an attempt to investigate role of *unc-22* (Fire *et al.*, 1998). Subsequently this technology has become widely used fundamental genomics tool for characterizing functions of many of newly identified genes in insects from genome sequencing projects (Hannon, 2002; Kuttenkeuler and Boutros, 2004; Chen *et al.*, 2007). The basic components of the RNAi machinery, namely dsRNA specific ribonucleases Dicer, which first cleaves long dsRNAs into short interfering RNAs (siRNAs), and the RNA-induced silencing complex (RISC), which acts as an effector complex for the targeting mRNAs by sequence specific degradation, are evolutionarily conserved across virtually all eukaryotic taxa.

### 1.8.1 RNA interference mechanism in insects

In insects in general and *D. melanogaster* in particular, two RNA silencing pathways are known to exist which are mediated by siRNAs and miRNAs (Tomoyasu *et al.*, 2008). RNAi is the specific downregulation of gene expression by double-stranded RNA (dsRNA). The specificity is based on the sequence of one strand of the dsRNA corresponding to part or all of a specific gene transcript. RNAi is a post-transcriptional control mechanism involving sequenc-specific degradation of a target mRNA. This degradation is mediated by the production of small interfering RNAs (siRNAs) from the dsRNA, which is cleaved by dsRNA-specific endonucleases referred to as dicers (from the dicer gene identified in *D. melanogaster* (Bernstein *et al.*, 2001; Hammond, 2005; Jaskiewicz and Filipowicz, 2008). The siRNAs are 21 bp dsRNA fragments carrying two nucleotide overhangs at the 3' end of each strand; one strand of the siRNA acts as guide strand and is assembled into an RNA-induced silencing complex (RISC) in conjunction with the argonaute multi-domain protein. Argonaute protein contains an RNaseH-like domain responsible for target degradation (Martinez *et al.*, 2002; Filipowicz, 2005) (**Fig. 1.11**).

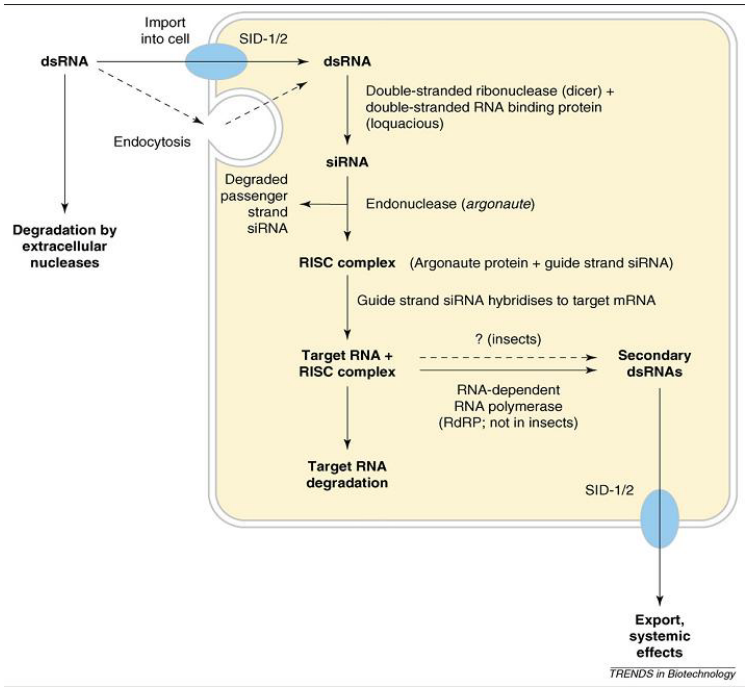



**Figure 1.11** Mechanism of RNA interference in insects. (Adopted from Price and Gatehouse, 2008)

The process is closely related to post-transcriptional gene regulation by micro-RNAs (miRNAs), where the outcome is inhibition of translation initiation, and shares many of the same components. In plants and nematodes, RNAi have shown systemic effects on gene expression, so that gene knockout spreads throughout the organism and persists over development. The basis of this effect is the presence of an RNA-dependent RNA polymerase (RdRP) that is able to interact with the RISC complex and generate new dsRNA based on the partially degraded target template by using the hybridised siRNA strands as primers. The synthesized dsRNA is then acted on by the dicer enzymes to generate new siRNAs (secondary siRNAs), amplifiying silencing signal. In this way, once a dsRNA is introduced into a cell, can induce gene silencing and persist through development as well as generation. Though RNAi holds great promise in future of developing efficient pest control strategies, its success is limited by certain factors as detailed in **Table 4.**

**Table 4: Possible causes of RNAi insensitivity in insects**

| | |
|---|---|
| **Species-related** | supplied dsRNA are efficiently degraded |
| | low amplification and spreading of the RNA signal |
| | core RNAi genes less responsive after dsRNA treatment |
| **Tissue-related** | target tissue is hardly permeable to dsRNAs |
| | Components of the RNAi machinery are mildly expressed in the tissue |
| **Gene-related** | particular dsRNA is efficiently degraded |
| | gene efficiently overcomes RNAi by increasing mRNA expression |
| | target mRNA is too transient |
| | target mRNA is inaccessible to RNases |
| | Sequence polymorphism in target mRNA |

(Adopted and modified from Xavier Bell´es, 2010)

## 1.8.2 RNAi in insect pest management

RNAi has been exploited across eukaryotes for applications ranging from functional genomics to provision of valuable crop traits, such as resistance against viruses, bacteria and nematodes. However, in insects this technology is in its budding state and recently many experimental studies are being done to identify potential target genes from pest. These will be further utilized for crop protection by producing

transgenic expressing dsRNA targeting specific insect genes. Recently most of the RNAi studies in insects involve use of sequence-specific dsRNA as effector to induce silencing in recipient insects (Huvenne and Smagghe, 2010).

As of now one of the most common methods of delivering dsRNA is direct injection of the dsRNA into target tissues or developmental stages of insects (Misquitta and Paterson, 1999; Bettencourt *et al.*, 2002; Bucher *et al.*, 2002; Rajagopal *et al.*, 2002; Amdam *et al.*, 2003; Gatehouse *et al.*, 2004; Tomoyasu and Denell, 2004). The mechanism of dsRNA uptake in insects may vary in different species; two mechanisms have been proposed for dsRNA uptake (i) RNA channel transporter SID-1first discovered in the nematode *C. elegans* (Winston *et al.*, 2002), and this protein appears to be conserved in many but not all insect taxa (**Fig. 1.12**) (Gordon and Waterhouse, 2007) and (ii) Receptor mediated endocytosis (Saleh *et al.*, 2006; Ulvila *et al.*, 2006).

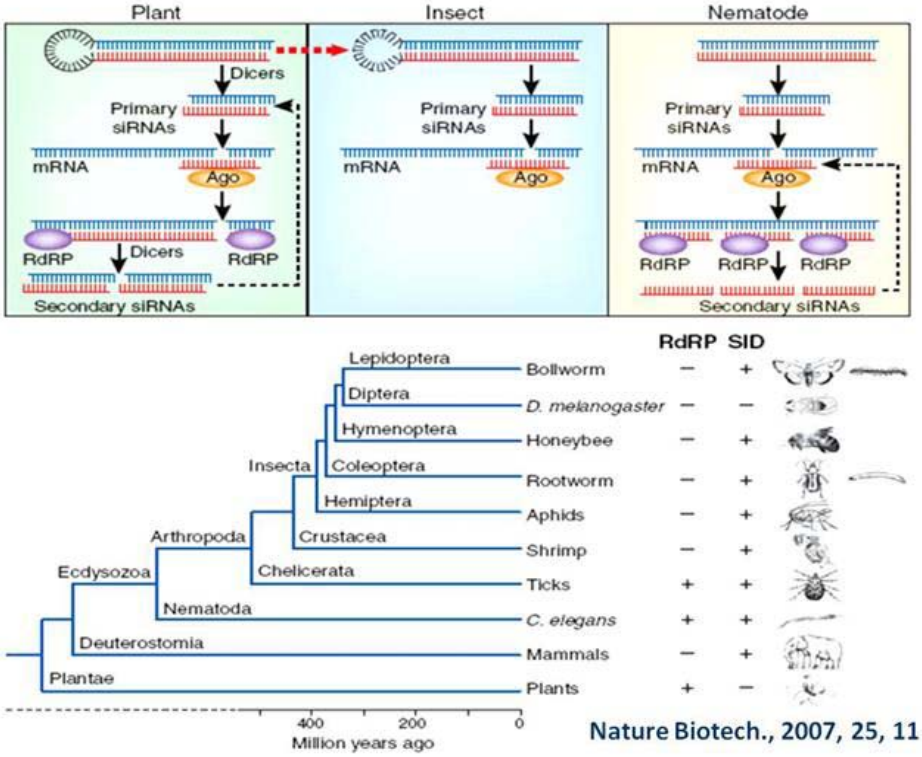



**Figure 1.12** Comparative RNAi mechanism and their genes in plants and animals. Comparison of RNAi in plants, insects and nematodes. Evolutionary relationships of insects, higher animals and plants, indicating the presence or absence of genes encoding RdRP and the RNA channel transporter SID12.

Dipteran insects lack an ortholog for SID-1, and yet dsRNA can enter cultured *D. melanogaster* S2 cells (Caplen *et al.*, 2000; Clemens *et al.*, 2000; Bettencourt-Dias and Goshima, 2009) or into tissues of fruit flies injected with dsRNA (Dzitoyeva *et al.*, 2001). RNAi can be induced simply by soaking the animals in a solution of dsRNA as observed in nematodes (Tabara *et al.*, 1998) and flatworms (Orii *et al.*, 2003). Gene silencing was also observed in *Drosophila* dechorionated embryos by soaking them in dsRNA (Eaton *et al.*, 2002). In addition to this, oral delivery of dsRNA first demonstrated in *C. elegans* (Timmons *et al.*, 2001), is now being widely used in some of the insect species for RNAi studies. Knockdown of targeted genes in light brown apple moth larvae (*Epiphyas postvittana*; Turner *et al.*, 2006), the hemipteran *Rhodnius prolixus* (Araujo *et al.*, 2006), the tsetse fly (*Glossina morsitans morsitans*; Walshe *et al.*, 2009), the termite (Reticulitermes flavipes; Zhou *et al.*, 2008) and diamondback moth (*Plutella xylostella*) larvae (Bautista *et al.*, 2009) was obtained by supplying dsRNA in artificial diets. In these studies, delivery of the dsRNA into the gut resulted in knockdown of a gene's expression in different tissues, indicating that the RNAi was systemic; whereas no observable systemic RNAi was found in the tsetse fly. Oral delivery of dsRNA targeting a gut-specific aminopeptidase in case of *S. litura* failed to induce RNAi in that insect's gut cells (Rajagopal *et al.*, 2002), suggesting this mode of delivery may not be suitable for all species.

Baum *et al.*, (2007) showed that 14 of 290 diet-delivered different dsRNAs caused significant mortalities at doses 5.2 ng/cm$^2$ in Western corn rootworm larvae (WCR, *Diabrotica virgifera*). In another study, Mao *et al.*, (2007) showed that knockdown of cotton bollworm gut-specific cytochrome P450 gene, CYP6AE14, increased sensitivity of insects to gossypol in artificial diets (**Fig. 1.13**).

Besides these, bacterial feeding is a non-invasive and convenient technique of dsRNA delivery. Producing dsRNA in genetically engineered *Escherichia coli* strain HT115 has become reliable and economic way to produce large quantities of dsRNA. *E. coli* mediated delivery of dsRNA has been reported in *C. elegans* (Timmons and Fire, 2001), *Entamoeba histolytica* (Solis *et al.*, 2009) and *S. exigua* (Tian *et al.*, 2009). Few successful RNAi experiments have been carried out in a number of lepidopteran species. Knockdown of a pigment gene following dsRNA injection into *B. mori* embryos (Quan *et*

*al.*, 2002), a putative *Bacillus thuringiensis* toxin receptor in *S. litura* larvae (Rajagopal *et al.*, 2002) and an immune response protein in the tobacco hornworm *M. sexta* (Eleftherianos *et al.*, 2006).

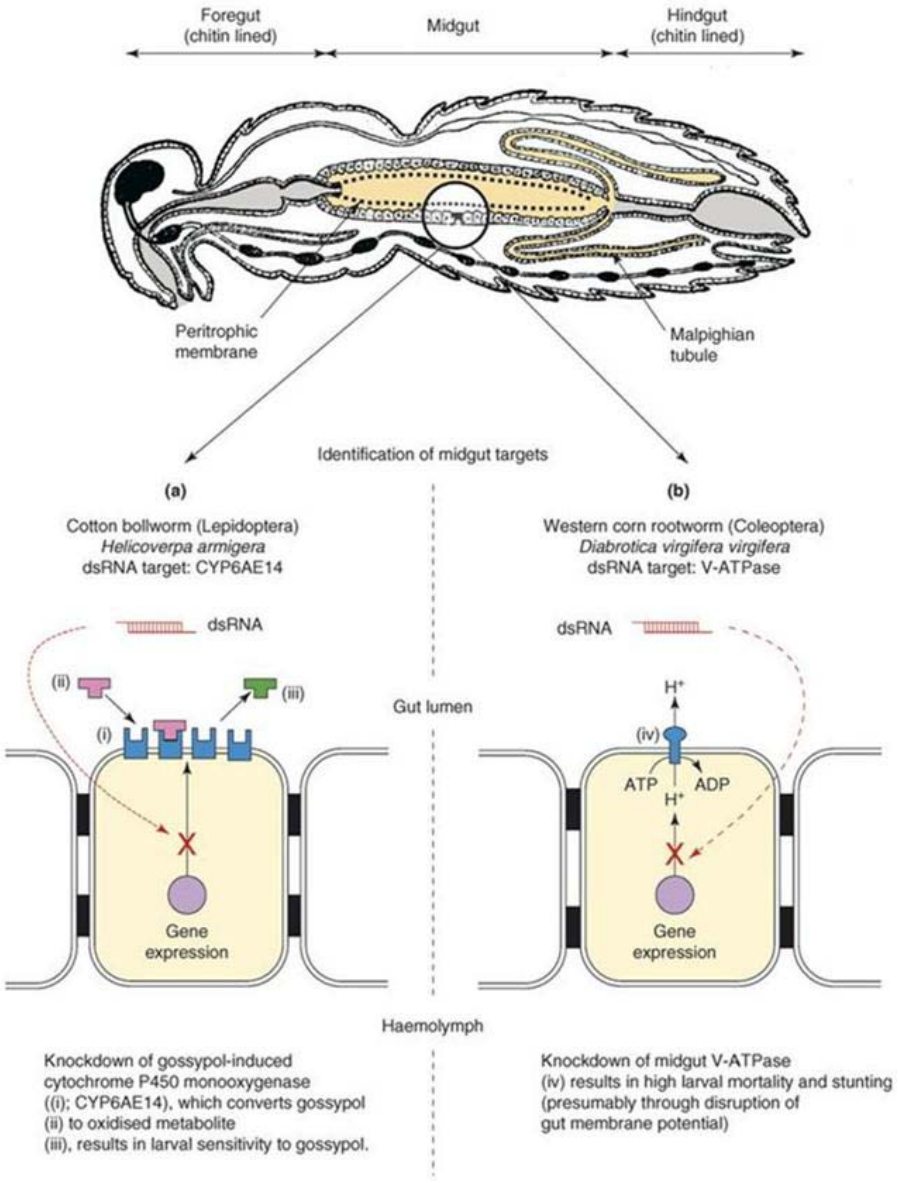



**Figure 1.13** Overview of RNAi approaches for insect-resistant transgenic plants with emphasis on two successful studies by Baum *et al.*, 2007 and Mao *et al.*, 2007.

Effects of diet delivered various concentrations of midgut and non-midgut gene of *H. armigera* were recently elaborated by Asokan *et al.*, 2013. Zhao *et al.*, (2013) showed that RNAi-mediated knockdown of catalase in *S. litura* lead to low survival rates in

larvae. Success of RNAi in insects majorly depends upon selection of target gene and efficient delivery of dsRNA. Many of the studies are being carried to screen potential candidate genes for developing species-specific targets (dsRNA) for pest control. Examples involve targeting of chitin synthase in *S. exigua* (Chen *et al.,*2008), targeting V-ATPase transcripts in fruit flies, flour beetles, pea aphids and tobacco hornworm (Whyard *et al.,* 2009), acetylcholinestarase in *H. armigera* (Kumar *et al.,* 2009), transgenic tobacco expressing EcR dsRNA from *H. armigera* (Zhu *et al.,* 2012), HaHMAG-CoA reductase in *H. armigera* (Wang *et al.,*2013), etc. He *et al.,* 2013 showed that diet delivered fluorescent nanoparticle conjugated dsRNA i.e. FNP /CHT10-dsRNA can induce specifc and potent silencing leading to mortality in insects. Terenius *et al.,* (2011) have reviewed successes and failures of RNAi in Lepidoptera elaborating the primary guidelines for experimental design. Moreover, many studies are done for optimization of various aspects of RNAi experiments in insects; one such study is recently reported in *H. armigera* by Yang *et al.,* 2014. The ever expanding RNAi studies in insect pests thus become an effective tool to identify novel target genes. However, in spite of these exciting possibilities, some significant challenges remain. Are all target genes equally amenable to this approach? Will this technology be applicable to other insect groups? Will RNAi be overcome by sequence polymorphisms in pest populations?

RNAi has opened up a new line of thinking in designing a futuristic approach that could result in paradigm shift in IPM strategies. Considering the known characteristics of *H. armigera* and the available methods of its control, we propose to develop a novel and efficient pest control strategy. With this background we attempted to address following questions in this thesis, (i) which are key candidate proteases (trypsin- and chymotrypsin-like) involved in *H. armigera* polyphagy (ii) which enzymes (proteins) are involved in adaptation of *H. armigera* to non-host plant (*Cassia tora*) and synthetic pesticide (chlorpyrifos) (iii) can we utilize RNAi approach to silence identified candidate genes of *H. armigera* and understand their role in insect growth and development. Answering these questions might provide us with deeper insights into polyphagy and adaptability in *H. armigera* and possible potential target genes for RNAi technology.

## 1.9 Organization of thesis

The main highlights from earlier studies on polyphagy and adaptability of *H. armigera* as well as RNAi studies in insect pest and their applicability are summarized in the present chapter 'Review of literature'. These important findings and information generated were used further to devise the objectives of the thesis. The work carried out in doing so has been further organized into following chapters:

### Chapter 2:

### Differential protease activity augments polyphagy in *Helicoverpa armigera*

This chapter describes gene expression patterns of diverse *H. armigera* trypsin and chymotrypsin isoforms, when larvae were fed on various host plants such as okra, rose, pigeon pea and maize. These patterns were studied for larval, pupal and moth stages of *H. armigera* development. Zymographic profiles of serine proteases was also studied, characterized and the differential protease isoform from rose and pigeon pea fed larvae were identified by nano LCMS approach. These important findings were published in **Insect Molecular Biology (2013) 22, 258–272.**

### Chapter 3:

### Molecular responses of *H. armigera* upon exposure to host and non-host plants, and synthetic pesticide

This chapter describes the biochemical and molecular approaches employed in studying the adaptability of *H. armigera* to non-host plant as well as synthetic pesticide. Here, we have identified differential proteins from *H. armigera* gut, heamolymph and frass in response to *Cassia tora* (non-host plant). These were further studied for gene expression and biochemical responses, the findings were published in **Journal of Proteome Research (2011) 10, 5128–5138**. We also studied the complex biochemical and physiological response of *H. armigera* against synthetic pesticide chlorpyrifos (organophosphate). Based on GC-MS studies we tried to propose the possible chlorpyrifos breakdown pathway in *H. armigera*. These findings were published in **Insect Science (2014, In Press)**.

## Chapter 4:

## Interruption of *Helicoverpa armigera* development upon silencing of diverse genes by dsRNA feeding

This chapter describes the gene silencing study of identified candidate genes of *H. armigera*. The chosen target genes were based on our findings from previous chapters. Here, we tested total of 15 dsRNA's, each targeting a different gene for silencing in *H. armigera*. dsRNA was supplied through artificial diet. We checked for systemic silencing and observed for any phenotypic abnormalities in dsRNA fed larvae. For most of the selected genes we observed prominent silencing effects at transcript as well as biochemical level. The manuscript is **under review**.

## Chapter 5:

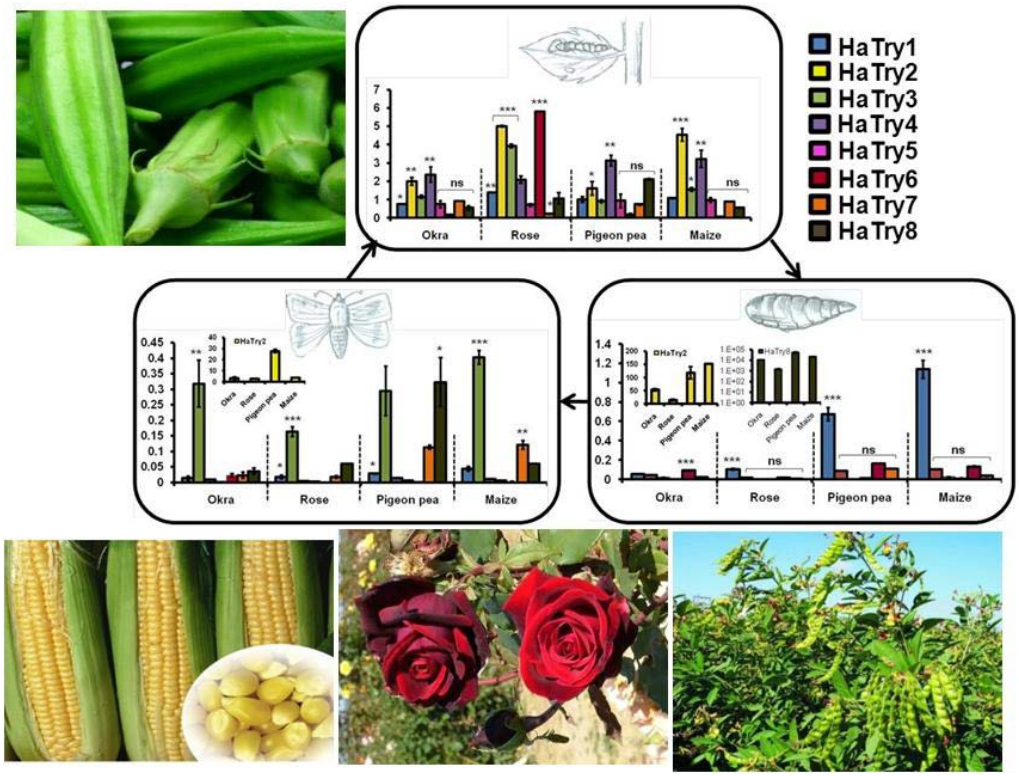
## General discussion and future directions

This chapter describes key highlights of the present research work. Various aspects of *H. armigera* polyphagy and adaptability, and feasibility of RNAi have been discussed. High adaptability of *H. armigera* drives the efficacy of its survival on various host plants and resistance to toxins. *H. armigera* uses complex and distinct physiological and biochemical mechanisms to overcome plant antifeedants and toxins. Selective silencing of key enzymes (proteins) of *H. armigera* shows adverse effect on its growth and development, highlights potential of RNAi in pest control. Along with these certain future leads that can be further explored are also discussed here.

Finally, all the literature referred, to develop protocols and to infer our results has been listed in the **Bibliography** chapter.

# Chapter 2

## Differential protease activity augments polyphagy in *Helicoverpa armigera*




**The research work described in this chapter has been published as full-length research article in Insect Molecular Biology (2013), 22, 258–272.**

# Chapter 2

## Differential protease activity augments polyphagy in *Helicoverpa armigera*


### Abstract

*Helicoverpa armigera* (Lepidoptera: Noctuidae) and other polyphagous agricultural pests are extending their plant host range and emerging as serious agents in restraining crop productivity. It is one of the most devastating crop pests mainly due to its feeding habits and its ability to undergo diapauses. The larvae of this insect pest feed on principally reproductive structures and growing points such as cotton buds and bolls, corn ears, tobacco buds, and sorghum heads, pulses pods etc, leading to dramatic reduction in agricultural yield. Dynamic regulation, coupled with a diversity of digestive and detoxifying enzymes, play a crucial role in the adaptation of polyphagous insects. To investigate the functional intricacy of serine proteases in the development and polyphagy of *H. armigera*, we profiled the expression of eight trypsin-like and four chymotrypsin-like phylogenetically diverse mRNAs from different life stages of *H. armigera* reared on nutritionally distinct host plants. These analyses revealed diet- and stage-specific protease expression patterns. The trypsins expressed showed structural variations, which might result in differential substrate specificity and interaction with inhibitors. Protease profiles in the presence of inhibitors and their mass spectrometric analyses revealed insight into their differential activity. These findings emphasize the differential expression of serine proteases and their consequences for digestive physiology in promoting polyphagy in *H. armigera*.

## 2.1 Introduction

Polyphagous insect pests such as *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) represent one of the most important biotic stresses influencing crop productivity (Sharma *et al*., 2000; Ferry *et al*., 2004). Several chemical pesticides and biotechnological approaches such as transgenic Bt cotton are used to control the pest (Hilder *et al*., 1987; Wu *et al*., 1997), but the absence of resistance to *H. armigera* in host plants, the lack of adequate control measures, and the resurgence of pesticide/toxin resistance together make field management of this pest challenging (Ryan, 1990; Karban and Baldwin, 1997; Tabashnik *et al*., 2008, 2009; Dunse *et al*., 2010a). The discovery and design of broadly applicable insecticidal molecules are, therefore, critical for the long-term control of this insect pest. The inhibition and regulation of vital insect processes such as development, digestion and adaptation may help to reduce crop damage and enhance productivity.

In insects, proteases are a major group of hydrolytic enzymes categorized as endo- and exo-proteases and are predominantly involved in digestive processes, proenzyme activation, metamorphosis, release of physiologically active peptides and complement activation (Terra, 1988; Terra and Ferreira, 1994; Borovsky and Mahmood, 1995; Huang *et al*., 2010). Serine proteases, namely, trypsins and chymotrypsins are abundant in the digestive tract of *H. armigera* (Srinivasan *et al*., 2006). Twenty-one trypsin-like, 14 chymotrypsin-like, two elastase-like, and several aminopeptidase and carboxypeptidase genes were found in the gut tissue of *H. armigera* reared on a high-protein diet free of inhibitors (Bown *et al*., 1997, 1998, 2004; Gatehouse *et al*., 1999; Chougule *et al*., 2005; Angelucci *et al*., 2008). These enzymes are released extracellularly into the gut lumen and are active at an alkaline pH (Johnston *et al*., 1991; Purcell *et al*., 1992). Serine endopeptidases belong to the S1 family (chymotrypsin family) of peptidases which contain a catalytic triad of histidine, aspartic acid and serine (http://merops.sanger.ac.uk/). In the case of trypsins, the Asp (189), Gly (216) and Gly (226) residues contribute to a negatively charged S1 (ratio of residues flanking to the catalytic site) site; thus, they are highly specific for the positively charged side chain of arginine or lysine in the substrate. Similarly, residues of S1 sites form a deep hydrophobic pocket of chymotrypsin and make it preferable for phenylalanine,

tryptophan, and tyrosine at P1 on the substrate (Srinivasan *et al*., 2006). Dynamic expression and an ability to regulate gut proteases augment the insect's adaptability to diverse dietary protein/anti-nutritive/toxic constituents. The midgut enzyme profile of Lepidoptera larvae reared on an artificial diet (AD) or transgenic plants containing proteinase inhibitors (PIs) show that the insects are capable of up-regulating normal gut proteases (Christeller *et al*., 1992). Moreover, they also induce several novel inhibitor-resistant/degrading proteases (Jongsma *et al*., 1995; Bown *et al*., 1997, 2004; Broadway, 1997; Gatehouse *et al*., 1997; Jongsma and Bolter, 1997; Wu *et al*., 1997; Giri *et al*., 1998; Mazumdar-Leighton and Broadway, 2001a, b; Zavala *et al*., 2008; Dunse *et al*., 2010a, b).

Previous studies have indicated that the insects possess the strength to modify gut protease, amylase and lipase expression in accordance with both natural and PI-containing diets (Harsulkar *et al*., 1999; Patankar *et al*., 2001; Chougule *et al*., 2005; Tamhane *et al*., 2005; Kotkar *et al*., 2009, 2012; Sarate *et al*., 2012). Further exploration is required to determine how or whether the sensitivity of *H. armigera* protease complements plant defence molecules. We studied the diversity of *H. armigera* gut trypsins and chymotrypsins through insect developmental stages (larvae, pupae and adult) reared on four nutritionally diverse host plants, namely, okra (OK; *Abelmoschus esculentus*), rose (RO; *Rosa rubiginosa*), pigeon pea (PP; *Cajanus cajan*) and maize (MZ; *Zea mays*), using real-time quantitative PCR and enzyme activity assays. We assessed protease activities in developmental stages and characterized those using synthetic inhibitors and diverse recombinant PIs from *Capsicum annuum* (CanPIs). A nano-Liquid Chromatography Mass Spectrometry-elevated energy (nano- LCMSE) approach was used for the identification of differentially expressed proteases from larvae feeding on RO and PP and was confirmed by gene expression analysis. By superimposing predicted structures, we observed divergence in the catalytic and binding sites of differentially expressed trypsin isoforms. The present study enhances the understanding of digestive physiology of *H. armigera* with emphasis on dynamics of gut protease expression and their interactions with different substrates and inhibitors.

## 2.2 Material and Methods

### 2.2.1 Materials

Synthetic substrates N-a-benzoyl-DL-arginine *p*-nitroanilide (BA*p*NA), and azocasein, PIs TPCK and TLCK, bovine trypsin, acetonitrile and sequencing-grade modified trypsin were obtained (Sigma Chemical Co., St. Louis, MO, USA). X-ray films and Kodak 163 DA developer were purchased from Kodak (Chennai, India). MassPrep predigested standard protein rabbit glycogen phosphorylase B (GP) was purchased from Waters Corporation (http:// www.waters.com). Highly pure chemicals for ADs, natural diets and the rest of the insect rearing materials were purchased locally.

### 2.2.2 Insect culture and tissue harvest

*H. armigera* larvae were collected from the chickpea fields of Mahatma Phule Krishi Vidyapeeth, Rahuri, MS, India, and reared on AD under laboratory conditions, i.e. humidity 60%, temperature 28 °C and photoperiod of 16h light: 8h dark for one generation as described earlier (Nagarkatti & Prakash, 1974). Neonates from subsequent generations were reared on AD for two days and then transferred onto host plants (OK, RO, PP and MZ) and maintained for their complete life cycles. Tissues of fourth-instar larvae, pupae, and adults (three independent biological replicates) were harvested separately and snap-frozen for further biochemical and molecular analyses.

### 2.2.3 Sequence analysis and primer designing

Complete coding and amino acid sequences of pro-enzymes (full-length, inclusive of signal peptide) of 21 *H. armigera* trypsin-like and nine chymotrypsin-like proteases were retrieved from the National Center for Biotechnology Information (http:// www.ncbi.nlm.nih.gov). Complete coding sequences of *HaTry* and chymotrypsin (*HaChy*) were used in multiple sequence alignment (MSA) by CLUSTAL W2 program (http://www.ebi.ac.uk/Tools/ msa/clustalw2) using the Gonnet protein weight matrix and the clustering was done using a neighbour-joining algorithm. For gene expression analysis using quantitative real-time PCR, eight *H. armigera* trypsin and four chymotrypsin genes were chosen based on their sequence homology and divergence.

Primers were designed manually within non-homologous regions of these genes, around 400bp downstream from the start codon (**Table 1**). Only the coding sequences were used to design specific primers with G + C content of 40 to 60% and a melting temperature of 60 °C.

**Table 1: List of primers used for real-time PCR analyses**

| Name | Genbank ID | Primer sequence 5'-3' | For/Rev |
|---|---|---|---|
| **HaActin** | AF286059 | GATCGTGCGCGACATCAAG | F |
| | | GCCATCTCCTGCTCGAAGTC | R |
| **HaTry1** | EU982841 | GAGGACACAGATGTGGAGGGG | F |
| | | GAACACACGGAATTCAGCCACG | R |
| **HaTry2** | EU770391 | GCGTAAAGGATGCGGTTGG | F |
| | | CAGGATGGCAACCATCCATG | R |
| **HaTry3** | EU325548 | CGACCACACTGACGCGAG | F |
| | | GCACGCCACTGGACATGG | R |
| **HaTry4** | EF600059 | GTGCTACCCCTTCTGATTC | F |
| | | AACTTGTCGATGGAGGTGAC | R |
| **HaTry5** | EF600054 | GGTCTCTGCTAACCTCCACC | F |
| | | CTGGATGCCAGGGACGTGC | R |
| **HaTry6** | Y12276 | TGGCTGGGGTGACACTTTCT | F |
| | | GTCTCCCTGGCACTGGTC | R |
| **HaTry7** | Y12271 | CAGAGGATTGTGGGTGGTTCG | F |
| | | GCGGTGAGGATAGCCCTGTT | R |
| **HaTry8** | Y12286 | GGGCTACTGGTGCCTTCAACG | F |
| | | CAGAGTCATACACGTCACCGACG | R |
| **HaChy1** | HM209422.1 | CGACTTGTCAGGTGGTCAGGCTG | F |
| | | GCGATTCTGGTACCGCCGGAGAAC | R |
| **HaChy2** | EU325550.1 | GACTTGTCAGGTGGCCAGGCTG | F |
| | | GCGATTCTGGTACCGCCGGAGAAC | R |
| **HaChy3** | GU323796.1 | TGACTTGTCAGGTGGCCAAGCTG | F |
| | | GCGATTCTGGTACCGCCGGAGAAC | R |
| **HaChy4** | Y12273 | CACCATCTTCATCTTCCAATCCGTGTGC | F |
| | | GTGTTGATACGAGTACCACCGAAGAAC | R |
| **HaTry22** | AF261988 | GTGATCTACGCTGAAGCG | F |
| | | GGAGTCCTCAGCCTGATG | R |
| **HaChy10** | AF233729 | CAGGCCGGTCTTCTGACAC | F |
| | | CATCGAACCAGCAGTGTG | R |
| **HaChy11** | AF233728 | CTCCTTCACTGTAGTCCTC | F |
| | | CATCTCTGGTGCTGATGC | R |

All primer pairs were optimized for specific and efficient amplification. Most of the primers were designed specific to individual isoform(s), while few of them were designed for the entire clade, as these groups of isoforms were 99% similar (e.g. *HaTry6* and *HaTry7*). The genes were referred as *HaTry1* to *8* and *HaChy1* to *4*; the primers were named accordingly and used in mRNA expression studies.

### 2.2.4 Quantitative real-time PCR

Total RNA for one biological replicate was isolated from pools of five individuals of fourth-instar larvae, pupae and adult *H. armigera*, using Trizol reagent (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. The total RNA was treated with RNase-free DNAase I (Promega, Madison, WI, USA) to eliminate genomic DNA contamination. The quality and quantity of RNA were determined by agarose gel electrophoresis and spectrophotometric analysis using Nanodrop (Thermo Scientific, Waltham, MA, USA). Synthesis of first-strand cDNA was carried out in 20-ml reactions using 2 mg of total RNA with reverse transcription (Promega) as per the manufacturer's instructions. Three independent RNA preparations representing three independent b iological replicates were used. cDNA was synthesized in triplicate and recombined to balance cDNA sets to control for pipetting error and to produce larger volumes. Real-time RT-PCR was performed using 7900HT Fast Real- Time PCR System (Applied Biosystems, Foster, CA, USA) to examine transcript abundance of selected *HaTry* and *HaChy* mRNAs. Efficiency for each gene amplification was assessed by constructing standard plots using 5X serial dilutions of cDNA, and those with efficiencies of 97–100% were used for further analysis. The mRNA expression levels were normalized using b-actin as the reference gene. Quantitative real-time PCR was performed in 10 µl reactions (biological and technical triplicates) containing 5 µl of 2X concentrate SYBR mix (Fast Universal SYBR Green, Roche, Berlin, Germany), 0.5 ml of forward and reverse primer each (10 mm, ie 500 nm in reaction) and 1 µl of cDNA (10 ng) template. The cycling parameters used were 95 °C for 10 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. At the end of each run, dissociation curve analysis of the amplified product was carried out to evaluate the specificity. This involved denaturation at 95 °C for 15 s, cooling to 60 °C for 15 s and then gradual heating at 0.01 °C/sec to a final

temperature of 95 °C. The gene expression ratios of target genes were calculated using a standard curve (logarithmic) calculation similar to the ddC (delta delat Ct; arithmetic) method; for each primer pair including actin, a standard curve was obtained using a 5X serially diluted pool of cDNA. These were further used to calculate the relative ratios/ quantity of target transcripts compared with actin and was expressed as means of three biological replicates along with the standard error.

### 2.2.5 Extractions of *H. armigera* gut proteases and biochemical assays

*H. armigera* gut tissues were homogenized and mixed with 0.2 M glycine-NaOH pH 10 (1:3 w/v), and kept at 4 °C for 2 h. The homogenate was centrifuged at 4 °C for 20 min at 10 000 *g;* supernatant was used as HGPs. Enzymatic assays using azocasein and benzoyl-arginyl-*p*-nitro-anilide (BA*p*NA; Erlanger *et al*., 1961) as substrates were performed in order to estimate protease-like and trypsin-like activities, respectively. BA*p*NA was used at a final concentration of 1 mM in a 1 ml assay buffer; 0.2 M glycine-NaOH (pH 10) was used as assay buffer. Assays were carried out at 37 °C for 20 min. The reactions were terminated by adding 200 ml of 30% acetic acid and absorbance was measured at 410 nM. Assays were done in triplicate with appropriate blanks. An azocasein assay was carried out using 1% (w/v) solution of the substrate in assay buffer. HGPs were added to 200 ml substrate and incubated for 30 min at 37 °C, and the reaction was terminated by adding 300 ml of 5% trichloro-acetic acid. The precipitated proteins were centrifuged at 10 000 *g* for 10 min and 0.5 ml of supernatant was added to 0.5 ml of 1 M NaOH. The absorbance of this solution was measured at 450 nM and the HGPs responsible for an increase in 1.0 OD per min was defined as one proteinase unit.

### 2.2.6 Visualization of protease activity profiles

Activity profiles of proteases from different tissues were visualized using gel-X-ray film contact print technique (GXCT), as described earlier (Pichare and Kachole, 1994; Harsulkar *et al*., 1998). In brief, equalized units of protease activity, 0.02 U from each sample, were loaded onto the native polyacrylamide gel electrophoresis (PAGE) gel. Post-electrophoresis gels were rinsed with distilled water, equilibrated with 0.2 M glycine-NaOH (pH 10.0) for 10 min and exposed to X-ray film at intervals of 15 min, 30

min, 1 h, 2 h and 3 h. Visualization was also attempted for the extracts of *H. armigera* pupae and adults. Similarly, sodium dodecyl sulphate (SDS) PAGE gels were also processed for activity visualization after SDS was removed from the gel with washes of 2.5% triton X-100 (in distilled water). The gels were then incubated in 0.2 M glycine-NaOH buffer (pH 10) for 10 min and overlaid on X-ray film for 2 h. The films were washed with warm water and HGP activity bands were visualized as hydrolyzed gelatin on the X-ray film (Harsulkar *et al*., 1998).

Extracts of fourth-instar larvae were further characterized to determine the identity of differential proteases, using tissues from larvae fed on contrasting diet sources (RO and PP). Protease isoforms were visualized after pretreatment with specific and irreversible synthetic inhibitors of trypsin and chymotrypsin proteases, namely, TLCK and TPCK, as well as rCanPIs containing inhibitory domains for trypsin and/or both trypsin/chymotrypsin (TI/CI), namely CanPI-7 (4IRD, 2TI+2CI), -13 (1IRD, TI), -15 (1IRD, TI) and IRD-9 (CanPI-22, IRD-TI). Heterologous expression and characterization of the rCanPI activity is described previously (Tamhane *et al*., 2007; Mishra *et al*., 2010), further the most effective CanPI candidates were selected for this study. Activity units for all of the above-mentioned PIs were determined as described earlier (Telang *et al*., 2003), and those showing optimum inhibition (20 mg of rCanPI and 200 mM of TLCK and TPCK) were used for pretreatment with HGPs and visualization of protease isoforms. RO and PP were individually mixed with rCanPIs and synthetic inhibitors, incubated at RT for 15 min and then subjected to 8% native PAGE under a constant voltage of 250 V. Appropriate controls such as HGP from inhibitor-free RO- and PP-fed insects were included. These gels were processed after electrophoresis to visualize the protease profiles as mentioned above.

### 2.2.7 LCMS$^{E}$ analyses

The differentially expressed proteases from larvae that fed on RO and PP, namely RO2, RO3, PP3 and PP4 (Please see **Fig. 2.7A**), were characterized further by nano-liquid chromatography mass spectrometry analysis. Protein bands were excised from the native gel based on the activity by the GXCT method and processed for trypsin in-gel digestion according to Shevchenko *et al*. (2007). Gel pieces were washed twice with

deionized water, destained with a 1:1 solution of 50% acetonitrile and 50 mM $NH_4HCO_3$ followed by dehydration in 100% acetonitrile (ACN). The dried, shrunken gel pieces were reduced with 10 mM dithiothretol for 45 m at 56 °C and alkylated with 55 mM iodoacetamide in dark at ~24 °C for 40 min. Gel pieces were dehydrated and then digested with trypsin (Sigma, St Louis, MO, USA) at 37 °C overnight. The resulting peptides were extracted twice by adding 20 ml of a solution containing 5% trifluoro-acetic acid and 50% acetonitrile for 15 min, respectively. The extracts were dried in a SpeedVac (Labconco, Kansas City, MO, and USA) and then reconstituted in 10 ml of 4% aqueous ACN containing 0.1% formic acid for subsequent analysis. Mass spectrometric analysis was performed using ultra performance liquid chromatography (nano-acquity, Waters Corp., Milford, MA, USA) coupled to a SYNAPT high definition mass spectrometer (Waters Corp.). The liquid chromatography conditions and mass methods followed during the analysis were as described earlier for data-independent acquisition and identification of proteins (Dawkar *et al*., 2011). FASTA sequences for *Helicoverpa* proteases from NCBI were used as a databank in the workflow for Protein Lynx Global Server analysis.

### 2.2.8 Structural superimposition of *H. armigera* trypsins

Homology modelling was done to predict structures of *HaTry1*, *HaTry3*, *HaTry4*, *HaTry6* and *HaTry8* (GenBank ID: EU982841, EU325548, EF600059, Y12276 and Y12286, respectively). These trypsins were selected on the basis of sequence divergence obtained from phylogenetic analysis and differential expression profiles. A sequence similarity search was performed using PSI-BLAST against a database of known protein structures with default parameters. The three-dimensional models for all the *HaTry*s were generated using a protein structure prediction server (PS)2 (http://ps2.life.nctu.edu.tw/), which implements an approach to comparative modeling by satisfying spatial restraints, and were extracted from the alignment of the target sequence with the multiple template structures. Predicted models were validated by PROCHECK (Laskowski *et al*., 1993) and ProSA(http:// prosa.services.came.sbg.ac.at/prosa.php) (Wiederstein and Sippl, 2007).

The structures were aligned structurally by TM align (http:// zhanglab.ccmb.med.umich.edu/TM-align), using *HaTry4* as a reference. The sequence

alignments were obtained by CLUSTALW2 (Thompson *et al*., 1994) and the result was confirmed using the Align-2D command within the MODELLER program (Sali and Blundell, 1993). Align-2D generate an alignment of sequences with structures using a variable gap-opening penalty that favours gaps in exposed regions and avoids gaps within secondary structure elements.

### 2.2.9 Docking analyses

A docking study was performed to determine the binding energy and interaction of CanPI-7 with trypsin and chymotrypsin. Predicted structures of differentially expressed trypsins were refined by minimizing energy and relaxing restraints. In order to perform molecular docking, models of *H. armigera* trypsin and different substrates/inhibitors were submitted to the Patchdock online server (http://bioinfo3d.cs.tau.ac.il/PatchDock/) following the standard package protocols (Schneidman-Duhovny *et al*., 2005). Binding energy obtained for each complex was normalized by standard values (standard binding energy of trypsin and substrate/inhibitor interaction) and represented in a heat map format using MeV software packages (http://www.tm4.org/mev/). The gradient ruler from 0 to 1.5 is an indicator of interaction strength.

### 2.2.10 Statistical analyses

The data from biochemical assays and quantitative real-time PCR were collected in triplicate; statistical significance was determined by single factor ANOVA. For data sets showing $F$cal > $F$crit at a 0.01 further *post hoc* analysis was performed using Tukey–Kramer's multiple comparisons honestly significant difference (HSD) test to find out the critical difference by GRAPHPADINSTAT version 3.00 (GraphPad Software, San Diego CA, USA, http://www.graphpad.com). Groups were formed based on similarity in the critical difference values and were represented as significant by * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$, respectively. $P > 0.05$ was not significant.

## 2.3 Results

### 2.3.1 Phylogenetic diversity of *H. armigera* trypsins and chymotrypsins

*H. armigera* trypsins (*HaTry*s) and *H. armigera* chymotrypsins (*HaChy*s) are proteases about 260 amino acids long (with the exception of *HaTry1* at ~301 and *HaTry2* at ~428 amino acids long). The phylogenetic analysis of trypsin-like protease sequences showed that they group into approximately five clades; *HaTry8*, *HaTry1*9 and *HaTry11* group into a distant clade (**Fig. 2.1A**). Multiple sequence alignment of *HaTry*(s) revealed that there is minimal conservation of the sequences and substitution of active site residues in certain isoforms [*HaTry2*, *HaTry8* and *HaTry1*9 (**Fig. 2.1B**].

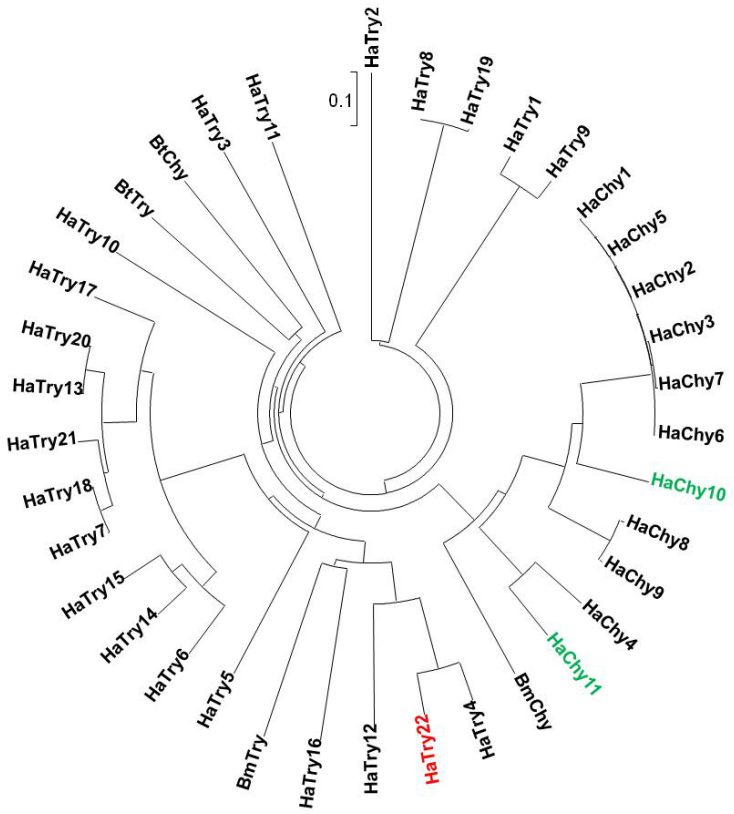



**Figure 2.1A** Phylogram of trypsin-like and chymotrypsin-like serine proteases (mature protein/proenzyme) from *Helicoverpa armigera* (GenPept accession numbers are mentioned in Table 2) generated by CLUSTAL W2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2) using the Gonnet protein weight matrix and the clustering were done using a neighbour-joining algorithm. Trypsin-like sequences (BmTry-Q1HPT9 &BtTry-P00760) and chymotrypsin-like sequences (BmChy-Q1HPW8

& BtChy-Q7M3E1) from *Bombyx mori* and *Bos taurus* were also included in the alignment; this served as the basis for similarity and divergence. Red and green color (See colour version online) indicates the trypsin and chymotrypsin isoforms identified from larvae fed on rose and pigeon pea, respectively.

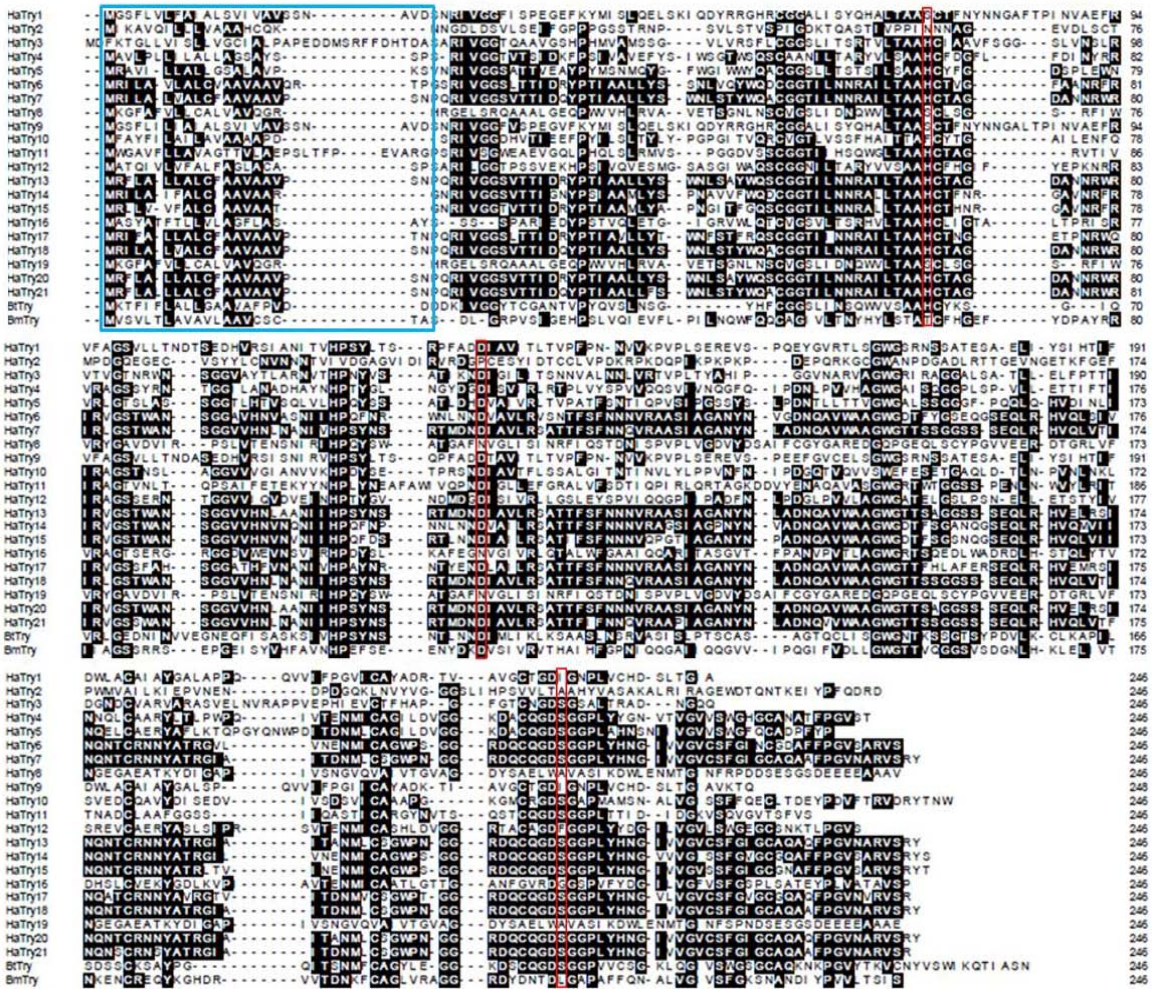


**Figure 2.1B** Multiple sequence alignment of 21 *H. armigera* trypisn (*HaTry*) (mature protein sequences).Trypsin-like sequences (BmTry-Q1HPT9 & BtTry-P00760) from model *Bombyx mori* and *Bos taurus* were also included in the alignment; this served the basis for similarity and divergence. Shaded regions represent conserved sequences, while the active site residues - namely, His (57), Asp (102) and Ser (195) -- are highlighted by red rectangles and the predicted signal peptides are highlighted by blue rectangles.

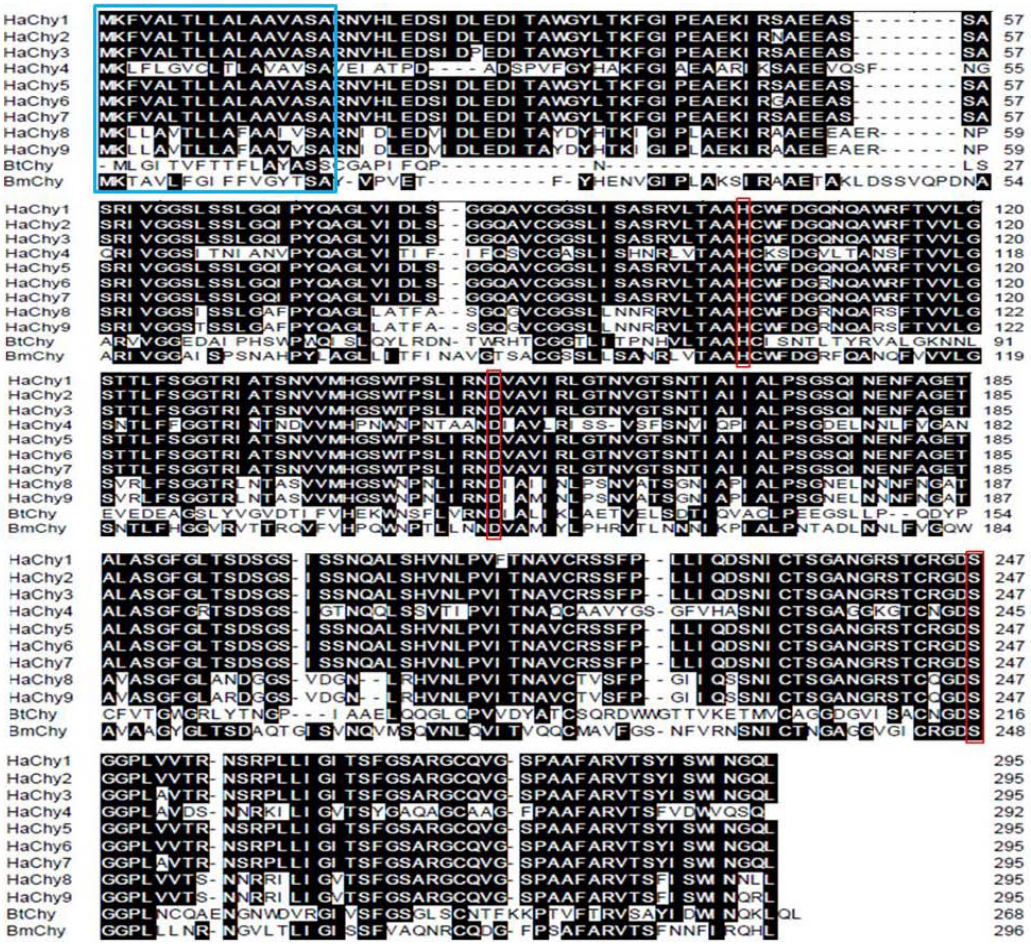


**Figure 2.1C** Multiple Sequence alignment of 9 *H. armigera* chymotrypsins (*HaChy*) (mature protein sequences). Chymotrypsin-like sequences (BmChy-Q1HPW8 & BtChy-Q7M3E1) from model *Bombyx mori* and *Bos taurus* were also included in the alignment; this served the basis for similarity and divergence. Shaded regions represent conserved sequences, while the active site residues - namely, His (57), Asp (102) and Ser (195) -- are highlighted by red rectangles and the predicted signal peptides are highlighted by blue rectangles.

Based on these analyses and earlier studies on the *HaTry* expression, eight diverse *HaTry*s out of 21 were selected for further study. The sequence similarity matrix for these selected genes showed similarity from ~17 to 76%. *HaTry8* is most diverged from the rest, followed by *HaTry2*, *HaTry* 3 and *HaTry* 1 (**Fig. 2.1D**). Likewise, the residues of molecular substrate-binding S1 pocket, namely Asp (189), Gly (216), and Gly (226) were found to be substituted by different amino acids in *HaTry1*, *HaTry2*, *HaTry3* and *HaTry8*

isoforms. The essential nucleophile Asp (195) was replaced by Val (226) in *HaTry1*, Lys (222) in *HaTry2*, Gly (228) in *HaTry3* and Tyr (206) in *HaTry8*, indicating the presence of non-synonymous substitutions. The nine full-length reported *HaChy* isoforms showed relatively more sequence conservation, and only *HaChy4* formed a very distant clade &BtChy-Q7M3E1) from *Bombyx mori* and *Bos taurus* were also included in the alignment; this served as the basis for similarity and divergence. Red and green color indicates the trypsin and chymotrypsin isoforms identified from larvae fed on rose and pigeon pea, respectively.

**Fig 2.1D**

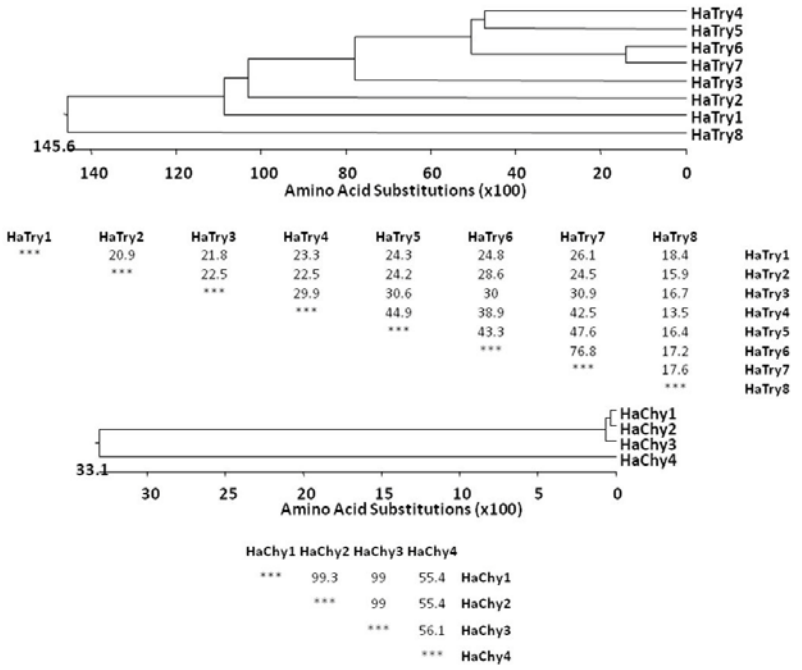



**Fig 2.1E**

**Figure 2.1D and E** Phylogeny and sequence distances matrix of the 8 *HaTry*s (**2.1D**) as well as 4 *HaChy*s (**2.1E**) generated out of the multiple sequence alignment. The matrix depicts the percent similarity amongst the sequences.

## 2.3.2 Variations in the structure and specificity of *H. armigera* trypsins

Based on the *HaTry* phylogeny and gene expression (**Fig. 2.1A, B and D and 2.3**), *HaTry4*, *HaTry1*, *HaTry3*, *HaTry6* and *HaTry8* isoforms were selected for structure

prediction and alignment. The predicted *HaTry4* model (**Fig. 2.2A**) presents the classic fold of trypsin-like enzymes, with two juxtaposed b-barrel domains and the catalytic residues bridging the barrels. The active site of *HaTry4* consists of a triad of His (69), Asp (114) and Ser (211). PROCHECK analysis revealed that the predicted *HaTry4*, *HaTry1*, *HaTry3*, *HaTry6* and *HaTry8* structures had 98% amino acid residues in the allowed φ and ψ conformational region. These structures were then used for structural alignment with *HaTry4* (**Fig. 2.2**). The superimposition of the structurally equivalent Ca atoms of *HaTry1*, *HaTry3*, *HaTry6* and *HaTry8* with *HaTry4* showed that *HaTry1* and *HaTry3* had structures that were distinct from those of *HaTry4*, with a root mean square deviation (RMSD) of 6.55 and 6.37 Å, respectively.

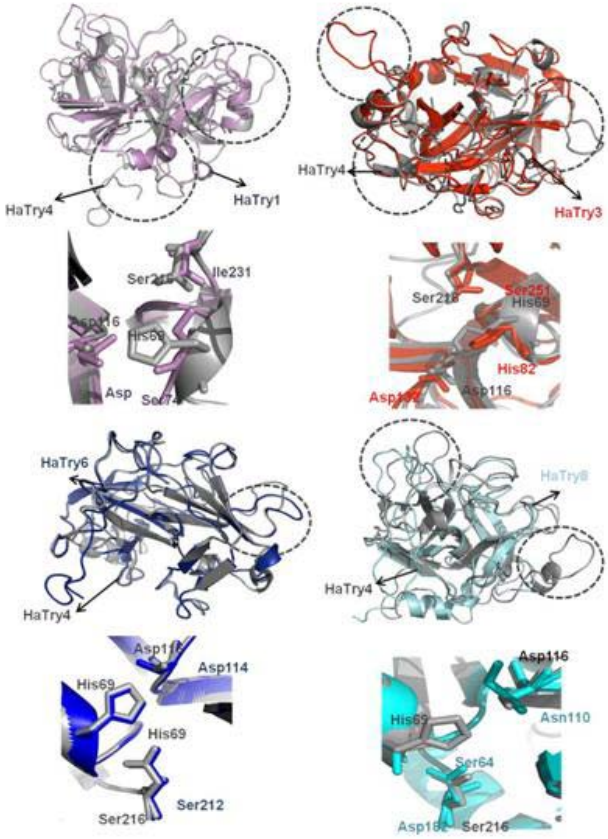



**Fig 2.2A**

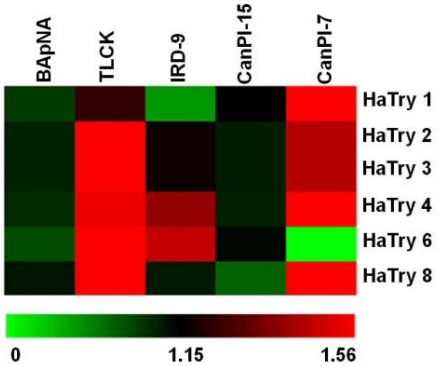



**Fig 2.2B**

**Figure 2.2** Structural and functional divergence of *H. armigera* trypsins. **(A)** Superimposition of the predicted structures of *H. armigera* trypsins. *HaTry2* (green) and *HaTry3* (red) show distinct structures compared to *HaTry* 4 (gray), whereas *HaTry6* (blue) and *HaTry8* (cyan) were structurally closely related to *HaTry4*; their active site

conformations were also mapped. *HaTry4* was used as a template for superimposition. The striking differences among the structures are highlighted by dotted circles. **(B)** Heat map of relative free binding energy of *HaTrys* (*HaTry* 1, 2, 3, 4, 6 and 8) with different substrate/inhibitors.

The structure of *HaTry4* was closely related to *HaTry6* and *HaTry8*, with RMSD values of 1.40 and 1.91 Å, respectively. The predicted structures of *HaTry1* and *HaTry3* exhibited noticeable differences in terms of askew loops and helices as indicated by dotted circles (**Fig. 2.2A**). Their active sites showed a significant difference in conformation compared with the active sites of *HaTry4*, *HaTry6*, and *HaTry8*. Docking simulations suggest that structural differences in trypsins might reflect differential binding energies with substrates/inhibitors. In the case of BA*p*NA and CanPI-15, relatively weak binding energies were observed as compared with N-a-*p*-tosyl-l-lysine chloromethyl ketone (TLCK), CanPI-7 and inhibitory repeat domain (IRD)-9 (Fig. 2.2B). Many of the residues of the primary binding sites in the predicted trypsin-like enzymes were conserved, as expected (His, Asp, Ser; **Fig. 2.2A**); however, changes which could affect enzyme-substrate and enzyme inhibitor interactions occur in residues adjacent to these active site residues which might lead to significant differences in binding energy.

### 2.3.3 Temporal expression of *H. armigera* trypsin transcripts in response to various diets

The quantitative real-time PCR analyses showed that expression levels of *HaTry* and *HaChy* in response to diet and throughout the insect development were complex. The expression of *HaTry*(s) across developmental stages, when *H. armigera* were reared on OK, RO, PP and MZ was relatively high compared with that of *HaChy*(s) (**Figs 2.3 and 2.4**). All *HaTry*s accumulated more at the fourth-instar larval stage, with *HaTry4* the highest, followed by *HaTry2*, *HaTry3* and *HaTry1*; in contrast, *HaTry5*, *HaTry7* and *HaTry8* showed weak expression (**Fig. 2.3A**). The expression of *HaTry*s was found to be highest in larvae that were fed on RO, followed by MZ, PP and OK. The expression of *HaTry6* was six-fold higher than that of other genes in RO-fed larvae, while the expression of *HaTry6* was low in larvae that were fed on other diets. *HaTry2* and *HaTry3*

transcripts were also highly expressed in RO-fed larvae, while *HaTry2* was high in MZ-fed larvae. The expression of *HaTry4* transcripts was nearly constant in all larvae regardless of diet, while the expression of *HaTry5*, *HaTry7* and *HaTry8* was low.

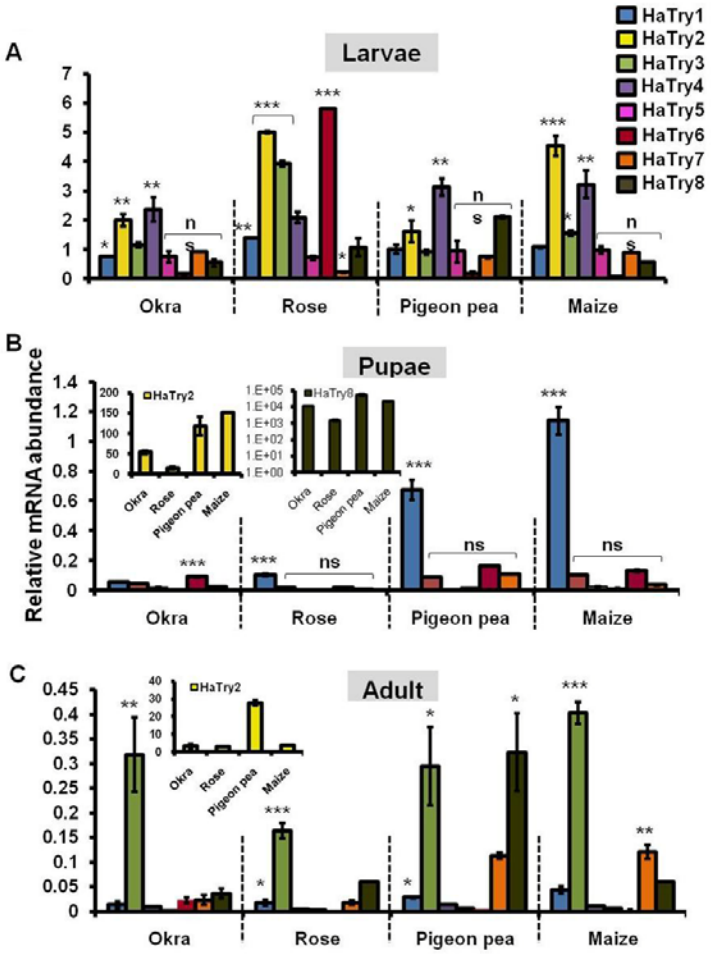



**Figure 2.3** Relative mRNA abundance of trypsin-like serine proteases (*HaTry1* to *HaTry8*) of *H. armigera* larvae (**2.3A**), pupae (**2.3B**) and adult (**2.3C**) reared on okra (OK), rose (RO), pigeon pea (PP) and maize (MZ). Expression values were calculated using a relative standard curve method. The amplification efficiency was 97 to 100% for reference gene (β-actin) and trypsin(s). Data represent mean values of three independent biological replicates along with standard error (±). Post-hoc analyses using Tukey-Kramer multiple comparisons test were performed for statistical significance and represented as * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$; ns for $p>0.05$.

The pupal stage showed enormously high expression of *HaTry8* and *HaTry2*, followed by moderate expression of *HaTry1*, while the expression of other isoforms was

barely detectable (**Fig. 2.3B**). *HaTry1*, *HaTry6* and *HaTry7* isoforms showed the highest expression in pupae of the larvae reared on PP and MZ, and were significantly lower in pupae of the larvae reared on RO and OK. The expression level of *HaTry*s at the adult stage was the lowest regardless of diet (**Fig. 2.3C**). In the adult stage, *HaTry2* showed the highest expression followed by *HaTry3*. Adults of larvae reared on PP showed the highest expression of *HaTry8*.

### 2.3.4 Temporal expression of *H. armigera* chymotrypsin transcripts in response to various diets

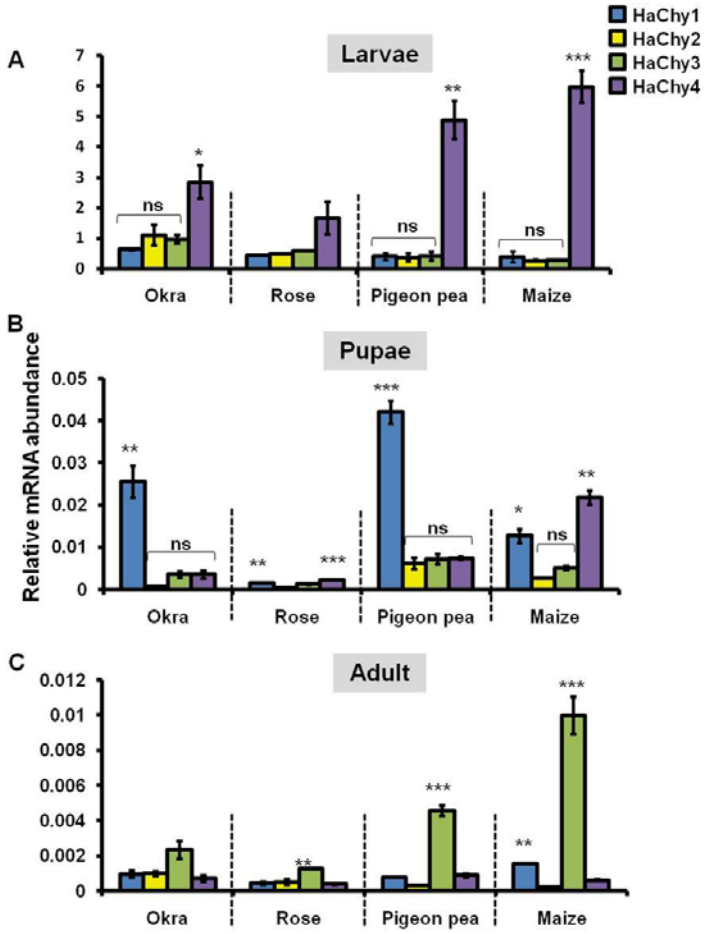



**Figure 2.4** Relative mRNA abundance of chymotrypsin-like serine proteases (*HaChy1* to *HaChy4*) of H. *armigera* larvae (**2.4A**), pupae (**2.4B**) and adult (**2.4C**) reared on okra (OK), rose (RO), pigeon pea (PP) and maize (MZ). Expression values were calculated using a relative standard plot curve. The amplification efficiency was 97 to 100% for

reference gene (β-actin) and chymotrypsin(s). Data represent mean values of three independent biological replicates alongwith standard error (±). Post-hoc analyses using Tukey-Kramer multiple comparisons test were performed for statistical significance and represented as * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$; ns for $p>0.05$.

*HaChy*s showed the highest transcript at the larval stage, reduced transcript levels at the pupal stage, and the lowest levels at the adult stage (**Fig. 2.4**). *HaChy4* was expressed at the highest levels in larvae reared on MZ, followed by those reared on PP, OK and RO (**Fig. 2.4A**). The pupal stage showed higher expression for *HaChy1*, with maximum expression in pupae of the larvae reared on PP, followed by OK, MZ and RO (**Fig. 2.4B**). The expression of *HaChy*s3 was highest in adults of the larvae reared on MZ followed by those reared on PP (**Fig. 2.4C**).

## 2.3.5 RT-PCR analyses

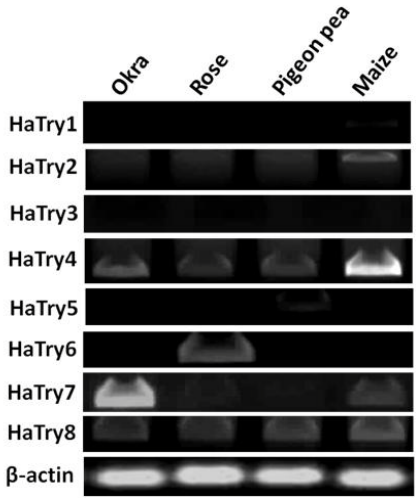



**Figure 2.5** Semi-quantitative RT-PCR analysis of *HaTry* 1 to 8 of fourth-instar larvae fed on okra (OK), rose (RO), pigeon pea (PP) and maize (MZ). The expression levels were normalized with respect to β-actin.

Relative expression of eight isoforms of *HaTry* was also determined by semi-quantitative PCR. For this, the cDNAs from 4$^{th}$ instar larvae reared on okra, rose, pigeon

pea and maize were used. The cDNA amount was normalized using β-actin as endogenous reference gene. The results from RT-PCR analyses (**Fig. 2.5**) corroborate with that of real time PCR analyses. *HaTry4* showed highest expression in larvae fed on chosen diets. *HaTry8* was also highly expressed for all the diets followed by *HaTry2*, 7 and 3. Whereas, *HaTry6* was uniquely expressed in rose fed larvae and *HaTry5* in pigeon pea fed larvae. While, expression of *HaTry1* was barely detectable.

### 2.3.6 Qualitative dynamics of protease activities

Protease activity was found to be five-fold higher in larvae than in adults and pupae (**Fig. 2.6**). Adults of the larvae reared on RO showed significantly higher protease activity than adults of the larvae reared on other diets; however, a several-fold difference in total protease activity was noted in all the stages of *H. armigera* development while trypsin-like protease activity remained constant. Conversely, a diet-specific trend was noted at all stages. Total proteolytic activity (U/mg insect tissue) was highest in larvae fed on PP, twofold lower in those fed on MZ and RO, and three-fold lower in those fed on OK. Trypsin-like activity was highest in pupae fed on PP followed by RO and the trend remained the same for the adult.

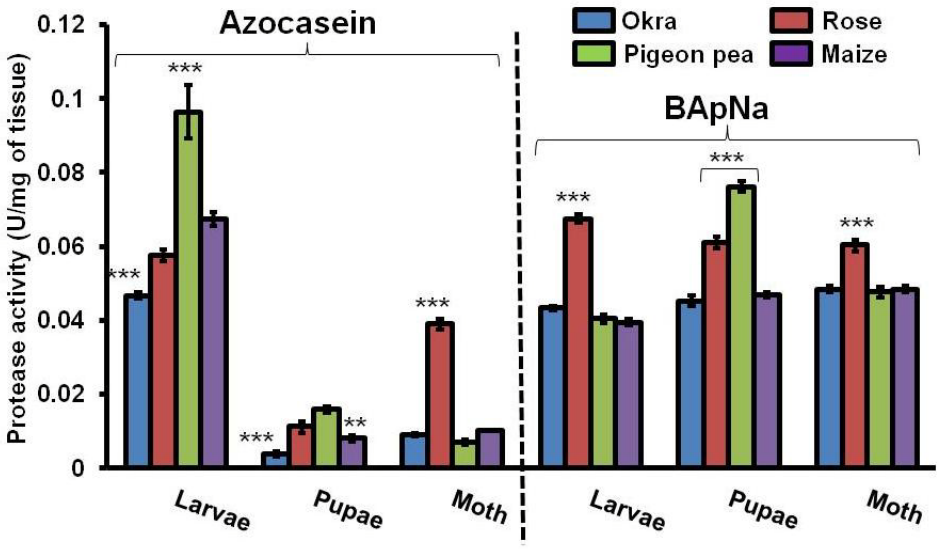



**Figure 2.6** Total protease (Azocasein) and trypsin-like activity (BapNa) U/mg of tissue. Activity units were determined for the larval, pupal and adult stages fed on okra (OK), rose (RO), pigeon pea (PP) and maize (MZ). Data represent mean values of three

independent biological replicates alongwith standard error (±). Post-hoc analyses using Tukey-Kramer multiple comparisons test were performed for statistical significance and represented as * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$; ns for $p>0.05$

### 2.3.7 In gel visualization of dynamics of protease activities

The protease profile of the fourth-instar larvae revealed qualitative and quantitative differences. Lower protease activity was detected in pupal and adult stages. Larvae fed on PP show seven protease isoforms ($P_{PP1}$ to $P_{PP7}$), followed by six isoforms in MZ ($P_{MZ1}$ to $P_{MZ6}$), five isoforms in OK ($P_{OK1}$ to $P_{OK5}$) and four isoforms in RO ($P_{RO1}$ to $P_{RO4}$). The protease isoforms common in all the diets were $P_{OK\ 2,\ 3,\ 4}$; $P_{RO3}$; $P_{PP4,\ 5,\ 6}$ and $P_{MZ3,\ 4,\ 5}$; while some were unique, e.g. $P_{OK5}$; $P_{RO2,\ 4,\ 5}$; $P_{PP2,\ 7}$ and $P_{MZ6}$ (**Fig. 2.7A**).

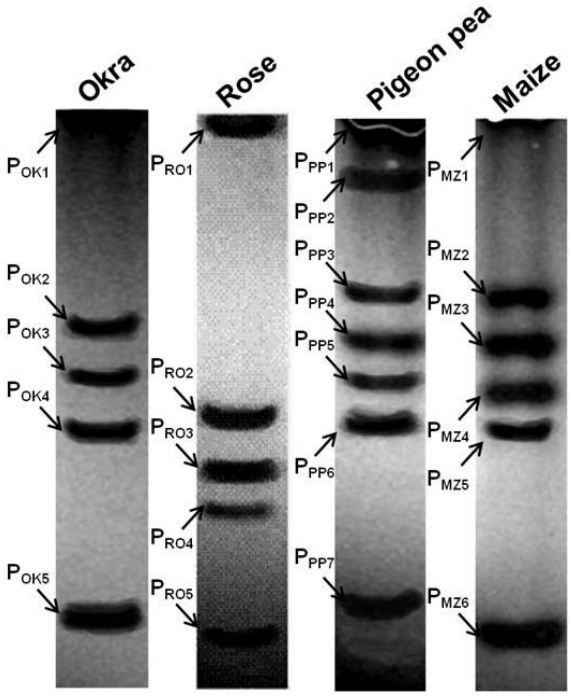



**Figure 2.7A** Differential protease activity visualized using GXCT, untreated gelatin on X-ray film was acted as the substrate. Protein extract from larvae fed on okra, rose, pigeon pea and maize was separated by native (8%) PAGE and visualized for the protease activity profile.

*H. armigera* gut protease (HGP) activity profile was checked with SDS-PAGE also. In this case gel was run under non-denaturing conditions. It was found that *H. armigera* proteases are differentially expressed in response to different host plants. Most of the isoforms were visible in case rose fed larvae, followed by those fed on pigeon pea, okra and maize. The qualitative and quantitative differences were quite clear amongst various *H. armigera* proteases. As in figure **2.7B** rose fed larvae showed relatively more number and intense bands as compared to rest of the diets. While larvae fed on pigeon pea showed less number of bands, followed by those fed on okra and maize. Diet-specific trend was observed for larvae fed with all chosen diets. This can be observed by the appearance of unique isoform in rose fed larvae, whereas few isoforms were commonly expressed across all diet fed larvae. Eventhough some of the protease isoforms were commonly expressed, difference in their intensity was observed suggesting their differential expression.

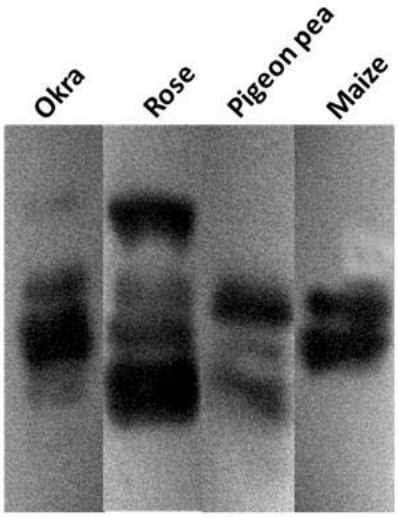



**Figure 2.7B** Activity profile of *Helicoverpa* gut proteases (HGPs) of fourth-instar larvae fed on okra (OK), rose (RO), pigeon pea (PP) and maize (MZ). Isoforms of *H. armigera* gut proteaseswere separated by SDS-PAGE (12%) and visualized using gel X-ray contact print technique (GXCT); untreated X-ray film was used as a substrate.

### 2.3.8 Characterization of HGPs using proteinase inhibitors (rCanPI and synthetic inhibitors)

Proteases were further categorized into trypsin/chymotrypsin-like isoforms by treating *H. armigera* gut protease (HGP) with specific protease inhibitors such as TLCK and N-tosyl-l-phenylalanine chloromethyl ketone (TPCK). TLCK inhibit $P_{RO2}$ and $P_{RO3}$ isoforms (**lane 2, Fig. 2.8A**), but they are not inhibited by TPCK treatment (**lane 3, Fig. 2.8A**). $P_{PP3}$ and $P_{PP4}$ isoforms of PP-HGP were inhibited by both TLCK and TPCK. Some isoforms appeared after PP-HGP was incubated with TLCK and TPCK, and were absent in the control, as indicated by red arrows (**Fig. 2.8A, lanes 5 and 6**). The protease profile for recombinant *C. annuum* PIs (rCanPIs) treated RO- and PP-HGP (CanPI-7, CanPI-13, CanPI-15 and IRD-9; Fig. 2.8B) showed complex interaction of PI with HGPs (**Fig. 2.8C, D**). rCanPI exhibited effective inhibition of PP-HGP isoforms, while RO-HGPs were weakly inhibited. IRD-9 showed the maximum inhibition of PP-HGP, followed by CanPI-13 and CanPI-7, but in the case of RO-HGP inhibition was relatively stronger for CanPI-13, followed by CanPI-7 and IRD-9 (**Fig. 2.8D**). The gel X-ray contact print technique (GXCT) showed complete inhibition of prominent isoforms $P_{RO2}$ and $P_{RO3}$, but new isoforms appeared (indicated by red arrows in **Fig. 2.8C, lane 3**) after incubation of RO-HGPs with CanPI- 13. The protease profiles of PP-HGPs pretreated with IRD-9 showed complete inhibition of PP-HGPs without generating any new protease isoforms. All CanPIs inhibited prominent PP-HGP isoforms, while some new isoforms were observed in PP-HGP pretreated with CanPI-13 and CanPI-15 (marked by red arrows in **Fig. 2.8C, lanes 3 and 4**) and would indicate protease isoforms generated due to complex *in vitro* protease-PI interaction. Differentially inhibited and newly generated isoforms were identified by a nano-LCMSE approach (**Fig. 2.8, Table 2**).

PRO2, PRO3 were identified as trypsins and PPP3, PPP4 were identified as chymotrypsinogens as detailed in **Table 2**. The identified chymotrypsinogens (accession numbers AAF71516 and AAF1515) were from *Agrotis ipsilon* as the peptides showed the best match to these sequences compared with similar sequences from *Helicoverpa* sp. It was found that the identified peptides also map to reported *HaChy* with less coverage and few residues were unique to the *A. ipsilon* sequence (**Appendix I**)

**Table 2: List of proteins identified by LCMS$^E$ analyses**

| Protein | Genbank ID | Description | MW (Da) | pI (pH) | PLGS Score | Peptides | Coverage (%) |
|---|---|---|---|---|---|---|---|
| **$P_{PP3}$** | AAF71516 | AiC5 chymotrypsinogen *Agrotis ipsilon* | 29890 | 8.3013 | 70.2864 | 12 | 60.5536 |
| **$P_{PP4}$** | AAF71515 | AiC2 chymotrypsinogen *Agrotis ipsilon* | 29475 | 8.1861 | 215.6177 | 3 | 19.5122 |
| **$P_{RO2}$** | AAF74750 | putative trypsin precursor Hz3 *Helicoverpa zea* | 16413 | 6.5771 | 132.3745 | 5 | 21.3559 |
| **$P_{RO3}$** | AAF74750 | putative trypsins precursor Hz3 *Helicoverpa zea* | 16413 | 6.5771 | 73.073 | 1 | 12.4183 |

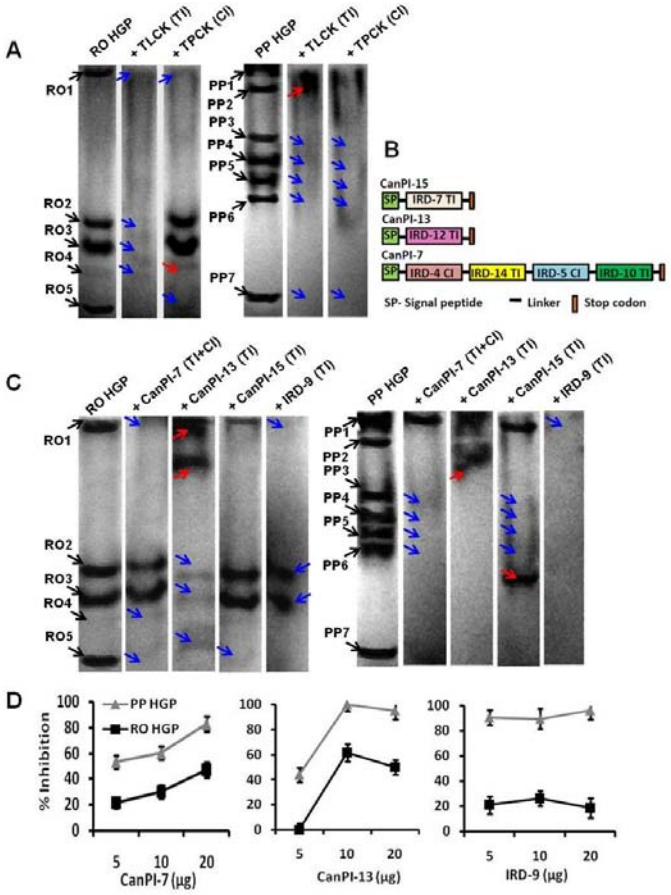



**Figure 2.8** Activity profiles of *H. armigera* gut proteases (HGPs) reared on rose (RO) and pigeon pea (PP) when pre-treated with specific protease inhibitors. Protease profile upon pretreatment with synthetic inhibitors TLCK and TPCK and recombinant PIs;

rCanPI-7, -13, -15 and IRD-9 are represented in (**A)** and (**C),** respectively. (**B)** Schematic structure of the selected recombinant CanPIs and (**D)** shows the differential inhibition of RO and PP HGPs by rCanPIs.

### 2.3.9 Expression analysis of the newly identified proteases

The differential protease isoforms identified by LCMS$^{E}$ from RO ($P_{RO2}$ $P_{RO3}$) and PP HGP ($P_{PP3}$ and $P_{PP4}$) were checked for homology with the rest of the reported *HaTry*s and *HaChy*s. The phylogenetic tree generated from mRNA sequences showed that trypsin-like PRO-2 and -3 (AAF74750) form shares 87% similarity with *HaTry4*, while the PPP-3 (AAF71516) and PPP-4 (AAF71515) were homologous to *HaChy2* (73% similarity) and *HaChy4* (75% similarity) respectively. $P_{RO-2,}$ $P_{RO-3}$, $P_{PP-3}$ and $P_{PP-4}$ were named *HaTry2*2, *HaChy1*0 and *HaChy1*1, and were marked in the phylogenetic tree (**Fig. 2.1A**, isoforms in red and green colour). Reverse-transcription (RT)-PCR analysis was performed for protease isoforms *HaTry2*2, *HaChy1*0 and *HaChy11* from *H. armigera* fed on PP and RO. The real-time quantification of *HaTry2*2 isoforms exhibited relatively high expression in RO-fed larvae and low in PP-fed larvae. By contrast, the relative expression of *HaChy1*0 and *HaChy11* was higher in RO-fed larvae than in PP-fed larvae (**Fig. 2.9**).

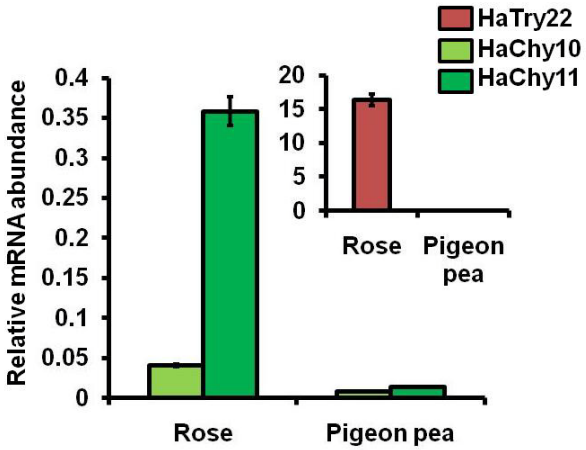



**Figure 2.9** Relative mRNA abundance of the newly identified protease isoforms from rose and pigeon pea fed larvae; β-actin was used as internal reference gene.

## 2.4 Discussion

### 2.4.1 Dynamics of protease gene expression in insect developmental stages

Twenty-one reported variants of *HaTry* isoforms grouped into approximately five clades. While 9 *HaChy* isoforms grouped into only four clades. Phylogenetic analyses of *HaTry* and *HaChy* revealed that they are highly homologous sequences with few diverged variants. Multiple sequence alignment of *HaTry* and *HaChy* isoforms showed that few of the isoforms have gained synonymous substitutions at their active site residues. Such sequence diversity might impart differential functionality to variant isoforms of *HaTry* and *HaChy*. On the basis of their percent similarity they were studied for expression patterns in *H. armigera* larvae when fed on chosen diets. During this study representative 8 *HaTry* isoforms and 4 *HaChy* isoforms were selected.

The present study showed that trypsin and chymotrypsin isoforms of *H. armigera* are under dynamic flux and that they switch their functional specificity according to diet and insect developmental stage. The present study also confirms that, in the larval stage, which is the important assimilatory phase in an insect's life, specific trypsin expression patterns are observed (Chougule *et al*., 2005). High accumulation of *HaTry8* and 2 at the pupal stage suggested that these isoforms might be involved in the developmental processes leading to the transition from larvae to pupae. Moreover, *HaTry*s might have an alternative functional role as a preparatory reserve for further developmental stages. Adults exhibited a thousand-fold down-regulation of *HaTry* isoforms compared with larvae and ten-fold compared with pupae, except *HaTry2*, irrespective of the diet on which the insects were raised. This indicates that *HaTry2* might play a major role in the metamorphosis from pupae to adult and maintenance of adult stage digestive processes. Like *HaTry*s, chymotrypsin also showed dynamic expression patterns throughout different stages of the insect's life cycle. Expression of chymotrypsin isoforms was found to be developmental stage-specific, rather than being diet-specific as with trypsins.

The pattern observed for chymotrypsins was like that of *HaChy4* expression, dominating in the larval stage, with *HaChy1* in the pupal and *HaChy3* in the adult stages. This expression pattern indicates that chymotrypsins might have fundamental roles in immunity, development and metamorphosis (Terra and Ferreira, 1994; Borovsky and

Mahmood, 1995). The quantitative and qualitative difference in the protease gene expression reflects the dynamic regulation of protease expression throughout different stages of insect development and in response to various diets. This also suggests the involvement of serine proteases in functions other than digestion, such as development and metamorphosis. The complexity in *HaTry* and *HaChy* expression also indicated that larval gut proteases are dynamically regulated such that larvae can overcome different plant antifeedants/metabolites. Doing so insect evolves strength to tackle proteins/plant metabolites from previously non-preferred host plant and thereby increase their diet-breadth.

### 2.4.2 Multifaceted proteases: bio-potency for polyphagy

Signaling mechanisms that govern the differential regulation of protease genes in insects are not well understood and thus the molecular basis of larval responses remains enigmatic. In some Lepidopteran species it is known that certain neuropeptides act as regulatory switches governing digestive protease expression (Harshini *et al*., 2002; Huang *et al*., 2010). In *H. armigera,* flexibility in digestive proteases and dynamic gene expression was observed when insects were fed with PIs and/or heterogeneous plant metabolites, which reflect the evolving breadth of diet in polyphytophagous Lepidoptera (Gatehouse *et al*., 1997). Insect proteases display broader substrate specificity and differ significantly in their interaction with inhibitors (Belew *et al*., 1975; Johnson *et al.*, 1989; Peterson *et al*., 1995; Chougule *et al*., 2005). Recently it was found that protease activity of *H. armigera* larvae is strongly influenced by dietary protein content (Sarate *et al*., 2012). The gut protease activity of *H. armigera* fed on PP and RO differed quantitatively and qualitatively: total proteolytic activity was higher in larvae fed on PP than in larvae fed on RO. Trypsin-like activities are predominant in insects feeding on RO. Several distinct protease activity isoforms were detected in the gut when larvae were fed on nutritionally diverse diets such as RO, PP, OK and MZ, suggesting specific protease isoforms were involved in adaptive metabolism.

It was intriguing observation that relatively HaTry expression was highest in RO fed larvae than those fed with PP, MZ and OK, respectively. The possible reason for such expression pattern could be that eventhough the protein content is low in RO,

composition might be complex. Therefore, RO fed larvae is utilizing most of the protease isoforms to derive the required nutrition out of available proteins. However, in case of PP fed larvae the ability of larvae to digest the available protein could be efficient with comparatively lesser number of protease isoforms. Further characterization of the protease complement from *H. armigera* fed on RO and PP revealed their functional differentiation (**Fig. 2.8**), e.g. protease isoforms from RO-fed larvae were not inhibited when treated with synthetic trypsin inhibitor (TI) or rCanPI-13. The protease isoforms of PP-HGP were strongly inhibited by IRD-9 (TI), CanPI-7 [chymotrypsin inhibitor (CI) and TI], CanPI-13 (TI) synthetic trypsin, and chymotrypsin inhibitors, while they were weakly inhibited by CanPI-15 (TI). The difference in the electrophoretic mobility of protease isoforms was also evident in that PP-HGP and RO-HGP showed different interaction with inhibitors. This indicates the differential sensitivity of proteases to inhibitors/substrates, stability of protease-PI and protease-substrate complexes (Christeller and Shaw, 1989).

Native gel-resolved, significantly variant protease activity isoforms from RO- and PP-fed *H. armigera* were sliced, trypsinized and identified by Liquid chromatography-tandem mass spectrometry as trypsin-like and chymotrypsin-like, respectively when screened using a custom *H. armigera* protease database. The identified proteases were not amongst the initial 21 reported sequences of trypsins and five of chymotrypsin. They were named as *HaTry22*, *HaChy10/11* and from their best hits primers were designed for real-time gene expression studies. The protease activity inhibition with TI- or CI-specific inhibitors and trypsins/chymotrypsin gene expression data of RO- and PP-fed larvae correlate with the RO- and PP-specific expression of the identified proteases. Dunse *et al*., (2010b) showed that the substitution of four amino acids (L, A, N and F) in loop 35 of *H. punctigera* chymotypsin 2A (HpCh2A) with the amino acids V, I, D and L from HpCh5 converted the NaPI-susceptible chymotrypsin to NaPI-resistant chymotrypsin. Most of these substitutions had a major impact on the structure and substrate selectivity of the enzymes, these substitutions play a major role by influencing sensitivity to PIs (Bown *et al*., 1997).

Structural comparison of predicted structures of *H. armigera* trypsins indicated differences in active site, S1 binding pocket and distal loops in *HaTry4*, *HaTry1*, *HaTry3*,

*HaTry6* and *HaTry8*. These structural changes may result in different substrate specificity. Crystallographic analysis suggests that the broad enzyme specificity is due to the backbone flexibility of the S1 site, particularly in the region of residue 216 and subsequent amino acids, which shift position significantly in different enzyme-substrate analogue structures (Bone *et al*., 1989, 1991). Consequently, it appears that the substrate specificity in trypsin and chymotrypsin illustrate the role of distal portions of the protein structure (Perona *et al*., 1995; Page &and Di Cera, 2010; Niu *et al*., 2011). The substrate-binding pocket of trypsins is composed of residues between Asp189 and Ser195 and those adjacent to Gly216 and Gly226. However, loops at the surface of the protease, flanking the substrate-binding site (residues 185–188 and 221–224) have also been shown by mutagenesis experiments to be implicated in substrate discrimination and rate of catalysis (Hedstrom *et al*., 1992), therefore, they might play a role in inhibitor-protease interaction. Understanding molecular mechanisms by which the distal elements influence substrate specificity in trypsins will provide insight into the role of the protein scaffold in enzyme catalysis. Comparison of the free energy of different trypsin-substrates/inhibitor interactions and their comparison using heat map analysis provided insights into the structure-function relationship in *H. armigera* gut proteases.

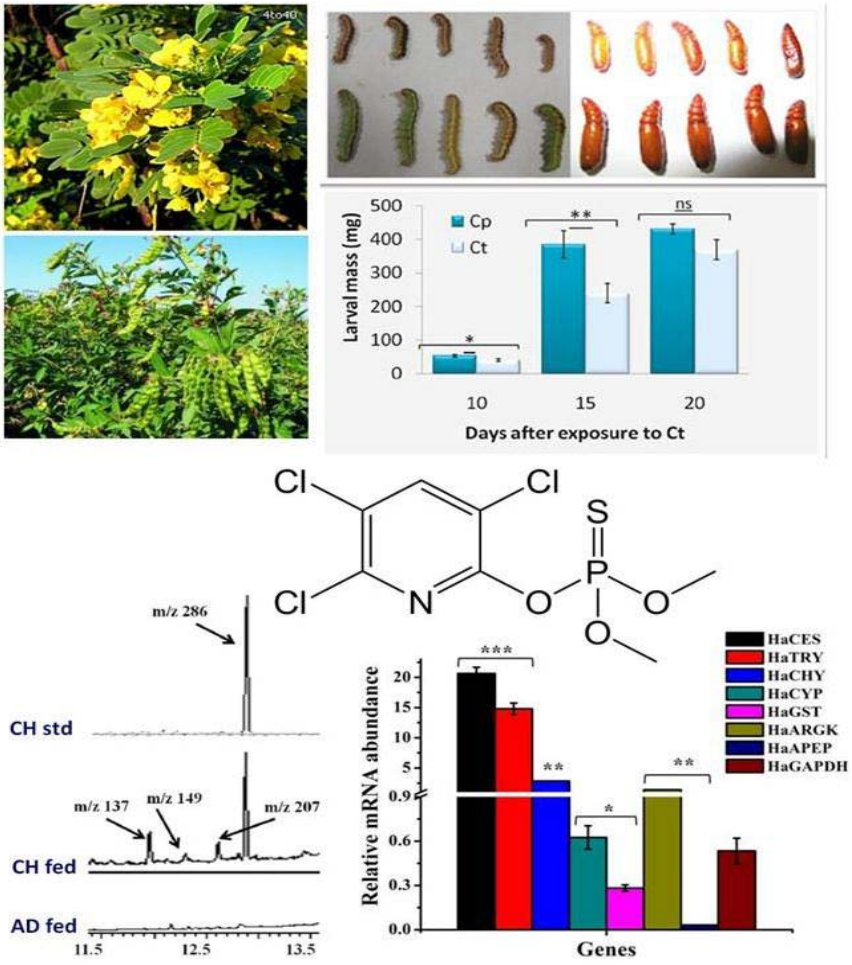
In nutshell, the present study provides enhanced understanding of the dynamics of the *H. armigera* gut physiology, especially digestive proteases, when the insects are fed on diverse natural host plants. In order to get further insights into complex regulatory mechanisms in serine proteases function more comprehensive study involving global scenario of all of the *HaTry* and *HaChy* isoforms is necessary. Similarly, comparative study of *H. armigera* and other Lepidopteran major pests will provide the possible evolutionary relationships amongst serine proteases and their importance for pest's dynamic physiology. Such a study might provide important findings about intricacy in functionality of proteases especially *in vivo* in insects. Knowing this will be beneficial to design effective strategies to tackle polyphagous insect pests. The increasing host range of such a polyphagous insect pest can be dealt with by a thorough study of the protease regulatory mechanism followed by its appropriate targeting.

## 2.5 Conclusion

In summary present study provides enhanced understanding of the dynamics of the *H. armigera* gut physiology when challenged to diverse natural host plants. Importantly it was evident from this study that other than digestion, proteases play major role in metabolism and metamorphosis. Specifically *HaTry*s were predominant throughout the diets and stages indicating their effective role in *H. armigera* physiology, on the other hand *HaChy*s were weakly involved in the digestive/detoxifying mechanism as well as the developmental pathways. Proteases from *H. armigera* exhibited complex interactions with rCanPI's and attempt to modify the neutralizing effect of PI's; also the activity levels differ according to the dietary protein sources for example HGP isoforms from RO-fed larvae were relatively unique and less sensitive to rCanPI's than those of PP-fed larvae. These observations indicated few intriguing facts (i) known, characterized protease inhibitors when used in these simple assays can help in characterizing the protease isoforms, (ii) recombinant PIs even with minor sequence variations interact very differently with the proteases from insects feeding on different dietary sources conversely also indicating the extent of complexity of the gut protease complement (iii) through some unknown *in vitro* interaction(s) between the proteases and the inhibitors newer isoforms are generated which probably could be tapped for identifying the mechanism of regulation of protease complement (iv) well characterized PIs, known to exert anti-metabolic influence on pest feeding on one crop plant cannot be directly applied to other crop plant for managing the same pest (v) Specific and stable RNA silencing of *HaTry*'s can be applied effectively to suppress the Lepidopteran pests.

# Chapter 3

## Molecular responses of *H. armigera* upon exposure to host, non-host plants and synthetic pesticide




**The part A of research work described in this chapter has been published as full-length research article in Journal of Proteome Research (2011), 10, 5128–5138 and Part B in Insect Science, 2014 (*In press*).**

# Molecular responses of *Helicoverpa armigera* upon exposure to host, non-host plants and synthetic pesticide

## Part A: Assimilatory potential of *H. armigera* reared on host (Chickpea) and non-host (*Cassia tora*) diets


## Abstract

Adaptation to plant allelochemicals is a crucial aspect of herbivore chemical ecology. To understand an insect ecology, we studied an effect of non-host *Cassia tora* seed based diet (Ct) on growth, development, and molecular responses in *Helicoverpa armigera*. We employed a comparative proteomic approach to investigate the differences in gut, hemolymph, and frass proteome of *H. armigera* reared on a normal (chickpea seed-based, Cp) and non-host Ct diet. In this study, a total of 46 proteins were identified by nano-LCMS$^{E}$. Among them, 17 proteins were up-regulated and 29 proteins were down-regulated when larvae were exposed to the Ct diet. Database searches combined with GO analysis revealed that gut proteases engrossed in digestion, proteins crucial for immunity, adaptive responses to stress, and detoxification were down-regulated in the Ct fed larvae. Proteins identified in *H. armigera* hemolymph were found to be involved in defense mechanisms. Moreover, proteins found in frass of the Ct fed larvae were observed to participate in energy metabolism. Biochemical and quantitative real-time PCR analyses of selected candidate proteins showed differential gene expression patterns and corroborated with the proteomic data. Our results suggest that the non-host (Ct) diet could alter expression of proteins related to digestion, absorption of nutrients, adaptation, defense mechanisms, and energy metabolism in *H. armigera*


## 3.1A Introduction

The larvae of herbivorous insects feed actively on plants to gather nutritional components required for development and progression into the reproductive adult phase. *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), a devastating polyphagous pest of many important crop plants throughout the world, is responsible for heavy economic losses (Sharma *et al.,* 2001). The larvae of this pest principally feed on reproductive structures of crop plants such as cotton-buds, balls, kernels, pods etc leading to dramatic reduction in the agricultural yield. This and its other special attributes like high fecundity, ability to undergo diapauses, high migratory abilities in moths have made it one of the serious crop pests. The adaptation and detoxification mechanism in *H. armigera* increases its resistance to non-host plants making this pest difficult to manage in the field. Considerable research has demonstrated the potential of insects to adapt to diverse phytochemicals and toxins (Gill *et al.,* 1992; Tabashnik *et al.,* 1994 and; Bauer *et al.,* 1995). Knowing which certain effective control measures have been developed, but these were found to overcome by this pest.

Plants respond to herbivore attack with highly evolved, elegantly regulated arrays of direct and indirect defenses. Direct defenses are those that involve production of metabolites, which directly retard the growth and development of herbivores and indirect defenses involve the production of metabolites that indirectly protect plants by attracting the herbivores' natural enemies, usually parasitoids and predators (Pare *et al.,* 1999; Walling, 2000; Kessler and Baldwin, 2002; Wu and Baldwin, 2010). During feeding, insect herbivores regularly encounter plant allelochemicals. The effect of allelochemicals on nutrient regulation and insect herbivore performance is modified depending on food nutrient composition (Behmer, 2009). Alterations in insect gut physiology or biochemistry can disrupt this sequential process and result in toxin resistance. However, as a result of co-evolution, herbivorous insects adapt to plant defenses by evasion and/or detoxification (Gatehouse, 2002).

Insects are also able to compromise defense strategies by exploiting signaling mechanisms. The corn earworm employs signaling molecules such as jasmonate and salicylate from its plant host to activate four of its cytochrome P450 genes, making the induction of detoxifying enzymes rapid and specific (Li *et al.*, 2002). The toxin resistance observed in the field strain was due to multiple resistance mechanisms, including

increased detoxification of these toxins by microsomal oxidases, glutathione S-transferases, hydrolases, reductases and acetylcholinesterases. Resistance appeared to be better correlated with detoxification enzyme activities in larval tissues, suggesting that the larval body is an ideal tissue source for comparing detoxification capability for adaptation and/detoxification in insects (Yu *et al.*, 2003). Recently many studies are being carried out to understand the insect's biochemical and physiological strengths to develop resistance against toxins of plant allelochemicals. Insect's ability to develop complex adaptive mechanism against diverse plant allelochemicals and absence of resistance to pest in crop plants is increasingly affecting the current pest management strategies at field level. Therefore, there is need to design more effective and robust pest control strategies.

To develop effective toxin resistance management strategies, a complete understanding of the physiological and genetic mechanisms by which insects become resistant to these insecticidal proteins and/or phytochemicals is needed. The purpose of this study is to understand adaptation and/detoxification capability in larvae of *H. armigera*. This study involved comparative biochemical and molecular approach to investigate the proteomic differences in gut, hemolymph and frass of *H. armigera* reared on normal host diet chickpea, *Cicer arietinum* (Cp) and non-host *Cassia tora* (Ct) diet. Comparative biochemical and molecular approach provide with key factors underlying adaptation and/detoxification mechanisms in this pest.

## 3.2A Materials and Methods

### 3.2.1 Materials

Bovine trypsin, chymotrypsin, elastase, benzoyl-DL-arginyl-p-nitroanilide (BApNA), N-Succinyl-Ala-Ala-Pro-Leu-nitroanilide (SAAPLpNA), acetonitrile, bovine serum albumin (BSA) and sequencing grade modified trypsin were procured from Sigma Chemicals, St Louis, MO. MassPrep predigested standard protein rabbit glycogen phosphorylase B (GP) was purchased from Waters Corporation (www.waters.com). All other chemicals of analytical grade were procured locally.

### 3.2.2 Rearing of insects

Actively feeding *H. armigera* larvae collected from fields were transferred and maintained on a chickpea based (Cp) diet (Nagarkatti and Praksah, 1974). The Cp diet (650 mL) was composed of chickpea seed meal, 77 g; wheat germ, 5.6 g; dried yeast powder, 19.2 g; casein, 12.8 g; ascorbic acid, 4.6 g; methyl para-hydroxy benzoate, 1.5 g; sorbic acid, 0.8 g; streptomycin sulfate, 0.2 g; cholesterol, 0.2 g; formaldehyde, 1 mL; multivitamin drops, 0.8 mL; vitamin E, 12 g, and agar, 10 g. To ensure greater genetic homogeneity among test populations, insects were maintained on a control diet for three generations. To understand the effect of Ct diet on growth and development of *H. armigera*, a set of insects was fed on Cp diet up to second instar and then transferred on Ct diet. Ct diet was fed to larvae for about 10-12 days continuously and fourth instar larvae were harvested for collecting tissue samples.

### 3.2.3 SDS-PAGE analysis of *H. armigera* proteins

Proteins from gut and frass were extracted according to Schuster and Davies (1983) with slight modifications. Tissue (500 mg) was ground using a mortar and pestle in liquid nitrogen, mixed with 20 mL extraction buffer (0.7 M sucrose; 0.5 M Tris; 30 mM HCL; 50 mM EDTA; 0.1 M KCl; 2% [v/v] mercaptoethanol) and vortexed for 10min. The homogenate was centrifuged at 4 °C for 13 000 g and the supernatant was transferred to a new falcon tube, to this an equal volume of water saturated phenol was added. Phenol phase was re-extracted with an equal volume of extraction buffer by shaking for 10 min and centrifugation at 13 000 g at 24 °C. The re-extracted phenol phase was precipitated with 5 volumes of 0.1 M ammonium acetate in methanol at 20 °C overnight. The precipitate was washed thrice with 0.1 M ammonium acetate in methanol and once with 100% acetone. The pellets were air-dried and resuspended in lysis buffer containing 8 M urea, 2 M thiourea, 4% (v/v) CHAPS and 50 mM DTT. The resulting suspension was centrifuged and stored at 80 °C for later use. Protein from the hemolymph was extracted by TCA-acetone precipitation (Xiang *et al.*, 2010). Hemolymph (80 µL) was mixed with 1 mL TCA-acetone and the mixture was kept at 4 °C for 2 h. Protein pellet was finally dissolved in lysis buffer.

Protein concentration was determined by Bradford's method (Bradford, 1976). Sample proteins (80 µg each) were loaded on 15% SDS polyacrylamide constant

separation gel with a 4% stacking gel and electrophoresed at 20 °C using an vertical electrophoresis apparatus (Bangalore Genei, India) constant current of 50 Amp/gel until the bromophenol blue front reached the bottom of the gel. The gels were visualized with Commassie Brilliant Blue (CBB) staining and scanned at 300 dpi using high resolution image scanner (Biorad GS 800, USA). In-gel digestion was performed as reported by Oostveen *et al.*, (1997). Protein bands were excised from the CBB-stained gels, washed twice with milli-Q water, destained with a 1:1 solution of 50% acetonitrile and 50 mM $NH_4HCO_3$ and then dehydrated in 100% acetonitrile (ACN) until the gel pieces were shrunken. The dried gel pieces were reduced with 10 mM dithiothretol for 45 min at 56 °C and alkylated with 55 mM iodoacetaamide in dark at RT for 40 min. Gel pieces were dehydrated and then digested with trypsin (Sigma, St Louis, MO) at 37 °C overnight. The resulting peptides were extracted twice by adding 200 µL of a solution containing 5% trifluoroacetic acid and 50% acetonitrile for 15 min, respectively. The peptides were dried in a SpeedVac (Labconco, Kansas City, MO) and then reconstituted in 10 µL of 4% aqueous ACN containing 0.1% formic acid for subsequent analysis.

### 3.2.4 Liquid chromatography mass spectrometry analysis

All the samples were analyzed by nanoscale capillary $LCMS^E$ using a NanoAcquity ultra performance liquid chromatography (UPLC) system (Waters Corporation) coupled to a Q-TOF MALDI-SYNAPT High Definition Mass spectrometer (Waters Corporation). The nano-LC separation was performed using a bridged-ethyl hybrid (BEH) $C_{18}$ reversed phase column (1.7 µm particle size) with an internal diameter of 75 µm and length of 150 mm (Waters Corporation). The binary solvent system used comprised 99.9% water and 0.1% formic acid (mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). The samples were initially preconcentrated and desalted online at a flow rate of 5 µL/min using a Symmetry $C_{18}$ trapping column (internal diameter 180 µm, length 20 mm) (Waters Corporation) with a 0.1% B mobile phase. Each sample (total digested protein) was applied to the trapping column and flushed with 0.1% solvent B for 3 min at a flow rate of 15 µL/min. After each injection, peptides were eluted into the NanoLockSpray ion source at a flow rate of 300 nL/min using a gradient of 2-40% B over 50 min. The lockmass calibrant peptide standard, 600

fmol/µL glu-fibrinopeptide B, was infused into the NanoLockSpray ion source at a flow rate of 300 nL/min and was sampled during the acquisition at 30 s intervals. The mass spectrometer was operated in V-mode at a resolution of at least 9000 full width at half height (fwhh). For $LCMS^E$, full scan (m/z 50_2000) $LCMS^E$ data were collected using the "expression" mode of acquisition, which acquires alternating 1 s scans of normal and elevated collision energy (Silva *et al.,* 2005 and 2006). Data were collected at a constant collision energy setting of 4 V during low-energy MS mode scans, whereas a step from 20 to 40 V of collision energy was used during the high-energy $MS^E$ mode scans.

### 3.2.5 Data processing and database searching

The continuum $LCMS^E$ data were processed and searched using Protein Lynx Global Server 2.4 (PLGS; Waters Corporation) software. Protein identifications were obtained by searching either insecta database (www.uniprot.org) or insect+fabaceae family database constructed separately (for raw datafiles of frass proteome). $LCMS^E$ data were searched with a fixed carbamidomethyl modification for Cys residues, along with a variable modification for oxidized Met residues. The Ion Accounting search algorithm within PLGS was developed specifically for searching data-independent $MS^E$ data sets, and a detailed description of the algorithm was recently published by Li and coworkers (2009). The Ion Accounting search parameters that were used to search the data independent analysis (DIA) data included precursor and product ion tolerance (automatic setting), minimum number of peptide matches (1), minimum number of product ion matches per peptide (5), minimum number of product ion matches per protein (9) and maximum number of missed tryptic cleavage sites. The false positive rate was 4%. Search results of the proteins and the individual MS/MS spectra with confidence level at or above >95% were accepted. Triplicate replications were performed for each sample and a comparison between Cp and Ct feeding groups was conducted.

### 3.2.6 Preparation of extracts and enzymatic analyses

Five hundred milligrams of gut tissue and frass of *H. armigera* were ground to a fine powder and extracted in 500mL of 200mM Glycine-NaOH buffer (pH 10) for 2 h at 4 °C. The extract was then centrifuged at 12 000 g (4 °C; 10 min) and the clear supernatant was used as a source of enzyme for all solution assays. *H. armigera* hemolymph was directly used as an enzyme source. *H. armigera* larvae were fed on Cp and Ct diets in vials and the resulting frass from third and fourth instar larvae were collected. Frass samples were extracted in 0.2M Glycine-NaOH buffer pH 10, in a similar way as mentioned above. These extracts were used for protease estimation assays. Protease activity was measured by azocaseinolytic assays (Brock *et al.,* 1982). One proteinase unit was defined as the amount of enzyme that increased the absorbance by 1.0 OD under the given assay conditions. Bovine trypsin and trypsin/chymotrypsin like activity were estimated using enzyme-specific chromogenic substrates, BApNA and SAAPLpNA, as reported in our earlier communication (Patankar *et al.*, 2001; Telang *et al.,* 2003). For the inhibitor assays, a suitable volume of seed extract was added to the *H. armigera* gut protease (HGP) extract or to the respective proteinase and incubated at room temperature (25 °C) for 30 min.

The residual proteinase activity was estimated as described above. One proteinase unit was defined as the amount of enzyme that increased absorbance by 1 OD and one PI unit was defined as the amount of inhibitor that caused inhibition of 1 unit of proteinase activity under the assay conditions. Glutathione-S-transferase (GST) activity was determined according to Habig *et al.*, (1974), using a 100 mM phosphate buffer pH 6.5, containing organic solvent at a final concentration of 0.25% ethanol and benzene substrate (1,2-dichloro-1-dinitrobenzene). A unit of activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product per min under assay conditions. NADPH-cytochrome P450 (P450) spectrophotometric assays for P450 activity was performed using standard 1 cm disposable cuvettes in a total reaction volume of 1 mL. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) reductase activity was determined using 300 μM DPPH and HGP extract in 100 mM potassium phosphate (pH 7.6). Absorbance reductions at 520 nm were determined after adding 100 μM NADPH. An extinction coefficient of 4.09 $mM^{-1}$ $cm^{-1}$ was used to calculate the number of moles of DPPH

reduced per mol of enzyme (Yim *et al.*, 2005). Each enzyme assay was performed in triplicates for Cp and Ct feeding group.

### 3.2.7 Gene expression analysis by Real-time PCR

Tissues (gut and hemolymph) of fourth instar *H. armigera* were harvested by dissecting the larvae mid-ventrally under sterile conditions, snap frozen in liquid nitrogen and stored at 80 °C. Total RNA was isolated from pools of 5 replicates of gut and hemolymph respectively using Trizol reagent (Invitrogen, Carlsbad, CA) based on the manufacturer's instructions. The total RNA was treated with RNase-free DNAase I (Promega, Madison, WI, USA) to eliminate genomic DNA contamination. The quality and quantity of RNA was determined by agarose gel electrophoresis and spectrophotometric analysis using Nanodrop (Thermo Scientific, Waltham, MA), respectively. Synthesis of the first strand cDNA was carried out in 20 μL reactions using 2 μg of total RNA with Reverse transcription system (Promega, Madison, WI) as per the manufacturer's instructions.

Quantitative Real-Time PCR (qRT-PCR) was performed using 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster, CA) to examine the transcript abundance of the identified candidate proteins. Relative mRNA expression of trypsins (*HaTry*1 Acc. No.Y122277, Y12275, Y12276, AF261980; *HaTry*2 Acc. No. EF600059; *HaTry*3 Acc. No. Y12271, Y12283, Y12270, Y12269 and *HaTry*4 Acc. No. EU770391), chymotrypsins (*HaChy*1 Acc. No. HM209422.1; *HaChy*2 Acc. No. HM209422.1, EU325550.1, GU323796.1, Y12273 and *HaChy*3 Acc. No. Y12273) and GST (*HaGST*, Acc. No. EU289223) was quantified using Faststart Universal SYBR Green Master (Rox) 2X concentrate (Roche Diagnostics, Gmbh, Germany). From the available sequences for *H. armigera* trypsin and chymotrypsin (NCBI) sets of primer pairs were designed based on sequence identity; representing various isoforms of each of these genes listed in **Table1**. The relative expression levels of target genes were calculated using the relative standard plot method (as described in **section 2.2.4, chapter 2**) and were expressed as mean standard errors of three biological replicates.

### 3.2.8 Statistical Analysis

Data was analyzed by one-way analysis of variance (ANOVA) with Tukey's-Kramer multiple comparisons test. Data points were considered significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.0001$.

**Table 1: List of primers used for real time PCR analyses**

| Primer Name | Sequence 5'→3' | Gene bank Acc. No. | Gene Name |
|---|---|---|---|
| **HaActin** | F: GATCGTGCGCGACATCAAG<br>R: GCCATCTCCTGCTCGAAGTC | AF286059 | B-actin |
| **HaTry1** | F: TGGCTGGGGTGACACTTTCT<br>R: GTCTCCCTGGCACTGGTC | Y12277<br>Y12275<br>Y12276<br>AF261980 | putative serine protease mRNA |
| **HaTry2** | F: GTGCTACCCCTTCTGATTC<br>R: AACTTGTCGATGGAGGTGAC | EF600059 | protease (SerProxmRNA -5) |
| **HaTry3** | F: CAGAGGATTGTGGGTGGTTCG<br>R: GCGGTGAGGATAGCCCTGTT | Y12271<br>Y12283<br>Y12270<br>Y12269 | putative serine protease mRNA |
| **HaTry4** | F: GCGTAAAGGATGCGGTTGG<br>R: CAGGATGGCAACCATCCATG | EU770391 | serine protease-like protein 1 mRNA |
| **HaChy1** | F: CGACTTGTCAGGTGGTCAGGCTG<br>R: CGATTCTGGTACCGCCGGAGAAC | HM209422.1 | Chymotrypsin |
| **HaChy2** | F: CGGTGACTCTGGTGGCCCTC<br>R: GTGACTCTGGCGAAGGCAGCAGG | HM209422.1<br>EU325550.1<br>GU323796.1<br>Y12273 | Chymotrypsin mRNA |
| **Hachy3** | F:CACCATCTTCATCTTCCAATCCGTGTGC<br>R:GTGTTGATACGAGTACCACCGAAGAAC | Y12273 | Chymotrypsin |
| **HaGST** | F: CATGACAGTCGCACGATTCTG<br>R: AGTAATGTTTCGGCCCCTAGTG | EU289223 | glutathione S-transferase |

## 3.3A Results

### 3.3.1 Effect of Cp- and Ct-based diets on growth and development of *H. armigera*

To evaluate the effect of *C. tora* seed components on *H. armigera*, larvae were reared on a diet prepared with *C. tora* seed powder (Ct) and a host diet prepared from chickpea flour (Cp). There was a 2- to 3-fold reduction in larval mass in the larvae fed on the Ct diet (**Fig. 3.1A**).

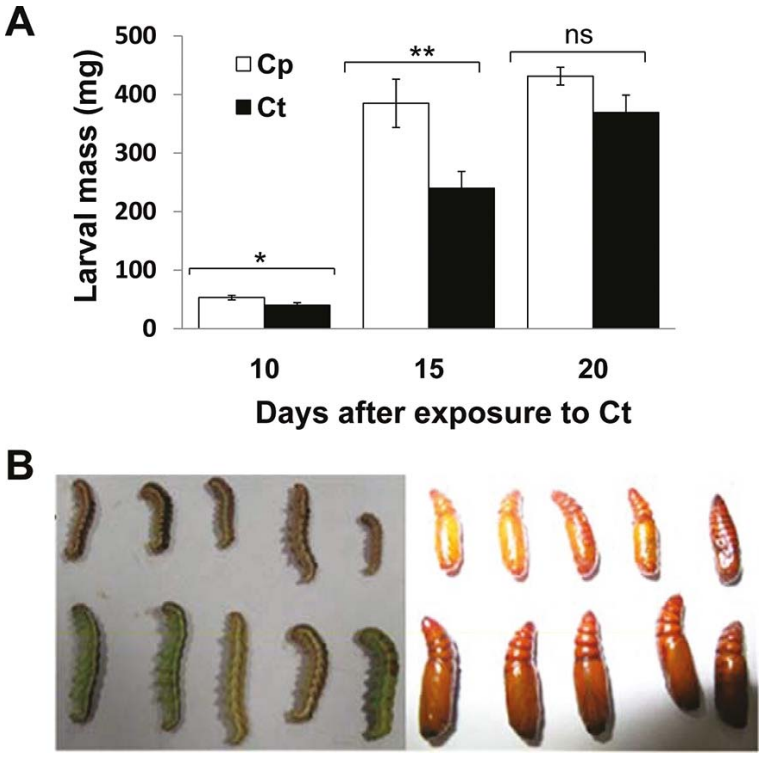



**Figure 3.1** Development of *H. armigera* reared on Cp and Ct diets. (**A**) Larval mass fed on Cp (hollow bars) and Ct diet (black bars). Graph shows average mass from each set of 15 larvae. Larvae were critically weighed on every fifth day. (**B**) Photograph of larvae grown on a Ct diet showing stunted growth (upper row) and Cp diet showing normal growth (lower row). Standard mean errors are indicated. * and ** indicate that values are significantly different from each other at $p < 0.01$ and $p < 0.005$, respectively.

Food intake was dramatically reduced in the larvae grown on the Ct diet compared to the Cp diet. Over 85% of the larvae fed on Ct diet showed stunted growth.

Furthermore, pupation was also delayed for more than 8-10 days in larvae showing stunted growth and 32% mortality was also observed in the Ct fed *H. armigera*. From larval mass gain profile it was clear that Ct is exhibiting stronger retardation of growth until 15 days post feeding. Beyond this larva is either ready to overcome the inhibitory effects of Ct or managed to survive by pupation. Ct diet effectively altered development of *H. armigera* larvae as compared to that of Cp diet. Critical reduction in larval as well as pupal size was observed; and morphology of Ct fed larvae and pupae was altered as compared to that of control (Cp diet) (**Fig. 3.1B**). These results indicate that Ct seeds contain strong *H. armigera* growth inhibitory compounds (proteins/metabolites), and during the effect of the Ct diet on growth, development and larval age was found to be critical.

### 3.3.2 Inhibition potential of chickpea and *C. tora* protease inhibitors

The inhibition potential of chickpea and *C. tora* protease inhibitors (seed extracts) against gut and frass protease activity of *H. armigera* larvae was measured. Proteases extracted from gut of Cp fed larvae showed 35% inhibition by chickpea PIs, while those of *C. tora* PIs showed 73% inhibition of total protease activity. Chickpea PIs were able to inhibit only 41% of the frass protease activity, while *C. tora* PIs were able to inhibit 64% of the total protease activity (**Table 2**). The gut and frass proteases of larvae showed significant difference in the expression of individual proteases.

**Table 2: Inhibition of proteinase activity of *H. armigera* larvae reared on cp and ct diet assessed against chickpea and *C. tora* seed PIs[a]**

| | **Inhibition of protease (%) activity by[b]** | |
|---|---|---|
| Source of proteases | Chickpea PIs | C. tora PIs |
| Gut | 35 ± 1.01 | 73 ± 2.21[c] |
| Frass | 41 ± 2.19 | 64 ± 2.6[c] |

[a] Inhibitory activity was estimated by taking various concentrations of inhibitor to obtain maximum inhibition of proteinase activity. Activities were estimated using azocasein as a substrate, as described in materials and methods.

[b] Values are mean of three experiments ± SEM.

[c] Significantly different from control (Cp PIs) at $p < 0.02$.

### 3.3.3 Differential SDS-PAGE protein profile of Ct and CP fed *H. armigera*

SDS-PAGE protein profiles of gut, hemolymph and frass of *H. armigera* reared on Cp and Ct diets were as shown in **Fig. 3.2**. It was observed that upon loading equivalent amount of proteins onto SDS-PAGE, significant quantitative differences were seen for larval proteins of CP and Ct fed larvae. In that, heamolymph (**Fig. 3.2B**) showed more number of proteins followed by gut (**Fig. 3.2A**) and frass (**Fig. 3.2C**). SDS-PAGE protein profile for respective tissues exhibited differential accumulation of larval proteins in Ct and Cp fed larvae. These differences were both qualitative and quantitative. The gel was sliced uniformly and then subjected for identification of proteins by nano-LCMS analyses.

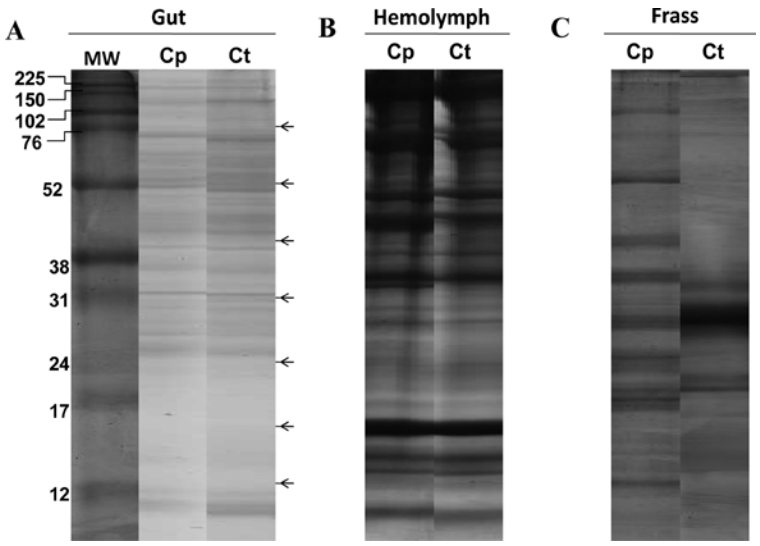



**Figure 3.2** SDS-PAGE pattern of proteins in *H. armigera* gut (**A**), hemolymph (**B**) and frass (**C**) of larvae reared on Cp and Ct diets. Eighty micrograms of protein were loaded in each well and a 15% resolving gel was used for the separation of proteins. Eight slices of each lane were excised as marked by arrows in the figure for trypsin digestion

### 3.3.4 Identification of differentially expressed proteins in *H. armigera* upon exposure to the Ct diet

Overall 46 proteins were successfully identified, among which 25 proteins were from gut, 15 from hemolymph and 6 from frass (**Tables 3, 4, and 5**).

**Table 3: List of identified proteins by LCMS$^{E}$ in gut of *H. armigera***

| Protein name | Acc. no. | MW (kDa) | pI | PLGS Score | Total Peptides | Unique Peptides | Coverage (%) | Fold ↓/↑ on Ct[a] |
|---|---|---|---|---|---|---|---|---|
| Myosin heavy chain | B5M9A2 | 96 | 4.8 | 1076 | 24 | 01 | 20.81 | 5.22$^{\uparrow}$ |
| Very high density lipoprotein | A8CMX | 172 | 5.4 | 472 | 37 | 29 | 19.36 | 2.94$^{\uparrow}$ |
| ATP synthase subunit | Q1HPT0 | 54 | 5.1 | 7132 | 49 | 01 | 50.87 | 2.21$^{\uparrow}$ |
| Carboxyl choline esterase | D5G3G4 | 31 | 6.2 | 3278 | 22 | 10 | 42.75 | 1.96$^{\uparrow}$ |
| Sterol carrier protein | D1GJ60 | 57 | 8.2 | 741 | 22 | 15 | 40.74 | 1.37$^{\uparrow}$ |
| Lipase | B1NLE6 | 30 | 4.9 | 696 | 11 | 11 | 18.15 | 1.20$^{\uparrow}$ |
| Thioredoxin peroxidase | B2KSE9 | 22 | 5.9 | 3196 | 33 | 15 | 51.30 | 1.19$^{\uparrow}$ |
| Arylphorin subunit | Q9U5K4 | 84 | 6.7 | 788 | 15 | 02 | 9.02 | 1.05$^{\uparrow}$ |
| Chymotrypsin like protease | O18450 | 30 | 8.5 | 2436 | 18 | 01 | 35.93 | 1.06$^{\downarrow}$ |
| Phosphoglycerate mutase | Q3S2I9 | 28 | 6.3 | 489 | 8 | 02 | 15.68 | 1.08$^{\downarrow}$ |
| Polycalin | B6CMG | 101 | 4.4 | 304 | 17 | 01 | 12.40 | 1.20$^{\downarrow}$ |
| Acyl CoA dehydrogenase | D2XMK | 45 | 8.4 | 1541 | 53 | 00 | 35.86 | 1.35$^{\downarrow}$ |
| Trypsin | C7SB25 | 32 | 6.0 | 489 | 12 | 04 | 22.59 | 1.37$^{\downarrow}$ |
| 3-hydroxyisobutyrate | B3GQU6 | 33 | 9.3 | 927 | 15 | 08 | 14.59 | 1.40$^{\downarrow}$ |
| β-1,3-glucanase | B1NLE1 | 41 | 5.9 | 748 | 12 | 07 | 29.06 | 1.46$^{\downarrow}$ |
| Fatty acid binding protein | B6CMG | 14.7 | 6.7 | 2119 | 36 | 07 | 56.81 | 1.51$^{\downarrow}$ |
| Arginine kinase | D3Y4D1 | 39 | 5.7 | 19835 | 91 | 00 | 53.80 | 1.77$^{\downarrow}$ |
| NADPH-cytochrome-P450 | E0A3A7 | 77 | 5.3 | 239 | 12 | 08 | 21.83 | 2.18$^{\downarrow}$ |
| Heat shock protein | C7ED93 | 61 | 5.5 | 772 | 16 | 10 | 22.55 | 2.37$^{\downarrow}$ |
| Acetoacetyl CoA thiolase | D8VKG | 28 | 5.7 | 537 | 9 | 02 | 19.84 | 2.51$^{\downarrow}$ |
| Activated C kinase | A9LST1 | 35 | 7.2 | 769 | 18 | 00 | 32.28 | 3.31$^{\downarrow}$ |
| Triosephosphate isomerase | Q5XUN | 26 | 5.6 | 1221 | 9 | 06 | 17.33 | 3.73$^{\downarrow}$ |
| Aldehyde dehydrogenase | B0FBK1 | 21 | 5.5 | 532 | 19 | 04 | 40.68 | 3.80$^{\downarrow}$ |
| Midgut aminopeptidase | Q7Z266 | 114 | 5.1 | 1364 | 23 | 02 | 27.12 | 4.29$^{\downarrow}$ |
| Diverged serine protease | O18439 | 27 | 5.6 | 2855 | 35 | 19 | 29.68 | 7.88$^{\downarrow}$ |

[a] Fold change equals the difference in concentrations observed between the two diets.

↑ Protein fold increase on Ct diet.

↓ Protein fold decrease on Ct diet.

In gut proteome of *H. armigera*, 17 proteins related to digestion, immunity, energy production and apoptosis mechanism were up-regulated and 8 proteins involved in carbohydrate metabolism, lipid metabolism and energy transfer were down-regulated (**Table 3**); in hemolymph, 9 up-regulated proteins are reported to be involved in immunity, RNA processing, mRNA directed protein synthesis and metamorphosis, while 6 down-regulated proteins were implicated in energy transfer, hydrolysis, defense mechanisms and amino acid storage related functions (**Table 4**); and in frass lipase, β-

1,3-glucanase and aminopeptidase were up-regulated and serine proteases were down-regulated in larvae reared on Cp and Ct diets, respectively (**Table 5**). Overall, 17 proteins were up-regulated while the rest 29 proteins were down-regulated in Ct fed *H. armigera*. Myosin heavy chain fragment, very high density lipoprotein, ATP synthase subunit, carboxyl choline esterase and lipase with other three proteins were up-regulated in *H. armigera* gut reared on Ct as compared to the larvae fed on Cp diet. Other digestive enzymes, such as serine proteases, exhibited differential accumulation based on dietary PIs. We found decreased expression of 17 gut proteins related to the various mechanisms in Ct fed *H. armigera*. Phosphoglycerate mutase, down-regulated in Ct fed larvae, is implicated to have vital role in the chemical reactions and pathways resulting in the breakdown of a monosaccharide into pyruvate, with the concomitant production of ATP. Digestive enzymes viz. trypsin and chymotrypsin involved in the hydrolysis of proteins into smaller polypeptides and/or amino acids by cleavage of their peptide bonds, were also down-regulated in Ct fed *H. armigera*, whereas proteins involved in the mechanism of apoptosis (activated C kinase), energy transfer (polycalin), immunity related functions (midgut aminopeptidase), cell redox homeostasis (acyl CoA dehydrogenase), stress response (heat shock protein) and protein involved in detoxification mechanism (P450) were down-regulated. *H. armigera* hemolymph proteins are known to be involved in immunity related functions. Scolexin catalyzes the hydrolysis of internal, α-peptide bonds in a polypeptide chain; promoting protein fragment is implicated in lipid recognition, particularly in the recognition of pathogen related products; prophenoloxidase is a larval storage protein (LSP), which may serve as a store of amino acids for synthesis of adult proteins; cathespin 1 like protease serve a variety of functions *in vivo* and *in vitro*, mainly for the proper folding of the newly synthesized enzymes; Cytochrome c oxidase is a key enzyme in aerobic metabolism; while neuroglain and odorant binding protein are known to play a vital role in immunity related functions. These proteins were found to be more abundant in Ct-reared larvae than in Cp-fed larvae. Proteins engaged in defense mechanism GST, imaginal disk growth factor and cationic protein were under-expressed in the larvae reared on Ct as compared to those reared on Cp diet. Other down-regulated proteins included apolipoprotein, found in many insect species and functions in transport of diacylglycerol (DAG) from the fat body lipid storage depot to flight muscles in the

adult life stage; ribosomal protein, catalyze mRNA-directed protein synthesis in all organisms and arylphorin subunit, a larval storage protein (LSP), which may serve as a store of amino acids for synthesis of adult proteins. A decline in carboxylesterase and lipoprotein synthesis in Ct-fed larvae was also observed. Anal droppings (frass) of *H. armigera* showed accumulation of essential enzymes involved in defense and digestion related mechanisms. Lipase, β-1, 3-glucanase and aminopeptidase were found to be induced in *H. armigera* and serine proteases showed down-regulation in Ct fed larvae.

**Table 4: List of identified proteins by LCMS$^{E}$ in hemolymph of *H. armigera***

| Protein name | Acc. no. | MW (kD | pI | PLGS Score | Total Peptides | Unique Peptides | Coverage (%) | Fold ↑/↓ on Ct[a] |
|---|---|---|---|---|---|---|---|---|
| Scolexin | C1JE15 | 30 | 6.2 | 1414 | 5 | 02 | 14.93 | 5.23$^{\uparrow}$ |
| Promoting protein | B6A8I6 | 84 | 7.7 | 3930 | 8 | 06 | 68.83 | 4.90$^{\uparrow}$ |
| Prophenoloxidase | Q2VIY6 | 80 | 5.7 | 3373 | 80 | 14 | 57.50 | 4.35$^{\uparrow}$ |
| Cathepsin 1 like protease | B9UCR2 | 62 | 4.8 | 2349 | 39 | 26 | 22.00 | 1.99$^{\uparrow}$ |
| Histone | B4IM99 | 22 | 11. | 2104 | 16 | 00 | 30.54 | 1.60$^{\uparrow}$ |
| Odorant binding protein | D2SNX8 | 15 | 6.4 | 4735 | 16 | 04 | 40.81 | 1.21$^{\uparrow}$ |
| GI23657 OS Drosophila | B4KDL6 | 47 | 9.9 | 371 | 5 | 01 | 6.02 | 1.05$^{\downarrow}$ |
| Apolipophorin | P86356 | 38 | 4.6 | 5769 | 1 | 01 | 37.14 | 1.28$^{\downarrow}$ |
| Cationic protein | D9MNH4 | 11 | 8.3 | 6947 | 15 | 13 | 17.92 | 1.28$^{\downarrow}$ |
| Ribosomal protein | Q962T9 | 18 | 11. | 1664 | 5 | 01 | 27.71 | 1.42$^{\downarrow}$ |
| Carboxylesterase | D5KX96 | 60 | 5.0 | 510 | 35 | 01 | 32.72 | 1.54$^{\downarrow}$ |
| Very high density | A8CMX8 | 172 | 5.4 | 2473 | 191 | 139 | 47.10 | 1.81$^{\downarrow}$ |
| Glutathione-S-transferase | Q964D6 | 24 | 7.4 | 1613 | 6 | 02 | 42.12 | 2.00$^{\downarrow}$ |
| Putative uncharacterized | B1NLE0 | 25 | 5.0 | 6547 | 53 | 24 | 66.52 | 6.16$^{\downarrow}$ |
| Imaginal disk growth | Q0QJJ5 | 47 | 7.3 | 541 | 39 | 12 | 20.78 | 10.11$^{\downarrow}$ |

[a] Fold change equals the difference in concentrations observed between the two diets.

↑ Protein fold increase on Ct diet.

↓ Protein fold decrease on Ct diet.

**Table 5: List of identified proteins by LCMS$^{E}$ in frass of *H. armigera***

| Protein name | Acc. no. | MW (kDa) | pI | PLGS Score | Total Peptides | Unique Peptides | Coverage (%) | Fold ↑/↓ on Ct[a] |
|---|---|---|---|---|---|---|---|---|
| Lipase | A9X7K9 | 31 | 8.79 | 1932 | 16 | 12 | 31.50 | 17.14$^{\uparrow}$ |
| β-1,3-glucanase | B1NLE1 | 41 | 5.97 | 823 | 30 | 00 | 29.86 | 14.20$^{\uparrow}$ |
| Aminopeptidase | Q8MU78 | 114 | 5.10 | 414 | 4 | 01 | 4.83 | 3.80$^{\uparrow}$ |
| Chymotrypsin | D7RZZ7 | 30 | 8.02 | 1729 | 17 | 01 | 19.66 | 3.58$^{\downarrow}$ |
| Trypsin | B6CME8 | 29 | 8.19 | 1130 | 3 | 01 | 16.12 | 4.80$^{\downarrow}$ |
| Serine protease | A9P3S4 | 27 | 5.92 | 320 | 7 | 01 | 32.03 | 12.0$^{\downarrow}$ |

[a] Fold change equals the difference in concentrations observed between the two diets.

↑ Protein fold increase on Ct diet.

↓ Protein fold decrease on Ct diet.

### 3.3.5 Serine protease, P450 and GST activities in *H. armigera* reared on Cp and Ct diets

Biochemical assays revealed that serine protease, P450 and GST activities were significantly reduced in Ct fed *H. armigera* larvae as compared to control (Cp fed) larvae. Activity of gut trypsin, P450 and GST was down-regulated on Ct (**Figs 3.3 and 3.4 A, B**), except gut chymotrypsin, which showed almost similar activity in both Cp and Ct fed larvae (**Fig. 3.3**).

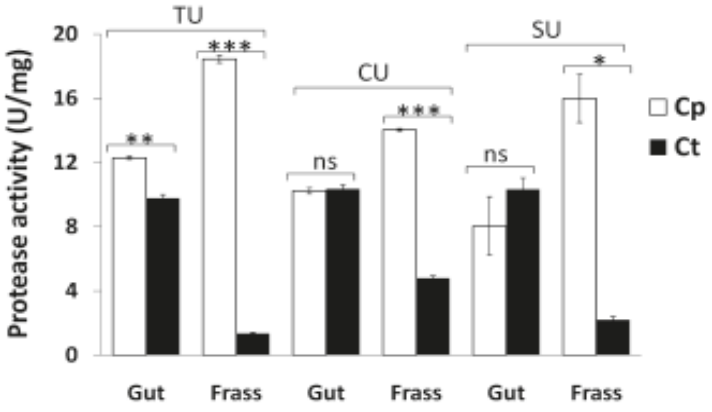



**Figure 3.3** Trypsin (TU), chymotrypsin (CU) and serine protease (SU) activity in gut and frass derived from *H. armigera* larvae fed on Cp (hollow bars) and Ct diet (black bars). Standard mean errors are indicated. ***, ** and * indicates that values are significantly different from each other at $p < 0.0001$, $p < 0.001$ and $p < 0.05$, respectively.

Total protease, trypsin and chymotrypsin activity were reduced by 7.26, 13.66, and 2.92 fold in frass of *H. armigera* fed on Ct, respectively. GST activity was down-regulated by 0.37 fold in hemolymph of Ct fed larvae while activity reduction in P450, reported to be involved in detoxification mechanisms, was observed in the larval gut (1.23 fold) of Ct fed *H. armigera*.

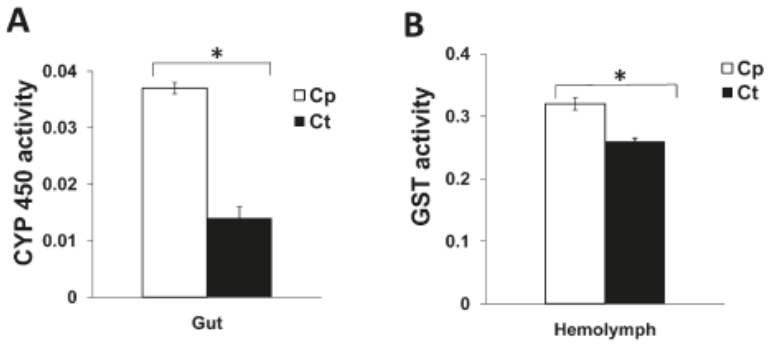



**Figure 3.4 (A)** Reduction of DPPH by cytochrome P450 (P450) (expressed in nmol reduced DPPH/mg of protein) from *H. armigera* reared on Cp and Ct diet. (**B**) Variation of gluthatione S-transferase (GST) activity (nmol/mg of protein) of *H. armigera* reared on the Cp and Ct diets. Standard mean errors are indicated. * indicate that values are significantly different from each other at $p < 0.05$

### 3.3.6 Gene expression patterns of candidate proteins exhibiting differential expression in *H. armigera* upon exposure to Ct diet

To obtain information about key genes regulating the metabolic pathways, as well as defense mechanisms of *H. armigera*, qRT-PCR analyses were performed with cDNA reverse transcribed from mRNA isolated from different *H. armigera* tissues. The results of qRT-PCR indicate tissue-specific expression profiles of selected isoforms of the identified candidate proteins. Trypsin isoforms (**Fig. 3.5**) exhibited complexity in expression profiles. *HaTry*1 followed by *HaTry*2, *HaTry*3 and *HaTry*4 was found to be most abundant in gut tissue of Cp fed insects and showed nearly 4X higher expression than the rest of the isoforms; while in gut tissue of Ct-fed insects, *HaTry*2 was found to be abundant, followed by *HaTry*3, *HaTry*4, and *HaTry*1. Gut tissue of Ct fed larvae showed 7X downregulation of the *HaTry*1 gene than Cp fed larvae, while *HaTry*2 (3X), 3 (4X) and 4 (0.3X) were up-regulated in Ct-fed larvae. Chymotrypsin isoforms also (**Fig. 3.5**) showed expression patterns resembling trypsins in gut tissue of Cp and Ct-fed *H. armigera*. A nearly constant level of expression was observed for *HaChy*1 in gut tissue of Cp and Ct fed insects, while *HaChy*2 and *HaChy*3 showed 2X and 3X up-regulation in gut tissue of Ct-fed larvae than Cp-fed larvae, respectively. Interestingly, high levels of transcripts of several digestive proteases were also detected in frass. Hemolymph was

only analyzed for relative expression of glutathione-S-transferase (*HaGST*) (**Fig. 3.5**). *HaGST* from hemolymph of Ct-fed larvae showed down-regulation (2X) compared with Cp-fed *H. armigera* larvae.

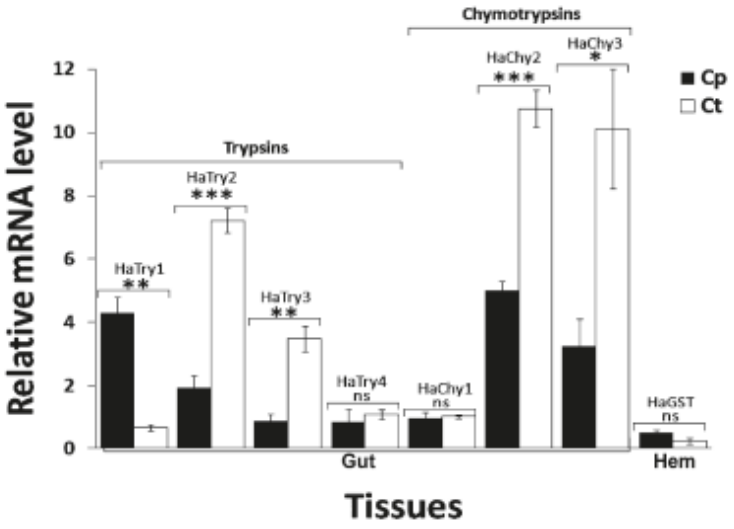



**Figure 3.5** Quantitative Real-Time RT-PCR analysis of selected trypsin (*HaTry*), chymotrypsin (*HaChy*) and glutathione-S-transferase (*HaGST*) genes with RNA extracted from gut and hemolymph of *H.armigera* larvae reared on Cp and Ct diets respectively. The Y-axis represents the relative gene expression ratio calculated using the standard relative plot method. Error bars represent standard deviation in three biological replicates. *, ** and *** indicate that values are significantly different from each other at $p < 0.02$, $p < 0.005$ and $p < 0.0001$, respectively.

## 3.4A Discussion

### 3.4.1 Ct diet retarded growth and development of *H. armigera*

Earlier studies on PIs have dealt with the identification and characterization of specific protease inhibitors (Giri *et al.*, 1998; Francis *et al.*, 2002, 2005). Proteins like vicilins and arcelins isolated from several legumes (Yunes *et al.*, 1998) slow down larval development of insects. Especially, arcelin (Arc) seed proteins have been shown to have an inhibitory effect on the larval development of the Mexican bean weevil, *Z. subfasciatus*, (Osborn *et al.*, 1988; Cardona *et al.*, 1990) and some wild bean accessions containing Arc are highly resistant to the bean weevil (*Acanthoscelides obtectus*). The

present study demonstrated the efficacy of *C. tora* seeds containing PIs in retarding the growth of *H. armigera* and in inhibiting the activity of HGP. However, it has also been reported that, the insects develop resistance to chickpea PIs, phytochemicals by detoxification mechanism and plant defense proteins might be potentially recognized as substrates by insect gut proteases (Pickett *et al.,* 1989; Yu *et al.,* 2003) for example, multicystatin of potato tubers by *Diabrotica* larval proteinases; (Orr *et al.,* 1994) oryzacystatin by black vine weevil proteinases (Michaud *et al.,* 1995,1996) trypsin inhibitors of chickpea by gut proteinases of podborer (Giri *et al.,* 1998) and oryzacystatin and soybean Bowman-Birk TI by beetle larvae (Girard *et al.,* 1998). Detoxification enzymes are known to be involved in metabolism of and resistance to phytochemicals and insecticides (Agosin *et al.,* 1985; Oppenoorth *et al.,* 1985). Arc, phytohemagglutinins (PHA) and α-amylase inhibitors (αAI) comprise the bean-lectin family although αAIs exhibit no sugar-binding sites and Arcs show weak agglutinin properties.

Despite some common features, these related proteins differ in their mode of action. PHA binds to glycans of the intestinal mucosa and acts as a mitogen; αAI inhibits the activity of α-amylases, whereas Arc has been suggested to bind to the peritrophic matrix of the gut of insects and interfere with nutrient absorption (Hamelryck *et al.,* 1996; Chrispeels *et al.,* 1998). More generally, polyphagous insects can selectively express a broad range of enzymes that assist in digesting xenobiotics including secondary metabolites from plants (Li *et al.,* 2000). However, in the present study *C. tora* seeds containing metabolites or allelochemicals might have inhibited the activity of several crucial enzymes thereby reducing the growth and development of *H. armigera*. Furthermore, the differential responses of *H. armigera* to different PIs and metabolites of chick pea and *C. tora* suggested that adaptation of *H. armigera* to one group of PIs or plant secondary metabolites does not mean insensitivity to other PIs or other secondary metabolites. A specific inhibitor and metabolite combination would significantly delay the growth and generation advance of *H. armigera* in the field.

### 3.4.2 Ct diet disturbed the energy metabolism in *H. armigera*

Plant-insect interactions so far have been investigated using gene expression studies. However, there has been lack of more proteomic studies focusing on insect adaptation toward the plant defense. Although proteomics has emerged as an enormously powerful tool for gaining insights into various physiological changes at the cellular level, relatively few attempts has been made to apply this technique to study insect adaptation and response to toxic substances, as well as phytochemicals (Sharma *et al.,* 2004). The differential expression of various proteins reflects the overall change in cellular structure and metabolism after exposure to diverse nutritional and chemical compounds. In the present study, proteins identified in *H. armigera* gut might have a role in digestion and defense as well as detoxification of phytochemical compounds with the help of enzymes including serine proteases, Myosin heavy chain, β-1,3-glucanase, lipase and P450 (**Table 3**). Among these proteins, serine proteases, Myosin heavy chain, β-1, 3-glucanase and lipase are reported to be involved in digestion and energy metabolism, whereas P450 is involved in oxidation of various toxic substances.

The substrates of P450 enzymes include metabolic intermediates of lipids as well as phytochemical substances, such as secondary metabolites and many other toxic chemicals including pesticides (Rawlings and Barrett, 1994; Shewry *et al.*, 1995; McCart and Ffrench-Constant, 2003; Chao *et al.,* 2010). Observations from this study showed that the expression of trypsin, β-1, 3-glucanase and P450 were down-regulated in gut of *H. armigera* fed on Ct, while Myosin 1 heavy chain and lipase were up-regulated. The present proteomic data together with previous studies demonstrated that the decreased expression of these enzymes might be due to their inhibition by metabolites or proteins present in non-host diet. In addition, down-regulation of heat shock protein in *H. armigera* larvae fed on Ct than Cp diet probably suggest inability of Ct fed larvae to give response to stress condition. However, production of high levels of heat shock proteins can also be triggered by exposure to different kinds of environmental stress conditions, such as infection, inflammation exercise and exposure of the cell to toxins (Santoro, 2000). Low ability to digest nutrients in *H. armigera* reared on the Ct diet maybe due to the weakened activity of enzymes responsible for digestion of nutrients and defense mechanisms in the gut. Nonetheless, energy metabolism related proteins viz. very high

density lipoprotein, ATP synthase and sterol carrier protein were up-regulated in Ct-fed larvae and might overcome the effects of non-host diet. Indeed, whole gut of *H. armigera* is reported for several crucial functions (Giri *et al.,* 1998; Harsulkar *et al.,* 1999).

### 3.4.3 Nutrient imbalance modifies the immune system of *H. armigera*

Enzymatic analysis is an approach to recognize, how particular chemicals are involved in interactions with organisms and their surroundings. Plants have evolved a variety of defense mechanisms in order to reduce insect attack, while insects have evolved strategies to overcome these plant defenses using detoxification enzymes (Berenbaum, 1995; Taniai *et al.,* 1996). Although the insecticidal properties of Arc variants against bruchid pests have been demonstrated, their precise mechanism of action is still unknown: Arc could be toxic as was postulated by Osborn *et al.,* (1988) or it could be indigestible and induce larval starvation (Minney *et al.,* 1990). However, GST, imaginal disk growth factor, cationic protein and odorant binding protein (OBPs) play vital roles in the immune system of insects due to their antimicrobial property (Francis *et al.,* 2005; Hariharan *et al.,* 2006). These proteins, except odorant binding protein, were down-regulated significantly in Ct fed *H. armigera* hemolymph. Indeed, OBPs are thought to deliver odors to olfactory receptors and thus may be the first biochemical step in odor reception capable of some level of odor discrimination (Gadenne *et al.,* 2001; Takken *et al.,* 2001). OBPs are also associated with plant-volatile-sensitive olfactory sensilla (Vogt *et al.,* 1999).

In the present study, up-regulation of OBP in Ct-fed larvae might be the reason to search for host food by its odor reception mechanism. This new field of study will greatly contribute to the understanding of insect chemical communication mechanisms, particularly with agricultural pests and disease vectors, and could result in future strategies to reduce their adverse effects. The exact role of OBPs as the first line of discrimination has still not been demonstrated. Gene Ontology (GO) studies showed that OBP interacted selectively and noncovalently with major histocompatibility complex molecules and also interacted with a poisonous substance that causes damage to biological systems. GST and cationic protein processes were involved in the development or functioning of the immune system, an organismal system for calibrated responses to

potential internal or invasive threats. Also, these enzymes are involved in all processes that reduce or remove the toxicity of nitrogenous compounds that are dangerous or toxic. Imaginal disk growth factor is reported to increase the mass of imaginal discs by cell proliferation prior to metamorphosis. Imaginal discs are epithelial infoldings in holometabolous larvae that develop into adult structures (legs, antennae, wings etc.), during metamorphosis. This is evidence to show that although almost all of the above enzymes were under expressed in Ct fed larvae; *H. armigera* was trying to adapt to the non-host diet with the help some other enzymes like OBP and neuroglain.

### 3.4.4 Insect frass is a rich repository of biological information

Insects exhibit a range of intriguing behavioral and morphological adaptations related to waste disposal in a range of contexts. Some insects, for example, make use of their own excrement as a physical or chemical barrier against natural enemies, while others actively distance themselves from their waste material. Anal droppings of insect herbivores are a rich repository of biological information (Weiss, 2006). It is well established that frass is an important source of compounds involved in host selection by insect parasitoids (Vinson, 1976). The proteomic analysis reported herein shows that the insect frass is enriched in defense-related proteins. Many of these proteins were previously shown to accumulate in *Manduca sexta* midgut and have established roles in anti-insect defense. Excretion of active enzymes from insects led us to hypothesize that insect frass may be a useful source of material to identify many digestion and defense-related proteins (Chen *et al.*, 2005). To test this idea, we employed a proteomic approach to catalogue and quantify proteins in frass from *H. armigera* larvae reared on Cp and Ct diets. Aminopeptidase, lipase and β-1, 3 glucanase were the most abundant proteins found in frass from Ct reared *H. armigera* larvae. Another three differentially regulated proteins were also catalogued in frass. Down-regulation of serine proteases in the frass of Ct-reared larvae might be due to inhibitory proteins present in *C. tora* seeds. Lepidopteran larvae secrete an active β-1, 3-glucanase into the midgut lumen that may function in digestion of β-1, 3-glucans released by commensal or invading bacteria, suggesting a role in immune response (Pauchet *et al.*, 2009). In our study, β-1, 3-glucanase was found to be present in the gut as well as frass of *H. armigera*. However,

this enzyme was under expressed in gut while up-regulated (12-fold) in frass from Ct-fed larvae.

All these observations suggested that there is an enigma of the actual role of β-1, 3-glucanase, and could be either involved in immune defense proteins or digestive enzymes. These findings provide insight into the evolution of plant anti-insect proteins and establish a robust experimental approach to identify hyper stable proteins that serve important roles in plant protection against biotic stress. Frass of insect pest might have an application in biological control strategies, either as a direct deterrent or to increase the search efficiency of natural enemies.

### 3.4.5 Enzymatic activities in *H. armigera* are up on diet switch

Enzymes are regulated by different kinds of compounds in several biological systems, (Akerman *et al.,* 2003) and an insect's feeding preference depends on the allelochemical blend in diets and plants. P450 is well documented in several herbivores for the metabolism of a broad range of secondary metabolites from the various plants (Cohen *et al.,* 1992). Gut proteases and P450were found to be down-regulated in Ct-fed *H. armigera*. Activity of serine proteases in Ct-fed *H. armigera* was down-regulated, this could be a reason for stunted growth, and low survival rate, while down-regulation of P450 could be evidence to show that larvae is not yet adapted to *C. tora* seed metabolites to digest them. GST activity in hemolymph of Ct-fed larvae was down-regulated as compared to that of Cp-fed larvae. GST from Ct-reared larvae was down-regulated, which might be due to allelochemicals susceptibility of non-host diet. It seems that frass analysis also showed reduced activity of all proteases in Ct-fed *H. armigera*. The furanocoumarins in *Papilio polyxenes* were detoxified by P450 monooxygenase and GST (Hung *et al.,* 1995). In general, polyphagous insects can selectively express a broad range of enzymes that assist in the detoxification of numerous xenobiotics, including allelochemicals from plants (Li *et al.,* 2000). Beside these enzymes, plenty of potential proteins could also be influenced by the presence of plant defense mechanisms.

### 3.4.6 Differential gene expression or complex blend of isoforms: what are the underlying mechanisms?

Genes such as proteases (trypsin, chymotrypsin, aminopeptidase, carboxypeptidase etc) and GST played a vital role in food digestion and immunity to a certain extent, and metabolism of toxic compounds in insects, respectively (Cohen *et al.*, 1992). Insects are believed to be self-protected from the toxicity due to the presence of multiple isoforms of GST and P450 (Zheng *et al.*, 1994). However, the biological and physiological role of different diets in insect physiology has long been a mystery. It is interesting to know whether all these genes are involved in adaptation of *H. armigera* to non-host plants, or do they also have other functions? The expression profiles of selected candidate genes confirm the biochemical and proteomics findings, but not in totality, since there are many isoforms of trypsins, chymotrypsins, aminopeptidases, carboxypeptidases whereas GST has fewer (Harsulkar *et al.*, 199). There are many isoforms of trypsin and chymotrypsin known to be involved in digestive processes in lepidopteran pests. It is clear that there is tremendous complexity in trypsin and chymotrypsin expression, suggesting their role in building the adaptive response of the insect. Trypsin-like proteases play a predominant role in digestion as well as metamorphosis and to some extent in immunity, as evident from the expression studies and insect performance. Diverse isoforms of trypsins and chymotrypsins enhance the digestive flexibility of the insects. The cumulative effect of proteases at enzymatic/ protein level could be low because possibly not all the proteins could be translated and function in digestion. Some isoforms might be less efficient to combat altered diet components and it is difficult to compare proteins with transcripts since they possess high similarity. Furthermore, we did not checked expression of all the trypsin and chymotrypsin like genes in present analysis.

Hence there is difference at transcript expression and enzymatic as well as protein level. These proteases act under cascade and are tightly regulated exhibiting a switch in expression. Trypsin was found to play a more major role than chymotrypsin. In addition to this, GST and P450 maintained the metabolism of the insects and their survival. Also different isoforms of trypsin and chymotrypsin were up or downregulated in Cp and Ct

diets indicating their complexity and strength in neutralizing the different phyotochemicals. A few isoforms of GST reported to show differential regulation on different diets. HaGST showed lower expression in hemolymph of Ct-fed larvae indicating that *C. tora* seed metabolites might interfere in *H. armigera* adaptability by inhibiting the activity of GST.

## 3.5A Conclusion

This study employed a proteomic approach to investigate the proteome of gut, hemolymph and frass of the *H. armigera* reared on Cp and Ct diets. Biochemical, proteomics and qRT-PCR results revealed that proteases, P450 and GST, alter their expression on diet switch in *H. armigera*. However, the outcomes suggested that non-host Ct diet retards the growth and development of *H. armigera* with reduction in activity of many vital enzymes that are involved in digestion and defense mechanisms. These results support a hypothesis about some possible functions of various enzymes in insect physiology and raise the necessity for some future detailed studies in adaptation to plant defense compounds in insects and related metabolisms with detailed molecular biology approaches. This study demonstrated the insect's performance on host and non-host diet and the key factors underlying the adaptive response; their protein and transcript flux, by priming the insect against non-host diet components. Information about dissecting adaptation mechanisms and regulation of the metabolic pathways in polyphagous insects will pave a way for the development of new pest control strategies.

# Part B: The expression of proteins involved in digestion and detoxification are regulated in *Helicoverpa armigera* to cope up with chlorpyrifos insecticide

## Abstract

*Helicoverpa armigera* is a key pest in many vital crops, which is mainly controlled by chemical strategies. To manage this pest is becoming challenging due to its ability and evolution of resistance against insecticides. Further, its subsequent spread on non-host plant is remarkable in recent times. Hence, decoding resistance mechanism against phytochemicals and synthetic insecticides is a major challenge. The present work describes that the digestion, defense and immunity related enzymes are associated with chlorpyrifos resistance in *H. armigera*. Proteomic analysis of *H. armigera* gut tissue upon feeding on chlorpyrifos containing diet (CH) and artificial diet (AD) using nano-liquid chromatography mass spectrometry identified up-regulated 23-proteins in CH fed larvae. Database searches combined with gene ontology analysis revealed that the identified gut proteins engrossed in digestion, proteins crucial for immunity, adaptive responses to stress, and detoxification. Biochemical and quantitative real-time polymerase chain reaction analysis of candidate proteins indicated that insects were struggling to get nutrients and energy in presence of CH, while at the same time endeavoring to metabolize chlorpyrifos. Moreover, we proposed a potential processing pathway of chlorpyrifos in *H. armigera* gut by examining the metabolites using gas chromatography mass spectrometry. *H. armigera* exhibit a range of intriguing behavioral, morphological adaptations and resistance to insecticides by regulating expression of proteins involved in digestion and detoxification mechanisms to cope up with chlorpyrifos. In these contexts, as gut is a rich repository of biological information; profound analysis of gut tissues can give clues of detoxification and resistance mechanism in insects.

## 3.1B Introduction

Managing field pests has become increasingly challenging due to the ability of pests to adapt and evolve into resistant strains against insecticides. Evolution of insecticide resistance and its subsequent spread in the insects is remarkable (Heckel, 2012). Many species have been heavily exposed to insecticides (organophosphates, DDT, pyrethroids and carbamates), resulting in resistant pest populations (Dawkar *et al.*, 2013).

Constant efforts are being taken to develop new insect pest control strategies, which involve study of ecology, physiology and biochemistry of target insects.

*Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is one of the most important polyphagous lepidopteran insect pests found on more than 300 plant species worldwide. Damage by this pest to various crop plants ranges from 50% to 90% hence, making it the most important yield reducer (Blossey and Hunt-Joshi, 2003). Since the last 50 years, the most popular and reliable means of *H. armigera* control is the use of varied mechanistic classes of insecticides, which gave rise to physiological, ethological and ecological complications (Sharma, 2006). These include high polyphagy, cosmopolitan nature, high migratory potential (adults), facultative diapause and high fecundity along with its ability to develop resistance to insecticides (Metcalf, 1989; Enayati *et al.*, 2005). The purpose of present investigation was to study the responses of *H. armigera* to synthetic organophosphate insecticide, chlorpyrifos (CH). Here, we studied the effect of CH on *H. armigera* growth and development and the fate of CH in insect gut. We employed comparative proteomic, enzymatic and quantitative real time polymerase chain reaction (qRT-PCR) analyses to understand the biochemical and molecular responses of *H. armigera* upon exposure to CH. Enzymatic activity and relative gene expression analyses of some candidate proteins indicated possible biochemical process involved in metabolizing CH. The feasible intermediates of CH were identified by gas chromatography-mass spectrometry (GC-MS) in gut extracts of CH fed larvae. Based on these results, we propose possible processing of chlorpyrifos in *H. armigera*. Altogether these findings suggest that *H. armigera* regulates simultaneously the proteins related to digestion and detoxification mechanisms to adjust the nutrient requirements and potentially neutralize insecticide molecules.

## 3.2B Materials and Methods

### 3.2.1 Materials

Azocasein, acetonitrile, bovine serum albumin (BSA), sequencing grade modified trypsin, tricine, methanol and lucine p-nitroanilide were procured from Sigma Chemical Co., St. Louis, MO, USA. MassPrep predigested standard protein rabbit glycogen phosphorylase B (GP), Rapigest and yeast enolase were purchased from Waters

Corporation, Milford, MA, USA. Organophosphate insecticide Deviban (chlorpyrifos 20% EC) (*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate) ($C_9H_{11}Cl_3NO_3PS$; MW 350.6) was procured from Devidayal Agro Chemicals Ltd., Panchmahal, Gujarat, India. All other chemicals used were of analytical grade.

### 3.2.2 Rearing of insects and feeding bioassays

Actively feeding *H. armigera* larvae were collected from fields in Mahatma Phule Krishi Vidyapeeth, Rahuri, India. These were maintained on chickpea based artificial diet (AD) (Nagarkatti and Prakash, 1974). AD containing 6, 8, 12 and 16 ppm of chlorpyrifos was prepared and referred as CH diet. Feeding bioassays on AD and CH diet were carried out using neonates. Growth performance of *H. armigera* larvae was assessed by determining average mass of AD and CH fed larvae (25 larvae each) at interval of 5 days. Upon continuous feeding of *H. armigera* on AD and AD-CH diet gut tissues were harvested from $4^{th}$ instar larvae and stored at -80 °C untill further use.

### 3.2.3 Sample preparation for proteomic analysis

Proteins were extracted from *H. armigera* gut according to Schuster and Davies (1983) with slight modifications. Protein pellet was dissolved in 0.1% Rapigest (500 µl) and concentration was estimated by Bradford assay (1976). This complex protein mixture was concentrated to 100 µl using MW 5000 cut off spin column and reduced with DTT and alkylated with iodoacetamide. After overnight digestion with trypsin, digested proteins were concentrated by using MW 3000 cut off spin column and used for nano LC-MS analyses.

### 3.2.4 Liquid chromatography-mass spectrometric analyses

Digested protein samples were subjected to a NanoAcquity ultra performance liquid chromatography (UPLC) coupled to MALDI-SYNAPT HDMS (Waters Corporation, Milford, MA, USA). The nano-LC separation was performed using a bridged-ethyl hybrid (BEH) C18 reversed phase column (1.7 µm particle size) with an internal diameter of 75 µm and length of 150 mm. Each sample of total digested protein

was injected into the trapping column and flushed with 0.1% solvent A (0.1% formic acid containing water) for 3 min at a flow rate 15 µl/min. Upon each injection, peptides were eluted into the NanoLockSpray ion source at a flow rate of 300 nl/min using a gradient of 2 to 40% B (0.1% formic acid containing acetonitrile) over 110 min. The lockmass calibrant peptide standard, 500 fmol/µl glu-fibrinopeptide B, was infused into the nano lock spray. The mass spectrometer was operated in V-mode at a resolution of at least 9000 full- widths at half height. Full scan (*m/z* 50-2000) LC-$MS^E$ data was acquired using the "expression" mode which involves alternating 1 sec scans of normal and elevated collision energy (Silva *et al.*, 2005, 2006). Data was collected at a constant collision energy setting of 4 V during low-energy MS mode scans, whereas a step from 14 to 40 V of collision energy was used during the high-energy $MS^E$ mode scans.

### 3.2.5 Data processing and database searching

The continuum LC-$MS^E$ data was processed and analyzed using Protein Lynx Global Server 2.5.2 software (PLGS; Waters Corporation, Milford, MA, USA). Protein identifications were obtained by searching Lepidopteran database (www.uniprot.org). LC-$MS^E$ data was analyzed with a fixed carbamidomethyl modification for Cys residues, along with a variable modification for oxidized Met residues. Ion Accounting search parameters used for data independent analysis (DIA) were as described earlier in **section 3.2.5 of part A**, in this chapter. Replicates were performed for each sample and a comparison between AD and CH feeding group was conducted.

### 3.2.6 Enzymatic analysis

The extraction was performed by homogenizing gut tissue of *H. armigera* in Glycine-NaOH buffer (200 mM, pH 10) followed by stirring at 4$^o$C for 2 h. The extract was centrifuged at 12,000 g at 4$^o$C for 10 min and the clear supernatant was used as a source of enzymes for all solution assays. Protease activity was measured by azocaseinolytic assay (Brock *et al.*, 1982). One protease unit was defined as the amount

of enzyme that increased the absorbance by 1.0 OD under the given assay conditions. Enzymatic activity of NADPH-cytochrome P450 reductase (CYP) was performed as described above (**section 3.2.6, part A)**, (Yim *et al.*, 2005).

Aminopeptidase activity was assayed using L-leucine-*p*-nitroanilide as the substrate by continuous spectrophotometric rate determination. The final assay was carried out in 1.00 ml reaction mix containing 20 mM Tricine, 0.4% (v/v) methanol, 0.18 mM L-leucine-*p*-nitroanilide, 0.005% (w/v) bovine serum albumin, and gut extract. The reaction mix was equilibrated at 25°C and the increase in *A*405 nm (obtained by maximum linear rate for both test and blank) was recorded for 5 min. Units of aminopeptidase activity were calculated as one unit of activity which is the amount of enzyme that causes an increase in optical density at 405 nm of 1 unit/min (Prescott and Wilkes, 1976). Arginine Kinase activity was determined by Blethen method (Blethen, 1970) using 300 mM Phospho(enol) pyruvate, 200 mM Adenosine 5'-Triphosphate (ATP), 500 mM L-Arginine (ARG), ß-Nicotinamide Adenine Dinucleotide (7.5 mM), PK/LDH Enzyme (PK/LDH), Glycine buffer (100 mM), contain 2-mercaptoethanol (10 mM), Glycine Buffer (pH 8.6, 250 mM), Magnesium sulfate (200 mM), KCl (2 M). One unit activity will convert 1.0 µmol of L-arginine and ATP to N -phospho-L-arginine and ADP per minute. Enzyme assays were performed in triplicates.

## 3.2.7 Quantitative real time PCR analysis

Differentially expressed *H. armigera* proteins were checked for relative transcript accumulation. Total RNA extraction, first strand cDNA synthesis and relative quantification of *H. armigera* Cytochrome P450 (*HaCYP*), Glutathione S-transferase (*HaGST*) Carboxylesterase (*HaCES*), Chymotrypsin (*HaCHY*), Trypsin (*HaTRY*), Arginine Kinase (*HaARGK*), Glyceraldehyde-3-Phosphate Dehydrogenase (*HaGAPDH*) and Aminopeptidase (*HaAPEP*) mRNA was performed according to protocol described in Dawkar *et al*., 2011 and Chikate *et al.*, 2013. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used for total RNA extraction and first strand cDNA was synthesized by random priming using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). SYBR chemistry, 7900HT Fast Real Time PCR System (Applied Biosystems, Foster, CA, USA) and ΔΔCt method was utilized for quantifying

mRNA. β-actin was used as endogenous reference gene for normalization of mRNA expression. AD fed larvae was considered as calibrator to calculate the relative fold expression of target transcripts. The primers used for real time PCR analyses are mentioned in **Table 1**.

**Table 1: List of primers used for real time PCR analyses**

| Name | Primer Sequence 5'-3' | For/Rev | Accession no. | Protein ID |
|---|---|---|---|---|
| **HaACT** | GATCGTGCGCGACATCAAG | F | AF286059 | - |
| | GATCGTGCGCGACATCAAG | R | | |
| **HaTRY** | GTGCTACCCCTTCTGATTC | F | EF600059 | Q9NB81 |
| | AACTTGTCGATGGAGGTGAC | R | | |
| **HaCHY** | CGACTTGTCAGGTGGTCAGGCTG | F | HM209422 | ADI32883.1 |
| | GCGATTCTGGTACCGCCGGAGAAC | R | | |
| **HaGST** | CAGATGAGGCTCTGCTCAAGAA | F | EF033109 | A0MSN0 |
| | TGATGTCGACGGCGTCTATAGT | R | | |
| **HaAPEP** | GTATCACACGACCCCGGAAAT | F | AAN04900 | Q8MU78 |
| | GCATCGACAGCTGGAAGAGAGT | R | | |
| **HaCYP** | CAAGCTCGTGATGACAAAGG | F | BAC22B17 | Q8MTD0 |
| | GCGAAGAGGTTACGTGAGAA | R | | |
| **HaCES** | GTCGCTCAATTTGCCAGTTG | F | EF547544 | D5G3E6 |
| | GCCTTGTTCTTATTCGTGCG | R | | |
| **HaARGK** | AAGGAGGTGTTCGACGCTCTTA | F | ADD22718 | D3Y4D1 |
| | CTCAACACCAGACTGGATGCAA | R | | |
| **HaGAPDH** | TGCTGAATACGTCGTTGAATCC | F | AEB26314 | E2EHM1 |
| | TTCTTAGCACCACCCTCTAAATGAG | R | | |

## 3.2.8 Metabolite extraction and identification by GC-MS

*H. armigera* gut metabolites were extracted using deionized water and dichloromethane (1:2 v/v). Solvent phase was collected for further analysis. Identification of intermediate metabolites of CH degradation was carried out using gas chromatography mass spectrometry (GC-MS). Clarus 500 (Perkin Elmer, Waltham, MA, USA) instrument with Rtx-5MS (Restek Corporation, Bellefonte, PA, USA) capillary column (30 m × 0.32 mm i.d. × 0.25 μm film thickness) was used for analyses; column temperatures were programmed from 50 °C for 3 min, raised to 310 °C at 20 °C $min^{-1}$ and held isothermal for 5 min. Injector temperature was 200 $^{o}$C, inlet line temperature was 180 °C and source temperature was 150 °C. Helium was used as carrier gas at a flow rate 1 ml/min. Mass spectra were obtained at 70 eV with a scan time of 0.2 s for *m*/*z* 30-300. The unique peaks and compounds were identified by searching these mass spectra in

the National Institute of Standards and Technology (NIST)/the National Bureau of Standards (NBS)/WILEY 8th Ed. Standards and Technology (NIST/NBS/WILEY8ST) mass spectral library.

### 3.2.9 Statistical analysis

Data was analyzed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test. Data points were considered significant at $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***); and are indicated in respective figures.

## 3.3B Results

### 3.3.1 CH retards growth of *H. armigera* larvae

The $LD_{50}$ concentration of CH was found to be 8 ppm. The adverse effect of insecticide on larval growth performance was evident from feeding bioassays on CH diet at 8 ppm concentration. Food intake was dramatically reduced in the larvae grown on the CH diet compared to that of AD. All larvae fed on CH diet showed stunted growth, as evident from the prolonged molting instars compared to those fed on control AD. Two to 3-fold reductions in average mass was observed in CH fed larvae than that of AD fed larvae (**Fig. 3.1**)

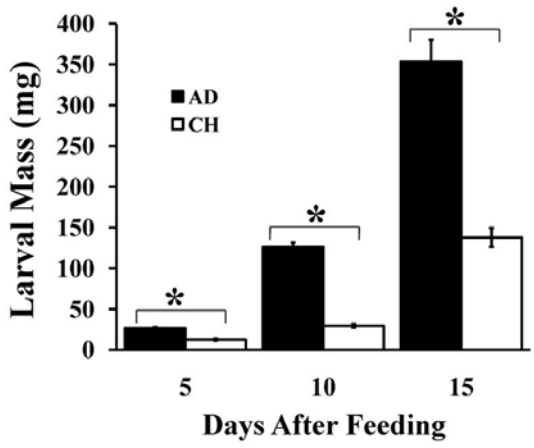


**Figure 3.1** Development of *H. armigera* on AD and CH diet. The experiment was performed in triplicate. Larvae were critically weighed on every fifth day. Standard mean errors are indicated. * indicates that values are significantly different from each other at $*p < 0.05$

**Table 2: List of identified proteins by LC-MS$^E$ in gut of AD and CH fed *H. armigera***

1 ↑ Protein fold increase on CH diet

2 ↓ Protein fold decrease on CH diet

3 Fold change equals the fold difference in concentration observed between two diets

## 3.3.2 Proteins identified in *H. armigera* gut upon exposure to CH diet

Proteomic analysis of *H. armigera* gut tissue using nano-Liquid chromatography mass spectrometry revealed upregulation of 23-proteins (fold change from 1.5 to 8.4) upon CH-exposure. These proteins were identified with high confidence, number of

| Protein name | Acc. No. | MW (kDa) | *p*I | PLGS score | Total peptides | Coverage (%) | Fold ↑/↓ On CH |
|---|---|---|---|---|---|---|---|
| Elongation factor | Q25152 | 45.07 | 8.63 | 537 | 14 | 46 | 8.35↑ |
| Very high density lipoprotein | A8CMX8 | 172.7 | 5.42 | 54 | 23 | 23 | 8.27↑ |
| Serine protease | O18447 | 26.93 | 9.11 | 927 | 7 | 42 | 4.86↑ |
| Catalase | H9U5T8 | 56.83 | 7.97 | 56 | 9 | 17 | 4.43↑ |
| Cryptochrome | I1ZEF8 | 92.28 | 8.11 | 46 | 14 | 22 | 3.80↑ |
| Aminopeptidase | Q8MU78 | 114.0 | 5.10 | 55 | 12 | 16 | 3.69↑ |
| Heat shock protein | C0KJJ4 | 74.81 | 5.62 | 60 | 15 | 24 | 3.40↑ |
| DNA metabolism protein | A4KXB5 | 158.7 | 9.05 | 31 | 12 | 12 | 3.27↑ |
| Glycogen synthase | F2Q6J8 | 76.14 | 5.81 | 32 | 6 | 21 | 2.74↑ |
| Carboxyl choline esterase | D5G3E6 | 82.31 | 4.47 | 53 | 17 | 39 | 2.60↑ |
| Arginine kinase | D3Y4D1 | 39.86 | 5.70 | 1395 | 19 | 49 | 2.41↑ |
| Juvenile hormone esterase | E7E1H4 | 51.58 | 8.11 | 30 | 6 | 23 | 2.11↑ |
| Glyceraldehyde 3P dehydrogenase | E2EHM1 | 24.27 | 6.08 | 1276 | 10 | 62 | 2.06↑ |
| ATP synthase subunit | D9ILX6 | 55.13 | 5.04 | 785 | 28 | 64 | 2.05↑ |
| Thioredoxin peroxidase | B2KSE9 | 21.95 | 5.9 | 327 | 12 | 65 | 2.01↑ |
| Fatty acid binding protein | B6CMG0 | 14.73 | 6.73 | 318 | 5 | 43 | 1.88↑ |
| Acetyltransferase | D2SNV8 | 16.44 | 8.89 | 221 | 3 | 26 | 1.76↑ |
| V ATPase A | E2IV54 | 33.15 | 5.22 | 538 | 10 | 50 | 1.74↑ |
| Vacuolar V H ATPase $\beta$-subunit | E7BZ73 | 54.84 | 5.09 | 241 | 19 | 50 | 1.58↑ |
| Cytochrome P450 | Q8MTD0 | 57.9 | 7.62 | 34 | 7 | 20 | 1.50↑ |
| Thioredoxin peroxidase | B2KSE9 | 21.95 | 5.9 | 170 | 13 | 66 | 1.47↑ |
| Vitellogenin | A7XS62 | 198.6 | 8.83 | 25 | 22 | 19 | 1.90↑ |
| Acyl CoA binding protein | B6A8H5 | 9.46 | 9.13 | 730 | 3 | 44 | 1.52↑ |

peptides 3 to 28 and protein coverage from 12 to 66% (**Table 2**). Elongation factors and very high-density lipoproteins were up-regulated by >8-fold followed by serine proteases and catalase (4.4-fold) in CH fed as compared to AD fed larvae. Other proteins such as aminopeptidase, CYP, heat shock protein, carboxyl choline esterase, arginine kinase, juvenile hormone esterase, thioredoxin esterase and glyceraldehydes-3-phosphate dehydrogenase were upregulated by 2 to 3-fold in CH fed larvae. Many known detoxifying enzymes *viz*. CYP, thioredoxin peroxidase, acetyltransferase and malate dehydrogenase were also exhibiting their higher expression in CH fed *H. armigera*.

### 3.3.3 Quantitative changes in *H. armigera* enzyme activities in response to CH

Biochemical analysis of candidate enzymes namely, CYP, arginine kinsae, serine protease and aminopeptidase was performed. Overall, candidate enzyme activities were found to be significantly upregulated in CH fed *H. armigera* larvae as compared to AD fed larvae (**Fig 3.2**). The activity of CYP and arginine kinase was distinctly higher in CH fed larvae than that of AD fed larvae. Serine protease and aminopeptidase were also significantly up-regulated in CH fed larvae. Activity of CYP (**Fig 3.2A**) was 2-fold higher; arginine kinase (**3.2B**) was 4-fold higher in CH than AD fed larvae. Similarly, serine protease (**3.2C**) and aminopeptidase (**3.2D**) activity was 1-fold higher in CH fed larvae as compared to that of AD fed larvae.

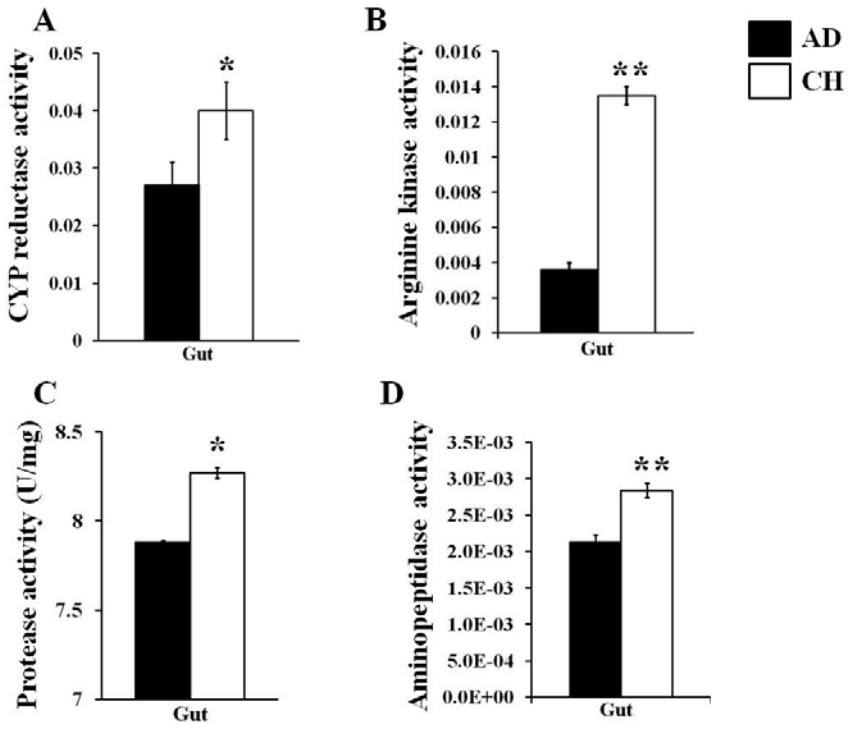



**Figure 3.2** Activity of selected *H. armigera* enzymes based on proteomic data. **(A)** NADPH-cytochrome P450 reductase (CYP) activity in *H. armigera* reared on AD and CH diet (expressed in nmol reduced DPPH/mg of protein) **(B)** Arginine kinase activity (U/ mg of protein) **(C)** Serine protease activity **(D)** Aminopeptidase activity (U/ mg of protein). Standard mean errors are indicated. *indicates that values are significantly different from each other at *$p$< 0.05 and **$p$<0.01.

### 3.3.4 Expression dynamics of candidate genes in *H. armigera*

Overall out of 8 selected candidate genes, 4 showed upregulation of mRNA, whereas rest 4 showed downregulation in CH fed larvae as compared to AD fed larvae (**Fig. 3.3**). Out of 4 up-regulated genes, carboxylesterase (*HaCES*) mRNA was abundantly expressed (20-fold higher), followed by trypsin (*HaTRY*; 14-fold higher), chymotrypsin (*HaCHY*; 2.8-fold higher) and arginine kinase (HaARGK; 2 fold higher) in CH fed as compared to AD fed larvae. On the other hand, glutathione S-transferase (*HaGST*), aminopeptidase (*HaAPEP*), cytochrome p450 (*HaCYP*) and glyceraldehyde-3-phosphate dehydrogenase (*HaGAPDH*) mRNA were downregulated in CH fed larvae. Amongst these 4 mRNAs, *HaAPEP* was barely detected. The selected proteins were

found to be abundant and specifically up-regulated in gut proteome of CH larvae as against AD fed, indicating their role in metabolizing of chlorpyrifos. The relative quantification of these selected mRNA is consistent with the proteomic data.

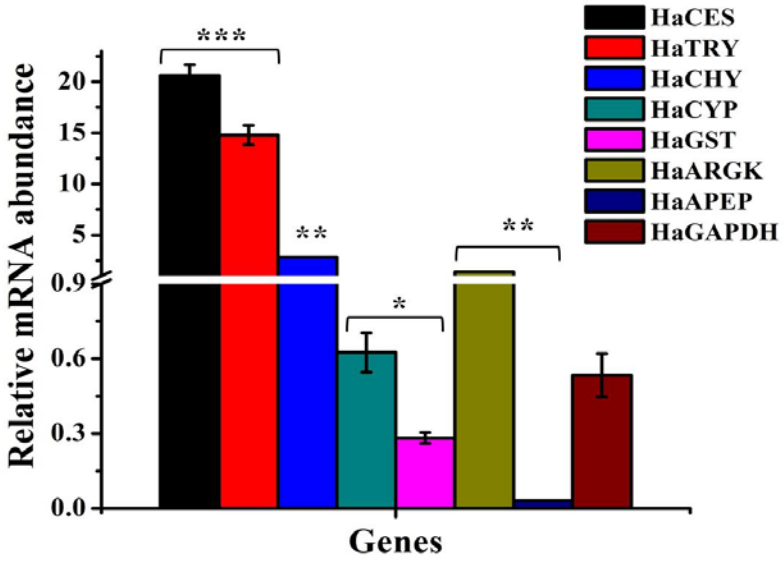



**Figure 3.3** Quantitative Real-Time PCR analysis of selected candidate genes from *H. armigera* reared on CH diet. Relative mRNA abundance of trypsin (*HaTRY*), chymotrypsin (*HaCHY*), glutathione S-transferase (*HaGST*), carboxylesterase (*HaCES*),arginine kinase (*HaARGK*), aminopeptidase (*HaAPEP*), cytochrome P450 (*HaCYP*) and glyceraldehyde-3-phosphate dehydrogenase (*HaGAPDH*) was determined by comparative CT method. β-actin was used as endogenous reference gene and AD fed larvae was considered as calibrator. Error bars represent standard deviation in three biological replicates. *indicates that values are significantly different from each other at *$p$<0.05, **$p$< 0.01and ***$p$< 0.001.

### 3.3.5 CH related metabolites in *H. armigera* gut

GC-MS analysis was carried out to investigate the chlorpyrifos breakdown metabolites in *H. armigera* gut extract. The total ion current chromatograms of standard chlorpyrifos, CH and AD fed gut extract of larvae were as shown in **Fig. 3.4 A, B and C**, respectively. Chlorpyrifos was detected at retention time (RT) 13 min (*m/z*= 286). Appearance of three distinct peaks at RT of 12.42 (*m/z* 207), 12.29 (*m/z* 149) and 11.85 min (*m/z*135) along with 13 min (CH) was observed in CH fed larvae gut extract (**Fig.**

**3.4B**). As expected, the TIC for AD fed larvae did not show any peak at these RT (**Fig. 3.4C**). These unique peaks were combined for mass spectra and identified using mass spectral library (National Institute for Standard Technology, NIST), Gaithersburg, MD, USA). Based on these identified metabolites, we proposed a potential processing of chlorpyrifos in *H. armigera* (**Fig. 3.4D**). At first two subsequent steps, CYP monooxygenase (CYP4M6) might be involved in release of two ethylene molecules followed by formation of oxon derivative (P=O), which could be further hydrolyzed to 2, 3, 5-trichloro-6-methoxypyridine by hydrolases.

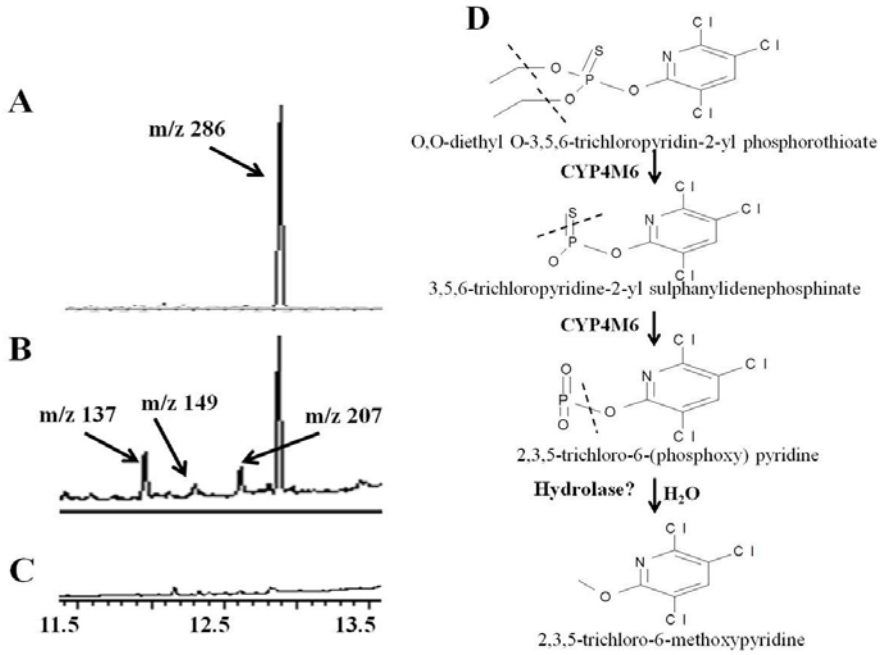



**Figure 3.4** Total Ion Current chromatogram (TIC) of gut metabolites from AD and CH fed *H. armigera* obtained by GC-MS analyses and proposed CH breakdown pathway in *H. armigera*. **(A)** TIC of CH (*m/z* = 286) **(B)** TIC of CH fed *H. armigera* gut metabolites showed three distinct peaks (*m/z* were 135, 149 and 207) **(C)** TIC of AD fed *H. armigera* gut metabolites **(D)** Proposed CH breakdown pathway in *H. armigera* showing probable intermediate metabolites of CH detoxification

## 3.4B Discussion

Gut proteome of *H. armigera* fed on CH diet showed significant up-regulation of plethora of enzymes (**Table 2**). Biochemical data of candidate proteins such as proteases, CYP, Arg kinase indicated that insects are struggling to obtain required nutrients and energy in presence of CH at the same time endeavoring to metabolize CH, which was also evident from gene expression profiles (Dawkar *et al.,* 2011; Chikate *et al.,* 2013). Furthermore, digestive enzymes *viz*. trypsin and chymotrypsin-like proteases found to be significantly up-regulated in CH fed *H. armigera*, although the growth of CH fed larvae was retarded. It is known that over expression of proteases is costly, and thus growth and development of larvae is severely affected (Broadway, 1995).

Generally, polyphagous insects can selectively express a broad range of enzymes that assist in the detoxification or neutralization of numerous natural and synthetic toxins (Li *et al.,* 2004; Sharma *et al.,* 2004). Being polyphagous nature, *H. armigera* has dynamic ability to survive on several hosts and also neutralize effect of deterrents/toxicants. Its ability to metabolize and detoxify insecticides has rapidly acquired resistances to synthetic insecticides and natural plant toxins. In this scenario, one example is resistance to CH, which has been documented in India, Pakistan, Egypt and in many other countries (Dawkar *et al.,* 2013). The present data suggest that in *H. armigera* not only detoxification related enzymes but also enzymes involved in digestion and energy metabolism mechanism play important role in adaptation. For example, biosynthesis, metabolism, transport, detoxification and digestion related proteins were found to be up-regulated in CH fed larvae. Several gut enzymes of insects such as proteases, aminopeptidases, carboxypeptidases, CYP, glucosyltransferases, GST and esterases were reported in various vital functions (Patankar *et al.,* 2001; Ferry *et al.,* 2004; Dawkar *et al.,* 2011).

Elongation factor, glycogen synthase, ATP synthase, V ATPase-A, DNA metabolism protein, Vacuolar V-type proteins are known to be involved in metabolic processes and was up-regulated in CH fed insects. Other highly expressed proteins *viz*. Fatty acid-binding protein, acetyltransferase, very high density lipoprotein are reported for transport mechanism, however vitellogenin enables the directed movement of lipids into, out of or within a cell, or between cells. Moreover, serine protease, aminopeptidase

are involved in digestion; cryptochrome in DNA repair; heat shock protein played vital role during stress conditions; arginine kinase in phosphorylation; Juvenile hormone esterase are essential sesquiterpenes that control insect development and reproduction. All the above mentioned enzymes were up-regulated in CH fed larvae.

Although, activity of many enzymes in CH fed *H. armigera* was up-regulated, probably they might play synergistic role. However, cytochrome P450 monooxygenases play a significant role in the detoxification of host plant allelochemicals and synthetic insecticides in Lepidoptera (Sasabe *et al.,* 2004). It has been widely known that CYP families are capable of metabolizing insecticides. Several studies associating P450 transcript levels and enzyme activities have suggested that P450s from other subfamilies also participate in the detoxification of other classes of plant allelochemicals. Reports on epoxidation of the cyclodiene insecticides aldrin and heptachlor by the CYP6A1 protein (Andersen *et al.,* 1994), hydroxylation of the pyrethroid insecticides deltamethrin and permethrin by the CYP6D1 protein (Scott, 1999) and metabolism of cypermethrin, diazinon and aldrin by the CYP6B8 protein (Li *et al.,* 2004a) support the role of the CYP6 family in insecticide metabolism. Insect's each line of defense is capable of metabolizing diverse compounds detoxification mechanisms contributing insects to resistance/tolerance to toxic allelochemicals and insecticides that provides a competitive advantage to this generalist insect.

"Endurance", the term used by Gordon (1961), fits with the current study. The "endurance" factor may be important in lowering the susceptibility of larvae and as they grow larger, they become more resistant. Under severe selection pressure, polyphagous holometabolous insects often show extraordinarily high and generalized tolerance to insecticides (Candas *et al.,* 2003; Heckel, 2012; Stevens *et al.,* 2013). Another study showed that *H. armigera* midgut lumen proteome was diverse and complex suggesting high adaptability to different toxins (Pauchet *et al.,* 2008). As knowledge of nutrition increased, it has become clear that, as a result of natural selection, all potential food sources may become imbalanced to some extent (Gordon, 1959; Perry *et al.,* 2011; Stevens *et al.,* 2013). There is a positive correlation between food preference and the nutritive value of diet eaten by the grasshopper, *Melanoplus mexicanus* (Ibanez *et al.,* 2013).

It has been important to investigate basis of insecticide processing and/ resistance in insects. There are many reports available on metabolizing synthetic insecticides by microbes and insects (Gordon, 1961; Motoyama and Dauterman, 1980; Nauen and Denholm, 2005; Pedra *et al.,* 2005; Nakamura *et al.,* 2007; Ranson *et al.,* 2011). Two xanthotoxin-inducible P450s from highly divergent subfamilies (*CYP6B* and *CYP321A*) contribute to the resistance of *H. zea* larvae to toxic furanocoumarins and insecticides (Sasabe *et al.,* 2004). The four CYP6B transcripts that have been characterized in the polyphagous *H. zea* (*CYP6B8*, *CYP6B28*, *CYP6B9* and *CYP6B27*) are also induced in response to ubiquitous plant intermediates, such as chlorogenic acid (Li *et al.,* 2002a). Detoxification of pyrethroids has also been shown to be P450-mediated (Li *et al.,* 2000b). Enhanced resistance to the pyrethroid cypermethrin after exposure to furanocoumarins (Li *et al.,* 2000b) and induction of CYP6B mRNA expression after exposure to furanocoumarins is also reported (Li *et al.,* 2000a, 2002c). Although from long time there is an argument for an evolutionary relationship between allelochemical-inducible CYP genes and insecticide-metabolizing P450 sequences. Here, we report that the toxicity of organophosphate insecticides can be altered by regulating enzymes involved in metabolism of CH. CH possess a P=S group, which could be activated by CYP to the oxon (P=O) enhancing the inhibition of acetylcholinesterase (AChE) (Chambers and Carr, 1995). Mutch and Williams (2006) reported that CH was metabolized to CH-oxon by CYP enzymes. In accordance with these reports we propose the possible CH metabolizing pathway in *H. armigera*.

Even though many vital enzymes are highly expressed in *H. armigera* upon exposure to CH, larvae showed growth retardation and here we may get the information of genesis of resistance in *H. armigera*. In response to CH, *H. armigera* larvae upregulates cocktail of detoxification enzymes, which subsequently leads to sequential breakdown of chlorpyrifos? Enzymes involved in energy metabolism (arginine kinase), digestion (serine protease), transport of fatty acid (fatty acid binding protein) etc. were significantly upregulated in larvae fed on CH diet, suggesting their role in stress and possibly metabolizing insecticide molecule. The possible detoxification of chlorpyrifos in *H. armigera* might be by CYP and carboxylesterases; these enzymes might impart potential adaptive capability to insect against insecticides through evolution. The present

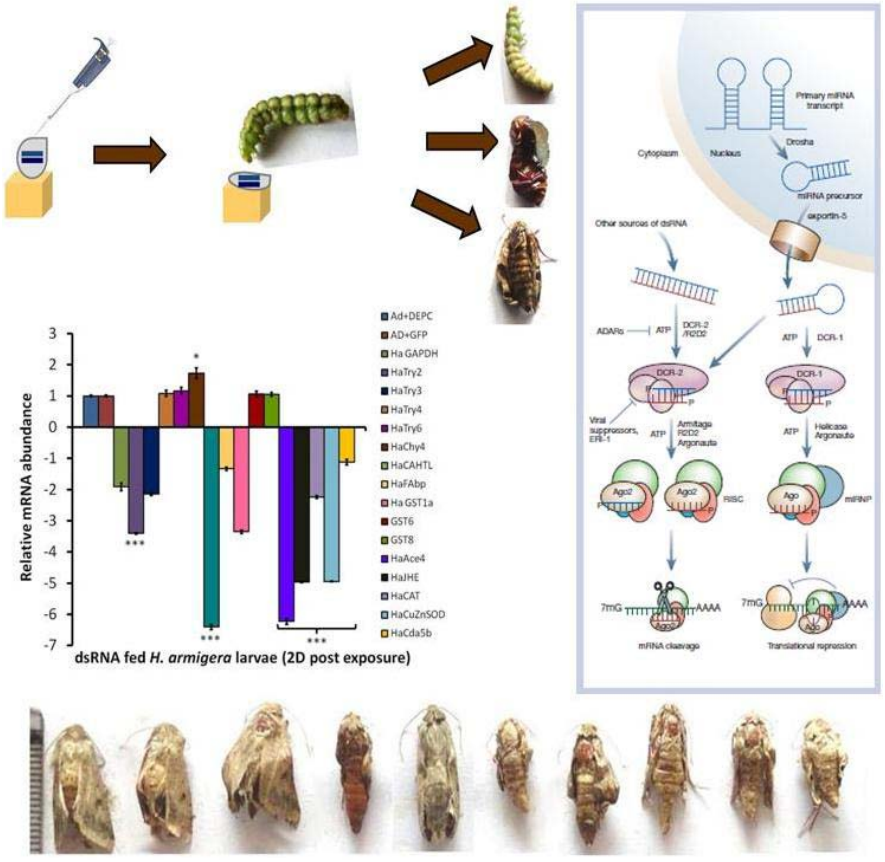
study therefore, offers an added interest to understand the adaptive functionality of proteins in *H. armigera* upon exposure to organophosphate like CH.

## 3.5B Conclusion

Chlorpyrifos based diet significantly retarded *H. armigera* growth and development; which was evident by reduction of mass gain in CH fed larvae. The larval molting intervals were found to be delayed leading to delayed pupation. Interestingly key enzymes such as carboxyl esterases, cytochrome P450 and arginine kinase were upregulated in CH diet fed larvae. This indicated that larvae attempt to metabolize chlorpyrifos to less harmful metabolites by cascade of above mentioned enzymes. However, it was noticeable that alongwith enzymes involved in detoxification pathways, proteases showed significantly higher activity. The possible explanation for this could be that larvae accelerate its digestive proteases to obtain available proteins from AD, eventhough it was supplemented with chlorpyrifos. Moreover, probable involvement of proteases in chlorpyrifos detoxification cannot be ignored and their functionality in doing so remains unknown. qRT-PCR analyses of selected genes in Ct and CH diet fed larvae corroborated with proteomics data, suggesting the complex biochemical mechanism underlying chlorpyrifos tolerance. Proteins involved in digestion, energy and lipid metabolism, detoxification were found to be differentially expressed; such that larvae struggle to survive and overcome the effect of chlorpyrifos simultaneously.

# Chapter 4

## Interruption of *Helicoverpa armigera* development upon silencing of diverse genes by dsRNA feeding




**The research work described in this chapter has been communicated for publication.**

# Chapter 4

## Interruption of *Helicoverpa armigera* development upon silencing of diverse genes by dsRNA feeding


### Abstract

*Helicoverpa armigera* is one of the major crop pests and is less amenable to current pest control methods. RNA interference (RNAi) is emerging as a potent insect pest control strategy. With this scenario, we tested 15 dsRNAs' targeting various *H. armigera* enzymes/proteins namely, proteases like trypsin (*HaTry2, 3, 4* and *6*), chymotrypsin (*HaChy4*) and cysteine proteases like cathepsin (*HaCATHL*); glutathione *S*-transferases (*HaGST1a, 6 and 8*); esterases (*HaAce4, HaJHE*); catalase (*HaCAT*); super-oxide-dismutase (*HaCu/ZnSOD*); fatty acid binding protein (*HaFabp*) and chitin deacetylase 5b (*HaCda5b*) for gene silencing. Highest mortality was observed in *HaAce4* dsRNA fed larvae (85%) followed by *HaJHE*; *HaCAT*; *HaCuZnSOD*; *HaFabp* and *HaTry3* whereas remaining showed less mortality. Overall dsRNA fed larvae showed significant reduction in larval mass gain till 48 h post dsRNA feeding. Out of 15 dsRNAs, most of them resulted into malformed larvae, pupae and moth at a dose of 60µg/day. However, the extent of malformation was more in moths as evident from distinct abnormal phenotypes. Significant downregulation of cognate mRNA expression and reduction in trypsin and GST-like enzyme activities were observed post dsRNA feeding. These findings might provide important insights for designing potent dsRNA as species-specific pesticide.

## 4.1 Introduction

*Helicoverpa armigera* is one of the major Lepidopteran insect pests, due to its polyphagous and cosmopolitan nature. Larvae of this pest feed on a wide range of economically important crops such as cotton, pulses, vegetables, cereals altogether over 300 plant species. It preferably feeds on high nitrogen containing growing plant parts like cotton buds, balls, legume pods, corn etc. and there by leads to dramatic reduction in agricultural yield. Annually US$ 2 billion are spent on insecticides inspite of which *H. armigera* infestation causes heavy economic losses worldwide (US$ 7.5 billion) (Sharma *et al.,* 2006). Currently widely accepted methods for *H. armigera* control are spraying of synthetic insecticides and use of transgenic *Bacillus thuringiensis* (Bt) toxin plants (Peferoen, 1997; Schnepf *et al.,* 1998; Heckel, 2012). However, due to extensive and unplanned spraying of insecticides, this pest have become resistant to most of the insecticides by mutations in the target receptor or overexpression of detoxifying enzymes (Metcalf 1989; Fournier *et al.,* 1992; Park *et al.,* 1997; Enayati *et al.,* 2005; Dawkar *et al.,* 2011, 2013). Heavy use of chemical insecticides leads to ecological havoc including human health by contaminating environments (Dietz and Van Der Straaten, 1992; Edwards, 1993). Transgenic plants producing Bt toxins have proven to be successful in controlling few major insect pests but their long-term and large area of cultivations has lead to sporadic cases of development of resistance against Bt toxin (Bottger *et al.,* 1964; Edwards, 1993; Tabashnik *et al.,* 2008, 2013). Therefore, it is essential to develop alternative novel effective strategies to control insect pests without affecting the environment and under these circumstances RNAi offers great potential in developing species-specific pesticide.

RNA interference (RNAi) is an evolutionarily conserved mechanism of gene regulation across all eukaryotes. Pioneering observations were recorded in plants (Napoli *et al.,* 1990). However, the underlying mechanism still remained unknown. The mechanism of RNAi was discovered in nematode *Caenorhabditis elegans*, in an attempt to investigate role of *unc-22* (Fire *et al.,* 1998). Subsequently this technology has become widely used fundamental genomics tool for characterizing functions of many of newly identified genes in insects from genome sequencing projects (Hannon, 2002; Kuttenkeuler and Boutros, 2004). Recently most of the RNAi studies in insects involve

use of sequence-specific dsRNA as effectors to induce silencing in recipient insects (Huvenne and Smagghe, 2010). Direct injection of the dsRNA is one of the most common methods of delivering dsRNA into target tissues or developmental stages of insects (Bucher *et al.,* 2002; Rajagopal *et al.,* 2002; Amdam *et al.,* 2003; Tomoyasu and Denell, 2004).

In addition to this, oral delivery of dsRNA first demonstrated in *C. elegans*, is now being widely used in some of the insect species for RNAi studies (Timmons *et al.,* 2001). Knockdown of targeted genes in light brown apple moth larvae *Epiphyas postvittana* (Turner *et al.,* 2006), tsetse fly *Glossina morsitans morsitans* (Walshe *et al.,* 2009) and diamondback moth, *Plutella xylostella* larvae (Bautista *et al.,* 2009) was obtained by supplying dsRNA in artificial diet. In these studies, delivery of the dsRNA into the gut resulted in knockdown of a gene's expression in different tissues, indicating that the RNAi was systemic; whereas no observable systemic RNAi was found in the tsetse fly. Oral delivary of dsRNA targeting a gut-specific aminopeptidase in case of *Spodoptera litura* failed to induce RNAi in that insect's gut cells (Rajagopal *et al.,* 2002), suggesting this mode of delivery may not be suitable for all species. Baum *et al.,* (2007) studied effect of diet-delivered 290 different dsRNAs on Western corn rootworm larvae (WCR, *Diabrotica virgifera*) and found that 14 of these caused significant mortalities at doses 5.2 ng/cm$^2$. Transgenic corn targeting A subunit of the V-type ATPase proton pump showed significant reduction in WCR-feeding damage. In another study, Mao *et al.,* (2007) showed that knockdown of cotton bollworm gut-specific cytochrome P450 gene, CYP6AE14, increased sensitivity of insects to gossypol in artificial diets. *E. coli* mediated delivery of dsRNA has been reported in *C. elegans* (Timmons *et al.,* 2001), and *S. exigua* (Tian *et al.,* 2009). Few successful RNAi experiments have been reported in number of Lepidopteran species (Rajagopal *et al.,* 2002; Eleftherianos *et al.,* 2006). Effects of diet delivered various concentrations of midgut and non-midgut gene of *H. armigera* were recently elaborated by Asokan *et al.,* (2013). Zhao *et al.,* 2013 showed that RNAi-mediated knockdown of catalse in *S. litura* led to low survival rates in larvae.

Success of RNAi in insects majorly depends upon selection of target gene and efficient delivery of dsRNA. Many of the studies are being carried out to screen potential candidate genes for developing species- specific targets (dsRNA) for pest control.

Examples involve targeting of chitin synthase in *S. exigua* (Chen *et al.*, 2008), targeting V-ATPase transcripts in fruit flies, flour beetles, pea aphids and tobacco hornworm (Whyard *et al.*, 2009), acetylcholinesterase in *H. armigera* (Kumar *et al.*, 2009), transgenic tobacco expressing EcR dsRNA from *H. armigera* (Zhu *et al.*, 2012), HaHMAG-CoA reductase in *H. armigera* (Wang *et al.*,2013), etc. He *et al.*, 2013 showed that diet delivered dsRNA conjugated to fluorescent nano particle i.e. FNP/CHT10-dsRNA can induce specific and potent silencing leading to mortality in insects. Terenius *et al.*, 2011 have reviewed successes and failures of RNAi in Lepidoptera, elaborating the primary guidelines for experimental design. Moreover, many studies are done for optimization of various aspects of RNAi experiments in insects; one such study is recently reported in *H. armigera* by Yang *et al.*, (2014). The ever expanding RNAi studies in insect pests thus become an encyclopedia for design and selection of newer target genes for efficient pest control.

In this respect, we screened 15 dsRNAs' targeting major classes of enzyme namely, proteases, glutathione *S*-transferases, esterases, catalase/SOD, fatty acid binding protein and chitin deacetylase for gene silencing in *H. armigera* by supplying them with AD. We tested effect of 60µg/day dose of dsRNA (applied thrice at 24 h interval) for selected genes on *H. armigera* growth and development. We found that, out of 15 dsRNAs' mortality was higher in *HaAce4* dsRNA fed larvae (85%) followed by those fed with *HaJHE*; *HaCAT*; *HaCuZnSOD*; *HaFabp* and *HaTry*3 whereas the rest dsRNAs' showed less mortality. The reduction of target transcript expression was also checked and found to be significantly decreased in dsRNA fed larvae as compared to larvae fed on AD diet. Biochemical studies also indicated the downregulation of cognate gene products in dsRNA fed *H. armigera*. The present work can provide potential dsRNAs' that can be used for species-specific insect pest control.

## 4.2 Materials and Methods

### 4.2.1 Insect rearing

Actively feeding *H. armigera* larvae collected from fields were transferred and maintained on artificial diet (Nagarkatti and Prakash, 1974). To ensure greater genetic

homogeneity among test populations, insects were maintained on artificial diet for three generations. Subsequently, larvae were collected and maintained on AD to get 2$^{nd}$ instar larvae for further dsRNA feeding bioassays. Collection and rearing of new batch of larvae was done in nuclease-free insect culture vials. Proper precautions were taken while diet preparation and larval maintenance so as to minimize the interference by RNases during further feeding experiments.

### 4.2.2 Synthesis of dsRNA

Based on earlier studies in our lab and literature, we selected major enzyme/protein classes in *H. armigera* for gene silencing by feeding them specific dsRNA. These involved 15 mRNAs' belonging to proteases (6), glutathione *S*-transferases (3), esterases (2), catalase (1)/SOD (1), fatty acid binding protein (1) and chitin deacetylase (1) (detailed in **Table 1**). Green fluorescent protein (GFP) was used as negative control while glyceraldehyde phosphate dehydrogenase from *H. armigera* (GAPDH) was used as positive control. Sequence analyses for each of these target mRNA class was done using CLUSTAL W and primers containing gene-specific sequence adapted to T7 promoter sequence in inverted orientation were designed. The primer sequences, amplicon sizes used for preparation of dsRNAs targetting each chosen mRNA are listed in **Table 2**.

cDNA of AD and pigeon pea fed larvae was used for amplification of target regions. These were cloned into P$^{GEMT}$, confirmed and then linearized plasmids were used as template for *in vitro* synthesis of dsRNA. The protocol used for *in vitro* dsRNA synthesis was based on MegaScript RNAi kit instruction manual (Ambion, Austin, Texas, USA). Once synthesized dsRNAs were purified and assessed for their quality and quantity by agarose gel electrophoresis and Nanodrop 1000 spectrophotometer (Thermo Scientific). Stability of dsRNA when coated on AD cube was assessed for different time intervals. These dsRNAs were then used for feeding bioassays of *H. armigera*.

**Table 1: Functions of selected target genes from *Helicoverpa armigera***

| Class | Genes | Accession No. | Function | Malformation of moths (%) |
|---|---|---|---|---|
| Protein visualization | *GFP* | L29345 | Localization of protein in living cell | - |
| Glycolytic enzyme | *HaGAPDH* | JF417983 | Conversion of glyceraldehyde 3-phosphate to D-glycerate 1, 3-bisphosphate | 1.79 |
| Serine proteases | *HaTry2* | EU770391 | Protein digestion, constitute about 95% of the total digestive activity | 16.07 |
| | *HaTry3* | EU325548 | | 47.50 |
| | *HaTry4* | EF600059 | | 12.50 |
| | *HaTry6* | Y12276 | | 23.86 |
| | *HaChy4* | GU323796 | | 14.77 |
| Cysteine protease | *HaCAHTL* | EU528473 | Insect molting and metamorphosis | 32.95 |
| Lipid transport protein | *HaFabp* | EU325560 | Lipid binding and transport activity | 44.64 |
| Glutathione *S*-transferase | *HaGST1a* | HM209431 | Catalyses xenobiotics, including pesticides in the mercapturic acid pathway, elimination of toxic compounds | 25.00 |
| | *HaGST6* | GQ149104 | | 31.94 |
| | *HaGST8* | FJ546089 | | 54.17 |
| Esterases | *HaAce4* | JF894118 | Hydrolyzes the neurotransmitter acetylcholine to terminate synaptic transmission | - |
| Esterases | *HaJHE* | HM588760 | Regulates larval to adult transition in insects | 87.50 |
| Catalase | *HaCAT* | JQ009332 | Catabolism of hydrogen peroxide | 12.50 |
| Superoxide dismutase | *HaCuZnSOD* | JQ009331 | Detoxification of cellular oxidative stresses | 25.00 |
| Hydrolases | *HaCda5b* | GQ411191 | Convert chitin, a β-1,4-linked N-acetylglucosamine polymer, into its deacetylated form, chitosan, a natural glucosamine polymer | 12.50 |

**Table 2: List of primers used for *in vitro* dsRNA synthesis**

| Primer Name | Primer sequence (5'→3') | F/R | Accession No. |
|---|---|---|---|
| **HaAce4_iF** | TAATACGACTCACTATAGGGAGAGTGGAGACTCAACGAAGATC | F | JF894118 |
| **HaAce4_iR** | TAATACGACTCACTATAGGGAGACTCTTAGACCACATAATGAACTC | R | |
| **HaCAT_iF** | TAA TAC GAC TCA CTA TAG GGA AGACCATGGCTCAAGCAGAGAGC | F | JQ009332 |
| **HaCAT_iR** | TAA TAC GAC TCA CTA TAG GGA ATTGTGGTCCATCACGTTGGTAG | R | |
| **HaCathL_iF** | TAA TAC GAC TCA CTA TAG GGA ATATGACGCTAGGCTCGAAGGTC | F | EU528473 |
| **HaCathL_iR** | TAA TAC GAC TCA CTA TAG GGAGACTAGGGCAACCTTCAAAGCTCC | R | |
| **HaCda5b_iF2** | TAA TAC GAC TCA CTA TAG GGA GA CTGCACTCCATCAGCCATAAG | F | GQ411191 |
| **HaCda5b_iR2** | TAA TAC GAC TCA CTA TAG GGA GA ATCATGCAATCTTGCTCCGAAG | R | |
| **HaChy4_iF** | TAATACGACTCACTATAGGGAGAGCTGGTCTTGTGATCACCATC | F | Y12273 |
| **HaChy4_iR** | TAATACGACTCACTATAGGGAGAGATGGTCACAGAGCTCAACTG | R | |
| **HaCu/ZnSOD_iF** | TAA TAC GAC TCA CTA TAG GGA GATCTGCGGGTGCTCACTTCAAC | F | JQ009331 |
| **HaCu/ZnSOD_iR** | TAA TAC GAC TCA CTA TAG GGA GAGATAACACCGCAGGCAATACG | R | |
| **HaFabp_iF** | TAA TAC GAC TCA CTA TAG GGA GATCACCTCCTCCACCTTCAAGAC | F | EU325560 |
| **HaFabp_iR** | TAA TAC GAC TCA CTA TAG GGA GAACATCCTTGGCGGTCATCACAG | R | |
| **HaGAPDH1_iF** | TAATACGACTCACTATAGGGAGACAAGGCTGGTGCTGAATACG | F | JF417983 |
| **HaGAPDH1_iR** | TAATACGACTCACTATAGGGAGACAGAGGGTCCATCCACTG | R | |
| **HaGFP1i_F** | TAATACGACTCACTATAGGGAGA CAAGATACCCAGATCATATGAAAC | F | L29345 |
| **HaGFP1i_R** | TAATACGACTCACTATAGGGAGA GCTTCCATCTTTAATGTTGTGTC | R | |
| **HaGST1a_iF** # | TAATACGACTCACTATAGGGAGAGGAGACAATATCCAAGAGG | F | HM209431 |
| **HaGST1a_iR** | TAATACGACTCACTATAGGGAGACGTTGATCCTCTGCAAGAG | R | |
| **HaGST6_iF** | TAATACGACTCACTATAGGGAGAGACAAACTGACCTTGGCAGAC | F | GQ149104 |
| **HaGST6_iR** | TAATACGACTCACTATAGGGAGAACTGCGCTACCATAGCTCTG | R | |
| **HaGST8_iF** | TAATACGACTCACTATAGGGAGAGAGAACAGCACAAAGAAG | F | FJ546089 |
| **HaGST8_iR** | TAATACGACTCACTATAGGGAGAGCCAAGAACTTCTCAGCG | R | |
| **HaJHE_iF** | TAA TAC GAC TCA CTA TAG GGA GA CCACCAAGATCTACACGGACC | F | HM588760 |
| **HaJHE_iR** | TAA TAC GAC TCA CTA TAG GGA GA ATTCCTGTTCCGCTCATCAAG | R | |
| **HaTry2_iF** | TAATACGACTCACTATAGGGAGAATGGAGAGACCAAATTCGGAG | F | EU770391 |
| **HaTry2_iR** | TAATACGACTCACTATAGGGAGACCAGTAGCGAAGCACCTG | R | |
| **HaTry3_iF** | TAATACGACTCACTATAGGGAGAGTTCAGTGGTGGCTCGCTG | F | EU325548 |
| **HaTry3_iR** | TAATACGACTCACTATAGGGAGAGCGTGGAATGTGCAGACCTC | R | |
| **HaTry4_iF** | TAATACGACTCACTATAGGGAGACGACATCAACTACCGTCGTG | F | EF600059 |
| **HaTry4_iR** | TAATACGACTCACTATAGGGAGACAGAGGACCACCAGAGTC | R | |
| **HaTry6_iF** | TAATACGACTCACTATAGGGAGAGCAGTCCACAACGTTGCTTCG | F | Y12276 |
| **HaTry6_iR** | TAATACGACTCACTATAGGGAGACGATGCCGTTGTGGTAGAGAG | R | |

# This primer pair was designed targeting six GST isoforms HM209429, HM209427, HM209428, HM209430, EF591059 and one mentioned in above table

### 4.2.3 dsRNA feeding bio-assays

In order to understand the effect of chosen dsRNAs' (functions of gene mentioned in **Table 1**) on growth and development of *H. armigera* feeding bioassays were carried out as described below. We checked both delivery methods, droplet feeding (Turner *et al.*, 2006) and delivery of dsRNA through AD to find out better one. In case of droplet feeding, we utilized 400 ng and1µg of dsRNA targeting *HaTry2* and *7*, prepared in 10% sucrose solution. Appropriate dilutions were made and 2 µl of each dsRNA droplet were fed to 2$^{nd}$ instar larvae separately. Prior to feeding, larvae were starved for 44 h; intake of entire droplet was ensured visually. Sucrose solution (10% w/v in Nuclease Free Water) devoid of dsRNA was used as control. Post feeding the larval mass from each set was measured for 24, 48 and 72 h; in parallel few were harvested to perform further analyses.

In other experiments, dsRNA feeding was carried out using AD; dsRNA solution of appropriate concentration was applied onto surface of AD cube (~1 cm$^2$). dsRNA was allowed to diffuse into diet and fed to insects. In this way, dsRNA (60 µg) feeding was carried out for selected 17 genes and AD coated with DEPC treated water was used as non-dsRNA control, separately. dsRNA targeting GFP was used as negative control and one targeting GAPDH as positive control. Prior to experiments stability of dsRNA on AD was checked. dsRNA applications were done thrice at 24 h intervals for all target genes, fresh AD cube with fresh 60 µg application were used each time. Following to this, larvae were reared on normal AD and observed for their growth and development. During dsRNA feeding experiments, 25 larvae from each of the treatment groups were harvested in liquid nitrogen at 2, 4 and 8 days post dsRNA exposure (DPE) and stored at -80 °C. Silencing effects were observed in terms of larval mass gain, percent mortality, phenotypic abnormalities, egg laying capacity and reduction of target transcript expression as well as enzyme activities.

### 4.2.4 Real time PCR analyses

In order to evaluate the efficiency of silencing, relative transcript accumulation of selected target genes was checked for respective treatment groups. Overall 18 treatment groups were considered; in these AD+ DEPC water and AD+GFP dsRNA were negative

controls while AD+*HaGAPDH* was positive control. Total RNA for one biological replicate was isolated from pools of 10 larvae using Trizol reagent (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. Synthesis of first-strand cDNA, real-time RT-PCR was performed as described in Chikate *et al.,* 2013. The relative expression fold was calculated using ΔΔ CT method. β-actin was used as endogenous reference gene and control group AD+DEPC water was used as calibrator. The PCR reactions were performed in biological and technical triplicates. The list of primers used for real time PCR analyses is mentioned in **Table 3**.

### 4.2.5 Enzymatic assays

Protein extract was prepared from control and dsRNA fed group larvae using 0.2 M glycine-NaOH (pH 10.0). This was then used as enzyme source for calculating glutathione *S*-transferase (GST) and trypsin-like activity units. GST activity was determined according to Habig *et al.,* (1974). The amount of enzyme that catalyzed the formation of 1 nmol of product per min under assay conditions was defined as one GST unit. Enzymatic assay using benzoyl-arginyl-*p*-nitro-anilide (BA*p*NA) as substrate was performed in order to estimate trypsin-like activity (Erlanger *et al.,* 1961). Trypsin-like activity was measured from control and dsRNA fed larvae as described in **section 2.2.5, chapter 2**.

### 4.2.6 Statistical analysis

Data was analyzed by one-way analysis of variance (ANOVA) and Post hoc analyses using a Tukey-Kramer multiple comparisons test. Data points were considered significant at $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***); and are indicated in respective figures.

**Table 3: List of primers used for real time PCR analyses of selected target genes**

| Primer Name | Primer sequence- 5'-3' | F/R | Accession No. |
|---|---|---|---|
| ***HaAce4* F** | ATTGTCCCCTCTGTCAAGGAAC | F | JF894118 |
| ***HaAce4* R** | GATATAATAGCCCAAGGTGCGG | R | |
| ***HaCAT* F** | CCCCGAGGATTTGCTGTTAAA | F | JQ009332 |
| ***HaCAT* R** | GATGAAGAAAATTGGCGTGTTGT | R | |
| ***HaCAT*HL F** | CTGGGCCACTTACAACGAAGAT | F | EU528473 |
| ***HaCAT*HL R** | GATCCCAACCAGGAGTTGAACT | R | |
| **HaCda5b F** | GCCTTCATCAGATTCCTCAACC | F | GQ411191 |
| **HaCda5b R** | CACCCAGTCAATCACTTCGCT | R | |
| **Hachy 4F** | TGACTTGTCAGGTGGCCAAGCTG | F | GU323796.1 |
| **Hachy 4R** | GCGATTCTGGTACCGCCGGAGAAC | R | |
| **HaCu/ZnSOD F** | CTGACCCTGATGACCTTGGAG | F | JQ009331 |
| **HaCu/ZnSOD R** | GATAACACCGCAGGCAATACG | R | |
| **HaFabp F** | CTTGAAGCAGGTGCAGAAGTCA | F | EU325560 |
| **HaFabp R** | ACAGCCTTCATCTCTTCGGGT | R | |
| **HaGAPDH 1F** | TGCTGAATACGTCGTTGAATCC | F | JF417983 |
| **HaGAPDH 1R** | TTCTTAGCACCACCCTCTAAATGAG | R | |
| **HaGFP F** | CACTGGAGTTGTCCCAATTCTTG | R | L29345 |
| **HaGFP R** | CCTTCACCCTCTCCACTGACAG | F | |
| **HaGST 1F** # | CAAAATGAAGGGTCTGGGAGAA | F | HM209431.1 |
| **HaGST 1R** | CGTTCAAAGCGTATGTCTTCGA | R | |
| **HaGST 6F** | CTCGTGGCGACTGTGTCCACTA | F | GQ149104.1 |
| **HaGST 6R** | AGTCGCTTTCACCAGCTCAAACC | R | |
| **HaGST 8F** | TCTTTACCCAGCTGATCCGAAA | F | FJ546089.1 |
| **HaGST 8R** | AAAGCTGGAAACAGAATCCCAC | R | |
| ***HaJHE* F** | GCAAAAACGTGCTGAGACTGG | F | HM588760 |
| ***HaJHE* R** | TCCTTGATGATGCTGCTCTGAC | R | |
| **HaTry 2F** | GCGTAAAGGATGCGGTTGG | F | EU770391 |
| **HaTry 2R** | CAGGATGGCAACCATCCATG | R | |
| **HaTry 3F** | CGACCACACTGACGCGAG | F | EU325548 |
| **HaTry 3R** | GCACGCCACTGGACATGG | R | |
| **HaTry 4F** | GTGCTACCCCTTCTGATTC | F | EF600059 |
| **HaTry 4R** | AACTTGTCGATGGAGGTGAC | R | |
| **HaTry 6F** | TGGCTGGGGTGACACTTTCT | F | Y12277 |
| **HaTry 6R** | GTCTCCCTGGCACTGGTC | R | |

**#** This primer pair was designed for six GST isoforms HM209429, HM209427, HM209428, HM209430, EF591059 and one mentioned in above table

## 4.3 Results

### 4.3.1 Effect of droplet feeding of dsRNA on *H. armigera* growth and development

Droplet feeding of dsRNA was not significant in RNA silencing of target genes. Single dose of 400ng of *HaTry* 7 and 2 dsRNA feeding was found to be ineffective in inducing RNA silencing of target genes (**Fig. 4.1**). Larval mass gain profile showed no significant difference among control and dsRNA fed larvae even after 72 h.(**Fig. 4.1A**). No specific reduction of transcript accumulation of *HaTry* 7 and 2 were observed upon 24, 48 and 72h post dsRNA feeding (**Fig. 4.1B**). In this experiment droplet feeding was done only once, followed by which larvae were reared on normal AD. The possible reason for low efficiency of silencing could be supply of low dose of dsRNA and single feeding. However, the presence of multiple isoforms of *HaTry*'s and their higher sequence homology must also be taken care of while designing the target dsRNAs. This is why the target transcripts might not be effectively reduced at given dose of dsRNA.

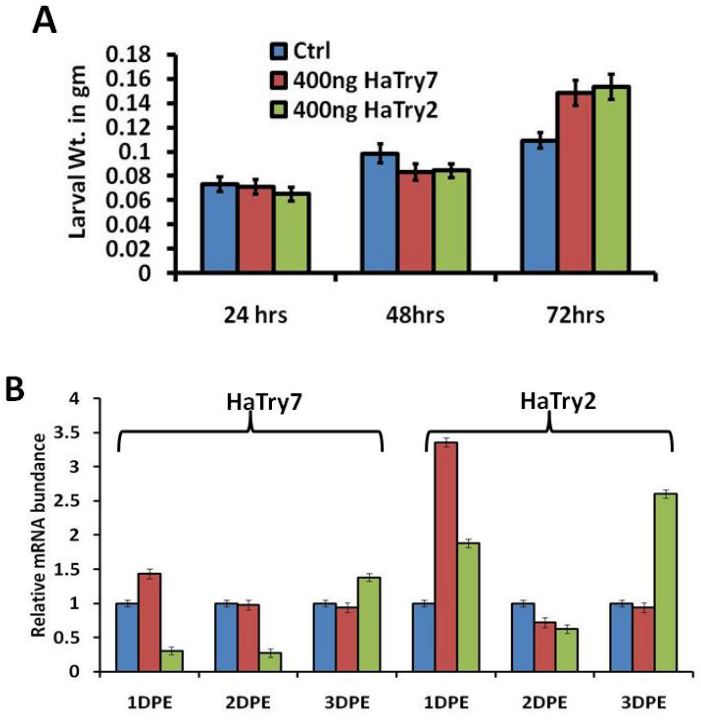



**Figure 4.1** Effect of droplet feeding (single dose) of 400ng dsRNA to *H. armigera*. Larval mass gain profile upon feeding *HaTry*7 and 2 specific dsRNA, respectively

(**4.1A**). Relative transcript accumulation of *HaTry*7 and 2 upon dsRNA feeding (**4.1B**); relative expression level was determined by real time PCR analyses using non-dsRNA control as calibrator and β-actin as endogenous reference gene.

In another experiment higher dose i.e. 1µg of dsRNA targeting *HaTry* 7 and 2 was checked for RNA silencing by droplet feeding (**Fig. 4.2**). Feeding was done only once and then larvae were reared on normal AD. In terms of growth no significant difference was observed between control and dsRNA fed larvae, this was evident from larval mass gain profile at 24, 48 and 72h post dsRNA feeding (**Fig. 4.2A**). Interestingly the total protease activity of dsRNA fed larvae was found to be significantly reduced as compared to that of control, 72h post feeding (**Fig. 4.2B**). However, significant reduction of *HaTry*7 transcript was observed while *HaTry*2 mRNA remained constant post 72h of dsRNA feeding (**Fig. 4.2 C**).

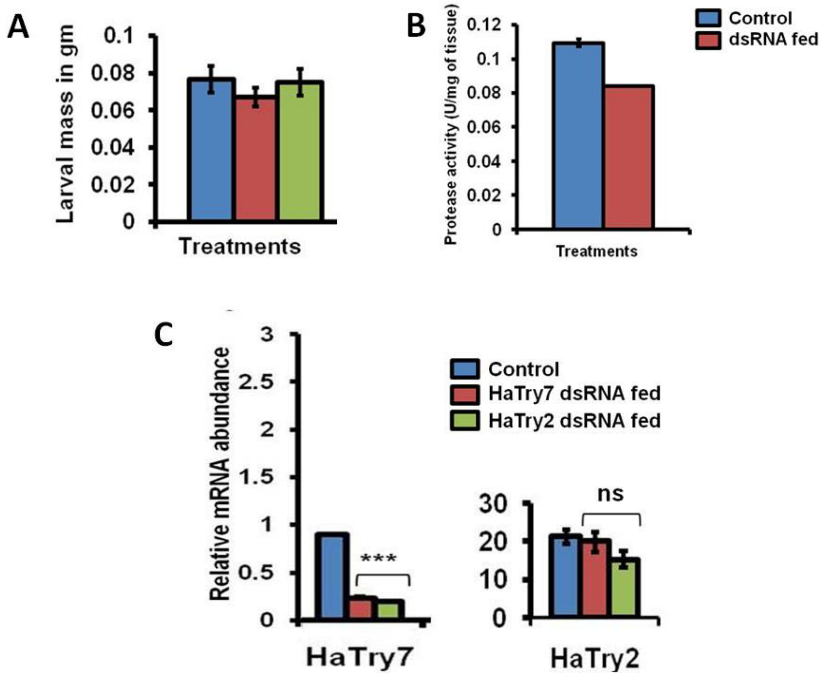



**Figure 4.2** Effect of droplet feeding (single dose) of 1µg dsRNA to *H. armigera*. Larval mass gain profile upon feeding *HaTry*7 and 2 specific dsRNA, respectively (**4.2A**). Total protease activities of dsRNA fed larvae compared to control (**4.2B**). Relative transcript accumulation of *HaTry*7 and 2 upon dsRNA feeding (**4.2C**); relative expression level was

determined by real time PCR analyses using non-dsRNA control as calibrator and β-actin as endogenous reference gene.

### 4.3.2 Altered *H. armigera* growth and development in response to dsRNA supplied with AD

Feeding dsRNA along with AD induced specific silencing of cognate mRNAs. The dsRNA amount 60 µg/day was found to effectively interrupt *H. armigera* growth and development. The effect of silencing was observed in terms of larval mass, growth performance and development upon feeding AD containing dsRNA. Out of 15 dsRNAs, *HaAce4* dsRNA fed larvae showed highest mortality (85%) followed by those fed with *HaJHE*; *HaCAT*; *HaCuZnSOD*; *HaFabp* and *HaTry3* whereas the rest dsRNAs' showed less mortality (**Fig. 4.3**).

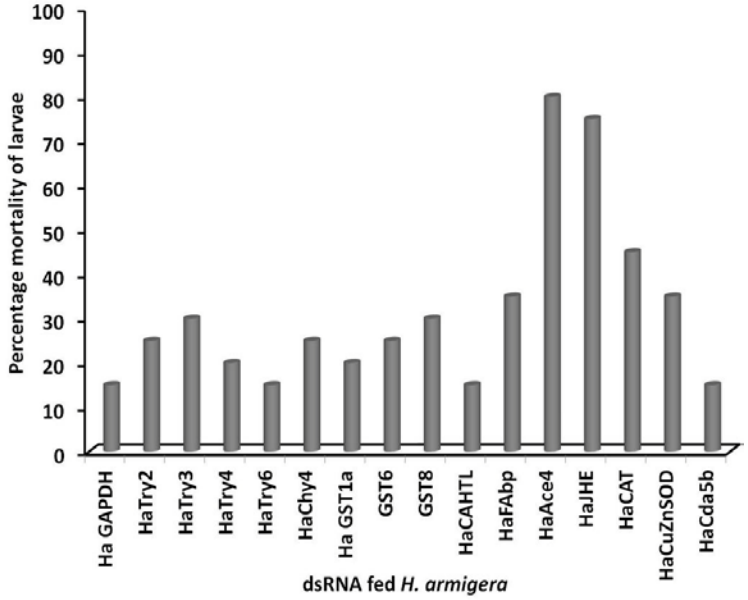



**Figure 4.3** Percentage mortality of *H. armigera* after reared on AD containing dsRNA of various genes. dsRNA for each gene was applied thrice onto surface of AD cube after interval of 24 h. Following to these larvae were reared on normal AD and observed till moth formation.

The effect of non-dsRNA control and negative control (GFP dsRNA) was found to be similar and negligible. Larval growth was found to be significantly retarded in most of the dsRNA fed larvae as compared to control. This was evident from reduction in mass

gain of dsRNA fed larvae unlike control group larvae post 48, 96 and 144 h of dsRNA feeding (**Fig. 4.4A, B and C**).

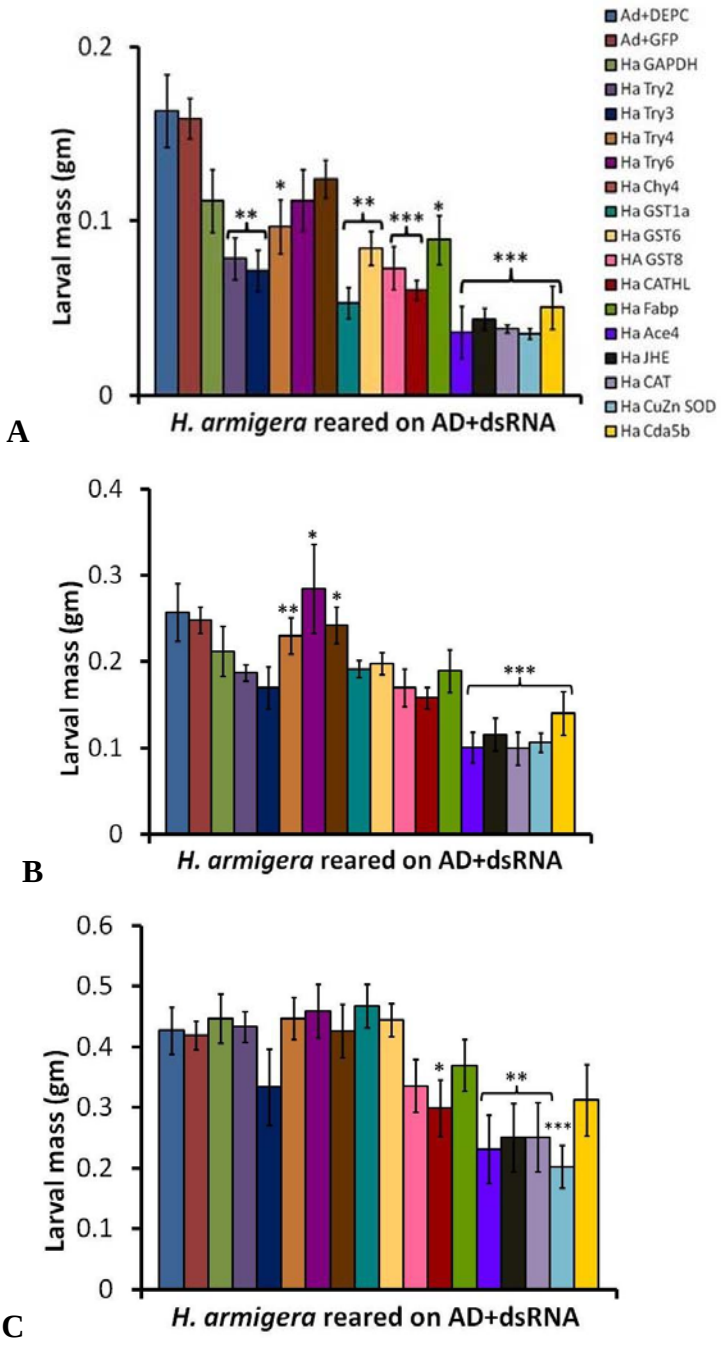



**Figure 4.4** Larval mass gain (gm) of *H. armigera* post dsRNA feeding. Mass of dsRNA fed larvae post 48 h **(4.4A)** showed significant reduction in mass gain as compared to control. Reduction of mass gain was significant for *HaAce4*, *HaJHE*, *HaCAT*,

*HaCuZnSOD* and *HaCda5b* dsRNA fed larvae post 96 (**4.4B**) and 144h (**4.4C**), respectively. The mass gain profile of larvae was observed till pupae at interval of 48h.

### 4.3.3 Phenotypic abnormalities in *H. armigera* larvae, pupae and moth upon dsRNA feeding

The silencing effect of dsRNA supplied with AD was persistent till all the stages of larvae. Not only larval mass were affected but developmental abnormalities were observed in most of dsRNA fed larvae as compared to control. Three doses of dsRNA (60 µg) were found to induce gene silencing resulting into the distinct abnormal phenotypes of larvae (**Fig. 4.5A**), pupae (**Fig. 4.5B**) and moth (**Fig. 4.5C**).

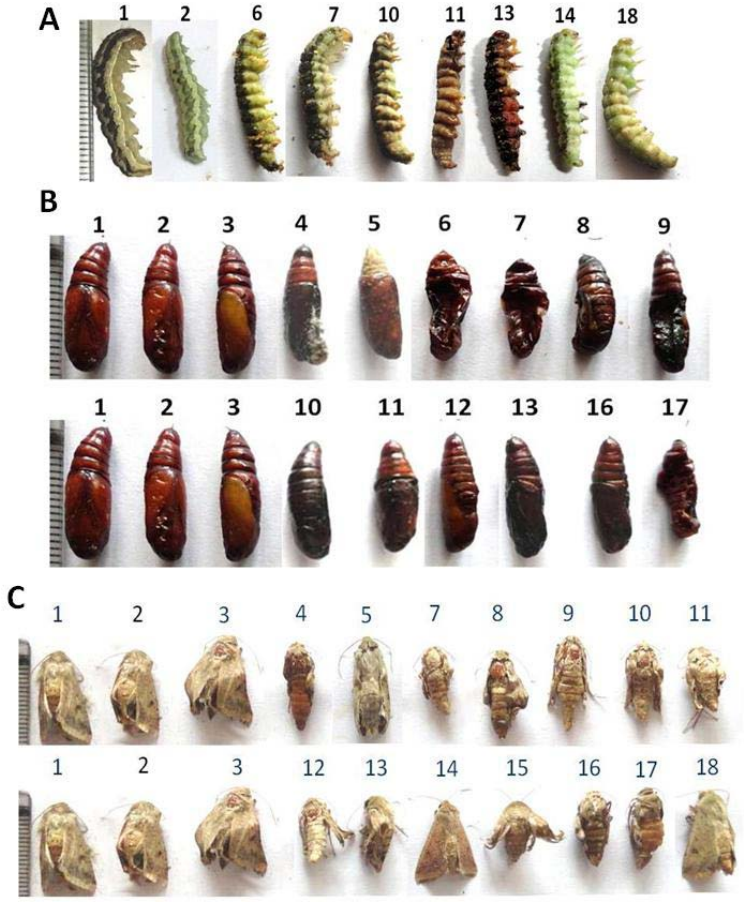



**Figure 4.5** Developmental abnormalities in *H. armigera* larvae, pupae and moth upon dsRNA feeding. dsRNA targeting each different gene was fed separately and non-dsRNA (AD+DEPC), unrelated dsRNA (AD+GFP) were used as controls. The extent of

abnormalities and malformation for dsRNA fed larvae **(4.5A)**, pupae **(4.5B)** and Moth **(4.5C)** was remarkable than that of control larvae.

*HaTry4*, *HaTry6*, *HaFabp*, *HaGST1a*, *HaGST8*, *HaAce4* and *HaCda5b* dsRNA fed larvae showed abnormal growth. Whereas at pupal stage *HaTry2*, *3*, *4* and *6*; *HaChy4*; *HaCATHL*; *HaCAT*; *HaCuZnSOD* dsRNA fed larvae formed malformed pupae. The extent of malformation was relatively more in moths as compared to larvae and pupae. The percentage malformation of moths was highest in *HaJHE* (87.5%) dsRNA fed larvae followed by *HaGST8*, *HaTry3*, *HaFabp*, *HaCuZnSOD* and remaining showed lesser malformation (**Table 1**). Moreover, in some cases delayed pupation was observed upon dsRNA feeding as compared to that of control group. Interestingly we found that female moth's egg laying capacity was also significantly affected in most of dsRNA fed larvae. For example density of eggs was lowest in larvae fed on *HaAce4*, *HaJHE* and *HaGST1* dsRNA and lower in *HaTry2*, *3*, *4* and *6*, *HaFabp*, *HaCAT*, *HaCuZnSOD*, *HaGST6* and *8*, *HaCda5b* dsRNA fed larvae. While female moths of control group were highly fecund. This could be indicative of penetrance of silencing signal and its efficacy.

### 4.3.4 Silencing of cognate mRNAs' of *H. armigera*

Real time PCR analyses showed that specific and effective silencing was induced for selected target genes of *H. armigera*. The suppression of target transcripts was significant 2 days post dsRNA feeding while at 8 days post feeding, these mRNAs were found to restore their expression comparative to control group. Overall significant downregulation of cognate mRNA's was observed for 11 genes (*HaGAPDH*, *HaTry2*, *3*; *HaCATHL*; *HaFabp*; *HaGST1a*, *6*; *HaAce4*; *HaJHE*; *HaCAT*; *HaCuZnSOD* and *HaCda5b*; while *HaTry4*, *6*; *HaCHy4* and *HaGST8* showed slightly higher expression compared to that of control (**Fig. 4.6**). There was 6.5-fold downregulation in *HaCATHL* and *HaAce4* mRNA, while it was 5-fold lower expression for *HaJHE* and *HaCuZnSOD* mRNA as compare to control larvae. The expression of *HaTry2* and *HaGST1a* mRNA was 3.5-fold lower as compared to control one whereas those for *HaGAPDH*, *HaTry3*, *HaFabp*, *HaCAT* and *HaCda5b* were only 2-fold lower as compared to the control. The relative transcript accumulation was nearly constant for non-dsRNA control and negative

(non-related dsRNA) GFP dsRNA control (**Fig. 4.6**). Relative mRNA expression for most of the target genes, 8 days post dsRNA feeding was found to be reinstate, compared to that of control group. This indicated that dsRNA feeding has induced specific interference of cognate mRNAs with variable efficacy.

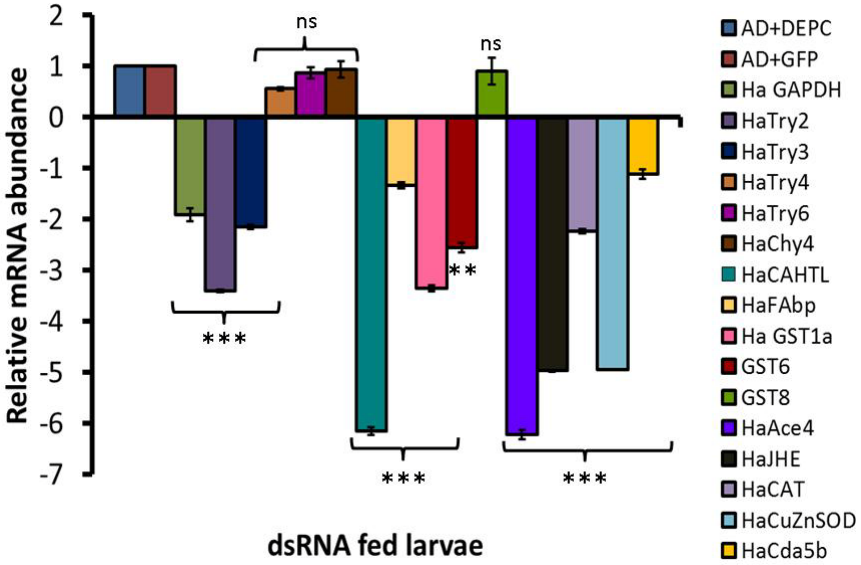



**Figure 4.6** Relative transcript accumulation of selectively silenced candidate genes from *H. armigera* was determined by real time PCR analyses. The relative expression fold was calculated by ΔΔ CT method using β-actin as endogenous control and AD+DEPC as calibrator. Significant downregulation was observed for cognate mRNAs after 2 days post dsRNA feeding. Standard mean errors are indicated. * and ** indicate that values are significantly different from each other at $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$, respectively.

### 4.3.5 Reduction of target enzyme activities

Biochemical assays for GST and trypsin-like activities were performed for respective dsRNA fed and control *H. armigera*. Significant reduction of GST (**Fig. 4.7A**) and trypsin-like (**Fig. 4.7B**) enzyme activities was observed in dsRNA fed larvae compared to that of control, 8 days post dsRNA feeding. *HaGST1a, 6* and *8* dsRNA fed larvae showed GST activity units nearly similar to that of control 2 days post dsRNA feeding. However, activity units of GST were found to be 2.5-fold lower in *HaGST1a* and *6* while 1 fold lower in *HaGST8* dsRNA fed larvae 8 days post dsRNA feeding.

Similarly, *HaTry2*, *3*, *4* and *6* dsRNA fed larvae showed activity units almost similar to control following 2 days post dsRNA feeding. However, trypsin activity units were 2-fold lower in *HaTry3* and *4* and 1.5 fold lower in *HaTry2* and *6* dsRNA fed larvae 8 days post dsRNA feeding. Both GST and trypsin-like activity units were found to be nearly constant in AD+DEPC-water and AD+GFP dsRNA fed larvae. Amongst the controls, GST activity was higher at 8 days while trypsin-like activity units were higher at 2 days post feeding.

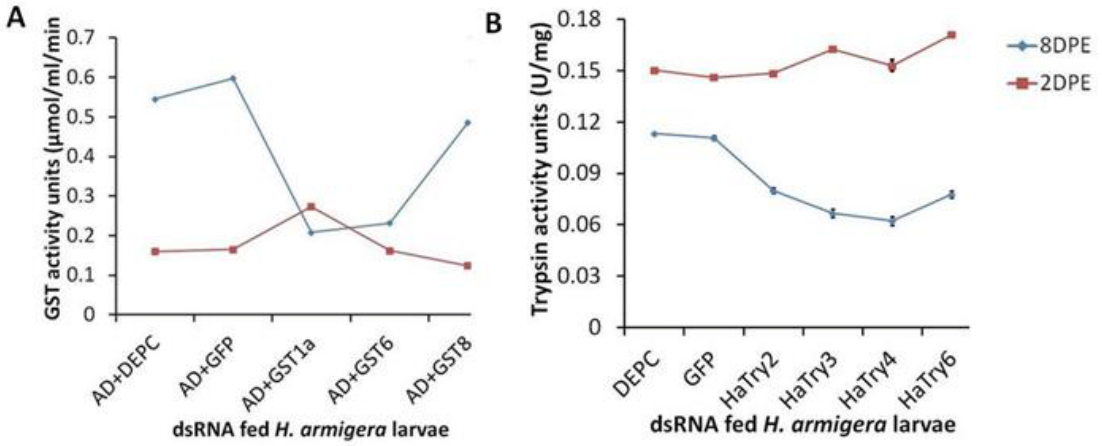



**Figure 4.7** Enzyme activity units for glutathione *S*-transferase **(4.7A)** and trypsin **(4.7B)** in dsRNA fed *H. armigera* larvae. Specific substrates namely benzene substrate (1, 2-dichloro-1-dinitrobenzene) and benzoyl-DL-arginyl-pnitroanilide (BA*p*NA) were used for respective enzymes assays of GST and trypsin

### 4.3.6 Silencing effect at translational level

The efficacy of silencing upon dsRNA feeding was checked at protein level for one of the target gene juvenile hormone esterase (*HaJHE*). Whole larval proteins from AD fed and *HaJHE* dsRNA fed larvae were extracted and incubated with fluorescently labeled azadirachtin. Subsequently native PAGE of these samples were carried out and visualized. Interestingly it was found that, lanes corresponding to AD fed larval proteins showed stronger fluorescence while it was very weak for *HaJHE* dsRNA fed larvae (**Fig. 4.8**). Weak or no fluroscence was observed in case of JHE silenced larval proteins; indicating non-availability of JHE for azadirachtin binding. This indicated that JHE was

not available in sufficient amounts in JHE silenced larvae as compared to AD fed larvae and showed weak fluorescence on native PAGE. On the other hand, AD fed larval proteins with labeled azadirachtin showed prominent fluorescence indicating presence of sufficient JHE and its strong interaction with azadirachtin.

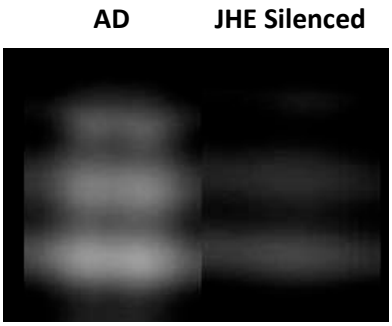



**Figure 4.8** Native PAGE profile of AD and JHE dsRNA fed larval proteins. Silencing effect was evident at biochemical level as shown by native PAGE profile of AD and JHE dsRNA fed larval proteins in presence of fluorescently labeled azadirachtin.

## 4.4 Discussion

Till date many of the studies are being reported in insects to explore RNA silencing to develop efficient pest control strategy and successes of which are variable (Terenius *et al.,* 2011). Particularly, Lepidopteran insects are known to be relatively impervious to dsRNA induced silencing, though, few studies described the variable efficacy of RNA silencing (Rajagopal *et al.,* 2002; Chen *et al.,* 2008; Kumar *et al.,* 2009; Tian *et al.,* 2009; Zhu *et al.,* 2012; Wang *et al.,* 2013). With this knowledge, we attempted to explore the gene silencing and identification of possible potential target genes in *H. armigera*, which in future could be utilized for pest control. In the present study, the efficacy of RNA silencing for various *H. armigera* enzymes/proteins was evaluated by feeding sequence-specific dsRNA along with AD. The class of target genes selected here represented varied functions such as digestion, detoxification, metamorphosis and lipid transport etc (**Table 1**); which is one of the key factors influencing the silencing effect. This study showed that dsRNA delivery through AD induces specific gene silencing upon multiple doses of dsRNA. Ingestion of dsRNA is a natural, non-invasive and convenient method and thus can be preferred to induce

silencing in *H. armigera*. We observed that, out of 15 target genes most were effectively silenced upon systemic dosing of dsRNA. Interestingly, we observed that many of the selected genes are showing efficient silencing leading to abnormal phenotypes at pupal and moth stages of dsRNA fed larvae. In these esterases i.e. acetylcholinesterase (*HaAce4*) and juvenile hormone esterase (*HaJHE*) were found to be most effective showing highest mortality and severe phenotypic abnormalities (pupal head, curly wings in moth).

Similar observations were earlier shown in different insect by Kumar *et al.,* 2009; He *et al.,* 2012; Dimitrios *et al.,* 2013. By comparing insect phenotype **Fig. 4.3** and **4.4 (A, B, C,** and **D)** and **4.6**, we can conclude that the effect of silencing is variable even though the dsRNA concentration and larval stage used for feeding were same in each group. Most of the observations are correlating in terms of reduction in larval mass gain, percentage mortality and phenotypic abnormalities. However, in certain cases the phenotype is visible but there is no significant change in mass gain and transcript abundance 48 h post dsRNA feeding. These observations might suggest that fed dose of dsRNA is effective in inducing silencing but the effect is differential based on the type of target genes and the insect's developmental stages. One of the noticeable finding was that the retardation of growth in few dsRNA fed larvae was seen to restore while few of them already suffered silencing and developed abnormal phenotypes later. In parallel, droplet feeding method was also evaluated for silencing two of the selected genes but no significant effect on retardation of growth or cognate mRNA expression was observed. However, in droplet feeding experiments, 1 µg of dsRNA showed significant downregulation of *HaTry7,* whereas *HaTry2* was not affected. There has been previous report describing the variable efficacy of silencing upon dsRNA feeding through droplet, which also varies according to insect system (Turner *et al.,* 2006). In order to obtain potent and specific silencing, there is need to optimize the dose of target gene and dsRNA delivery method in an insect system.

Specifically it has been observed that less evolved insects show robust systemic RNAi response inheritable via germ-line transmission (Bucher *et al.,* 2002; Lynch and Desplan, 2006); while more evolved insects like Lepidopterans appear to be refractory to systemic RNAi (Terenius *et al.,* 2011). Although the suppression of mRNA was not

observed 8 days post dsRNA feeding, there was significant reduction in GST and trypsin-like activities. This indicated that translation of target gene was influenced by silencing. The silencing effect at biochemical level was confirmed by observing the native PAGE profile of AD and AD+JHE dsRNA fed larval proteins with fluorescently labeled azadirachtin. This data gives additional evidence to support our earlier observations. In summary it can be proposed that chosen candidate genes might provide suitable potential target gene for *H. armigera* control after its extensive validation. It is apparent from this study that depending upon the function of gene and its regulatory mechanism, the silencing effect was variable. However, high through put screens would be more useful in deciding the target genes; for many insects this remains a key question to choose the target genes for silencing. The basis of selection becomes challenging as it is essential to consider the roles of genes.

In insects, research on RNAi has largely focused on the non-cell autonomous (environmental and systemic) RNAi response. Most investigations of RNAi in insects have involved delivery of *in vitro* synthesized dsRNAs into embryos or the hemocoel by microinjection. However, microinjection is not a useful means to deliver dsRNA for pest control. Further, the potential utility of RNAi for insect pest control demonstrated that the systemic RNAi can be induced in insects by oral administration of dsRNA (Araujo *et al.,* 2006; Turner *et al.,* 2006). In the later study third instar larvae of the light brown apple moth *Epiphyas postvittana* were fed dsRNAs targeting transcripts encoding a larval gut enzyme and a pheromone binding protein (PBP) in adult antennae. It was found that PBP transcript levels were significantly reduced in adult moths and indicated that the ingested dsRNA was taken up by larval midgut cells and mobilized to cells in the eye/antennal disc, where it was persisted for at least 18 days. Recently Sapountzis *et al.,* (2014) reported that gene expression knockdown effect in each single body compartment was dependent on the administration method used and showed clear relationship between gene knockdown and body compartment-specific RNAi response. RNAi studies of non-model insects, involved use of dsRNA produced through *in vitro* enzymatic reverse transcription or chemical synthesis. However, this is unrealistic for field application for pest control.

A recent study establishes methodological advances that address the above limitations by combining high throughput genome-wide searching for candidate target genes and screening for optimal ones with bioassays (Wang *et al.*, 2011). Currently good quality full genome sequences are available for only a few pests of agricultural significance (such as the red flour beetle *T. castaneum* and the pea aphid *A. pisum*).While in 2011 the i5k initiative was launched with the objective of sequencing the genomes of 5000 insect and related arthropod species (species known to be important to worldwide agriculture, food safety, medicine, and energy production) over the following 5 years (http://arthropodgenomes.org/ wiki/i5K) (Gu *et al.*, 2013). Such comprehensive genomic information will not only facilitate the selection of the most desirable targets, but will also ensure the specificity and potency of RNAi in pest control. Genomic information will also permit cross-referencing among ecologically interacting species such as predators and natural enemies so as to avoid off-target effects. Furthermore, the above mentioned methods of RNAi induction, including topical application of dsRNA, bacteria or plant virus based RNAi systems, are all amenable to streamlined high throughput screening. Most desirable feature of RNAi approaches for crop protection is the exquisite selectivity of RNAi. This selectivity can be exploited to devise RNAi-based pest management strategies that have no effect on non-target species, thus permitting their integration into existing integrated pest management programs.

Development of pest management strategies based on RNAi must take into consideration potential pitfalls and limitations, most notably, the ability of a pest species to develop resistance to an RNAi. Such an example has been shown, where the ability of a dsRNA to produce a useful phenotypic effect could be overcome by sequence polymorphisms in the target gene of a pest (Gordon and Waterhouse, 2007). It is therefore imperative to evaluate the extent of sequence polymorphism in specific target genes in pest populations. Price and Gatehouse (2008) recently reviewed that another biochemical pathway or a paralogous gene with partially overlapping function could compensate for the loss of function of an RNAi-induced phenotype. The potential to develop this type of resistance can be minimized by careful design of dsRNAs targeting the expression of well understood target genes. Other strategies to minimize the possibility of resistance development and to enhance the efficacy of RNAi include

employment of combinations of dsRNAs targeting different genes and the use of more than one delivery method. Although understanding of RNAi in insects is still limited, recent advances suggest that this field holds outstanding promise for developing a new generation of pest control tools.

## 4.5 Conclusion

Feeding dsRNA along with AD was found to induce silencing of cognate mRNAs in *H. armigera*. Effective dose was found to be 60 µg of dsRNA; however, the silencing efficacy was variable based upon the type of target gene. Retardation of growth was observed in dsRNA fed larva as compared to that of control. In most of dsRNA fed larvae the silencing was found to be prominent leading to malformed pupa and adult of *H. armigera*. Significant downregulation of target transcripts was observed 2 days post dRNA feeding as compared to control. However, the target transcripts were found to reinstate at 8 days while some of dsRNA fed larvae suffered the silencing effect and developed into abnormal phenotypes. Gene silencing was also observed at biochemical level; where we checked the native profile of AD fed and *HaJHE* dsRNA fed larval proteins in presence of fluroscently labeled azadirachtin. Interestingly JHE silenced insect protein showed weak fluorescene unlike AD fed larval protein indicating the unavailability of JHE for interaction with azadirachtin. In summary the chosen candidate genes might serve potential and specific targets for *H. armigera* control; after their precise validation.

# Chapter 5

## General Discussion and Future Directions

# Chapter 5

## General discussion and future directions

Polyphagous insect pests such as *H. armigera* represent one of the most important biotic stresses influencing crop productivity. Several chemical pesticides and biotechnological approaches such as transgenic Bt cotton are used for its control, but the absence of resistance to *H. armigera* in host plants, the lack of adequate control measures, and the resurgence of pesticide/toxin resistance together make field management of this pest challenging. These synthetic toxins are highly hazardous to human health and our ecosystem. *H. armigera* being polyphagous is highly adaptive to the kind of ingested food and has to deal with several host plant natural toxins and synthetic insecticides used in agriculture. The adaptation and inactivation mechanisms in this pest are progressively making it resistant to existing as well as newly developed pesticides, rendering them ineffective. Moreover the insect is spreading to previously non-host plants, thus expanding its damage impact zone. The damage by this pest from different crops now ranges from 50 to 90% making it the most important yield reducer.

In this respect previous studies were done to understand diversity of digestive proteases and gut complement complexity and their role in adaptation of *H. armigera* to various host plants. With this background, several questions as detailed in "Genesis of thesis" were tried to address in the present work as follows: differential protease activity augments polyphagy in *H. armigera;* molecular responses of *H. armigera* upon exposure to host and non-host plants, and synthetic pesticide; silencing of candidate genes identified from above studies in *H. armigera* depicts their role in insect growth and development. The present study have shed light into some of interesting aspects of polyphagy and adaptability in *H. armigera* and feasibility of gene silencing, and has further raised questions on design and development of potent dsRNA strategy for *H. armigera* control.

## 5.1 Multifaceted proteases offer bio-potency for polyphagy in *H. armigera*

To understand the role of diverse *H. armigera* serine proteases in polyphagy, feeding bioassays were performed on distinct host plants and biochemical and molecular responses of insect with respect to serine proteases were studied. Phylogenetic analyses of *H. armigera* serine proteases revealed that they are highly homologous sequences with few diverged variants. Multiple sequence alignment of particularly, *HaTry* and *HaChy* isoforms showed that few of the isoforms have gained synonymous substitutions at their active site residues. Such sequence diversity might impart differential functionality to variant isoforms of *HaTry* and *HaChy*. During this study on the basis of their percent similarity representative 8 *HaTry* and 4 *HaChy* isoforms were selected.

The present study showed that trypsin and chymotrypsin isoforms of *H. armigera* are under dynamic flux and that they switch their functional specificity according to diet and insect developmental stage. Further study confirms that, in the larval stage, which is the important assimilatory phase in an insect's life cycle, specific trypsin expression patterns are observed (Chougule *et al*., 2005). High accumulation of *HaTry8* and *2* at the pupal stage suggested that these isoforms might be involved in the developmental processes leading to the transition from larvae to pupae. Moreover, *HaTry*s might have an alternative functional role as a preparatory reserve for further developmental stages. Adults exhibited a thousand-fold down-regulation of *HaTry* isoforms compared with larvae and ten-fold compared with pupae, except *HaTry2*, irrespective of the diet on which the insects were raised. This indicates that *HaTry2* might play a major role in the metamorphosis from pupae to adult and maintenance of adult stage digestive processes. Expression of chymotrypsin isoforms was found to be developmental stage-specific, rather than being diet-specific as with trypsins. The pattern observed for chymotrypsins was like that of *HaChy4* expression, dominating in the larval stage, with *HaChy1* in the pupal and *HaChy3* in the adult stages. This expression pattern indicates that chymotrypsins might have fundamental roles in immunity, development and metamorphosis (Terra and Ferreira, 1994; Borovsky and Mahmood, 1995). The complexity in *HaTry* and *HaChy* expression also indicated that larval gut proteases are

dynamically regulated. Doing so insect evolves strength to tackle plant defensive proteins/antifeedants and metabolites from previously non-preferred host plant and thereby increase their diet-breadth.

Signaling mechanisms that govern the differential regulation of protease genes in insects are not well understood and thus the molecular basis of larval responses remains enigmatic. In some Lepidopteran species it is known that certain neuropeptides act as regulatory switches governing digestive protease expression (Harshini *et al*., 2002; Huang *et al*., 2010). In *H. armigera*, flexibility in digestive proteases and dynamic gene expression was observed when insects were fed with PIs and/or heterogeneous plant metabolites, which reflect the evolving breadth of diet in polyphytophagous Lepidoptera (Gatehouse *et al*., 1997). The gut protease activity of *H. armigera* fed on pigeonpea (high-protein diet) and rose (low-protein diet) differed quantitatively and qualitatively: total proteolytic activity was higher in larvae fed on pigeonpea than in larvae fed on rose. Several distinct protease activity isoforms were detected in the gut when larvae were fed on nutritionally diverse diets such as rose, pigeonpea, okra and maize, suggesting specific protease isoforms were involved in adaptive metabolism.

It was intriguing observation that relatively *HaTry* expression was highest in rose fed larvae than those fed with other diets in present study. The possible reason for such expression pattern could be that even though the protein content is low in rose, composition might be complex. Therefore, rose fed larvae is utilizing most of the protease isoforms to derive the required nutrition out of available proteins. However, in case of pigeonpea fed larvae the ability of larvae to digest the available protein could be efficient with comparatively lesser number of protease isoforms. The difference in inhibition of protease isoforms from pigeonpea and rose –HGP by rCanPIs and synthetic proteinase inhibitors indicated the differential sensitivity of proteases to inhibitors, stability of protease-PI complexes. It is found that, these substitutions of amino acid residues play a major role by influencing sensitivity of proteases to PIs (Bown *et al*., 1997, Dunse *et al*. 2010b). Structural comparison of predicted structures of *H. armigera* trypsins indicated differences in active site, S1 binding pocket and distal loops in *HaTry4*, *HaTry1*, *HaTry3*, *HaTry6* and *HaTry8*. These structural changes may result in different substrate specificity. However, loops at the surface of the protease might play a

role in substrate discrimination and rate of catalysis (Hedstrom *et al*., 1992). Comparison of the free energy of different trypsin-substrate/inhibitor interactions and their comparison using heat map analysis provided insights into the structure-function relationship in *H. armigera* gut proteases. Thus the present study provides enhanced understanding of the dynamics of the *H. armigera* gut physiology, especially digestive proteases, when the insects are fed on diverse natural host plants. However, important question arises here, about complex regulatory mechanisms of serine protease function and their diverse functionality. Similarly, comparative study of *H. armigera* and other Lepidopteran major pests will provide the possible evolutionary relationships amongst serine proteases and their importance for pest's dynamic physiology. Knowing this will be beneficial to design effective strategies to tackle polyphagous insect pests.

## 5.2 Adaptability of *H. armigera* against plant anti-feedants

In order to identify key proteins or enzymes involved in adaptability of *H. armigera*, we studied molecular responses of larvae fed on host chickpea (Cp) and non-host *Cassia tora* (Ct) diets. The study demonstrated the efficacy of *C. tora* seeds in retarding growth of *H. armigera*. The anti-feedant effect of Ct diet was evident from reduction in mass gain of Ct fed larvae as compared to that of Cp fed larvae. Moreover, altered morphologies of pupae and stunted growth were observed in Ct fed larvae. We checked for inhibitory potential of *C. tora* PIs and found that these PI's showed relatively stronger inhibition of HGPs than those of chickpea PIs. However, it has been reported that the insects develop resistance to chickpea PIs by modulating the protease specificity and overexpressing PI-insensitive proteases (Giri *et al.,* 1998; Telang *et al.,* 2005). Phytochemicals detoxification mechanisms in insects are documented and majorly detoxification enzymes are known to be involved in metabolism of phytochemicals (Agosin *et al.,* 1985; Oppenoorth *et al.,* 1985; Pickett *et al.,* 1989). Particularly, polyphagous insects can selectively express a broad range of enzymes that assist in digesting xenobiotics including secondary metabolites from plants (Li *et al.,* 2000). In our study *C. tora* seed metabolites and PIs together might have inhibited the activity of several crucial enzymes, which consequently resulted into retardation of growth and development of *H. armigera*. Furthermore, results suggested that adaptation of *H.*

*armigera* to one group of PIs or plant secondary metabolites does not mean insensitivity to other PIs or secondary metabolites. A specific inhibitor and metabolite combination would significantly delay the growth and generation advance of *H. armigera* in the field.

Effect of Ct diet on *H. armigera* was analyzed at molecular level by using comparative proteomic approach to understand proteomic differences in gut, heamolymph and frass. Proteins identified in *H. armigera* gut mainly functioned in digestion, energy metabolism and defense (detoxification of phytochemical compounds). Specifically expression of trypsin, β-1, 3-glucanase and P450 were down-regulated in gut of Ct fed larvae, while myosin 1 heavy chain and lipase were up-regulated. Among these proteins, serine proteases, β-1, 3-glucanase and lipase are reported to be involved in digestion whereas P450 is involved in oxidation of various toxic substances. The decreased expression of these enzymes might be due to their inhibition by metabolites or proteins present in Ct diet. In addition, down-regulation of heat shock protein in Ct fed larvae probably suggests its inability to overcome the stress. Nonetheless, energy metabolism related proteins viz. very high-density lipoprotein, ATP synthase and sterol carrier proteins were up-regulated in Ct-fed larvae and might serve in overcoming the effects. These proteins might be involved in maintaining the steady energy levels under stress conditions. GST, imaginal disk growth factor and cationic protein play vital roles in the immune system of insects due to their antimicrobial property (Francis *et al.,* 2005; Hariharan *et al.,* 2006). GST and cationic protein processes were involved in the development or functioning of the immune system. Imaginal disk growth factor is reported to increase the mass of imaginal discs which further develop into adult structures (legs, antennae, wings etc.) in holometabolous larvae during metamorphosis. These proteins were significantly down-regulated in Ct fed *H. armigera* hemolymph. Although most of the above enzymes were downregulated in Ct fed larvae; thus larvae was struggling to adapt and overcome the anti-nutritive effects of non-host phytochemicals by upregulating key proteins involved in energy metabolism, adaptation, signaling and immunity. OBP (odorant binding protein) was up regulated in Ct fed larvae. Gene Ontology (GO) studies showed that OBP interacted selectively and non-covalently with major histocompatibility complex molecules. Up-regulation of OBP in Ct-fed larvae indicated its major role in seeking host food by odor reception and differentiation.

The proteomic analysis reported herein shows that the insect frass is enriched in defense-related proteins. Aminopeptidase, lipase and β-1, 3 glucanase were the most abundant proteins found in frass of Ct fed larvae. For example, β-1, 3-glucanase was found to be present in the gut as well as frass of Ct fed larvae, but this enzyme was down regulated in gut while up-regulated (12-fold) in frass. All these observations suggested that there is an enigma of the actual role of β-1, 3-glucanase, and could be either involved in immunity or digestive functions. P450 is well documented in several herbivores for the metabolism of a broad range of plant secondary metabolites (Cohen *et al.,* 1992). Gut proteases and P450 were found to be down-regulated in Ct-fed larvae. This could be a reason for stunted growth, low survival rate and inability of larvae to adapt to Ct diet. GST activity in hemolymph of Ct-fed larvae was downregulated compared to that of Cp-fed larvae. The retardation of GST activity might be due to difference in allelochemicals of non-host diet and inefficiency of their metabolism by insect. Proteases (trypsin, chymotrypsin, aminopeptidase, carboxypeptidase, etc) and GST played a vital role in food digestion and immunity to a certain extent, and metabolism of toxic compounds in insects, respectively (Cohen *et al.,* 1992).

Insects are believed to be self-protected from the toxicity due to the presence of GST and P450 (Zheng *et al.,* 1994). However, the biological and physiological role of different diets in insect physiology has long been a mystery. The expression profiles of selected candidate genes confirm the biochemical and proteomics findings, except for certain isoforms of trypsins, chymotrypsins, aminopeptidases, carboxypeptidases and GSTs (Harsulkar *et al.,* 1999; more references covering other than serine proteases and GSTs). Many isoforms of trypsin and chymotrypsin are known to be involved in digestive processes in Lepidopteran pests (Purcell *et al.,* 1992). It is clear that there is tremendous complexity in trypsin and chymotrypsin expression, suggesting their role in building the adaptive response of the insect against non-host diet (Harsulkar *et al.,* 1999; Telang *et al.,* 2005). Trypsin-like proteases play a predominant role in digestion as well as metamorphosis and to some extent in immunity, as evident from the expression studies and insect performance (Ref). Diverse isoforms of trypsins and chymotrypsins enhance the digestive flexibility of the insects. These proteases act under cascade and are tightly regulated exhibiting a switch in expression. In addition to this, GST and P450 maintained

the metabolism of the insects and their survival. This study demonstrated the insect's performance on host and non-host diets, and the key factors underlying the adaptive response; their specific protein and transcript flux involved in priming the insect against non-host diet components.

## 5.3 *H. armigera* detoxifies synthetic pesticide by complex biochemical pathway

Molecular responses of *H. armigera* against a synthetic pesticide chlorpyrifos was studied by using comparative biochemical and proteomic approach and possible chlorpyrifos detoxification pathway was derived. The gut proteome of *H. armigera* fed on CH diet showed significant up-regulation of plethora of enzymes. Biochemical data of candidate proteins such as proteases, CYP, Arg kinase indicated that insects are struggling to obtain required nutrients and energy in presence of CH at the same time endeavoring to metabolize CH, which was also evident from gene expression profiles (Dawkar *et al*., 2011; Chikate *et al*., 2013). Furthermore, digestive enzymes *viz*. trypsin and chymotrypsin-like proteases found to be significantly up-regulated in CH fed larvae, although the growth of larvae was retarded. It is known that over expression of proteases is costly, and thus growth and development of larvae is severely affected (Broadway, 1995). Generally, polyphagous insects can selectively express a broad range of enzymes that assist in the detoxification or neutralization of numerous natural and synthetic toxins (Li *et al*., 2004; Sharma *et al*., 2004). Being polyphagous, *H. armigera* has dynamic ability to survive on several hosts and neutralize effect of various deterrents/toxicants. Its ability to metabolize and detoxify has rapidly acquired resistances to synthetic insecticides and natural plant toxins. In this scenario, one example is resistance to CH, which has been documented in India, Pakistan, Egypt and in many other countries. The present data suggest that in *H. armigera* not only detoxification related enzymes but also enzymes involved in digestion and energy metabolism mechanism play important role in adaptation. For example, biosynthesis, metabolism, transport, detoxification and digestion related proteins were found to be up-regulated in CH fed larvae. Elongation factor, glycogen synthase, ATP synthase, V ATPase-A, DNA metabolism protein,

Vacuolar V-type proteins are known to be involved in metabolic processes and was up-regulated in CH fed insects. Other highly expressed proteins *viz*. fatty acid-binding protein, acetyltransferase, very high density lipoprotein are reported for transport mechanism. Moreover, serine protease, aminopeptidase are involved in digestion; cryptochrome in DNA repair; heat shock protein played vital role during stress conditions; arginine kinase in phosphorylation; Juvenile hormone esterase are essential sesquiterpenes that control insect development and reproduction. All the above mentioned enzymes were up-regulated in CH fed larvae.

Particularly, cytochrome P450 monooxygenases play a significant role in the detoxification of host plant allelochemicals and synthetic insecticides in Lepidoptera (Sasabe *et al*., 2004). It has been widely known that CYP families are capable of metabolizing insecticides. Insect's each line of defense is capable of metabolizing diverse compounds contributing enhanced resistance/tolerance of insects to toxic allelochemicals and insecticides; this in turn provides a competitive advantage to the generalist insect. "Endurance", the term used by Gordon (1961), fits with the current study. The "endurance" factor may be important in lowering the susceptibility of larvae and as they grow larger, they become more resistant. Under severe selection pressure, polyphagous holometabolous insects often show extraordinarily high and generalized tolerance to insecticides (Candas *et al*., 2003; Heckel, 2012; Stevens *et al*., 2013). Another study showed that *H. armigera* midgut lumen proteome was diverse and complex suggesting high adaptability to different toxins (Pauchet *et al*., 2008). It has been important to investigate basis of insecticide processing and/ resistance in insects. Since long there is an argument for an evolutionary relationship between allelochemical-inducible CYP genes and insecticide-metabolizing P450 sequences. Here, we report that the toxicity of organophosphate insecticides can be altered by regulating enzymes involved in metabolism of CH. CH possess a P=S group, which could be activated by CYP to the oxon (P=O) enhancing the inhibition of acetylcholinesterase (AChE) (Chambers and Carr, 1995). In accordance with these reports we propose the possible CH metabolizing pathway in *H. armigera*.

Even though many vital enzymes are highly expressed in *H. armigera* upon exposure to CH, larvae showed growth retardation and here we may get the information

of genesis of resistance in *H. armigera*. In response to CH, *H. armigera* larvae upregulates cocktail of detoxification enzymes, thia subsequently may lead to sequential breakdown of chlorpyrifos. Enzymes involved in energy metabolism (arginine kinase), digestion (serine protease), transport of fatty acid (fatty acid binding protein) etc. were significantly upregulated in larvae fed on CH diet, suggesting their role in stress and possibly metabolizing insecticide molecule. The possible detoxification of chlorpyrifos in *H. armigera* might be by CYP and carboxylesterases; these enzymes might impart potential adaptive capability to insect against insecticides through evolution. The present study therefore, offers an added interest to understand the adaptive functionality of proteins in *H. armigera* upon exposure to organophosphate like CH.

## 5.4 Potential of RNAi approach for developing strategies for *H. armigera* control

The efficacy of RNA silencing for various *H. armigera* enzymes/proteins was evaluated by feeding sequence-specific dsRNA along with AD. The class of target genes selected here represented varied functions such as digestion, detoxification, metamorphosis and lipid transport etc; which is one of the key factors influencing the silencing effect. This study showed that dsRNA delivery through AD induces specific gene silencing upon multiple doses of dsRNA. It suggested that ingestion could be potential delivery method to induce RNAi in *H. armigera* and offers added advantage of natural, non-invasive and convenient method. We observed that out of 15 target genes most of them were effectively silenced upon systemic dosing of dsRNA. Parallely, droplet feeding method was also evaluated for silencing for two of the selected genes but no significant effect on retardation of growth or cognate mRNA expression was observed. However, in droplet feeding experiments, 1 µg of dsRNA was supplied to larvae and significant downregulation of *HaTry7* was observed, whereas *HaTry2* was not affected. There has been previous report describing the variable efficacy of silencing upon dsRNA feeding through droplet, which also varies according to insect system (Turner *et al.*, 2006).

Till date many of the studies are being done in insects to explore RNA silencing to develop efficient pest control strategy and successes of which are variable (Terenius *et*

*al.,* 2011). Particularly, Lepidopteran insects are known to be relatively impervious to dsRNA induced silencing, though, few studies are described for the variable efficacy of RNA silencing (Rajagopal *et al.,* 2002; Chen *et al.,* 2008; Kumar *et al.,* 2009; Tian *et al.,* 2009; Zhu *et al.,* 2012; Wang *et al.,* 2013). Interestingly, we observed that many of the selected genes are showing efficient silencing leading to abnormal phenotypes at pupal and moth stages of dsRNA fed larvae. In these esterases i.e. acetylcholinesterase (*HaAce4*) and juvenile hormone esterase (*HaJHE*) were found to be most effective showing highest mortality and severe phenotypic abnormalities (pupal head, curly wings in moth). Similar observations were earlier shown in different insects (Kumar *et al.,* 2009; He *et al.,* 2012; Kontogiannatos *et al.,* 2013;). We can conclude that the effect of silencing is variable even though the dsRNA concentration and larval stage used for feeding were same in each group. Most of the observations are correlating in terms of reduction in larval mass gain, percentage mortality and phenotypic abnormalities. However, in certain cases the phenotype is visible but there is no significant change in mass gain and transcript abundance after 48 h post dsRNA feeding. These observations might suggest that fed dose of dsRNA is effective in inducing silencing but the effect is differential based on the type of target genes and the insect's developmental stages. One of the noticeable finding was that the retardation of growth in few dsRNA fed larvae was seen to restore while few of them already suffered silencing and developed abnormal phenotypes later.

Specifically it has been observed that less evolved insects show robust systemic RNAi response inheritable *via* germ-line transmission (Bucher *et al.,* 2002; Lynch and Desplan, 2006); while more evolved insects like Lepidopterans appear to be refractory to systemic RNAi (Terenius *et al.,* 2011). Although the suppression of mRNA was not observed 8 days post dsRNA feeding, there was significant reduction in GST and trypsin-like activities. This indicated that silencing is induced at translational level also. The silencing effect at biochemical level was confirmed by observing the native PAGE profile of AD and AD+JHE dsRNA fed larval proteins with fluorescently labeled azadirachtin. There was remarkable difference in fluorescence intensity amongst the larval proteins from AD fed larvae as compared to AD+JHE dsRNA fed larvae. Fluorescence signal from AD fed larval proteins was stronger than that of AD+JHE dsRNA larval proteins.

This data gives an additional evidence of the silencing at translational level. It is apparent from this study that depending upon the function of gene and its regulatory mechanism, the silencing effect was variable. Development of pest management strategies based on RNAi must take into consideration potential pitfalls and limitations, most notably, the ability of a pest species to develop resistance to RNAi. Such an example has been shown, where the ability of a dsRNA to produce a useful phenotypic effect could be overcome by sequence polymorphisms in the target gene of a pest (Gordon and Waterhouse, 2007). Price and Gatehouse (2008) reviewed that another biochemical pathway or a paralogous gene with partially overlapping function could compensate for the loss of function of an RNAi-induced phenotype. The potential to develop this type of resistance can be minimized by careful design of dsRNAs targeting the expression of well understood target genes. Other strategies to minimize the possibility of resistance development and to enhance the efficacy of RNAi include employment of combinations of dsRNAs targeting different genes and the use of more than one delivery method. Although understanding of RNAi in insects is still limited, recent advances suggest that this field holds outstanding promise for developing a new generation of pest control tools. As per the discussion stated above, following important leads from this study could be further pursued to investigate finer details about *H. armigera* polyphagy and adaptability, and thereby develop efficient pest control strategy.

## 5.5 Future Directions:

i. Understanding explicit details of complexity and flexibility of *H. armigera* proteases and other factors involved in its adaptation and surveillance

ii. Exploring complex regulatory mechanisms underlying diverse serine proteases function and their flexibility

iii. Use of established gene silencing protocol and its feasibility to screen more potential targets for species-specific RNAi

iv. Validation of identified potent species specific dsRNA for *H. armigera* control

v. Design of generic guidelines for use of dsRNA to develop delivery vehicles or formulations.

vi. Identified target genes (dsRNA) could be applied for insect pest control individually or synergistically through *in planta* expression.

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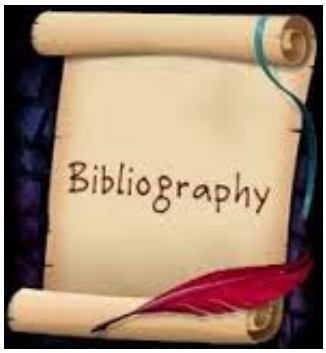


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# APPENDIX I

**_Helicoverpa_ chymotrypsin Sequences Homologous to _Agortis_ Sequence AAF71516 showing the mapped peptides**

```
gi|297340770|gb|ADI32883.1|        MKFVALTLLALAAVASARNVHLEDSIDLEDITAWGYLTKFGIPEAEKIRS 50
gi|297340768|gb|ADI32882.1|        MKFVALTLLALAAVASARNVHLEDSIDLEDITAWGYLTKFGIPEAEKIRS 50
gi|297340764|gb|ADI32880.1|        MKFVALTLLALAAVASARNVHLEDSIDLEDITAWGYLTKFGIPEAEKIRS 50
gi|282721216|gb|ADA83701.1|        MKFVALTLLALAAVASARNVHLEDSIDPEDITAWGYLTKFGIPEAEKIRS 50
gi|297340766|gb|ADI32881.1|        MKFVALTLLALAAVASARNVHLEDSIDLEDITAWGYLTKFGIPEAEKIRG 50
gi|171740891|gb|ACB54940.1|        MKFVALTLLALAAVASARNVHLEDSIDLEDITAWGYLTKFGIPEAEKIRN 50
gi|54310844|gb|AAV33658.1|         ------------------VHLEDSIDLEDITAWGYLTKFGIPEAEKIRN 31
gi|8037817|gb|AAF71516.1|          MKFLALALLALTVFASAKHVSFEDAIDLEDITAYGYLSKIGAPLAEKIRQ 50
                                                * :**:** *****:***:*:* * *****

gi|297340770|gb|ADI32883.1|        AEEAS-SASRIVGGSLSSLGQIPYQAGLVIDLSGGQAVCGGSLISASRVL 99
gi|297340768|gb|ADI32882.1|        AEEAS-SASRIVGGSLSSLGQIPYQAGLVIDLSGGQAVCGGSLISASRVL 99
gi|297340764|gb|ADI32880.1|        AEEAS-SASRIVGGSLSSLGQIPYQAGLVIDLSGGQAVCGGSLISASRVL 99
gi|282721216|gb|ADA83701.1|        AEEAS-SASRIVGGSLSSLGQIPYQAGLVIDLSGGQAVCGGSLISASRVL 99
gi|297340766|gb|ADI32881.1|        AEEAS-SASRIVGGSLSSLGQIPYQAGLVIDLSGGQAVCGGSLISASRVL 99
gi|171740891|gb|ACB54940.1|        AEEAS-SASRIVGGSLSSLGQIPYQAGLVIDLSGGQAVCGGSLISASRVL 99
gi|54310844|gb|AAV33658.1|         AEEAS-SASRIVGGSLSSVGQIPYQAGLVIDLAGGQAVCGGSLISASRVL 80
gi|8037817|gb|AAF71516.1|          AEEQQDSHVRIVGGSLSSLGQFPHQAGLLTQFAGGQGVCGGSLIKANRVV 100
                                   *** . *  *********:**:*:****: :::***.*******.*.**:

gi|297340770|gb|ADI32883.1|        TAAHCWFDGQNQAWRFTVVLGSTTLFSGGTRIATSNVVMHGSWTPSLIRN 149
gi|297340768|gb|ADI32882.1|        TAAHCWFDGQNQAWRFTVVLGSTTLFSGGTRIATSNVVMHGSWTPSLIRN 149
gi|297340764|gb|ADI32880.1|        TAAHCWFDGQNQAWRFTVVLGSTTLFSGGTRIATSNVVMHGSWTPSLIRN 149
gi|282721216|gb|ADA83701.1|        TAAHCWFDGQNQAWRFTVVLGSTTLFSGGTRIATSNVVMHGSWTPSLIRN 149
gi|297340766|gb|ADI32881.1|        TAAHCWFDGRNQAWRFTVVLGSTTLFSGGTRIATSNVVMHGSWTPSLIRN 149
gi|171740891|gb|ACB54940.1|        TAAHCWFDGQNQAWRFTVVLGSTTLFSGGTRIATSNVVMHGSWTPSLIRN 149
gi|54310844|gb|AAV33658.1|         TAAHCWFDGQNQAWRFTVVLGSTTLFSGGTRIPTSNVVMHGSWTPSLIRN 130
gi|8037817|gb|AAF71516.1|          TAAHCWFDGQNQGRSVIVVLGSVNLFSGGNRQTSTNIVMHGSWNPSLIRN 150
                                   *********:**.  . *****..*****.* .::*:******.******

gi|297340770|gb|ADI32883.1|        DVAVIRLGTNVGTSNTIAIIALPSGSQINENFAGETALASGFGLTSDSGS 199
gi|297340768|gb|ADI32882.1|        DVAVIRLGTNVGTSNTIAIIALPSGSQINENFAGETALASGFGLTSDSGS 199
gi|297340764|gb|ADI32880.1|        DVAVIRLGTNVGTSNTIAIIALPSGSQINENFAGETALASGFGLTSDSGS 199
gi|282721216|gb|ADA83701.1|        DVAVIRLGTNVGTSNTIAIIALPSGSQINENFAGETALASGFGLTSDSGS 199
gi|297340766|gb|ADI32881.1|        DVAVIRLGTNVGTSNTIAIIALPSGSQINENFAGETALASGFGLTSDSGS 199
gi|171740891|gb|ACB54940.1|        DVAVIRLGTNVGTSNTIAIIALPSGSQINENFAGETALASGFGLTSDSGS 199
gi|54310844|gb|AAV33658.1|         DVAVIRLGTNVATSNTIAIIALPSGSQINENFAGETALASGFGLTSDTGS 180
gi|8037817|gb|AAF71516.1|          DVAVIRIN-NVGLNNNINVIPLATGS--ESYAGENAVASGFGLTRDGGS 196
                                   ******:. **. .*.* :*.*.:**   *.:***.*:******* * **

gi|297340770|gb|ADI32883.1|        ISSNQALSHVNLPVFTNAVCRSSFPLLIQDSNICTSGANGRSTCRGDSGG 249
gi|297340768|gb|ADI32882.1|        ISSNQALSHVNLPVITNAVCRSSFPLLIQDSNICTSGANGRSTCRGDSGG 249
gi|297340764|gb|ADI32880.1|        ISSNQALSHVNLPVITNAVCRSSFPLLIQDSNICTSGANGRSTCRGDSGG 249
gi|282721216|gb|ADA83701.1|        ISSNQALSHVNLPVITNAVCRSSFPLLIQDSNICTSGANGRSTCRGDSGG 249
gi|297340766|gb|ADI32881.1|        ISSNQALSHVNLPVITNAVCRSSFPLLIQDSNICTSGANGRSTCRGDSGG 249
gi|171740891|gb|ACB54940.1|        ISSNQALSHVNLPVITNAVCRSSFPLLIQDSNICTSGANGRSTCRGDSGG 249
gi|54310844|gb|AAV33658.1|         ISSNQALSHVNLPVITNAVCRNSFPLLIQDSNICTSGANGRSTCRGDSGG 230
gi|8037817|gb|AAF71516.1|          VSG--ALSHVTLPVITNAVCRSSFPLIIQDSNICVNGAGGRSTCQGDSGG 244
                                   :*.  *****.***:******.****:*******..**.*****:*****

gi|297340770|gb|ADI32883.1|        PLVVTRNSRPLLIGITSFGSARGCQVGSPAAFARVTSYISWINGQL 295
gi|297340768|gb|ADI32882.1|        PLVVTRNSRPLLIGITSFGSARGCQVGSPAAFARVTSYISWINGQL 295
gi|297340764|gb|ADI32880.1|        PLAVTRNSRPLLIGITSFGSARGCQVGSPAAFARVTSYISWINGQL 295
gi|282721216|gb|ADA83701.1|        PLAVTRNSRPLLIGITSFGSARGCQVGSPAAFARVTSYISWINGQL 295
gi|297340766|gb|ADI32881.1|        PLVVTRNSRPLLIGITSFGSARGCQVGSPAAFARVTSYISWINGQL 295
gi|171740891|gb|ACB54940.1|        PLVVTRNSRPLLIGITSFGSARGCQVGSPAAFARVTSYISWINGQL 295
gi|54310844|gb|AAV33658.1|         PLVVTRNNRPLLIGITSFGSARGCQVGSPAAFARVTSYISWINGQL 276
gi|8037817|gb|AAF71516.1|          PLTVVRSGRPILIGITSFGSARGCQVGSPAAFARVTSFAAWINAQ- 289
                                   **.*.*..**:*************************: :***.*
```

**Accession nos. are described in the table below,**

| Accession | Protein name |
|---|---|
| **ADI32883.1** | chymotrypsin [Helicoverpa armigera] |
| **ADI32882.1** | chymotrypsin [Helicoverpa armigera] |
| **ADI32881.1** | chymotrypsin [Helicoverpa armigera] |
| **ADI32880.1** | chymotrypsin [Helicoverpa armigera] |
| **AAV33658.1** | chymotrypsinogen [Helicoverpa punctigera] |
| **ACB54940.1** | chymotrypsin [Helicoverpa armigera] |
| **ADA83701.1** | chymotrypsin [Helicoverpa armigera] |
| **AAF71516.1** | AiC5 chymotrypsinogen [Agrotis ipsilon] |

## Curriculum Vitae

**Yojana R. Chikate**

| | |
|---|---|
| Father & Mother | : Rohidas & Chaya |
| Date of Birth | : 16th March, 1987 |
| Gender | : Female |
| Place of Birth | : Kinhai (Satara) |
| Nationality | : Indian |
| Hobbies | : To play Hockey and Badminton, Captain of School Hockey team, Winner at Zonal level and Selected for State Level Hockey tournament. |

### EDUCATION

| | |
|---|---|
| **2009-2014** | **Ph.D. Biotechnology** *(Appear)*, CSIR-National Chemical Laboratory, University of Pune, under the supervision of Dr. Ashok P. Giri, topic entitled *"Development of efficient strategies for control of Lepidopteran insect pest"* |
| **2009** | **M.Sc. Biotechnology,** Vidya Pratishthan's Arts, Science and Commerce College, Baramati**,** University of Pune, India |
| **2007** | **B.Sc. Biotechnology,** Vidya Pratishthan's Arts, Science and Commerce College, Baramati, University of Pune, India. |

### EXPERTISE

**Technical Summary**

1. Techniques in Molecular biology and Biochemistry
2. Proteomics and Mass Spectrometry
3. Plant tissue Culture Techniques
4. Microbiological Techniques

**Area of Interest**

1. Plant insect Interactions
2. Molecular Biology and Biochemistry
3. Genomics and Proteomics
4. Plant Secondary Metabolic Pathways

### ACHIEVEMENTS

| | |
|---|---|
| **2009** | University topper in **M.Sc. Biotechnology**, University of Pune, India. |
| **2009** | Awardee for Department of Biotechnology, New Delhi, India fellowship in Biotechnology Eligibility Test. |

| | |
|---|---|
| **2009** | Qualified National Level Biotechnology Talent Search Exam (NLBTSE), organized by Pharma Helpline Foundation; Jaipur, Rajasthan. |
| **2008** | Awardee for **CSIR**-National Eligibility Test (JRF), India |

## EXPERIENCE

| | |
|---|---|
| **Feb 2012 to Oct 2012** | **Visiting Student at Weizmann Institute of Science** (Department of Plant Sciences), **Rehovot, Israel;** Studied glycoalkaloid metabolism pathway in solanaceous plants. |

## PUBLICATIONS

1. **Chikate Y R**, Dawkar V V, Tilak P V, Barbole R S, Gupta V S, Giri A P (2014) Interruption of *Helicoverpa armigera* development upon silencing of diverse genes by dsRNA feeding**. (Under review).**

2. Dawkar V V, **Chikate Y R**, More T H, Gupta V S, Giri A P (2014) The expression of proteins involved in digestion and detoxification are regulated in *Helicoverpa armigera* to cope up with chlorpyrifos insecticide. **Insect Science (In Press).**

3. Dawkar V V, **Chikate Y R,** Lomate P R, Dholakia B B, Gupta V S and Giri A P (2013) Molecular insights into detoxification mechanisms of field crop Lepidopteron insect pests. **Journal of Proteome Research 12, 4727−4737.**

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5. **Chikate Y R,** Tamhane V A, Joshi R S, Gupta V S and Giri A P (2013) Differential protease activity augments polyphagy in *Helicoverpa armigera.* **Insect Molecular Biology 22, 258–272.**

6. Dawkar V V, **Chikate Y R,** Gupta V S, Slade S E and Giri A P (2011) Assimilatory potential of *Helicoverpa armigera* reared on host (Chickpea) and non-host (*Cassia tora*) diets. **Journal of Proteome Research 10, 5128–5138.**

## REFERENCES


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