GENOMIC ANALYSIS OF THE GENES EXPRESSED IN THE EUROPEAN CORN BORER (*Ostrinia nubilalis*) GUT AND THEIR EXPRESSION RESPONSES TO BT TOXINS

by

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M.A., Northwest A&F University, China, 2001

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2011

Abstract

European corn borer, *Ostrinia nubilalis*, is one of the most destructive insect pests of corn in the Midwest corn belt of the United States. The crystal protein toxin (Cry1Ab) expressed by the bacterium, *Bacillus thuriginesis* (Bt), specifically targets *O. nubilalis* gut and functions as "stomach poison". Transgenic corn expressing Cry1Ab can effectively control *O. nubilalis* larval infestation. However, *O. nubilalis* has the potential to develop resistance to Bt toxins which prompts concerns that transgenic corn will lose its control efficacy. Previous studies found that *O. nubilalis* gut serine proteases and membrane proteins were involved in Bt toxicity and resistance. Therefore, this study was to identify and characterize gut transcripts potentially involved in Bt toxicity and resistance, and to compare their transcriptional responses to the ingestion of Cry1Ab protoxin and transgenic corn leaves expressing Cry1Ab toxin. We identified and characterized 34 cDNAs encoding putative trypsins, chymotrypsins, and trypsin- and chymotrypsin-like protease homologs from *O. nubilalis* gut-specific expressed sequence tags (ESTs). Blast and phylogenetic analysis of their deduced amino acid sequences indicated that 15 were putative trypsins belonging to Try-G2 and Try-G3 groups (none of them was grouped in Try-G1), another 15 were putative chymotrypsins in one large group (CTP-G1), and the remaining four were serine protease homologs in Try-G4 and CTP-G2 groups, respectively. The existence of diverse trypsins, chymotrypsins and serine protease homologs in *O. nubilalis* could be an adaptation to different food sources and also a defense mechanism against plant-specific protease inhibitors and Cry toxins from transgenic corn. The expressions of four putative trypsins (*OnTry4*, *OnTry5*, *OnTry6* and *OnTry14*) were up-regulated in *O. nubilalis* larvae after the ingestion of Cry1Ab protoxin. The differential expressions of these protease transcripts may implicate a link to Cry1Ab intoxication. To better understand the basic physiology of insect gut and Bt toxin interactions, we developed a high-resolution 8×15K cDNA microarray chips based on the larval gut specific ESTs. Each microarray contains 12,797 probes representing 2,895 unique larval gut transcripts. The expressions of 174 transcripts were differentially regulated at least 2-fold (P-value ≤ 0.05) after the larvae fed Cry1Ab protoxin for 6 hours. Among them, 13 transcripts, putatively encoding eight serine protease, three aminopeptidase, one alkaline phosphatase, and one cadherin-like protein, were identified and further validated their expression ratios by quantitative PCR (qPCR). Three trypsin transcripts were up-regulated by more than 5-

fold in larvae fed Cry1Ab protoxin. Sequence analysis suggests that they may have role in protoxin activation and toxin degradation. The transcriptional responses of laboratory-selected Cry1Ab resistant (R) and susceptible (S) strains of *O. nubilalis* to the ingestion of transgenic corn (MON811) leaves expressing Cry1Ab toxin were also examined. Even though R-strain larvae showed 200-fold resistance to Cry1Ab protoxin as compared with S strain, the larvae from both strains eventually died after fed transgenic corn leaves. However, the survival time of R-strain larvae was significantly different from that of S-strain larvae. The median lethal time (LT_{50}) for the early third-instar larvae of R- and S-strains were 5.4 and 3.6 days, respectively. Furthermore, we identified 398 and 264 transcripts from the larvae of the S and R strains, respectively, with a significantly increased or decreased expression (expression ratio cut off $\geq$2.0 fold with *p*-value $\leq$0.05) as compared with those in the larvae fed on non-transgenic corn leaves. The number of transcripts and their expression ratios of S-strain larvae are larger than these of R-strain larvae. These significantly differentially expressed transcripts may play important roles in influencing Cry1Ab toxicity from toxin degradation, toxin binding, to intracellular defense. Seventeen transcripts including serine protease and aminopeptdiase in S strain and nine in R strain were further analyzed by qPCR to validate their expression ratios. This study not only revealed information about the difference in the transcriptional responses of these genes to Cry1Ab between Bt-resistant and susceptible strains of *O. nubilalis*, but also provided new insights into potential interactions of the protoxin, toxin from transgenic corn with important proteins in the gut of *O. nubilalis* larvae.

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Acknowledgements

First of all I would like to thank Drs. Kun Yan Zhu and Lawrent (Larry) L. Buschman not only for their excellent supervision but also for their encouragements when I was in the tremendous hard time after I lost my husband. They always opened their arms and gave their support to me no matter what had happened in my life. I am also thankful for all the inspiring discussions and encouraging comments for the dissertation. And, I will never forget their encouragements for attending meeting and participation in student presentations.

I like to thank Drs. Brenda Oppert, Susan Brown, and Ming-Shun Chen. They were always there no matter what kind of help I needed. They gave to me generous suggestions for my research project and shed a light on my future. Especially, Dr. Brenda Oppert served temporally as my major supervisor when Dr. Zhu was out of town, and she gave me lots of great ideas on my presentation on Kansas Research forum in 2009.

I want to say thanks to my former supervisor, Dr. Jerry Reiger, (Center of Biotechnology Institution in University of Maryland). He helped in revising my proposal and preparation of manuscripts writing. He also continues sending me notes to encourage me to finish my study.

I would like to thank Dr. Tom Philips and Dr. David Margolies, our former and current department heads, and all department colleagues for their encouragements and generous supports which make me realize I am a lucky person. I could not find appropriate words to describe how much I appreciate their kindness.

I appreciate very much for Dr. Michael C. Smith's helps. He not only allowed me to use his green house spaces, but also recommended me a wonderful psychologist helping me healing my emotional suffering after the loss of my dear husband. I would like to thank Drs. Brian McCornack, and Ludek Zurek for their generosity letting me use their insect rearing room and Bio-Rad iCycler all the time.

My deepest and sincere thanks go to my lab members: Chitvan Khajuria, Yanhui Lu, Sharon Starkey, Guanghui Tang, and Xin Zhang. They were always my primary help providers in all what I needed. I will always carry them deeply in my heart.

I owe thanks to my little boy, Vinny Sun. He is my hope and my strength. He pushes me to move forward no matter how hard it would be. He always stands beside me no matter what had happened. Thank God, Vinny is the precious gift He ever gave me.

The most important appreciation belongs to my dear husband Qihong. I know he is watching me from the heaven, and he is also proud of my accomplishments.

Dedication

To my husband, Qihong Sun

Chapter 1 - Literature Review

1. European corn borer

In the Midwestern corn-belt area of North America, *Ostrinia nubilalis* is one of the most destructive insect species of corn. Newly hatched first generation *O. nubilalis* larvae tunnel in the tender central whorl and feed on developing leaf tissue, and yield can be reduced substantially. However, the newly hatched larvae of second generation feed on pollen in the leaf axil or in reproductive tissues. The third and fourth instar larvae of the second generation feed on corn tasseling tissue and tunnel into the midribs and stalks to complete their larval development. *O. nubilalis* damage in the stalk and ear causes poor ear development, broken stalks and dropped ears, resulting in substantial yield losses (Mason et al., 1996). *O. nubilalis* attacks stems of plants with diameters large enough for the larvae to enter (Showers et al., 1989). Therefore, most *O. nubilalis* development occurs hidden inside the plant tissue. Because *O. nubilalis* are hidden inside the plant, and have multiple generations (2-3 generations in Kansas) in a growing season in North America, control with common chemical insecticides sprayed on the plant surface is inconsistent. Skill and persistence is required to control *O. nubilalis* with conventional insecticides (Showers et al., 1989; Ostlie et al., 1997). In addition, *O. nubilalis* flight is extended over three weeks and a single insecticide application does not last long enough to cover the entire flight and many can escape insecticide treatments. However, if treatments are well-timed, they can provide acceptable control (Showers et al., 1989).

2. *O. nubilalis* management

2.1 Traditional O. nubilalis *management strategy*

In the cornfield, *O. nubilalis* populations can be reduced by chopping up corn stalks and ploughing the corn residue into a deep furrow to prevent moths from emerging to continue reproduction. In addition, crushing or burning corn stubble is thought to reduce the population of overwintering larvae. In addition to physical control methods, chemical and biological methods have also been used in the Midwest corn-belt. For example, spraying synthetic pyrethroid insecticides is effective against *O. nubilalis* larvae when timed correctly, and mortality can reach

75%. However, spraying insecticides kills beneficial organisms, like ladybirds (*Coccinella septempunctata*), lacewings (Chrysoperla *sp.*), and predaceous mites that feed on *O. nubilalis* eggs and young larvae. Releasing biological control organisms like chalcid wasps is another strategy, which has been tried but was mostly unsuccessful (Showers et al., 1989). Another biological control agent, *Bacillus thuringensis* (Bt) (trade name "Biobit, MVP, Steward, Thuricide, Dipel"), has also been used to control lepidopteran insect pests. Compared to the synthetic pyrethroid insecticides, the Bt formulation is less effective against lepidopteran larvae, but the harm to beneficial organisms is limited. However, the success of Bt control efficacy is as low as 30% because it easily degrades under sunlight. No matter how efficient the technologies are, it is essential to have appropriate timing of the treatment for successful control of field populations of *O. nubilalis*. In addition, intensive scouting is extremely important for effective control of *O. nubilalis* in the field (Showers et al., 1989).

2.2 Transgenic Bt crop control strategy

The genetic information of Bt Cry toxins has been transferred into corn, one of many genetically modified organisms (GMOs). This particularly successful event, Bt corn, can produce a Bt toxin continuously to kill caterpillars. Bt corn has been grown widely in the Midwest corn-belt since 1996 and has saved Midwest farmers billions of dollar in the past decade (Witkowski et al., 1997; Hutchison et al., 2010).

3. *Bacillus thuringiensis* (Bt)

Bacillus thuringiensis (Bt) is a gram positive, spore-forming, soil-dwelling bacterium, initially isolated from dying larvae of *Bombyx mori* by Shigetane Ishiwatari. Ernst Berlin first described the ability of Bt to kill certain insects in the larval stage (Berlin, 1915). Sporulation of this bacterium produces insecticidal crystalline inclusions, called δ-endotoxins or crystal (Cry) proteins. Cry proteins have been used as biopesticides to control lepidopteran larvae in the field since 1938. There are many different subspecies of Bt bacteria with insecticidal activities against different insect species. For example, *B. thuringiensis israelensis* (Bti) has been used as a biological control agent for dipteran insect pests, such as mosquitoes, fungus gnats, and blackflies. Bti was approved by WHO as water-dispersible granule in 2004. Bti contains the pBtoxis plasmid encoding numerous Cry and Cyt toxins, including Cry4, Cry10, Cry11, Cyt1

and Cyt2, and is available as a commercial biopesticide for pest control in public water systems (WHO. 2009).

The gene encoding Cry1Aa was first cloned into a plasmid and its amino acid sequence was reported in 1984 (Schnepf et al., 1985). There are over 100 *cry* genes encoding different types of Cry toxins with insecticidal and nematicidal specificities (Crickmore et al., 1998). Bt toxins are classified by their amino acid sequence and insecticidal specificity. Currently, 55 different types of Cry- and 2 Cyt-toxin groups have been identified (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt).

3.1 Cry toxin structure

Cry1 proteins are produced as 130 kDa protoxins in the sporulation stage of the bacterium. Protoxins are dissolved in the alkaline environment of the lepidopteran larval gut, and proteases cleave the protoxin to yield an active toxin that is 60-70 kDa. This activation involves the removal of the N-terminal peptide and approximately half of remaining protein from the C-terminus to produce the activated Cry toxin (Chroma et al., 1990). The active Cry toxin is composed of three functional domains. Domain I is a seven helix bundle; domain II is triple anti-parallel β-sheets; and domain III is a β-sheet sandwich (Schnepf et al., 1998).

Domain I consists of seven alpha helices and is responsible for inserting into the midgut lipid raft, in which helix α5 is encircled by six other amphipathic α helices (Grochulski et al. 1995). Two mechanisms have been proposed for domain I insertion into the midgut raft. In one model, α5/α6 functions like a penknife and inserts itself into the membrane with oligmerization of several toxin molecules forming an aqueous pore (Hodgman et al., 1990). In the other model, the helix pair α6/α7 initiates the membrane insertion of pairs of helices to form helical hairpins (Li et al., 1991).

Domain II consists of three β sheets, which are assumed to interact with gut receptors and contribute to toxin specificity. This domain has the lectin-fold structure, which is assumed to determine carbohydrate affinity because lectin can specifically recognize carbohydrate determinants on the receptor (Sankaranarayanan et al., 1996). There are several other factors that

implicate the function of domain II residues in toxin specificity and high-affinity carbohydrate binding (Dean et al., 1996).

The C-terminal domain III consists of two twisted antiparallel β sheets, called the β sandwich, that also has been implicated in determining specificity. For example, swapping domain III between toxins by recombination can result in changes in specific activity (Masson et al., 1994; de Maagd et al., 1996). Domain III is involved in binding to putative receptors of target insects, and it plays a crucial role in specificity through receptor recognition (Lee et al., 1995).

The three domains of Cry toxin and their mode of action have been extensively studied (Li et al., 1991; Ounjai et al., 2007). Crystal structures are available for some commonly used crystal proteins in the protein database (www.rcsb.org/pdb/explore/explore.do/structureID).

3.2 The mode of action of Bt Cry toxin

Two models for the mode of action of Cry toxins have been proposed: (1) pore-formation model; and (2) signal transduction model. The pore-formation model proposes that there are two or more different Cry toxin binding-proteins and the gut membrane glycolipid that are involved in Bt toxin processing (Bravo et al., 2007), including a cadherin-like protein, a glycosylphosphatidyl-inositol (GPI) anchored aminopeptidase-N, or a GPI-anchored alkaline phosphatase, and the glycolipid membrane itself. In the pore-formation model, the Bt protoxin is first ingested, solubilized and activated by proteolytic action at the N- and C-termini. The activated Cry toxin binds to Bt-R1 (cadherin-like protein), which promotes additional proteolytic cleavage at the N-terminal end of the toxin-α1 helix, and in turn facilitates the formation of the pre-pore oligomerized toxin. The oligomerized toxin binds to secondary receptors (GPI anchored membrane proteins), such as aminopeptidase or alkaline phosphatase. Finally, the toxin oligomer inserts into the bi-lipid raft of the membrane and forms a pore that causes the cell to lose integrity and the unbalanced osmotic pressure causes the cell to burst (Gill et al., 1992; Bravo et al., 2007).

An alternative model was called signal transduction model and it proposed that Cry toxins are not pore-forming toxins, but instead activate a cascade signal pathway mediated by the interaction with a transmenbrane cadherin (Zhang et al., 2006). The initial steps in both models

are similar, from ingestion, solubilization and activation of the protoxin to the interaction with the primary cadherin receptor (BT-R). However, in the signal transduction model, this primary binding with cadherin directly triggers a cascade of intracellular reactions, including stimulation of the membrane coupled G protein receptor (GTP-GDP receptor) and adenylyl cyclase (AC), and increasing the levels of cAMP to activate protein kinase A (PKA). Activated protein kinase A accelerates cell death through sequential cytological changes, including membrane blebbing (appearance of ghost nuclei), cell swelling and lysis (Zhang et al., 2006). This model states that insect cell death occurs without the participation of oligomeric structures that form lytic pores of Cry toxin or the participation of other receptors, like GPI-anchored proteins.

Since then, multiple publications have provided evidence supporting both models from protoxin solubilization, activation to cell membrane binding. In the case of gut protease activation, the slower protoxin hydrolysis in gut extracts of one Bt resistant strain, *Plodia interpunctella*, was demonstrated and the resistance was due to the absence of a major gut protease and resulted in the increased resistance to the toxin (Oppert et al., 1997). Other studies demonstrated that midgut proteases of *Manduca sexta* and *Spodoptera frugiperda* can activate 130 kDa Cry1Ab δ-endotoxin to form 60-65 kDa toxin (Miranda et al., 2001). Disruption of a cadherin gene in *Heliothis virescens* leading to toxin resistance demonstrates the importance of cadherins as Bt toxin receptors (Gahan et al., 2001). Other data have demonstrated that cadherin is Bt toxin essential receptor. An anti-cadherin antibody can reduced Cry1Ab and Cry1Ac toxicity in *H. virescens* (Xie et al., 2005). Cry1Ac resistance of *Pectinophora gossypiella* was also genetically linked to three different cadherin deletion alleles (Morin et al., 2003).

Studies supporting the signal transduction model have demonstrated that the susceptibility of *M. sexta* to Bt Cry1Ab toxin correlates directly to the expression of cadherin (BT-R) in this insect (Griko et al., 2008) and cell death was due to toxin exocytosis, not caspase cascade programmed cell death. Cry1Ab binding with BT-R promotes exocytotic transport of BT-R from intracellular membrane vesicles to the plasma membrane and accelerate H5 cell death. Blocking the activation of AC/PKA signal transduction by either EDTA or PKA inhibitors can prevent cell death (Zhang et al., 2008).

However, the pore-formation model proposes that the interaction with cadherin facilitates further proteolytic cleavage, removal of one α helix from the toxin and that this results in the oligomerization of the toxin. The toxin oligomer binds to secondary receptors, which are proteins anchored to the membrane, by a glycosylphosphatidylinositol (GPI)-anchor, such as aminopeptidase N or alkaline phosphatase. Both APN and ALP molecules have high binding affinity with the oligomerized Cry1Ac toxin in *M. sexta* (Knight et al., 1995 and McNall et al., 2003). The larvae of a *S. exigua* strain that are resistant to Cry1C were shown to lack the mRNA transcript encoding a GPI-anchored aminopeptidase N1 (Herrero et al., 2005). Silening midgut aminopeptidase of *S. litura* by dsRNA confers resistance to Cry1Ac (Rajagopal et al., 2003). One *H. virescens* strain that is resistant to Cry1Ac showed lower binding affinity to a glycosylphosphatidylinositol (GPI)- anchored alkaline phosphatase than to that of the susceptible strain (Jurat-Fuentes et al., 2004).

4. Transgenic Bt crops

Bt crops have been engineered with genes encoding Bt toxins that are toxic to lepidopteran and coleopteran insect pests. For example, Bt corn expressing Cry1Ab (toxic to lepidopteran pests) and Cry3Bb (toxic to coleopteran pests) are intended to control *O. nubilalis* and the western corn rootworm (*Diabrotica virgifera*), respectively. Bt potatoes expressing Cry3Aa were produced to control the Colorado potato beetle (*Leptinotarsa decemlineata*) (Reed et al., 2001). Today, more than 10 different Cry toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1C, Cry1D Cry1E, Cry1F, Cry2Aa Cry2Ab, Cry3A, Cry3B, and Cry34/Cry35) are available as commercial pesticides (Bravo et al., 2007). More than 20 different Bt engineered crops have been tested, and more than 10 different transgenic crops (rice, corn, cotton, soybean, tobacco, potato, squash, canola, tomato and papaya) have been used commercially worldwide with considerable success (Toenniessen et al., 2003). In 2010, the global area of biotech crops continued to soar and reach 148 million hectares or 365 million acres, about 87-fold increase since the commercialization began in 1996 (Clive., 2010).

5. Insect resistance to Bt toxins

Insects have the potential to develop resistance to nearly every type of insecticides, including sprayable Bt formulations and genetically modified Bt crops. Insecticide resistance is usually present as genetic variation in large insect populations. If the population is large with sufficient genetic diversity, there are likely to have some individuals thate will be unaffected by a given

insecticide. These unaffected (or resistant) individuals are different from susceptible individuals. The unaffected individuals may have target molecules (enzymes or receptors) that are not sensitive (site insensitivity-mediated resistance) to the applied insecticides, or they may have a mechanism to break down insecticides (metabolic pathway-mediated resistance), or have a behavioral avoidance trait to avoid feeding poisoned food or contacting insecticide treated surface. Thus, the unaffected individuals can survive from insecticide treatments if they lack the enzymes that are needed for solubilization or activation, or if they gain another enzyme activity that can hydrolyze the insecticide, or if they lack target receptors. When an insecticide is applied, these individuals survive and pass their resistance traits to the following generations. Over time, a greater proportion of the overall insect population will be unaffected by the insecticide. Even though Bt is different from other insecticides in its mode of action and usage, insects still have the potential to develop resistance to Bt toxins.

The development of resistance to *Bt* toxins is unfortunate because Bt toxins are more pest-specific, much more effective and environmentally safer than conventional insecticides. However, resistance to Bt was first found in *P. interpunctella* in storage bins of Bt-treated grain (McGaughey, 1985). The first report of resistance selected in the field was in the diamondback moth (*Plutella xylostella*) (Tabashnik et al., 1997); and the resistant individuals of the cotton bollworm (*Helicoverpa armigera*) were reported in cotton in China (Li et al., 2007; Liu et al., 2010). Meanwhile, multiple Bt resistant strains of lepidopteran insect species have successfully been selected in the laboratory, including *P. interpuctella* (McGaughey., 1992), *P. gossypiella* (Tabashnik et al., 2006), *P. xylostella* (Zhao et al., 2003), *L. decemilineate* (Cooper et al., 2007), *Trichoplusia ni* (Kain et al., 2004), *Helicoverpa zea* (Liu et al., 2008), *Ephestia kuehniella* (Rahman et al., 2004), *O. nubilalis* (Huang et al., 1999; Stodola et al., 2006) and other insects (reviewed in Bravo et al., 2008).

Bt resistance may occur through various mechanisms. The activity of insect gut proteases may be reduced, resulting in a reduction in protoxin solubilization and/or the activation of Bt protoxins to active toxins. Changes in gut enzymes may also increase the degradation of Bt toxin or they can sequester the Bt protoxin or toxin. Either a reduction in the number of toxin receptors on the brush border membrane or binding affinity to Bt toxins may also decrease in a resistant insect.

5.1 Insect gut serine protease-mediated resistance

After the susceptible insects ingest Cry1 protoxin, the protoxins (130 kDa) are solubilized and sequentially cleaved by alkaline gut proteases to yield active toxins (60-65kDa) (Chroma et al., 1990). The N-terminal peptide and approximately half the remaining protein from the C-terminus are removed to form the active toxins in the protoxin activation process. An important factor in insect resistance to Bt is the altered capacity for processing protoxin to Cry toxin. The gut serine proteases that are involved in Bt protoxin activation have been described for several insect species, including *P. interpunctella* (Oppert et al., 1994), *O. nubilalis* (Li et al., 2004), and *S. litura* (Barkhade and Thakare, 2011). In *P. interpunctella*, Bt-resistant larvae had a slower Bt protoxin processing rate (more than 300 times slower) than susceptible larvae (Oppert et al., 1997). Serine proteases involved in the degradation of Bt toxin have also been described in *Helicoverpa armigera* (Shao et al., 1998), *Mamestra brassicae* (Lightwood et al., 2000), *M. sexta* and *S. frugiperda* (Miranda et al., 2001), and *Lymantria dispar* (Audtho et al., 1999). The midgut juice of *M. sexta* and *S. frugiperda* can also inactivate Cry1Ab protoxin by splitting the protoxin into 30-kDa fragments, and the appearance of 30-kDa fragments correlates with a decrease in pore formation and insecticidal activity (Miranda et al., 2001). Gut juice from *L. dispar* cleaved Cry2Aa1 protoxin at the C-terminus producing an active 58-kDa toxin, but continued incubation of the toxin with gut juice resulted in further cleavage, producing a 49-kDa protein with reduced toxicity (Audtho et al., 1999). Radiolabeled Cry2Aa1 protoxin was used to demonstrate the excessive cleavage and reduced Bt toxicity *in vivo* (Glick et al., 2009).

The serine proteases, including trypsin, chymotrypsin, and elastase, have been described in the activation process of Cry1 protoxins. For example, the 135-kDa Cry1Ab protoxin can be digested to 63-kDa by three mammalian proteases (bovine trypsin, bovine chymotrypsin, porcine elastase) (Kaiser-Alexnat et al., 2004). The data implies that the same enzymes are involved in activating protoxins, although insect proteases can have different properties from those in other organisms.

The evidences that insect gut proteases affect Bt toxicity in insect gut come from many studies. In *P. interpunctella,* resistant larvae have reduced trypsin and/or chymotrypsin activity (Oppert et

al., 1997; Candas et al., 2003). When Cry1Ac protoxin was used as a substrate to examine serine protease activities of the castor semi-looper (*Achaea janata*) midgut homogenate, Budatha et al. (2008) identified one elastase-like and three trypsin-like proteases were active against the protoxin. Another study found decreased expression at the transcript level of a gene encoding trypsin in larvae of a Bt-resistant *O. nubilalis* strain compared with that of a susceptible strain (Li et al., 2005). Knockdown of a trypsin gene, *SfT6*, in *S. frugiperda* larvae reduced susceptibility to Bt CryCa1 protoxin (Rodriguez-Cabrera et al., 2010). In addition, in *H. virescens* larvae, gut extracts from a resistant strain had reduced trypsin-like and chymotrypsin-like activity (Karumbaiah et al., 2007).

5.2 Other gut enzymes

There are a few reports of selective interactions of insect gut enzymes with toxins. Esterases in *C. fumiferana* were reported to precipitate Cry1A toxins and attenuate toxicity (Milne et al., 1998). An *H. armigera* Cry1Ac-resistant strain larvae had increased production of gut caboxylesterase. The overproduced caboxylesterase was assumed to bind to and sequester Cry1Ac toxin. However, it is unclear whether carboxylelastase involved in Bt toxin sequestration at present (Gunning et al., 2005).

5.3 Transmembrane protein-mediated resistance

Toxin binding proteins and receptors in the insect gut have been studied in many Bt susceptible insects (Bravo et al., 2007). Receptor-mediated resistance may be caused by altered affinity or reduced number of protein receptors in epithelial membrane. This type of resistance was first reported in Cry1A-resistant *P. interpunctella*, in which high levels of resistance were selected in the laboratory from insects collected from grain bins (van Rie et al., 1990).

Cadherins (CADRs) are trans-membrane proteins with a cytoplasmic domain and an extracellular domain. Cry toxin binding to a cadherin receptor is an essential step in both proposed Bt mode of actions, and was first demonstrated as a high affinity receptor for Cry1A in *M. sexta* (Vadlamudi et al., 1993). The expression of a CADR gene in lepidopteran cells increases the sensitivity to Cry1A toxin exposure (Nagamatsu et al., 1999). Proteins in the *M. sexta* brush border membrane vesicles precipitated with Cry1Ab toxin and demonstrated that the

CADRs can specifically interact with the monomeric form of the toxin (Bravo et al., 2004). Genetic studies of five different species (*H. virescens, P. xylostella, H. armigera*, *P. gossypiella* and *M. sexta*) identified mutations in the cadherin receptor that were associated with resistance to the Cry1A toxin (Jurat-Fuentes et al., 2006; Higuchi et al., 2007; Yang et al., 2007; Fabrick et al., 2007; Gomez et al., 2003; Xie et al., 2005).

In a laboratory selected Cry1Ac-resistant *H. virescens* strain, a single mutation was found to be responsible for 40-80% of Cry1Ac resistance (Gahan et al., 2001). This mutation was linked to retrotransposon insertion in the CADR gene that resulted in reduction of toxin binding affinity. Laboratory selected Cry1Ac resistant strains of *P. gossypiella* and *H. armigera* were also found to have CADR alleles associated with Cry1Ac resistance (Xu et al., 2005). Thus, there exists a tight linkage between Cry1Ac resistance and the cadherin locus, and this is the basis for DNA monitoring of Bt resistance in field populations of the cotton insect pest complex (Xu et al., 2005). Because Bt toxicity is primarily determined by univalent binding to membrane cadherin (Zhang et al., 2006), the susceptibility of a target insect to a Bt toxin may be closely correlated with the level of cadherin expression in the insect as observed in *M. sexta* (Griko et al., 2008).

However, mutations in a 12-cadherin-domain protein conferred some Cry1Ac resistance in *H. virescens* but did not block toxin binding in vitro assays (Jurat-Fuentes et al., 2004). It is possible that there exist other trans-membrane proteins that serve as Cry toxin binding partners. Gahan et al. (2010) found an inactivating mutation of the ABC transporter ABCC2 of *H. virescens* that was genetically linked to Cry1Ac resistance and was also correlated with a loss of Cry1Ac binding to membrane vesicles. Thus, ABC protein may also play a key role in the mode of action of Bt toxins.

5.4 Membrane anchored protein-mediated resistance

Cry toxins bind to lepidopteran aminopeptidase through the Cry toxin domain III. Domain III specifically recognizes N-acetylgalactosamine (GalNAc) moieties and facilitates the subsequent interaction of the Cry domain II loop regions (Knight et al., 1994). Five isoforms of APN have been identified from *H. armigera*, *M. sexta, S. exigua* and *L. dispar* (Rajagopal et al., 2002, 2003; Herrero et al., 2005; Lee et al., 1996; Knight et al., 1995). Cooper et al. (1998) found that

the oligomeric form Cry toxin can bind to APNs in *M. sexta*. Rajagopal et al. (2002) demonstrated that APN1 of *H. armigera* can bind to Cry1Aa, Cry1Ac and Cry1Ab activated toxins. Oligomeric Cry1Ac specifically recognized a 120 kDa peptide APN in *L. dispar* (Lee et al., 1996). In *S. litura*, biochemical analyses (immunoblotting, ligand blotting, and lectin blotting) also demonstrated that APNs could bind to Cry toxins (Budatha et al., 2007). Thus, these studies indicate that APNs are potential receptors of Cry1 Bt toxins.

APNs can bind Bt toxin in the gut, and their functional roles were demonstrated by RNA intereference (RNAi) in several studies. The injection of dsRNA for one APN gene in *S. litura*, that shares 97% amino acid sequence identity to APN-N4 of *S. exigua*, resulted in reduced transcript levels and decreased susceptibility to Cry1C toxin (Rajagopal et al., 2002). Knockdown of three APN genes (*DsAPN1*, *DsAPN2* and *DsAPN3*) in larvae of *Diatraea saccharalis,* each also resulted in a decreased susceptibility to Cry1Ab (Yang et al., 2010). Moreover, APN-N1 gene was not expressed in a lab-selected Cry1Ac resistant colony of *S. exigua* (Herrero et al., 2005). All of these results support the hypothesis that aminopeptidase N are receptors for Bt Cry toxin and can influence insect susceptibility to Cry toxins.

Membrane bound alkaline phosphatase (ALP), also known as GPI-anchored ALP, in the larval BBM are also characterized as putative functional receptors of Bt toxin oligomers (Jurat-Fuentes et al., 2004; Fernandez et al., 2006). The resistance to Cry1Ac in *H. virescense* larvae that showed a reduced toxin binding to HvALP in immunoblot analysis is a result of reduced HvALP product on gut membrane (Jurat-Fuentes et al., 2004). Proteomic and genomic detection also found reduced levels of midgut membrane-bound ALPs as a common feature in Cry-resistant strains of *H. virescens*, *H. armigera*, and *S. frugiperda* as compared with the susceptible larvae (Jurat-Fuentes et al., 2011).

5.5 Glycolipid-mediated resistance

Glycolipid components are also considered as a factor that influences Bt toxicity in nematodes (Griffitts et al., 2001 and 2005). Knockdown of *bre* genes, encoding glycosyltransferase proteins in the glycolipid biosynthesis in *C. elegans*, rendered worms' resistance to Cry5Ba (Griffittes et al., 2005). Therefore, *bre* gene products are required for the uptake of toxin into intestinal cells

(Griffitts et al., 2001 and 2003). BLAST searches of *C. elegans* bre-2 (GenBank accession no: AY533304) identified one galactosyltransferase gene-CG8668 from *D. melanogaster*. By genetic mapping, a gene (GenBank accession no: EF579961) similar to CG8688 from *D. melanogaster* was identified in *P. xylostella* and is likely to be involved in glycolipid synthesis (Baxter et al., 2008).

Griffitts et al. (2005) also noticed wild-type *C. elegans* contain multiple high polar glycolipid species, but were lacking in *bre* mutants. Thus, Griffitts et al. (2005) proposed that the gut glycolipid component was the factor influencing Bt toxicity in *C. elegans*. In one Cry1Ac-resistant strain of *P. xylostella*, larval gut neutral glycolipid content was substantially lower than that of susceptible strain (Karumaraswami et al., 2001). Levels of putative hexasaccharylceramide and trisaccharylceramide in the resistant strain were half that of the susceptible strain. These data support the hypothesis that gut membrane glycolipid component can be a factor influencing Bt toxicity in lepidopteran insects too.

6. Other Bt toxin-triggered defense responses

Ingestion of Bt toxin may also trigger other cellular defense pathways. In a whole-organism approach of *C. elegans*, several differentially-expressed defense pathways were discovered, including the p38 mitogen-activated protein kinase (MAPK) pathway (Huffman et al., 2004), the unfolded protein response (UPR) pathway (Bischof et al., 2008), the hypoxic response pathway (Bellier et al., 2009), and insulin/insulin-like intrinsic cellular defense pathways (Chen et al., 2010). Therefore, Bt Cry toxin triggers multiple genes involved complex defense responses. For example, in the insulin and insulin-like pathway, the reduced expression of *DAF-2*, encoding insulin-like receptor, by RNAi confers resistance of *C. elegans* to Cry5B toxin more than 10-fold, and *daf* mutants have more than 12-fold resistance to Cry5B than the wild type. Mutants of *age*, *aap*, *pdk* genes, encoding PI3 kinase and pyruvate dehydrogenase kinase (PDK), respectively, in the *daf* pathway, also confer resistance to Cry5Ba in *C. elegans* (Chen et al., 2010).
Recently, a global functional analysis of *C. elegans* genes by RNAi screening was published. They found 106 *C. elegans* genes that were very important for cellular protection against Cry5B attack. They are called *hpo* genes because knock-downs showed hypersensitive to Cry5B phenotype. In the complicated defensive mechanisms, JNK pathway converges in parallel on

p38-regulated MARK pathway to regulate the transcriptionally-induced defense response to Cry5B (Kao et al., 2011).

7. Bt toxicity and resistance in *O. nubilalis*

Resistance to Dipel formulations has been associated with decreased expression of gut trypsin genes in larvae of *O. nubilalis* (Li et al., 2005). Siqueira et al. (2006) also demonstrated that resistance to Cry1Ab protoxin was associated with the decreased levels of Cry1Ab binding to gut proteins. These observations indicate that Bt resistance mechanisms in *O. nubilalis* are multifaceted and complicated. Bt resistance in *O. nubilalis* may also be associated with increased toxin degradation (Li et al., 2004), or toxin binding proteins with low binding affinity (cadherin-like proteins, gut aminopeptidases, or alkaline phosphatases) (Bel et al., 2009). In order to clarify which genes or gene families play key roles in Bt toxicity and resistance, one approach is to screen a large number of gut transcripts to identify the differentially expressed genes which may be potentially involved in Bt toxicity or resistance. However, while this approach can identify overall changes in gene expression in intoxicated or resistant larvae, mutational differences in specific Bt toxin interaction genes may not be identified through this approach.

8. Microarray analysis of Bt resistance

DNA microarray can be used to screen a large number of transcripts that are differentially expressed in organisms, and it has been widely used to determine changes in gene expression under certain conditions. Microarrays offer a quick, accurate and large scale screening technique that has been widely used in different insect model systems such as silkworm (*B. mori*) (Xia et al., 2007), fruit fly (*D. melanogaster*) (Gupta, 2003), red flour beetle (*T. castaneum*) (Morris or et al., 2009; Oppert et al., 2010), pea aphid (*A. pisum*) (Wilson et al., 2006), and mosquito (*A. gambiae*) (David et al., 2005; Kambris et al., 2009). Commercially-available microarrays have been used to determine mosquito (*A. gambiae*) detoxification gene responses to insecticide exposure (David et al., 2005). In genome wide transcript analyses of the silkworm, *B. mori*, gene expression profiles have been obtained for responses to injury (Liu et al., 2009), B. mori nucleopolyhedrovirus (Sagisaka et al., 2009), and cytoplasmic polyhedrosis virus in *B. mori* (Wu et al., 2011) and bacteria *Bacillus bombyseptieus* (Huang et al., 2009). In other insect species, custom arrays based on specific cDNA sequences or EST databases have been developed for gene expression analysis (Kijimoto et al., 2009 and Baker et al., 2009). In microarray

experiments related to Bt, differential gene expression was studied in *C. fumiferana* under Bt Cry toxin exposure (Côté et al., 2005).

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Chapter 2 - Identification and characterization of 34 cDNAs encoding putative serine proteases in the gut of the European corn borer, *Ostrinia nubilalis*

Abstract

In lepidopteran insects, the serine proteases trypsin and chymotrypsin are the primary digestive enzymes and involved in *Bacillus thringiensis* (Bt) protoxin/toxin hydrolysis. In this study, 34 cDNAs encoding putative trypsins, chymotrypsins, and trypsin- and chymotrypsin-like protease homologs were identified and characterized from the gut-specific expressed sequence tags from the European corn borer, *Ostrinia nubilalis*. Blast and phylogenetic analysis of their deduced amino acid sequences indicated that 15 were putative trypsins belonging to Try-G2 and Try-G3 groups (none of them was grouped in Try-G1), another 15 were putative chymotrypsins in one large group (CTP-G1), and the remaining four were serine protease homologs in two groups (Try-G4 and CTP-G2). The existence of diverse trypsins and chymotrypsins in *O. nubilalis* is an adaptation to diverse food sources and also a defense mechanism against different plant-specific protease inhibitors. However, two trypsin-like proteases (OnTry12 and OnTry13) and two chymotrypsin-like proteases (OnCTP15 and OnCTP16) belong to two new groups (Try-G4 and CTP-G2, respectively). OnTry12 shows 69 % identity to serine protease 31 (SP31) from *Mamestra configurata* whereas OnTry13 shows 63% identity to trypsin (HaFLS00713) from *Helicoverpa armigera*. SP31 and HaFLS00713 were associated with remodeling of cuticle in the gut peritrophic matrix and are involved in molting. Both OnCTP15 and OnCTP16 were grouped in CTP-G2 with four chymotrypsin like proteases from *Manduca sexta* (Ms-AM419170 and Ms-AM690448), *H. armigera* (Ha-EU325548), and *Spodoptera exigua* (Se-AY820894) by bootstrap value (=99), and they were important in molting from larva to pupa in three species. RT-PCR results indicated that 34 putative trypsins, chymotrypsins and serine protease homologs are highly expressed in gut tissues, but they have different expression patterns in different regions of the gut. *OnTry-G2* is highly expressed in the *O. nubilalis* larval foregut and midgut, whereas *OnTry-G3* is highly expressed in the midgut and hindgut. Real-time quantitative PCR was used to evaluate the expression of protease transcripts in the larval gut of third instars with or without

exposure to Cry1Ab protoxin. *OnTry5* and *OnTry6* were significantly up-regulated (*P*-value≤0.05) in the interval from 2 to 24 h after Cry1Ab protoxin ingestion. Four transcripts encoding putative chymotrypsins (*OnCTP2*, *OnCTP5*, *OnCTP12* and *OnCTP13*) were up regulated at least 2-fold (*P*-value ⩽0.05) after 24 h of Cry1Ab protoxin exposure. The data are the first in-depth study of gut transcripts encoding proteases in *O. nubilalis* larvae, and differential expressions of some protease transcripts may implicate a link to Cry1Ab intoxication.

Keywords: gene expression; *Ostrinia nubilalis*; chymotrypsin; Cry1Ab protoxin; serine protease; trypsin

1. Introduction

The European corn borer, *Ostrinia nubilalis*, is an important insect pest of corn, *Zea mays*, in the Midwest corn belt of the United States (Showers et al., 1989). Currently, *O. nubilalis* is controlled mostly by transgenic corn expressing *Bacillus thurigiensis* (Bt) Cry1Ab and Cry1F toxins (Witkowski et al., 1997). Although transgenic Bt corn is effective in controlling *O. nubilalis*, this technology would be threatened by populations of *O. nubilalis* resistant to transgenic toxins (Bates et al., 2005). At present, resistance management relies on the high dose/refuge strategy, with non-Bt corn planted in a refuge to conserve susceptible alleles in the field (Shelton et al., 2000).

Cry protoxins are naturally occurring insecticidal proteins produced by Bt, a soil inhabiting bacterium. The mode of action of Cry protoxins in susceptible insects includes solubilization of the crystalline protein, proteolytic processing of protoxin to the activated toxin, and binding of activated toxin to midgut cadherin receptors. In one model, this binding facilitates toxin to recognize secondary membrane GPI-anchored protein and insert into the midgut epithelial cell membrane to form unselective pores (Bravo et al., 2008). In another model, this specific binding of toxin to cadherin triggers the intracellular signal pathway that leads to gut cell lysis (Zhang et al., 2006). Any physiological modification to this cascade of events can result in a reduction in insect susceptibility to the toxin. For example, changes in the expression or type of proteolytic digestive enzymes, such as trypsin and chymotrypsin, can alter the toxicity of Bt toxins by reducing toxin solubility and reducing the activation of protoxin or can increase the degradation of toxin (Oppert et al., 1997).

The lepidopteran larval gut represents a complex proteolytic environment containing serine proteases (trypsins, chymotrypsins, and elastases), cysteine proteases (cathepsin B or L), aminopeptidases and carboxypeptidases, all involved in the digestion of protein (Srinivasan et al., 2006). Trypsins can contribute up to 95% of the total digestive activity in the lepidopteran larval gut (Patankar et al., 2001). However, in other insects, such as the cockroach, some beetles, mosquito larvae, wasps and hornets, chymotrypsins are the major enzymes in protein digestion (Nation, 2001). For example, chymotrypsins play a major role in proteolytic activity of the

homopteran *Eurgaster integriceps* (Hosseininaveh et al., 2009). In lepidopterans, most trypsins and chymotrypsins have properties related to the extreme alkaline pH in the larval gut lumen, and they are known to digest dietary proteins into short peptides in preparation for amino acid absorption by the midgut (Srinivasan et al., 2006).

The phenomenon of plant expressed protease inhibitors (PIs) to defend against feeding by phytophagous insects was described in the 1950's (Lipke et al., 1954). Scientists have developed transgenic plants with specifically enhanced PIs to suppress the feeding by insects (Hilder et al., 1987; Johnson et al., 1989). However, insects, like *Tenebrio molitor*, *Diatraea saccharalis, Heliothis virescens, Spodoptera frugiperda*, and *Helicoverpa zea* have already developed the ability to overcome PIs (Volpicella et al., 2003; Lopes et al., 2004) by expressing many alternative proteases that have different sensitivities to host plant PIs (Mazumda-Leighton and Broadway, 2001a; 2001b) or by increasing the proteolytic inactivation of PIs mediated by insect gut proteinases (Giri et al., 1998). For example, Valpicella et al. (2003) purified two different types of trypsins, HzTrypsin-C and HzTrypsin-S, from *Helicoverpa zea*. Both were major trypsins involved in digesting plant material, but HzTrypsin-C was very sensitive to plant inhibitors whereas HzTrypsin-S was insensitive to plant inhibitors. This indicates that HzTrypsin-S was adapted as a defensive against protease inhibitors of plants. Similarly, two classes of genes encoding trypsins were identified from *S. frugiperda*. One class was constitutively expressed before or after exposure to soybean trypsin inhibitors (SBTI) while the second class was not expressed before exposure to SBTI, but was significantly induced upon ingestion of SBTI (Brioschi et al., 2007). Those studies indicate that there exists a diversity of serine proteases in the insect gut and that this is an adaptation to the food source and it can be used as a defense against plant-produced protease inhibitors.

Some trypsins and chymotrypsins are also involved in other physiological functions, such as haemolymph coagulation, fibrinolysis, embryonic development, fertilization, and apoptosis (Halfon et al., 1998; Page and Di Cera, 2008; Sui et al., 2009), as well as larval molting (Brohen et al., 2008; 2009) and immunity (Kanost et al., 2004). For example, one chymotrypsin from *M. sexta* binds to the extracellular C-terminal domain of zymogenic chitin synthase 2 and proteolytically activates this chitin synthase-2 (Broehan et al., 2008). Broehan et al (2009) also

identified two chymotrypsins from *Tribolium castaneum* (TcCTLP5 and TcCTLP6) that were involved in insect molting. Some chymotrypsins are also responsible for mediating specific protein-protein interactions (e.g., proteolytic activation of prophenoloxidase) and can be involved in the Toll signaling pathway (Jiang et al., 2008). The involvements of trypsin and chymotrysin in Bt Cry toxicity were also reported in Indianmeal moth (Chroma et al., 1990; Oppert, 1999).

There are diverse genes that encode putative trypsins and chymotrypsins in lepidopteran insects. Some genomic DNA (or cDNA) sequences for trypsins and chymotrypsins have been reported for *M. sexta* (Peterson et al., 1994), *Choristoneura fumiferana* (Wang et al., 1999), *Plodia interpunctella* (Zhu et al., 2000), *Helicoverpa armigera* (Mazumdar-Leighton et al., 2000), *H. zea (*Mazumdar-Leighton et al., 2001a), *Agrotis ipsilon* (Mazumdar-leighton et al., 2001b and 2001c), *Sesamia nonagrioides* (Diaz-Mendoza et al., 2005), *Ostrinia furnacalis* (Xu et al., 2006), *Bombyx mori* (Niu et al., 2006), *Spodoptera litura* (Zhan et al., 2011), *Bombyx mandarina* (Arunkumar et al., 2008), *S. frugiperda* (Rodriguez-Cabrera et al., 2010), *Mamestra configurata* (Erlandson et al., 2010), and *O. nubilalis* (Coates et al., 2008). In lepidopterans, genes encoding proteases are often duplicated, presumably in response to the evolutionary selection pressure of plant inhibitors (Jongsma and Bolter., 1997). Regarding the mode of action of Bt toxins, serine proteases play key roles in activating and/or degrading Bt Cry-toxins (Oppert, 1999). However, in *O. nubilalis*, genes encoding gut proteases have not been thoroughly studied, and it is unclear which proteases are involved in Cry protoxin activation.

In our previous studies of *O. nubilalis* gut trypsins, we demonstrated that there was a reduced trypsin activity in a Bt resistant strain of *O. nubilalis* that was associated with reduced Cry1Ab protoxin activation (Li et al., 2004). Further, we identified a transcript encoding trypsin (OnTry23) with reduced expression levels in this Bt resistant strain compared with a susceptible strain (Li et al., 2005). In this study, we describe 34 full-length transcripts encoding putative trypsins, chymotrypsins, and serine protease homologs from an *O. nubilalis* gut specific cDNA library. Transcripts were systemically analyzed in *O. nubilalis* larvae for tissue-specific expression patterns and transcriptional responses after ingestion of Cry1Ab protoxin. These data

provide the first comprehensive study of protease genes in the gut of *O. nubilalis* larvae and the first insights into how the Cry toxins influence their transcriptional changes.

2. Material and methods

2.1 Insect rearing

The Bt-susceptible strain (Lee) of *O. nubilalis* was obtained from French Agricultural Research Inc. (Lamberton, MN). Larvae were reared under long-day conditions (L:D=16:8) at 26°C on artificial diet (Bio-Serv. Inc. Frenchtown, NJ). Adults were reared in metal net cages covered with wax paper under long-day conditions with 70% humidity, and were routinely fed 2% sucrose water to provide supplementary nutrition. The eggs were collected from the wax paper every day and kept in insect rearing cups with high humidity (≥ 80%) until hatching. Newly hatched larvae were immediately transferred to artificial diet and reared to third or fifth instar for testing. The larval developmental stage was determined by moving them to a new rearing dish after each molt.

2.2 cDNA sequencing

The cDNA sequences for 34 putative trypsins, chymotrypsins and their homologs were identified from *O. nubilalis* gut-specific cDNA library using Blast2go analysis (Khajuria et al., 2009). Ten were full length cDNAs, and the remaining were amplified from partial cDNA sequences, using 5' and 3' rapid amplification of cDNA end (RACE) (Clontech SMART^{TM} RACE cDNA amplification kit, Mountain View, CA). Total RNA samples from the gut tissues of fifth-instar larvae were used for cDNA synthesis, and RACE was conducted with one gene specific primer and another 5' or 3' universal primer using the TOUCHDOWN PCR programs. The specific PCR product was sub-cloned into plasmid T-vector and sequenced using an ABI 3700 DNA sequencer at the KSU DNA Sequencing Facility (Manhattan, KS). Full-length cDNA sequences were confirmed by gene specific primer pairs (Table 2-1) in both directions.

2.3 Sequence analysis and phylogentic tree construction

Homology analyses of cDNAs were performed by Blast. Amino acid sequences were deduced from cDNA sequences using the online translation tool from ExPASy Proteomics Server

(Gasteige et al., 2003). Signal peptide sequences were predicted by SignalP (Bendtsen et al., 2004). Multiple sequence alignments of cDNA sequences from *O. nubilalis* were performed with published sequences from *Bombyx mori*, *Bombyx mandarina*, *Agrotis ipsilon*, *Spodoptera frugiperda*, *Mamestra configurata*, *Spodoptera litura*, *Manduca sexta*, *Ostrinia furnacalis*, *Sesamia nonagriorides*, *Galleria mellonella*, *Plodia interpunctella*, *Helicoverpa zea*, *Helicoverpa armigera*, *Helicoverpa punctigera, Choristoneura fumiferana*, *Lacanobia oleracea*, and *Heliothis virescens* using ClustalW2 (Katoh et al., 2002). Dendrograms were constructed according to the neighbor-joining method and confidence bootstrap values were determined using bootstrap analysis with the separation supported (5000 of 5000 bootstrap) re-sampling steps using MEGA 4 (Tamura et al., 2007).

2.4 RT-PCR analysis of serine protease tissue-specific expression pattern

Because the expression of transcripts encoding proteases were consistent in all larval developmental stages of *O. nubilalis* (data is not shown), we used early fifth-instar larvae to detect tissue-specific expression patterns. Eight different tissues were dissected from fifth-instar larvae, including foregut, hindgut, midgut, haemolymph, Malpighian tubules, carcass, fatbody and silk gland. The tissue samples from five larvae were pooled and homogenized in 500µl Trizol reagent (Invitrogen Inc. Federick, MD) for total RNA extraction and analyzed by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc. USA) at 260 nm. First strand cDNA was synthesized using Fermentas cDNA synthesis kit (Fermentas Inc. Glen Burnie, MD). The gene specific primers were designed using Beacon Designer 7 (Primer Biosoft Inc. Palo Alto, CA) (Table 2-1). Subsequently, RT-PCR was performed using 0.5 µl cDNA as template with 25 cycles of 95°C for 30s, 56°C for 30s and 72°C for 30s. *O. nubilalis* ribosomal protein S3 (RPS3) was used as a reference gene to detect the relative expression of 34 putative trypsin, chymotrypsin and serine protease genes. Ten microlites of each PCR product was loaded onto 1.5% agarose gel containing ethidium bromide to examine tissue-specific expression patterns.

2.5 Preparation of Cry1Ab protoxin and determination of median lethal concentration of Cry1Ab protoxin

Cry1Ab protoxin was prepared from *Escherichia coli* (strain ECE54) expressing the *cry1Ab* gene, based on the method described by Lee et al. (1992), and stored at -80°C as a suspension.

The bacterial strain was provided by the *Bacillus thuringiensis* Genetic Stock Center, Ohio State University (Columbus, OH).

The medial lethal concentration (LC_{50}) of Cry1Ab protoxin was determined in a 7-d bioassay at room temperature. In this assay, third-instar larvae were first starved for 24 h, and larvae (n=16) were fed artificial diet containing each of six different concentrations of Cry1Ab protoxin (0, 0.04, 0.2, 1, 5, 25 µg/ml). Each concentration was repeated six times for a total of 576 larvae in the bioassay. Fresh artificial diet containing Cry1Ab protoxin was replaced every other day, and surviving individuals were recorded every day. The bioassay data were analyzed for the LC_{50} value by probit regression using GLM procedure (PROC GLM, SAS Institute, 1996). The LC_{50} for Cry1Ab protoxin for early third-instar larvae of *O. nubilalis* was 0.25 µg/ml (95% CI= 0.14-0.33 µg/ml). Mortality was not observed in control larvae fed artificial diet only.

2.6 qRT-PCR analysis of trypsin-like protease transcripts under Cry1Ab treatment

After a total of 120 early third-instar larvae were starved for 24 h at 26°C, they were individually transferred onto artificial diet containing 0.25 µg/ml Cry1Ab protoxin (LC_{50}) or no protoxin for 2, 6, 12 and 24 h. The treatment was carried out with three biological replicates; each with five individuals. The gut tissues were dissected from each of the five individual, pooled, and homogenized in Trizol reagent for total RNA extraction. The first strand cDNA of each replicate was synthesized using a cDNA synthesis kit (Fermentas Inc. Glen Burnie, MD). Gene specific primer pairs were used (Table 2-1) in qRT-PCR with 1 µl cDNA (1:25 dilution) as template in a 25-µl reaction (SYBR Green, Fermentas) with two technical replicates. PCR was performed with 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s using a Bio-Rad IQ thermocycler (Bio-Rad Laboratories Inc. Hercules, CA). Relative expression differences were normalized to the RPS3 transcript level and analyzed using the comparative C_t method ($\Delta\Delta C_t$) (Paffla., 2001). The significant differential expression of transcripts from *O. nubilalis* larvae with or without exposure to Cry1Ab was analyzed by ANOVA and Fisher's least significant difference (LSD) multiple comparison.

3. Results

3.1 Analysis of cDNAs and their deduced amino acid sequences

A total of 34 putative serine protease transcripts were identified from our *O. nubilalis* gut-specific cDNA library. Full-length cDNA sequences were obtained using 5`and 3` RACE and confirmed by sequencing in both directions. The lengths of the cDNA sequences ranged from 684 to 1185 bp. The cDNAs of nine genes, including *OnTry1*, *OnTry2*, *OnTry3*, *OnTry21*, *OnTry22*, *OnTry23*, *OnCTP1*, *OnCTP2* and *OnCTP3,* appeared to be identical to those already available in the NCBI database (Li et al., 2005; Coates et al., 2008) (Table 2-2A; 2-2B). The remaining 25 genes were newly identified in this study.

Among the 34 deduced amino acid sequences, 15 are putative trypsins; 15 are putative chymotrypsins; and four are trypsin and chymotrypsin-like protease homologs (OnTry12, OnTry13, OnCTP15 and OnCTP16). Thirteen putative trypsins (except OnTry8 and OnTry9) and 14 putative chymotrypsins (except OnCTP10) have typical and conserved sequence motifs for trypsins and chymotrypsin motifs, including N-terminal putative activation residues (Arg or Lys) for cleavage and formation of mature trypsins and chymotrypsins, three catalytic triad residues (His, Asp, and Ser), and six Cys for three disulfide bonds to maintain protein's tertiary structure (Figure 2-1a and 2-1b) (Page and Di Cera, 2008).

Both trypsins and chymotrypsins have an S1 binding pocket which is almost identical in primary sequences and backbone tertiary structures, but an important difference is that residue 189 is a negatively charged Asp in trypsin and a polar Ser in chymotrypsin. This residue lies at the bottom of the S1 binding pocket and determines different S1 pocket chemical properties. This difference was once used to explain the different specificity of trypsin and chymotrypsin (Steitz et al., 1969). However, loops regions of trypsin and chymotrypsin have significant effect on enzyme activity, substrate specificity, and substrate binding specificity (Ma et al., 2005). Generally, chymotrypsins are more flexible in substrate specificity and binding sites than trypsins (Hedstrom et al., 1992), and cleave proteins on the carboxyl side of amino acid residues like Tyr, Phe, Trp, His, Thr, and Gly in *M. sexta* (Peterson et al., 1995). Chymotrypsins usually have Ser or Gly in the S1 pocket. Among 14 putative chymotrypsins, OnCTP2, OnCTP9, OnCTP11, and OnCTP12 had Ser in S1 pocket, while the remaining 10 putative chymotrypsins had Gly at the S1 pocket (Fig. 2-1B). This substitution (Ser/Gly) in the S1 pocket of OnCTPs may have different substrate recognition and binding properties because these CTPs required

certain cofactors and need to be activated by other chymotrypsins (Jiang et al., 1998). Moreover, OnCTP6 has one substitution on the catalytic triad where Ser was replaced by Thr, but we also considered this predicted protein a chymotrypsin (Xu et al., 2007). Two putative trypsins (OnTry8 and OnTry9) and one putative chymotrypsin (OnCTP10) lack one of these criteria, like conserved catalytic residues, or substrate binding residue, and may be non-functional.

3.2 Comparison of serine proteases in O. nubilalis and other lepidopteran insects

The deduced amino acid sequences of 34 putative trypsins, chymotrypsins and serine protease homologous genes, including those from other lepidopteran insects, were used to construct a phylogenetic tree (Table 2-3). The neighbor-joining method was applied to the predicted amino acid sequence identity alignment to classify those serine proteases and homologs into six groups (bootstrap value ≥73) with four trypsin groups (Try-G1, Try-G2, Try-G3 and Try-G4) and two chymotrypsin groups (CTP-G1 and CTP-G2) (Figure 2-2). Among putative trypsin sequences, there were three main branches (Try-G1, Try-G2 and Try-G3). *OnTry10*, *OnTry2* and *OnCTP14* could not be grouped with any of above groups because the bootstrap value (< 50) is too low to be grouped in any of above groups. *OnTry12* and *OnTry13*, *OnCTP15* and *OnCTP16* were trypsin- and chymotrypsin-like protease homologs and grouped in Try-G4 and CTP-G2, respectively, with novel functions.

3.3 Tissue- specific expression profiles of 34 serine protease genes

Expression patterns of *O. nubilalis* protease and homolog genes were analyzed by RT-PCR in eight different tissues: foregut, hindgut, midgut, haemolymph, Malpighian tubules, carcass, fatbody and silk gland. All were highly expressed in the gut (Figure 2-3a and 2-3b). Putative trypsins of Try-G3 (*OnTry11*, *OnTry3*, *OnTry23*, *OnTry22* and *OnTry21*) were highly expressed in the midgut and hindgut, while Try-G2 (*OnTry1*, *OnTry4*, *OnTry5*, *OnTry6*, *OnTry7*, *OnTry8*, *OnTry14* and *OnTry9*) were highly expressed in the foregut and midgut. These data further support the phylogenetic grouping of two main branches (Try-G2, Try-G3). *OnTry1* and *OnTry8* were also expressed in other tissues, including silkgland, fatbody, and Malpighian tubules.

Of the 15 putative chymotrypsins and chymotrypsin-like protease genes, nine were expressed in the foregut and midgut (*OnCTP1b*, *OnCTP3*, *OnCTP4*, *OnCTP7*, *OnCTP8*, *OnCTP10*,

OnCTP14, *OnCTP16*, and *OnCTP17*). Only *OnCTP12* was expressed in both the midgut and hindgut, but not in the foregut. Two putative chymotrypsins (*OnCTP2* and *OnCTP5*) were expressed in all three gut tissues; three (*OnCTP6*, *OnCTP9*, and *OnCTP13*) were midgut-specific genes. Expression of chymotrypsin transcripts were not detected in other tissues. The expressions of two putative chymotrypsins (*OnCTP11and OnCTP15)* and one trypsin-like serine protease (*OnTry13*) were too low to be detected by RT-PCR.

3.4 Transcriptional response of 34 serine proteases to Cry1Ab protoxin ingestion

The expression protease transcripts were analyzed for *O. nubilalis* larvae fed artificial diet with and without Cry1Ab protoxin after four different feeding periods (2, 6, 12, and 24 h). The expression levels of four trypsins, *OnTry4*, *OnTry5*, *OnTry6* and *OnTry14*, were correlated to increasing protoxin exposure (Table 2-4). However, the expression of *OnTry2* was down-regulated in all Cry1Ab exposure time points. The expressions of 15 putative chymotrypsins were not affected by exposure to Cry1Ab for 2 to 12 h. However, four putative chymotrypsins (*OnCTP2*, *OnCTP5*, *OnCTP12* and *OnCTP13*) were up-regulated at least 2-fold (*P*-value$\leqslant$0.05) after 24 h of exposure to Cry1Ab (Table 2-4).

4. Discussion

4.1 Characterization of trypsin and chymotrypsin genes

We identified 15 transcripts encoding putative trypsins, in which two (OnTry8 and OnTry9) lack one or more typical trypsin motifs. Based on phylogenetic analysis, these two homologs were in the group Try-G2, suggesting they may have arisen from mutation events that have been described in other insect species (Lopes et al., 2009).

With the exception of OnCTP10, the remaining putative chymotrypsin transcripts in the CTP-G1 group have predicted proteins that contain conserved catalytic triads (His, Asp and Ser), six Cys forming disulfide bridges, and the conserved activation sites at the N-terminal of the mature enzyme. Among 14 putative chymotrypsins, 11 have the Ser/Gly substitutions in the S1 pocket (Figure 2-1b). OnCTP10 has an Asn/Ser replacement in the S1 pocket, and we have classified this gene product as a protease homolog.

OnTry12 and OnTry13 were trypsin-like protease homologs. OnTry12 and OnTry13 have orthologs in *M. configurata* (accession No: FJ205434) and *H. armigera* (accession No: EU325547) (Campbell et al., 2008, Liu et al., 2009). A trypsin-like protease gene (accession No: EU325547) in *H. armigera* was constitutively expressed and had activity in the insect cuticle during larval feeding, molting and metamorphosis, but its expression was influenced by 20-hydroxyecdysone (20E) and the juvenile hormone (JH) analog methoprene (Liu et al., 2009). The authors suggested that this enzyme is involved in the remodeling of cuticle, and orthologs in *O. nubilalis*, OnTry12 and OnTry13, may have similar function.

OnCTP15 and OnCTP16 were predicted as chymotrypsin-like serine protease homologues, and were associated with orthologs from *M. sexta* (accession No: AM419170 and AM690448), *H. armigera* (accession No: EU325548), and *S. exigua* (accession No: AY820894). In SeC34 (accession No: AY820894), the highly conserved motive 'DSGGP' in the catalytic domain around the active site of chymotrypsin is changed to 'DSGSA'. It also has differential inhibitor preferences comparing to chymotrypsins, e.g. the commonly chymotrypsin inhibitor, TPCK and EDTA, hardly inhibit its activity at all the concentrations tested. This gene was specifically expressed in the midgut of last instar larvae prior to the onset of pupation (Herrero et al., 2005). Another chymotrypsin from *H. armigera* (accession No: EU325548), has the similar sequence and activity properties, was associated with insect gut peritrophic matrix formation (Campbell et al., 2008). In this study, OnCTP15 and OnCTP16 have the same substitutions on the active site (DSGGP were replaced by DSDSA or DSGSA, respectively) may also be important during larval to pupal molting.

4.2 Diversity of trypsins, chymotrypsins, and serine proteases in lepidopterans

The presence of multiple protease genes can be traced back to multi-copy protease genes that arose due to gene duplication and subsequent diversification events (Baptista et al., 1998). Mutation events in gene sequence may lead to amino acid alterations that influence the structural and functional properties of proteases, although these functional differences in insects are not fully understood. Host plants produce proteinase inhibitors to suppress insect proteinase activity as a defense mechanism against insect infestation. However, lepidopteran trypsins evidently have evolved to hydrolyze this kind of protein inhibitor and thereby avoid inhibition (Giri et al.,

1998). It means that insects feeding on food sources containing protease inhibitors are able to express trypsins that can hydrolyze protein inhibitors. The insects overcome the negative effects of plant inhibitors by expressing alternative inhibitor-insensitive proteases and/or up-regulation of other proteases (Giri et al., 1998; Mazumda-Leighton and Broadway, 2001a and 2001b; Lopes et al., 2004). Jongsma (1997) emphasized the co-evolution of plants and herbivores, including plants expressing protease inhibitors as a defense against insects, and the well-adapted species that overcome the effects of the plant inhibitors. Therefore, the multiple, varying protease-encoding genes in many lepidopteran species is an adaption to diverse food source and an adaptive mechanism for reducing the deleterious effects of plant protease inhibitors.

Indeed, the existence of multiple trypsins and chymotrypsins has been noted in the analysis of these enzymes in lepidopteran species, *H. armigera* (Bown et al., 1997). For example, trypsins from *H. armigera, S. nonagrioides*, and *H. zea* were identified and divided into three major groups based on their sensitivities to soybean protease inhibitors and substrate preferences (Bown et al., 1997; Lopes et al., 2004; Díaz-Mendoza et al., 2005). In *M. configurata*, more than 30 serine proteases or homologs were identified, including eight trypsins, nine chymotrypsins and 13 serine protease homologs (Hegedus et al., 2003). Similarly, in this study, we identified 34 serine protease transcripts from an *O. nubilalis* gut specific cDNA library, encoding 15 putative trypsins, 15 putative chymotrypsins, and two trypsin-like and two chymotrypsin-like protease homologs. Most trypsins from lepidopteran insects were found in our classification of Try-G2 and Try-G3 groups. However, all putative chymotrypsins were found in the large group CTP-G1. One chymotrypsin gene (*SlCTLP*) from *S. litura* (accession No. GQ354838) was food-inducible and was suppressed by starvation (Zhan et al., 2010). Therefore, the OnCTPs from CTP-G1 may play similar important role in protein digestion. Therefore, the existence of multiple serine proteases in *O. nubilalis* may be an adaptation to different food sources, as was described for *S. frugiperda*, a polyphagous insect, feeding on at least 10 different families of plants. *O. nubilalis* not only infests corn, but also uses potato, sweet pepper as temporary or alternative hosts (Ebora et al., 1994; Barlow et al., 2004). The alternative hosts expressing different protease-inhibitors, but *O. nubilalis* can express alternative serine proteases, which can overcome different protease-inhibitor inhibition. For example, *O. nubilalis* can overcome chicken ovomucoid, corn trypsin inhibitor, potato protease inhibitor from the artificial diet (Larocquea and Houseman., 1990).

Previous study also found that Try-G2 trypsins from *H. armigera* are insensitive to Kunitz-type soybean trypsin inhibitor, while Try-G3 trypsins from *H. armigera* are sensitive to Kunitz-type soybean trypsin inhibitor (Bown et al., 1997; Lopes et al., 2004). This indicates that the diversity of trypsins must be an adaptation against protease inhibitors of plants and getting used to the diverse food source. In *O. nubilalis*, the tryspin genes in these two groups may have their specific roles in the defense against plant protease inhibitors.

The activities of insect serine proteases are also regulated by native protease inhibitors, such as serpins, which form covalent bonds with the serine protease. The wild-type serpin 1 from *Arabidopsis thaliana* has the limited potency to inhibit trypsin activities of *O. nubilalis* gut extracts suggesting *O. nubilalis* can overcome the inhibition of serpin 1 (Breusegem et al., 2010). The diverse serine proteases of *O. nubilalis* not only can overcome plant expressed protease-inhibitors, but also can limit native serpin inhibition.

4.3 Tissue specific expression patterns of trypsin and chymotrypsin genes in O. nubilalis

Thirty-four serine protease transcripts had slightly different expression patterns for the eight different tested tissues (foregut, hindgut, midgut, haemolymph, Malpighian tubules, carcass, fatbody and silk gland) (Figure 2-3a and 2-3b). As expected, all of them were highly expressed in midgut tissues, suggesting an important function in dietary protein digestion (Johnston et al., 1991; Purcell et al., 1992). Fifteen putative trypsins of *O. nubilalis* are grouped in two major groups (Figure 2-2). The trypsins of Try-G2 were highly expressed in the foregut and midgut, whereas those in Try-G3 were highly expressed in midgut and hindgut. The differential expression pattern implies that these trypsins may have different functions in food digestion. The tissue specific expression patterns of 15 trypsins were further supported by their sensitivity or insensitivity to Kunitz-type soybean trypsin inhibitor (Lopes et al., 2004). Among the trypsin genes of *A. ipsilon*, *H. armigera*, and *H. zea*, Try-G1 and Try-G3 were sensitive to protease inhibitors and absent or down-regulated in the presence of the inhibitor, whereas trypsins in Try-G2 were insensitive to protease inhibitors and overexpressed or newly expressed in the presence of the inhibitor (Bown et al., 1997; Mazumdar-Leighton and Broadway, 2001a; Erlandson et al., 2010).This is a kind of adaptation for food source containing protease inhibitors. The foregut-midgut (Try-G2) trypsins, which are insensitive protease inhibitors, are the first access to and

digest food sources containing protease inhibitors. This facilitates the midgut-hindgut (Try-G3) trypsins, which are protease inhibitor sensitive, to further digest. It may also be possible that foregut-midgut trypsins play roles in activation of other zymogens, like trypsinogen, chymotrypsinogen and proelastase (de Haen and Gertler. 1974), or in initiating a cascade of events and subsequently regulate late trypsin expression (Barillas-Mury et al., 1995). Indeed, in the female mosquito adults, *Aedes aegypti*, multiple trypsin transcripts expression levels are sequentially induced with feeding periods, and an early trypsin may play a role in initiating a cascade of events with the subsequent expression of late trypsins (Lu et al., 2006; Venancio et al., 2009). The foregut-midgut specific trypsins of *O. nubilalis* may have the same roles in midgut-hindgut trypsinogen, chymotrypsinogen and proelastase activation.

We also found that *OnTry1* and *OnTry8* were also slightly expressed in silk glands, and may function on silk production (Cohen et al., 1993). *OnTry1* and *OnTry21* were expressed in all the tested tissues suggesting that they may be involved in other important physiological functions, such as insect molting (Sui et al., 2009).

4.4 Trypsins and chymotrypsins function in Bt toxicity

The diversity of serine proteases would be an example of adaptive evolution leading to a diversification of digestive enzymes. The adaptation of *O. nubilalis* to anti-nutrients in plants may be an advantage to survive sublethal concentrations of Cry toxins from transgenic corn, possible under certain environmental conditions. Understanding the functions of serine proteases in *O. nubilalis* will be important in understanding mechanisms of resistance that might develop in *O. nubilalis*.

The down-regulated serine proteases could be implicated in Bt resistance if they are involved in the solubilization and activation of Bt protoxins (Oppert et al., 1997). Several studies have shown that trypsins hydrolyze Bt protoxins or toxins, and have been genetically linked to Bt resistance (Oppert et al., 1997; Herreo et al., 2001; Li et al. 2004; 2005). For example, midgut enzymes from a Bt resistant strain of *H. virescens* were reported to activate the protoxin slower but to degrade the toxin faster than the enzymes from a susceptible strain (Forcada et al., 1996, 1999). Another study also found that when Bt protoxin was used in mosquito control, trypsin

was the major gut enzyme that activated the 130 kDa Cry4B protoxin into a 65 kDa toxin (Puntheeranurak et al., 2004). However, activated toxin was able to efficiently control mosquito larvae because it was resistant to hydrolysis by midgut proteases. In our lab, previous studies also noted that soluble trypsin-like proteinase activity of a resistant *O. nubilalis* strain was approximately half that of a susceptible strain (Li et al., 2004). Reduced trypsin-like activity was attributed to reduced expression of the transcript OnT23 in Bt-resistant *O. nubilalis* (Li et al., 2005).

In this study, four putative trypsins (*OnTry4*, *OnTry5*, *OnTry6* and *OnTry14*) were up-regulated in *O. nubilalis* larvae after the ingestion of Cry1Ab protoxin. If these genes products are involved in the activation of Bt protoxins, the up-regulation of these genes could lead to an increased susceptibility of the larvae to Bt toxins. On the other hand, if these genes products are involved in the degradation of Bt protoxins and toxins, the up-regulation of these genes may lead to decreased susceptibility of larvae to Bt toxins. In a resistant strain of Indianmeal moth (*P. interpunctella*), the lack of a major gut protease activity, PiT2 (accession No: AF064526), was responsible for about 90% of the resistance to Cry1Ab protoxin in a *B. thuringiensis* subsp. *entomocidus*-resistant colony (Oppert et al., 1994; 1996 and 1997). In the fall armyworm (*S. frugiperda*), a trypsin SfT6 (accession No: FJ940726) was demonstrated by RNAi that it plays a role in Bt Cry1Ca1 susceptibility because the knockdown of SfT6 can reduce the susceptibility to a Bt Cry1Ca1 protoxin (Rodriguez-Cabrera et al., 2010). In phylogenetic tree analysis, OnTry4, OnTry5, OnTry6 and OnTry14 in *O. nubilalis*, which were up-regulated after 6 h Cry1Ab protoxin exposure, were in the same group with SfT6 and PiT2 and share 79%, 69%, and 68% identities and are in the same group (Try-G2) with. It is possible that those genes may contribute to protoxin activation.

The functional diversity of chymotrypsins may also be an adaptive mechanism that could allow insects survive on Cry toxins expressed in Bt transgenic plants. Trypsins are important in Bt protoxin activation (Oppert et al., 1997), however, chymotrypsin may be more important in Bt protoxin solubilization and degradation (Oppert., 1999). For example, protoxin insensitivity was found in *H. armigera* because the activated toxin was degraded by midgut juice (Shao et al., 1998). Chymotrypsin inhibitor (TPCK) showed the strong inhibitory effects against *H. armigera*

gut juice and prevented further degradation of activated toxin. Thus, chymotrypsin played a major role in toxin degradation in *H. armigera*. Similarly, larval gut juice of *C. fumiferana* can reduce or even eliminate toxin rendering *C. fumiferana* less susceptible to Bt toxins than *B. mori* (Pang and Gringorten, 1998).

In our study, we found four putative *OnCTPs* (*OnCTP2*, *OnCTP5*, *OnCTP12*, and *OnCTP13*) in *O. nubilalis* that were significantly up-regulated after 24-hour of exposure to Cry1Ab. The chymotrypsins encoded by those transcripts may contribute to the degradation of activated Cry1Ab toxin in *O. nubilalis*, especially OnCTP2 and OnCTP12 which were up-regulated more than 10 fold after 24 h exposure to Cry1Ab. OnCTP2 and OnCTP12 also have high pI values (above 9.0) which is an adaptation to the high pH of lepidopteran midgut contents. Because chymotrypsin degrade activated toxin or/and receptor bound toxin, it might be a reason why chymotrypsin up-regulation (after 24 h exposure) were much later than trypsin up-regulation (2 h to 6 h exposure). Further research is needed to clarify the role of these putative chymotrypsins in Bt protoxin toxicity in *O. nubilalis*.

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Figure Legends

Figure 2-1a-b. Multiple alignments of deduced amino acid sequences of 15 putative trypsins (a) and 15 chymotrypsins (b) from *O. nubilalis* larvae using Clustal W2. The predicted signal peptide is highlighted in orange; the catalytic triads and conserved regions are squared in red; the conserved catalytic triads are marked with blue arrow at the top; the autocatalytic site is marked with green arrow at the top; and the conserved residue in the S1 pocket (or trypsin-determination residue) is marked with purple arrow.

Figure 2-2. Phylogenetic analysis of autocatalytic amino acid sequences of 34 serine proteases from *O. nubilalis* with serine proteases from *P. interpunctella*, *C. fumiferana*, *H. armigera*, *M. sexta*, *B. mori*, *A. ipsilon*, *S. nonagrioides*, *S. frugiperda, S. litura*, *H. zea, B. mandarina, H. punctigera*, *M. configurata*, *S. exigua*, *L. oleracea*, *G. mellonella*, *O. furnacalis*. Bootstrap values are obtained by neighbor-joining method using 5000 replications. The blue "▲" indicated the trypsin genes that have already been submitted to the NCBI database; and the red "▲" indicates new cDNA sequences that were revealed from this study and will be submitted to GenBank. The GenBank accession numbers of the sequences used in this analysis are listed in Table 2-4.

Figure 2-3a-b. Expression of 31 serine protease genes in eight different tissues including foregut (FG), hindgut (HG), midgut (MG), haemolymph (HL), Malpighian tubules (MT), carcass (CA), fatbodies (FB) and silk gland (SG) from the ECB larvae. The expression levels of *OnTry13*, *OnCTP15* and *OnCTP11* were too low to be detected by RT-PCR. *O. nubilalis* ribosomal protein S3 (*RPS3*) was used as a reference gene.

Table 2-1. Sequences of primers used in RT-PCR and qRT-PCR analyses.

Gene	Primer sequence (5`-3`)	Product Length (bp)	Group
OnTry1	ATGCGTACCTTCATCGTTCTAC GCCATCTCAGGGTATTGGTTAATG	116	Try-G2
OnTry4	ACCTGTCCATCATCCGAACC TCAGACGACGATCCTCCTTG	157	
OnTry5	GGACAGTTCTCTGAGCAGTTAC ACAGCATGTTGTCAGTGATGG	109	
OnTry6	ATTCTCAACAACAGGGCTATTTTG TGTAGTCAGGGTGGTTAATGATTC	148	
OnTry7	CATCACGGAGAACATGCTTTG CGTTGACACCAGGGAAGAAG	158	
OnTry8	TGTTTCATCGGTACTGTCACTG GAGGATCACTCGTCTGTTAAGG	193	
OnTry9	GAGTGGGGTCTTCCTTCAGG CAGCAATGTCGTTGTTAAGCG	105	
OnTry14	GCATCATACCCGTCACATCTAC GTGAAGTTGCCGTACTGAGTC	148	
OnTry11	CTGGTGGAGTTATTGCCTACG GTGGTTTGCTGGATGGATGG	133	Try-G1
OnTry3	TCAGACTGGTCACCCCTTTC TCACGGCATAGGTTGTTGTTG	196	
OnTry22	ATGGCGTCCTCGTTGGTG TGGTGCCTCCCACAATGC	82	
OnTry23	GAGACCACCATCAACGAGTATC ATGTTAGCAGCACACGACTG	92	
OnTry21	CAACTACGCCACTCTCTCATC GACGCCAGGGAAGCCATC	175	
OnTry10	CACAAAGTCCTGGAGGAAGATTC GTTCACGCCTGTCTGTTGC	103	
OnTry2	CACAAAGTCCTGGAGGAAGATTC GTTCACGCCTGTCTGTTGC	125	
OnTry12	GCCAGCATTACACCTTCCG TCGCAGTTCTCGTAGTAAGAC	128	Try-G4
OnTry13	CATCATCATCCACCCAGACTATG GGCACCGTCCTCTTCCTC	187	
OnCTP1b	ACCTGCCTACCAGCGTTTC CCGAAGCCTGAAGCAATAGC	112	CTP-G1
OnCTP4	GCTGGTTCCCTCTACTGGTC GAGATGGTGTTGGAGAAGGC	79	
OnCTP5	TTGCGGGATACGGGAAGAC GGAGATTGACCGAGTGGAGAG	85	
OnCTP3	TGTGATCCAGCCCATCTCTC CAGAAGTGCGTCCGAATCC	95	

OnCTP17	CCCCTTCGTCCACGCTAG GTCACACCAACCAAGAGTCTC	123	
OnCTP7	TTGCGGGATACGGGAAGAC GGAGATTGACCGAGTGGAGAG	75	
OnCTP6	TCGGGACAACTGGTCTAGC CGCACTCGTCGTTAGGTATC	114	
OnCTP8	GCCGCTGGATTTGGAAAGAC GAGGGTGCTCGGGAATACG	135	
OnCTP9	TCAGTGGAACCCGTGGAAC CAGTGCGATTGGTTGGATGG	94	
OnCTP10	CCTACTGAGGATGCGAATAACG TGGGTTGGCTGGGTTTGG	96	
OnCTP11	ATAGAGCACCCGAATTACAACG GTAGGTTTGCGAGCCAGTG	123	
OnCTP12	CCAGATCAACCGCATCGC TTCCTGAAGAAGCCAGTAAACC	110	
OnCTP2	GAGGAGGGCACGGACTTC TTCCTGTGTTCAAGGTGATGAC	106	
OnCTP13	TCTTCTCAACCACGACTTAACG ATTACTTGAAGCGACACAAATCTG	117	
OnCTP14	GTAAGACTGGTCGGTGGTAAAG TCGGCTCCAAGAACACAATG	149	
OnCTP15	CCATAGGTGAGAAGGAATGTGC GTTGGTCTTCAGCGATACTAGAG	164	CTP-G2
OnCTP16	TCCTCGCCTGTGGTGTTC GATGGTGGTCACGGTCAAC	156	

Table 2-2a-b. Characteristics of 34 serine protease and homologs

Table2-2a: characteristics of 15 putative trypsins and 2 trypsin homologous cDNAs and their deduced protein sequences from *O. nubilalis* larval midgut.

Trypsin and trypsin-like genes	ORF (bp)	Amino acids	Molecular mass (kDa)	Isoelectric point (pI)	Accession No
OnTry3	789	263	28.2	5.47	AY953064.1
OnTry10	753	251	26.8	8.33	TBS*
OnTry11	786	262	28.8	4.48	TBS
OnTry21	768	256	25.9	4.78	EU673450.1
OnTry22	771	257	27.4	7.64	EU673451.1
OnTry23	843	281	29.5	7.14	AY513650.2
OnTry1	768	256	27.3	8.99	AY953063.1
OnTry4	771	257	28.0	9.19	TBS
OnTry5	774	258	27.4	8.55	TBS
OnTry6	684	228	24.9	8.35	TBS
OnTry7	774	258	28.1	6.04	TBS
OnTry8	774	258	27.4	9.45	TBS
OnTry14	858	285	31.4	7.07	TBS
OnTry9	783	261	27.7	6.70	TBS
OnTry2	1185	395	42.1	7.39	AY513652.2
OnTry12	924	308	32.7	5.04	TBS
OnTry13	1005	335	36.2	4.96	TBS

Table2-2b: Characteristics of 15 putative chymotrypsins and 2 chymotrypsin homologous cDNAs and their deduced protein sequences from O. nubilalis larval midgut.

Sequence name	ORF (bp)	Amino acids (aa)	Molecular weight (kDa)	Isoelectric point (pI)	NCBI accession No.
OnCTP1b	867	289	30.6	7.66	AY953053
OnCTP2a	840	280	29.3	9.12	AY953056
OnCTP3	861	287	29.6	7.67	EU673454
OnCTP4	882	294	30.9	5.48	TBS*
OnCTP5	891	297	31.4	5.51	TBS*
OnCTP6	852	284	30.1	8.51	TBS*
OnCTP7	849	283	29.7	8.79	TBS*
OnCTP8	894	298	31.8	6.36	TBS*
OnCTP9	888	296	31.5	7.04	TBS*
OnCTP10	843	281	30.9	5.36	TBS*
OnCTP11	903	301	32.1	9.16	TBS*
OnCTP12	840	280	29.6	9.71	TBS*
OnCTP13	939	313	33.8	8.75	TBS*
OnCTP14	738	246	27.3	5.97	TBS*
OnCTP15	834	278	30.5	5.87	TBS*
OnCTP16	843	281	29.5	7.41	TBS*
OnCTP17	969	323	33.6	5.94	TBS*

"*"To be submitted to GenBank .

Table 2-3. GenBank accession numbers for insect putative trypsins, chymotrypsins and their homologs that were used in phylogenetic analysis.

Organisms	GenBank accession NO
Bombyx mori	DQ443145, AB117641, AB003670, DQ443284, DQ311255
Agrotis ipsilon	AF261974, AF261975, AF261973, AF261971 AF161970, AF233730, AF233729, AF233728
Spodoptera frugiperda	FJ940726, AY251276
Mamestra configurata	FJ205402, FJ205440, FJ205428, FJ205434, FJ205424, FJ205413,
Spodoptera litura	EF635223, GQ354838,
Spodoptera exigua	AY820894
Manduca sexta	AM690450, L34168, AM419170, AM690448, AM690449
Ostrinia furnacalis	DQ356009, DQ486902
Sesamia nonagriorides	AY587147, AY587146, AY587157, AY587161, AY587152, AY587155, AY587150, AY587163,
Onstrinia nubilalis	AY953063, AY513650, EU673451, EU673450, AY953064, AY513652, AY953056, EU673454, AY953053, AY953054,
Galleria mellonella	AY040819
Plodia interpunctella	AF064526, AF173496, AF015610, AF173498
Helicoverpa zea	AF261984, AF261983, AF261980, AF261981, AF261982, AF261985, AF233731, AF233733, AF233732
Helicoverpa armigera	Y12269, Y12283, Y12270, Y12276, Y12275, Y12277, EF600059, EF600054, EU325547, EU325548, Y12287, AF045139, Y12279, HM209421, Y12273,
Choristoneura fumiferana	L04749

Lacanobia oleracea	AJ007706
Heliothis virescens	EF531637, EF531642, EF531635, EF531634, EF531638, EF531621, EF531624, EF531625, EF531623, EF531626,
Bombyx mandarina	AY945210, EU672968
Helicoverpa punctigera	AY618890, AY618892, AY618893, AY618895, AY618889

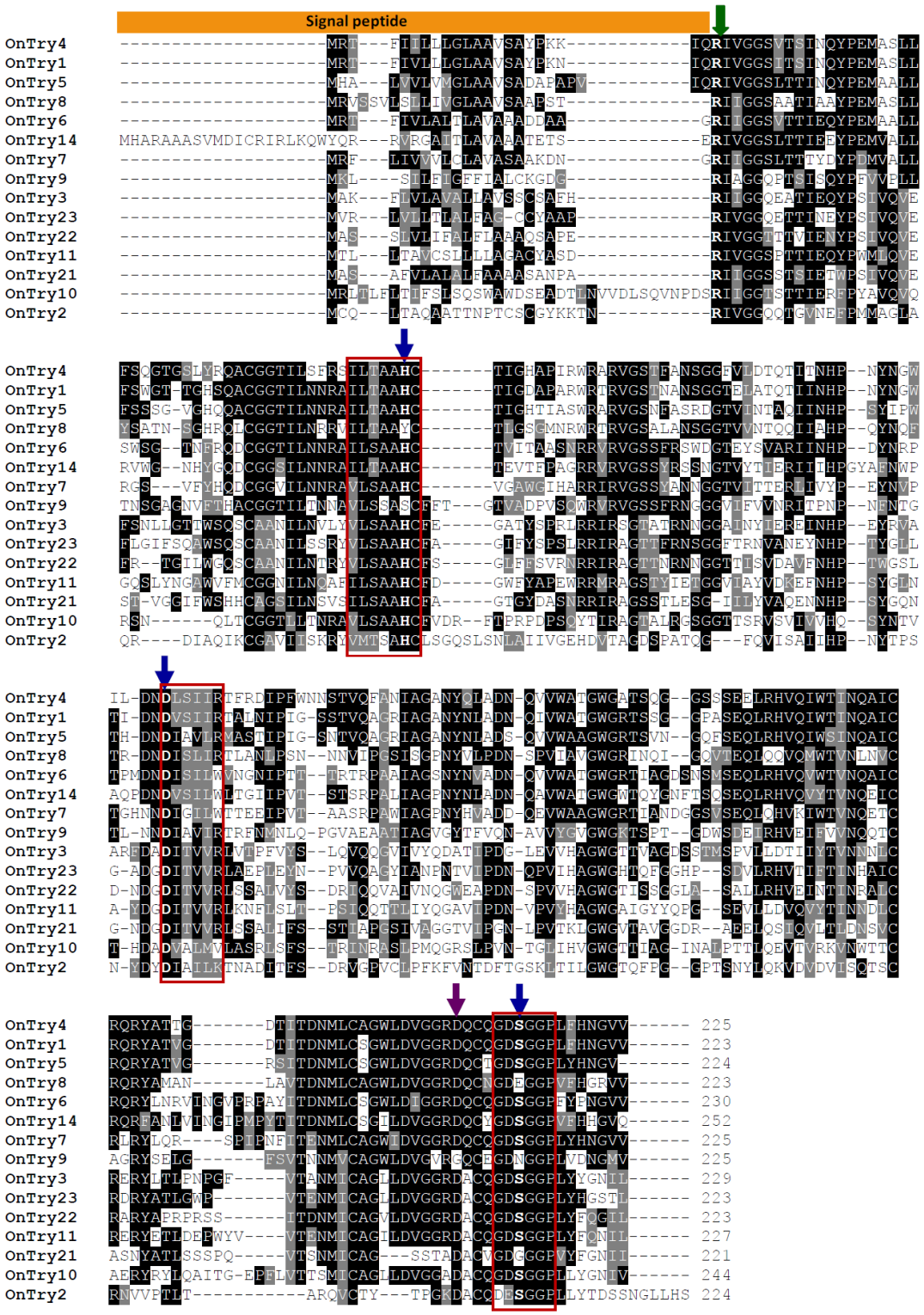
Table 2-4. Relative expression ratio of putative trypsins, chymotrypsins and their homologous genes in the gut of early third-instar larvae of O. nubilalis fed Cry1Ab protoxin at the LC50 concentration relative to larvae unfed the protoxin at different feeding periods.

Gene name	Relative expression ratio in larvae exposed to Cry1Ab (h) (*p*-value)			
	2	6	12	24
OnTry2	-1.79* (0.0329)	-3.34*(0.0012)	-2.31* (0.0175)	-4.51*(0.0003)
OnTry4	1.56	1.36	2.92* (0.0005)	3.14* (0.0045)
OnTry5	2.99*(0.047)	5.73* (0.045)	6.26* (0.0062)	25.18*(0.0063)
OnTry6	11.06*(0.049)	9.22*(0.0169)	4.70*(0.0447)	32.81*(0.0214)
OnTry14	-2.67	3.54*(0.0186)	2.02	14.20*(0.0499)
OnCTP2	-1.10	-1.60	1.80	11.22*(0.0012)
OnCTP5	-6.14	3.00	2.13	4.43*(0.0256)
OnCTP12	-4.57	2.03	2.65	19.12*(0.0163)
OnCTP13	-3.23	-2.01	-1.03	3.39*(0.0041)

The asterisk "*" indicates that the given gene expression significantly different at four feeding periods (*P*-value ≤0.05), whereas the positive "+" and negative "–" signs indicate up- or down-regulation, respectively.

Figure 2-1a-b: Alignment of 15 putative trypsin and 15 putative chymotrypsin sequences deduced from cDNAs of *O. nubilalis*.

a



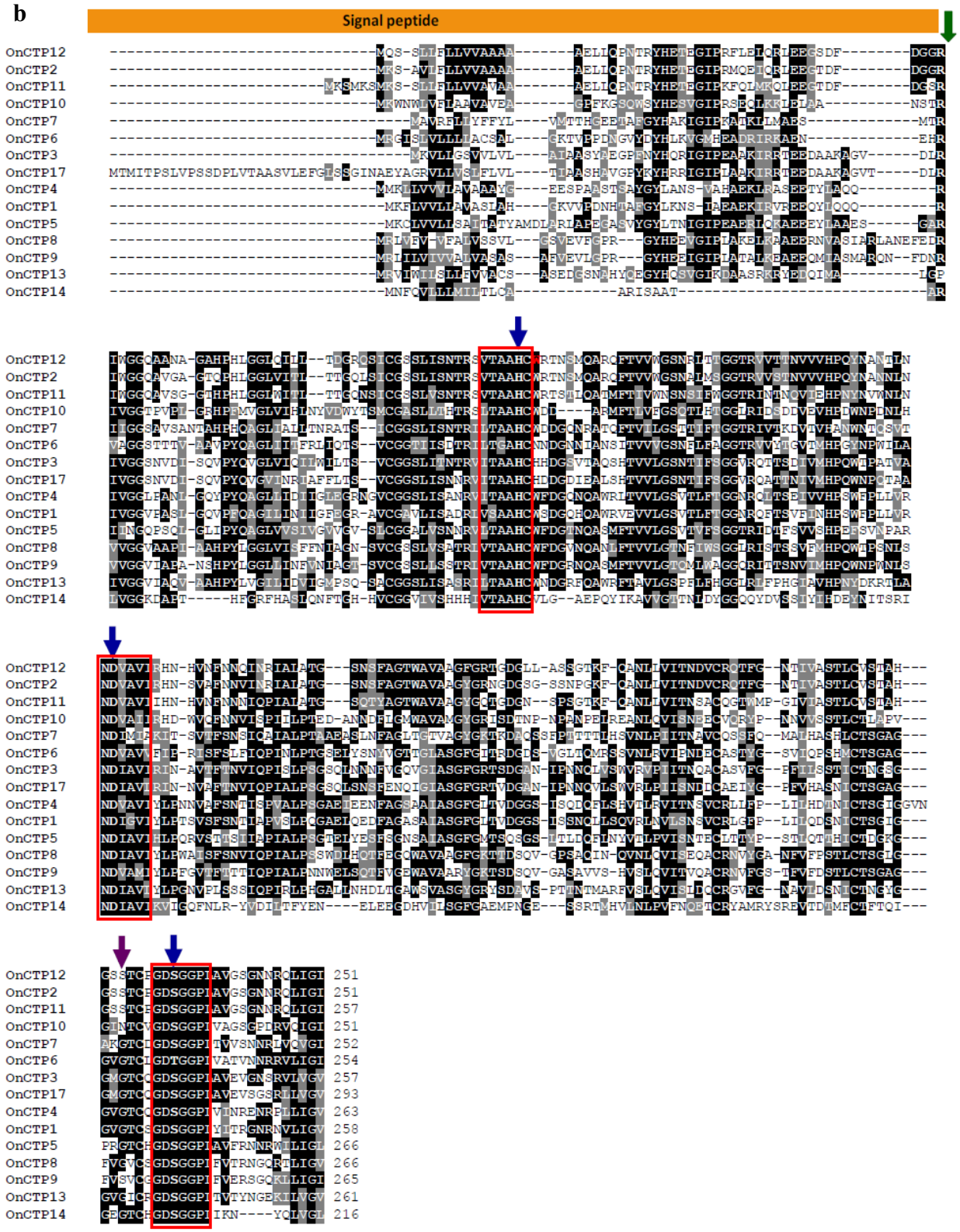

b
Signal peptide
OnCTP12
OnCTP2
OnCTP11
OnCTP10
OnCTP7
OnCTP6
OnCTP3
OnCTP17
OnCTP4
OnCTP1
OnCTP5
OnCTP8
OnCTP9
OnCTP13
OnCTP14

Figure 2-2. Phylogenetic analysis of trypsins, chymotrypsins and serine protease homologs.

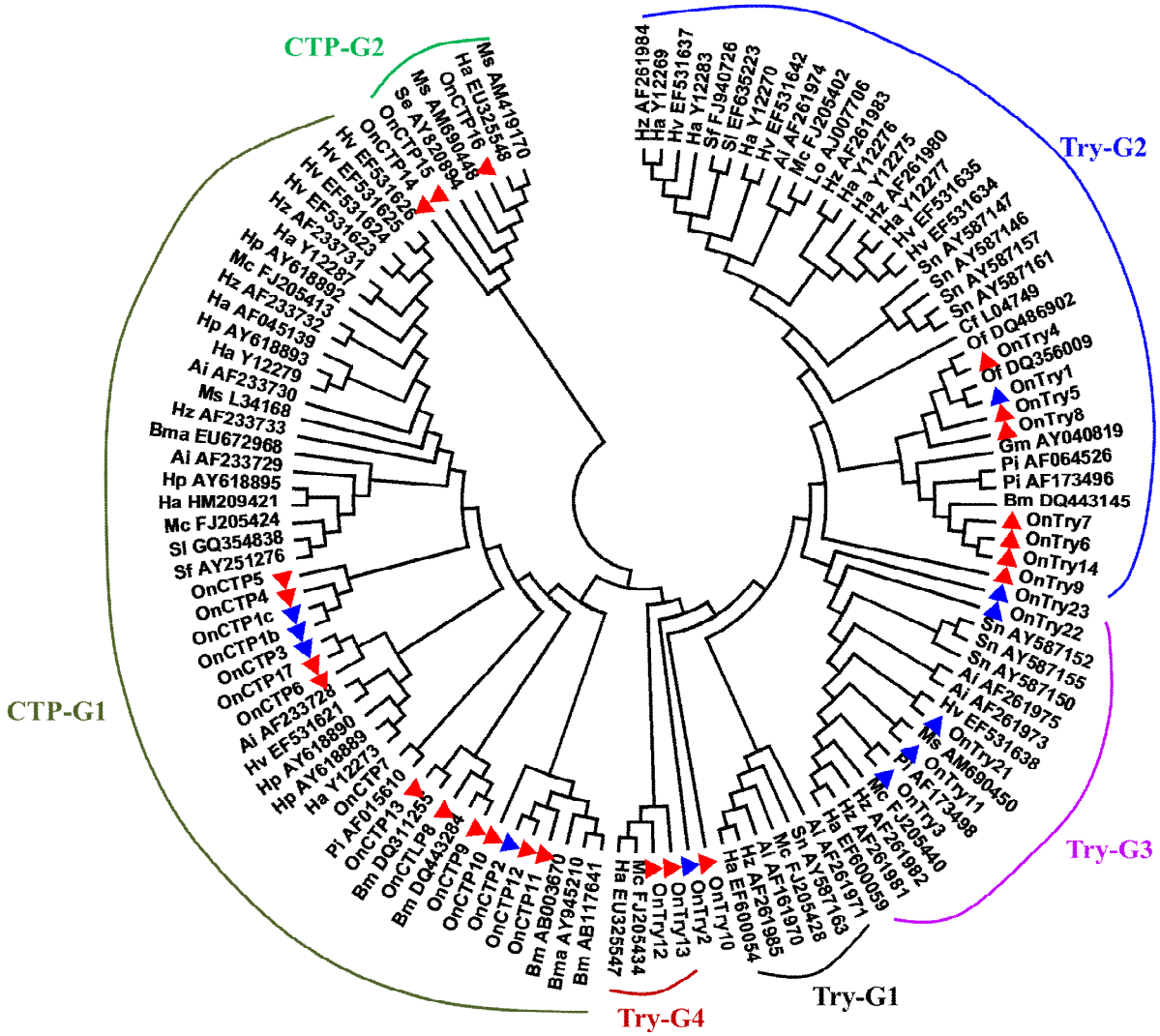
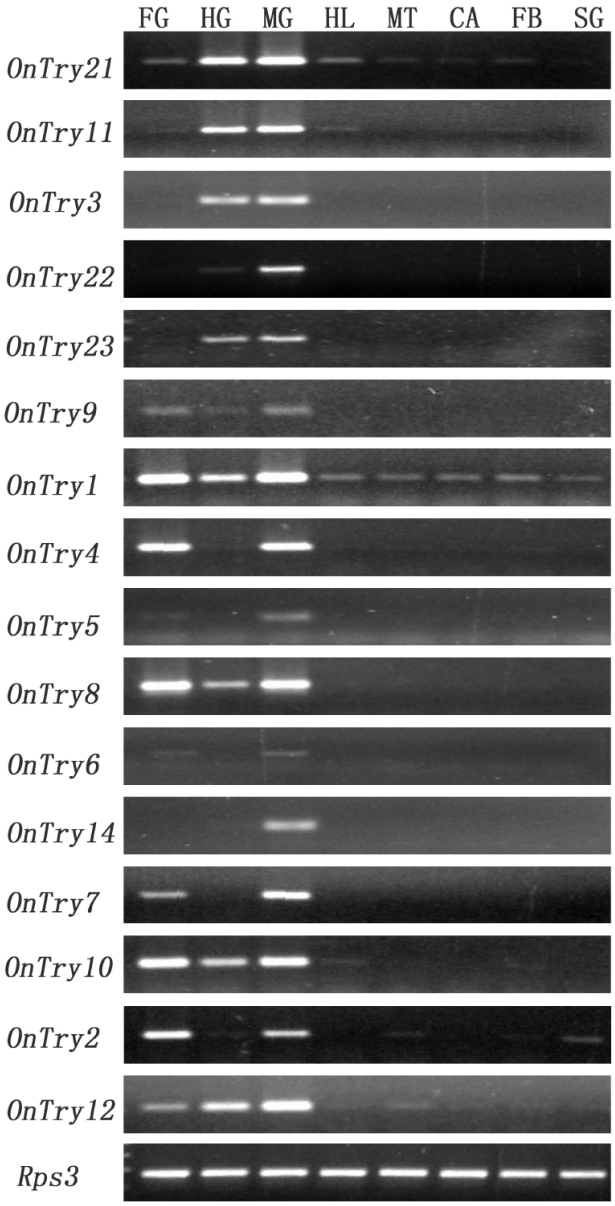
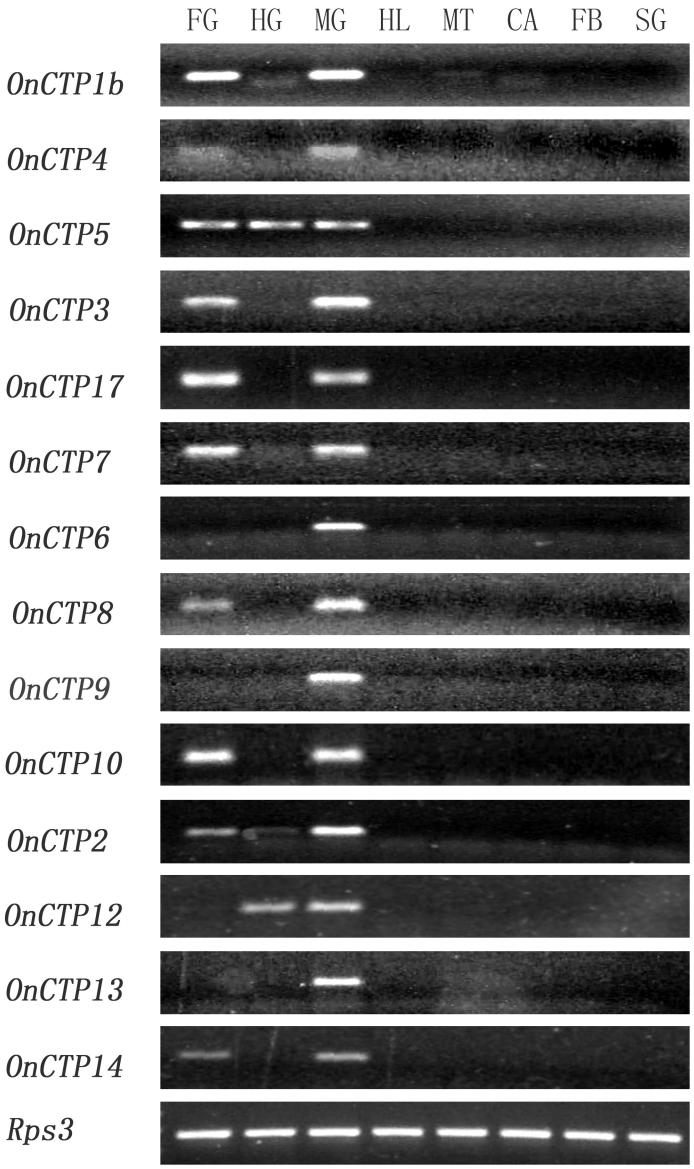


Figure 2-3a-b. 31 serine protease genes relative transcription levels in eight different tissues

a



b


FG
HG
MG
HL
MT
CA
FB
SG
OnCTP1b
OnCTP4
OnCTP5
OnCTP3
OnCTP17
OnCTP7
OnCTP6
OnCTP8
OnCTP9
OnCTP10
OnCTP2
OnCTP12
OnCTP13
OnCTP14
Rps3

Chapter 3 - Changes in gene expression in the larval gut of *Ostrinia nubilalis* in response to *Bacillus thuringiensis* Cry1Ab ingestion

Abstract

The European corn borer, *Ostrinia nubilalis*, is the target pest for transgenic corn expressing insecticidal toxins from *Bacillus thuringiensis* (Bt). We developed a cDNA microarray based on 15,000 cDNA elements representing 2,895 unique genes identified from the *O. nubilalis* larval gut. This microarray was used to monitor gene expression in early third instar Bt-susceptible *O. nubilalis* larvae after 6 h feeding on diet with or without Cry1Ab protoxin. We identified 174 genes from the gut for which the expression was changed more than 2-fold in larvae exposed to Cry1Ab. Thirteen of these genes encode proteins that have been reported in Bt toxicity studies, include eight serine proteases, three aminopeptidases, one alkaline phosphatase, and one cadherin. The expression of trypsin-like protease genes was variable (three were up-regulated and one was down-regulated), but those encoding the three potential Bt-binding proteins, aminopeptidase, alkaline phosphatase and cadherin, were consistently up-regulated after the ingestion of Cry1Ab protoxin. This study is the first large-scale survey of Cry1Ab protoxin induced transcriptional responses in *O. nubilalis* gut tissue, providing a platform for functional studies of toxin-insect interactions.

Key word: Bt-binding protein, European corn borer, Cry1Ab protoxin, microarray, serine protease, transcriptional response.

1. Introduction

The European corn borer, *Otrinia nubilalis*, primarily infests corn, and is responsible for important economic losses in North American agriculture (Showers et al., 1989). Crystal proteins derived from *Bacillus thuringiensis* (Bt) that specifically target insect gut tissue have been used as bio-insecticides to successfully control several lepidopteran, dipteran, and coleopteran insect pests for 15 years (Clive, 2010). Transgenic Bt crops are examples of successful control of insect pests in the field through genetic engineering (Witkowski et al., 1997).

After insects ingest Bt protoxin, there are multiple gut genes products involved in Bt toxicity, from protoxin solubilization, activation, to gut membrane binding, insertion and gut intracellular defense. An initial important factor influencing Bt protoxin toxicity is altered capacity for processing protoxin to Cry toxin. Serine proteases, like trypsins, are important in Bt protoxin activation (Oppert, 1999). Several studies have shown that trypsins hydrolyze Bt protoxins or toxins, and have been genetically linked to Bt resistance (Oppert et al., 1997; Herrero et al., 2001; Li et al., 2004; 2005). For example, midgut enzymes from a Bt resistant strain of *H. virescens* were reported to activate the protoxin slower but to degrade the toxin faster than the enzymes from a susceptible strain (Forcada et al., 1996, 1999). In our lab, previous studies also noted that soluble trypsin-like proteinase activity of a resistant *O. nubilalis* strain was approximately half that of a susceptible strain (Li et al., 2004). Reduced trypsin-like activity was attributed to reduced expression of the transcript OnT23 in Bt-resistant *O. nubilalis* (Li et al., 2005). However, chymotrypsins, may be more important in degradation of activated Bt toxin. For example, a chymotrypsin inhibitor (TPCK) was able to inhibit the degradation of activated toxin by enzymes in *H. armigera* gut juice (Shao et al., 1998).

Bt toxin binding proteins in the gut brush border membrane are another major factor influencing Bt toxicity. In a resistant insect, there may be a reduction in the number of binding proteins and/or a change in the affinity of the toxin for the binding protein. Trans-membrane proteins, like cadherins, have been proposed as toxin binding proteins that facilitate the sequential insertion of toxin in the membrane (Vadlamudi et al., 1993). Genetic studies of *H. virescens*

identified mutations in midgut cadherin and an ABC transporter that conferred resistance to Cry1Ac toxin (Gahan et al., 2001, 2010).

Two models have been proposed to explain the mode of action of Bt toxins on the insect gut cell membrane. In a pore-formation model, toxin initially binds to the high-affinity receptor cadherin, which facilitates a pre-pore oligomeric toxin capable of binding to glycosylphosphatidylinositol-anchored membrane proteins, such as aminopeptidase or alkaline phosphatase, which promotes the insertion of the bound toxin oligomer into the lipid raft of the gut membrane to form pores and ultimately causing the gut cell to burst. In a signal transduction model, monomeric Cry toxins bind to gut cell membrane cadherins, but in this model, cadherin binding directly triggers intracellular signaling pathways involving stimulation of G coupled protein and adenylyl cyclase (AC), increased cAMP levels, and activation of protein kinase A (PKA). The induction of AC and PKA results in cytological changes and cell blebbing, swelling and lysis (Zhang et al., 2006).

Because Bt toxin is capable of binding to multiple gut cell membrane proteins, binding may also trigger multiple intracellular defense mechanisms. In Cry5B induces several cellular defense mechanisms in *Caenorhabditis elegans*, and multiple gut cellular gene products were proposed as defense factors against Cry5B damage, including p38 mitogen-activated protein kinase pathway (Huffman et al., 2004), the unfolded protein response pathway (Bischof et al., 2008), the hypoxia and hypoxic response pathway (Bellier et al., 2009), and the DAF-2 insulin/IGF-1 signaling pathway (Chen et al., 2010). Indeed, more than 106 *C. elegans* genes were described as *hpo* genes to confer resistance to Bt Cry5B (Kao et al., 2011). Similarly, the interaction between Cry Bt protoxin/toxin and *O. nubilalis* gut genes products is complicated with multiple proteins involved.

While Cry toxin have been effective control agents with insecticidal specificity toward diverse insect pests in field, *O. nubilalis* larvae have the potential to develop resistance within a few generations by continuously exposing larvae to artificial diet containing Cry1Ab protoxin (Siqueira et al., 2006). The resistance mechanism to Bt toxin is multifaceted, mirroring the complicated mode of action, involving multiple genes products (Bravo et al., 2008). Changes in *O. nubilalis* that have resulted in resistance include decreased Bt protoxin activation (Li et al.,

2005), decreased sensitivity or the expression level of Bt toxin binding proteins, or increased expression of other intracellular defense proteins in the larval gut cell (Siqueira et al., 2006; Khajuria et al., 2009; Zhang et al., 2006; Kao et al., 2011).

Microarray analysis is a widely used method to identify and analyze insect genes that are differentially expressed under certain conditions, such as insecticide exposure, bacterial infection, injury, etc. For example, microarrays have been used to identify gut gene expression responses of *C. fumiferana* under Bt Cry toxin exposure (Côté et al., 2005) and detoxification gene responses of *A. gambiae* to insecticides exposure (David et al., 2005). In this study, we used a gut specific microarray to examine gene expression responses in Bt-susceptible *O. nubilalis* larvae after 6-h feeding on diet with or without Cry1Ab protoxin.

2. Experimental procedures

2.1 European corn borer

The Bt-susceptible strain (Lee) of *O. nubilalis* was obtained from French Agricultural Research Inc. (Minnesota, USA). Larvae were reared at 26°C using artificial diet (Bio-Service Inc. NJ), and adults were reared in a metal cage under long-day conditions (L: D =16: 8) and 70% humidity, and routinely fed 2% sucrose water to provide supplementary reproductive nutrition. The eggs were collected from the wax paper every day and kept in insect rearing cups with high humidity ($\geqslant$ 80%) until hatching. Newly hatched larvae were immediately transferred to artificial diet and reared to third or fifth instar for testing. The larval developmental stage was determined by moving larvae to a new rearing dish after each molt.

2.2 Cry1Ab protoxin bioassay

The protoxin, at the concentration that kills 50% of early third-instar *O. nubilalis* larvae (LC_{50}=0.25 µg/ml diet) in a 7-day bioassay (Chapter 2), was mixed into artificial diet. Thirty third-instar larvae were starved for 24 h to ensure that they would feed immediately on the experimental diets. Half (fifteen larvae) were fed artificial diet containing (0.25 µg/ml Cry1Ab) protoxin and the other half were fed control diet without Cry1Ab. After feeding for 6 h (no mortality observed), larvae were dissected, and whole gut tissues were removed for total RNA

extraction (3 replicates for each control and treatment group and 5 individuals in each replicate). Six hours was chosen as the time when intoxication of *O. nubilalis* larvae would be evident based on their feeding behavior. Total RNA was independently prepared from the gut tissue from each replicate using TRIzol reagent (Invitrogen Inc. Frederick, MD) according to the manufacture's protocols. The quantity and quality of the total RNA samples were determined by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc. USA) and Agilent 2100 bioanalyzer.

2.3 Microarray analysis

A high-resolution 8×15K multi-pack expression microarray for single-color detection was designed using Agilent's probe design algorithms (Aglient Technologies Inc. Santa Clara, CA). Five oligonucleotide probes from each of 2,895 unique ESTs from the *O. nubilalis* larval gut were computationally designed, and potential cross-hybridization probes were discarded. In total, 12,972 usable probes were obtained from the *O. nubilalis* ESTs, which represent 2,895 unique gut transcripts. Agilent also provided the background control and the standard control probes. Agilent's sureprint inkjet technology was employed to uniformly deposit all oligo probes (60 mers) onto special prepared glass slides. Each glass slide contained eight identical microarray chips.

Cyanine-3-labeled cRNAs were synthesized from Agilent one-color microarray-based gene expression kit. Dye-incorporation ratio was determined using a NanoDrop 2000 spectrophotometry. The cRNAs samples with the ratio cyanine-3 labeled cRNA ≥10pmol/ug were used for hybridization. Cyanine-3 labeled cRNAs (600 ng) of each sample was hybridized to the microarray chip (six samples, including 3 samples from control diet treated and 3 samples of Cry1Ab protoxin diet treated, were hybridized with six individual microarray chips) and incubated at 65˚C for 17 h. Slides were scanned using an Axon GenePix 4000B (Molecular Devices Inc. Sunnyvale, CA) microarray scanner at a 532 nm wavelength. The signal intensity of each hybridized spot was qualified and quantified with Agilent Feature Extraction Ver. 9.5 software (Aglient Technologies Inc. Santa Clara, CA). Features were flagged using the software algorithm. Normalization was carried out with Agilent GeneSpring GX11 based on the default normalization setting for a one-color experiment. Expression ratios were calculated to compare

the protoxin expression in the treated groups relative to the control groups without the protoxin. Statistical difference between values was determined by student's t-test. The gene expression differences with *P*-value ≤0.05 and expression ratios ≥2.0 were considered significant differences. Gene ontologies of all significantly and differentially expressed genes were analyzed using Blast2go (http://www.blast2go.org) at level 2.

2.4 Quantitative real-time PCR validation

The total RNA for microarray analysis also was used for quantitative real-time PCR. One microgram of total RNA was reverse-transcribed in a 20-µl reaction mixture using Fermentas ReverAid^{TM} First Strand cDNA synthesis kit (Fermentas Inc. Glen Burnie, MD). Quantitative real-time PCR was performed in a Bio-Rad iCycler (Bio-rad Inc, Hercules, CA) using Fermentas SYBR green qPCR kit (Fermentas Inc. Federick, MD). The PCR program was set with an initial denaturation of 94˚C for 5 min, followed by 45 cycles at 94˚C for 10 sec, 56˚C for 10 sec and 72˚C for 10 sec. The specific primers for 13 candidate genes and the endogenous control gene (ribosome protein L18) were designed using Beacon 7 Designer^{TM} (Table 3-1). Relative expression differences were normalized to ribosome protein L18 (RPL18) transcript level, and analyzed by the comparative C_T method ($\Delta\Delta$ C_T) (Paffla, 2001).

3. Results

3.1 Gene expression profiles of O. nubilalis larvae fed on Cry1Ab protoxin

Among the 2,895 unique *O. nubilalis* gut transcripts, 174 gut transcripts were identified for as differentially expressed in larvae fed diet containing Cry1Ab (*P*-value ≤0.05 with expression ratios ≥2.0). These data included 80 down-regulated and 94 up-regulated transcripts (Figure 3-1). Among the 174 significantly and differentially expressed transcripts, 106 had BLAST results (e value <1.0e-3), but 68 did not (Table 3-2); thus, their functions are unknown.

3.1.1 Gut transcripts products may be involved in Bt toxin solubilization, activation, degradation or potential sequestration

Serine proteases constitute the most abundant group of genes (eight genes) that were differentially expressed when exposed to Cry1Ab (Table 3-2), including five trypsin-like transripts (contig4768, contig0389, contig1207, contig0243, and ECB-C-18_B11) and three chymotrypsin-like transcripts (contig3704, contig5740, contig0770), in which three trypsins and three chymotrypsins were up-regulated. There were five carboxylesterase transcripts (contig0115, contig3820, J-ECB-07_G03, J-ECB-09_D02, and ECB-27_F04) that were also differentially expressed after Cry1Ab protoxin ingestion. These genes may be involved in Bt toxicity mechanisms, either by activation, degradation, or sequestration of Cry1Ab protoxin/toxin.

3.1.2 Bacterial recognition molecules and antimicrobial peptides

One peptidoglycan recognition protein (PGRP) gene (EST id: contig2223) was identified with five-fold increased expression ratio under Cry1Ab toxin exposure. Two antimicrobial peptide transcripts, antibacterial protein (J-ECB-60_D07) and hinnavin II antibacterial peptide (gi_133905829), were up-regulated ~3-fold, and 7-fold, respectively. Up-regulation of these transcripts may be a defense mechanism to respond the septicemia that results from pore formation during Cry toxin activity. Moreover, three transcripts, encoding proteins similar to caspase-4 and ctl-2 antioxidant enzyme (contig0492 and contig5143) were up-regulated, and these transcripts may be involved in immune and intracellular daf-2 insulin pathways in response to defense Cry toxin attacking (Table 3-2).

3.1.3. Potential gut membrane toxin binding proteins

One cadherin-like transcript (J-ECB-25_B09) was up-regulated ~2-fold in this analysis. Cadherins serve as a high-affinity receptor for Cry1Ab in *O. nubilalis* (Flannagan et al., 2005). In this array analysis, we founded that transcripts encoding three aminopeptidases and one alkaline phosphatase were differentially regulated after 6 h protoxin exposure. An alkaline phosphatase (contig5858) and aminopeptidase (contig4776) transcript were up-regulated ~2-fold; however, two aminopeptidase transcripts (contig4879 and ECB-V-05-D12) were down-regulated ~2-fold (Table 3-2). Aminopeptidases have been proposed as to be Cry1Ab toxin binding proteins in *O. nubilalis* (Khajuria et al., 2009).

3.1.4 Other genes

Fifty-one transcripts identified in the microarray are potentially involved in diverse metabolic processes, such as xenobiotic, amino acid, carbohydrate, lipid, gut chitin metabolism (Table 3-2). Expression levels of most genes related to metabolism were down-regulated, probably due to the stress after the ingestion of Cry1Ab protoxin. Eleven transcripts (α-amylase, glycoside hydrolase, glucose phosphate dehydrogenase, glucosyltransferase, enolase, and hydroxybutyrate dehydrogenase), involved in carbohydrate metabolic pathways (from glycogen to glucose and ketone body metabolism), were down-regulated. Similarly, six transcripts involved in lipid metabolic pathways, such as alkaline ceramidase-like enzyme, lipase and desaturase, were also down-regulated.

3.2 Verification of gene expression by quantitative PCR

We validated selected serine protease transcripts with significantly differential expression ratios in the microarray using quantitative PCR. The transcription levels of six genes putatively encoding protein digestion enzymes, including three trypsins (contig4768, contig0389, contig1207) and three chymotrypsins (contig3704, contig5740, contig0770), were up-regulated after Cry1Ab ingestion. The expression levels of two chymotrypsins (contig1207 and contig0389) and two trypsins (contig3704 and contig4768) expression levels were increased by more than 6.0 fold. The expression of two serine protease genes (contig0234 and ECB-C18-B11) was also validated to be down-regulated by more than 2.0 fold (Figure 3-2).

Five transcripts putatively encoding Cry1Ab binding proteins were also differentially expressed in response to the ingestion of Cry1Ab protoxin (according to pore-formation model). These transcripts encoded putative cadherin (J-ECB-25_B09), alkaline phosphatase (contig5858), and aminopeptidases (contig4776, contig4879, ECB-V05_D12) that were also validated by qPCR. Three transcripts (one cadherin-like, one alkaline phosphatase-like and one aminopeptidase) were up-regulated by approximately 2.0 fold, whereas the expression levels of two aminopeptidases (contig4879, ECB-V05_D12) were down-regulated by more than 2.0 fold (Figure 3-2).

4. Discussion

This study was conducted to demonstrate how *O. nubilalis* gut transcripts were influenced by Cry1Ab protoxin. We wanted to use microarray analysis because microarray can simultaneously screen the expression changes of many gut transcripts in reponses to Cry1Ab protoxin. In this microarray containing 2,895 *O. nubilalis* gut transcripts, only 6.0% were differentially expressed after ingestion of Cry1Ab protoxin. However, at this point the analysis was limited by functionally-annotated transcripts/proteins in the database, as only 106 transcripts had homolog descriptions in the database.

4.1 Transcripts may be involved in Bt protoxin solubilization, activation and degradation

After insects feed on Cry protoxin, the solubilized inactive protoxins are cleaved by alkaline gut proteases to yield active toxins (Chroma et al., 1990). The dissolved and activated toxin monomer can interact with gut receptors. Thus, Bt protoxin activation or degradation are primary factors influencing protoxin Bt toxicity (reviewed in Oppert, 1999). According to Bt mode of action, gut serine proteases are first involved in Cry protoxin solubilization, releasing and activation. As expected, *O. nubilalis* gut serine proteases, including trypsins and chymotrypsins, were either up-regulated or down-regulated and potentially could be involved in the proteolysis of Cry1Ab protoxin. Li et al. (2005) reported that larvae of the KS-SC Bt-resistant strain had relatively lower trypsin activity than the susceptible strain, and a trypsin transcript (OnTLP23) was expressed at lower levels in the resistant strain relative to a susceptible strain. This trypsin transcript belonged to the Try-G3 group (Chapter 2), which includes representative enzymes that are sensitive to Kunitz-type soybean trypsin inhibitor. In previous study, the expressions of transcripts related to OnTry25 were not significantly different between resistant and susceptible strains. These observations imply that OnTry23 may be involved in resistance to Cry1Ab by decreasing protoxin activation in some Cry1Ab-resistant *O. nubilalis* strains, but its expression is normally not changed in susceptible insects. However, in this study, we found that the other three trypsin transcripts were significantly up-regulated (>3.0-fold) after 6 h of Cry1Ab protoxin ingestion. Based on phylogenetic analysis, these transcripts (contig4786, contig3704, and contig0770) correspond to OnTry5, OnTry6 and OnTry14 from the Try-G2 group (Chapter 2), similar to OnTry1, or OnTry25 (OnTry25 and OnTry1 come from gene polymorphisms with 97% nucleotide identity). They are in the group that contains enzymes that are insensitive to

Kunitz-type soybean trypsin inhibitor (Lopes et al., 2004). In a resistant strain of *P. interpunctella*, the lack of a major gut protease activity, PiT2 (accession No: AF064525), was responsible for about 90% of the resistance to Cry1Ab protoxin in a *B. thuringiensis* subsp. *entomocidus*-resistant colony (Oppert et al., 1994; 1996 and 1997). In phylogenetic analysis (Chapter 2), OnTry5, OnTry6, and OnTry14 in *O. nubilalis* share 78%, 69% and 68% amino acid sequence identities with PiT2 and were in the same group with PiT2 and. It is possible that OnTry5, OnTry6 and OnTry14 may also contribute to protoxin activation.

While insect gut trypsin mainly participate in Bt protoxin activation, chymotrypsin may be more important in toxin degradation (review in Oppert, 1999). In our study, we observed the up-regulation of three transcripts encoding chymotrypsin proteases (contig3704, contig5740, contig0770). Therefore, up-regulation of these transcripts may be an attempt by the insect to defend Cry1Ab attack by accelerating toxin degradation.

4.2 Transcripts that encode potential Bt toxin binding proteins

In many susceptible insects studied to date, the binding of active monomeric toxin to the transmembrane protein cadherin is an essential step of Bt toxin mode of action (reviewed in Bravo et al., 2008). In a signal transduction model, this binding directly triggers the intracellular protein kinase A pathway and leads to cell lysis. However, in a pore-formation model, toxin binding results in additional toxin processing to form toxin oligomers, which have high binding affinity for N-acetylgalactosamine residues on N-aminopeptidases (APNs) and possibly alkaline phosophatase (ALPs). It is unclear why the binding protein-cadherin-like protein (J-ECB-25-B09) was up-regulated, but worthy to further clarify cadherin activity after Cry1Ab protoxin ingestion.

In this study, one transcript encoding APN was up-regulated and two were down-regulated. APNs have been proposed as receptors of Bt Cry toxin in several lepidopteran species, such as *M. sexta* (Knight et al., 1994), Asian corn borer (*O. furnacalis*) (Xu et al., 2011), sugarcane borer (*Diatraea saccharalis*) (Yang et al., 2010), and cotton bollworm (*Helicoverpa armigera*) (Rajagopal et al., 2002). The injection of dsRNA for an APN gene in *S. litura* resulted in reduced transcript levels and decreased susceptibility to Cry1C toxin (Rajagopal et al., 2002). Moreover, the APN-N1 gene was not expressed in a lab-selected Cry1Ac resistant colony of *S. exigua*

(Herrero et al., 2005). Thus, the down-regulation of two APN transcripts could be another defense mechanism to reduce Cry1Ab binding in *O. nubilalis*. However, the up-regulated APN transcript in this study (contig4776) belongs to the APN2 group. Its role in Cry1Ab toxicity is needed to clarify in *O. nubilalis*.

4.3 Influences of Cry toxin on the gut chitin metabolic pathway

The ingestion of Cry toxin by insects can not only trigger a change in the transcription of the genes potentially involved in Bt toxicity or Bt degradation, but can also affect the expression other proteins that may be involved in gut defense and repair mechanisms. Indeed, ingestion of Cry toxins destroys the epithelial membrane of the insect gut, which leads to the gut contents leaking into the haemolymph and promoting septicemia (Broderick et al., 2006). Therefore, under Cry toxin exposure, larvae may invoke other gut enzymes to repair cell damage and keep an integrated and functional gut system.

In this category, chitin synthase (EC 2.4.1.16) is an enzyme that catalyzes the polymerization of chitin from UDP-*N*-acetylglucosamine (UDP-GlcNAc) monomers and has a role in cell wall biogenesis (Hogenkamp et al., 2005). However, chitinase (EC 3.2.1.14) is a digestive enzyme to hydrolyze glycosidic bonds in chitin. Therefore, chitin synthase and chtinase have the opposite functions in chitin metabolic pathway. Other studies indicated that chitinase can potentiate the efficacy of Bt toxin (Kramer et al., 1998). Thus, the up-regulation of chitin synthase and down-regulation of chitinase is repair mechanism to keep gut system integrity.

4.4 Gut cellular defense pathways

In the *C. elegan*-Cry5B interaction, functional analyses of cellular defense pathways have been reported (Kao et al., 2010). They identified 106 genes, called *hpo* genes, because the knock-downs showed hypersensitive to Cry5B phenotype, were important for cellular protection against an attack. In *C. elegans, ctl-2* gene functions as an antioxidant enzyme that protects cells from reactive oxygen species, and *ctl-2* expression is negatively regulated by DAF-2 mediated insulin signaling and its activity is required in *daf-2* mutant *C. elegans*. The DAF-2 mediated insulin signaling pathway has been identified as one of the cellular defense mechanisms in *C. elegans* (Chen et al., 2010). In this study, we found that several transcripts related to cellular defense pathways, like *ctl-2*, were down regulated.

Similarly, a plasma membrane glycoprotein encoded by *C. elegans scav*-4 gene, serves as hyposensitive or immune protein against Cry5B attack. In this study, we identified one peptidoglycan recognition protein 2 transcript that was up-regulated ~5-fold, and two other antibacterial protein transcripts (antibacterial protein and hinnavin II antibacterial peptides) were also up-regulated more than 2-fold. All those transcripts may also play same role in *O. nubilalis* immunity responses against Cry1Ab attack.

The results of this study indicate that the use of a cDNA microarray is a productive way to examine insect transcripts' responses to insecticidal agents. The technique can efficiently identify novel transcripts that may be involved in insect-insecticide interactions. Our study provides new insights into the molecular mechanism of Bt and insect interaction and molecular basis of Bt resistance in *O. nubilalis*. However, the usefulness of our data has been limited by the availability of information about the genes in the databases, which may be improved with increased information about gut transcripts and proteins in lepidopterans.

Acknowledgement

We would like to thank Mandar Deshpande of the KSU Integrated Genomics Facility for his technical support, Nanyan Lu of the KSU Bioinformatics Center for her help in data analysis, Dr. Blair D. Siegfried at the University Nebraska-Lincoln for providing us with Cry1Ab protoxin, and Dr. Zurek Ludek and Dr. Brain McCornack for providing us with accesses to the Bio-Rad iCycler equipment and insect rearing room, respectively.

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Figure Legends:

Figure 3-1. Gene expression profiles from the gut of *O. nubilalis* larvae fed Cry1Ab protoxin. The gene transcriptional responses were compared in larvae fed a diet containing Cry1Ab protoxin and larvae fed a normal diet to obtain the expression ratio (cut-off ≥2.0) and significance (*P*-value ≤0.05). The *P*-value and expression ratios were transformed to negative log and $\log_2$ scale, respectively, in the plot. The solid datapoints represent down-regulated gut genes (total 88) and clear datapoints represent up-regulated genes (total 94).

Figure 3-2. Microarray (spotted bar) and qRT-PCR (outlined diamond bar) analyses of 13 differentially regulated transcripts, including those encoding putative cadherin (CAD) (EST ID: J-ECB-25B09), alkaline phosphatase (ALP) (EST ID: contig5858), aminopeptidase (APN) (EST ID: contig4776, contig4879, ECB-V05_D12), trypsin and trypsin-like serine protease transcripts (TLP) (EST ID: contig4786, contig3704, contig0770, contig0243, ECB-C18-B11), and chymotrypsin and chymotrypsin-like serine protease transcripts (CLP) (EST ID: contig0389, contig1207, contig5740). The fold change of each gene in the microarray and qRT-PCR analysis are marked on the top of each column.

Table 3-1. Sequences of primers used in RT-PCR analysis.

Gene name	Primer sequences	Product size (bp)	EST ID
Trypsin-like serine protease	GGACAGTTCTCTGAGCAGTTAC ACAGCATGTTGTCAGTGATGG	109	contig4786
Trypsin-like serine protease	ATTCTCAACAACAGGGCTATTTTG TGTAGTCAGGGTGGTTAATGATTC	148	contig3704
Trypsin-like serine protease	GCATCATACCCGTCACATCTAC GTGAAGTTGCCGTACTGAGTC	148	contig0770
Trypsin precursor	GCCAGCATTACACCTTCCG TCGCAGTTCTCGTAGTAAGAC	128	contig0243
Silk gland derived trypsin serine protease	CACAAAGTCCTGGAGGAAGATTC GTTCACGCCTGTCTGTTGC	125	ECB-C-18-B11
Chymotrypsin-like serine protease	GGTGCTTGTTAGTATGTT AAACTTCTTTAATTGCTCAG	116	contig0389
Chymotrypsin-like serine protease	ATAGAGCACCCGAATTACAACG GTAGGTTTGCGAGCCAGTG	123	contig1207
Chymotrypsin-2	CCCCTTCGTCCACGCTAG GTCACACCAACCAAGAGTCTC	123	contig5740
Aminopeptidase N	TTCCAAACACATTTTCTTG AAGCGTATTGTCCTCTAT	118	contig4776
Aminopeptidase N	CAGTAGCGATAACATCAC CCAGTCAAGTCTTCTCTA	183	contig4879
Aminopeptidase N	GTCAACGAAATTGTCATC AGTCATATTCTGGCTGTA	109	ECB-V-05-D12
Cadherin-like protein	CTATGTGTTCTCAATCCAA TCGTCGATGTTGACTATC	75	J-ECB-25-B09
Alkaline phosphatase	CGGATTATCTGCTGGGTTTATTTG AGTGTGGGCTCGGTAACG	79	Contig5858

Table 3-2. Summary of 106 significantly differentially expressed (fold change ≥2.0 and p-value≤0.05) transcripts with blast results from *O. nubilalis* larvae in response to the ingestion of Cry1Ab protoxin.

EST id	gene homologs	Expression Ratio (Cry1Ab-treated/Control)
Bt toxin solubilization, activation, degradation or sequestration		
Contig[0243]	trypsin precursor	-2.45
Contig[0389]	serine protease	8.39
Contig[0770]	trypsin-like serine protease	3.16
Contig[1207]	serine protease	7.67
Contig[3704]	trypsin-like serine protease	6.36
Contig[4768]	trypsin-like serine protease	10.75
Contig[5740]	chymotrypsin-2 (chymotrypsin ii)	3.26
ECB-C-18_B11	silk gland derived serine protease	-2.63
Contig[0115]	carboxylesterase [*Loxostege sticticalis*]	3.69
Contig[3820]	carboxylesterase [*Helicoverpa armigera*]	-2.21
J-ECB-07_G03	carboxylesterase [*Loxostege sticticalis*]	3.58
J-ECB-09_D02	carboxylesterase [*Spodoptera litura*]	-3.31
ECB-27_F04	carboxyl/cholinesterase 4A [*Bombyx mori*]	2.06
Potential Bt toxin binding partners		
J-ECB-25_B09	cadherin-like protein	2.85
ECB-V-05_D12	aminopeptidase n	-2.55
Contig[4776]	aminopeptidase n	2.17
Contig[4879]	aminopeptidase n1	-2.31
Contig[5858]	alkaline phosphatase	2.23
Signal transduction		
Contig[0492]	caspase-4 [*Lymantria monacha*]	2.36
ECB-10_C01	pyridoxal kinase [*Bombyx mori*]	-2.38
Contig[5143]	ctl2 antioxidant enzyme [*Aedes aegypti*]	2.32
transporter		
Contig[0814]	sodium-bile acid cotransporter	-5.03

Contig[1314]	potassium coupled amino acid transporter [*Manduca sexta*]	-4.68
Contig[4763]	sodium-bile acid cotransporter [*Aedes aegypti*]	-4.39
Contig[5743]	amino acid transporter [*Bombyx mori*]	-5.40
ECB-21_C09	sugar transporter protein 3 [*Bombyx mori*]	2.52
J-ECB-39_E12	sugar transporter [*Culex quinquefasciatus*]	-2.65
J-ECB-55_E04	monocarboxylate transporter [*Aedes aegypti*]	-3.40
Transcription factor and gene expression		
Contig[3833]	DNA-binding nuclear protein p8 [*Simulium guianense*]	4.89
Contig[4800]	endonuclease-reverse transcriptase [*Bombyx mori*]	2.49
ECB-V-26_F03	histone H3.2-like [*Meleagris gallopavo*]	-2.04
Contig[3869]	Cellular repressor of E1A-stimulated genes 1 [*Tribolium castaneum*]	-3.16
Contig[5038]	MluI cell cycle box [MCB] Binding Factor 2 [*Samia cynthia*]	2.41
Metabolism		
1. Xenobiotics metabolism		
Contig[0004]	glutathione *S*-transferase [*Choristoneura fumiferana*]	-3.53
Contig[2246]	glutathione *S*-transferase 16 [*Helicoverpa armigera*]	-2.59
Contig[0012]	microsomal glutathione transferase [*Heliothis virescens*]	-2.19
ECB-C-03_D08	cytochrome P450 monooxygenase cyp6ab4 [*Bombyx mandarina*]	2.62
Contig[5080]	cytochrome P450 monooxygenase cyp4m5	2.81
J-ECB-21_A02	aliphatic nitrilase [*Bombyx mori*]	-2.43
2. **Lipid metabolism**		
J-ECB-35_D11	alkaline ceramidase-like isoform 1 [*Bombus terrestris*]	-3.09
Contig[0029]	gastric lipase-like protein [*Epiphyas postvittana*]	-2.08
Contig[0140]	pancreatic lipase-like protein [*Epiphyas postvittana*]	-4.66
Contig[1081]	pancreatic lipase-like protein [*Epiphyas postvittana*]	-2.58
Contig[1486]	C-5 sterol desaturase-like [*Acyrthosiphon pisum*)	-4.96
Contig[1897]	C-5 sterol desaturase-like [*Acyrthosiphon pisum*]	-4.48
3. **Carbohydrate metabolism**		
Contig[4242]	alpha-amylase 2 [*Diatraea saccharalis*]	-2.21

Contig[4425]	enolase [*Antheraea pernyi*]	-2.36
ECB-V-12_H04	enolase [*Bombyx mori*]	-2.26
ECB-28_F02	glucose phosphate dehydrogenase [*Axia margarita*]	-2.83
Contig[5232]	glycoside hydrolases [*Aedes aegypti*]	-2.31
Contig[4123]	hydroxybutyrate dehydrogenase [*Heliothis virescens*]	-2.28
ECB-V-05_G12	ecdysteroid UDP-glucosyltransferase[*Bombyx mori*]	-2.27
ECB-V-08_G03	UDP-glucosyltransferase [*Bombyx mori*]	-2.30
ECB-12_E11	UDP-glucosyltransferase [*Bombyx mori*]	-2.08
ECB-V-19_F07	UDP-glucosyltransferase [*Bombyx mori*]	-2.41
ECB-V-22_H08	UDP-glucosyltransferase [*Bombyx mori*]	-2.80
4. **Amino acid metabolism**		
Contig[4515]	gamma-glutamyl hydrolase [*Aedes aegypti*]	-2.50
J-ECB-24_G10	methyltransferase [*Mesobuthus caucasicus*]	2.14
Contig[5690]	methyltransferase-like protein [*Latrodectus hesperus*]	2.92
Contig[1237]	farnesoic acid *O*-methyltransferase [*Ceratitis capitata*]	2.90
J-ECB-30_A09	farnesoic acid *O*-methyltransferase [*Ceratitis capitata*]	2.91
Contig[5679]	phosphoserine aminotransferase [*Antheraea pernyi*]	-2.18
ECB-09_B04	asparagine synthetase [*Bombyx mori*]	3.06
5. Gut chitin metabolism		
Contig[0188]	chtinase 8 [*Drosophila melanogaster*]	-2.74
ECB-V-28_H03	chitin synthase 2 [*Manduca sexta*]	2.16
ECB-C-05_D05	glucosamine-fructose-6-phosphate aminotransferase 2	2.24
6. **Other metabolic enzymes**		
Contig[0077]	caboxypeptidase 4 [*Mamestra configurata*]	-2.25
Contig[0009]	putative carboxypeptidase A-like [*Nasonia vitripennis*]	-2.19
Contig[0019]	glutamate carboxypeptidase-like isoform 1 [*Apis mellifera*]	-3.16
J-ECB-33_G12	juvenile hormone epoxide hydrolase-like protein 1 [*Bombyx mori*]	-3.00
Contig[0557]	juvenile hormone epoxide hydrolase [*Spodoptera exigua*]	-2.17
Contig[1953]	NADP-dependent oxidoreductase [*Bombyx mori*]	-3.27

Contig[3531]	aldo-keto reductase [*Aedes aegypti*]	-2.23
Contig[4521]	aldo-keto reductase [*Bombyx mori*]	-4.40
J-ECB-37_E05	oxidoreductase [*Acromyrmex echinatior*]	-2.37
Contig[4410]	methionine-R-sulfoxide reductase [*Tribolium castaneum*]	-2.46
Contig[3814]	alcohol dehydrogenase [*Bombyx mori*]	-2.07
gi_133906638	retinol dehydrogenase [*Heliothis virescens*]	-3.61
Contig[5542]	acetyltransferase [*Heliothis virescens*]	-2.98
J-ECB-39_F07	cytidylate kinase [*Bombyx mori*]	-2.73
Anti-bacterial related protein		
J-ECB-60_D07	antibacterial protein [*Heliothis virescens*]	2.84
gi_133905829	hinnavin II antibacterial peptides [*Pieris rapae*]	7.13
Contig[2223]	peptidoglycan recognition protein 2 [*Manduca sexta*]	5.04
Others		
Contig[0347]	fatty acid binding protein [*Bombyx mori*]	7.79
ECB-V-18_A08	fatty acid binding protein [*Helicoverpa zea*]	-4.19
Contig[0566]	fatty acid binding protein [*Spodoptera litura*]	-2.17
Contig[1640]	fatty acid-binding protein-like [*Bombus terrestris*]	-2.99
Contig[2896]	lipid storage droplet 2-like [*Bombyx mori*]	2.28
Contig[0407]	sensory appendage protein 3 [*Manduca sexta*]	-17.04
J-ECB-08_B02	sensory appendage protein 3 [*Manduca sexta*]	-9.26
ECB-19_G03	Nose resistant to fluoxetine protein 6 [*Harpegnathos saltator*]	2.56
ECB-V-07_D03	Nose resistant to fluoxetine protein 6 [*Harpegnathos saltator*]	2.66
Contig[5724]	silk protein P25 [*Corcyra cephalonica*]	33.34
Contig[4952]	fibroin light chain [*Corcyra cephalonica*]	53.91
ECB-02_H03	saposin-like protein [*Bombyx mori*]	-2.02
Contig[5293]	trypsin inhibitor [*Bombyx mori*]	90.23
Contig[5386]	tetraspanin E118 [*Bombyx mori*]	2.33
ECB-C-04_H06	tetraspanin D107 [*Bombyx mori*]	-2.70
Contig[5414]	ubiquitin C	2.747
Contig[1085]	cuticular protein 25, RR-1 family [*Anopheles gambiae*)	-4.92

J-ECB-12_F09	globin 1 [*Bombyx mori*]	-2.54
J-ECB-29_G03	IST1 homolog [*Bombus terrestris*]	2.18
J-ECB-32_D06	tetratricopeptide repeat domain 27 (TTC27) [*Culex quinquefasciatus*)	-2.44
J-ECB-47_A02	hepatocyte growth factor-regulated tyrosine kinase substrate [*Tribolium castaneum*]	3.09
gi_133905779	Vanin-like protein 1 [*Culex quinquefasciatus*]	-3.36
ECB-19_B09	Vanin-like protein 1 [*Acromyrmex echinatior*]	2.30

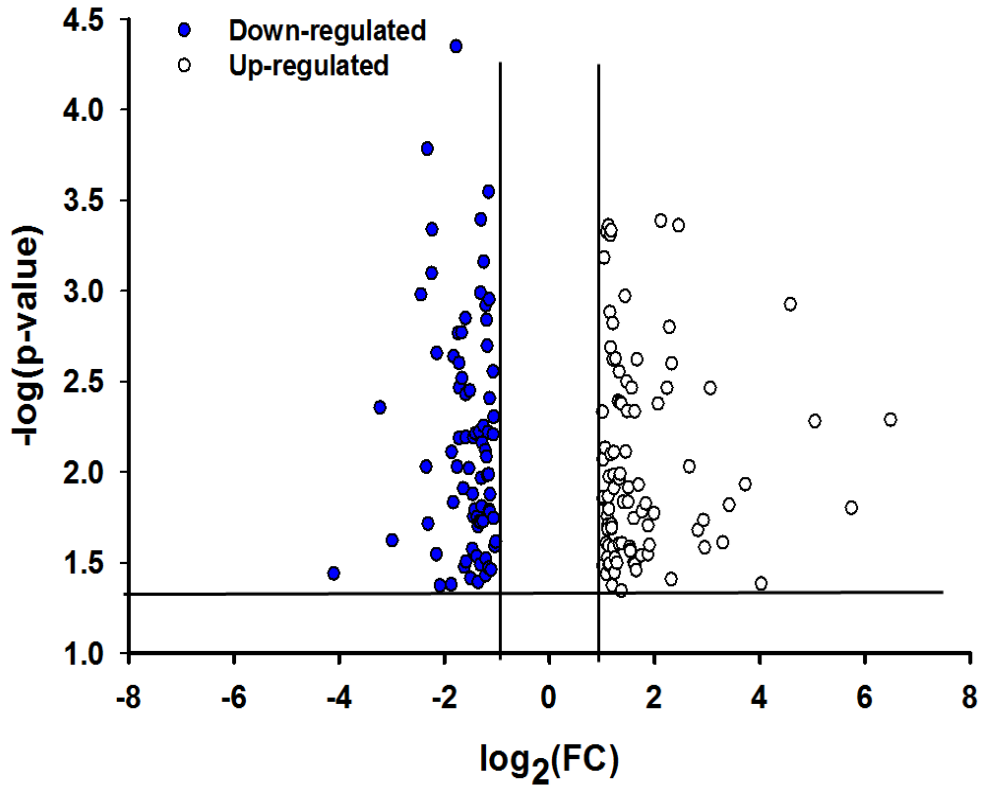

Figure 3-1. Gene expression profiles in the gut of *O. nubilalis* larvae fed on Cry1Ab protoxin

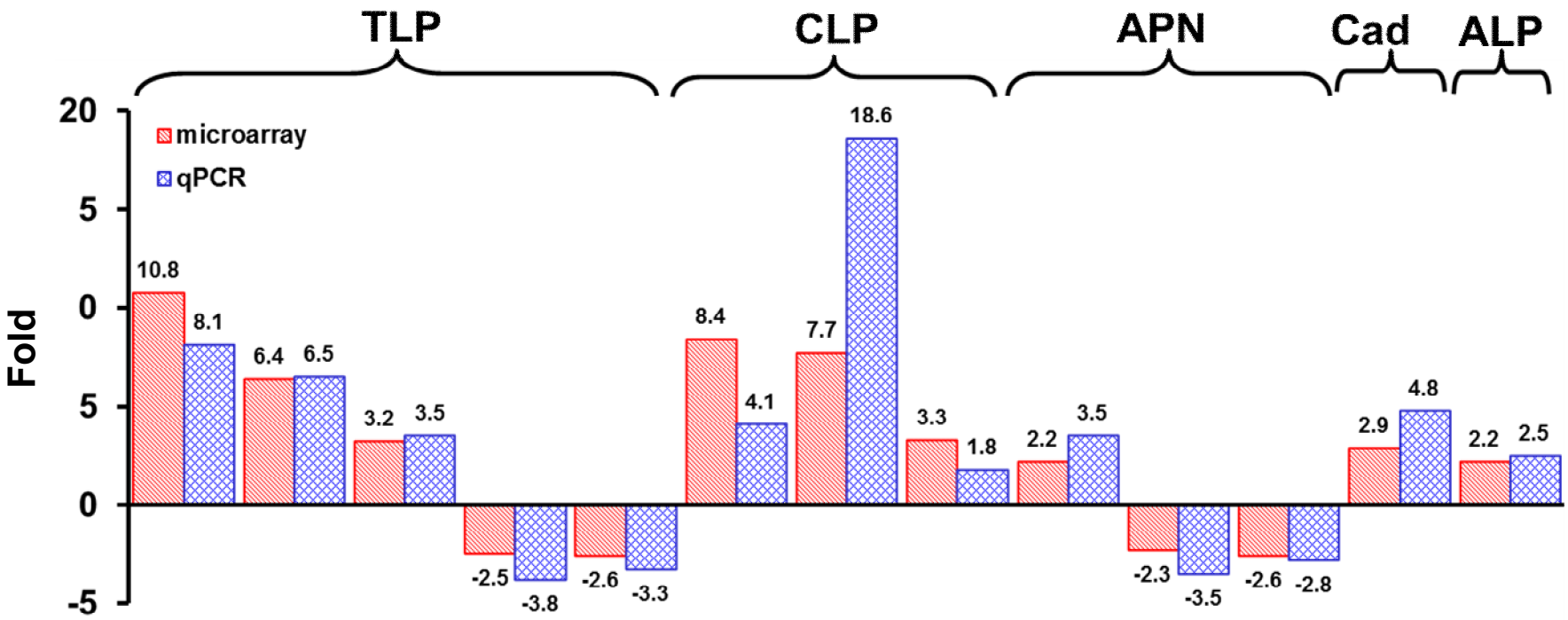

Figure 3-2. Microarray and qRT-PCR analyses of 13 differentially expressed genes.

Chapter 4 - Changes in gene expression in Bt-resistant and susceptible *Ostrinia nubilalis* larvae after ingestion of transgenic Cry1Ab corn leaves

Abstract

Transgenic corn expressing insecticidal Cry1Ab toxin from *Bacillus thuringiensis* (Bt) is effective in controlling the European corn borer, *Ostrinia nubilalis*. However, understanding the response of *O. nubilalis* to transgenic corn may help to develop strategies to prevent resistance in *O. nubilalis*. In this study, we developed a cDNA microarray from an *O. nubilalis* gut-specific EST database, containing 15,000 cDNA elements representing 2,895 unique sequences. We used this microarray to examine gene expression in early third-instar *O. nubilalis* larvae of a laboratory-selected resistant (R) strain and a susceptible (S) strain. The larvae of both strains were starved for 24 h and then fed transgenic Cry1Ab corn (MON810-event) leaves for 6 h. We identified 398 transcripts from the S strain and 264 transcripts from the R strain with significantly increased or decreased expression (expression ratio ≥2.0 fold; p-value ≤0.05) as compared with the control larvae fed the leaves of a non-transgenic corn isoline. When S strain larvae were fed transgenic corn leaves, the number of differentially expressed transcripts and their expression ratios were larger than those R strain larvae fed transgenic corn. Annotated transcripts included those involved in gut defense mechanisms, such as those encoding chitin-modifying and diverse cellular defense proteins. In addition, 17 transcripts in the S strain and 9 transcripts in the R strain were identified that are potentially involved in Bt toxicity or/and resistance, including serine protease and aminopeptidase transcripts. Interestingly, when larvae fed on artificial diet, two aminopeptidase genes (contig1398 and contig4776) have opposite trends in R and S-strain larvae. One aminopeptidase transcripts (contig4776) had a 9-fold higher expression in R-strain than in S-strain larvae, but another aminpeptidase transcript (contig1398) had 4-fold higher expression in S-strain than that of S-strain larvae. However, when R- and S-strain larvae were fed transgenic corn leaves for 6 h, these two aminopeptidase transcripts (contig4776 and contig1398) were down-regulated in S strain larvae, but were up-regulated in R-strain compared to control larvae. This study represents a large-scale evaluation of gut transcriptional responses in *O. nubilalis* larval exposed or unexposed to transgenic corn

expressing Cry toxins. This study also provides an insight into the interactions of transgenic corn and its target insect.

Key word: Bt-binding protein, European corn borer, gene transcriptional response, microarray, serine protease, transgenic Cry1Ab corn.

1. Introduction:

Transgenic corn, *Zea mays*, expressing insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) has become widely adopted in U.S. agriculture. It has been used to suppress European corn borer, *Ostrinia nubilalis*, and has saved Midwest farmers billions of dollars in the past decade (Hutchison et al., 2010). In the field it can kill 99% of first generation *O. nubilalis* (Witkowski et al., 1997). However, if individuals of *O. nubilalis* tolerate transgenic corn, their survival will likely increase when continuously exposed to transgenic corn, prompting concerns that transgenic corn will lose its control efficacy. However, the EPA requires that farmers follow the high dose/refuge insect resistance management strategy, where non-Bt crops are planted near Bt crops in order to maintain a genetically susceptible *O. nubilalis* population. This strategy is designed to prevent or delay the development of resistance in *O. nubilalis* (Onstad et al., 2008). Other resistance management strategies have also been applied to improve the efficacy and improve insect resistance management, including introducing multiple Bt genes (Cao et al., 2002) and combining Bt gene with other insecticidal protein genes (Maqbool et al., 2001) in individual crop events. The potential for the development of resistance has been demonstrated in laboratory-selected *O. nubilalis* colonies. Selection with Dipel-ES resulted in 73-fold resistance to Cry1Ab (LC_{50}) after seven generations of selection (Huang et al. 1997), and selection with Cry1Ab protoxin resulted in 14-fold resistance after 5- to 7-generation exposures (Chaufaux et al., 2001).

Studies in our laboratory on the Dipel-selected *O. nubilalis* strain have demonstrated that the activity of serine proteases, such as chymotrypsin and trypsin, were reduced in the resistant strain suggesting the reduced protease activity in this strain could be a factor in resistance by reducing the rate of protoxin activation (Huang et al., 1999; Li et al. 2004). Bt protoxin activation is one of key steps in Bt toxicity (Oppert et al., 1997). However, this Dipel-selected resistant strain was almost as susceptible to transgenic Bt corn (MON810-event) as the susceptible strain. This suggests that Cry1Ab toxin expressed in corn tissue was already functionally active and did not require solubilizaion and activation by insect gut serine proteases (Li et al., 2007). Therefore, the protease-based resistance in the laboratory-selected *O. nubilalis* did not pose a threat to transgenic plants.

Other studies have determined that resistance based on receptors like cadherin-like protein, aminopeptidase N (APN) and alkaline phosphatase (ALP) are a much more severe threat in transgenic plants. For example, cadherin-mediated resistance was found in a field population of *H. armigera*. Three cadherin alleles (r1, r2 and r3) were found to be associated with Cry1Ac resistance in a field population of *P. gossypiella* and *H. armigera* (Morin et al., 2003 and Yang et al., 2007). In a laboratory selected *H. virescence* strain, a deletion mutation in a cadherin gene was identified and genetically linked with Cry1Ac resistance (Gahan et al., 2007). Aminopeptidase-mediated resistance has also been studied in lepidopteran species. Cry1Ab resistance associated with the aminopeptidase gene N was identified in the laboratory-selected resistant strain of *O. furnacalis* (Xu et al., 2011). The Cry1Ac resistance in *H. arimgera* was associated with a deletion mutation in the aminopeptidase gene N1 (Zhang et al., 2009). Recently, alkaline phosophatase has also been proposed as a potential gene involved in Bt toxicity and/or resistance in a laboratory selected resistant strain of *H. virescens* (Jurat-Fuentes et al., 2004). The resistant larval brush border membrane had the reduced specific alkaline phosphatase activity which contributes the reduced amount of Cry1Ac binding in *H. virescens*.

Binding assays indicated that Bt δ-endotoxin can bind three aminopeptidase proteins and one cadherin protein in the brush border membrane vesicles of *O. nubilalis* (Hua et al., 2001 and Flannagan et al., 2005). Squierira et al (2006) also found that proteins in larval gut extracts of a laboratory-selected Cry1Ab resistant *O. nubilalis* strain demonstrated a lower binding affinity for Cry1Ab toxin than that of susceptible strain, but there was no evidence of a genetic linkage between Bt resistance and a cadherin-like protein. The different expression patterns of several aminopeptidase genes in a Cry1Ab selected resistant strain and susceptible strain of *O. nubilalis* were studied in our laboratory. Khajuria et al. (2011) suggested that changes in two amino acid residues of the aminopeptidase-P like gene (*OnAPP*) involving Glu^{305} to Lys^{305} and Arg^{307} to Leu^{307} were associated with Cry1Ab resistance in two resistant strains of *O. nubilalis*.

The ingestion of Bt Cry toxin has also been found to trigger complicated defense mechanisms in insect gut cells. Therefore, cellular defense genes may also contribute to insect tolerance or resistance to Cry toxin. In the nematode *C. elegans*, several cellular defense mechanisms were

proposed to protect against Cry5B attack, and multiple genes were identified to contribute to Cry5B toxin resistance. For instance, the ingestion of Cry5B toxins triggered multiple *C. elegans* cellular defense pathways, such as the p38 mitogen-activated protein kinase pathway (Huffman et al., 2004), the unfolded protein response pathway (Bischof et al., 2008), and the hypoxia and hypoxic response pathway (Bellier et al., 2009). Chen et al. (2010) proposed that the reduction of the DAF-2 insulin/IGF-1 signaling pathway confers resistance to Bt Cry5B in *C. elegans.* They noted that the *daf*-2 mutants have more than 10-fold resistance to Cry5B as compared with the wild-type. Kao et al. (2011) found 106 *C. elegans* genes that were important for cellular protection against Cry5B attack using global RNAi screening. Those genes were called *hpo* genes because their knock-down caused *C. elegans* to be hypersensitive to Cry5B. Therefore, the resistance mechanism to Bt Cry toxin could be complicated and multifaceted with multiple proteins involved, or might be as simple as a mutation in one major toxin binding protein.

Any changes in gut proteins of *O. nubilalis*, from proteases, membrane binding partners to intracellular defense gene products, either through differential expression or mutation, may contribute to Bt Cry toxin resistance. Even though cadherin and aminopeptidase were proposed as major factors influencing Bt toxicity and resistance in *O. nubilalis* (Squerira et al., 2006; Xu et al., 2011), there are multiple gut proteins that may also contribute as defense mechanisms and increase larval survival in response to the ingestion of Cry toxins.

In this study, we used microarrays to evaluate differences in gene expression for Cry1Ab resistant and susceptible *O. nubilalis* strains when they were exposed or unexposed to transgenic Cry1Ab corn. We hypothesize that multiple proteins in the insect gut respond to the active Cry1Ab toxin expressed by transgenic corn, and some of these genes may serve as potential candidates for further analysis regarding their involvement in Bt toxicity and/or resistance in *O. nubilalis*.

2. Experimental material and methods

2.1 Insect larvae rearing and transgenic corn planting

A Bt-resistant (R, also known as Sky) strain and a susceptible (S, also known as Meads) strain of European corn borer were obtained from the University of Lincoln-Nebraska (Lincoln, NE) and maintained at Kansas State University (Manhattan, KS) in which the R-strain had been selected from the same S-strain by artificial diet containing Cry1Ab protoxin (0.3 µg/ml) for multiple generations in laboratory. The eggs were collected from wax paper each day and kept in insect rearing cups with high humidity (above 80%) until hatching. Newly hatched larvae were immediately transferred to artificial diet without Cry1Ab protoxin and reared to the third instar for testing. The larval developmental stage was determined by moving them to a new rearing dish after each molt.

Transgenic Cry1Ab corn Pioneer 34P88 (MON810 event) and its isoline 34P86 seeds (Pioneer Hybrid, Johnston, IA) were planted in the greenhouse and grown to the six-leaf stage. Fresh corn leaves were collected and cut into approximately 4-cm^2 pieces with leave vein to feed the insects in the bioassay.

2.2 Determination of median lethal time of O. nubilalis fed transgenic Cry1Ab

The median lethal time (LT_{50}, the time that 50% of larvae died) bioassay was conducted with three replicates, with each replicate contining 48 and 56 early third-instar larvae of the R-strain and S-strain, respectively. To ensure that the larvae fed on the leaves immediately, they were starved for 24 hour and then transferred to rearing cups with transgenic or non-transgenic corn leaves. Leaf tissues were replaced daily, and the surviving larvae were recorded over the next seven days. Larvae were held at 26°C and 16L: 8D photoperiod. Results of the bioassays of R- and S-strain were analyzed by probit regression analysis using GLM procedure (SAS Institute, 1996) for LT_{50}.

2.3 Microarray analysis

A group of 40 early third-instar larvae of each strain were starved 24 hours, and then 20 larvae from each strain were transferred to rearing cups containing transgenic or non-transgenic corn leaves for 6 hours, respectively. The surviving larvae in the four groups (each group represented

one biological replicate with five individual larvae) were dissected to processed for total RNA extraction. The total RNA of each replicate (five individuals) was extracted using TRIzol reagent according to the manufacture's protocol (Invitrogen Inc. Frederick, MD). RNA quantity and quality were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc. Wilmington, DE) and an Agilent 2100 bioanalyzer.

Two *O. nubilalis* cDNA microarray slides (each containing 8 microarrays) were used in this study to detect larval gut transcriptional responses, one for the susceptible strain and the other for the resistant strain. Microarrays were high-resolution 8×15K multi-pack expression arrays for single-color detection was designed using Agilent's probe design algorithms (Aglient Technologies Inc. Santa Clara, CA). Five oligonucleotide probes from each of 2,895 unique ESTs from *O. nubilalis* larval gut were computationally designed and potential cross-hybridization probes were discarded. In total, 12,972 usable probes were obtained from *O. nubilalis* ESTs, which represent 2,895 unique gut transcripts.

Cyanine-3-labeled cRNAs were synthesized from Agilent one-color microarray-based gene expression kit. The dye-incorporation ratio was determined using a NanoDrop 2000 spectrophotometry. Eight RNA samples were used for cRNA synthesis, and eight cRNAs samples with cyanine-3 labeled cRNA ≥10pmol/ug were used for hybridization. Cyanine-3 labeled cRNAs (600 ng) of each sample were hybridized to microarray chips (eight samples with 4 treated and 4 control samples were hybridized with eight microarray chip, individually) and incubated to hybridize the chip at 65˚C for 17 h. Slides were scanned using Axon GenePix 4000B microarray scanner under 532nm wavelength. The signal intensity of each hybridized spot was qualified and quantified with Agilent Feature Extraction Ver. 9.5 software (Aglient Technologies, Inc. Santa Clara, CA). Features were flagged using the software algorithm. Normalization was carried out with GeneSpring GX11 (Agilent) based on the default normalization setting for one-color experiment. Expression ratios were calculated to compare the expression gut transcripts of treated groups (larvae fed transgenic corn) relative to control groups (larvae fed non-transgenic corn). Statistical differences in transcript expression were determined with multiple testing corrections (Benjamin and Hochberg false-discovery) (Benjamin et al.,

1995). Transcripts expression changes with p-value ≤0.05 and expression ratio ≥2.0 (cut-off) were considered significantly different.

2.4 Real-time quantitative PCR analysis

All significant differentially expressed candidate genes were identified based on Bt toxin model of action as being potentially involved in Bt toxicity and/or resistance. The expression ratios of all candidate transcripts were further validated by real-time quantitative PCR (qPCR). In this validation, 1 μg of total RNA from each microarray replicate sample was reverse-transcribed into a 20 μl reaction mixture with Fermatas ReverAid^{TM} First Strand cDNA synthesis kit (Fermentas). qPCR was performed on a Bio-Rad iCycler using a SYBR green qPCR kit (Fermentas Inc. Glen Burnie, MD). The PCR program was set with an initial denaturation of 94˚C for 5min, followed by 45 cycles at 94˚C for 10sec, 56˚C for 10sec and 72˚C for 10 sec. The specific primers for 19 genes and the reference gene ribosome protein L18 (RPL18) were designed using Beacon 7 Designer^{TM} (Primer Biosoft Inc. Palo Alto, CA) (Table 4-1). Relative expression differences were normalized to the RLP18 transcript level and analyzed using the comparative C_t method ($\Delta\Delta$ C_t) (Paffla., 2001).

2.5 qRT-PCR analysis of two aminopeptidases expression in R- and S-strain

The qPCR was also used to evaluate the relative expression of two aminopeptidase transcripts (contig4776 and contig1398) in the R- and S-strain larvae fed artificial diet. Three biological RNA samples of each strain of were prepared from fifteen third instar larvae without exposure to any toxin (five individuals in each replicate).

3. Results

3.1 Bioassays with R- and S-strains fed transgenic Bt or non-transgenic corn.

Third-instar larvae of *O. nubilalis* resistant strain (R-strain) have 200-fold resistance to Cry1Ab protoxin (data not shown) as compared with the susceptible strain (S-strain), and this R-strain were selected by Cry1Ab protoxin for multiple generation in laboratory, but both strains eventually died after feding on transgenic corn leaves. The survival of R-strain larvae was significantly longer than that of S-strain larvae. The median lethal times (LT_{50}) for the early

third-instar larvae of R- and S-strains were 5.4 and 3.6 days, respectively (Table 4-2). There was no mortality for larvae feeding on non-transgenic corn from either strain.

3.2 Gene expression profiles of S- and R-strain larvae fed transgenic corn leaves

The gene expression profiles for *O. nubilalis* revealed that there were more genes that were differentially expressed in S-strain larvae than in R-strain (14 vs 9%, respectively) after ingestion of transgenic corn leaves. There were 398 transcripts that were differentially expressed (expression ratio ≥2.0 with p value ≤0.05) in S-strain, and only 264 transcripts that were differentially expressed (expression ratio ≥2.0 with *P* value ≤0.05) in R-strain (Figure. 4-1), comparing larvae fed transgenic vs nontransgenic leaves. It is interesting to note that 150 genes differentially regulated gene were common to both R- and S-strains after feeding on transgenic corn leaves for 6 h.

We further examined differentially expressed transcripts for their possible biological functions based on the descriptions of their homologs in the databases. However, nearly one third of these transcripts (122 out of 398 in S-strain and 95 out of 264 in R-strain) did not have functional annotations (Table 4-4). Similarly, most transcripts were down-regulated more in the S-strain larvae than in the R-strain larvae. For example, trypsin (contig0243), sodium-bile amino cotransporter (contig 0814), glutathione *S*-transferase (contig0004), carboxypeptidase (ECB-V29-E10), and aquaporin (J-ECB-06-D11) were down-regulated approximately 10-fold in the S-strain larvae, but were down-regulated by less than five folds in the R-strain larvae (Table 4-4). Because R-strain larvae have been selected by Cry1Ab protoxin multiple generations, the suppression of these genes may represent an adaptation to Cry1Ab in R-strain larvae. When S-strain larvae were exposed to transgenic corn, the down-regulation of these genes was more dramatic because the S-strain larvae are more susceptible to the toxin.

3.3 Comparison of transcripts which are potentially involved in Bt toxicity

Transcripts encoding serine proteases, like trypsin, chymotrypsin and their homologs, were all down-regulated in both R- and S-strain larvae fed transgenic corn leaves. More serine protease transcripts were down-regulated in the S-strain (12) than these in the R-strain (6). This suggests that the down-regulation may be a response to Bt toxin and plant inhibitor stress. However, three

serine protease inhibitors were up-regulated in S-strain larvae fed transgenic corn leaves whereas this was not observed in the R-strain (Table 4-4).

According to pore-formation model, seventeen candidate transcripts that were potentially involved in Bt toxicity and/or resistance were identified in the S-strain larvae fed transgenic corn leaves for 6 h (Figure.4-3a). Among them, 12 were serine proteases (contig0130, contig0147, contig4021, contig0573, contig0243, contig0039, contig3466, contig0578, contig3118, contig0293, contig2151, ECB-C18-B11) and 5 were aminopeptidases (contig1398, contig4776, contig5112, ECB-V02-D07 and ECB-V05-D12). All candidate genes encoding putative serine proteases and aminopeptidases were down regulated after larval ingestion of transgenic corn leaves.

In R-strain larvae, there were 9 transcripts that were potentially involved in Bt toxicity and/or resistance (according to pore-formation model) (Figure. 4-3b). Five were serine proteases (contig1519, contig0113, contig0243, contig0039, contig0578, and contig3118), and four were aminopeptidases (contig1398, contig4776, contig5112, J41F04). All five serine protease genes and one of the aminopeptidase genes (contig5112) were down-regulated in the larvae fed transgenic corn leaves. However, three aminopeptidase genes (contig1398, contig4776, and J41_F04) were up-regulated.

Six of the genes potentially involved in Bt toxicity and/or resistance (according to pore-formation model) had significant changes in expression in both R and S-strain larvae after being fed transgenic corn leaves for 6 h (Figure. 4-4). These include the transcripts putatively encoding three trypsins and three aminopeptidases (contig0243, contig0039, contig0578, contig5112, contig4776 and contig1398). Among these six genes, one aminopeptidase (contig5112) and three trypsin genes (contig0243, contig0039, contig0578) were down-regulated, while two aminopeptidases (contig4776 and contig1398) were up-regulated in R-strain larvae in response to the ingestion of transgenic corn leaves (Figure. 4-4).

3.4 Insect defense mechanisms against Cry toxin attack

It is also noteworthy that seven differentially regulated transcripts that are involved gut chitin metabolic process were significantly down-regulated after larvae ingested active Cry1Ab toxin. They include chitin deacetylase 1 (contig0233), chitin deacetylase 5 (contig0505), (Table 4-4). In particular, chitinase 8 (contig0188) was down-regulated in the S-strain larvae more than 6-fold. The down-regulation of chitin deacetylase and other chitinase genes could be a defense against Cry toxin by increasing the chitin content in the peritrophic matrix to repair the damaged gut tissue and keep gut system integrity.

In this study, more than 300 transcripts showed significant and differential expression in either R- or S-strain larvae or both after exposure to transgenic corn expressing Cry1Ab. According to the studies of Cry5B and *C. elegans* system, many are potential *hpo* genes and may be involved in cellular defense in the *O. nubilalis*, e.g. cytochrome b5 reductase (contig0218), transcription regulator factor (Rho GTPase-activating protein, ECB-09_F07), GTP-binding protein (contig3760), protein kinase receptor (J-ECB-37_F07), and ATP binding protein (J-ECB-33_E10). These transcripts were up-regulated in either the R- or in the S-strain larvae that were fed transgenic corn leaves for 6 h (Table 4-4).

3.5 Expression patterns of two aminopeptidase transcripts in R- and S-strain larvae

When we compared relative expression ratios of the two aminopeptidase transcripts (contig4776 and contig1398) in R and S-strain larvae, we found opposite expressions in R- and S-strain larvae. Contig4776 had a 9-fold higher expression in R-strain than in S-strain larvae, but contig1398 had 4-fold higher expression in S-strain (Fig 4-5).

4. Discussion

4.1 Gut protease transcript expression changes after ingestion of transgenic corn

A previous study in this laboratory noted that the Dipel resistant larvae of *O. nubilalis* could not survive on Bt Cry1Ab transgenic corn (Li et al., 2007). In this study, Cry1Ab protoxin-selected resistant stain larvae also did not survive on transgenic corn tissues, but they did live longer, presumably because they were resistant to Cry1Ab. In Chapter 3, we reported a microarray study of susceptible *O. nubilalis* larvae that were fed Cry1Ab protoxin, and we identified five serine

protease genes that were up-regulated, four in particular (contig0389, contig1207, contig3704, contig4768) were up-regulated more than 5-fold. However, in this study, the expression of these serine protease transcripts were unchanged when *O. nubilalis* larvae were fed transgenic corn expressing Cry1Ab. This suggests that there is a difference in the responses of *O. nubilalis* larvae to the two types of toxins (Cry1Ab protoxin versus the Cry1Ab toxin expressed in transgenic corn) and also implicates a difference in the responses of larvae to two type of food sources (transgenic corn containing protease inhibitors versus artificial diet without any protease inhibitor). Li et al (2007) reported that Cry1Ab protoxin was rapidly hydrolyzed by enzymes in the corn leaf cytoplasm so it rapidly degrades the protoxin into peptide fragments with molecular masses ranging from 132 kDa to 74 kDa and eventually 58 kDa. This suggests that the transgenic toxin is functionally activated. Therefore, insect populations that have reduced proteinase activity such as the Dipel selected strain may not pose a threat to the efficacy of commercial transgenic Bt corn.

It is notable that the regulation of 12 carboxylesterase transcripts changed after larvae were fed transgenic Cry1Ab corn leaves. In the S-strain larvae, there were 10 carboxylesterase transcripts that were either down- or up-regulated, but in the R-strain there were only three carboxylesterase that were down-regulated. The interaction between Cry toxin and carboxylesterases has not been clarified, but in a previous study, the authors proposed that carboxylesterase may be involved in Cry toxin sequestration (Guning et al., 2005). If so, the up-regulation of two carboxylesterase transcripts (J-ECB-09_D02, and Contig0115) in the S-strain could be such a defense mechanism to reduce the toxicity of Cry1Ab toxin.

The gene expression profiles revealed that there were many transcripts that were differentially expressed in *O. nubilalis* after exposure to transgenic corn expressed Cry1Ab (Figure. 4-2). In Chapter 3, when susceptible larvae were fed Cry1Ab protoxin in artificial diet, there were 174 transcripts that were differentially expressed after feeding on the protoxin. In this chapter we report that there were 398 transcripts that were differentially expressed in S-strain larvae and 264 transcripts that were differentially expressed in R-strain larvae after ingestion of transgenic corn leaves (activated toxin). It is clear that the activated toxin was associated with a much larger number of transcripts that were differentially expressed than was the protoxin. There were 150

genes that were differentially regulated in both R- and S-strains after feeding on transgenic corn leaves, but only 48 of these were differentially regulated under three different treatment combinations (S larvae fed Cry1Ab protoxin, S-larvae fed transgenic corn and R-larvae fed transgenic corn). Among the 48 gut transcripts, six of them had a different regulation patterns for larvae fed the artificial diet containing Cry1Ab protoxin versus larvae fed transgenic corn leaves expressing Cry1Ab toxin. Three transcripts (ECB-02_H03, ECB-14_E05 and ECB-C-04_H06) were down-regulated after the larvae were fed Cry1Ab protoxin, but they were up-regulation after the larvae were fed transgenic corn leaves with activated Cry1Ab toxin. In contrast, three transcripts (ECB-11_E06, ECB-21_C09 and ECB-C-05_D05) were up-regulated in the larvae fed Cry1Ab protoxin and down-regulated in larvae fed transgenic corn leaves (activated Cry1Ab toxin) (Table 4-3). Overall, when S-strain larvae were fed transgenic corn, the gut transcript expression ratios were higher than these of S larvae fed Cry1Ab protoxin and R larvae fed transgenic corn leaves. For example, a trypsin homolog (EST id: contig0243) and a carboxylesterase (EST id: ECB-17_F12) had higher expression ratios in all three different treatment combinations, but the largest expression ratio was found in S larvae fed transgenic corn leaves (Table 4-3).

4.2 Aminopeptidase transcripts expression in R- and S-strain larvae

Aminopeptidases are involved in binding Cry1Ab to the midgut in *M. sexta* to form toxin oligomers (Bravo et al., 2004; Pacheco et al., 2009) and in *B. mori* (Ibiza-Palacios et al., 2008). In this study, we identified three aminopeptidase transcripts (contig5112, contig4776 and contig1398) that were changed in expression in both R and S-strain larvae after being fed transgenic corn leaves. However, if they were fed artificial diet without any toxin, APN2 (contig4776) had 9-fold higher transcription levels in S-strain larvae than that of R-strain larvae; but when S- and R-strain larvae were fed transgenic corn expressing Cry1Ab toxin and diet containing Cry1Ab protoxin, it was down-regulated by 2-fold in S- strain larvae fed transgenic corn, but up-regulated by 2~3-fold in both S-strain larvae fed on Cry1Ab protoxin and R- strain larvae fed transgenic corn, as compared with those fed non-Bt corn and non protoxin. Blast analysis found that this aminopeptidase shares 95% identity to aminopeptidase N2 of *O. furnacalis* (GenBank accession No: ACB47287), which was reported to be involved in Bt

toxicity in *O. furnacalis* (Xu et al., 2011). It is necessary to clarify APN2 (contig4776) role in Cry1Ab toxicity in *O. nubilalis*.

APN1, (contig1398), had 4-fold higher expression in S-strain than that of R-strain larvae when they were fed artificial diet without any toxin. There is a possibility that the reduced expression of this gene could serve as a resistance mechanism by reducing the amount of toxin binding receptors. This aminopeptidase shares 98% identity with aminopeptidase N1 of *O. furnacalis* (GenBank accession No: ACX85727), and 61% identity with that (accession No: ACC68682) of *H. armigera* (Zhang et al., 2009). Those aminopeptidases have been identified as major proteins associated with binding to Cry1 toxins and have been proposed as Cry1 toxin resistance genes in *O. furnacalis* and *H. armigera* (Xu et al., 2011; Zhang et al., 2009). We speculate that the ortholog (contig1398) that we identified in *O. nubilalis* may play a similar role in Bt toxicity and resistance, perhaps by reducing the number of binding receptors.

4.3 Other cellular defense mechanisms triggered by Cry1Ab

The changed expression of chitin related transcripts is a notable example of *O. nubilalis* gut defense mechanism against Cry1Ab attack. The differentially regulated transcripts, like chitin deacetylase and chitinase 8 were some important genes involved in chitin metabolism in peritrophic matrix of larval midgut. Chitin deacetylases (EC 3.5.1.41) are enzymes that catalyze chitin to form chitosan and acetate, and are major enzymes that degrade gut chitin (Araki et al., 1974). This is thought to weaken the insects' PM and possibly facilitate access of Bt toxins to the gut membrane. Thus, the down-regulation of chitin deacetylase and other chitinase genes could be a defense against Cry toxin by increasing the chitin content in the PM. Thus the up- or down-regualtion of chitin-related enzymes is one protection mechanism to defend Cry1Ab insertation on gut membrane.

The heat shock protein, 70 gene (contig0227), was also up regulated in both strains after larvae fed transgenic Bt corn leaves. Heat shock proteins are considered an arthropod defense factor against bacterial and virus infection (Washburn et al., 2001; Lee et al., 2000). Heat shock protein 70 genes have been well characterized as stress-induced transcripts and they are also called unusual stress gene (Kimura et al., 2001). Therefore, the up-regulation of the heat shock

protein 70 gene after ingestion of transgenic corn expressing Cry1Ab toxin may serve as a defense against Cry toxins or it may also simply indicate the stress that the larvae experienced after feeding on Bt toxin.

In summary, our studies revealed a number of genes whose expression levels were altered by the ingestion of transgenic corn leaves expressing Cry1Ab toxin in R- and S-strains of *O. nubilalis*. In *C. elegans* more than 106 genes (called *hpo* genes) are proposed to be involved in Bt Cry5B toxin defense after using large scale RNAi screening (Kao et al., 2011). In this study, we also identified some possible *hpo* genes that may be involved in cellular defense in *O. nubilalis*. Our results also provide new insight into the gut gene responses to Bt toxin. This supports the proposed hypothesis that insects defense against Cry toxin attack involves multiple gut proteins, from gut lumen digestion enzymes, gut cell membrane binding partners, to intracellular defense factors. Any changes in those complicated mechanisms can contribute to insect ability to survive in the presence of Cry toxin. Nevertheless, further studies will be necessary to clarify the functional roles that these differentially expressed genes contribute to Cry1Ab toxicity and resistance.

Acknowledgement

We would like to thank Mandar Deshpande of the KSU Integrated Genomics Facility for his technique support, Nanyan Lu of the KSU Bioinformatics Center for her help in data analysis, Dr. Blair D. Siegfried at the University Nebraska-Lincoln for providing us with Bt-resistant strain (Sky) and susceptible strain (Meads) of the ECB, and Dr. Zurek Ludek and Dr. Brain McCornack for providing us with accesses to the Bio-Rad iCycler equipment and insect rearing room, respectively.

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Figure legends

Figure 4-1. The total number of significantly up- or down-regulated transcripts ($p \leq 0.05$) in the gut of *O. nubilalis* in Cry1Ab resistant (R-) and susceptible (S-) strain larvae fed transgenic Cry1Ab corn leaves for 6-h. The R-strain was Cry1Ab protoxin selected and the S-strain was unselected isoline.

Figure 4-2. A Venn diagram showing the number of transcript variations between R- and S-strain larvae of *O. nubilalis* after fed on transgenic Cry1Ab corn leaves, and the S-strain larvae after fed on artificial diet containing Cry1Ab protoxin.

Figure 4-3a. Microarray analysis (diagonal lined bars) and qPCR analyses (cross hatched bars) for 17 differentially expressed transcripts in the larval gut of S-strain larvae after fed on transgenic corn leaves expressing Cry1Ab toxin. These genes were considered candidate genes, that were potentially involved in Bt toxicity and/or resistance. Up and down bars indicate up and down regulated transcripts.

Figure 4-3b. Microarray analysis (diagonal lined bars) and qPCR analyses (cross hatched bars) for nine differentially expressed transcripts in the larval gut of R-strain after fed transgenic corn leaves expressing Cry1Ab toxin. Those genes were considered candidate genes that were potentially involved in Bt toxicity and/or resistance. Up and down bars indicate up and down regulated transcripts.

Figure 4-4. Comparisons of expression changes for six candidates genes that may potentially be involved in Bt toxicity and/or resistance in the larval gut of R-strain (cross hatched bars) and S-strain (spotted bars) in response to the ingestion of transgenic Cry1Ab corn leaves.

Figure 4-5. Relative transcriptional levels of two aminopeptidase transcripts (EST ID: contig1398 and contig4776) in the larval gut of R-strain (spotted bar) and S-strain (open bar).

Table 4-1. Sequences of primers used in quantitative PCR analysis

Putative gene name	EST ID	Primer sequence	Product size (bp)	strain
Chymotrypsin-like protease	contig0130	TCGGGACAACTGGTCTAGC	71	S-strain
		CGCACTCGTCGTTAGGTATC		
Chymotrypsin-like protease	contig0147	GCTGGTTCCCTCTACTGGTC	79	S-strain
		GAGATGGTGTTGGAGAAGGC		
Silk gland derived trypsin	ECB-C-18-B11	CACAAAGTCCTGGAGGAAGATTC	125	S-strain
		GTTCACGCCTGTCTGTTGC		
Chymotrypsin-like protease	contig4021	ACCTGCCTACCAGCGTTTC	112	S-strain
		CCGAAGCCTGAAGCAATAGC		
Chymotrypsin-like protease	contig0573	TCAGTGGAACCCGTGGAAC	94	S-strain
		CAGTGCGATTGGTTGGATGG		
Trypsin-like protease	contig3466	GAGTGGGGTCTTCCTTCAGG	105	S-strain
		CAGCAATGTCGTTGTTAAGCG		
Trypsin-like protease	contig3118	AACTACGACGGAGAAAAG	114	S-strain
		GCACTGCTCATTATCAATA		
Trypsin-like protease	contig0293	CTCGTAGAAGAAGATGATG	194	S-strain
		TCGTTAGAGTCTTCGTTA		
Serine protease	contig2151	GTAAGACTGGTCGGTGGTAAAG	149	S-strain
		TCGGCTCCAAGAACACAATG		
Aminopeptidase N	ECB-V-02-D07	AACTTACCTTCTGGCTAT	88	S-strain

		TCTGGCTATAACTTCGTA		
Aminopeptidase N	ECB-V-05-D12	GTCAACGAAATTGTCATC	109	S-strain
		AGTCATATTCTGGCTGTA		
Chymotrypsin-like protease	contig1519	CGAACTTATCCAATGACAT	192	R-strain
		TCACTTGGTTGATTTGTG		
Trypsin	Contig0113	TTCTACTGTGAACATCCT	96	R-strain
		TCCAAAGATTCAAATCCC		
Aminopeptidase N	J-ECB-41_F04	AAGGACTAACTGCTATGA	150	R-strain
		CTGCCAAGTTGATTCTTA		
Trypsin-like protease	contig0578	CATCCGAGATTCTCTTATG	120	R- and S-strains
		CAGTGTTATTGTAGAACTCT		
Trypsin-like protease	contig0039	ATGCGTACCTTCATCGTTCTAC	116	R- and S-strains
		GCCATCTCAGGGTATTGGTTAATG		
Trypsin precursor	contig0243	GCCAGCATTACACCTTCCG	128	R- and S-strains
		TCGCAGTTCTCGTAGTAAGAC		
Aminopeptidase N	contig1398	TCTGTAGTCTGGTTCACATTATCC	84	R- and S-strains
		ACTCACCTCCGCTGTATCC		
Aminopeptidase N	contig4776	TTCCAAACACATTTTCTTG	118	R- and S-strains
		AAGCGTATTGTCCTCTAT		
Aminopeptidase N	contig5112	CTTCAACAGCCCACTGGAGAG	92	R- and S-strains
		ACGCAAGACATATTAGGTAACAGC		

Table 4-2. Probit analysis of larval responses in R- and S-strains to the ingestion of transgenic Cry1Ab corn leaves

Strains	Number of larvae	Slope and SE	LT_{50} (95% Confidence interval) (days)	P-value>ChiSq
R-strain	144	0.74±0.06	5.38 (4.79-6.00)	0.1614
S-strain	175	1.32±0.16	3.68 (3.47-3.88)	0.1312

Table 4-3. The gut transcript expressions were differentially regulated in all three strains under three different treatment combinations.

The larvae of two S strain (Lee and Meads) were fed artificial diet containing Cry1Ab protoxin and transgenic corn expressing Cry1Ab toxin whereas the larvae of a laboratory-selected CrylAb protoxin resistant strain (Sky) were fed transgenic corn expressing Cry1Ab toxin. "*" S on protoxin and S on corn represent Lee and Meads larvae feed diet contaning protoxin and transgenic corn leaves, respectively; R on corn is Sky strain larvae fed on transgenic corn leaves.

EST ID	Sequence description	S on protoxin*	S on corn*	R on corn*
BM2_B12	hypothetical protein TcasGA2_TC011405 [*Tribolium castaneum*]	-2.07	-2.24	-2.3
Contig[0004]	glutathione S-transferase [*Choristoneura fumiferana*]	-3.53	-8.76	-2.95
Contig[0009]	putative carboxypeptidase A-like [*Nasonia vitripennis*]	-2.19	-3.85	-2.09
Contig[0019]	glutamate carboxypeptidase-like isoform 1 [*Apis mellifera*]	-3.14	-6.64	-2.28
Contig[0243]	trypsin [*Helicoverpa armigera*]	-2.45	-9.02	-5.89
Contig[0814]	sodium-bile acid cotransporter [*Tribolium castaneum*	-5.03	-14.14	-4.21
Contig[1081]	pancreatic lipase-like protein [*Epiphyas postvittana*]	-2.57	-4.83	-5.01
Contig[1314]	amino acid transporter [*Bombyx mori*]	-4.68	-3.8	-2.51
Contig[1486]	C-5 sterol desaturase-like [*Acyrthosiphon pisum*)	-4.96	-7.75	-2.79
Contig[1897]	C-5 sterol desaturase-like [*Acyrthosiphon pisum*]	-4.48	-7.03	-2.13
Contig[1953]	NADP-dependent oxidoreductase [*Bombyx mori*]	-3.27	-2.71	-2.51
Contig[4527]	conserved hypothetical protein [*Culex quinquefasciatus*]	-7.89	-15.05	-5.28
Contig[4763]	sodium-bile acid cotransporter [*Aedes aegypti*]	-4.39	-10.83	-6.56
Contig[5679]	phosphoserine aminotransferase [*Antheraea pernyi*]	-2.18	-4.08	-3.26
Contig[5743]	amino acid transporter [*Bombyx mori*]	-5.4	-3.96	-2.55

ECB-02_H03	saposin-like protein [*Bombyx mori*]	-2.02	2.84	2.87
ECB-14_E05	N/A	-3.62	3.27	2.56
ECB-C-04_H06	tetraspanin D107 [*Bombyx mori*]	-2.7	4.16	2.99
ECB-C-11_A06	hypothetical protein EAI_08582 [*Harpegnathos saltator*]	-2.86	-4.22	-3
gi_133905779	Vanin-like protein 1 [*Culex quinquefasciatus*]	-3.35	-9.2	-5.67
gi_133906638	retinol dehydrogenase [*Heliothis virescens*]	-3.61	-10.53	-10.95
J-ECB-39_E12	sugar transporter [*Culex quinquefasciatus*]	-2.65	-9.57	-4.35
J-ECB-55_E04	monocarboxylate transporter [*Aedes aegypti*]	-3.4	-3.62	-3.88
Contig[1263]	N/A	4.74	2.32	2.91
Contig[1573]	N/A	2.07	2.27	2.76
Contig[4776]	SXSS-APN2 [*Ostrinia furnacalis*]	2.17	-2.59	2.31
Contig[5143]	ctl2 [*Aedes aegypti*]	2.32	2.25	2.17
Contig[5262]	N/A	5.52	3.58	4.86
Contig[5386]	tetraspanin E118 [*Bombyx mori*]	2.33	2.34	2.04
Contig[5414]	Ubiquitin-63E, isoform A [*Drosophila melanogaster*]	2.73	2.49	2.04
Contig[5929]	N/A	2.6	3.98	2.45
Contig[6000]	N/A	2.14	2.48	2.82
ECB-11_E06	GD21009 [*Drosophila simulans*	2.21	-5.13	-2.69
ECB-14_H12	N/A	2.13	5.29	5.16
ECB-17_F12	carboxylesterase [*Helicoverpa armigera*]	16.41	24.46	6.87
ECB-18_B07	N/A	3.99	2.71	2.44
ECB-21_C09	sugar transporter protein 3 [*Bombyx mori*]	2.52	-4.9	-2.12
ECB-C-05_D05	glucosamine-fructose-6-phosphate aminotransferase 2(*Culex quinquefasciatus*]	2.24	-2.88	-2.1
ECB-V-07_D03	MIP19203p [*Drosophila melanogaster*]	2.66	6.6	6.08

ECB-V-16_B02	N/A	2.36	2.35	2.07
ECB-V-25_C10	hypothetical protein [*Aedes aegypti*]	2.29	5.91	6.27
gi_133907290	N/A	4.37	5.14	3.9
J-ECB-25_H03	N/A	4.21	6.25	4.84
J-ECB-29_G03	IST1 homolog [*Bombus terrestris*]	2.18	5.72	6.66
J-ECB-39_H09	Extracellular domains-containing protein [*Acromyrmex echinatior*]	2.15	3.36	2.67
J-ECB-47_A02	growth factor-regulated tyrosine kinase substrate (hgs) [*Tribolium castaneum*]	3.09	3.77	3.78

Table 4-4. The gut transcript expression ratios in the larvae of R- and S-strains of *O. nubilalis* after fed transgenic Cry1Ab corn leaves for 6 h.

EST ID	Sequence description	Expression ratios	
		S-strain	R-strain
Trypsin, chymotrypsin, serine protease inhibitors and cysteine B like proteases			
Contig[0039]	trypsin serine protease [*Ostrinia furnacalis*]	-3.63	-2.01
Contig[0113]	trypsin [*Helicoverpa armigera*]		-4.67
Contig[0130]	chymotrypsin-like protease C3 [*Heliothis virescens*]	-2.95	
Contig[0147]	chymotrypsin [*Helicoverpa armigera*]	-2.28	
Contig[0243]	trypsin [*Helicoverpa armigera*]	-9.02	-5.89
Contig[2151]	larval chymotrypsin-like protein precursor [*Aedes aegypti*]	-3.71	
Contig[0293]	serine protease 24 [*Mamestra configurata*]	-3.22	
Contig[0573]	chymotrypsin-like serine protease [*Bombyx mori*]	-2.47	
Contig[0578]	chymotrypsin-like protease [*Helicoverpa armigera*]	-4.68	-2.52
Contig[1519]	chymotrypsin-like serine protease [*Bombyx mori*]		-2.26
Contig[3118]	putative chymotrypsin [*Antheraea assama*]	-3.05	
Contig[3466]	trypsin [*Manduca sexta*]	-2.88	
ECB-C-18_B11	trypsin-like proteinase T2a precursor [*Ostrinia nubilalis*]	-3	
Contig[4021]	chymotrypsin-like serine protease [*Ostrinia nubilalis*]	-2.57	
ECB-C-13_A03	chymotrypsin-C-like [*Meleagris gallopavo*]		-2.3
J-ECB-52_A02	serine protease inhibitor 3 [*Tabanus yao*]	10.71	
J-ECB-08_D11	putative protease inhibitor 4 [*Lonomia obliqua*]	19.48	
Contig[1913]	serpin-2 [*Bombyx mandarina*]	2.13	
Contig[3603]	cathepsin B-like cysteine proteinase [*Helicoverpa assulta*]	2.98	
Aminopeptidase			
Contig[1398]	aminopeptidase N isoform 1 [*Ostrinia furnacalis*]	-2.32	2.05
Contig[4776]	Cry1Ab-RR resistance protein APN2 [*Ostrinia furnacalis*]	2.59	2.31
Contig[5112]	aminopeptidase N3 [*Bombyx mori*]	-2.9	-2.32
ECB-V-02_D07	aminopeptidase N3 [*Ostrinia nubilalis*]	-3.5	
ECB-V-05_D12	aminopeptidase N 3a [*Ostrinia nubilalis*]	-3.28	
J-ECB-41_F04	aminopeptidase [*Helicoverpa armigera*]		2.09

Carboxylesterase and carboxypeptidase			
Contig[0266]	carboxyl/choline esterase CCE006d [*Helicoverpa armigera*]carboxylesterase -11	-2.61	
Contig[0115]	carboxylesterase [*Loxostege sticticalis*]	25.18	
Contig[1179]	carboxylesterase-like protein [*Helicoverpa armigera*]		-2.84
Contig[3820]	carboxylesterase [*Helicoverpa armigera*]	-2.67	
Contig[4729]	carboxylesterase [*Bombyx mori*]	-17.96	
Contig[5372]	carboxylesterase CarE-11 [*Bombyx mori*]	-2.69	
Contig[5691]	carboxyl/choline esterase [*Helicoverpa* armigera]	-10.91	-4.57
ECB-V-21_D08	carboxylesterase [*Helicoverpa armigera*]	-4.55	
J-ECB-07_G03	carboxylesterase [*Loxostege sticticalis*]	16.37	
J-ECB-09_D02	carboxylesterase [*Spodoptera litura*]	-2.86	
Contig[5791]	carboxyl/choline esterase CCE021b [*Helicoverpa armigera*]		-2.87
ECB-27_F04	carboxyl/cholinesterase 4A [*Bombyx* mori]	-2.71	
Contig[2666]	midgut carboxypeptidase [*Loxostege sticticalis*]		-2.82
Contig[3400]	carboxypeptidase [*Aedes aegypti*]	-2.79	
Contig[3637]	carboxypeptidase C [*Culicoides sonorensis*]	-2.35	
Contig[3784]	midgut carboxypeptidase [*Loxostege sticticalis*]	-2.21	
ECB-V-29_E10	midgut carboxypeptidase 1 [*Trichoplusia ni*]	-17.64	-4.19
J-ECB-42_B07	zinc carboxypeptidase A 1 [*Culex quinquefasciatus*]	-2.37	
Contig[0077]	caboxypeptidase 4 [*Mamestra configurata*]	-2.12	
Contig[0009]	putative carboxypeptidase A-like [*Nasonia vitripennis*]	-3.85	-2.09
Contig[0019]	glutamate carboxypeptidase-like isoform 1 [*Apis mellifera*]	-6.65	-2.28
Contig[3603]	cathepsin B-like cysteine proteinase [*Helicoverpa assulta*]	2.98	
Chitin related transcripts			
Contig[0308]	peritrophin type-A domain protein 2 [*Mamestra configurata*]		-5.72
Contig[0505]	chitin deacetylase 5b [*Helicoverpa armigera*]	-3.3	-2.43
Contig[0188]	chtinase 8 [*Drosophila melanogaster*]	-6	
Contig[4654]	peritrophic membrane chitin binding protein [*Loxostege sticticalis*]	-2.39	
Contig[0233]	chitin deacetylase 2 [*Mamestra brassicae*]	-2.6	-2.44
ECB-C-05_D05	glucosamine-fructose-6-phosphate aminotransferase 2 [*Culex quinquefasciatus*]	-2.88	-2.1
Contig[4598]	glucosamine-6-phosphate N-acetyltransferase [*Bombyx mori*]		2.26
Signal transduction			

Contig[3760]	small GTP-binding protein [*Bombyx mori*]	2.24	
ECB-14_B10	protein kinase c inhibitor [*Bombyx mori*]		3.48
J-ECB-37_F07	receptor for activated protein kinase C [*Helicoverpa armigera*]	2.76	
J-ECB-33_E10	ATP-binding cassette sub-family B member 1 [*Trichoplusia ni*]	2.59	
J-ECB-10_B09	Oxidative stress-induced growth inhibitor 1 [*Camponotus floridanus*]	-2.36	
ECB-V-27_D10	Hypoxia up-regulated protein 1 [*Acromyrmex echinatior*]		-2.59
Contig[5194]	signal sequence receptor [*Biston betularia*]		-2.18
ECB-30_C02	signal sequence receptor beta subunit [*Bombyx mori*]		-2.35
Transcription regulator factors			
Contig[5330]	Rho GTPase activating protein, putative [*Pediculus humanus corporis*)		2.94
Contig[3833]	DNA-binding nuclear protein p8 [*Simulium guianense*]		3.78
ECB-09_F07	Rho GTPase-activating protein 12-like [*Acyrthosiphon pisum*]	2.7	2.76
ECB-V-14_D07	reverse transcriptase	2.3	2.22
Heat shock protein			
Contig[0227]	heat shock cognate 70 [*Plutella xylostella*]	2.36	2.06
Contig[2669]	heat shock cognate 70 protein [*Trichoplusia ni*]		-2.65
gi_133905913	heat shock cognate 70 protein [*Loxostege sticticalis*]		-2.97
J-ECB-29_F11	heat shock protein hsp23.7 [*Bombyx mori*]		-14.3
Transporter			
Contig[0814]	sodium-bile acid cotransporter	-14.14	-4.21
Contig[1314]	potassium coupled amino acid transporter [*Manduca* sexta]	-3.8	-2.52
Contig[4763]	sodium-bile acid cotransporter [*Aedes aegypti*]	-10.83	-6.56
Contig[5496]	putative sugar transporter [*Lutzomyia longipalpis*]	-2.66	-2.36
Contig[5743]	putative amino acid transporter [*Bombyx mori*]	-3.96	-2.55
ECB-16_F07	sodium-dependent phosphate transporter [*Aedes aegypti*]	-5.51	2.83
ECB-21_C09	sugar transporter [*Culex quinquefasciatus*]	-4.9	-2.12
ECB-22_H11	Sodium- and chloride-dependent glycine transporter 2 [*Harpegnathos saltator*]		-2.45
ECB-V-21_D11	monocarboxylate transporter [*Aedes aegypti*]		-2.21
ECB-V-22_E06	GDP-fucose transporter, putative [*Nasonia vitripennis*]	2.02	2.39
gi_133906576	ATP-binding cassette transporter subfamily B [*Bombyx mori*]		-2.39
J-ECB-39_E12	sugar transporter [*Culex quinquefasciatus*]	-9.56	-4.34
J-ECB-52_H10	monocarboxylate transporter 14-like [*Bombus terrestris*]	-3.3	-2.13

J-ECB-42_F09	zinc transporter foi-like isoform 2 [*Apis mellifera*]		6.84
J-ECB-55_E04	monocarboxylate transporter [*Aedes aegypti*]	-3.62	-3.88
Contig[3828]	transport protein Sec61 gamma subunit [*Bombyx mori*]		-2.07
Xenobiotics detoxification enzyme			
Contig[4722]	ctyochrome P450 CYP6AB4 [*Bombyx mandarina*]	-2.93	
Contig[5056]	cytochrome P450 CYP332A1 [*Bombyx mori*]		2.14
Contig[5080]	cytochrome P450 monooxygenase Cyp4M5 [*Bombyx mori*]		3.84
ECB-C-03_D08	CYP6AB4 [*Bombyx mandarina*]		2.62
J-ECB-10_F11	cytochrome P450 [*Cnaphalocrocis medinalis*]	2.38	
J-ECB-21_B02	DIMBOA-induced cytochrome P450 [*Ostrinia furnacalis*]	-3.34	
Contig[0004]	glutathione S-transferase [*Choristoneura fumiferana*]	-8.76	-2.95
Contig[0012]	microsomal glutathione transferase [*Heliothis virescens*]		-2.59
Antibacterial related proteins			
gi_133905829	hinnavin II [*Pieris rapae*]	11.84	
Contig[5720]	immune-related Hdd13 [*Hyphantria cunea*]	-2.41	
Contig[4668]	peptidoglycan recognition protein B [*Samia cynthia ricini*]		-2.84
J-ECB-60_D07	antibacterial protein [*Heliothis virescens*]		3.49
Other metabolic enzymes			
BM2_M13R_D02	lipase-like protein [*Helicoverpa armigera*]	-2.9	
Contig[0016]	gastric lipase-like protein [*Epiphyas postvittana*]	-2.56	
Contig[0029]	gastric lipase-like protein [*Epiphyas postvittana*]	-5.44	
Contig[0140]	pancreatic lipase-like protein [*Epiphyas postvittana*]		-11.58
Contig[0450]	insect intestinal lipase 6 [*Mamestra configurata*]	-4.71	
Contig[0923]	insect intestinal lipase 6 [*Mamestra configurata*]	-7.96	-10.45
Contig[1081]	pancreatic lipase-like protein [*Epiphyas postvittana*]	-4.83	-5.02
Contig[2857]	pancreatic lipase 2 [*Mamestra configurata*]	-3.19	
Contig[4980]	gastric lipase-like protein [*Epiphyas postvittana*]		-2.69
Contig[5664]	lipase [*Helicoverpa armigera*]	-4.33	
ECB-07_C04	Lipase 1 [*Camponotus floridanus*]	-4.64	
J-ECB-56_D02	Gastric triacylglycerol lipase [*Camponotus floridanus*]	-4.57	
Contig[1486]	C-5 sterol desaturase-like [*Acyrthosiphon pisum*)	-7.75	-2.79
Contig[1897]	C-5 sterol desaturase-like [*Acyrthosiphon pisum*]	-7.03	-2.13

ECB-V-05_B10	acyl-CoA-delta9-3a-desaturase [*Dendrolimus punctatus*]		-2.19
ECB-C-20_B10	carbohydrate kinase-like [*Tribolium castaneum*]	-2.37	
J-ECB-61_C03	UDP-glucosyltransferase [*Bombyx mori*]	2.57	2.08
ECB-V-19_F07	UDP-glucosyltransferase [*Bombyx mori*]	2.87	
J-ECB-30_H09	beta-glucosidase precursor [*Spodoptera frugiperda*]	-4.16	2.01
ECB-C-14_E07	beta-glucosidase precursor [*Spodoptera frugiperda*]	-5.25	
J-ECB-15_G11	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [*Nasonia vitripennis*]	2.65	2.42
Contig[0075]	glucosidase [*Bombyx mori*]	-3.44	
Contig[0700]	beta-fructofuranosidase 2 [*Manduca sexta*]	-2.98	
Contig[0911]	juvenile hormone epoxide hydrolase [*Helicoverpa armigera*]	-2.39	
Contig[4515]	putative gamma-glutamyl hydrolase [*Aedes aegypti*]	-2.56	-2.49
Contig[4896]	epoxide hydrolase [*Aedes aegypti*]	-2.65	
Contig[5232]	glycoside hydrolases [*Aedes aegypti*]	-29.36	
Contig[4425]	enolase [*Antheraea pernyi*]	-2.36	
J-ECB-46_D03	ubiquitin carboxyl-terminal hydrolase 14-like isoform 1 [*Bombus terrestris*]	2.42	
J-ECB-33_G12	juvenile hormone epoxide hydrolase-like protein 1 [*Bombyx mori*]	-6.2	
J-ECB-50_D10	beta-glucosidase precursor [Spodoptera frugiperda]	-5.16	
J-ECB-58_H02	platelet-activating factor acetyl-hydrolase isoform 1b alpha subunit [*Aedes aegypti*]	2.16	
BM2_B12	hydroxysteroid (17-beta) dehydrogenase 8 [*Sus scrofa*]	-2.24	-2.3
Contig[0022]	alcohol dehydrogenase	-6.41	-2.36
Contig[0030]	aldehyde dehydrogenase	-2.88	
Contig[0231]	short-chain dehydrogenase		-3.25
Contig[1778]	acyl-coa dehydrogenase	-2.5	-2.63
Contig[3532]	isocitrate dehydrogenase	-3.13	
Contig[3814]	alcohol dehydrogenase [*Bombyx mori*]	-3.07	
Contig[4310]	3-hydroxybutyrate dehydrogenase type 2	-2.11	
Contig[5289]	isovaleryl coenzyme A dehydrogenase [*Heliothis virescens*]	-5.68	
ECB-01_M13R_G12	zinc-containing alcohol dehydrogenase [*Bombyx mori*]		2.17
ECB-14_M13R_D05	aldehyde dehydrogenase [*Heliothis virescens*]	-3.09	
gi_133906638	retinol dehydrogenase [*Heliothis virescens*]	-10.53	-10.95
Contig[0362]	carbonyl reductase	-4.82	-2.14
Contig[1032]	gamma-interferon inducible lysosomal thiol reductase [*Glossina morsitans*)	-2.48	

Contig[2754]	carbonyl reductase	2.51	
Contig[4521]	aldo-keto reductase		-27.07
Contig[5655]	lysosomal thiol reductase IP30 isoform 2 [*Bombyx mori*]		-2.29
Others			
Contig[0218]	NADPH cytochrome b5 reductase 1 [*Helicoverpa armigera*]	-2.14	-2.02
Contig[5933]	Abhydrolase domain-containing protein 4 [*Harpegnathos saltator*]	2.38	
Contig[3484]	mitochondrial cytochrome c [*Bombyx mori*]		-2.65
Contig[1432]	catalase [*Bombyx mori*]	-2.36	
Contig[1310]	uridine phosphorylase [*Aedes aegypti*]	-2.3	
Contig[5679]	phosphoserine aminotransferase	-4.08	-3.26
Contig[0098]	JHBP-like protein [*Diploptera punctata*]	-3.76	
Contig[0165]	actin depolymerizing factor	2.26	
Contig[0241]	conserved hypothetical protein [*Culex quinquefasciatus*]	2.16	
Contig[0382]	beta-1,3-glucanase [*Helicoverpa armigera*]	-2.21	
Contig[0407]	sensory appendage protein 3 [*Manduca sexta*]		-57.87
Contig[0535]	hypothetical protein AaeL_AAEL009036 [*Aedes aegypti*]	-2.71	-
Contig[0810]	calreticulin [*Galleria mellonella*]		-3.37
Contig[0827]	Ankyrin repeat domain protein [Wolbachia endosymbiont of (*Culex quinquefasciatus*]	-3.26	
Contig[0924]	carbonic anhydrase [*Aedes aegypti*]	-2.12	
Contig[0976]	ferritin [*Manduca sexta*]	-2.33	
Contig[1250]	aquaporin	-2.47	
Contig[1305]	angiotensin converting enzyme [*Spodoptera littoralis*]	-2.19	
Contig[1573]	hypothetical conserved protein	2.27	2.76
Contig[1640]	fatty acid-binding protein-like [*Bombus terrestris*]	-2.76	
Contig[1780]	26S protease regulatory subunit 4 [*Culex quinquefasciatus*]	2.23	
Contig[1868]	Rtnl1, isoform B [*Drosophila melanogaster*]	2.74	2.21
Contig[1880]	AKH/corazonin-like hormone precursor [*Heliothis virescens*]	5.46	4.09
Contig[1953]	NADP-dependent oxidoreductase [*Bombyx mori*]	-2.71	-2.51
Contig[2576]	unknown [*Picea sitchensis*]	-3.03	
Contig[2896]	lipid storage droplets surface binding protein 2	2.98	
Contig[2947]	high-affinity copper uptake protein	-5.88	
Contig[3004]	beta lactamase domain	-5.38	

Contig[3035]	anterior fat body protein	-12.82	
Contig[3237]	dipeptidyl peptidase 4	-2.74	
Contig[3266]	unknown [*Picea sitchensis*]	-3.02	
Contig[3372]	macrophage migration inhibitory factor	4.26	
Contig[3454]	alpha1,3-fucosyltransferase C [*Apis mellifera*]	2.3	
Contig[3515]	PREDICTED: hypothetical protein [*Nasonia vitripennis*]	-3.17	-2.37
Contig[3585]	glutathione peroxidase	2.14	
Contig[3619]	takeout/JHBP-like protein [*Diploptera punctata*]	-3.65	
Contig[3674]	bcr-associated protein, bap [*Aedes aegypti*]	3.15	
Contig[3708]	muscle protein 20-like protein [*Papilio* xu*t*hus]	2.15	
Contig[3740]	FK506-binding protein [*Bombyx mori*]		-3.83
Contig[5365]	tetraspanin 2A, isoform A [*Drosophila melanogaster*]	2.22	
Contig[5386]	tetraspanin E118 [*Bombyx mori*]	2.34	2.04
ECB-C-04_M13R_H06	tetraspanin d107	4.16	2.99
Contig[3869]	creg1 precursor(Cellular repressor of E1A-stimulated genes 1) [*Tribolium castaneum*]		-2.18
Contig[4000]	ryanodine receptor-like protein [*Phlebotomus papatasi*]	2.28	
Contig[4287]	beta-tubulin [*Bombyx mori*]	2.56	
Contig[4526]	proteasome 26S non-ATPase subunit 4 [*Bombyx mori*]	2.04	
Contig[4527]	conserved hypothetical protein [*Culex quinquefasciatus*]	-15.05	-5.28
Contig[4714]	similar to X box binding protein-1 CG9415-PA [*Tribolium castaneum*]	2.73	2.28
Contig[4784]	leucine repeat-rich protein [*Heliconius melpomene melpomene*]		2.83
Contig[4916]	hypothetical protein TcasGA2_TC002334 [*Tribolium castaneum*]		3.6
Contig[5038]	MBF2 [*Samia cynthia*]		2.39
Contig[5045]	ubiquitin-conjugating enzyme rad6 [*Aedes aegypti*]	2.02	
Contig[5050]	HMG176 isoform A [*Mamestra configurata*]	2.94	4.92
Contig[5114]	xaa-pro dipeptidase peptidase [Ae*d*es *aegypti*]	-2.08	
Contig[5119]	similar to CG3625 CG3625-PB isoform 2 [*Tribolium castaneum*)	-2.63	-3.78
Contig[5123]	Abl interactor 2 [*Harpegnathos saltator*]		2.41
Contig[5133]	myosin light chain 2 [*Antheraea pernyi*]	2.98	
Contig[5136]	ceramidase [*Aedes aegypti*]	-2.9	
Contig[5143]	ctl2 [*Aedes aegypti*]	2.25	2.17
Contig[5148]	Coronin-2B [*Harpegnathos saltator*]	2.79	

Contig[5168]	tropomyosin-1 [*Bombyx mori*]	3.64	
Contig[5228]	putative C1A cysteine protease precursor [*Manduca sexta*]	2.31	3.42
Contig[5259]	dipeptidyl-peptidase [*Aedes aegypti*]	-3.1	
Contig[5301]	insect intestinal mucin 3 [*Helicoverpa armigera*]	-2.4	
Contig[5397]	astacin [*Mamestra configurata*]	-4.06	
Contig[5632]	GH18999 [*Drosophila grimshawi*]		5.4
Contig[5707]	myosin light polypeptide 9 isoform B [*Bombyx mori*]	2.28	2.53
Contig[5715]	alkaline nuclease [*Bombyx mori*]	-3.7	-2.54
Contig[5729]	SEC14-like protein 2-like [*Apis mellifera*]	-4.23	-2.79
Contig[5744]	363_100_1 protein [*Mamestra con*figurata]		-2.67
Contig[5800]	larval cuticle protein 14 [*Manduca sexta*]	3.14	
Contig[5826]	troponin I [*Loxostege sticticalis*]	2.57	
Contig[5837]	hypothetical protein TcasGA2_TC002510 [*Tribolium castaneum*]	2.54	3.09
Contig[5838]	canopy-1-like [*Apis mellifera*]		-2.83
Contig[5872]	suppressor of profilin 2 [*Bombyx mori*]	2.73	2.2
Contig[5929]	unknown [*Picea sitchensis*]	3.98	2.45
Contig[5975]	SocE [*Bacillus cereus* W]		-2.94
Contig[6042]	chemosensory protein [*Papilio xuthus*]	6.71	
ECB-01_M13R_G02	actin-related protein ARP2/3 complex subunit ARPC2 [*Glossina morsitans morsitans*]	2.45	2.1
ECB-02_M13R_H03	saposin-like protein [*Bombyx mori*]	2.84	2.87
ECB-03_M13R_E12	DUF233 protein [*Heliothis virescens*]	9.52	
ECB-11_M13R_E02	Lipoyltransferase 1, mitochondrial [*Acromyrmex echinatior*]	-3.47	-2.34
ECB-11_M13R_E06	GD21009 [*Drosophila simulans*]	-5.13	-2.69
ECB-12_M13R_D10	putative WD repeat domain 13 (Wdr13) [*Heliconius melpomene*]		2.27
ECB-14_M13R_B03	ubiquitin conjugating enzyme E2 [*Bombyx mori*]	2.54	2.72
ECB-15_M13R_C06	translocon-associated protein gamma isoform 2 [*Bombyx mori*]		-2.54
ECB-16_M13R_E08	mitochondrial aminotransferase [*Camponotus floridanus*]		-2.94
ECB-17_M13R_C10	protein disulfide isomerase [*Helicoverpa armigera*]		-2.6
ECB-18_M13R_C11	beta-tubulin [*Bombyx mori*]	3.74	
ECB-18_M13R_E05	thioredoxin [*Bombyx mori*]	2.35	
ECB-19_M13R_A07	astacin [*Mamestra configurata*]	-3.34	
ECB2_M13F_C08	farnesyl diphosphate synthase [*Bombyx mori*]	-2.96	-2.64

ECB-21_M13R_E01	CG12926-PA-like protein [*Helicoverpa armigera*]	-2.48	
ECB-23_M13R_E01	alpha-tocopherol transfer protein-like [*Bombus terrestris*]	3.63	2.87
ECB-25_M13R_G02	CG3862-PA-like protein [*Plutella xylostella*]	2.09	
ECB-26_M13R_F04	Arp2/3 complex p21 subunit [*Spodoptera frugiperda*]	2.42	
ECB-26_M13R_F05	myosin regulatory light chain smooth muscle minor isoform	2.26	2.57
ECB-27_M13R_B09	ankyrin repeat domain protein	-2.86	
ECB-C-02_M13R_F10	Rab32 [*Helicoverpa armigera*]	2.45	
ECB-C-03_M13R_G05	conserved hypothetical protein [*Culex quinquefasciatus*]	3.32	
ECB-C-04_M13R_A08	beta-1 tubulin [*Spodoptera frugiperda*]	3.33	
ECB-C-05_M13R_C05	Lysine-specific demethylase 6A [*Acromyrmex echinatior*]	6.49	5.5
ECB-C-06_M13R_B02	nidogen, putative [*Pediculus humanus corporis*]	-3.5	-2.68
ECB-C-11_M13R_A06	hypothetical protein EAI_08582 [*Harpegnathos saltator*]	-4.22	-3
ECB-C-12_M13R_E09	kinesin-associated protein, putative [*Aedes aegypti*]	2.14	
ECB-C-13_M13R_F05	heterogeneous nuclear ribonucleoprotein A1 [*Bombyx mori*]	2.28	
ECB-C-14_M13R_B06	glycolipid transfer protein [*Bombyx mori*]		2.55
ECB-C-14_M13R_D01	hypothetical protein AND_12479 [*Anopheles darlingi*]	-2.85	
ECB-C-17_M13R_F04	rrm-containing protein seb-4, putative [*Pediculus humanus corporis*]	-2.12	
ECB-C-20_M13R_C09	conserved hypothetical protein [*Culex quinquefasciatus*]	2.26	
ECB-V-02_M13R_E10	scavenger mRNA-decapping enzyme DcpS-like isoform 1 [*Apis mellifera*]	2.17	
ECB-V-05_M13R_D03	Adhesion-regulating molecule 1 precursor [*Pediculus humanus corporis*]	3.25	2.68
ECB-V-05_M13R_G12	ecdysteroid UDP-glucosyltransferase	3.54	
ECB-V-07_M13R_C07	GH18999 [*Drosophila grimshawi*]	3.37	2.19
ECB-V-07_M13R_D03	MIP19203p [*Drosophila melanogaster*]	6.6	6.08
ECB-V-08_M13R_G01	conserved hypothetical protein [*Culex quinquefasciatus*]	2.07	
ECB-V-08_M13R_G03	ecdysteroid UDP-glucosyltransferase	3.17	
ECB-V-09_M13R_A03	Mps one binder kinase activator-like 1 [*Harpegnathos saltator*]	2.84	
ECB-V-12_M13R_E11	Dipeptidyl peptidase 4 [*Harpegnathos saltator*]	-3.57	
ECB-V-14_M13R_C12	vacuolar protein sorting 37b	2.3	2.82
ECB-V-15_M13R_B06	visgun, isoform A [*Drosophila melanogaster*]	3.52	
ECB-V-15_M13R_E06	Receptor expression-enhancing protein 1 [*Harpegnathos saltator*]	2.18	
ECB-V-18_M13R_A08	fatty acid binding protein		-2.79
ECB-V-19_M13R_E04	sialic acid synthase-like [*Xenopus tropicalis*]		2.16

ECB-V-22_M13R_G04	CG42837 [*Drosophila melanogaster*]	-3.14	
ECB-V-23_M13R_A09	fatty acid transport protein [*Ostrinia scapulalis*]	-2.41	
ECB-V-23_M13R_C10	growth hormone-regulated TBC protein 1-A-like [*Bombus terrestris*]	2.27	2.1
ECB-V-23_M13R_H01	WW domain binding protein wBP-2 [*Glossina morsitans morsitans*]	2.42	2.15
ECB-V-25_M13R_C10	conserved hypothetical protein [*Culex quinquefasciatus*]	5.91	6.27
ECB-V-25_M13R_F09	similar to presqualene diphosphate phosphatase [*Tribolium castaneum*]	3.46	3.21
ECB-V-26_M13R_H03	AMP dependent CoA ligase [*Aedes aegypti*]	-5.28	
ECB-V-29_M13R_B10	farnesyltransferase/geranylgeranyltransferase type I alpha subunit [*Bombyx mori*]	2.28	
ECB-V-29_M13R_G10	acyl-CoA oxidase [*Heliothis virescens*]		-2.42
gi_133905779	vanin-like protein 1	-9.2	-5.67
gi_133906199	hypothetical protein TcasGA2_TC001925 [*Tribolium castaneum*]	3.17	2.58
gi_133906407	kynureninase [*Bombyx mori*]		-3.3
gi_133906419	lipophorin receptor [*Galleria mellonella*]	2.63	2.44
gi_133906904	GK17133 [*Drosophila willistoni*]	2.12	4.04
gi_133906913	similar to CG3823 CG3823-PA [*Tribolium castaneum*]	4.37	
J-ECB-04_M13R_E11	sorting nexin [*Culex quinquefasciatus*]		3.06
J-ECB-05_M13R_E12	actin 3 isoform, putative [*Tribolium castaneum*]		2.38
J-ECB-06_M13R_D11	aquaporin [*Bombyx mori*]	-9.45	-3.2
J-ECB-08_M13R_B02	sensory appendage protein 3 [*Manduca sexta*]		-71.78
J-ECB-11_M13R_C08	ADP ribosylation factor-like protein [*Phlebotomus papatasi*]	2.14	
J-ECB-14_M13R_D04	CG2765 CG2765-PA [*Tribolium castaneum*]		2.49
J-ECB-14_M13R_H06	similar to CG14661-PA [*Apis mellifera*]	6.28	
J-ECB-17_M13R_A10	GTP-binding protein yptV3 [*Culex quinquefasciatus*]	2.42	2.36
J-ECB-21_M13R_F05	Kunitz-type protease inhibitor precursor [*Galleria mellonella*]		-4.66
J-ECB-21_M13R_G07	Immunoglobulin superfamily member 10 [*Acromyrmex echinatior*]	2.55	2.33
J-ECB-24_M13R_F10	p94-like protein		3.83
J-ECB-24_M13R_G10	putative midgut protein [*Phlebotomus perniciosus*]	4.16	
J-ECB-25_M13R_A10	2-acylglycerol O-acyltransferase 1 [*Harpegnathos saltator*]	-2.23	
J-ECB-25_M13R_D01	myelin proteolipid [*Biston betularia*]	2.29	
J-ECB-25_M13R_G02	ribosomal protein L30 [*Manduca sexta*]	3.23	
J-ECB-29_M13R_G03	IST1 homolog [*Bombus terrestris*]	5.72	6.66
J-ECB-29_M13R_H09	Inhibitor of growth protein 3 [*Camponotus floridanus*]	2.59	

J-ECB-31_M13R_B02	26S proteasome non-ATPase regulatory subunit 11 [*Acromyrmex echinatior*]	2.19	
J-ECB-33_M13R_D08	pyridoxine 5'-phosphate oxidase [*Bombyx mori*]	-2.38	
J-ECB-33_M13R_G10	CG32512 CG32512-PA [*Tribolium castaneum*]	2.66	3.41
J-ECB-35_M13R_F06	heme oxygenase [*Bombyx mori*]	2.29	2.22
J-ECB-37_M13R_E05	oxidoreductase [*Acromyrmex echinatior*]	-2.77	
J-ECB-38_M13R_B03	inhibitor of growth protein 3	2.75	2.06
J-ECB-39_M13R_F07	cytidylate kinase	-2.21	
J-ECB-39_M13R_H09	conserved hypothetical protein [*Culex quinquefasciatus*]	3.36	2.67
J-ECB-40_M13R_D07	troponin C [*Bombyx mori*]	2.22	
J-ECB-41_M13R_A03	Transient receptor potential channel pyrexia [*Acromyrmex echinatior*]	3.23	3.65
J-ECB-41_M13R_H07	G10 protein [*Bombyx mori*]		2.05
J-ECB-42_M13R_A08	RGS-GAIP interacting protein GIPC [*Bombyx mori*]		2.24
J-ECB-42_M13R_C06	glucosylceramidase-like [*Monodelphis domestica*]	-3.45	
J-ECB-43_M13R_F12	EF-hand domain family, member D1 [*Nasonia vitripennis*]	8.91	7.79
J-ECB-47_M13R_A02	hepatocyte growth factor-regulated tyrosine kinase substrate [*Tribolium castaneum*]	3.77	3.78
J-ECB-49_M13R_C04	actin [*Spodoptera exigua*]	2.18	
J-ECB-49_M13R_H10	cystathionine gamma-lyase [*Bombyx mori*]	6.29	2.97
J-ECB-50_M13R_C10	S-adenosylmethionine decarboxylase proenzyme [*Camponotus floridanus*]		2.46
J-ECB-50_M13R_F10	elongation factor-1 alpha [*Corcyra cephalonica*]	-2.08	
J-ECB-53_M13R_A10	hypothetical protein TcasGA2_TC008350 [*Tribolium castaneum*]	2.21	
J-ECB-54_M13R_F04	troponin T transcript variant A [*Bombyx mandarina*]	4.03	
J-ECB-58_M13R_A02	cuticular protein RR-1 motif 23 [*Bombyx mori*]	19.61	
J-ECB-58_M13R_A11	innexin 2 [*Heliothis virescens*]		2.49
J-ECB-60_M13R_B03	pyroglutamyl-peptidase 1-like [*Apis mellifera*]	-2.33	
J-ECB-60_M13R_C07	probable 4-coumarate--CoA ligase 3-like isoform 2 [*Acyrthosiphon pisum*]	-5.18	
J-ECB-60_M13R_H07	disulfide-isomerase A6 [*Culex tarsalis*]		-2.48

Figure 4-1. The total number of significantly up- or down-regulated transcripts ($p \leq 0.05$) in the gut of *O. nubilalis* in Cry1Ab resistant (R-) and susceptible (S-) strain larvae fed transgenic Cry1Ab corn leaves for 6-h

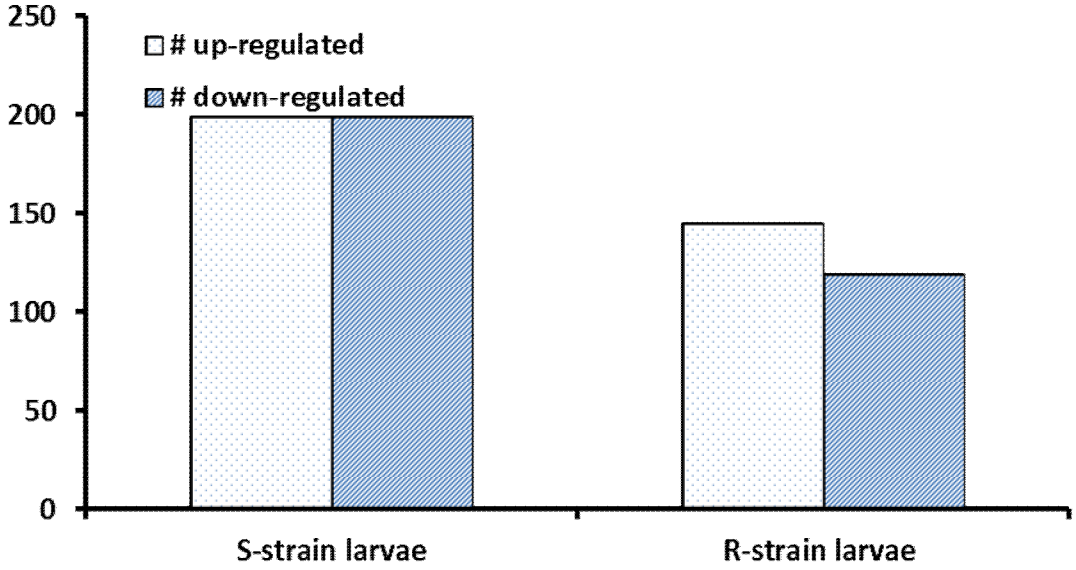


Figure 4-2. A Venn diagram.

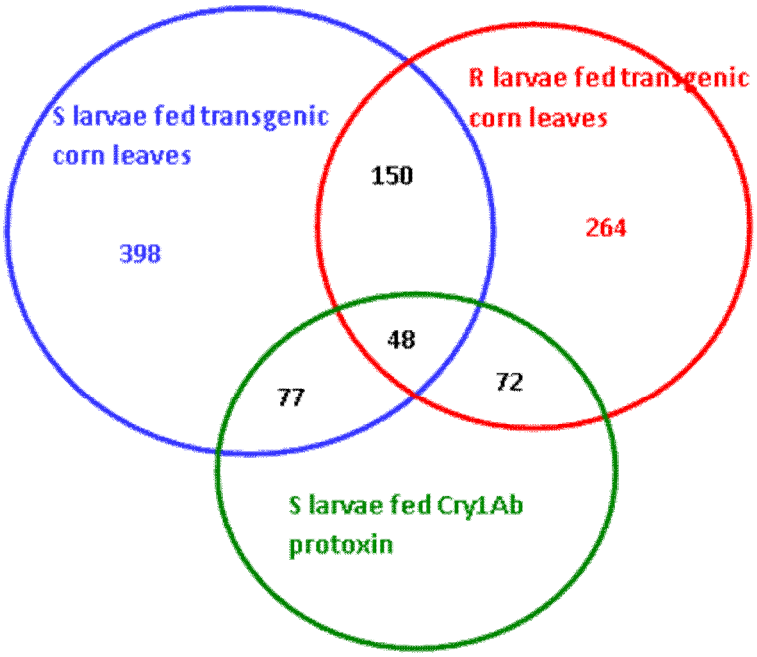


Figure 4-3a-b. Microarray analysis (diagonal lined bars) and qPCR analyses (cross hatched bars) for 17 and 9 differentially expressed transcripts in the larval gut of S- and R-strain larvae after fed on transgenic corn leaves expressing Cry1Ab toxin, respectively.

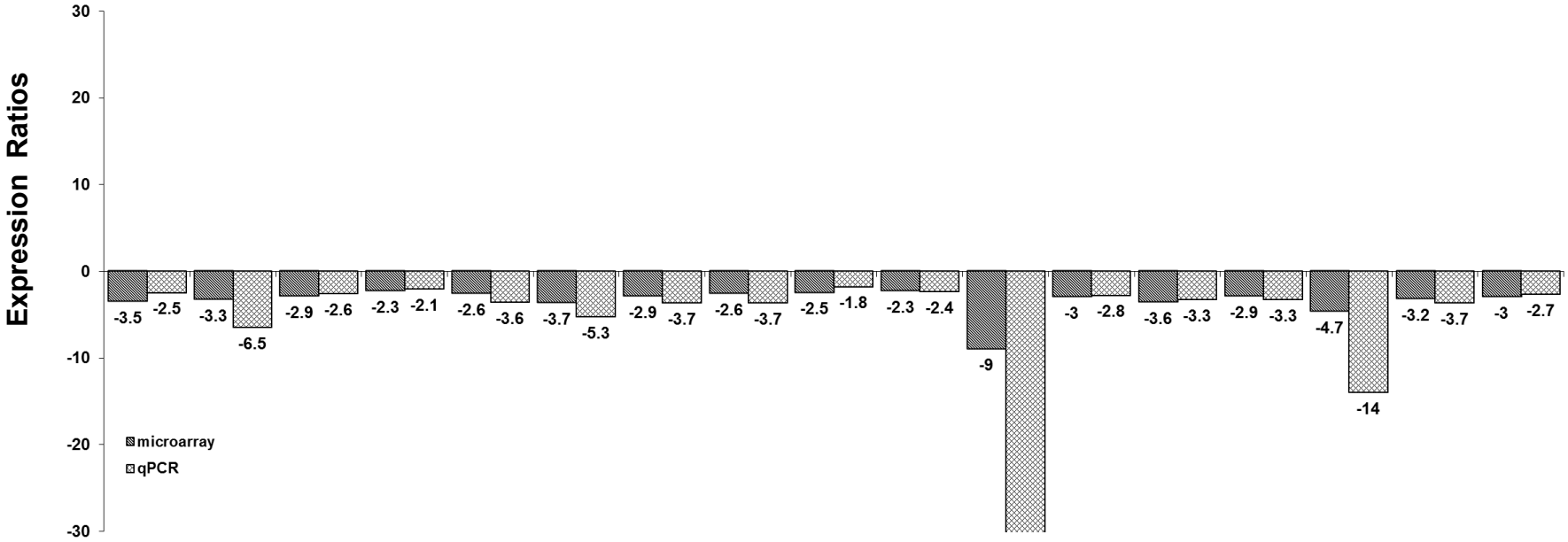


Fig 4-3a

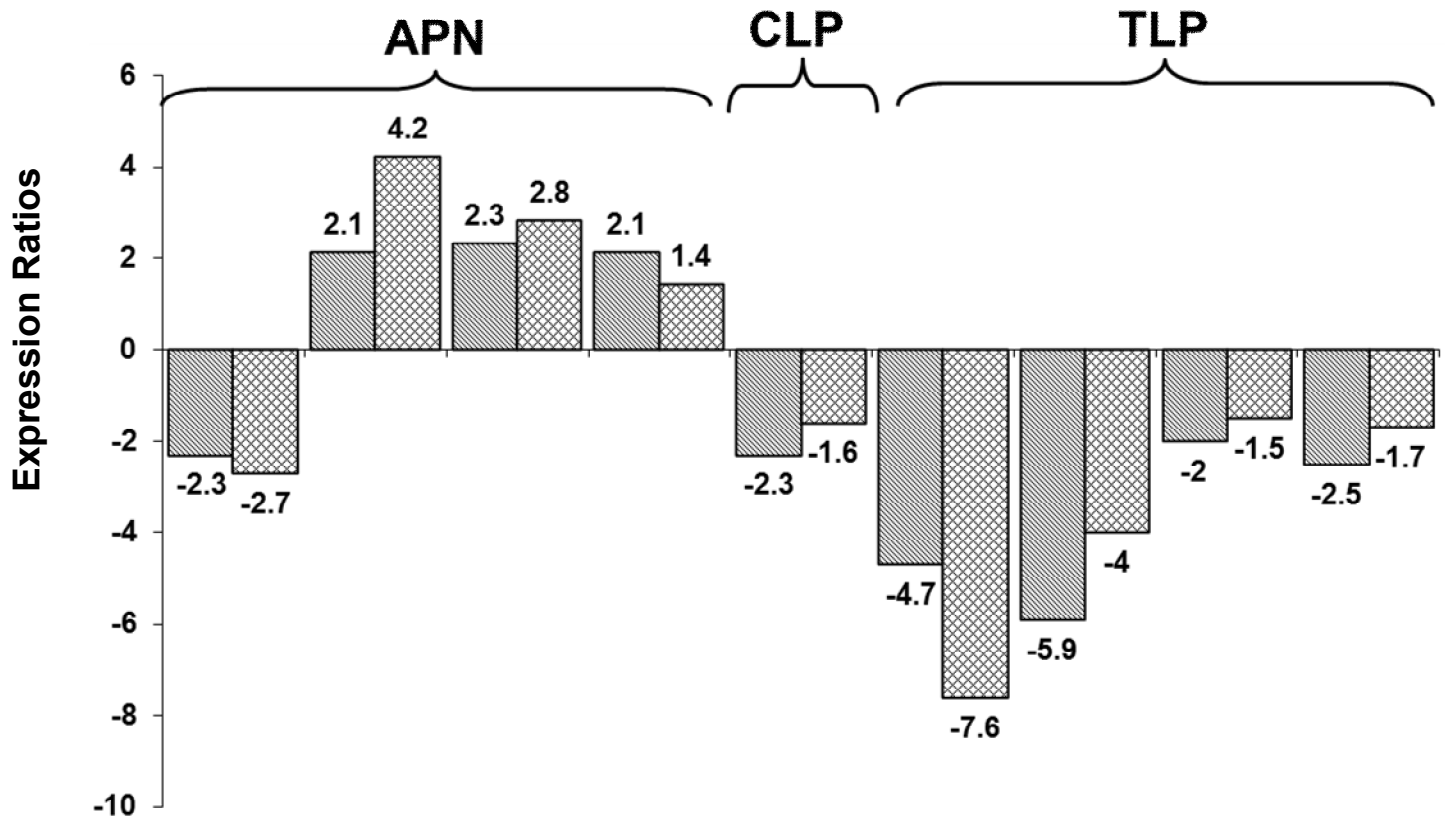

APN
CLP
TLP
Expression Ratios
6
4
2
0
-2
-4
-6
-8
-10
-2.3
-2.7
2.1
4.2
2.3
2.8
2.1
1.4
-2.3
-1.6
-4.7
-7.6
-5.9
-4
-2
-1.5
-2.5
-1.7

Fig. 4-3b.

Figure 4-4 . Comparisons of expression changes for six candidate genes that may potentially be involved in Bt toxicity and/or resistance in the larval gut of R- and S-strain in response to the ingestion of transgenic Cry1Ab corn leaves

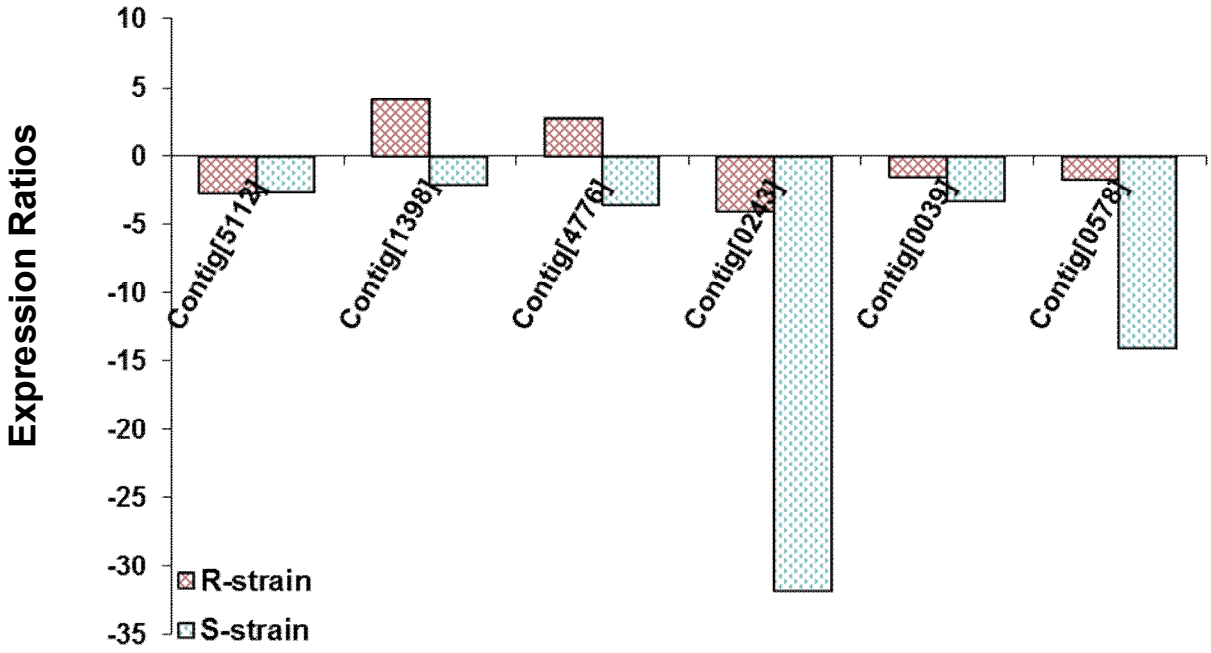
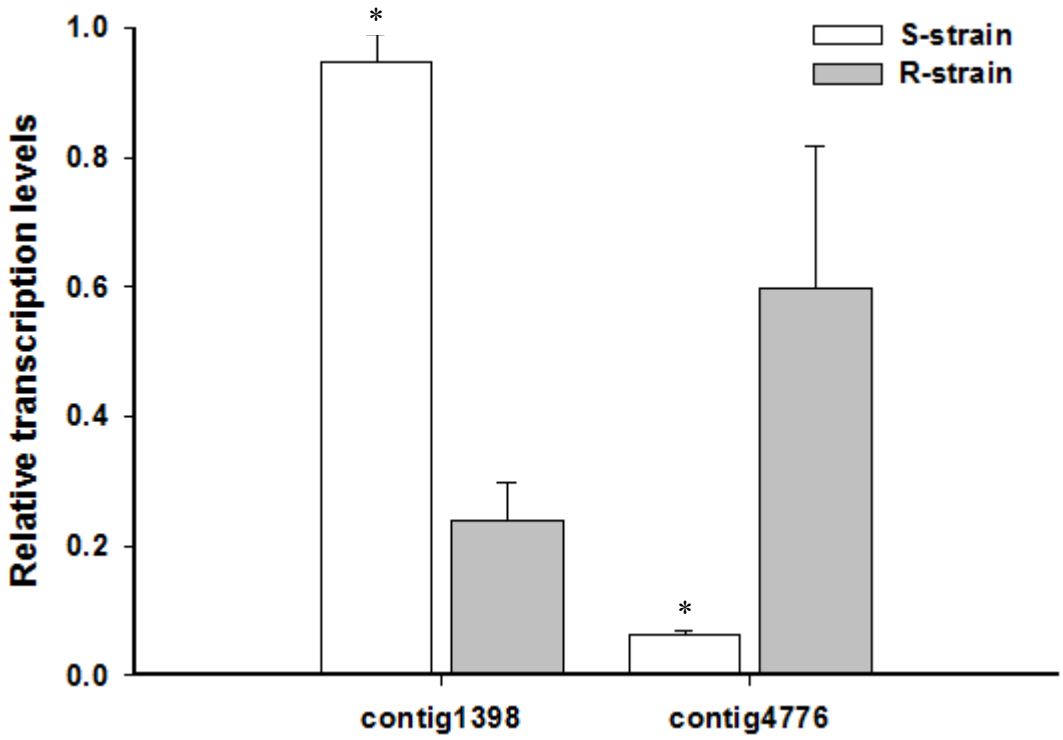


Figure 4-5. Relative transcriptional levels of two aminopeptidase transcripts (EST ID: contig1398 and contig4776) in the larval gut of R-strain and S-strain.



Summary

European corn borer, *Ostrinia nubilalis*, is one of the most destructive insect pests of corn, and its damage causes poor ear development, broken stalks and dropped ears, resulting in substantial yield losses. Larvae of *O. nubilais* are hidden inside the plant; therefore, it is hard to control them by spraying insecticides on the plant surface. *Bacillus thuringiensis* (Bt) expressing Cry toxins have insecticidal and target specific properties and transgenic Bt corn, which carries *cry* genes, can continually produce Cry toxins in plant tissues where *O. nubilalis* larvae fed. Such transgenic corn can efficiently control *O. nubilalis* infestation. After *O. nubilalis* larvae ingested Cry toxin, there are multiple interactions of gut gene products with Cry toxins, which influence the toxicity of Bt toxins. Insect gut serine proteases, like trypsins and chymotrypsins, first solubilize crystal protoxin and release the active toxin into gut lumen, and then multiple gut membrane binding partners are involved in unselective pore formation on gut membrane or cellular signal transduction resulting in gut cell lysis. Certainly, the Cry toxin attack also triggers intracellular defense.

In this study, we identified and characterized 34 putative serine protease genes from *O. nubilalis* gut specific cDNA library, including 15 encoding trypsins, 15 chymotrypsins, 2 trypsin-like and 2 chymotrypsin-like serine protease homologs. Blast and phylogenetic analysis of the deduced amino acid sequences indicated that 15 putative trypsins belong to Try-G2 and Try-G3 groups, whereas 15 putative chymotrypsins is within the same large group (CTP-G1). Two trypsin-like homologs (*OnTry12* and *OnTry13*) and two chymotrypsin-like homologs (*OnCTP15* and *OnCTP16*) belong to two new groups (Try-G4 and CTP-G2, respectively). The existence of diverse serine proteases in *O. nubilalis* is an adaptation to diverse food sources and also a defense mechanism against different plant-specific protease inhibitors, and an advantage to survive sub-lethal concentrations of Cry toxins from transgenic corn. However, two trypsin-like proteases (OnTry12 and OnTry13) and two chymotrypsin-like proteases (OnCTP15 and OnCTP16) belong to two new groups (Try-G4 and CTP-G2, respectively). OnTry12 shows 69 % identity to serine protease 31 (SP31) from *Mamestra configurata* whereas OnTry13 shows 63% identity to trypsin (HaFLS00713) from *Helicoverpa armigera*. SP31 and HaFLS00713 were associated with remodeling of cuticle in the gut peritrophic matrix and are involved in molting.

Both OnCTP15 and OnCTP16 were grouped in CTP-G2 with four chymotrypsin like proteases from *Manduca sexta* (Ms-AM419170 and Ms-AM690448), *H. armigera* (Ha-EU325548), and *Spodoptera exigua* (Se-AY820894) by bootstrap value (=99), and they were important in molting from larva to pupa in three species. Thus, our results may suggest that OnCTP15 and OnCTP16 might have similar functions during the insect molting as found in other insects.

Results from RT-PCR analysis indicated that all putative trypsins, chymotrypsins and homologs are highly expressed in gut tissues, but they have different expression patterns in different regions of the gut. The trypsins of Try-G2 were highly expressed in the foregut and midgut, whereas those in Try-G3 were highly expressed in midgut and hindgut. The differential expression pattern implies that these trypsins may have different functions in food digestion. According to the sensitivities of trypsins to protease inhibitors (Kunitz-type soybean trypsin inhibitor) from *H. armigera* and *M. configurata*, trypsins of Try-G2 are insensitive, while those of Try-G3 are sensitive (Bown et al., 1997; Erlandson et al., 2010). This might be one kind of adaptation for food source containing protease inhibitors in *O. nubilalis*. The foregut-midgut (Try-G2) trypsins, which are insensitive to protease inhibitors, are the first access to and digest food sources containing protease inhibitors. This facilitates the midgut-hindgut (Try-G3) trypsins, which are sensitive to protease inhibitors, to further digest. It may also possible that foregut-midgut trypsins play roles in activation of other zymogens, such as trypsinogen, chymotrypsinogen and proelastase (de Haen and Gertler. 1974), or in initiating a cascade of events and subsequently regulate late trypsin expression (Barillas-Mury et al., 1995).

Real-time quantitative PCR was used to evaluate the expression of protease transcripts in the gut of third-instar larvae fed artificial diet with or without Cry1Ab protoxin for 2 h, 6 h, 12 h, and 24 h. Transcripts of four putative trypsins (*OnTry4*, *OnTry5*, *OnTry6* and *OnTry14*) were up-regulated in *O. nubilalis* larvae after the ingestion of Cry1Ab protoxin. If these genes products are involved in the activation of Bt protoxins, the up-regulation of these genes could lead to an increased susceptibility of the larvae to Bt toxins. On the other hand, if these genes products are involved in the degradation of Bt protoxins and toxins, the up-regulation of these genes may lead to decreased susceptibility of larvae to Bt toxins. In a resistant strain of Indianmeal moth (*Plodia interpunctella*), the lack of a major gut protease activity, PiT2 (accession No: AF064525), was responsible for about 90% of the resistance to Cry1Ab protoxin in a *B. thuringiensis* subsp.

entomocidus-resistant colony (Zhu et al., 2000). In the fall armyworm (*Spodoptera falrugiperda*), a trypsin SfT6 (accession No: FJ940726) was demonstrated by RNAi that it plays a role in Bt Cry1Ca1 susceptibility because the knockdown of SfT6 can reduce the susceptibility to a Bt Cry1Ca1 protoxin (Rodriguez-Cabrera et al., 2010). In phylogenetic tree analysis, OnTry4, OnTry5, OnTry6 and OnTry14 in *O. nubilalis*, which were up-regulated after 6 h Cry1Ab protoxin exposure, were in the same group with SfT6 and PiT2 and share 79%, 69% and 68% with PiT2. It is possible that those genes may contribute to protoxin activation.

Four putative *OnCTPs* (*OnCTP2*, *OnCTP5*, *OnCTP12*, and *OnCTP13*) in *O. nubilalis* were significantly (*P*-value ≤0.05) up-regulated in the third-instar larvae in the interval from 2 to 24 h after Cry1Ab protoxin ingestion. The chymotrypsins encoded by those transcripts may contribute to the degradation of active Cry1Ab toxin in *O. nubilalis*, especially *OnCTP2* and *OnCTP12* which were up-regulated more than 10 fold after 24 h exposure to Cry1Ab protoxin. Because chymotrypsin degrade activated toxin or/and receptor bound toxin, it might be a reason why chymotrypsin up-regulation (after 24 h exposure) were much later than trypsin up-regulation (2 h to 6 h exposure).

In order to examine the transcriptional responses of many other genes expressed in larval guts of *O. nubilalis*, we developed a high-resolution 8×15K cDNA microarray chips based on the larval gut specific EST database. Each microarray contains 12,797 probes representing 2,895 unique gut transcripts. The microarray was used to detect gut genes transcriptional responses to the ingestions of Cry1Ab protoxin. A total of 174 significantly differentially expressed (fold change cut off ≥2.0 with *p*-value ≤0.05) genes were identified from larvae fed on artificial diet containing Cry1Ab protoxin. These differentially expressed genes include 80 down-regulated and 94 up-regulated. Of 174 genes, the serine proteases constitute the most abundant group of genes (8 genes) that were regulated when exposed to Cry1Ab, including 5 putative trypsin or trypsin-like, and 3 putative chymotrypsin or chymotrypsin-like proteases. These genes may play important roles in Bt toxicity either by activation, degradation or other cellular defense pathways. Especially, three trypsin transcripts (EST ID: contig4786, contig3704, and contig0770) were significantly up-regulated (>3.0-fold) after the larvae ingested Cry1Ab protoxin for 6 h. Based on phylogenetic analysis (see Chapter 2), these transcripts are OnTry5, OnTry6 and OnTry14 from the Try-G2 group, similar to OnTry1. In a Bt resistant strain of *P. interpunctella*, the lack

of a major gut protease activity, PiT2 (accession No: AF064525), was responsible for about 90% of the resistance to Cry1Ab protoxin in a *B. thuringiensis* subsp. *entomocidus*-resistant colony (Zhu et al., 2000). Because OnTry5, OnTry6 and OnTry14 in *O. nubilalis*, which share 79%, 69%, and 68% identities and are in the same group (Try-G2) with PiT2, were up-regulated after 6-h Cry1Ab protoxin exposure, it is possible that those trypsins may also contribute to Bt protoxin activation.

Cadherin was proposed as essential monomeric Bt toxin binding partners in pore-formation and signal transduction model. In our microarray analysis, one cadherin-like protein was up-regulated ~2-fold. Aminopeptidases and alkaline phosphatase were specifically proposed as oligomeric toxin binding partners in pore-formation model. Three aminopeptidases and one alkaline phosphatase transcripts were also regulated after larvae fed protoxin for 6 h. Alkaline phosphatase transcript and one aminopeptiase were up-regulated ~2-fold; however, the remaining two aminopeptidase transcripts were down-regulated ~2-fold.

We further examined transcriptional responses of the genes expressed in the larval guts to the ingestion of transgenic Bt corn expressing Cry1Ab toxin both in the laboratory-selected Cry1Ab protoxin resistant (Sky, R-strain) and susceptible (Meads, S-strain) strains of *O. nubilalis*. Even though the third-instar larvae of *O. nubilalis* resistant strain was 200-fold resistance to Cry1Ab protoxin compared with the susceptible strain (data is not showed in this paper), both of them cannot survive on transgenic corn expressed Cry1Ab for 6-d exposure. The survival time of R-strain larvae was significantly different from that of S-strain larvae and the median lethal time (LT_{50}) for the early third-instar larvae of R- and S-strains were 5.4 and 3.6 days, respectively.

We identified 398 and 264genes from the larvae of the S and R strains, respectively, with a significantly increased or decreased expression (fold changes ≥2.0 fold with p-value ≤0.05) as compared with those in the larvae fed on non-transgenic corn leaves. The number of transcripts and their expression ratios of S-strain larvae are larger than these of R-strain larvae because R-strain larvae have been continuously selected by Cry1Ab protoxin for multiple generations and the suppression of these genes may have been adapted in the R-strain larvae. For example, most transcripts involving in metabolic process were down-regulated in both strain, like trypsin, glutathione *S*-transferase, carboxypeptidase were down-regulated approximately 10-fold in S-

strain larvae, but only five or less folds in R-strain larvae. When larvae of R-strain were fed transgenic corn leaves expressed a high dose of Cry1Ab toxin, the down regulation of those genes were only slightly enhanced. However, when S-strain larvae were fed transgenic corn leaves, the down-regulation of these genes was more dramatic than those of R-strain because the S-strain larvae were more susceptible to the toxin.

When the susceptible larvae of *O. nubilalis* were fed the artificial diet containing Cry1Ab protoxin, five serine protease genes were up-regulated. Among them, four (EST id: contig0389, contig1207, contig3704, contig4768) were up-regulated by more than 5-fold. However, when the susceptible larvae of *O. nubilalis* were fed transgenic corn leaves expressing Cry1Ab, none of them showed either up- or down-regulations of these genes, suggesting the difference in gene expression responses to Cry1Ab protoxin and Cry1Ab toxin expressed in transgenic corn. Twelve serine protease transcripts of S-strain and 6 of R-strain larvae were down-regulated after fed transgenic corn leaves.

Meanwhile, two aminopeptidase transcripts (EST id: contig4776 and contig1398) showed opposite expression patterns in R- and S-strain larvae without any treatments. Transcript-contig4776 has 9-fold higher expression ratio in R-strain than in S-strain larvae, in contrast, contig1398 have 4-fold higher expression ratio in S-strain than that of R-strain. However, when R- and S-strain larvae were fed transgenic corn leaves, the expression ratios of contig4776 and contig1398 showed opposite ways in R- and S-strain larvae. They were both down-regulated in S-strain larvae, but up-regulated in R-strain larvae. There is a possibility that aminopeptidase-contig4776 may be one cellular defense factor against Cry1Ab toxin attack; however, the reduced expression of aminopeptidase-contig1398 in R-strain larvae could serve as a resistance mechanism by the reduced the amount of toxin binding partners. Indeed, aminpeptidase-contig1398 shares 98% identity to an aminopeptidase N1 of *O. furnacalis* (GenBank accession No: ACX85727), 61% to *H. armigera* (accession No: ACC68682). All those aminopeptidases were identified as the major proteins having high binding affinity to Cry1 toxins in these species, and proposed as Cry1 toxin resistance genes in *O. furnacalis* and *H. armigera* (Xu et al., 2011; Zhang et al., 2009).

Our studies revealed a number of genes whose expression levels were altered by the ingestion of transgenic corn leaves expressing Cry1Ab toxin in both *O. nubilalis* R- and S-strains larvae. Our results not only provided information of the difference in the transcriptional responses of these genes to the toxin between Bt-resistant and susceptible *O. nubilalis* strains, but also provided new insights of potential interactions of the protoxin, toxin from transgenic corn with important proteins in the gut of *O. nubilalis* larvae. Nevertheless, further studies would be necessary to clarify functional roles of these differentially expressed genes that influence the toxicity of Cry1Ab protoxin and Cry1Ab toxin in *O. nubilalis*.

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