GENOMIC, EXPRESSION AND FUNCTIONAL ANALYSIS OF GENES FROM LARVAL GUT OF THE EUROPEAN CORN BORER, *OSTRINIA NUBILALIS* (HÜBNER)

by

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B.S., Punjab Agricultural University, India, 2002
M.S., Punjab Agricultural University, India, 2004

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Genomic information for lepidopteran insects, particularly agricultural pest species, is very limited but urgently needed due to their economic importance and biodiversity. The huge economic losses ($ 1-2 billons / year) caused by the European corn borer (*Ostrinia nubilalis*, Hübner, ECB) makes this insect species one of the major pests of corn in the United States and western world. Management of ECB by conventional methods is limited but has had a great success by transgenic Bt (*Bacillus thuringiensis*) corn, which targets insect gut. However, the widespread use of Bt corn may lead to the development of Bt resistance in ECB. Knowledge of genes expressed in the insect gut is considered crucial for understanding basic physiology of food digestion, their interactions with Bt toxins and pathogens, and for discovering new targets for pest management.

A large database of 15,000 expressed sequence tags (ESTs) was established from the ECB larval gut. To our knowledge, this database represents the largest gut-specific EST database from a lepidopteran pest. Analysis of 10 aminopeptidase-like genes between Cry1Ab–resistant and –susceptible ECB larvae revealed that aminopeptidase P-like (*OnAPP*) gene is a strong candidate for its role in Bt toxicity and resistance. The RNA interference mediated reduction in the transcript level of *OnAPP* gene in ECB larvae resulted in their reduced susceptibily to Cry1Ab.

Analysis of the chitinase-like gene (*OnCht*) revealed its essential role in regulating chitin content of peritrophic membrane (PM). Our results suggest that *OnCht* may influence food digestion, nutrient absorption or movement of digestive enzymes through the PM and can be an important target for insect management. We also identified and characterized six genes involved

in the innate immune defense response in ECB and showed that the expression of these genes were induced when challenged with bacteria.

In addition to these results, this research generated significant genomic information for the development of microarray from the larval gut of ECB. The establishment of the feeding-based RNA interference technique could potentially help in delivering dsRNA orally to ECB for high throughput screening of effective genes to be targeted for insect pest management.

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Dedication

I dedicate this dissertation to my parents, Kewel Krishan Khajuria and Darshan Kumari, for their encouragement, unconditional love, and dedication to raising their children as good individuals. Thank you for everything!

CHAPTER 1 - Introduction

The genomic information on insects has increased tremendously during last several years. Lepidoptera, the second most biodiverse group of insect species after Coleoptera, represents more than 160,000 species including many of the most devastating pests of crops, forests and stored products (Pierce 1995). However, genomic information for lepidopteran insects, particularly agricultural pest species is limited but urgently needed due to their economic importance and biodiversity. Availability and identification of the DNA sequences for an organism is essential for understanding the gene functions and their involvement in various biological processes.

Corn or maize (*Zea mays* L.) is a widely grown crop in the world with annual production of 790 million metric tons in 2007-2008 (Corn Refiners Association 2008) and grown on more than 148 million hectares worldwide (USDA, NASS 2007). The huge economic losses ($ 1-2 billons -/- year) caused by the European corn borer (*Ostrinia nubilalis*, Hübner, ECB) makes this insect species one of the major pests of corn in the United States and western world (Lauer and Wedberg 1999, Hyde et al. 1999). Management of ECB by conventional methods has been limited but management by transgenic Bt (*Bacillus thuringiensis*) corn has been very successful (Walker et al. 2000). The main target for the Bt toxin is the insect midgut, where it is solubilized and cleaved by gut protease to produce activated toxin, which then binds with the specific receptor to produce toxicity (Gill et al.1992). Due to the widespread use of Bt corn, there are concerns regarding the development of Bt resistance in field populations of ECB. Therefore, identification and characterization of the genes and their products involved in the toxin-target

interactions is fundamental in sustaining the use of transgenic Bt technology in the integrated pest management.

In addition to Bt action and resistance, the knowledge of genes expressed in the insect gut is also considered crucial for understanding basic physiology of food digestion, its molecular composition, its interaction with pathogens, and for discovering new targets for novel toxins to be used in pest management.

The European corn borer (*Ostrinia nubilalis* Hübner)

Distribution, biology, and economic importance of Ostrinia nubilalis

The European corn borer (ECB, *Ostrinia nubilalis* Hübner) is widely distributed across central and southern Europe, throughout North America, Siberia, northern India, and western China (Caffrey and Worthley 1927, Showers 1993). In the United States, ECB was first reported in 1917 (Vinal 1917). However, it is thought to have been introduced multiple times to North America in shipments of broom corn imported from Italy and Hungry to eastern United States and Canada between 1909 to 1914 (Caffrey and Worthley 1927). The life cycle of ECB is composed of four developmental stages: egg, larva, pupa, and adult. The ECB larva passes through five instars and they are the most important stage that causes major physiological and economically damage to corn. The ECB female lays eggs on corn leaves, and young larvae feed in the whorl and move to leaf sheath and midribs. It is the third instar which bores into the stalk, ear, and shank (Mason et al. 1996). The injury caused by larval feeding and boring disrupts the translocation of essential nutrients and water needed for proper plant development (VanDyk 1996, Witkowski and Wright 1997). Starting in late summer or early autumn and ending late the next spring in the Midwest region of the United States, ECB larvae over-winter in the stalks of their host plants as diapausing fifth instars (Caffrey and Worthley 1927). The number of

generations of ECB in United States increases from one to four from the north to the south and there is considerable local adaptation to climate conditions. Most parts of the Corn Belt have two generations (Mason et al. 1996). In Kansas, there are two but occasionally three generations per year (Showers et al. 1989).

ECB is a polyphagous insect and can develop on 223 plant species (both monocotyledon and dicotyledon) (Lewis 1975). ECB is one of the major damaging pests of corn in the United States. The other crops attacked by ECB include bell pepper (*Capsicum annuum*), oats (*Avena sativa*), barley (*Hordeum vulgare*), artichoke (*Cynara scolymus*), soybean (*Glycine max*), sunflower (*Helianthus annuus*), and Solanaceous crops. Damaged plants are susceptible to breakage, ear drop, and secondary infections by fungus *Fusarium* species (VanDyk 1996). Fusarium species which develop on the larval wounds can produces mycotoxins, such as zearalenone, fumonisin, and trichothecium, which are harmful to humans and livestock and as a consequence, silage containing contaminated corn can be rendered unusable (Marasas et al. 1984, Munkvold and Desjardins, 1997).

Management of ECB

Conventional control measures

The conventional methods to manage ECB involve the combination of the resistant varieties of corn, insecticide applications, biological control agents, and seasonal cultural practices (Showers et al. 1989). The strategy was to use natural resistance in varieties such as DIMBOA, and then use economic thresholds (ET) with insecticides to manage first generation larvae (Pilcher and Rice 2001). Lot of effort has been devoted to develop resistant varieties against leaf feeding by first and second generation larvae in the past decades (Showers et al. 1989). However, these resistant varieties were not able to protect the corn plant from the stalk

tunneling by late larvae. Management for second generation larvae was more limited. The typical recommendation for management of ECB was the timely application of insecticides, such as bifenthrin, carbofuran, or permethrin (Mason et al. 1996). The main reason for ineffectiveness of the insecticide applications was the difficulty in getting proper timing of the spray application to obtain an economic benefit (Heinemann et al. 1992). Timing of the insecticide spray was critical, so intensive scouting efforts were needed to determine the best time for insecticide applications that would kill the early stage larvae before they bore into the stalk (Sloderbeck et al. 1984, Mason et al. 1996). Rice and Ostlie (1997) suggested that growers were reluctant to scout, and/or had concerns regarding the use of multiple insecticide applications and this led to the difficulty in managing the larvae with insecticides. Other control measures, such as cultural control and conservation of natural enemies (such as *Orius insidious*, *Chrysoperla* spp, several ladybird beetles, *Lydella thompsoni, Eriborus terebrans, Simpiesis viridula, Macrocentris grandii* and *Beauveria bassiana*) only play a limited role in the management of ECB (Mason et al. 1996).

Transgenic Bt corn

Bacillus thuringiensis (Bt) is a naturally occurring gram-positive, aerobic, motile, endospore forming bacteria (Lacey and Kaya 2000), which has been found worldwide with at least 82 different serovars (Lecadet et al. 1999). *Bacillus thuringiensis* produces crystalline inclusions of entomocidal protein protoxins (Pigott and Ellar 2007), which are active on insects in the orders of Lepidoptera, Diptera, and Coleoptera (Schnepf et al. 1998). The spores and crystals of Bt have been used as a biopesticide for almost 60 years in forestry management, agriculture, and vector-born disease control (Schnepf et al. 1998, Federici 2005). However, Bt commercial products have several limitations such as degradation with sunlight, being washed

away with rain, and it is not penetrating so insects which enters the plants are not affected. The importance of Bt toxins in the management of insect pests has increased dramatically with the developmental of transgenic plants with the ability to express the Bt toxin proteins (Valaitis et al. 2001, Shelton et al. 2002). The first genetically engineered crop plants containing a gene from *B. thuringiensis* variety *Kurstaki* (Berliner) were commercially produced and harvested in the United States during 1996 (Hilbeck et al.1998). Transgenic corn expressing Bt toxins have been very effective against the ECB. Higgins et al. (1996) reported that during field trials, Bt corn hybrids showed more than 99% control of the first generation of the ECB in whorl-stage corn. Several Bt commercial corn hybrids are available in North America to control ECB. Agrisure CB (Synenta) and YeildGard Corn Borer (Monsanto) both use the Cry1Ab gene whereas Herculex I (Pioneer Hi-Bred/DowAgroSciences) uses Cry1F genes for ECB control. Also corn hybrids such as HerculexXTRA (Pioneer Hi-Bred/DowAgroSciences), YeildGardPlus (Monsanto), and YeildGard VT Triple (Monsanto) contains more than one gene to control both corn borer and corn rootworm (Sloderbeck and Whitworth 2009). The acreage under the genetically modified (GM) crops has been increasing since 1996, and in 2008, 80 % of the total corn planted in the U.S. was GM corn, which includes 18 % for insect resistance (expressing Bt toxin), 40 % with stacked genes for both insect and herbicide resistance, and 23% for herbicide resistance (USDA-NASS 2008).

Mode of action of Bt toxin

The mode of action of Bt toxin changes relatively inert crystalline protoxin form into the cytotoxic form and involves several steps (Schnepf et al. 1998). After being ingested by the insect, Bt crystals were solubilized by gut proteases under alkaline and reducing condition of insect midgut (Huber et al. 1981). Gut proteases recognize cleavage sites on the protoxin and

cuts it to produce active toxin (Chestukhina et al. 1982, Choma et al. 1990), which then binds to specific receptors on the midgut epithelium. Binding of the activated Bt-toxin to midgut-specific receptors causes a toxin conformation change, that can allow for the insertion and formation of ion channels or pores in the midgut apical membrane, that leads to osmotic imbalance and eventually death of the insect (Gill et al. 1992, Schnepf et al. 1998).

Two models have been proposed to explain the mode of action of Bt toxins with the presence or absence of oligomerization of the cry toxin monomers (Pigott and Ellar 2007). The Bravo Model (Bravo et al. 2004) proposes oligomerization steps and suggests that both cadherin-like protein (Bt-R_1) and aminopeptidase N (APN) receptors are essential for Cry1 toxicity. In this model, the crystalline toxin is solubilized and protoxin is converted into activated toxin by gut proteases. The activated toxin then binds to Bt-R_1 to undergo conformational change that facilitates cleavage of helix α-1 by membrane bound proteases (Gomez et al. 2002). The resultant form of toxin oligomerizes to form tetrameric pre-pores which has increased affinity for APN and binds to it. The oligomeric pre-pore structure is then directed by the APN to detergent-resistant membranes (DRMs), or lipid rafts, which facilitates membrane insertion to form a lytic pore. These pores causes osmotic imbalance within the insect gut and this leads to insect death. The second model (Zhang et al. 2005) involves a signaling cascade and challenges that Cry1Ab kills cell exclusively by osmotic lysis (Pigott and Ellar 2007). This model proposes that monomeric Cry1Ab binds to BT-R_1 and initiates an Mg^{+2} –dependant signaling pathway that promotes cell death. Zhang et al. (2006) suggested that binding of the Cry1Ab with the receptor activates the signaling pathway which involves stimulation of G protein, adenylyl cyclase, increased cyclic AMP levels, and activation of protein kinase A, which leads to the destabilization of the cytoskeleton and ion channels and subsequent cell death. Pigott and Ellar

(2007) suggested a caution in assessing the Zhang model, as further work is needed to establish the connection between toxicity and the rise in cAMP level. However, all of this work has been done using a cell line and it will be interesting to see if the work can be replicated under *in vivo* conditions.

Resistance mechanisms to Bt toxins

According to the mode of action for Bt toxin, a target insect could potentially develop resistance to Bt protoxins or toxins via one or more changes in the Bt-receptor interaction pathway. The two most commonly identified Bt resistance mechanisms are protease-mediated and receptor-mediated resistance.

Changes in the proteolytic activation of Bt toxins

As discussed above, midgut proteases play an important role in the solubilization and activation of Bt protoxins. In some insects, changes in digestive protienases were found to be associated with resistance to Bt toxins (Oppert et al. 1994, Oppert et al. 1996, Oppert 1999). *P. interpunctella* display resistance to Bt subsp. e*ntomocidus* HD-198 and this resistance is associated with the absence of a major gut proteinase that activates Bt protoxins (Oppert et al. 1997). In *Spodoptera littoralis,* an increase in protease specific activity was found to be associated with an increase in toxin degradation that may account for loss of sensitivity of larvae to Cry1c (Keller et al. 1996). In ECB, reduced protease activity in a strain of Dipel-resistant larvae was associated with reduced activation of protoxin (Huang et al. 1999, Li et al. 2004). However, transgenic Bt corn expresses the Bt toxin as actived trypsin resistant core protein, so this mechanism may not be an important resistance mechanism against Bt corn (Li et al. 2004).

Receptor mediated Bt resistance

The activated toxins bind readily to specific receptors on the apical brush border of midgut microvillae of susceptible insects (Hofmann et al. 1988). Therefore, receptors on the brush border membrane are key factors in determining specificity of Cry toxins. Many receptors for cry toxin have been reported in the midguts of lepidopteran insects. A cadherin-like protein has been reported from the midgut of ECB (Flanngan et al. 2005), *Manduca sexta* (Vadlamudi et al. 1993), *Bombyx mori* (Nagamatsu et al. 1999), *Pectinophora gossypiella* (Morin et al. 2003), and *Heliothis armigera* (Xu et al. 2005), which acts as receptor to a cry toxin. Aminopeptidase N has been identified as Cry toxin receptor from *M. sexta* (Knight et al. 1994), *Heliothis virescens* (Luo et al. 1997), *Bombyx mori* (Yaoi et al. 1997), *H. armigera* (Rajagopal et al. 2003), *Plutella xylostella* (Nakanishi et al. 2002), and *Lymantria dispar* (Valaitis et al. 1997). Glycolipids from the midgut of the *M. sexta* have also been reported to bind with Cry1Aa, Cry1Ab, and Cry1Ac (Griffitts et al. 2005). Also, alkaline phosphatases have been reported to act as a Cry1Ac receptor in *M. sexta* (McNall et al. 2003, Sangadala et al. 1994) and *H. virescens* (English and Readdy 1989, Jurat-Fuentes and Adang 2004) and as a Cry11Aa receptor in *Aedes aegypti* (Fernandez et al. 2006). Some preliminary results show that Cry toxin can also bind to two new receptors. A receptor called BTR-270 which is a 270-kDa glycoconjugate was isolated from *L. dispar* and binds strongly to Cry1Aa, Cry1Ab, and Cry1Ba, weakly to Cry1Ac, and not at all to Cry1Ca, Cry2Aa, Cry2Ba, and Cry3Aa (Valaitis et al. 2001). Another receptor which has a molecular mass of 252 kDa and is called as P252 was isolated from *B. mori* brush border membrane vesicles (BBMV) (Hossain et al. 2004). This receptor was able to bind to Cry1Aa, Cry1Ab, and Cry1Ac.

Alteration of the binding site is the best characterized mechanism of resistance to Cry toxins and generally confers high resistance levels (Ferré and VanRie 2002). A decrease in Cry toxin binding ability to midgut receptors has also been reported in resistant strains of *P. xylostella* (Ferré et al. 1991, Tabashnik et al. 1994 , Masson et al. 1995, Eschriche et al. 1995 , Tang et al. 1996), *H. virescens* (MacIntosh et al. 1991, Lee et al. 1995), *Spodoptera exigua* (Moar et al. 1995) and *Leptinotarsa decemlineata* (Loseva et al. 2002). In ECB binding analysis indicated that resistance to Cry1Ab and Cry1Ac in a Bt-resistant strain was not associated with a loss of toxin binding (Li et al. 2004). Gunning et al. (2005) reported that esterases in the gut of *H. armigera* are responsible for its resistance to transgenic cotton containing a Cry1Ac gene. The level of esterases was higher in the gut of the resistant strain than in the susceptible strain. They also showed that esterases could bind to Cry1Ac protoxin and activated toxin, and therefore could help detoxify Bt toxins.

Insect functional genomics

The genomic information for insects has increased tremendously during last several years. Whole genomes have been sequenced for several insect species, including the fruit fly (*Drosophila melanogaster*) (Adams et al. 2000), African malaria mosquito (*Anopheles gambiae*) (Holt et al. 2002), yellow fever mosquito (*A. aegypti*) (Nene et al. 2007), honey bee (*Apis mellifera*) (Weinstock et al. 2006.), silkworm (*B. m*ori) (Mita et al. 2004, Xia et al. 2004), red flour beetle (*Tribolium castaneum*) (Richards et al. 2008), and 11 other *Drosophila* species (Crosby et al. 2007, Lin et al. 2007). Genome sequencing of other insect species, including pea aphid *(Acyrthosiphon pisum*), northern house mosquito (*Culex pipiens)*, three species of parasitoid wasp (*Nasonia* sp.), Hessian fly (*Mayetiola destructor*), blood sucking bug (*Rhodnius prolixus*), and body louse (*Pediculus humanus*), are currently in progress (Deng et al. 2006,

Grimmelikhuijzen et al. 2007, Sattelle et al. 2007). The red flour beetle is the only agricultural insect pest whose whole genome sequence has become available to date.

Sequencing of the expressed sequence tags (ESTs) has been recognized as an economical approach to identify a large number of expressed genes that can be used in gene expression and other genomic studies (Gerhold et al. 1996, Dimopoulos et al. 2000, Porcel et al. 2000). Indeed, ESTs have been generated from several lepidopteran insects including the silkworm (Mita et al. 2003), spruce budworm (*Choristoneura fumiferana*) (Li et al. 2003), cotton bollworm (*H. armigera*) (Dong et al. 2007), diamondback moth (*P. xylostella*) (Eum et al. 2004), tobacco hawkmoth (*M. sexta*) (Robertson et al. 1999, Zou et al. 2008), and fall armyworm (*S. frugiperda*) (Deng et al. 2006, Negre et al. 2006).

The advent of transgenic crops shifted the focus for identifying insecticide targets from the nervous system to the midgut (Siegfried et al. 2005). The gut of major agricultural insect pests can be a target for pesticide development, a source of transgenic resistance (Li et al. 2004) and can influence the durability of host plant resistance (Koiwa et al. 2000). Insect gut proteins are involved in various functions including food digestion, detoxification, and developmental regulation. The high throughput genomic projects focused on characterizing the gene expression profiles from the cell or tissues have been expected to uncover the fundamental insights into the biological process (Swaroop and Zack 2002). In order to identify cellular pathways and genes that are selectively turned on or off in response to extrinsic factors or intrinsic genetic programs, it is important to deduce the catalogue of mRNA expressed in the specific cell or tissue types at various stages of development, aging and disease (Yu et al. 2003).

It has been long recognized that the insect gut is an important target for developing new strategies for insect pest management. Until now, however, only a few studies have focused on

the development of gut-specific EST libraries of lepidopterans as a tool to identify candidate genes involved in the toxicity of insecticides and the development of insecticide resistance. Gut-specific EST libraries were reported for light brown apple moth (*Epiphyas postvittana*) (6,416 ESTs) (Simpson et al. 2007), bertha armyworm (*Mamestra configurata*) (30 serine protease-related sequences) (Hegedus et al. 2003), and *O. nubilalis* (1,745 ESTs) (Coates et al. 2008). The generation and identification of large numbers of transcriptomes from the insect gut will provide the better understanding of its molecular composition and it will provide tools to elucidate the various biological processes as well as identify novel targets for insect control.

RNA interference technology

The potential function of gene in an organism can be determined by disrupting the gene and observing the effect of this loss on the organism (Waterhouse and Helliwell 2002). One method to cause gene disruption is to down-regulate the gene expression. Several techniques has been attempted to target specific RNAs for degradation such as use of anti-sense oligonucleotides and ribozymes (Bantounas et al. 2004). But the discovery that double-stranded RNA (dsRNA) can trigger silencing of the homologous genes has provided a new very promising tool for studying gene function (Hannon 2002). This process is called as RNA interference in animals (Hannon 2002) and post-transcriptional gene silencing in plants (Baulcombe 2004). In animals, RNAi was first discovered in *Caenorhabditis elegans*, in which mRNA or antisense RNA injections had no effect on protein production, but double-stranded RNA successfully silenced the targeted gene (Fire et al. 1998). The main steps involved in the RNAi mechanism are as follows: 1) dsRNA typically more than 200 bp is delivered into the body of organism. Upon entering into the cell, it follows a cellular pathway called RNAi pathway. 2) dsRNA is recognized by RNaseIII-like enzyme called dicer and brake down the

dsRNA to small 21-23 nucleotide long fragments called as siRNA. This process occurs in the presence of ATP (Bernstein et al. 2001). 3) siRNA are then incorporated into the endonuclease containing complex called RNA-induced silencing complexes (RISCs). RISC undergo ATP dependent process to unwind the double stranded siRNA. 4) siRNA strand guide the RISC to its complementary RNA molecule and binds with it (Hammond et al. 2000, Nykanen et al. 2001 5) Endonuclease then cleaves the RNA molecule and cleaved RNA fragments are then degraded by exonucleases. In insects, RNAi has been successfully used in *D. melanogaster* (Misquitta and Paterson 1999, Dzitoyeva et al. 2001), *Musca domestica* (Stauber et al. 2000), *B. mori* (Dai et al. 2007), *S. litura* (Rajagopal et al. 2002), *E. postvittana* (Turner et al. 2006), *A. pisum* (Jaubert-Possamai et al. 2007, Mutti et al. 2006), *Blattella germanica* (Cruz et al. 2006, Martin et al. 2006), *Periplaneta Americana* (Marie et al. 2000), *A. albopictus* (Caplen et al. 2002), *Bemisia tabaci* (Ghanim et al. 2007), a culture cell line from *A.gambiae* (Levashina et al. 2001), *A. mellifera* (Amdam et al. 2003), *Schistocerca gregaria* (Dong and Friedrich 2005), *Rhodnius prolixus* (Araujo et al. 2006), *Diabrotica virgifera* (Baum et al. 2007), and *Tribolium castaneum* (Bucher et al. 2002, Tomoyasu et al. 2004). Mostly, the preferred dsRNA delivery method in insects is microinjection of *in vitro* synthesized dsRNA into the insect haemoceol (Dzitoyeva et al. 2001) but in some cases oral feeding of dsRNA has also been effective (Turner et al. 2006, Araujo et al. 2006, Baum et al. 2007).

RNAi for pest management

RNAi technique can be used in developing the transgenic plants which cause the down regulation of essential genes in the insect and thus causing insect death (Price and Gatehouse 2008). Baum et al. (2007) reported a significant level of protection by the transgenic corn which is engineered to express dsRNA directed against *D. virgifera* V-ATPase A gene. Another

approach used by Mao et al. (2007) also demonstrated the successful delivery of the dsRNA targeted against the cotton bollworm through the transgenic tobacco and Arabidopsis plants. Here, the authors first identified a cytochrome P450 (CYP6AE14) gene from the midgut of the cotton bollworm, whose expression is related to the gossypol (cotton secondary metabolite) tolerance. The transgenic plants expressing dsRNA against CYP6AE14 were developed and were fed to cotton bollworm. These insects showed the silencing of the CYP6AE14 gene and when transferred to the artificial diet containing gossypol, they became more sensitive to gossypol. RNAi technology has the advantage to use wide range of targets that can be exploited to suppress the pest population but there is a need to screen and identify the effective target genes. Another key to the success of this approach is developing a transgenic plant capable of continuous delivery of sufficient amount of intact dsRNA for uptake by insects (Price and Gatehouse 2008).

Role of peritrophic membrane in food digestion

Peritrophic membrane (PM) consists of chitin and glycoproteins and is an important physical barrier between the food bolus and the gut epithelial cells. It is also an attractive target for insect pest management strategies (Hegedus et al. 2009). Most insects have PM but it is generally absent in the insect orders, Hemiptera, Thysanoptera, and adult Lepidopera (Lehane 1997). Lepidoteran larvae have type 1 PM, that is 0.5-1.0 μm thick and is formed by midgut epithelial cells along the entire length of midgut (Mercer and Day 1952). Type II PM is found in the dipteran larvae, some lepidoptera, embiodae, and primitive orders (e.g., Dermaptera and Isoptera) and is formed from special tissues called cardia located anterior to the midgut (Binnington 1988, Peters et al. 1979, Hegedus et al. 2009). PM protects insect midgut epithelial cells from abrasive food particles, digestive enzymes, and pathogens and plays an important role

in the digestive process by compartmentalizing the midgut to make nutrient acquisition more effective. However, one of the significant mechanisms regarding PM that is still poorly understood is how digestive enzymes pass through the PM and reach endoperitrophic space (Hegedus et al. 2009). Several mechanisms have been proposed by which digestive enzymes secreted from midgut epithelium penetrate the PM to reach the food bolus: 1) secretion of digestive enzymes from the epithelial cells in the anterior region of midgut where the PM may not be fully formed (Caldeira et al. 2007, Cristofoletti et al. 2001, Neira et al. 2008); 2) special pores in the PM to allow the enzymes to pass (Ferreira et al. 1994, Ferreira et al. 1999, Santos and Terra 1986); 3) release of enzymes before the PM is formed (Villalon et al. 2003); and 4) formation of temparory pores to allow the enzymes movement (Shen and Jacobs-Lorena 2003, Toprak et al. 2008). Temperory pore formation has been suggested in *A. gambiae,* which is achevied by gut chitinase enzyme by partially degrading the chitin in the PM (Shen and Jacobs-Lorena 2003). Understanding the movement of nutrients and enzymes through the PM also has implications for insect pest management. For example, certain genes involved in this process could be targeted to disrupt the function of PM, thereby decreasing the efficiency of the digestive process hindering the movement of enzymes and nutrient uptake.

Immune defense response

Insects are continuously exposed to potentially pathogenic microorganisms and eukaryotic parasites, but only a few encounters result in infection (Gillespie et al. 1997). Insects possess a complex and efficient system of biological defense against pathogens and parasites which include: 1) the integument and gut as physical barriers to infection; 2) coordinated responses of several subpopulations of hemocytes when these barriers are breached; 3) the induced synthesis of antimicrobial peptides and proteins, primarily by the fat body (Gillespie et

al. 1997). Innate immune system recognizes microorganisms through a series of pattern recognition receptors that are highly conserved in evolution (Hoffmann et al. 1999, Janeway and Medzhitov 2002). Components of the insect innate immune system include antimicrobial peptides, macrophage-like hemocytes, melanization, wound healing and complement-like thioester proteins in the hemolymph (Ip 2006). These insect antimicrobial mechanisms are effective against bacteria, parasites and fungi. The induction of the immune related proteins for defense requires the host to recognize the invader as non-self (Hashimoto et al. 2007, Schmid-Hempel 2005). Several families of the proteins are reported to be involved in the recognition of the surface characteristics of microbes such as peptidoglycan recognition proteins (PGRPs), gram negative binding proteins (GNBPs) or β-1-3 glucan recognition proteins , lipoploysaccharides, and mannans (Medzhitov et al. 1997). PGRP genes have been reported from *D.* (Werner et al. 2000, Dziarski and Gupta 2006), *M. sexta* (Yu et al. 2002), *B. mori* (Ochiai and Ashida 1999), *Samia cynthia ricini* (Hashimoto et al. 2007, Onoe et al. 2007), *Trichoplusia ni* (Kang et al. 1998), and *A. gambiae* (Christophides et al. 2002). GNBP genes have been found in *B. mori* (Ochiai and Ashida 2000), *M. sexta* (Ma et al. 2000), and *P. interpunctella* (Fabrick et al. 2003). After the pathogen infects the insect haemocoel, the defense response causes the synthesis of a battery of antifungal/antibacterial peptides (Hetru et al. 1998, Lamberty et al. 1999). Most of the antimicrobial peptides, such as lysozyme, are produced in the fat body or haemocytes and then released into the haemolymph of insects, which then damages the microbial cell membranes (Dimarcq et al. 1998, Lamberty et al. 1999, Lopez et al. 2003). Research in insect immunity can be expected to result in improved use of entomopathogens in biological control, in discovery of antimicrobial molecules that can be exploited by humans, and in new strategies for management of insect vectors of human and animal disease (Gillespie et al. 1997). Identification and

expressional analysis of antibacterial genes in *O.nubilalis* will provide new insights and better understanding of the immune defense response in *O.nubilalis.*

Objectives

General goal of this study was to generate large genomic database from the gut of the ECB to be used for better understanding of gut physiology and its interaction with Bt toxins and pathogens. The specific objectives of this study were outlined as follows:

1. Establish and analyze expressed sequence tag (EST) database from the gut of the ECB larvae;
2. Establish feeding-based RNA interference technique to be used for gene functional analysis in ECB larvae;
3. Identify and characterize aminopeptidase-like genes from the ECB larvae and determine their involement in Bt toxicity and resistance;
4. Identify and characterize chitinase-like gene from the gut of ECB larvae and determine its involvement in the chitin regulation in the peritrophic membrane; and
5. Identify and characterize antibacterial response genes from the ECB larvae.

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CHAPTER 2 - Expressed Sequence Tags from Larval Gut of the European Corn Borer (*Ostrinia nubilalis*): Exploring Candidate Genes Potentially Involved in *Bacillus thuringiensis* Toxicity and Resistance

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Abstract

Background: Lepidoptera represents more than 160,000 insect species which include some of the most devastating pests of crops, forests, and stored products. However, the genomic information on lepidopteran insects is very limited. Only a few studies have focused on developing expressed sequence tag (EST) libraries from the guts of lepidopteran larvae. Knowledge of the genes that are expressed in the insect gut are crucial for understanding basic physiology of food digestion, their interactions with *Bacillus thuringiensis* (Bt) toxins, and for discovering new targets for novel toxins for use in pest management. This study analyzed the ESTs generated from the larval gut of the European corn borer (ECB, *Ostrinia nubilalis*), one of the most destructive pests of corn in North America and the western world. Our goals were to establish an ECB larval gut-specific EST database as a genomic resource for future research and to explore candidate genes potentially involved in insect-Bt interactions and Bt resistance in ECB.

Results: We constructed two cDNA libraries from the guts of the fifth-instar larvae of ECB and sequenced a total of 15,000 ESTs from these libraries. A total of 12,519 ESTs (83.4%) appeared to be high quality with an average length of 656 bp. These ESTs represented 2,895 unique sequences, including 1,738 singletons and 1,157 contigs. Among the unique sequences, 62.7%

encoded putative proteins that shared significant sequence similarities (E-value $\leq 10^{-3}$)with the sequences available in GenBank. Our EST analysis revealed 52 candidate genes that potentially have roles in Bt toxicity and resistance. These genes encode 18 trypsin-like proteases, 18 chymotrypsin-like proteases, 13 aminopeptidases, 2 alkaline phosphatases and 1 cadherin-like protein. Comparisons of expression profiles of 41 selected candidate genes between Cry1Ab-susceptible and resistant strains of ECB by RT-PCR showed apparently decreased expressions in 2 trypsin-like and 2 chymotrypsin-like protease genes, and 1 aminopeptidase genes in the resistant strain as compared with the susceptible strain. In contrast, the expression of 3 trypsin-like and 3 chymotrypsin-like protease genes, 2 aminopeptidase genes, and 2 alkaline phosphatase genes were increased in the resistant strain. Such differential expressions of the candidate genes may suggest their involvement in Cry1Ab resistance. Indeed, certain trypsin-like and chymotrypsin-like proteases have previously been found to activate or degrade Bt protoxins and toxins, whereas several aminopeptidases, cadherin-like proteins and alkaline phosphatases have been demonstrated to serve as Bt receptor proteins in other insect species.

Conclusion: We developed a relatively large EST database consisting of 12,519 high-quality sequences from a total of 15,000 cDNAs from the larval gut of ECB. To our knowledge, this database represents the largest gut-specific EST database from a lepidopteran pest. Our work provides a foundation for future research to develop an ECB gut-specific DNA microarray which can be used to analyze the global changes of gene expression in response to Bt protoxins/toxins and the genetic difference(s) between Bt- resistant and susceptible strains. Furthermore, we identified 52 candidate genes that may potentially be involved in Bt toxicity and resistance. Differential expressions of 15 out of the 41 selected candidate genes examined by RT-PCR, including 5 genes with apparently decreased expression and 10 with increased expression in

Cry1Ab-resistant strain, may help us conclusively identify the candidate genes involved in Bt resistance and provide us with new insights into the mechanism of Cry1Ab resistance in ECB.

Background

The genomic information on insects has increased tremendously during last several years. Whole genomes have been sequenced for several insect species, including the fruit fly (*Drosophila melanogaster*) [1], African malaria mosquito (*Anopheles gambiae*) [2], yellow fever mosquito (*Aedes aegypti*) [3], honey bee (*Apis mellifera*) [4], silkworm (*Bombyx m*ori) [5, 6], red flour beetle (*Tribolium castaneum*) [7], and 11 other *Drosophila* species [8,9]. Genome sequencing of other insect species, including pea aphid (*Acyrthosiphon pisum*), northern house mosquito (*Culex pipiens*), three species of parasitoid wasp (*Nasonia* sp.), Hessian fly (*Mayetiola destructor*), blood sucking bug (*Rhodnius prolixus*), and body louse (*Pediculus humanus*), are currently in progress [10-12]. The red flour beetle is the only agricultural insect pest whose whole genome sequence has become available to date. Lepidoptera, the second most biodiverse group of insect species after Coleoptera, represents more than 160,000 species including many of the most devastating pests of crops, forests and stored products [13]. The silkworm was the first lepidopteran insect to have its complete genome sequenced [6]. However, genomic information for other lepidopterans, particularly agricultural pest species is limited but urgently needed due to their economic importance and biodiversity. Sequencing of the expressed sequence tags (ESTs) has been recognized as an economical approach to identify a large number of expressed genes that can be used in gene expression and other genomic studies [14-16]. Indeed, ESTs have been generated from several lepidopteran insects including the silkworm [17], spruce budworm (*Choristoneura fumiferana*) [18], cotton bollworm (*Helicoverpa armigera*) [19], diamondback moth (*Plutella xylostella*) [20], tobacco hawkmoth (*Manduca sexta*) [21,22], and fall armyworm

(*Spodoptera frugiperda*) [10,23]. It has been long recognized that the insect gut is an important target for developing new strategies for insect pest management. Until now, however, only a few studies have focused on the development of gut-specific EST libraries of lepidopterans as a tool to identify candidate genes involved in the toxicity of insecticides and the development of insecticide resistance. Gut-specific EST libraries were reported for light brown apple moth (*Epiphyas postvittana*) (6,416 ESTs) [24], bertha armyworm (*Mamestra configurata*) (30 serine protease-related sequences) [25], and European corn borer (ECB, *Ostrinia nubilalis*) (1,745 ESTs) [26]. ECB is one of the most destructive pests of corn and can cause as much as $1 billion of economic loss annually in the United States alone [27,28]. ECB also represents a complex of stalk borers, such as the southwestern corn borer (*Diatraea grandiosella*) and the sugarcane borer (*Diatraea saccharalis*). These stalk borers share similar ecosystem and create similar damage to corn plants. Although ECB has been successfully managed using transgenic Bt corn hybrids (plants that express insecticidal toxins of *Bacillus thuringiensis* or Bt), there are increasing concerns about the potential development of Bt resistance in ECB because of the widespread use of Bt corn [28,29]. Indeed, several ECB colonies have developed resistance to Bt toxins under laboratory selection conditions [30,31]. The main target for Bt toxins is the insect midgut, where Bt protoxins are activated by gut proteases to produce acti vated Bt toxins. The activated toxins then bind to specific receptor(s) to confer toxicity [32]. This means that insect resistance to Bt toxins could be conferred by protease-mediated and receptor-mediated mechanisms [33-37]. Because Bt toxins and insect gut interactions are determined by many gene products in the insect gut, including many proteins/enzymes involved in Bt protoxin activation, toxin binding to receptors and toxin degradation, any change in these systems has the potential to affect a particular Bt's specificity and efficacy, and could lead to Bt resistance in insects. Our

goals are to develop a gut-specific EST database from ECB larvae and explore candidate genes that are potentially involved in insect-Bt interactions and Bt resistance. In this paper, we report the analysis and annotations of 15,000 ESTs derived from the gut of ECB larvae. We discuss the putative identities of the ESTs, their potential biological and molecular functions, and present comparative analyses of our ESTs with sequences from other insects. This work provides the opportunity for developing an ECB gut-specific microarray that can be used to study insect-Bt interactions and genetic basis of Bt resistance in ECB. Furthermore, we revealed 52 candidate genes that could be involved in Bt toxicity and resistance. Among the 41 selected candidate genes examined by RT-PCR, we found 5 genes with apparently decreased expressions and 10 with increased expressions in Cry1Ab-resistant strain of ECB as compared with the susceptible strain of ECB. Differential expressions of these genes in a Cry1Ab-resistant strain may suggest possible involvement of these genes in Cry1Ab resistance, and therefore provides us with new insights into the mechanism of Cry1Ab resistance in ECB. This study may serve as a model for studying Bt resistance mechanisms and for developing bio-pesticides for all closely related corn stalk borers.

Results and discussion

Development and analysis of the ECB gut ESTs

We first used pPCR-XL-TOPO plasmid vector to prepare a cDNA library using total RNA purified from the whole guts of fifth-instar larvae of ECB. After we sequenced a total of 1,152 cDNA clones, we found that the cDNA inserts in the vector were not sufficient long (average length: 441 bp). Therefore, we used lambda Uni-ZAP RX vector to prepare a second cDNA library using mRNA purified from the guts of fifth-instar larvae of ECB. This library provided us with much longer cDNA inserts (average length: 674 bp). Because of this

significantly improved quality of the ESTs generated from the lambda library, we used the lambda library for our further sequencing of ESTs. Among the 15,000 random cDNA clones sequenced, only <8% were from the plasmid library whereas >92% were from the lambda library (Table 2.1). Our analysis of the 15,000 sequences resulted in 13,066 readable sequences (i.e., 87.1% success rate). These sequences were first trimmed for removal of vector sequences and then were subjected to filtration to exclude the sequences of <100 bp. Further analysis, using Repeat- Masker and Organelle Masker programs [38], removed an additional 547 sequences. Thus, the total number of high quality sequences obtained was 12,519 (83.4%) with an average length of 656 bp (Table 2.1). These high quality sequences have been deposited in the EST database (dbEST) with GenBank accession numbers from GH987145 to GH999663 at the National Center for Biotechnology Information (NCBI). Redundancy and assembly analyses of the high quality sequences using Sequencher software (Gene Codes Corp., Ann Arbor, MI, USA) resulted in 2,895 unique ESTs, including 1,157 contiguous sequences (contigs) that consist of 2 or more sequences, and 1,738 singletons that represent single sequences. Putative identities of the unique sequences were determined by searching the nonredundant database in GenBank using BLASTx. Among the 2,895 unique sequences, 1,816 (62.7%) showed significant matches at *E*-values of $\leq 10^{-3}$, whereas the remaining 1,077 (37.3%) did not exhibit meaningful matches (Figure 2.1A). The majority of the contigs were assembled from 10 or fewer ESTs (Figure 2.1B). On average, however, each contig was assembled from 10.1 sequences due to a few highly redundant ESTs.

Transcript abundance

The abundance of transcripts for a particular gene of an organism can be estimated from the corresponding EST abundance in a cDNA library [39]. The most abundant ESTs in our

cDNA libraries were those encoding trypsinlike proteases and chymotrypsin-like proteases (Table 2.2). As this cDNA library was constructed from the gut of ECB, the high number of transcripts from the digestive enzymes was expected. The most abundant contig was #0038 which consisted of 525 ESTs, and it included 4% of the total sequences. This contig shared maximum sequence similarity with the trypsin-like protease, T25 precursor, characterized previously in ECB [40]. Other abundant transcripts were contigs #0026 and #0062. Contig #0026 included 197 ESTs and encoded a putative chymotrypsin-like protease. Contig #0062 included 137 ESTs and encoded a putative trypsin-like serine protease. The highly expressed genes in ECB larval gut may have important implications for their growth and development. However, care must be taken in making general conclusions about the redundancy of EST's because some artifacts could also be involved [41].

Identification of the ORF and putative secretary proteins

The 2,895 contigs and singletons were subjected to the ORF predictor software to identify the open reading frame (ORF) of the sequences. This was done to identify the novel gene candidates, which have clear coding capacity. Among 2,895 unique ESTs, 1,119 (38.7%) had ORFs of at least 450 bp. Among 1,119 ORFs, 994 putative protein sequences (88.8%) shared sequence similarity (E-value $\leq 10^{-3}$) with known proteins in the non-redundant (NR) protein database in GenBank, whereas 125 (11.2%) did not share significant similarity with any known protein in the same database (Figure 2.2A). Thus, at least 11.2% of the protein-coding genes in the gut of ECB are potentially new genes. The remaining 1,553 contigs and singletons (53.6%) had an ORF of <450 bp and 223 (7.7%) did not have an ORF. Among the ESTs with ORFs of <450 bp, 452 (29.2%) had matches in the NR protein database, whereas 1,011 (70.8%) did not have matches. Many sequences did not have ORF of atleast 450 bp because the sequences were

too short (approximately 650 sequences were less than 450 bp). The lack of the ORFs in other sequences can be due to frame shift errors, 5' truncation of cDNA clones and the ESTs that were not derived from mRNA [42]. To identify the secretory proteins, putative protein sequences were examined to identify potential secretion signal peptide using SignalP software [43]. A total of 439 (15.2%) putative proteins were predicted to contain signal peptides (Figure 2.2B). Among the putative secretory proteins, 298 sequences (67.9%) had matches with known proteins in the NR protein database, whereas 141 putative secretory proteins (32.1%) were unique, sharing no significant sequence similarity with any known protein. This information is valuable since secretory proteins are important components of biological processes in the gut [44,45].

Comparative analyses of ECB gut ESTs

The development of EST databases has been recognized as a rapid method of sampling an organism's transcriptome and is complementary to a whole genome-sequencing project [46]. Indeed, a large number of ESTs have been generated from other model organisms. The 2,895 contigs and singletons obtained from the larval gut of ECB were compared with the sequences from other organisms. The first hits (highest score) of the sequences in the NR database were taken into account to determine the most similar organism. The largest number of first hit sequences (390; 13.5%) came up with *B. mori* (Figure 2.3). This can be explained by the fact that the genome of *B. mori* has been sequenced and partially annotated, and that both ECB and *B. mori* are lepidopterans. The second largest number of first hit sequences (290; 10.0%) was with *T. castaneum*, followed by *Ae. aegypti* (109; 3.8%), *Culex pipiens* (91; 3.1%), *and A. gambiae* (81; 3.8%). Only 2.5% of the sequences (72) were found to be most similar to predicted protein sequences from ECB. This is simply due to the very small number of sequences currently available in NCBI database from ECB. In order to compare our ECB gut ESTs with the 1,745

ECB ESTs that are already available in NCBI database, we performed BLASTN searches. Among our 2,895 contigs and singletons, 1,279 (44.2%) had significant matches at a cutoff *E*-value of $\leq 10^{-3}$ whereas 1,616 (55.8%) did not show any significant matches in NCBI database using BLASTN search. We compared our ECB ESTs with the ECB ESTs available in NCBI dbEST database. We found 475 sequences (16.4%) that had significant matches with *E*-values less than *E*-150 (Figure 2.4A). Within this category, 88 ESTs (3.0%) had matches with *E*-values less than *E*-150, 23 (0.8%) had *E*-values between *E*-150 and *E*-100, 131 (4.5%) had *E*-values of *E*-100 and *E*-50, 152 (5.2%) had *E*-values of *E*-50 and *E*-20, and 81 (2.7%) had *E*-values between *E*-20 and *E*-5 (Figure 2.4B). A total of 2,420 ESTs (83.6%) had no hits with currently available midgut ESTs in NCBI database. Because *B. mori* genome has not been fully annotated, we have also compared our ESTs with all available *B. mori* ESTs using BLASTN. Among the 2,895 contigs and singletons, 579 (20.0%) had hits with *B. mori* sequences at *E*-value $\leq 10^{-3}$ (Figure 2.4A). The remaining 2,316 ESTs (80.0%) did not show a significant match with the *B. mori* sequences. Among the 579 unique ESTs which had hits in the database, 43 (7.4%) had matches with *E*-value less than *E*-150, 64 (11.1%) had *E*-values between *E*-150 and *E*-100, 156 (26.9%) had *E*-values between *E*-100 and *E*-50, 135 (23.3%) had *E*-values between *E*-50 and *E*-20, and 181 sequences (31.3%) had *E*-values between *E*-20 and *E*-5 (Figure 2.4B).

Gene ontology

Blast2GO software was used to obtain the gene ontology (GO) terms for the unique sequences by comparing them through the Gene Ontology Consortium [47]. Among the 2,895 contigs and singletons, 1,815 showed blast hits at *E*value $\leq 10^{-3}$ and 1,119 ESTs of the 1,815 were mapped. A total of 120 mapped ESTs showed both the GO terms and Enzyme Commission (EC) numbers. Figure 2.5 shows the EST functional categories, where the ECB unique ESTs

were assigned to putative biological processes, molecular functions, and cellular components. Within the biological process category, 24.0% belong to cellular processes, followed by 17.0% metabolic processes, 11.0% developmental processes, 11.0% multi-cellular processes, and 8.0% each for biological regulation and localization. In the molecular function category, the maximum GO terms (40.0%) are included in catalytic activity, followed by binding (31.0%), transporter activity (10.0%), and 5.0% each for enzyme regulation activity and structural molecular activity (9.0%). In cellular components category, cell part, cell, and organelle had 27.0%, 24.0%, and 18.0% of the GO terms, respectively. They were followed by organelle part (13.0%), macromolecular complex (11.0%), envelope (4.0%), and membrane-enclosed lumen (3.0%).

Identification of ESTs potentially relevant to the Bt toxicity and resistance

The mode of Bt action in insects includes the ingestion of Bt protoxins, solubilization of Bt protoxins in insect gut, proteolytic activation of protoxins, binding of toxins to Bt receptors, membrane integration, pore formation, cell lysis, and insect death [48]. According to this mode of action, a target insect could potentially develop resistance to Bt protoxins or toxins via one or more changes in the Bt-receptor interaction pathway. Indeed, the two most commonly identified Bt resistance mechanisms are protease-mediated and receptor-mediated resistance [49]. Our analysis of ESTs derived from the larval gut of ECB revealed a number of genes that are potentially involved in Bt toxicity and resistance (Table 2.3). Specifically, we identified 18 ESTs putatively encoding trypsin-like proteases and 18 ESTs putatively encoding chymotrypsin-like proteases with *E*-value ranges from 2e-26 to 3e-137 and *E*-value 3e-27 to 3e-149, respectively. Changes in the proteolytic activity of digestive enzymes can alter the toxicity of Bt protoxins or toxins through effects on crystal solubilization and/or activation of protoxins, as well as degradation of activated toxin [33, 50-56]. A previous study from our lab has shown that Bt

resistance in a Dipel-resistant strain of ECB was primarily associated with reduced trypsin-like protease activity [35,40]. These trypsin-like proteases were also revealed in our EST analysis. Thus, our analysis of the ESTs generated from the guts of ECB larvae revealed many more candidate genes that deserve further analysis for their roles in Bt toxicity and resistance in ECB. Our EST analysis also revealed 13 ESTs putatively encoding aminopeptidases (*E*-value 1e-64 to 1e-116), 1 encoding a cadherin-like protein (*E*-value 1e-35), and 2 encoding alkaline phosphatases (*E*-value 1e-115 to 1e-131). Aminopeptidase N, cadherin-like proteins, and alkaline phosphatases have been found to serve as Bt toxin binding receptors in other insect species [57-59]. To verify the function of aminopeptidase N as a receptor for Bt Cry1Ac toxin in *Spodoptera litura*, RNAi technology was used to reduce the expression of aminopeptidase N. This resulted in a significant reduction in the susceptibility of the insect to Cry1Ac toxin [60]. Gahan *et al*. [61] showed that in a resistant strain (YHD2) of *Heliothis virescens*, there was a disruption of a cadherin-superfamily gene by a retrotransposon-mediated insertion that resulted in high levels of resistance to the Bt toxin Cry1Ac. Fernandez *et al*. [62] also reported that a GPI (glycosylphosphatidyl-inositol)-anchored ALP (alkaline phosphatase) was an important receptor molecule involved in Cry11Aa interactions with midgut cells and toxicity to *Ae. aegypti* larvae. These studies demonstrate that aminopeptidases, cadherin- like proteins, and alkaline phosphatases can serve as Bt toxin receptors involved in Bt toxicity and resistance. Thus, identification of these candidate Bt receptor genes in this study will allow us to further examine whether receptor-mediated resistance is involved in Bt resistance in ECB.

Comparison of expression profiles between Cry1Ab susceptible and resistant strains of ECB

We performed RT-PCR to compare the expression patterns of the candidate genes relevant to Bt toxicity and resistance between Cry1Ab-susceptible and resistant strains of ECB. Among 41 selected genes from the 52 candidate genes, which included 15 that putatively code for trypsin-like serine proteases, 13 for chymotrypsin-like serine proteases, 10 for aminopeptidases, 2 for alkaline phosphatases, and 1 for cadherin-like protein, we found apparently decreased expressions in 2 trypsin-like and 2 chymotrypsin-like protease genes, and 1 aminopeptidase genes in the resistant strain as compared with the susceptible strain (Figure 2.6). Among these genes, 2 trypsin-like protease genes (contig [0907] and ECB-30-C08) were virtually absent in the resistant strain. In contrast, we found apparently increased expressions in 3 trypsin-like and 3 chymotrypsin-like protease genes, 2 aminopeptidase genes, and 2 alkaline phosphatase genes in the resistant strain. The most noticeable increases were found in 1 trypsin-like protease (contig [3395]), 3 chymotrypsin-like protease (ECB-V-25_E02, contig [0379], and ECB- 23_F02), 1 alkaline phosphatase (contig [5091]), and 1 aminopeptidase (ECB-D12) genes. Although RT-PCR is not quantitative, reproducible results of such differential expression patterns for these candidate genes in the Cry1Ab-susceptible and resistant strains of ECB may imply their potential roles in conferring or contributing to Cry1Ab resistance as well as genetic differences between the susceptible and resistant strains of ECB. Indeed, certain trypsin-like and chymotrypsin-like proteases have previously been found to activate or degrade Bt protoxins and toxins, whereas several aminopeptidases, cadherin-like proteins and alkaline phosphatases have been demonstrated to serve as Bt receptor proteins in other insect species. Thus, our results may help conclusively identify the candidate genes involved in Cry1Ab resistance and provide us with new insights into the mechanism of Cry1Ab resistance in ECB. Nevertheless, further

research will be needed to confirm their involvements and to elucidate their roles in Cry1Ab resistance in ECB.

Conclusion

Our study resulted in a gut-specific EST database containing 12,519 high-quality ESTs from a total of 15,000 ESTs sequenced in an agriculturally important lepidopteran pest. To our knowledge, this database represents the largest gut-specific EST database from a lepidopteran pest. Our analysis using ORF predictor software showed that approximately 11.2% of the protein coding genes in our database may be specific to ECB as these sequences have an ORF of at least 450 bp but did not have significant matches with known sequences in NCBI database. We have also identified 52 candidate genes that are relevant to Bt toxicity and resistance. These genes encode trypsin-like proteases, chymotrypsin-like proteases, aminopeptidases, cadherin-like protein, and alk6aline phosphatases. Furthermore, we showed differential expressions of 15 out of the 41 representative candidate genes that were examined by RT-PCR, including 5 genes with apparently decreased expressions and 10 with increased expressions in Cry1Ab resistant strain. These results may help us further narrow down the candidate genes possibly involved in Cry1Ab resistance, and provide us with new insights into the mechanism of Bt resistance in general in ECB. We are in the process of developing a microarray using our unique ESTs together with the ECB gut-specific sequences which are already available in the GenBank. The microarray technology will help us analyze the global change of gene expression in response to Bt protoxins/toxins. It will also allow us to analyze any genetic differences between Bt resistant and -susceptible strains of ECB. Our genomic information on ECB could also serve as a valuable resource for identifying critical/vulnerable genes from the gut of ECB that would make useful physiological targets for new toxins that could be developed for use in pest management.

Methods

Insects rearing and dissection

The KS-SC Bt-susceptible ECB colony was used for generating EST libraries. This colony originated from the egg masses collected from the cornfields near St. John, Kansas, in 1995. The colony has been reared since then on artificial diets in the laboratory at Kansas State University according to Huang et al. [63]. The resistant ECB strain originated from a field collection of 126 diapausing larvae obtained from non-Bt hybrids in Kandiyohi Co., MN in 2001. The resistant strain was initiated from 14 larvae that survived exposure to a diagnostic Cry1Ab concentration used to identify potential changes in susceptibility to Cry1Ab [64, 65]. To minimize inbreeding or founder effects, the resistant insects were backcrossed twice with the susceptible strain which originated from the same collection. Because the resistance was incompletely recessive and involved multiple factors [65], the F1 progeny were randomly mated to obtain recombination of resistance factors in the F2 progeny to allow selection of resistant genotypes. The insects were then subjected to selection at a Cry1Ab concentration corresponding to two- to three-fold the LC50 for the F1 progeny (150 ng/cm2) [66]. This selection event was designed to eliminate all the susceptible homozygotes and most of the heterozygotes. The resistant survivors from this selection event were then subjected to a second cycle of backcrossing, random mating, and selection. After six generations, the Cry1Ab concentration used in selections was gradually increased to achieve 750 ng/cm2 at generation F10, a concentration that kills virtually all F1 progeny. At generation F17, the resistance to Cry1Ab in the re-selected strain was in excess of 800-fold. The guts were dissected from fifth-instar larvae in DEPC (diethylpyrocarbonate)-treated distilled water and were stored in TRI reagent TM (Molecular Research, Inc., Cincinnati, OH) at -80°C until used.

cDNA library construction and sequencing

Total RNA was isolated from the whole guts of ECB larvae using TRI reagent™. The plasmid library was constructed using Creator SMART™ cDNA library construction kit from Clontech (Palo Alto, CA) following the manufacturer's protocols with one modification; instead of using the original phage vector, PCR fragments were cloned directly into a pPCR-XL-TOPO plasmid using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The λ-library was constructed using ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack III gold cloning kit (Stratagene, La Jolla, CA) according to the manufacturer's protocols. Briefly, double stranded cDNA was synthesized from poly(A) RNA, sizefractionated through a Sepharose CL-2B gel filtration column, and ligated into λ–Uni-ZAP XR vector. The ligated DNA was packaged with the Gigapack III gold packaging extract and the library was plated on LB/agar plates. Recombinant plasmid within the lambda Uni-ZAP XR vector was *in vivo* excised using the ExAssist helper phage and recircularized to generate subclones in the pBluescript SK phagemid vector. To sequence the clones, M13R and M13F primers were used for 5' and 3' sequencing, respectively. Plasmid DNA was isolated using Qiagen Bio Robot 3000 and sequenced using an ABI 3700 DNA analyzer.

EST analyses and annotations

The DNA sequences were preprocessed by using the online software EGassembler [38]. Specifically, sequence cleaning process was employed to trim the vector and adaptor sequences from the ESTs. RepeatMasker process was used to mask the interspersed repeats and low complexity regions of the sequences by using *Drosophila* Repbase repeat library. The sequences were further masked by using vector masking against NCBI's vector library and organelle masking against mitochondrial library. The preprocessed ESTs were then assembled by using

Sequencher software (Gene Codes Corp., Ann Arbor, MI). The ORF regions of the assembled ESTs were identified by using the ORF predictor software [67] and secretory proteins were identified by looking for signal peptide sequence using SignalP software [43]. Gene ontology (GO) annotation was derived using Blast2GO software http://www.blast2go.de/[68].

Comparative analysis of ESTs

The ECB unique ESTs were comparatively analyzed for their sequence similarities against other organisms. The organism associated with the EST showing the highest BLAST score in GenBank databases was selected. The ECB gut ESTs were also compared with sequences from the silkworm and ECB that are currently available in the database by using BLASTN with a cutoff *E*-value of 10^{-3}.

Expression profiling by RT-PCR

Forty-one out of the 52 candidate genes were selected for comparing their apparent gene expression profiles between the Cry1Ab-susceptible and resistant strains of ECB by using RT-PCR. These genes were selected solely based on their representations among different gene groups from our EST analysis. After total RNA was isolated from four midguts dissected from one-day-old fifth-instar larvae of each strain (Cry1Ab-susceptible and resistant strains) of ECB by using TRI reagentTM (Sigma, St. Louis, MO), it was treated with TURBO™ DNase (Ambion, Austin, TX)to remove any genomic DNA contaminations. Three micrograms of total RNA was used for synthesis of first strand cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). cDNA prepared from total RNA was used as a template for RT-PCR. A minimum of two biological replications was used for all the PCR primer pairs. For all trypsin-like (except for ECB-30_C08) and chymotrypsin-like serine protease, alkaline phosphatase, and RPS3 genes, 25 PCR cycles were used whereas for aminopeptidase and

cadherin-like protein, 27 PCR cycles were used. For one trypsin-like serine protease gene (ECB-30_C08), however, 33 PCR cycles were used as the expression of this gene using fewer cycles was not visible on agarose gels. Each PCR was performed for above mentioned number of cycles, each consisting of 94°C for 30s, 55°C for 60s, and 72°C for 60s. The sequences of forward and reverse PCR primers, and expected size of PCR product for each of 41 candidate genes are provided in Table 2.4.

Authors' contributions

CK conducted the major part of this study including experimental design, construction of the cDNA libraries, EST analysis, RT-PCR analysis, and manuscript preparation. YCZ participated in experimental design, EST sequencing and preliminary analysis of EST data. MSC assisted in the development of the project, the establishment of the collaboration in EST sequencing, and manuscript preparation. LLB participated in experimental design, maintenance of the insect culture, and manuscript preparation. RAH participated in the development of the project and experimental design. JY assisted in EST sequencing and analysis. BDS and ALBC contributed materials and participated in data analysis and manuscript preparation. SM participated in experimental design and manuscript preparation. KYZ coordinated the project and participated in experimental design, EST analysis, and manuscript preparation. All authors read and approved the final manuscript.

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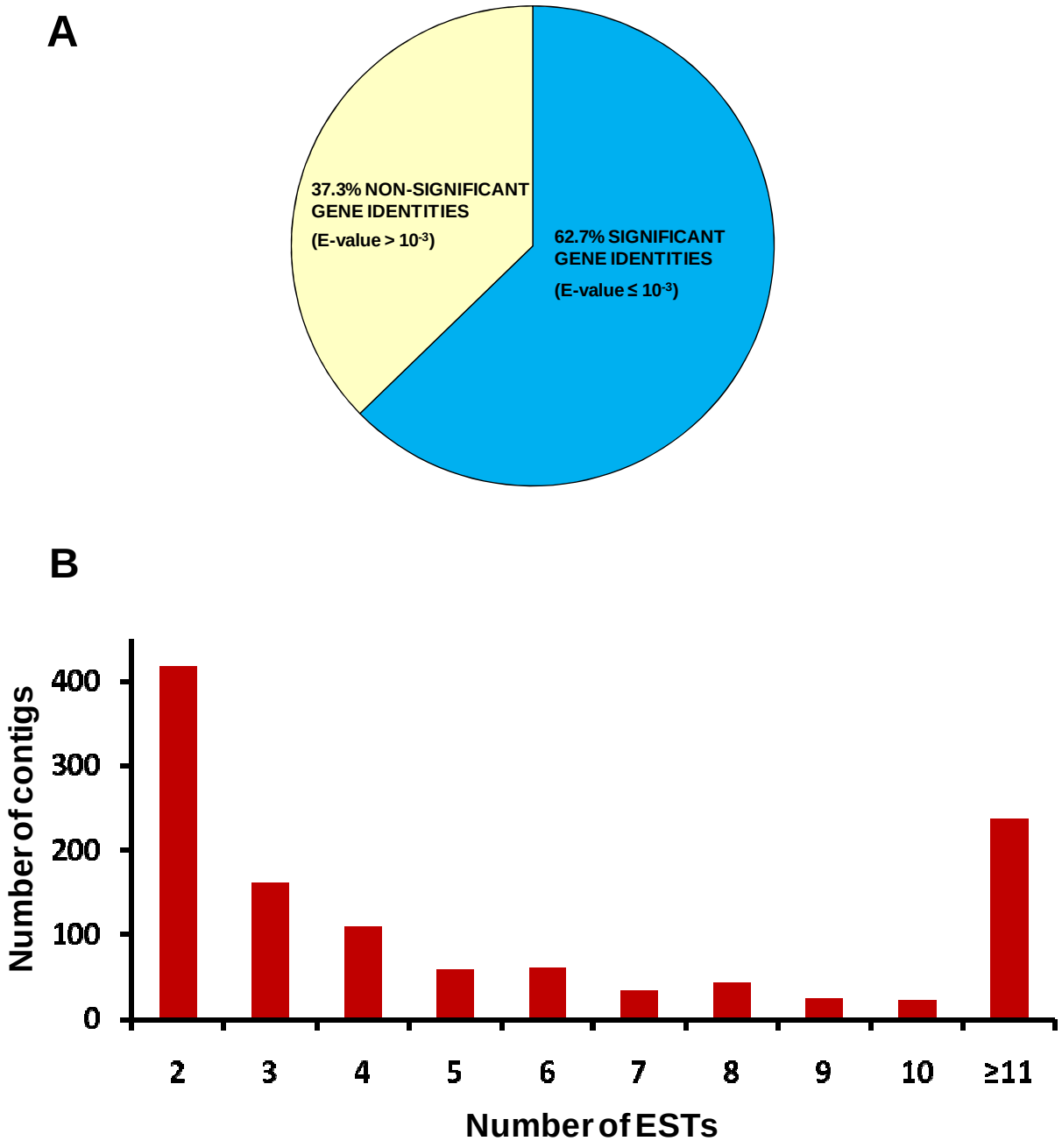

Figure 2.1 (A) Distribution of the 2,895 ECB gut-specific contigs and singletons with or without match in NCBI database using BLASTx. Sequences were defined as identical or similar cDNA sequences when they had E-values $\leq 10^{-3}$. (B) Distribution of ECB gut-specific ESTs in each contig.

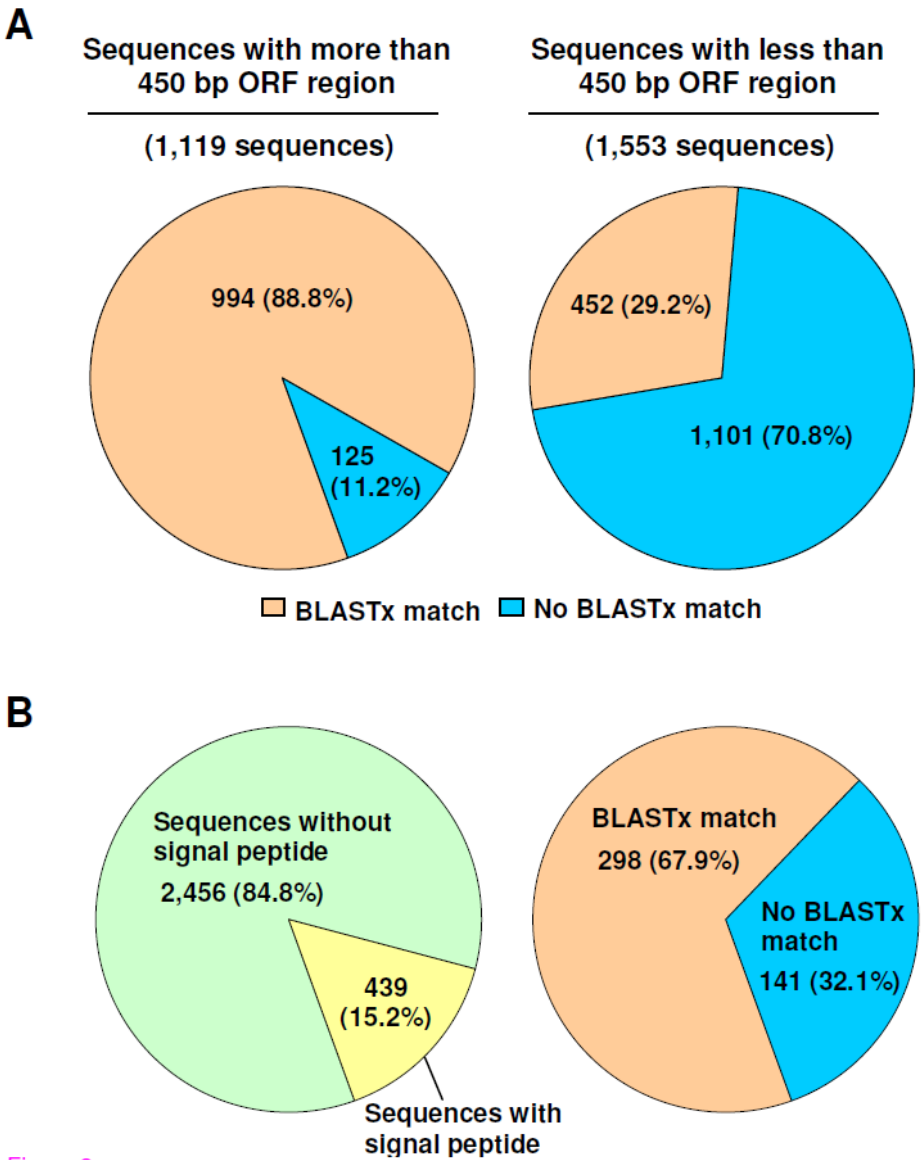

Figure 2.2 Open reading frame (ORF), secretory protein, and BLASTx results. (A) The proportion of the unique ESTs from ECB gut cDNA library with or without 450 bp of ORF region along with their matches in BLASTx using NCBI database. (B) Proportion of the unique ESTs with or without signal peptide along with their match in BLASTx using NCBI database.

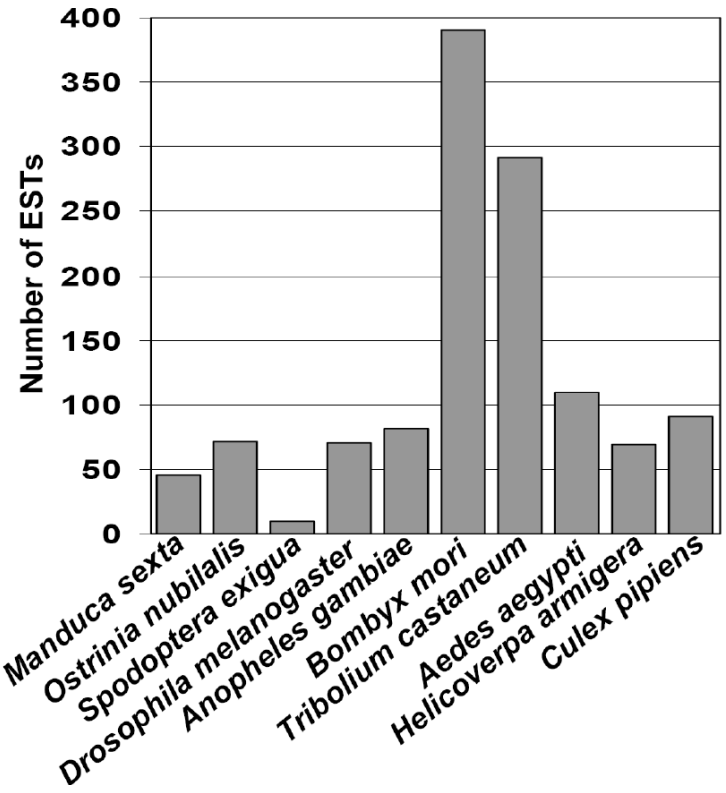

Figure 2.3 Similarity of ECB gut-specific ESTs with other insects. The first hit sequence (highest score) was used to determine the most similar organism.

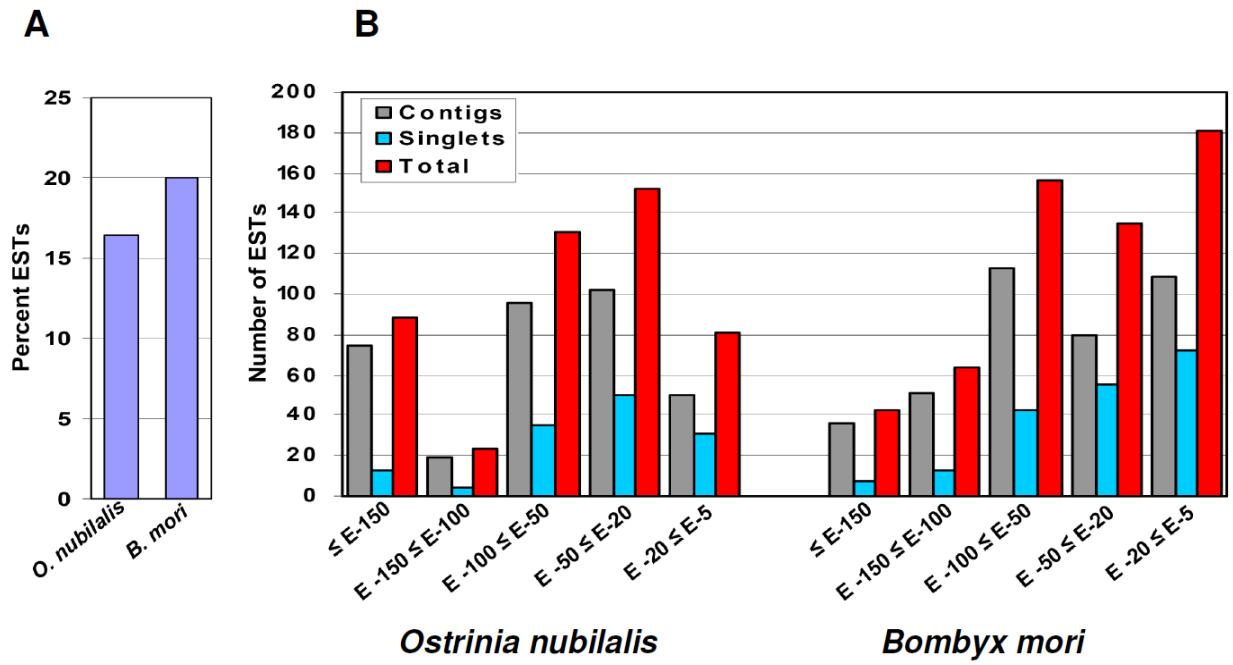

Figure 2.4 (A) Percentage of the 2,895 ECB gut-specific unique ESTs which shared matches with *O. nubilalis* and B. mori sequences with E-value ranging from E-150 to E-5 using BLASTN. (B) Comparative analyses of the 2,895 ECB gut-specific unique ESTs to B. mori sequences and other *O. nubilalis* sequences available in NCBI database using BLASTN.

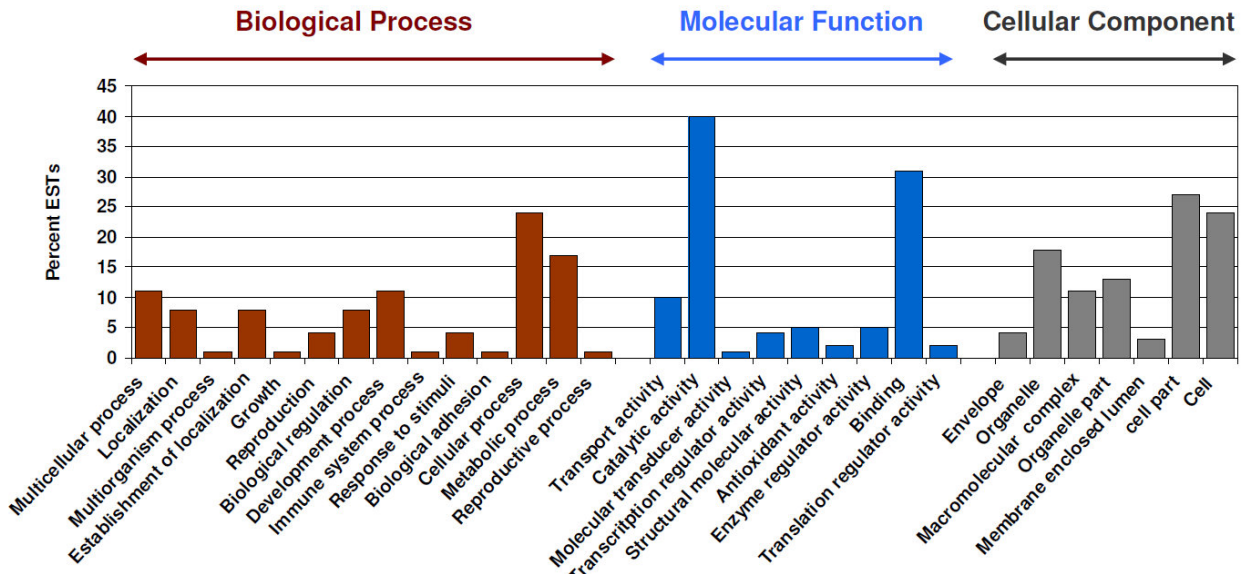

Figure 2.5 Distribution of the ECB gut-specific unique ESTs annotated at GO level 2. The Y-axis shows the percentage of the sequences. The x-axis shows 3 areas of annotation and with each area the sequences were further divided into subgroups at GO level 2.

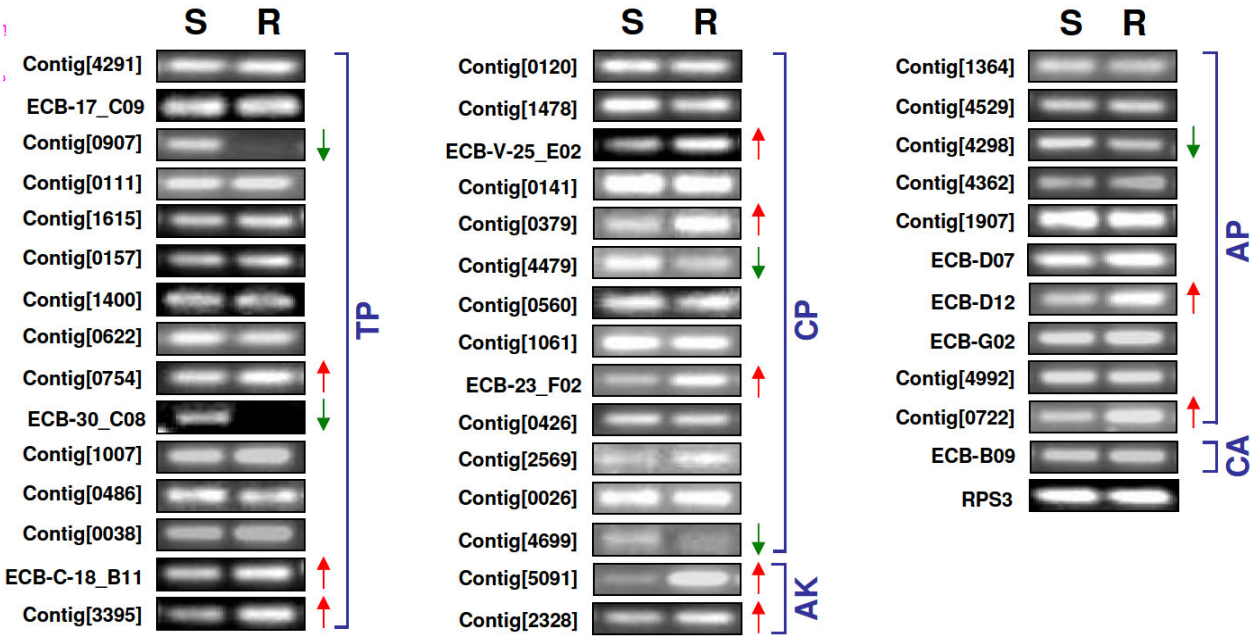

Figure 2.6 Expression profiles of 41 candidate genes relevant to Bt toxicity and resistance, which include 15 trypsin-like serine protease (TP), 13 chymotrypsin-like serine protease (CP), 2 alkaline phosphatase (AK), 10 aminopeptidase (AP), and 1 cadherin-like protein (CA) genes in the midgut of one-day-old fifth-instar larvae in Cry1Ab-susceptible (S) and resistant (R) strains of ECB as determined by RT-PCR. The arrows next to the gel pictures indicate the apparently decreased (↓) or increased (↑) expression of a particular gene in Cry1Ab-resistant strain as compared with the susceptible strain of ECB. The ribosomal protein S3 (*RPS3*) gene was used as a reference gene. At least two biological replications were used for each primer pair.

Table 2.1 Summary of the analysis of 15,000 ESTs from the guts of the European corn borer larvae

Library	Sequence direction	Number of clones sequenced	Chromatographs checked (EST number)		Sequence quality checked (EST number)		Average length (bp)	Number of contigs [b]	Number of singletons
			Good quality	Poor quality [a]	Good quality	Poor quality			
Plasmid	3'-end	1,152	764	388	722	42	441		
Uni-ZAP RX	5'-end	13,848	12,302	1,546	11,797	505	674		
Total	--	15,000	13,066	1,934	12,519	547	656	1,157	1,738

[a] The poor quality sequences were discarded and were not included in the analysis.
[b] The numbers of contigs and singletons were based on the analysis of all the ESTs sequenced from the two libraries.

Table 2.2 List of 20 largest contigs assembled from 15,000 ESTs from the guts of European corn borer larvae

Contig Identification	Putative identities	Number of ESTs	Length (bp)	% Total	*E*-value
Contig [0038]	Trypsin-like protease T25 precursor	525	942	4.1	3e-148
Contig [0026]	Chymotrypsin-like serine protease	197	1,321	1.5	1e-149
Contig [0062]	Trypsin-like serine protease	132	1,076	1.0	1e-131
Contig [0074]	Unknown	131	824	1.04	--
Contig [0059]	Trypsin-like serine protease	129	1,497	1.0	1e-117
Contig [0076]	Trypsin-like serine protease	129	1,133	1.0	1e-148
Contig [0077]	Unknown	97	652	0.77	--
Contig [0060]	Unknown	94	1,218	0.75	--
Contig [0125]	Ribosomal protein s13	87	888	0.69	1e-79
Contig [0092]	Trypsin-like serine protease	80	1,238	0.63	1e-149
Contig [0102]	Unknown	80	800	0.63	--
Contig [0040]	Thymosin isoform 1	78	1,447	0.62	1e-80
Contig [0243]	Trypsin-like serine protease	78	701	0.62	1e-120
Contig [0034]	Unkown	76	567	0.60	1e-60
Contig [0124]	Pancreatic triacylglycerol lipase	75	1,263	0.59	1e-99
Contig [0426]	Chymotrypsin-like serine protease	73	1,439	0.58	1e-129
Contig [0146]	Unknown	71	839	0.56	--
Contig [0997]	Unknown	71	574	0.56	--
Contig [0013]	Unknown	68	1,244	0.54	--
Contig [0175]	Phosphate mannosyltransferase	65	1,121	0.51	1e-20

Table 2.3 List of genes potentially involved in Bt toxicity and resistance as identified by EST analysis from the guts of the European corn borer larvae

	European corn borer					Silkworm[b]		
	EST ID	Matches	Organism[a]	% Identities	*E*-value	Matches	% Identities	*E*-value
Trypsin-like serine proteases								
1	Contig [0038]	AAR98918.1	*Ostrinia nubilalis*	254/256 (99%)	3e-148	AAB26023.1	144/233 (61%)	2e-78
2	Contig [0157]	ACB54937.1	*Helicoverpa armigera*	46/97 (47%)	8e-18	No match	--	--
3	Contig [0111]	ABF47507.1	*Ostrinia furnacalis*	248/257 (96%)	5e-135	AAB26023.1	136/231 (58%)	2e-64
4	Contig [0291]	AAX62039.1	*Ostrinia nubilalis*	257/258 (99%)	5e-137	AAB26023.1	100/240 (41%)	1e-43
5	Contig [0486]	ABU98624.1	*Helicoverpa armigera*	148/241 (61%)	1e-72	AAB26023.1	109/235 (46%)	4e-48
6	Contig [0754]	AAX62034.1	*Ostrinia nubilalis*	154/266 (57%)	7e-69	AAB26023.1	128/244 (52%)	3e-56
7	Contig [0622]	ABU98624.1	*Helicoverpa armigera*	145/249 (58%)	4e-82	AAB26023.1	101/238 (42%)	3e-50
8	Contig [0907]	ABU98619.1	*Helicoverpa armigera*	89/206 (43%)	6e-47	No match	--	--
9	Contig [1007]	AAR98918.1	*Ostrinia nubilalis*	218/252 (86%)	1e-120	AAB26023.1	119/229 (51%)	1e-57
10	Contig [1400]	ABU98619.1	*Helicoverpa armigera*	56/199 (28%)	2e-21	No match	--	--
11	Contig [1615]	ABF47507.1	*Ostrinia furnacalis*	101/189 (53%)	1e-64	AAB26023.1	95/183 (51%)	5e-50
12	Contig [3395]	AAX62032.1	*Ostrinia nubilalis*	129/209 (61%)	3e-72	AAB26023.1	117/208 (56%)	7e-59
13	Contig [4300]	AAX62035.1	*Ostrinia nubilalis*	79/84 (94%)	8e-43	AAB26023.1	43/72 (59%)	5e-16
14	Contig [4291]	AAX62032.1	*Ostrinia nubilalis*	181/236 (76%)	2e-105	AAB26023.1	138/233 (59%)	7e-75
15	ECB-30_C08	AAX62036.1	*Ostrinia nubilalis*	36/45 (80%)	1e-13	No match	--	--
16	ECB-17_C09	ABC87051.1	*Ostrinia furnacalis*	65/98 (66%)	5e-30	AAB26023.1	48/97 (49%)	4e-19
17	ECB-C-18_B11	AAR98920.2	*Ostrinia nubilalis*	198/204 (97%)	5e-114	No match	--	--
18	ECB-V-26_H09	ABC87051.1	*Ostrinia furnacalis*	35/50 (70%)	6e-12	NP_001040350	24/48 (50%)	3e-04
Chymorypsin-like serine proteases								
1	Contig [0026]	AAX62029.1	*Ostrinia nubilalis*	258/261 (98%)	5e-149	NP_001036903.1	163/259 (62%)	1e-86
2	Contig [0058]	AAX62029.1	*Ostrinia nubilalis*	228/261 (87%)	3e-120	No match	--	--
3	Contig [0120]	AAF71515.1	*Agrotis ipsilon*	174/287 (60%)	2e-84	NP_001040430.1	109/244 (44%)	2e-47
4	Contig [0141]	AAX62028.1	*Ostrinia nubilalis*	197/262 (75%)	1e-101	NP_001040430.1	130/261 (49%)	2e-52
5	Contig [0187]	AAX62026.1	*Ostrinia nubilalis*	193/202 (95%)	4e-97	No match	--	--
6	Contig [0299]	AAX62029.1	*Ostrinia nubilalis*	228/261 (87%)	6e-120	NP_001036903.1	166/259 (64%)	7e-82
7	Contig [0379]	AAX62030.1	*Ostrinia nubilalis*	111/242 (45%)	1e-55	NP_001036903.1	109/236 (46%)	8e-55
8	Contig [0426]	AAX62026.1	*Ostrinia nubilalis*	282/289 (97%)	1e-129	No match	--	--
9	Contig [0560]	NP_001040430.1	*Ostrinia nubilalis*	128/232 (55%)	1e-63	NP_001040430.1	171/272 (62%)	4e-93
10	Contig [0806]	AAX62029.1	*Ostrinia nubilalis*	202/208 (97%)	6e-137	No match	--	--
11	Contig [1061]	CAL92020.1	*Manduca sexta*	169/281 (60%)	1e-87	No match	--	--
12	Contig [1478]	NP_001040430.1	*Bombyx mori*	152/260 (58%)	5e-84	NP_001040430.1	152/260 (58%)	4e-84
13	Contig [2079]	AAL93243.1	*Aedes aegypti*	85/242 (35%)	7e-40	No match	--	--
14	Contig [2569]	AAF71518.1	*Helicoverpa zea*	119/240 (49%)	7e-49	NP_001040430.1	87/212 (41%)	1e-30
15	Contig [4479]	AAC36150.1	*Plodia interpunctella*	140/263 (53%)	3e-77	NP_001036826.1	117/270 (43%)	3e-51
16	Contig [4699]	AAX62029.1	*Ostrinia nubilalis*	195/261 (74%)	1e-102	NP_001036826.1	144/251 (57%)	9e-68
17	ECB-23_F02	CAM84318.1	*Manduca sexta*	88/209 (42%)	3e-36	No match	--	--
18	ECB-V-25_E02	AAX62031.1	*Ostrinia nubilalis*	32/32 (100%)	2e-11	NP_001040430.1	99/203 (48%)	6e-42
Aminopeptidases								
1	Contig [0722]	AAP37951.1	*Helicoverpa armigera*	72/193 (37%)	9e-29	BAA33715.1	60/160(37%)	2e-21
2	Contig [1364]	ABL01481.1	*Ostrinia nubilalis*	413/421 (98%)	0.0	NP_001037013.1	273/422 (64%)	2e-154
3	Contig [1716]	XP_560264.3	*Anopheles gambiae*	132/354 (37%)	3e-59	No match	--	--
4	Contig [1907]	ACB87202.1	*Ostrinia furnacalis*	370/374 (98%)	0.0	BAA33715.1	240/368 (65%)	8e-135
5	Contig [4362]	AAQ57405.1	*Helicoverpa armigera*	102/263 (38%)	2e-48	NP_001037013.1	89/266 (33%)	1e-42
6	Contig [4298]	ACB47287.1	*Ostrinia furnacalis*	291/297 (97%)	3e-167	NP_001036834.1	213/297 (71%)	6e-118
7	Contig [4992]	AAP37951.1	*Helicoverpa armigera*	168/246 (68%)	7e-99	BAA33715.1	85/238 (35%)	6e-38
8	Contig [4529]	ABV01346.1	*Ostrinia furnacalis*	342/356 (96%)	0.0	NP_001104835.1	226/350 (64%)	4e-125
9	ECB-G02	AAK85539.1	*Helicoverpa armigera*	196/262 (74%)	2e-114	No match	--	--
10	ECB-D07	ABQ51393.1	*Ostrinia furnacalis*	171/22 (75%)	1e-99	NP_001104835.1	159/225 (70%)	2e-95
11	ECB-D12	ABV01346.1	*Ostrinia furnacalis*	200/278 (71%)	1e-112	NP_001104835.1	155/275 (56%)	5e-80
12	ECB-C06	ABL01481.1	*Ostrinia nubilalis*	40/40 (100%)	9e-16	NP_001037013.1	23/39 (58%)	7e-07
13	ECB-F04	AAP37951.1	*Helicoverpa armigera*	117/208 (56%)	2e-61	BAA32475.1	67/190 (35%)	2e-24
Alkaline phosphatases								

1	Contig [5091]	NP_001037536.2	*Bombyx mori*	101/172 (58%)	3e-53	NP_001037536.2	101/172 (58%)	3e-53
2	Contig [2328]	BAF62124.1	*Bombyx mandarina*	176/260 (67%)	8e-103	NP_001037536.2	177/260 (68%)	2e-102
Cadherin-like protein								
1	ECB-B09	ABS59299.1	*Ostrinia furnacalis*	242/244 (99%)	7e-135	BAA99405.1	155/247 (62%)	1e-81

[a] Sequence with highest score in BLASTX search
[b] Match of ECB ESTs with silkworm sequence using BLASTX

Table 2.4 Sequences of PCR primers used to compare the gene expression profiles of trypsin-like and chymotrypsin-like serine proteases, alkaline phosphatases, aminopeptidases, and cadherin-like protein by RT-PCR between Cry1Ab-susceptible and resistant strains of European corn borer (*Ostrinia nubilalis*)

Name	Forward Primer	Reverse Primer	Product size (bp)
Trypsin-like serine proteases			
Contig[0111]	ACCTGTCCATCATCCGAACC	TCAGACGACGATCCTCCTTG	157
Contig[0486]	ATGGCGTCCTCGTTGGTG	TGGTGCCTCCCACAATGC	82
Contig[0754]	TGGGACTGTCTACACTATTGAAAG	GATGTGACGGGTATGATGCC	120
Contig[0622]	CTGGTGGAGTTATTGCCTACG	GTGGTTTGCTGGATGGATGG	133
Contig[0907]	GGCTACTCCTGCGGTCAC	CTGGACTGCTGCTGTATTGG	103
Contig[1007]	ATGCGTACCTTCATCGTTCTAC	GCCATCTCAGGGTATTGGTTAATG	116
Contig[1400]	ACGGAAGGTGGCACTCTC	TCTCTTGCGGAGGGATGTAG	154
Contig[1615]	ACCAGTTCACCAGGGACAAC	TGATGCTGCCAGGGATGAC	87
Contig[3395]	TGCTGGTGACTCAAACTCAATG	TGATGACTCGGTTCAAATAGCG	101
Contig[0157]	GCCAGCATTACACCTTCCG	TCGCAGTTCTCGTAGTAAGAC	128
Contig[4291]	CTCAACAACCGTGCTATCCTC	GCAGTGTTAATTACAGTTCCATCG	119
Contig[0038]	CATCACGGAGAACATGCTTTG	CGTTGACACCAGGGAAGAAG	158
ECB-30_C08	GATCACCATTTTGGAATTTTCG	GAGATACACGGGCGTTGC	192
ECB-17_C09	TGTTTCATCGGTACTGTCACTG	GAGGATCACTCGTCTGTTAAGG	193
ECB-C-18_B11	CACAAAGTCCTGGAGGAAGATTC	GTTCACGCCTGTCTGTTGC	125
Chymotrypsin-like serine proteases			
Contig[0026]	GAGGAGGGCACGGACTTC	TTCCTGTGTTCAAGGTGATGAC	106
Contig[0120]	TGTGATCCAGCCCATCTCTC	CAGAAGTGCGTCCGAATCC	95
Contig[0141]	GCTGGTTCCCTCTACTGGTC	GAGATGGTGTTGGAGAAGGC	79
Contig[0426]	ACCTGCCTACCAGCGTTTC	CCGAAGCCTGAAGCAATAGC	112
Contig[0560]	TCAGTGGAACCCGTGGAAC	CAGTGCGATTGGTTGGATGG	94
Contig[1061]	TCCTCGCCTGTGGTGTTC	GATGGTGGTCACGGTCAAC	156
Contig[1478]	GCCGCTGGATTTGGAAAGAC	GAGGGTGCTCGGGAATACG	135
Contig[2569]	TGCTTCTGGATTCGGAATGAC	GGAGATGACTGGAAGAGTAACG	85
Contig[4479]	TTGCGGGATACGGGAAGAC	GGAGATTGACCGAGTGGAGAG	75
Contig[0379]	CCTACTGAGGATGCGAATAACG	TGGGTTGGCTGGGTTTGG	96
Contig[4699]	CGTCCCTCTTGTGACAATGAAG	CCAGATCCTGCTGCCATCG	92
ECB-23_F02	TGGTGGAGCCTCTATCATCAG	GATTGCCATTCGTTGGTTGC	129
ECB-V-25_E02	ATCACCGCTGCTCATTGC	ACTCCTCCGCTGAAGATGG	92
Aminopeptidases			
Contig[0722]	GCACCCCATTCATTGTTCGC	GTATCTGGACGAGCCTGGAC	126

Contig[1364]	TCTGTAGTCTGGTTCACATTATCC	ACTCACCTCCGCTGTATCC	84
Contig[1907]	AATTCCAAACCTGGGCGTAC	GTTGTTCATGGCACTGTTGAC	89
Contig[4362]	ATCTGAAAAGCACCAACAGTCTTC	CTCTCGCCCTGATCGTCTTATG	156
Contig[4298]	ACCCTAACAGTAAGACAGTTTGAC	TGGCACTACAAGCAAGTAACG	197
Contig[4292]	AAGTCGTAAAGAGTAAACTGAGAG	GCCAGATCCAGCATGAAGTG	112
Contig[4529]	CTTCAACAGCCCACTGGAGAG	ACGCAAGACATATTAGGTAACAGC	85
ECB-G02	CGACTGGTTCAGGTATTGGTTC	AGGGTGATGCTTCAGACTACG	137
ECB-D07	CGCCGTGACCGTAACTGG	GTCGTCGCTAACAGAGAAGAG	195
ECB-D12	TGTATTGGCGGAGTCTGATTC	CCAGTCGTCATTGAGGAACC	93
Alkaline phosphatases			
Contig[5091]	ACTCGCTCATCGTGGTCAC	GTCGTCCTCCGTCGTCAC	200
Contig[2328]	CGGATTATCTGCTGGGTTTATTTG	AGTGTGGGCTCGGTAACG	79
Cadherin-like protein			
ECB-B09	GGTCATCAGCACGAAGAG	CAAGCATAGATACTAAGAACTGG	176

CHAPTER 3 - Identification of a Novel Aminopeptidase-like Gene that appear to be involved in Bt Toxicity and Resistance in European Corn Borer

Abstract

Studies to understand the Bt resistance mechanism in European corn borer (ECB, *Ostrinia nubilalis*) suggest that resistance may be due to changes in the midgut-specific Bt toxin receptor. In this study we identified and characterized 10 aminopeptidase-like genes in relation to Cry1Ab toxicity and resistance. The expression analysis for 10 aminopeptidase-like genes revealed that most of these genes were expressed predominantly in the larval midgut. No difference was found in the expression of these genes between Cry1Ab resistant and susceptible strains, which suggest that altered expression of these genes is unlikely to be responsible for resistance in these ECB strains. However, we found changes in two amino acids of the amino acid sequence of aminopeptidase-P like gene (*OnAPP*), Glu^{305} changed to Lys^{305} and Arg^{307} changed to Leu^{307} in two Cry1Ab resistant strains as compared with three Cry1Ab-susceptible strains. OnAPP is 701 amino acids long and has putative signal peptide at N-terminal, a predicted glycosylphosphatidyl-inositol (GPI)-anchor signal at the C-terminal, three predicted *N*-glycosylation sites at residues N178, N278 and N417, and an O-glycosylation site at residue T653. Using feeding based-RNA interference assay, we achieved 38 % reduction in the level of *OnAPP* transcript as compared with the control after 8 days. Furthermore, a Bt bioassay using insects fed diet containing *OnAPP* dsRNA resulted in reduced susceptibility to Cry1Ab by 25% as compared with larvae fed GFP dsRNA. These results strongly suggest that *OnAPP* gene may be involved in conferring Bt toxicity and resistance in two ECB strains.

Introduction

The insecticidal properties of the *Bacillus thuringiensis* (Bt) toxins have been extensively exploited for the insect pest control. The spores and crystals of Bt have been used as biopesticides for almost 60 years in the areas of forestry, agriculture, and vector-born disease control (Schnepf et al. 1998, Federici 2005). The importance of the Bt toxins in the management of the insect pests have increased dramatically by the development of transgenic plants with the ability of expressing the Bt toxin protein (Valaitis et al. 2001, Shelton et al. 2002). But there are concerns that wide-spread use of transgenic crops expressing Bt toxins may lead to the development of resistance in field populations of insects and shorten the life of Bt technology. Therefore, identification of the genes involved in the toxin interactions will be fundamental to developing effective resistance management stragies that will be useful in sustaining the transgenic Bt technology in integrated pest management.

The mode of action for Bt toxin in which the relatively inert crystalline protoxin form is changed into the cytotoxic form involves several steps (Schnepf et al. 1998). Two models have been proposed to explain the mode of action of Bt toxin. In both models the initial steps are identical, including solubilization of protoxin, activation of the soluble protoxin by the gut proteases into a Cry monomeric toxin, and binding of the toxin to the cadherin receptor (Soberon et al. 2009). The pore formation model (Bravo et al. 2004) suggests that cadherin causes toxin oligomerization and the oligomeric cry toxin then binds to the GPI-anchored receptors which help with toxin insertion into the membrane, making pores, which lead to osmotic imbalance within the insect gut and eventually death (Gill et al. 1992, Schnepf et al. 1998). In contrast, the signal transduction model (Zhang et al. 2005) proposes that monomeric Cry1Ab binds to cadherin and initiates an Mg^{+2} –dependant signaling pathway that promotes cell death. In addition to cadherin, there are many other cry toxin receptors that have been reported such as

GPI-aminopeptidase N, GPI-alkaline phosphatase, GPI-ADAM metalloprotease, glycolipids, glyco-conjugate, V-ATP synthase subunit, and actin (Krishnamoorthy et al. 2007, Valaitis et al. 2001, Ochoa-Campuzano et al. 2007, Pigot and Ellar 2007).

A number of insect species have developed resistance to Cry toxins when selected under laboratory conditions (Ferré and van Rie 2002). Two known mechanisms of Bt resistance have been identified in insects as proteinase-mediated and receptor-mediated resistance (Oppert et al.1997). However, the most common mechanism of Cry toxin resistance reported so far involves mutations that affect the assembly of cadherin receptor molecules (Ferré and van Rie 2002). The mutations in the cadherin gene have been shown to be genetically linked to Cry1A resistance in *Heliothis virescens, Pectinophora gossypiella,* and *Helicoverpa armigera* (Gahan et al. 2001, Morin et al. 2003, Soberon et al. 2007). In *Spodoptera litura*, reducing the expression of the aminopeptidase N gene with dsRNA resulted in reduced susceptibility to Cry1Ca toxin, suggesting it is involved in the toxicity (Rajagopal et al. 2002).

The European corn borer (ECB, *Ostrinia nubilalis* Hübner) is one of the most damaging pests of corn in United States and the western world. Transgenic corn expressing Bt toxins has been very successful in managing the ECB. Resistance to Cry toxins in ECB has developed under laboratory selection conditions (Siqueira et al. 2006, Siqueira et al. 2004, Bolin et al. 1999, Chaufaux et al. 2001). The resistance mechanism in the Dipel-resistant ECB has been linked to reduced proteases in the resistant strain as compare to the susceptible strain (Li et al. 2004, Li et al. 2005). In another study, comparison of the midgut protease between Cry1Ab resistant and susceptible strains showed no consistent difference (Siqueira et al. 2004) and they suggested that the resistance mechanism may involve modified midgut receptors (Siqueira et al. 2006). The difference in susceptibility to Cry1A toxins in the Europe-R ECB strain (Cry1Ab resistant) has

been linked to an altered receptor binding which is suggested by the reduced concentration of cadherin receptors in resistant strain as compared to susceptible strains (Siqueira et al. 2006). But in the same study, the other Cry1Ab resistant ECB strain (RSTT-R) did not show a similar mechanism and the authors suggest that some other factors may have more important contributions to resistance in this strain (Siqueira et al. 2006).

During the analysis of the expressed sequence tags (EST) from the gut of the European corn borer, we identified 10 cDNAs putatively encoding for aminopeptidase-like proteins which are reported to be receptors of Cry toxins. The major objective of this study was to explore the involvement of these genes in Bt toxicity or resistance in ECB. Our results show that a cDNA which encodes aminopeptidase P-like protein appears to be involved in Cry1Ab toxicity and resistance in ECB.

Results

cDNA sequence analysis

We searched our gut-specific ECB EST database, which consisted of 15,000 ESTs, for aminopeptidase-like genes (Khajuria et al. 2009). Ten ESTs shared similarity to known aminopeptidases. Nine of the ESTs (*OnAPN1* to *OnAPN9*) showed similarity with aminopeptidase-N (APN) like genes and one EST (*OnAPP*) showed similarity to aminopeptidase-P (APP) like gene (Table 3.1). Previous analysis of our EST database identified 13 ESTs with similarity to aminopeptidase-like genes but our further analysis from the 3'prime end sequencing shortlisted the number to 10 (Khajuria et al. 2009). Among the APN ESTs, four sequences showed 94-98% identies with ECB sequences already deposited in the NCBI database by Coates et al. (2008a), which suggests that these may be same sequences (Table 3.1). These clones have insert size ranging from 679 - 2143 bp. ESTs putatively encoding APN have percent

identity of 65 - 99% with other APN. The *OnAPP* cDNA showed the highest identity (42%) with APP from *Tribolium casteneum.* All the clones had partial cDNAs except for *OnAPP*, so further sequence analysis was performed for this gene only. According to Hidden Markov models, the signal peptide cleavage site was predicted to occur after Gly-19. Further analysis of the *OnAPP* gene revealed that this gene was possibly a membrane bound protein as glycosylphosphatidyl-inositol (GPI)-anchor signal was predicted at the C terminal end of this sequence (Figure 3.1). OnAPP also had three potential *N*-glycosylation sites at residues N178, N278 and N417 and one O-glycosylation site at residue T653. The predicted molecular mass of the active OnAPP protein was 72.7 kDa and it had a pI of 4.82.

Tissue and developmetal-stage specific expression

The mRNA level was assessed for all 10 aminopeptidase-like genes in six different tissues of the Bt susceptible 1-day old fifth instar larvae using real-time quantitative PCR (qPCR) (Figure 3.2). No detectable expression was observed in the fatbodies and salivary glands for all 10 genes. The expression of these genes was predominantly observed in the midgut tissues except for *OnAPN4* and *OnAPN6*. *OnAPN4* had highest expression in Malphigian tubules and *OnAPN6* had high expression in hindgut. Very low transcript levels were observed in the foregut for most genes except *OnAPN1*, *OnAPN4* and *OnAPN6* genes where there was no detectable expression. In addition to *OnAPN4*, three other genes, *OnAPN6*, *OnAPN7*, and *OnAPN8*, have detectable expression in Malphigian tubules. We also assessed the expression of *OnAPP* in the different parts of the midgut and found that it expressed equally in the anterior and posterior midgut but it expressed significantly lower in the center (Figure 3.3).

The expression of all 10 aminopeptidase-like genes from the ECB was also assessed by RT-PCR in different developmental stages including eggs, five larval instars, and pupae (Figure

3.4). Most of the stages had high expression in larval stages except for the *OnAPN6* which was predominantly expressed in eggs and had low intensity bands in the first-, third-, and fourth-instar larvae. In addition, transcripts of the *OnAPP*, *OnAPN2*, *OnAPN4*, and *OnAPN5* were detected in pupae, even though band intensity for *OnAPN2*, *OnAPN4*, and *OnAPN5* was lower than for the larval stage. *OnAPP* has expression in all the developmental stages with the highest expression in the first- and fifth-instar larvae and pupae. The expression of this gene increases from egg to first instar, then decreases in the second instar and remained low until pupation. *OnAPN1* has the highest expression in the first and second instars and its transcript was detected in eggs, third- and fourth-instar larvae. Transcripts of *OnAPN2*, *OnAPN5* and *OnAPN4* were detected in all developmental stages whereas transcripts of *OnAPN3*, *OnAPN7*, *OnAPN8* and *OnAPN9* were only detected in the larval stage.

Expression profiles between resistant and susceptible ECB

To identify the aminopeptidase-like genes which may have a potential role in the Bt toxicity and resistance, we analysed the expression of these genes using two pairs of Cry1Ab resistance and susceptible ECB strains (Figure 3.5). Our analysis showed that except for *OnAPP*, all other genes had no difference in the transcript levels for Cry1Ab resistant and susceptible strains of ECB. *OnAPP* had no or very low expression in the resistant strains compared with the susceptible strains. In order to make sure that the expression difference was not due to the mutations in the gene, we sequenced a region of the gene containing the primer sequences from both strains. We found that 8 out of 21 nucleotides in the forward primer sequence differed between the resistant larvae as compared with the susceptible larvae (Figure 3.6). This difference, however, was consistent across the two pairs of resistant strains and susceptible strains. Furthermore, the translated amino acid sequence of this region had two amino acids that

differed between resistant and susceptible ECB larvae (Figure 3.6). At position 305, the glutamic acid residue (E) has changed to lysine (K) and at position 307, the arginine residue (R) has changed to leucine (L) in resistant larvae as compared with the susceptible larvae.

RNA interference

To gain a better understanding of the role of the *OnAPP* gene in Bt toxicity in ECB larvae, we developed a feeding-based RNA interference (RNAi) technique to silence the expression of the *OnAPP* gene. Immediately after the development of larvae into the second instar, they were fed on an artificial diet mixed with *OnAPP* dsRNA. The dsRNA for green fluorescent protein (GFP) gene was used as control. After 4, 6 and 8 days, larvae were dissected to obtain midguts. Four midguts were pooled to assess the mRNA level in larvae fed the diets containing *OnAPP* dsRNA or *GFP* dsRNA. The transcript level for the *OnAPP* gene was reduced by 32.5 %, 26.6%, and 38.2 % after 4, 6, and 8 days, respectively, as compared with the larvae fed *GFP* dsRNA. This indicates that there was a statistically significant reduction of *OnAPP* mRNA levels in *OnAPP* dsRNA-fed larvae (Figure 3.7A). In order to determine how the dsRNA feeding affects the *OnAPP* mRNA in the individual larvae, we performed the same experiment again and after 8 days the midguts were dissected from individual larvae and *OnAPP* transcript level were determined. We found that expression of the *OnAPP* gene was reduced from 18.8 – 64.7 % in *OnAPP* dsRNA treated larvae as compared with *GFP* dsRNA treated larvae (Figure 3.7B). Futhermore, we exposed larvae fed artificial diet containing *OnAPP* dsRNA, *GFP* dsRNA, and water to the artificial diet containing Cry1Ab toxin for 7 days (6C). We found that mortality of larvae fed *OnAPP* dsRNA decreased by 23 and 25%, as compared with the larvae fed *GFP* dsRNA and water, respectively (Figure 3.7C).

Discussion

The genetic basis of insect resistance to Bt toxins has been suggested to be multigenic. Insects can develop resistance due to defective solubilization, deficient proteolytic activation, over-proteolysis (i.e. degradation of toxin), sequestration of toxin molecules by non-functional binding sites, defects in functional binding sites, defective pore formation and enhanced cellular repair (Griffitts and Aroian 2005). In several studies, altered binding sites have been associated with high resistance levels in insects. In ECB, there may be more than one independent resistance mechanism (Coates et al. 2008b). In Dipel-resistant ECB strain, the resistance mechanism has been associated with the reduced protease level in resistant larvae as compared with the susceptible larvae (Li et al. 2004). However, Cry1Ab resistant and susceptible strains of ECB had no consistent differences in activities of midgut proteases (Siqueira et al. 2004), but they showed that there was a reduction in the cadherin receptors in the resistance strain (Europe-R) as compared with the susceptible strain (Siqueira et al. 2006). Similar results were not found for the other Cry1Ab resistant strain (RSTT-R) in the same study. This evidence suggest that resistance in ECB could be due to changes in the midgut receptors which affect its binding with the Cry toxin (Siqueira et al. 2006). A recent study in ECB found no association between ECB Cry1Ab resistance with segregation of APN1, bre5 (Onb3GalT5), and cadherin allels in a Cry1Ab resistant ECB colony (Coates et al. 2008b). These reports suggest that there may be other proteins that play important roles in ECB resistance. In other insects several Cry toxin receptors have been reported such as cadherin, GPI anchored amiopetidase N, GPI anchored alkaline phosphatase, GPI-ADAM metalloprotease, glycolipids, glyco-conjugate, V-ATP synthase subunit, and actin (Soberon et al. 2009).

In this study, we identified and analyzed 10 aminopeptidase-like genes in Cry1Ab resistant and susceptible strains and found that aminopeptidase P-like gene was most likely

involved in Bt toxicity and resistance in ECB. Gene expression analysis for 10 aminopeptidase-like genes revealed that most of these genes were expressed predominantly in the midgut tissues except *OnAPN4* and *OnAPN6*, which expressed predominantly in the Malphigian tubules and hindgut, respectively. No expression was detected for any gene in fatbodies and salivary glands. These results are consistent with expression analysis of the four aminopeptidase N genes in *Trichopluisa ni* (Wang et al. 2005) and *Helicoverpa armigera* (Angelucci et al. 2008) where all four genes expressed predominantly in midgut tissues. In *T. ni*, however, two of the APN genes (APN1 and APN2) were also detected in Malphigian tubules and no expression was detected in fatbodies and salivary glands (Wang et al. 2005). In *Achaea janata* and *Spodoptera litura*, novel GPI anchored aminopeptidase N like genes were detected in the fatbodies with no expression in midgut tissues (Budatha et al. 2007a, Budatha et al. 2007b). We did not find any expression of aminopeptidase in the fatbodies. This may be because our cDNA library was constructed from the gut of the ECB. The aminopeptidase N genes in the midgut would have roles in the peptide digestion with various N-terminal residues (Hua et al. 1998, Bozic et al. 2003, Emmerling et al. 2001), but its role in the Malphigian tubules and fat bodies was unclear. It is suggested that fatbody APN may play a significant role in metamorphosis (Budatha et al. 2007a) whereas APN expression in Malphigian tubules may have a role in the hydrolysis of peptides in the lumen of Malphigian tubules (Wang et al. 2005). ECB aminopeptidase-like genes were predominantely expressed in larval stages except for *OnAPN6* which had the highest expression in eggs, suggesting it may have an important role during this stage of ECB. The *OnAPP* gene was highly expressed in pupae as well as in the first and fifth instars. This was also similar to a cytosolic APP from *Drosophila melanogaster*, where APP protein can be detected in the larval stage and its signal increases in pupae (Kulkarni et al. 2002).

Our gene expression analysis in Cry1Ab-resistant and -susceptible strains revealed that there were no differences in the expression of aminopeptidase-like genes between resistant and susceptible strains. Similar results for two resistant strains (RSTT-R and KS-R) and two susceptible (Europe-S and KS-S) strains strongly suggest that altered expression of these genes is unlikely to be responsible for resistance, but the possibility of the mutations in the sequences of these genes in resistant larvae deserve further investigation. Indeed, we found several nucleotide changes in the region from 912 to 930 bp of the *OnAPP* gene and these changes in the nucleotide sequence were similar in the two resistant strains and they lead to changes in two amino acids, Glu^{305} to Lys^{305} and Arg^{307} to Leu^{307}. Mutation in the APN gene in *H. armigera* has been reported to be associated with to Cry1Ac resistance in that species (Zhang et al. 2009). The APN-1 gene was absent in Cry1Ca resistant *S. exigua* larvae, and this suggested that this gene may be involved in Cry1Ca toxicity (Herrero et al. 2005). We did not find any report where APP-like genes had been implicated in the Bt toxicity and resistance. Instead, to our knowledge this is the first report where APP-like gene from an insect with predicted GPI-anchor signal peptide at the C-terminal has been identified. We found only one report from *D. melonogaster* where cytosylic form of APP had been characterized (Kulkarni et al. 2002). We also searched the NCBI database to find any APP with potential GPI anchor signal from insects but no results showed up. APP is a metalloprotease that releases the N-terminal amino acid residue from peptides with a penultimate proline residue (Ryan et al. 1994). APP has been biochemically characterized from bacteria (Yaron and Mlynar 1968, Mars and Monnets 1995), nematodes (Laurent et al. 2001), *D. melonogaster* (Kulkarni et al. 2002), plants (Hauser et al. 2001) and mammals (Simmons et al. 1992, Hooper et al 1990). The enzyme is active at high pH (8–9) and requires metal ions (typically Mn^{2+}) for optimal catalytic activity (Yaron and Mlynar 1968,

Yoshimoto et al. 1988). Membrane-bound forms of APP (mAPP) were first purified to homogeneity from porcine kidney following cleavage of its glycosylphosphatidylinositol (GPI) anchor by bacterial phospholipase C (Orawski et al. 1987). This purified mAPP was found to contain zinc but complete chemical sequencing of the protein and isolation of its cDNA revealed the absence of any typical zinc binding motifs found in other zinc metallopeptidases (Hyde et al. 1996). So, residues important in binding the zinc ion and in catalysis have been identified through molecular modelling and site-directed mutagenesis (Cottrell et al. 2000). While the physiological role of APP in insects is unclear, mammalian APP is involved in the protein turnover of collagen and the regulation of biologically active peptides, such as substance P and bradykinin (Cunningham and O'Connor 1997, Turner et al. 1997, Yaron and Naider 1993). Cry proteins have the ability to bind with receptors that are anchored to the membrane via a GPI moiety, which facilitates membrane insertion and pore formation (Soberon et al. 2009), but weather GPI anchored APP in ECB is a receptor of the Cry1Ab toxin will deserve further investigation.

We also silenced the expression of the *OnAPP* gene in susceptible ECB larvae by feeding *OnAPP* dsRNA to the insects and achieved 38 % reduction in the *OnAPP* transcript after 8 days. But our expression data using individual midguts revealed that there was a lot of variation among individuals regarding the reduction of *OnAPP* transcript following the dsRNA treatment. This variation may be due to the difference in the ability of individuals to take up dsRNA or may be due to the ability of individual insects to degrade the dsRNA in the midgut. Our Bt bioassay using insects fed *OnAPP* and *GFP* dsRNA resulted in reduced susceptibility of the fed larvae to Cry1Ab by 23-25 %. These data suggest that *OnAPP* gene may have role in Cry1Ab toxicity in the ECB, but further experiments are needed to find the precise nature of this mechanism. The

low reduction in the percent susceptibility in *OnAPP* dsRNA treated insects can be due to the small reduction of *OnAPP* transcript following dsRNA treatment and high variation of *OnAPP* transcript level among individuals. This also suggests that the *OnAPP* gene may not be solely responsible for resistance in the ECB and there are still other factors that may also be involved. Our results strongly suggest that *OnAPP* gene is a good candidate for further study to elucidate the Bt toxicity and the mechanism of resistance in ECB.

Materials and Methods

Insects rearing

The European corn borers used in this study for tissue and developmental stage expression and also for RNAi study were purchased as eggs and larvae (Lee French Laboratories, Lumberton, MN). Information regarding Cry1Ab resistant and susceptible ECB strains can be obtained from the research papers by Khajuria et al. (2009) and Siqueira et al. (2006).

cDNA sequence analysis

A gut-specific EST library was established from RNA isolated from fifth-instar ECB larvae as previously described and 15,000 clones were sequenced (Khajuria et al. 2009). The EST database consisting of 2,895 unique ESTs was searched for the genes encoding aminopeptidase-like genes. Ten clones from our EST library were identified, nine similar to aminopeptidase N and one similar to aminopeptidase P like genes. These clones were again sequenced from both ends using M13R and M13F primers in order to determine that these genes were unique. Signal P software was used to predict signal peptide (Bendtsen et al. 2004). The software ClustalW (Larkin et al. 2007) was used for multiple alignments and PredGPI was used to predict GPI anchor signal (Pierleoni et al. 2008). N-glycosylation sites were predicted by

NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and O-glycosylation sites were predicted by NetOGlyc 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/) (Julenius et al. 2005).

Tissue and developmental stage expression profiles

The feeding larvae of Cry1Ab susceptible colony (from Lee French Laboratories, Lumberton, MN.) were used in this analysis. Tissues were dissected in DEPC-treated water from one-day-old fifth-instar ECB larvae. Total RNA was isolated from different tissues (pooled from four animals) and different ECB developmental stages (pooled from four animals) using TRI reagent™ (Sigma, St. Louis, MO) and treated with TURBO™ DNase (Ambion, Austin, TX) to remove any genomic DNA contamination. One microgram of total RNA was used for synthesis of first strand cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). cDNA prepared from total RNA was used as a template for real-time qPCR or RT-PCR. The qPCR analysis was performed using SYBR green kit (Bio-Rad) and Bio-Rad iCycler iQs real-time PCR detection system at the Kansas State University Gene Expression Facility. qPCR cycling parameters included 95°C for 5 min, 40 cycles each consisting of 95°C for 30 sec, 55°C for 0.15 sec, and 72°C for 0.45 sec, followed by 95°C for 1 min and 55°C for 1 min. At the end of each quantitative PCR experiment, a melt curve was generated to rule out the possibility of primer-dimer formation. The relative expression analysis for qPCR was performed using ECB *RPS3* gene as an internal reference. For RT-PCR, 27 cycles were used for all genes including RPS3 gene, each cycle consisting of 94°C for 30s, 55°C for 60s, and 72°C for 60s. For qPCR analysis there were three biological replications, each with two technical replications. Primers for the genes were designed using Beacon Designer software (version 7). Primer sequences for the aminopeptidase-like genes are given in Table 3.2.

Expression profiles between Cry1Ab resistant and susceptible larvae

Transcript level for all 10 aminopeptidase-like genes were assessed in the midgut tissues from fifth instar larvae from each strain (Cry1Ab-susceptible and -resistant strains). Total RNA was isolated from four midguts pooled together using TRI reagent™ (Sigma, St. Louis, MO), and treated with TURBO™ DNase (Ambion, Austin, TX) to remove any genomic DNA contaminations. First strand cDNA preparation, and qPCR analysis were performed as described above. For qPCR analysis there were three biological replications, each with two technical replications.

RNA interference

dsRNA was prepared using the plasmid DNA as template by *in vitro* transcription for RNAi. The primers were designed using Beacon Designer software (version 7). T7 primer sequence was placed in front of both forward and reverse primers. The primer sequence to generate dsRNA for *OnAPP* gene were 5'- TAATACGACTCACTATAGGGTTTGGTCCT CACAGCACTTG and for 3'- TAATACGACTCACTATAGGGTTCACTGTGCCACTCG TCTC with product size of 333 bp. Similarly, for GFP, the primers used were 5'- TAATA CGACTCACTATAGGCCATTCTTTTGTTTGTCTGC and 3'- TAATACGACTCACT ATAGGGGCCAACACTTGTCAC with product size of 309 bps. The dsRNA was transcribed using the above gene specific primers and the AmpliScribe™ T7-Flash™ Kit (Epicentre Technologies, Madison, WI) according to the manufacturer's protocol. The dsRNAs were purified by phenol/chloroform extraction followed by ammonium acetate precipitation. Immediately after the development of larvae into second instar, they were individually fed the dsRNA mixed with fresh artificial ECB diet (Bio-serve). Three doses, each consisting of 10 μg of *OnAPP* dsRNA in 2 μl of water on day 0, 2, and 4 were added to the diet of each larva for a

total of 30 μg dsRNA /larva. The control larvae received the same amount of GFP dsRNA. After day six, larvae were transferred to normal artificial diet. Transcript levels of *OnAPP* in the midgut tissues of the larvae fed *OnAPP* and GFP dsRNA were determined on day 4, 6 and 8 by qPCR. Total RNA isolation, first strand cDNA preparation, and qPCR analysis were performed as described above. Three biological replications, each with two technical replications, were used for qPCR analysis.

To perform Cry1Ab bioassay, the RNAi experiment was performed as above and on day 6, larvae were exposed to Cry1Ab toxin at 2μg/ ml of diet and allowed to feed for 7 days. The mortality of the larvae was recorded on 7^{th} day after Bt treatment. Fifty larvae were used for each treatment and three independent experiments were performed for bioassay.

Statistical analysis

The gene expression and mortality analysis were subjected to one-way analysis of variance (ANOVA). Fisher's least significant difference (LSD) multiple comparisons were then used to separate the means among the treatments. All the statistical analyses were performed using ProStat software (Poly Software International Inc., Pearl River, NY).

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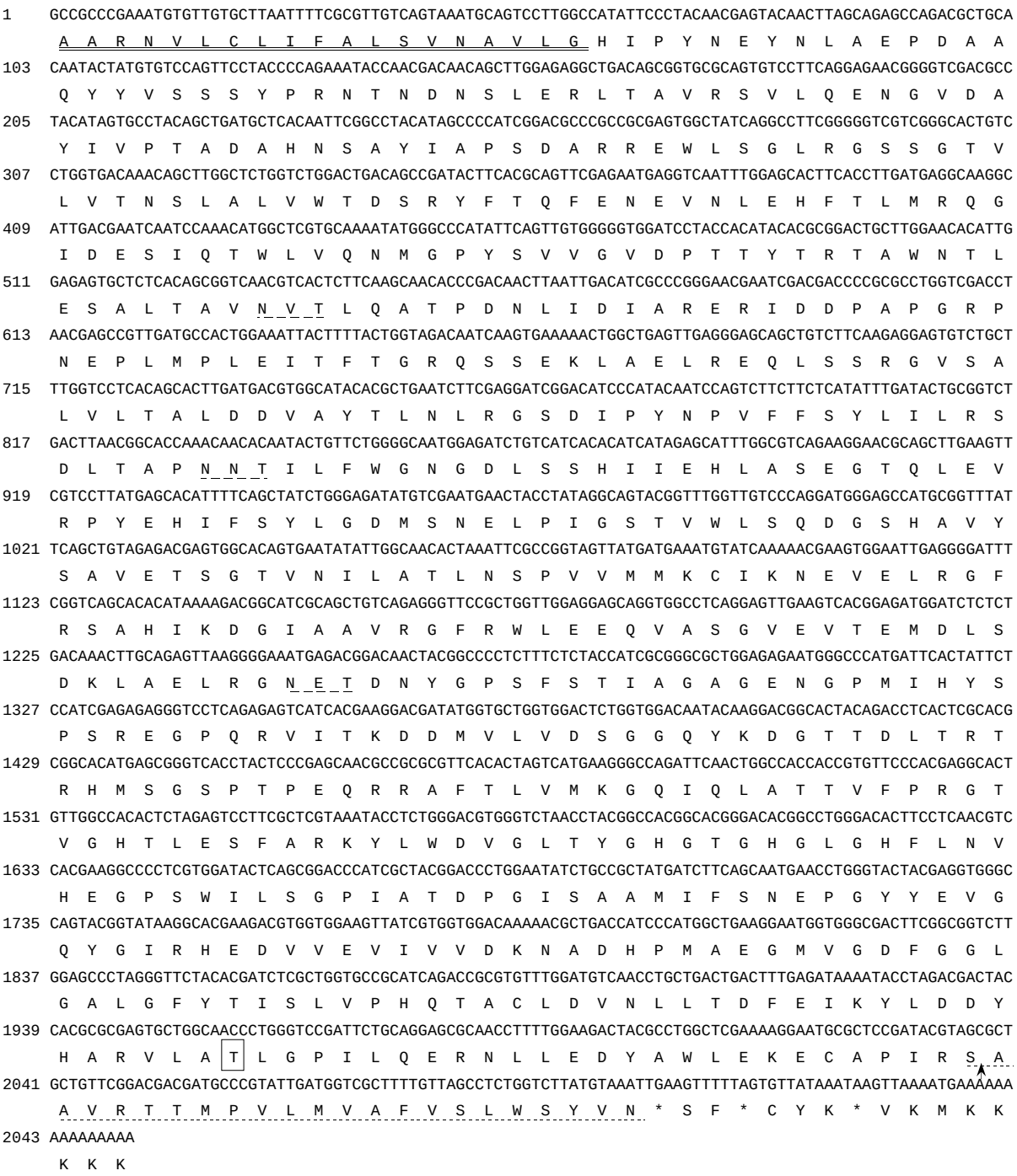

Figure 3.1 Sequence analysis of aminopeptidase P-like (*OnAPP*) gene from the Europeans corn borer larvae. The putative N-terminal signal peptide is double underlined. The GPI-anchored signal peptide is dot-underlined and the possible cleavage site of anchor moiety is indicated by arrow. The predicted O-glycosylated residue is boxed and the putative N-glycosylation sites are dash-underlined.

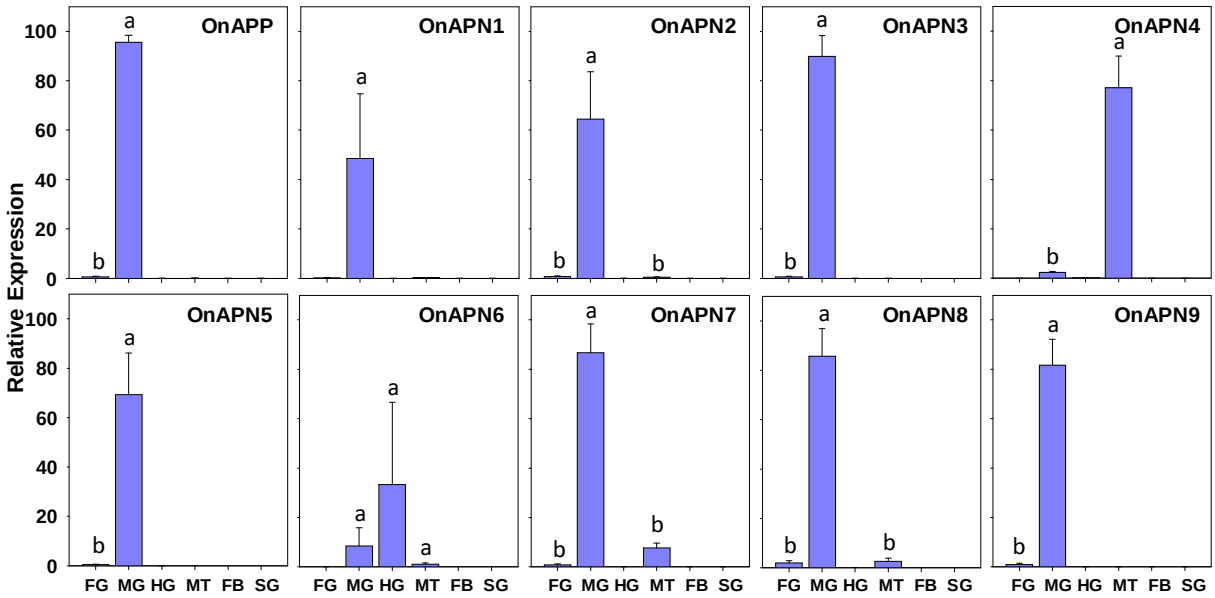

Figure 3.2 Expression profiles of 10 aminopeptidase-like genes in larval tissues of Cry1Ab-susceptible European corn borer strain. Gene expression were determined in foregut (FG), midgut (MG), hindgut (HG), Malphigian tubules (MT), fatbodies (FB), and salivary glands (SG) by Real-time PCR. The ribosomal protein S3 (*RPS3*) gene was used as a reference gene to calculate the relative expression levels. Standard error represented as error bars were determined from three biological replications and two technical replications. Different letters within a figure represent significant difference at P value ≤ 0.05.

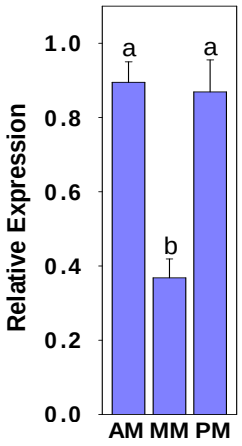

Figure 3.3 Expression level of aminopeptidase P-like (*OnAPP*) gene in the anterior midgut (AM), middle midgut (MM), and posterior midgut (PM) of the European corn borer larvae. Standard error represented as error bars were determined from three biological replications and two technical replications. Different letters within a figure represent significant difference at *P* value ≤ 0.05.

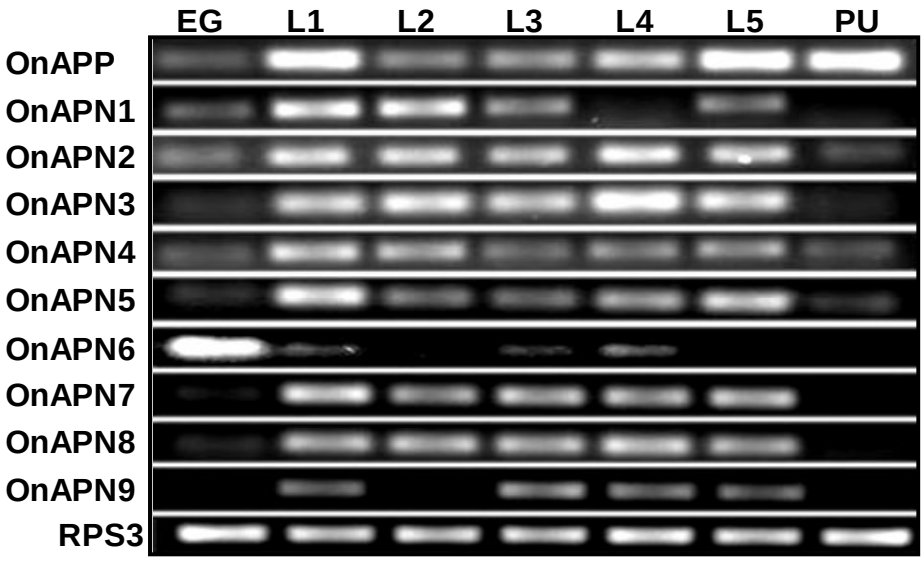

Figure 3.4 Expression profiles of 10 aminopeptidase-like genes in seven developmental stages of Cry1Ab-susceptible European corn borer: egg (EG), first instar (L1), second instar (L2), third instar (L3), fourth instar (L4), fifth instar (L5) larvae, and pupae (PU). The ribosomal S3 protein (*RPS3*) gene was used as a reference gene.

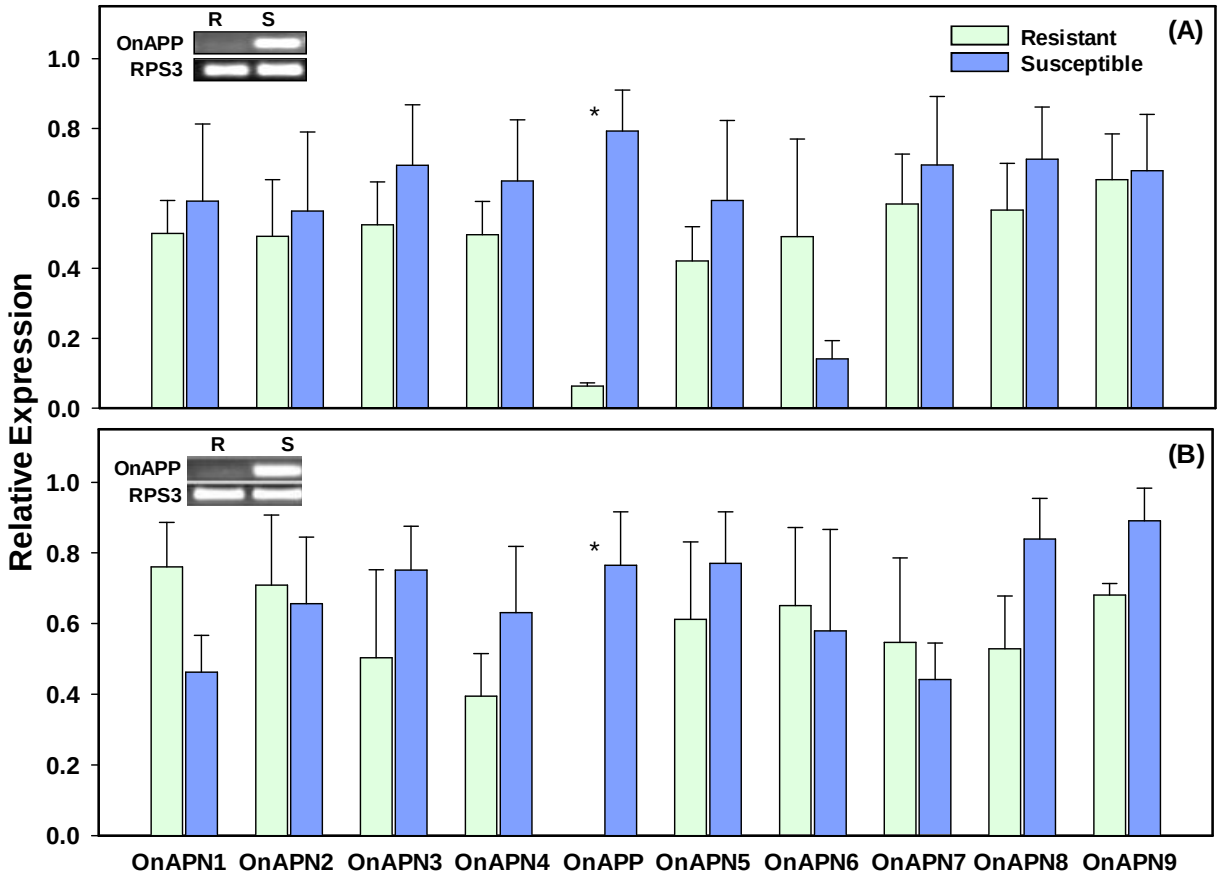

Figure 3.5 Expression profiles of 10 aminopeptidase-like genes in Cry1Ab resistant and susceptible strains of European corn borer larvae. Expression data was generated from two pairs of resistance and susceptible strains from (A) Iowa (RSTT-R and Europe-S) and (B) Nebraska (KS-R and KS-S). Bars represent relative expression for a particular gene between resistant and susceptible ECB strains and were constructed by using real-time PCR. There were three biological replications and two technical replications. Asterik (*) indicates the significant difference at p value < 0.01. Gel picture for RT-PCR for the *OnAPP* gene is given on the upper left corner. *RPS3* gene was used as reference gene.

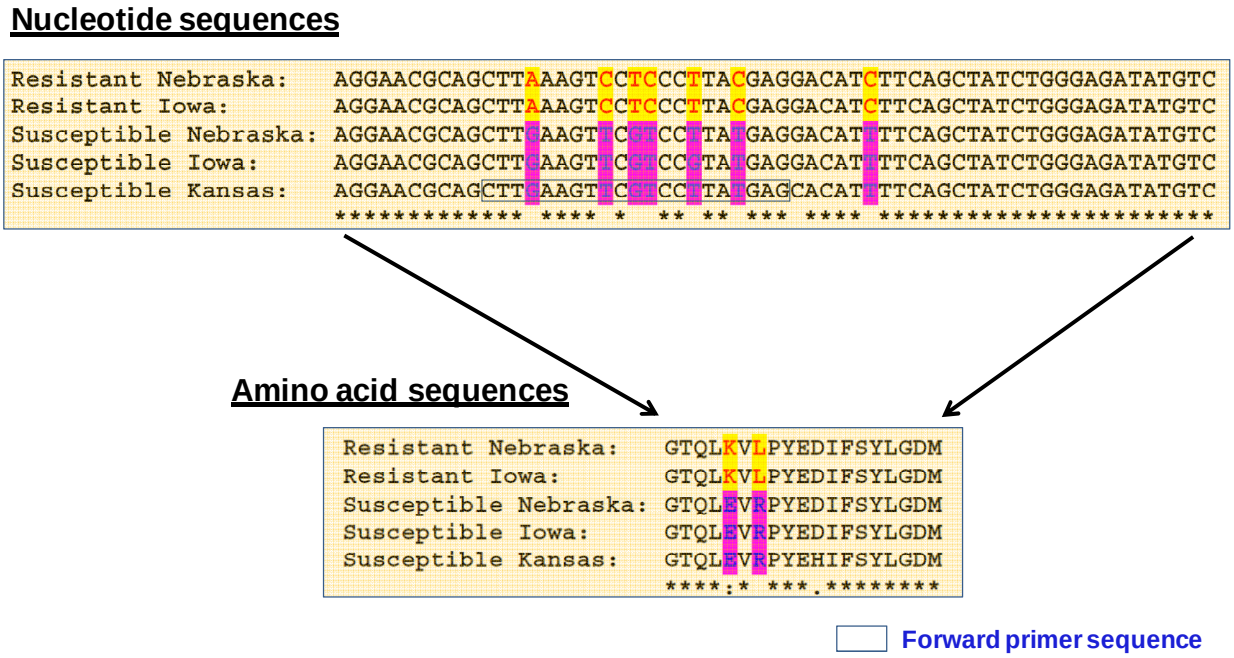

Figure 3.6 Comparisions of nucleotide and amino acid sequences between two resistant strains (resistant Nebraska: KS-R and resistant Iowa: RSTT-R) and three susceptible strains (susceptible Nebraska: KS-S, susceptible Iowa: Europe-S, and susceptible Kansas: Lee-S) of European corn borer.

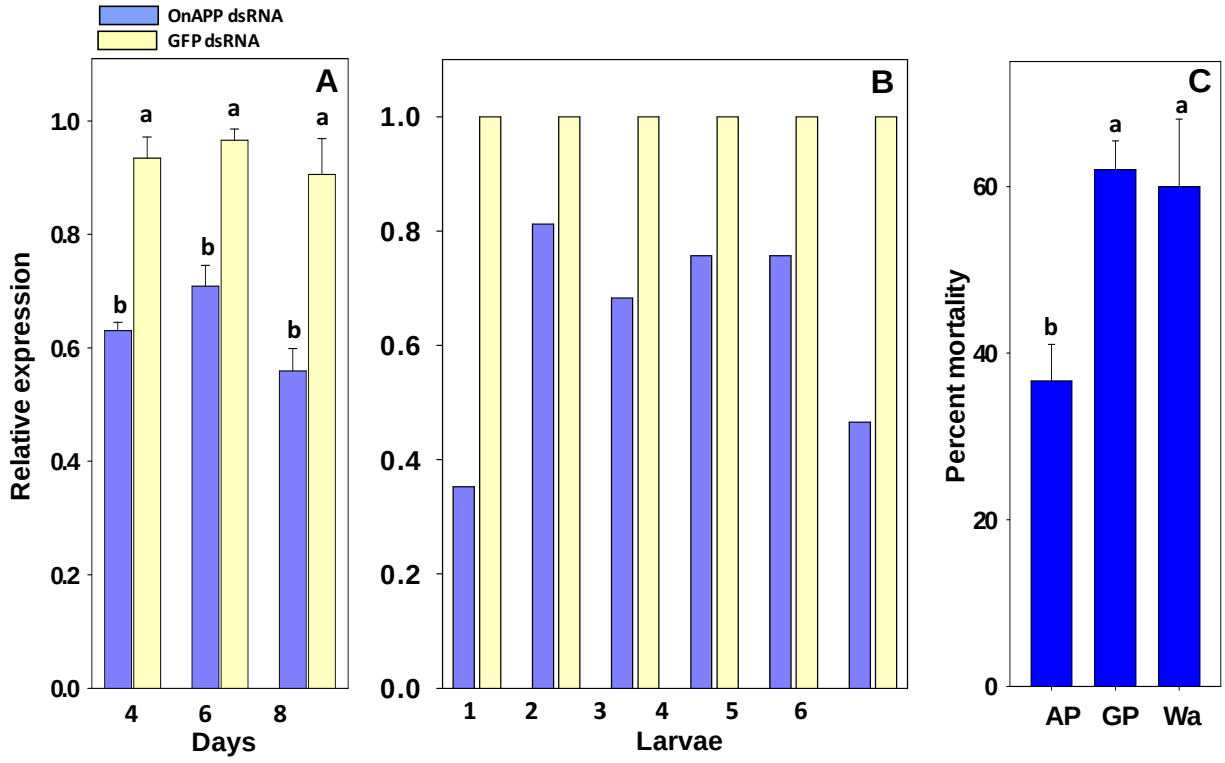

Figure 3.7 The expression of *OnAPP* gene in *OnAPP* dsRNA and GFP dsRNA treated larvae. (A) The expression of *OnAPP* gene in *OnAPP* dsRNA and GFP dsRNA treated larvae after 4, 6, and 8 days. (B) Expression of *OnAPP* was determined from individual midgut after 8 days of dsRNA feeding. (C) Percent mortality in *OnAPP* dsRNA, GFP dsRNA, and water treated larvae. Each bar represents mean ± standard error (n=3). Different letters represent significant difference with p value ≤ 0.05.

Table 3.1 Aminopeptidase-like genes identified from ECB gut EST database

	EST ID	Length (bp)	Matches	Organism	Protein type	% identity	E-value	Score (bits)
1.	OnAPP	2155	XP_974698.1	*Tribolium castaneum*	Aminopeptidase-P	42%	2e-124	451
2.	OnAPN1	924	ABL01482.1	*Ostrinia nubilalis*	aminopeptidase N2	95%	2e-30	139
3.	OnAPN2	991	ABL01481.1	*Ostrinia nubilalis*	aminopeptidase N1	98%	6e-105	386
4.	OnAPN3	2057	ABL01483.1	*Ostrinia nubilalis*	aminopeptidase N3	94%	1e-161	574
5.	OnAPN4	880	AAK85538.1	*Helicoverpa armigera*	aminopeptidase N	38%	2e-27	128
6.	OnAPN5	836	ABQ51393	*Ostrinia furnacalis*	aminopeptidase N	70%	4e-73	280
7.	OnAPN6	1904	ACA35025	*Helicoverpa armigera*	aminopeptidase N-6	65%	3e-124	450
8.	OnAPN7	829	ABL01484.1	*Ostrinia nubilalis*	aminopeptidase N4	98%	1e-88	332
9.	OnAPN8	1180	AAP37951.1	*Helicoverpa armigera*	aminopeptidase N2	68%	4e-99	351
10.	OnAPN9	679	ABL01483	*Ostrinia furnacalis*	aminopeptidase N 3	75%	3e-67	261

Table 3.2 PCR primer sequences used for gene expression comparison

Name	Forward primer	Reverse primer	Product size (bp)
OnAPP	CTTGAAGTTCGTCCTTATGAG	CACTGTGCCACTCGTCTC	138
OnAPN1	ACCCTAACAGTAAGACAGTTTGAC	TGGCACTACAAGCAAGTAACG	197
OnAPN2	TCTGTAGTCTGGTTCACATTATCC	ACTCACCTCCGCTGTATCC	84
OnAPN3	CTTCAACAGCCCACTGGAGAG	ACGCAAGACATATTAGGTAACAGC	85
OnAPN4	ATCTGAAAAGCACCAACAGTCTTC	CTCTCGCCCTGATCGTCTTATG	156
OnAPN5	TGTATTGGCGGAGTCTGATTC	CCAGTCGTCATTGAGGAACC	93
OnAPN6	GCACCCCATTCATTGTTCGC	GTATCTGGACGAGCCTGGAC	126
OnAPN7	AATTCCAAACCTGGGCGTAC	GTTGTTCATGGCACTGTTGAC	89
OnAPN8	AAGTCGTAAAGAGTAAACTGAGAG	GCCAGATCCAGCATGAAGTG	112
OnAPN9	CGCCGTGACCGTAACTGG	GTCGTCGCTAACAGAGAAGAG	93

CHAPTER 4 - A New Gut-Specific Chitinase Gene Essential for Regulation of Chitin Content of Peritrophic Membrane and Growth of European Corn Borer Larvae

Abstract

Chitinases belong to a large family of hydrolytic enzymes that break down glycosidic bonds in chitin. Gut-specific chitinases of insects have been hypothesized to control chitin turnover and porosity of peritrophic membrane (PM) in the gut, and therefore playing a crucial role in food digestion and nutrient absorption in insects. We identified a cDNA putatively encoding a unique chitinase (*OnCht*) in European corn borer (*Ostrinia nubilalis*). The *OnCht* gene was predominately expressed in larval midgut with no detectable expression either in eggs, pupae, or other larval tissues examined. We observed a significant increase in expression of *OnCht* associated with a decrease in expression of a gut-specific chitin synthase (*OnCHS-B*) gene in the larvae fed artificial diet. However, vice-versa was true only when larvae were not provided any food. Furthermore, there was a negative relationship between the *OnCht* gene expression and chitin contents in the midgut, suggesting that *OnCht* plays a crucial role in regulating chitin content of PM. By using a feeding-based RNAi technique, we were able to reduce the *OnCht* transcript levels by about 60% in the larvae. Consequently, these larvae showed significantly increased chitin content (26%) in the PM but reduced larval body weight (54 %) as compared with the larvae fed diet containing GFP dsRNA. Thus, for the first time, our studies provide strong evidence that *OnCht* plays an essential role in regulating chitin content of PM and affecting larval growth, presumably by influencing food digestion, nutrient absorption or movement of digestive enzymes through the PM.

Introduction

Chitin, a linear polymer of β-(1,4)-*N*-acetyl-D-glucosamine (GlcNAc), is an important structural component of the insect cuticle, cuticular lining of the foregut, hindgut, trachea, and the peritrophic membrane (PM) that lines the lumen of the midgut (1-2). For insects to grow and change from one developmental stage to another, chitin in the cuticle and the PM needs to be digested and replaced with new chitin (3). Chitin synthase genes (*CHS-A* and *CHS-B*, also known as *CHS-1* and *CHS-2*, respectively) are responsible for the synthesis of chitin in insects (4-6). CHS-A is responsible for chitin synthesis in the cuticle and CHS-B is responsible for chitin synthesis in the midgut cells which secrete the PM (4). Sequences of chitin synthases genes have been reported from several insects including dipteran insects such as *Lucilia cuprina* (6), *Anopheles gambiae, Aedes aegypti* (7) and *Drosophila melanogaster* (8), the lepidopteran *Manduca sexta* (9-10) and the coleopteran *Tribolium castaneum* (11). On the other hand, insect chitinases, which belong to the family 18 glycosyl hydrolases, are responsible for the degradation of chitin in the cuticle and PM (12). It was previously thought that there was only one chitinase-like gene in most insects (13). But now several fully annotated insect genomes are available and it has become clear that chitinases are encoded by a rather diverse family of genes and can be classified into five or more groups. There are currently a total of 22, 17, and 20 chitinase or chitinase-like genes in *Tribolium castenuem, Drosophila melanogaster*, and *Anopheles gambiae*, respectively (Zhang et al. unpublished).

Genes encoding chitinase and chitinase-like proteins have been characterized in several insect species, including *Manduca sexta* (14), *Bombyx mori*, *Hyphantria cunea* (15), *Spodoptera litura* (16), *Spodoptera frugiperda* (5), *Choristoneura fumiferana* (17), *Aedes aegypti* (18), *Anopheles gambiae* (19), *Glossina morsitans* (20), *Lutzomyia longipalpis* (21), *Chironomus tentans* (22), *Phaedon cochleariae* (23), *Tenebrio molitor* (24-25), and *Apriona germari* (26).

Structurally, chitinases can vary in their domain organization with varying arrangements of catalytic domains, chitin binding domains (ChBD), and serine/threonine-rich *O*-glycosylated linker interdomains (3). In addition to enzymatically characterized molting associated chitinases which have all the three domains, it has also been reported that there are other naturally occurring chitinases with only single catalytic domains that are also enzymatically active (20, 22, 23, 26). Among all the chitinase groups, group IV is most diverse and their genes are predicted to be expressed in fatbodies and/or midgut (3). Chitinases that are expressed in the gut presumably are responsible for digesting the chitin in the PM and have been reported in several insects, including *A. gambiae* (19), *L. longipalpis* (21), *P. cochleariae* (23), *T. molitor* (25), and *T. castaneum* (3).

Peritrophic membrane consists of chitin and glycoproteins and is an important physical barrier between the food bolus and gut epithelial cells. Lepidoteran larvae and many other insects have type 1 PM, that is 0.5-1.0 μm thick and is formed by midgut epithelial cells along the entire length of midgut (27). Type II PM is found in the dipteran larvae, some Lepidoptera, Embiodae, and primitive orders (e.g., Dermaptera and Isoptera) and is formed from special tissues called cardia located anterior to the midgut (28). There are many possible functions of the PM in insect midgut such as protecting the midgut epithelial cells from the abrasive food particles, digestive enzymes, and pathogens. PM also plays an important role in the digestive process by compartmentalizing the midgut to make nutrient acquisition more effective and allowing the reuse of hydrolytic enzymes (28).

The most significant unresolved mechanism regarding PM is how digestive enzymes and nutrients pass through the PM (28). Several mechanisms have been proposed by which digestive enzymes secreted from midgut epithelium penetrate the PM to reach the food bolus (29-37). A

few studies have suggested a role for gut chitinases in controlling the porosity of chitin-containing PM (19, 38). In *A. gambiae,* researchers proposed that the gut chitinase which is secreted into the blood meal by midgut epithelial cells may mediate the partial degradation of the chitin in the PM to increase its porosity (19, 28). Other suggested mechanisms include the release of enzymes before the PM is formed, allowing enzymes to pass through incompletely formed PM, and presence of special pores for enzyme movement (28). Understanding the movement of nutrients and enzymes through the PM also has implications for insect pest management. For example, certain genes involved in this process could be targeted to disrupt the function of PM, thereby decreasing the efficiency of the digestive process hindering the movement of enzymes and nutrient uptake.

In this paper, we report a unique gut-specific chitinase-like gene (*OnCht*) from the larvae of European corn borer (ECB, *Ostrinia nubilalis*), one of the most destructive pests of corn in North America and the western world. For the first time we provide strong evidence that *OnCht* plays an important role in regulating the chitin content of PM and subsequently affecting the growth and development of ECB larvae.

Results

Identification and Characterization of OnCht.

A cDNA sequence encoding a chitinase was identified from our expressed sequence tag (EST) library. The EST library was constructed from RNA isolated from the guts of fifth-instar ECB larvae and a total of 15,000 clones were sequenced (39). The identified deduced amino acid sequence showed significant similarities to other insect chitinases and chitinase-like proteins in the GenBank and was, therefore, named "*OnCht*". Because our ESTs were sequenced only from the 5' prime end, this clone was further isolated and sequenced again from both ends to obtain

the full length sequence. *OnCht* had an insert size of 1404 base-pairs (bp) with an open reading frame of 407 amino acid residues. The polyadenylation signal (AATAAA) was found 13 bp upstream of the poly (A) tract.

The deduced amino acid sequence was used to predict the signal peptide sequence using signal P software (40). According to Hidden Markov models, the signal peptide cleavage site was predicted to occur after Ala-18 (Figure 4.1). The predicted molecular mass of the active OnCht protein was 43.32 kDa and it had a pI of 4.38. The deduced OnCht protein sequence had high contents of leucine (9.1%), alanine (8.4%) and asparagine (8.1%). It had one catalytic domain extending from residue 22 to residue 368 and it did not have any chitin binding domain or serine/therionine rich linker regions as reported in several other chitinases. It had three predicted putative *N*-glycosylation sites, at residues N152, N273 and N313, and all the four highly conserved regions or signature motifs for insect chitinases (18). The conserved region I has the consensus sequence of KXXXXXGGW, where X is a non-specified amino acid. The conserved region II is known to be located in or near the catalytic site of the enzyme and has the consensus sequence of FDGXDLDWEYP. Glutamic acid (E) in this sequence is predicted to be the putative proton donor in catalytic mechanisms (41-43). Consensus sequences for conserved regions III and IV are MXYDXXG and GXXXWXXDXDD, respectively (Figure 4.1).

The deduced amino acid sequence of OnCht had a high level of amino acid sequence identities with other insect chitinases in GenBank. It has identities of 40.0, 39.1, and 37.5% with chitinase or chitinase-like proteins from *A. aegypti* (XP_001663099), *Lutzomyia longipalpis* (AAN71763) and *D. melanogaster* (NP_611542), respectively; all three are dipteran species. Phylogenetic tree was constructed by using the chitinase or chitinase-like protein sequences from *O. nubilalis* and several other insects (Figure 4.2). *OnCht* belongs to group IV chitinases which

includes many members that are expressed in insect fatbodies or gut tissues and appear to be induced in the larval or adult gut in response to feeding (3).

Tissue and Developmental Stage-specific Expression of OnCht.

The expression of *OnCht* gene in different ECB larval tissues was determined by real-time quantitative PCR (qPCR). The predominant expression of *OnCht* was in the larval midgut, with very little expression (≤3%) in the foregut and no detectable expression in the hindgut, fatbodies, salivary glands and carcass (Figure 4.3A). Within the midgut, there were significant differences in the mRNA levels in the anterior (75.8%), middle (23.9%), and posterior (0.3 %) parts of the midgut ($P<0.05$) (Figure 4.3B).

The expression of *OnCht* in ECB was also assessed by RT-PCR and qPCR in different developmental stages. Transcripts for *OnCht* were found in all the five larval instars, but no detectable expression was found in eggs, pupae, or adults (Figure 4.3C). During the larval stages, *OnCht* mRNA level was similar among the instars except for the third instar where the expression was significantly lower than that of other instars ($P<0.05$).

Feeding-Mediated Changes in Expression of OnCht and OnCHS-B.

We took advantage of an *OnCHS-B* cDNA partial sequence identified from our EST library to design primers for qPCR analysis of its expression along with *OnCht* in the larval midgut. When the larvae were maintained with food for 24 h, the expression in the midgut was 4.4-fold higher for *OnCht* but 2.5-fold lower for *OnCHS-B* (Figure 4.4) than for larvae maintained with no food ($P<0.05$). However, when the larvae maintained with food were transferred to a container with no food for another 24-h period, transcript levels decreased by 1.8-fold for *OnCht* , but increased by 1.8-fold for *OnCHS-B* (Figure 4.4) ($P<0.05$). In contrast, when the larvae that were maintained with no food were transferred to a container with food for

the next 24 h, their midgut *OnCht* transcript levels increased by 2.3-fold , but *OnCHS-B* transcript levels decreased by 2.3-fold ($P<0.05$) (Figure 4.4). The changes in expression of these two gut-specific genes in response to food suggest that *OnCht* expression is induced by larval feeding, presumably to reduce chitin contents in the PM (increasing its porosity). On the other hand, when no food is supplied, the chitin content increased, presumably due to increased chitin synthesis by OnCHS-B and/or decreased chitin degradation by OnCht.

Changes in Chitin Content in Relation to Insect Feeding

The chitin content of the PM and the whole midgut was measured directly for larvae maintained with or without food for 0, 12, 24, 36, and 48 h (Figure 4.5). The chitin content relative to midgut weight was 0.18, 0.14 and 0.33 µg/mg for larvae maintained with food for 0, 12 and 24 h, respectively. There were no significant differences among the chitin contents during these feeding periods ($P>0.05$) (Figure 4.5A). However, when the larvae that had been maintained with food for 24 h and then were maintained with no food for 12 and 24 h, their chitin contents increased by 3.1- and 3.4-fold, respectively ($P<0.05$) (Figure 4.5A). When another group of larvae was transferred from containers with food and maintained with no food for 12 and 24 h, chitin contents increased by 2.3- and 5.2-fold, respectively ($P<0.05$) (Figure 4.5B). When these larvae were transferred to a container with food, their midgut chitin contents decreased by 12- and 7.2-fold after 12 and 24 hr, respectively ($P<0.05$) (Figure 4.5B). In contrast, when the larvae were maintained continuously with food, their midgut chitin contents remained consistently low (Figure 4.5C).

To validate that our analysis of chitin contents based on the midgut weight was not biased due to the different sizes of larvae under the feeding and starvation conditions, we further calculated the same data for the chitin contents based on per larval midgut (Figure 4.6). This

analysis showed a similar pattern of the chitin contents between the fed and unfed larvae, indicating that even though the body sizes of the starved larvae was relatively smaller than those of the fed larvae, the chitin content of the starved larvae was still higher than that of fed larvae.

Then we separated the PM from the surrounding midgut and determined the chitin contents for the PM and the rest of the midgut tissue, separately. This was done to verify whether or not the dramatic changes in chitin contents of the midgut were due to changes in chitin content of the PM. Indeed, the chitin content of the PM for larvae maintained with no food was 3.4-fold higher than the chitin content of the PM of larvae maintained with food after 24 h (P<0.05) (Figure 4.7). In contrast, there was no significant difference in the chitin content for the midgut tissue for the two treatments (P>0.05). The low levels of chitin found in the midgut after the removal of the PM probably reflected the presence of tracheae on midgut. Furthermore, the diet on which larvae were reared was tested for chitin, and none was found (data not shown).

Effect of RNA Interference for OnCht on Larval Growth

To gain a better understanding of the function of *OnCht* gene and its role in regulating the chitin content of the PM of ECB larvae, we developed a feeding-based RNA interference (RNAi) technique to silence the expression of the *OnCht* gene. Immediately after hatching from eggs, the larvae were fed an artificial diet mixed with *OnCht* dsRNA. The dsRNA for green fluorescent protein (GFP) gene was used as a control. After 6 and 8 days, larvae were dissected to obtain midguts and assess the mRNA level in the larvae fed the two diets containing *OnCht* dsRNA or *GFP* dsRNA. The transcript level of *OnCht* gene in larvae was reduced by 63% and 64 % after 6 and 8 days, respectively, as compared with larvae fed the *GFP* dsRNA containing diet (P<0.05) indicating statistically significant reductions of *OnCht* mRNA levels in *OnCht* dsRNA-fed larvae (Figure 4.8A). When we compared the chitin contents of larvae fed *OnCht* or

GFP dsRNAs, the chitin contents increased by 26% in *OnCht* dsRNA-fed larvae as compared with GFP dsRNA-fed larvae ($P < 0.05$) (Figure 4.8B). We did not find significant differences between the chitin contents of midgut tissues (free of PM) isolated from *OnCht*- and *GFP*-dsRNA treated larvae.

The body weight of the larvae fed *OnCht* dsRNA and *GFP* dsRNA was also determined after 10 days. We found a 54% decrease in body weight for larvae fed the diet containing *OnCht* dsRNA as compared with those fed the same diet containing *GFP* dsRNA (Figure 4.8C & D).

Discussion

Chitinases are large and diverse enzymes and have received much attention from researchers in recent years due to their important biochemical functions in chitin metabolism. They are also potential targets for novel insect-specific pesticides for use in insect pest management (44). However, very little is known about the specific physiological functions of these enzymes in insect growth and development. Merzendorfer and Zimoch (45) suggested that insect gut-specific chitinases play a role in degrading the chitin present in the PM during molting. On the other hand, Shen and Jacobs-Lorena (19), Hegedus et al. (28), and You et al. (38) proposed that insect gut chitinases may help increase the porosity of the PM to facilitate the digestion process. Despite the great interest in understanding the physiological functions of these diverse chitinases, the regulatory function of chitin in the PM has been poorly studied in insects.

In this study, we identified and characterized a chitinase-like gene (*OnCht*) in the ECB and demonstrated for the first time, that *OnCht,* possibly along with *OnCHS-B,* play important roles in the regulation of chitin contents of the PM of the larval midgut. Because the expression of *OnCht* was only detected during larval feeding stages, and >97% of *OnCht* expression was found in the midgut (predominantly in the anterior midgut), we propose that this gene is designed

for larval midgut-specific expression in ECB. Low levels of expression of this gene were detected in the foregut but this could be due to contamination with anterior midgut tissue which may have occurred during the gut separations. The development- and tissue-specific expression patterns of *OnCht* in feeding ECB larvae support our proposal that this midgut-specific chitinase may be involved in chitin regulation for facilitating food digestion. In other insect species, more than one gut-specific chitinase-like gene has been identified. In *T. castaneum,* for example, several chitinase-like genes were found to be expressed at high levels in the larval gut (3). There may be other chitinase genes in the ECB larvae, which may be expressed during the insect molting. The presence of transcripts for only a single chitinase gene in our EST library and its high expression level in the anterior midgut (75.8 %) suggest that *OnCht* may play an important role in regulating PM chitin content and assembly in the anterior part of the midgut.

The chitin contents of the PM of insects usually accounts for approximately 3 to 13 % of their weight (46), but in some cases it can be significantly higher, as reported for *M. sexta*, where chitin contents were as high as 40 % of the dry weight of PM (47). However, it is unknown why there are such large variations in chitin contents of the PM among the insect species. In order to test our hypothesis that PM chitin contents are regulated during feeding, (presumably to alter the porosity of the PM to facilitate food digestion) we examined changes in transcript levels of *OnCht* and *OnCHS-B* in 1-day-old fifth-instar ECB larvae feeding on artificial diet. Our results suggest that expression of the *OnCht* and *OnCHS-B* genes are affected by feeding. When larvae were not provided food, the *OnCht* gene expression decreases significantly and *OnCHS-B* expression increases significantly relative to larvae maintained on food. These changes occur rapidly and reversibly. Similar functions of gut-specific chitinases were also suggested by other researchers for other insects (19, 28, 38). For example, the expression of gut-specific chitinase

genes in response to feeding has been reported in blood-feeding insects including *A. gambiae* (19) and *L. longipalpis* (21). In *L. longipalpis*, the expression of a midgut-specific chitinase gene was only found after blood feeding and reached peak expression at approximately 72 h post-blood meal.

Our studies clearly showed a negative correlation between *OnCht* gene expression and chitin content as well as a positive correlation between *OnCHS-B* gene expression and chitin content of the PM. Chitin contents increased significantly when larvae were maintained with no food as compared with larvae maintained on food for the same period of time. As expected, the chitin contents decreased dramatically when the starved larvae were allowed to feed. The relationship between chitin content in the PM of ECB and expression of *OnCht* and *OnCHS-B* genes under fed and starvation conditions suggests that these genes play important roles in the regulation of chitin contents in the PM.

The regulatory role of *OnCht* in altering chitin content of the PM of the larval midgut was further supported by our RNAi work. By using a feeding-based RNAi technique, we were able to reduce the *OnCht* transcript levels by 63-64% in larvae fed a diet containing *OnCht* dsRNA as compared with those of larvae fed a diet containing *GFP* dsRNA. Such a suppression of the *OnCht* transcript level in the larvae fed *OnCht* dsRNA resulted in a significant increase of chitin content (26%) in PM, suggesting that *OnCht* is involved in the regulation of the midgut chitin in ECB larvae, probably through a reduced rate of degradation of the chitin by this enzyme. Interestingly, the growth and development of these larvae were affected and there was a reduction in larval body weight (54 %) as compared with larvae fed GFP dsRNA, which is most likely due to defective food assimilation. The decreased porosity of the PM and/or loss of compartmentalization may hinder the digestion process of the larvae. Thus, our studies provided

strong evidence for the first time that *OnCht* plays an essential role in regulating chitin content of PM and affecting larval growth, presumably by influencing food digestion, nutrient absorption or movement of digestive enzymes through the PM.

Materials and Methods

European Corn Borer

The European corn borers used in this study were purchased as eggs and larvae from Lee French Laboratories, Lumberton, MN.

cDNA Sequence Analysis

A gut-specific EST library was established from RNA isolated from fifth-instar ECB larvae as previously described and 15,000 clones were sequenced (39). The EST database consisting of 2,895 unique ESTs was searched for the genes encoding chitinase and chitinase-like proteins and chitin synthase. Two clones from our EST library were identified, one similar to chitinase and another similar to chitin synthase B genes. These clones were again sequenced from both ends using M13R and M13F primers to obtain the sequences of the full length inserts. We found that the chitinase-like cDNA was full length but the chitin synthase cDNA was a partial clone consisting of only 506 bp. Signal P software was used to predict signal peptide (35). The software ClustalW (48) and MEGA4 (49) were used for multiple alignments and to construct a phylogenetic tree, respectively. Smart software (50) was used to predict domains in the amino acid sequences.

Tissue and Developmental Stage Expression Profiles

Tissues were dissected in DEPC-treated water from one-day-old fifth-instar ECB larvae. Total RNA was isolated from different tissues (pooled from four animals) and different ECB

developmental stages (pooled from four animals) by using TRI reagent™ (Sigma, St. Louis, MO). Only feeding larvae were used in this analysis. Total RNA was treated with TURBO™ DNase (Ambion, Austin, TX) to remove any genomic DNA contaminations. One microgram of total RNA was used for synthesis of first strand cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). cDNA prepared from total RNA was used as a template for real-time qPCR or RT-PCR. The qPCR analysis was performed using SYBR green kit (Bio-Rad) and Bio-Rad iCycler iQs real-time PCR detection system at the Kansas State University Gene Expression Facility. qPCR cycling parameters included 95°C for 5 min, 40 cycles each consisting of 95°C for 30 sec, 55°C for 0.15 sec, and 72°C for 0.45 sec, followed by 95°C for 1 min and 55°C for 1 min. At the end of each quantitative PCR experiment, a melt curve was generated to rule out the possibility of primer-dimer formation. The relative expression analysis for qPCR was performed by using the ECB *RPS3* gene as an internal reference. For RT-PCR, 27 cycles were used for both *OnCht* and *RPS3* gene, each cycle consisting of 94°C for 30s, 55°C for 60s, and 72°C for 60s. Three biological replications, each with two technical replications, were used for qPCR analysis and one biological replication was used for RT-PCR analysis. Primers for the genes were designed by using Beacon Designer software (version 7). The primers for *OnCht* are: 5'-TGCTATATTCTCCAGAACGAGTC (F) and 3'-GCCGTGGAAGTCATCAGTC (R) with product size of 195 bp; primers for *OnCHS-B*: 5'-GCCTGTTCCGTTGTCTATGC (F) and 3'-TCTCAATCTTCTCCATGCTATGTG (R) with product size of 93 bp.

Gene Expression Profiles under Feeding and Starvation Conditions

We divided 1-day-old fifth-instar larvae into two sets. The larvae in the first set were maintained with food (artificial diets) for 24 h and then with no food for next 24 h, whereas the

larvae in the second set were maintained with no food for 24 h and then with food for the next 24 h. The midguts from half the larvae were dissected after 24 h and the other half were dissected after 48 h. First strand cDNA was prepared as above and expression levels for *OnCht* and *OnCHS-B* were assessed using qPCR. Three biological replications (n = 4), each with two technical replications, were used in this analysis.

Chitin Content Assay

Chitin contents of the midgut (including PM), PM only or midgut only were determined using the method described by Zhang and Zhu (51). The larvae were divided into two sets as described above. The larvae were maintained with and without food. Zero hour referred to the start of the experiment. Due to differences in the size of insects which were maintained with food versus no food or due to the dsRNA treatment, we calculated chitin contents based on the wet weight of midgut tissue. For the samples where the whole midgut was used to assess the chitin contents, we dissected 10 extra larvae from each group to get midgut tissues and used its mean weight for normalization. For samples where midgut tissues and PM were separated, we used the same weight of midgut tissues for normalization of chitin contents in different samples. We also assessed the chitin content in 10 mg of artificial diet. Two or three independent biological replications, each with two-three technical replications, were used for each treatment.

RNA Interference

dsRNA was prepared using plasmid DNA as template by *in vitro* transcription for RNAi. The primers were designed using Beacon Designer software (version 7) and T7 primer sequence was placed in front of both forward and reverse primers. The primer sequence to generate dsRNA for *OnCht* gene were 5’-TAATACGACTCACTATAGGCGGAGGATGGAGCGAAG and for 3’-

TAATACGACTCACTATAGGACTCTCGCCTTCACTTAT with product size of 404 bp. Similarly, for GFP, the primers used were 5'-TAATACGACTCACTATAGGCCATTCTTTTGTTTGTCTGC and 3'-TAATACGACTCACTATAGGGGCCAACACTTGTCAC with product size of 309 bps. The dsRNA was transcribed by using the above gene specific primers and the AmpliScribe™ T7-Flash™ Kit (Epicentre Technologies, Madison, WI) according to the manufacturer's protocol. The dsRNAs were purified by phenol/chloroform extraction followed by ammonium acetate precipitation. Immediately after hatching, larvae were individually fed the dsRNA mixed with fresh artificial diet (Bio-serve). Three doses, each consisting of 10 μg of *OnCht* dsRNA in 2 μl of water on day 0, 2, and 4 were added to the diet of each larva for a total of 30 μg dsRNA /larva . The control larvae received the same amount of *GFP* dsRNA. After day six, larvae were transferred to normal diet. Transcript levels of *OnCht* in the midgut tissues of the larvae fed *OnCht* and *GFP* dsRNA were determined on day 6 and 8 by qPCR. Total RNA isolation, first strand cDNA preparation, and qPCR analysis were performed as described above. On day 10, the chitin contents of the midgut tissues and PM of larvae fed *OnCht* and GFP dsRNA were also determined as described above.

Statistical analysis

The gene expression and chitin content analyses were subjected to one-way analysis of variance (ANOVA). Fisher's least significant difference (LSD) multiple comparisons were then used to separate the means among the treatments. All the statistical analyses were performed using ProStat software (Poly Software International Inc., Pearl River, NY).

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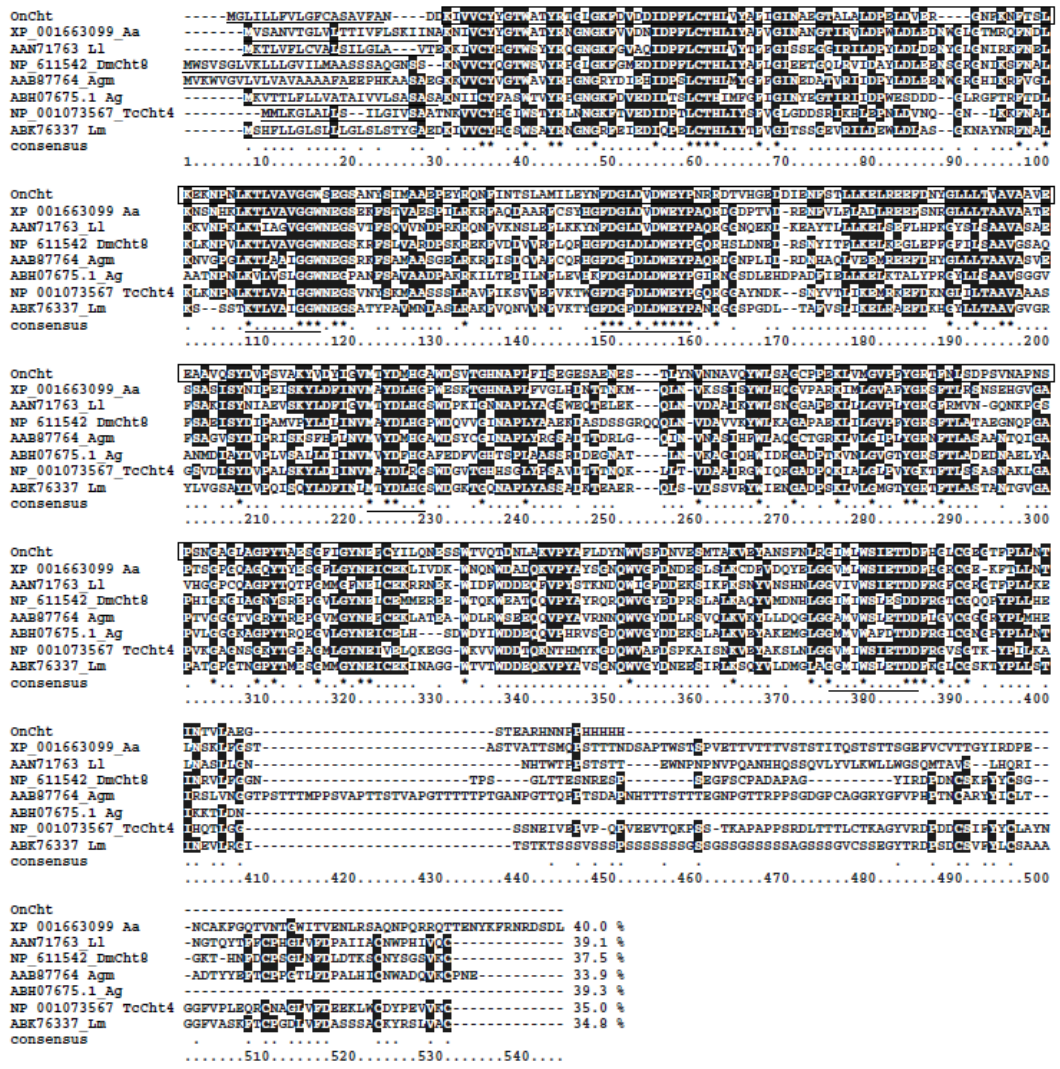

Figure 4.1 Multiple alignments of the amino acid sequences of *Ostrinia nubilalis* chitinase (*OnCht*) and other insect chitinases using CLUSTALW. The predicted signal peptides for all the sequences are marked by an underline at the start of each sequence. The catalytic domain of *OnCht* is shown in a rectangular box. The four conserved regions of insect chitinases are indicated by the underline. Fully conserved residues are indicated by a black background. Percent identities of all sequence with *OnCht* are given at end of each sequence. GenBank accession numbers and abbreviations of organism names are shown. The sequences used in this analysis were from *Aedes aegypti* (Aa)*; Drosophila melanogaster* (Dm)*; Anopheles gambiae* (Agm); *Tribolium castaneum* (Tc); *Lutzomyia longipalpis* (Ll)*; Apriona germari* (Ag); *and Locusta migratoria* (Lm).

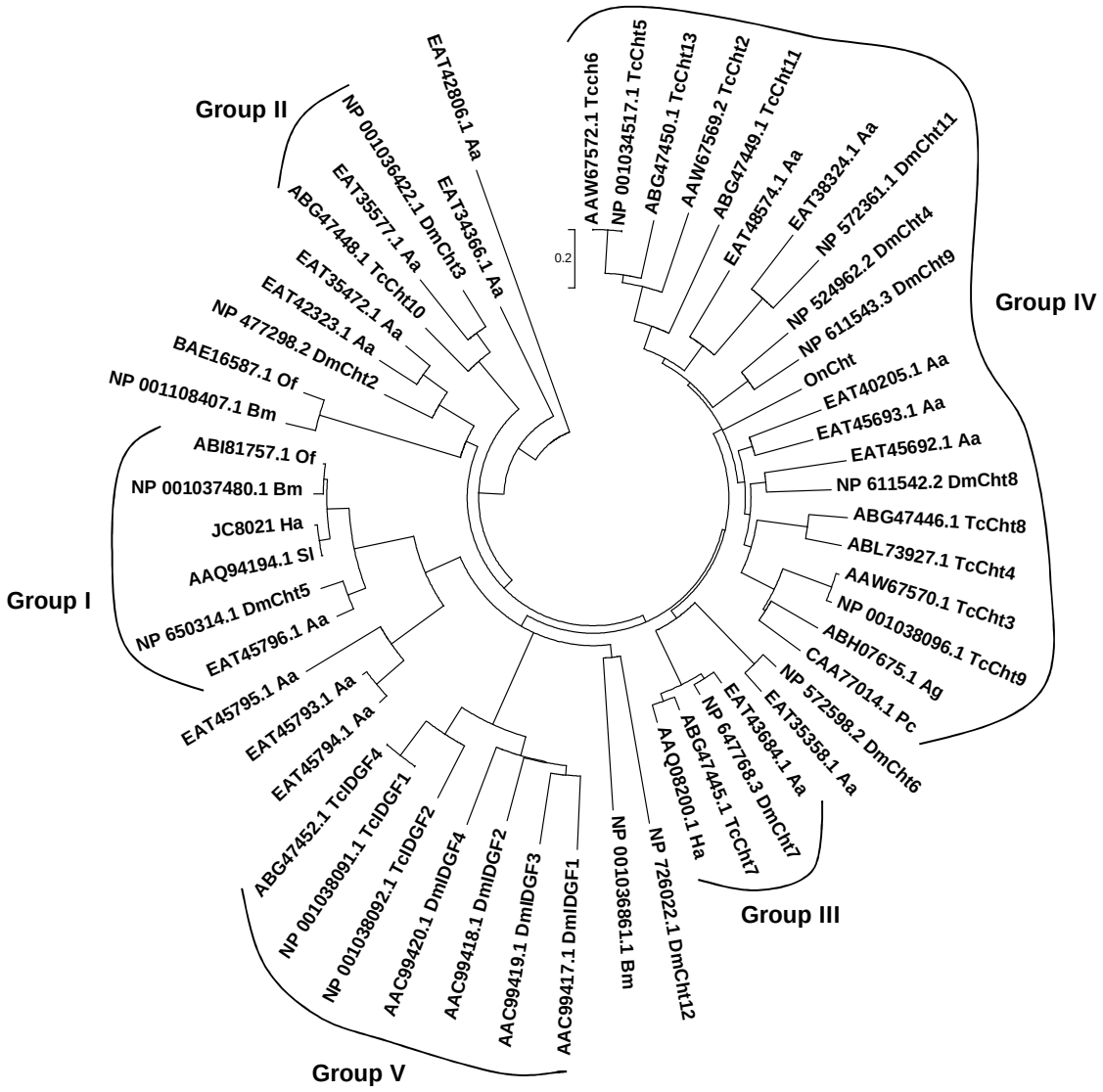

Figure 4.2 Neighbor-joining phylogenetic tree constructed from the full length amino acid sequences of 55 chitinase-like proteins from nine insects including *Ostrinia nubilalis* (*OnCht*). Chitinase groups are formed as described by Zhu et al. (3). GenBank accession numbers along with abbreviations of organism name are shown. Sequences used in the construction of the tree were from *Bombyx mori* (Bm)*; Helicoverpa armigera* (Ha)*; Spodoptera litura* (Sl)*; Aedes aegypti (Aa); Drosophila melanogaster* (Dm)*; Ostrinia furnacalis* (Of)*; Tribolium castaneum* (Tc); *Phaedon cochleariae* (Pc), and *Apriona germari* (Ag).

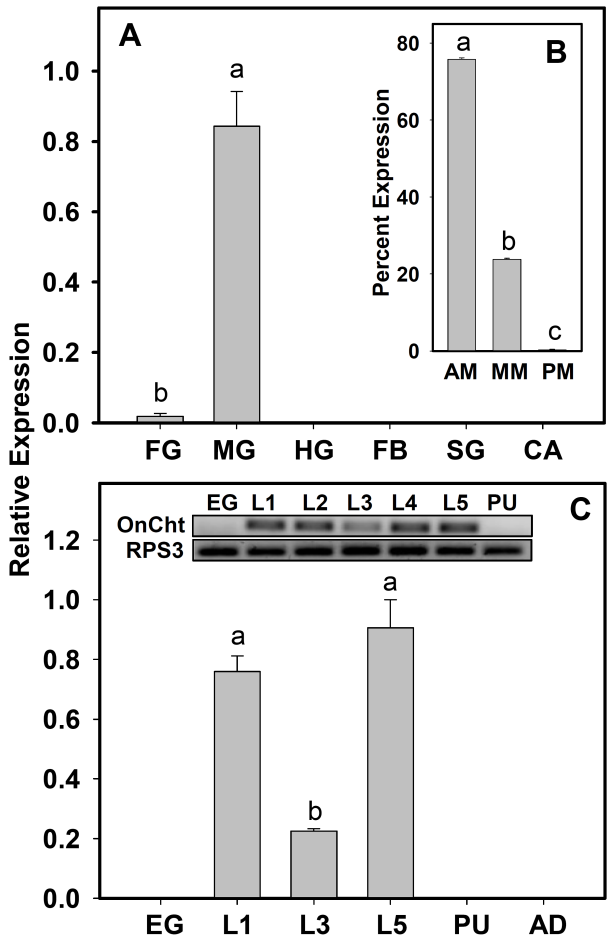

Figure 4.3 Expression of midgut-specific chitinase (*OnCht*) gene in different larval tissues, different parts of the midgut, and during different developmental stages by real-time PCR or RT-PCR. (A) Gene expression was determined in foregut (FG), midgut (MG), hindgut (HG), fatbodies (FB), salivary glands (SG), and carcass (CA). (B) Percent of *OnCht* transcripts in the anterior midgut (AM), middle midgut (MM), and posterior midgut (PM) compared to total transcripts for this gene. (C) Gene expression was studied for different developmental stages including egg (EG), first instar (L1), third instar (L3), fifth instar (L5) larvae, pupae (PU), and adults (AD). Gel picture from RT-PCR analysis showing expression of *OnCht* from second (L2) and fourth (L4) larval instar, in addition to other developmental stages is shown. Standard error bars were determined from three independent biological replications (n=4), with two technical replications each. Different letters within a figure represent significant difference at *P* value ≤ 0.05.

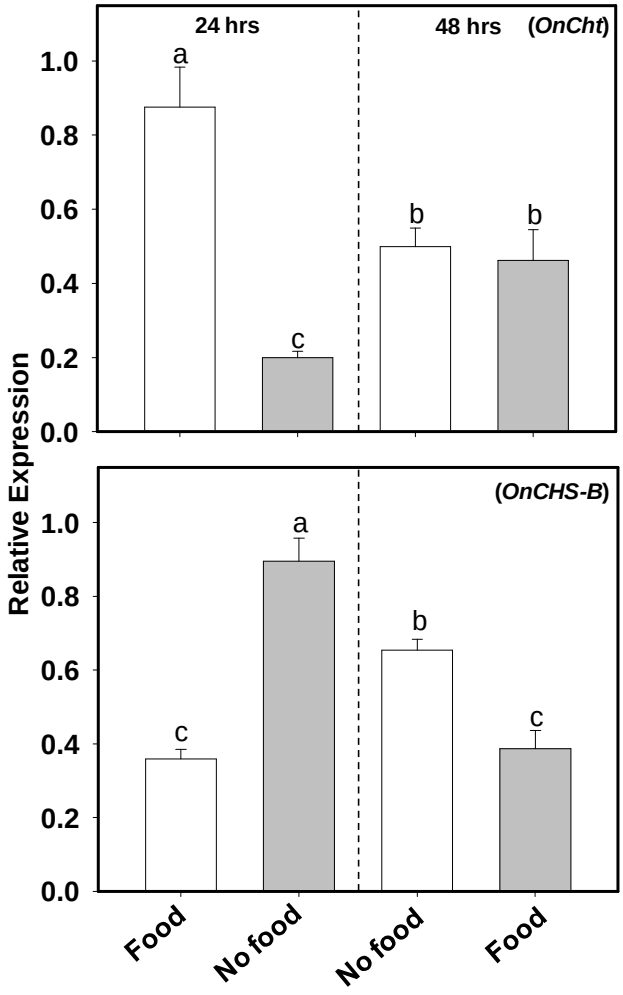

Figure 4.4 Relative expression of chitinase (*OnCht*) and chitin synthase-B (*OnCHS-B*) genes in the midgut of fifth-instar European corn borer larvae under food or no food conditions. Larvae in set 1 (empty bars) were fed for 24 h and then maintained with no food for next 24 h, whereas larvae in set 2 (filled bars) were maintained with no food for 24 h and then fed for next 24 h. mRNA level was assessed for both genes by qPCR after 24 and 48 h. Standard error bars were determined from three independent biological replications, with two technical replications each. Different letters with in a figure represent significant difference at P value ≤ 0.05.

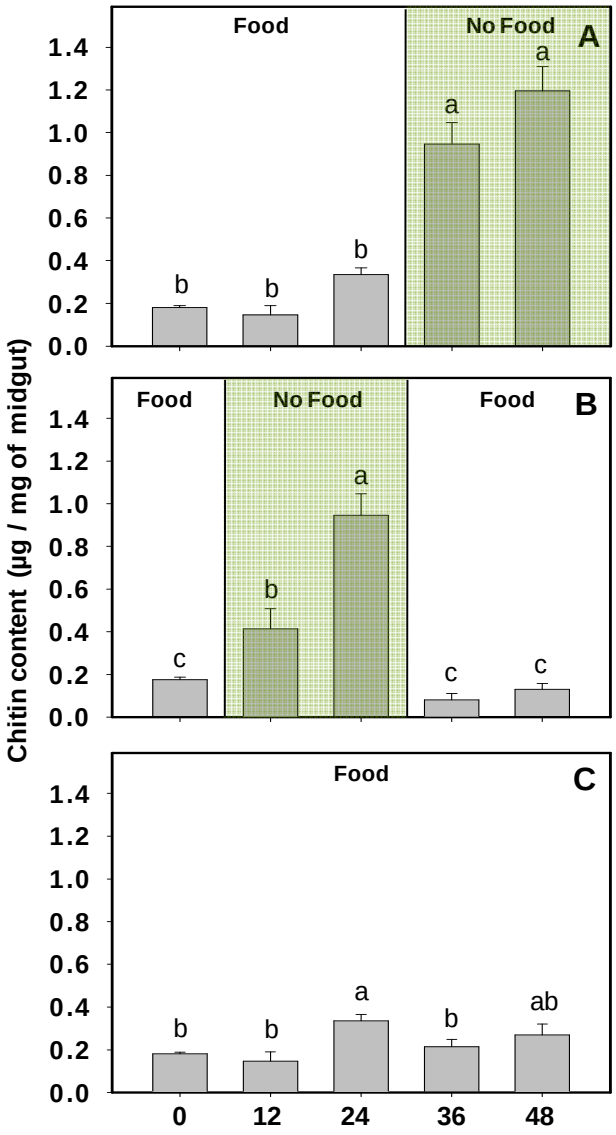

Figure 4.5 Chitin contents of midguts of fifth-instar European corn borer larvae relative to midgut weight under food or no food (background shaded) conditions. (A) Larvae were maintained on food for 24 h and with no food for next 24 h. (B) Larvae were maintained with no food for 24 h and allowed to feed for the next 24 h. (C) Larvae were maintained on food continuously. Zero (0) h refers to time at the start of the experiment when larvae were allowed to feed. Standard error bars were determined from three biological independent replications, with three technical replications each. Different letters within a figure represent significant difference at P value ≤ 0.05.

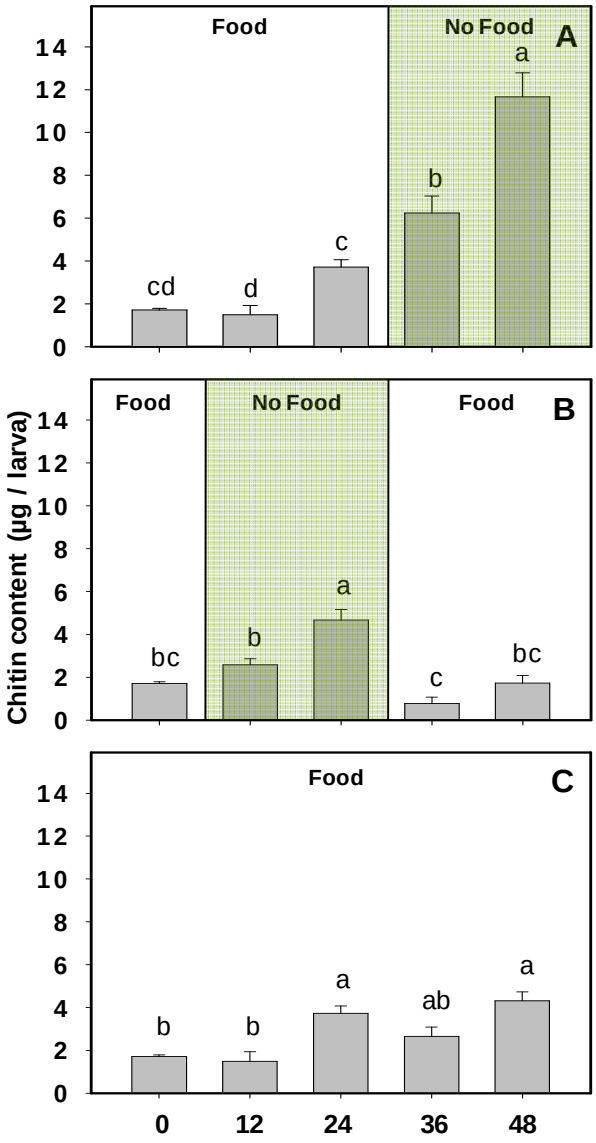

Figure 4.6 Chitin contents of midguts of fifth-instar European corn borer larvae under food or no food (background shaded) conditions. (A) Larvae were maintained on food for 24 h and with no food for next 24 h. (B) Larvae were maintained with no food for 24 h and allowed to feed for next 24 h. (C) Larvae were maintained on food continuously. Zero (0) h refers to the time at the start of the experiment when larvae were allowed to feed. Standard error represented as error bars were determined from three biological independent replications, each consisting of three technical replications. Different letters within a graph represent significant difference at P value ≤ 0.05.

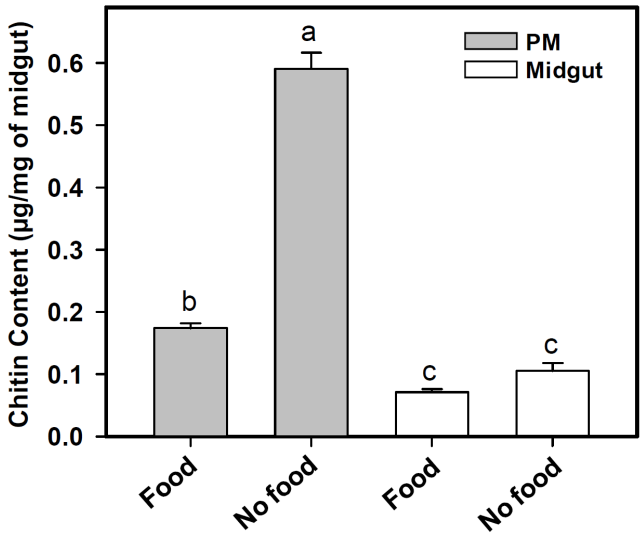

Figure 4.7 Chitin contents of peritrophic membrane (PM) and midgut tissues (MG) from the fifth-instar European corn borer larvae maintained under food and no food conditions. One set of larvae was maintained on food for 24 h and the other set with no food for the same period of time. Standard error bars were determined from three independent biological replications, with three technical replications each. Different letters represents significant difference at *P* value ≤ 0.05.

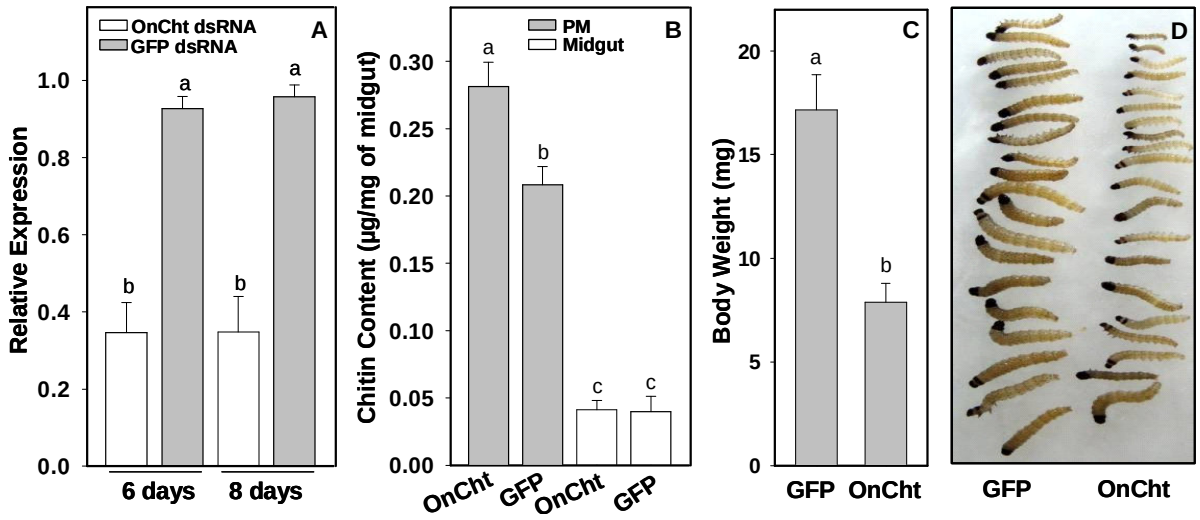

Figure 4.8 RNA interference of *Oncht* and its effect on midgut chitin contents and growth in European corn borer larvae. (A) Expression of midgut-specific chitinase gene (*OnCht*) in the European corn borer larvae after treatment with *OnCht* dsRNA and green fluorescent protein (*GFP*) dsRNA. Expression level of *OnCht* was determined by qPCR. Standard error bars were determined from 3 independent biological replications, with two technical replications each. (B) Chitin contents of the peritrophic membrane (PM) and midgut tissues (MG) from the European corn borer larvae treated with dsRNA for *OnCht* and *GFP*. Standard error bars were determined from three independent biological replications, with three technical replications each. (C) Mean body weight of the larvae treated with *OnCht* dsRNA and *GFP* dsRNA after 10 days of first dsRNA treatment. Each value represents mean ± standard error (n=27-30). (D) Picture of experimental larvae showing reduced body size in *OnCht* dsRNA treated larvae as compared with *GFP* dsRNA treated larvae. Different letters within a figure indicate significant difference with P value ≤ 0.05.

CHAPTER 5 - Characterization of Six Antibacterial Response Genes and Their Expression Responses to Bacterial Challenge in European Corn Borer

Abstract

We identified and characterized six antibacterial response genes from ECB larvae, including four peptidoglycan recognition proteins (PGRPs), one β-1-3 glucanse-1 (βglu-1), and one lysozyme. Tissue-specific analysis showed that all of these genes except for lysozyme had high mRNA levels in midgut tissues. All genes also showed expression in larval stage of ECB. None or low expression for these genes was detected in egg, pupa and adult. The expression of all six antibacterial response genes in fatbodies was up-regulated when ECB larvae were challenged with Gram-positive bacteria (*Enterobacter aerogenes*), however only PGRP-C and lysozymes were induced when challended with gram-negative bacteria (*Micrococcus luteus*). This study provides insight into the expressional pattern of antibacterial genes in ECB larvae and will lead to better understanding of the immune defense response in ECB.

Introduction

Innate immune response in insects is the main defense mechanism against microbial infection (Royet et al. 2005). The first step in the defense cascade of the host is to recognize the invading organism as non-self (Schmid-Hempel 2005) and then these follows induction of several immune related proteins in the body of the organism (Hashimoto et al. 2007). Several families of the proteins have been identified, which are involved in the recognition of the surface characteristics of microbes such as peptidoglycan recognition proteins (PGRPs), gram negative binding proteins (GNBPs) or β-1-3 glucan recognition proteins (βGRP), lipoploysaccharides (LPS), and mannans (Medzhitov et al. 1997). Once the pathogen has been recognized defense responses can be direct where a pattern recognition protein (PRR) mediates the killing of the microbe through encapsulation and phygocytosis or indirect where PRR triggers the activation of the serine protease cascades and intracellular immune signaling pathways leading to the induction of antimicrobial peptides such as lysozymes (Ferrandon et al. 2007, Warr et al. 2008). In *Drosophila melanogaster*, two immune signaling pathways have been characterized: 1) Toll pathway; 2) immunodeficiency (IMD) pathway. The activation of the Toll pathway is triggered predominantly by fungal and Gram-positive bacteria whereas the IMD pathway is activated mainly by Gram-negative bacteria (Hoffmann et al. 1996, Hoffmann and Reichhart 2002).

PGRPs molecules are conserved among insects and mammals (Kang et al. 1998). The first PGRP was discovered in the silkworm where it is present in the haemolymph and cuticle and binds with peptidoglycan (PGN) and Gram-positive bacteria. This recognition leads to the formation of melanin following the activation of prophenoloxidase (Yoshida et al. 1996). Subsequently, several additional PGRP genes have been found in insects. In *D. melanogaster* up to 19 different PGRP proteins has been in identified and are classified into short (S) and long (L) transcripts (Werner et al. 2000, Dziarski and Gupta 2006). PGRP genes have been characterized

in several other insect species including *Manduca sexta* (Yu et al. 2002), *Bombyx mori* (Ochiai and Ashida 1999), *Samia cynthia ricini* (Hashimoto et al. 2007, Onoe et al. 2007), *Trichoplusia ni* (kang et al. 1998), and *Anopheles gambiae* (Christophides et al. 2002). Structurally, PGRP has approximately 165 amino acids long carboxy-terminal type 2 amidase domain, also called the PGRP domain (Kim et al. 2003), and it is homologous to the bacteriophage and bacterial type 2 amidases (Kang et al. 1998, Werner et al. 2000, Liu et al. 2001, Dziarski 2004). The two closely spaced conserved cysteine residues that form a disulfide bond and are located in the middle of the PGRP domain and are considered crucial for PGRP functions and structures (Dziarski and Gupta 2006). In *Drosophila* PGRP-SA gene, the mutation in one of the conserved cysteine leads to the failure in activation of Toll pathway and to induce protective response against Gram-positive bacteria (Michel et al. 2001). However, a similar mutation in the human PGLYRP-2 leads to failure in its amidase activity (Wang et al. 2003).

The GNBP and βGRP proteins that are homologous were first reported from silkworm (Lee et al. 1996, Ochiai and Ashida 1988) and are involved in the recognition of the β-1-3 glucan presumably with two distinct glucan binding domains, N-terminal glucan recognition domain and C-terminal glucanse-like domain (Hoffmann 2003, Ochiai and Ashida 2000, Pauchet et al. 2009, Fabrick et al. 2004). The βGRPs has been identified from several insects including *B. mori* (Ochiai and Ashida 2000), *M. sexta* (Ma et al. 2000), and *Plodia interpunctella* (Fabrick et al. 2003) and has been reported to bind with β-1-3 glucan through N-terminal domain which is sufficient to activate the defense cascade (Ochiai and Ashida 2000, Fabrick et al. 2003, Ma et al. 2000). However, the C-terminal domain of *B. mori* βGRP does not have glucanase-like activity nor has affinity for β-1-3 glucan (Ochiai and Ashida 2000). Pauchet et al (2009) recently reported a new family of gut-specific genes from several lepidopteran species which have

glucanase-like activity and were named β-1-3 glucanse-1(βglu-1). These new proteins are related to but distinct from previously reported family of GNBP/ βGRP proteins found in lepidopterans.

After the pathogen infects the insect haemocoel the defense response causes the synthesis of a battery of antifungal/antibacterial peptides (Hetru et al. 1998, Lamberty et al. 1999). Most of the antimicrobial peptides are produced in the fat bodies or haemocytes and are released into the haemolymph of insects (Dimarcq et al. 1998, Lamberty et al. 1999, Lopez et al. 2003). Lysozymes are the widespread antimicrobial peptides and are integral part of the defense mechanism against bacteria and fungi (Dunn 1986, Fiolka et al. 2005). They are also the first anti-microbial factors to be isolated from the insect hemolymph (Powning and Davidson 1976). Lysozymes causes the lysis of bacterial cell wall by hydrolyzing the 1, 4-b-linkage between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycans which are present in the cell wall (Grunclova et al. 2003). In addition to the role of lysozymes in immune defense, they are also reported to have digestive role especially in insects that ingest large number of bacteria from decomposing matter, e.g. *Musca domestica*, *Anastrepha fraterculus* and *D. melanogaster* (Lemos and Terra 1991, Regel et al. 1998, Ursic-Bedoya et al. 2005)

Several studies on the antibacterial response genes in insects have been reported but this information is still limited in lepidopteran species. During analysis of the expressed sequence tags (EST) from the gut of the European corn borer (ECB, *Ostrinia nubilalis*), we identified clones that putatively encoded four PGRP genes, one GNBP gene and one lysozyme gene. In this study, we characterized the cDNA sequences from these genes, and studied the expression patterns of mRNA levels for the antibacterial response genes in different tissues and developmental stages. We have also assessed the transcript levels for these genes in the larvae

challenged with Gram-positive and –negative bacteria and discussed their role in antibacterial defense.

Results

Sequence analysis

We searched for immune related genes in our gut-specific ECB expressed sequence tag (EST) database which consisted of 15,000 ESTs (Khajuria et al. 2009). We found six clones, of which four showed high homology to PGRPs, one to βglu-1, and one to lysozyme. Because our cDNA libraries were only sequenced from the 5'-end, these clones were isolated and sequenced again from both ends to get the full length sequences. The insert sizes of 640, 649, 1254, 1931, 1312, 917 base pairs (bp), with open reading frame of 187, 196, 218, 231, 235, 120 amino acids were found in the clones named PGRP-A, -B, -C, -D, βglu-1, and lysozyme, respectively. All of these deduced amino acid sequences possess signal peptides except for PGRP-C (Figure 5.1 and Figure 5.2). The signal peptides were 18, 19, 18, 20, and 17 residue long in PGRP-A, -B, -D, βglu-1, and lysozyme, respectively. The active regions of PGRP-A, -B, -C, -D, βglu-1, and lysozyme are predicted to be 19.07, 19.37, 24.43, 24.30, 40.40, 13.56 kDa for molecular masses and 4.7, 5.59, 5.68, 5.61, 4.17, 8.95 for isoelectric points, respectively (Expasy ProtParam).

Multiple alignments and phylogenetic analysis

The multiple alignments analysis showed that ECB PGRP amino acid sequences had significant similarities with PGRP sequences from other insects. PGRP-A had highest percent identity (40%) with PGRP-SC2 from *D. melanogaster*, and had percent identities ranging of 29.5 - 36.0% with those of other lepidopteran insects. The ECB PGRP-B had highest identity (59.8%) with PGRP-B protein from the *S.cynthia ricini.* It has 28.4 - 45.5% identities with PGRP sequences from other lepidopteran insects and 36.6 % identity with PGRP-LB sequence from *D.*

melanogaster. The ECB PGRP-C and -D sequences had highest sequence identities of 55.6% and 60.3% with PGRP-D sequence from *S.cynthia ricini,* respectively, followed by 22.8 - 44.3% and 25.7 - 41.8% identities with sequences from other lepideopteran insects. Also ECB PGRP-C and -D had 39.3 and 38.5% identities with PGRP-LB sequence from *D. melanogaster,* respectively. All ECB PGRPs had 26.7 - 65.1% identities and were predicted to have amidase-like activity as they all have all the five conserved residues required for amidase activity. The phylogenetic tree was constructed by using all ECB PGRPs together with PGRP sequences from several insect species and found that ECB PGRP-B, -C, and –D, along with *S.cynthia ricini* PGRP genes, form a distinct cluster away from the PGRP genes from other lepidopteran insect species (Figure 5.3). The ECB βglu-1 gene had highest percent identity of 82.7% with similar sequence from *Helicoverpa armigera,* followed by 61.5 - 80.8% identities with sequences from other lepidopteran species. It also has percent identity of 57.9% with GNBP3 sequence from *A. gambiae,* 56.0% with GNBP from *A. aegypti*, and 54.6% and 51.6% with βGRP sequence from *Tribolium casteneum* and *Culex quinquefasciatus,* respectively (Figure 5.4). Phylogenetic analysis for this gene has already been reported by Pauchet et al. (2009) where they found that it could be grouped with other βglu-1 midgut-specific genes but was different from groups which were haemolymph specific. The ECB lysozyme also found to share high identities with other similar genes from several insects, with highest percent identity (65.0%) with a similar gene from *M. sexta*. It had 52.1 - 63.8% identities with other lepidopteran insects (Figure 5.2). Phylogentic analysis of ECB lysozyme showed that it forms cluster with lysozymes from other lepidopteran species (Figure 5.5).

Transcriptional pattern of ECB antibacterial response genes in larval tissues

The mRNA level was evaluated for all six antibacterial response genes in the tissues of the naive 1-day-old fifth-instar ECB larvae using RT-PCR. All the six genes had lowest expression or no expression in the salivary glands. PGRP-B gene expressed in all the tissues examined except in salivary gland. PGRP-A showed expression in midgut only whereas PGRP-C showed expression in epidermis, fatbodies, and midgut. PGRP-D and lysozyme showed expression in all the tissues where as βglu-1 predominanlty expressed in the midgut tissues (Figure 5.6A). Furthermore, by using real-time PCR we found similar results. PGRP-A, -C, and βglu-1 had significantly low or no expression in the fatbodies as compared with expression in the midgut. PGRP-B and -D showed no difference in the mRNA transcript level between fat bodies and midgut tissues. Also ECB lysozyme had signicantly high expression in fatbodies when compared with midgut (Figure 5.6B).

Transcriptional patterns of the ECB antibacterial response genes during development

The mRNA level was evaluated for all six antibacterial response genes in all the developmental stages of ECB. All of the six genes showed expression in the larval stage of ECB and had low or no detectable expression in eggs, pupae, or adults. PGRP-A and –B showed very similar expression pattern with expression in all larval instars except fifth instar. Also, no detectable expression for these genes was found in the eggs, pupae, and adults. PGRP-C showed expression in all larval instars (except for first instar) and adults. PGRP-D showed expression in all the developmental stages examined except for fourth instar larvae and adult whereas βglu-1 had no detectable expression in eggs. ECB lysozyme also showed expression in all the stages except for adult (Figure 5.7).

Expression profiles of the ECB antibacterial response genes during bacterial challenge

Real-time PCR was used to compare the expression profiles of the six antibacterial response genes in fatbodies of the fifth-instar ECB larvae, at various time points, after larvae were injected with *Enterobacter aerogenes* (Gram-negative bacteria) and *Micrococcus luteus* (Gram-positive bacteria) (Figure 5.8). We found that all the six genes were induced after *E. aerogenes* challenge; however, not all genes showed induced expression when challenged with *M.luteus* when compared with the control. All the genes except for PGRP-C showed highest induction after 12 hr of challenge with *E. aerogenes.* The PGRP-C showed highest expression within 6 hrs of *E. aerogenes* infection and remains similar after 12 hrs also. The expressions of most of these genes decreased after 24 hrs and are not significantly different from control. However, PGRP-C and lysozmes had significantly higher expression after 24 hrs of *E. aerogenes* challenge when compared with control. In case of *M. luteus* challenge, there was no significant induction of the PGRP-B, - A, -D, and βglu-1 genes when compared with the control. However, PGRP-C and lysozyme were induced after 6 and 12 hrs of challenge with *M. luteus*, respectively (Figure 5.6).

Discussion

We characterized six antibacterial response genes from the ECB larvae, including four PGRPs, one βglu-1, and one lysozyme. All the four PGRP genes, except for PGRP-C, were predicted to have a signal peptide sequence of 18 to 19 bp (Figure 5.1, 5.2, 5.4). ECB PGRP-B,-C, and -D formed a group along with PGRP genes from *D. melanogaster* (PGRP-LB), *A. agyepti* (ABF18154.1), *A. gambiae* (XP_321943.2), and *S.cynthia ricini* (Figure 5.3). ECB PGRP-B, -C, and -D showed higher homology with each other (41.5-65.0 % identity) and lower homology with ECB PGRP-A (25.0-30.7 % identity) and PGRP genes from other lepidopteran

insect species. These results are similar to those of the PGRP genes from *S. cynthia ricini* where PGRP-B, -C, -D have 31.4 -39.0 % identities with PGRP-A and other lepidopteran insects (Hashimoto et al. 2007). The five conserved amino acid residues required for amidase activity in PGRP genes are present in the ECB PGRP-B, -C, -D. In PGRP-A, however, third conserved residue His is replaced by Ala and at the position of the fifth conserved residue, Cys is replaced by Ser (Figure 5.1). The latter replacement has been linked to the receptor type PGRPs and considered a strong feature that the protein does not have amidase activity but is a receptor type PGRP (Onoe et al. 2007, Mellroth et al. 2003). We also characterized the β-1-3 glucanse-1gene, its full length sequence was found in our cDNA library, but while searching the GenBank we found that this sequence was already deposited in the NCBI database (accession no. ACI32836.1) (Pauchet et al. 2009). ECB βglu-1 gene has a signal peptide of 17 residues and possesses the GH16 (glycosyl hydrolase family 16) active site. This gene is distinctly different from the other classes of the βGRPs found in lepidopterans that have additional C-terminal domain but do not have glucanase-like activity. However, their functional differnces have not been known (Pauchet et al. 2009, Hoffmann 2003, Ochiai and Ashida 2000). ECB lysozyme shares high identity (52.1 - 65.0 %) with lysozyme from other lepidopteran insect species and they also clustered together in phylogenetic analysis (Figure 5.5). ECB lysozyme has 20 bp long signal peptide sequence and have the two active site residues (Glu and Asp) (Figure 5.2). In general, during phylogentic analysis, the lysozyme sequences tend to cluster according to their function (immune or digestive) or possible location of tissues where they express (fatbodies / haemocytes or digestive track) (Ursic-Bedoya and Lowenberger 2007). The ECB lysozyme which predominantly expresses in the fat bodies, tends to be closely related to the lysozyme sequences which have immune related role (*Rhodnius prolixus*-B, lepodopteran species) as

compared with lysozymes which are found in the digestive tract (*D. melanogaster*-X, *R. prolixus*-A, *Triatoma brasiliensis, T. infestans*) (Daffre et al. 1994, Ursic-Bedoya and Lowenberger 2007, Kollien et al. 2003, Araujo et al. 2006).

Analysis of tissue-specific expression in the current study revealed that all of the ECB antibacterial response genes except for lysozyme had high mRNA levels in midgut tissues and all genes had low or no detectable expression in the salivary glands. ECB PGRP-B, -C, -D, and lysozyme were expressed in several tissues whereas PGRP-A and βglu-1 was expressed mainly in the midgut. The *Drosophila* PGRP-SC1,-SC2, and -LB which have amidase activity are expressed in the gut of the naive larvae (Werner et al. 2000). The expression of these genes in the larval gut has been suggested to prevent the over-activation of the IMD pathway following the bacterial ingestion (Bischoff et al. 2004). Similarly, PGRP-B gene in *S. cynthia ricini* is expressed only in the gut of naive larvae and is induced in the fatbodies after injection of PGN (Hashimoto et al. 2007). PGRP-B shows strong expression in the epidermis which is similar to expression of PGRP genes from lepidopteran species and PGRP-SA gene in *D. melanogaster* (Werner et al. 2000, Ochiai and Ashida 1999, Marcu et al. 1998). Epidermis is the barrier to the infections and may have its own antibacterial defense response (Werner et al. 2000). ECB βglu-1 gene expresses predominantly in the gut tissues which is similar to βglu-1 genes from other lepidopteran insect species which have glucanase activity but their exact role in the defense response or digestion has yet to be established (Pauchet et al. 2009). The ECB lysozyme is expressed in all the tissue assessed with high mRNA level in the epidermis, fatbodies, and haemolymph. This expression pattern is similar to the lysozymes C-1 and C-7 in *A. gambiae,* which also shows expression in tissues such as fat bodies, midgut, and salivary glands (Li et al.

2005). Lysozyme has been reported to have digestive role in addition to its role in antimicrobial defense (Lemos and Terra 1991, Regel et al. 1998, Ursic- Bedoya et al. 2005).

The expression patterns of all six antibacterial response genes were assessed in all developmental stages. Large changes in the expression of antibacterial response genes occurred during the ECB development. PGRP-D and lysozyme become active in early stages of the development as their expression was detected in the egg and may play a role in initial recognition and defending eggs from microbial infection. No or Low mRNA level of PGRP genes was found in pupae and adults. βglu-1 was detected at low level in both pupa and adult where as PGRP-D was found in pupa and PGRP-C was found in the ECB adult.

The expression of all six antibacterial response genes in fatbodies was up-regulated when ECB larvae were challenged with Gram-positive bacteria (*E. aerogenes*), however PGRP-C and lysozymes were induced when challended with Gram-negative bacteria (*M. luteus*). All genes were up-regulated within 6 hrs after challenged with *E. aerogenes*, with maximum expression after 12 hrs and lowest or no expression after 24 hrs. Several studies have reported the up-regulation of PGRP genes when insects were exposured to bacteria or purified bacterial PGN (Kang et al. 1998, Ochiai and Ashida 1999, Werner et al. 2000, Dimopoulos et al. 2002, Christophides et al. 2002). The response of all six ECB genes was stronger with Gram-negative bacteria as compared with the Gram-positive bacteria. This response may be due to specificity of the type of the PGN, as Gram-negative bacteria have DAP-type PGN and most Gram-positive bacteria have Lys-type PGN (Dziarski and Gupta 2006). It has been reported that different stimuli lead to differential induction of the PGRP gene expression, suggesting the specificity of induction and effector function of different PGRPs (Christophides et al. 2002, Dimopoulos et al. 2002). Also, βglu-1 gene from *H. armigera* shows differential expression when exposed to

different stimuli (Pauchet et al. 2009). In addition to these genes, lysozymes which are usually considered a Gram-positive antibacterial factor are also effective against Gram-negative bacteria and fungi for e.g. arthropod c-type lysozymes (Li et al. 2005). This is the first study to characterize the antibacterial response genes in the ECB larvae. This study may lead to better understanding of the immune defense response in ECB.

Materials and Methods

Insects

The European corn borer used in this study was purchased as eggs and larvae from Lee French Laboratories, Lumberton, MN.

cDNA sequence analysis

Two cDNA libraries from the gut of fifth instar ECB larvae were constructed using: 1) Creator SMARTTM cDNA library construction kit from Clontech (Palo Alto, CA), and; 2) ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack III gold cloning kit following manufacturers instructions. Total of 15,000 ESTs from these libraries were sequenced from 5'end (Khajuria et al. 2009). cDNA libraries were analysed and searched for immune defense response genes. Six clones were found having putative identity to PGRPs, βglu-1, lysozyme. These clones were again sequenced from both ends using M13R and M13 F primers to obtain full length of the inserts. Signal P software was used to predict signal peptide (Bendtsen et al. 2004). ClustalW (Larkin et al. 2007, Thompson et al. 1994) and MEGA4 softwares (Tamura et al. 2007) were used for multiple alignments and to construct phylogenetic tree, respectively.

Tissue and developmental stage expression profiles

Total RNA was isolated from different tissues and different ECB developmental stage using TRI reagentTM (Sigma, St. Louis, MO). Tissues were dissected in DEPC-treated water from one-day-old fifth instar ECB larvae and pooled from four larvae. Total RNA was treated with TURBO™ DNase (Ambion, Austin, TX) to remove any genomic DNA contaminations. One microgram of total RNA was used for synthesis of first strand cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). cDNA prepared from total RNA was used as a template for real time PCR or RT-PCR. Two biological replications and two technical replications were used for real time PCR analysis and one biological replication was used for RT-PCR analysis. Realtime PCR included 95°C for 5 min, 40 cycles each consisting of 95°C for 30 sec, 55°C for 0.15 sec, and 72°C for 0.45 sec, followed by 95°C for 1 min and 55°C for 1 min. RPS3 genes was used as reference gene. For RT-PCR, 28 cycles were used for all genes including RPS3, each cycle consisting of 94°C for 30s, 55°C for 60s, and 72°C for 60s. Primers for these genes were designed by using Beacon Designer software (version 7) and their sequences are given in Table 5.1.

Bacterial challenge and expression profiles

Bacteria were streaked on the LB plate to get a pure colony and kept at 37° C overnight. The single colony was picked and grown in the LB-media overnight in the shaking incubator at 37° C and 200 rpm. Next day the bacterial solution was centrifuged at 4 C and 3000 rpm to get the pellet. The pellet was washed twice in the 0.15 M phosphate saline buffer (PBS) and centrifuged as above after each wash. The final pellet was dissolved in the 0.15 M PBS buffer. The OD value was adjusted to get OD = 0.4. The same procedure was performed for both kinds of bacteria (*E. aerogenes* and *M. luteus*). For injections, one-day-old fifth instar larvae were

anesthetized on ice for 30 minutes and then 5 ul of the bacteria+PBS solution was injected into the haemolymph of larvae. For control larvae, 5 ul of 0.15 M PBS buffer was injected. Seperated syringes were used for each treatment. After injections larvae were allowed to feed on the the artificial diet at 26° C. The fat bodies were dissected in DEPC-water after 6, 12, 24 hrs of injections. The RNA isolation, first-strand cDNA synthesis, and real-time PCR were performed as above.

Statistical analysis

The gene expression analysis in tissues and developmental stages were subjected to one-way analysis of variance (ANOVA). The expression analysis due bacterial exposure was subjected to two-way analysis of variance (ANOVA). Fisher's least significant difference (LSD) multiple comparisons were then used to separate the means among the treatments. All the statistical analyses were performed using ProStat software (Poly Software International Inc., Pearl River, NY).

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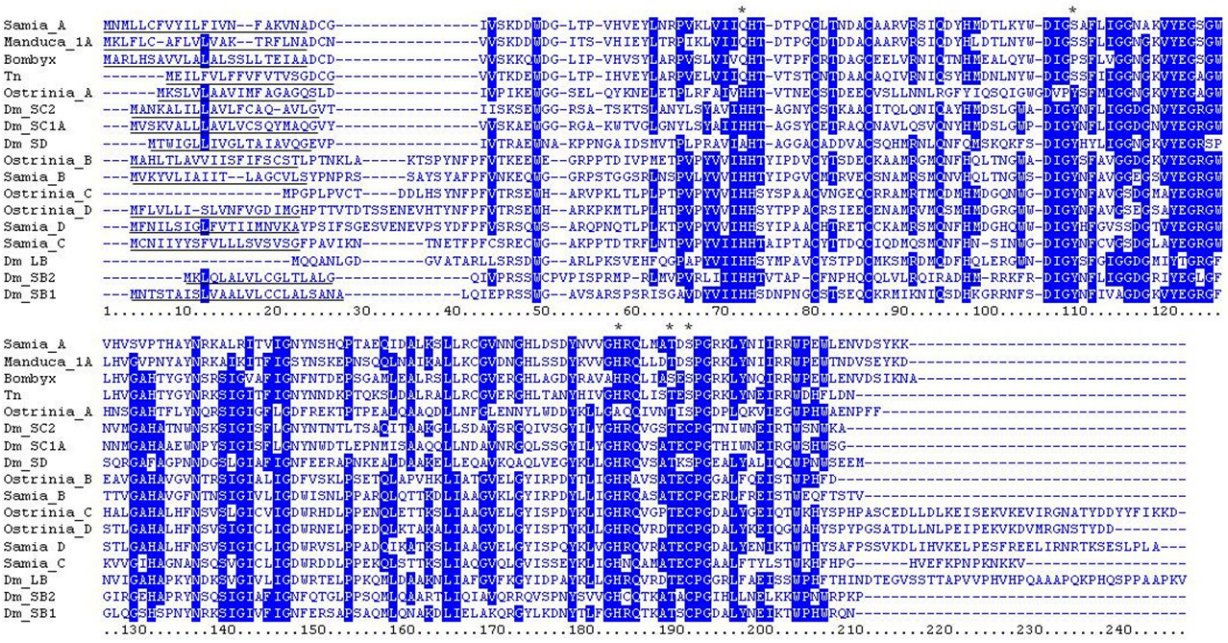

Figure 5.1 Multiple alignments of the amino acid sequences of *Ostrinia nubilalis* PGRPs and other insect PGRPs using CLUSTALW. Predicted signal peptide sequences are underlined. Positions of five residues required for the amidase activity are marked by asterik (*). No signal peptide was predicted for *O. nubilalis*-C and *Drosophila*-LB. Fully conserved residues are indicated by blue background. Sequences from the following insects were used in this analysis: *Samia cynthia ricini*-D (GenBank accession: BAF74637.1); *S. cynthia ricini*-B (BAF03520.1); *S. cynthia ricini*-A (BAF03522.1); *S.cynthia ricini*-C (BAF03521.1); *Bombyx mori* (NP_001036836.1); *Drosophila melanogaster*- SA (NP_572727.1); *D. melanogaster*- LB (AAN13506.1); *D. melanogaster*-SB2 (CAD89150.1); *D. melanogaster*-SB1 (CAD89135.1); *D. melanogaster*-SC2 (CAD89178.1); *D. melanogaster*-SC1A (CAD89162.1); *D. melanogaster*-SD (CAD89197.1); *M. sexta*-1A(AAO21509.1); *Trichoplusia ni* (AAC31820.1).

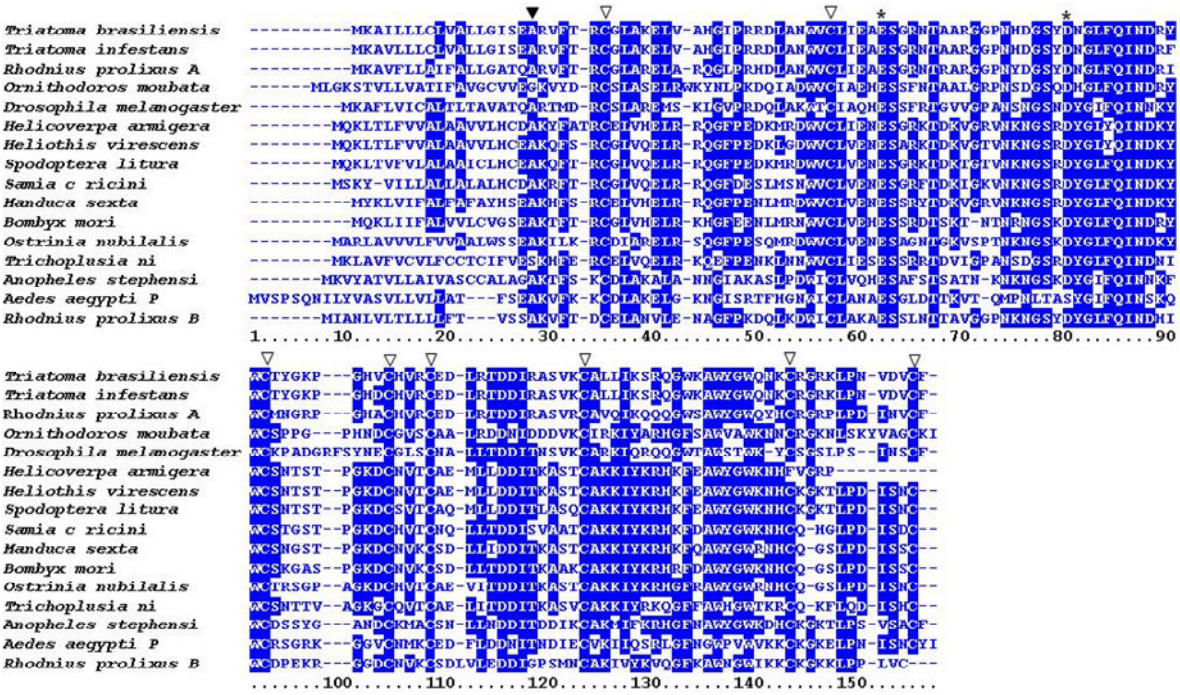

Figure 5.2 Multiple alignments of the amino acid sequences of *Ostrinia nubilalis* lysozyme and other insect lysozymes using CLUSTALW. The predicted cleavage site for signal peptide for all the sequences is marked by filled triangle (▼) above the alignement. The position of two catalytic residues, Glu and Asp are indicated by asterik (*). The eight conserved cysteine (C) residues involved in disulfide bridges are indicated by open triangle (▽) above the alignments. Fully conserved residues are indicated by blue background. The sequences used in this analysis were: *Heliothis virescens* (GenBank accession: AAD00078.1), *Spodoptera litura* (ACI16106.1), *Samia cynthia ricini* (BAB20806.1), *Manduca sexta* (AAB31190.2), *Bombyx mori* (NP_001037448.1), *Trichoplusia ni* (ABV68862.1), *Ornithodoros moubata* (AF425264.1), *Triatoma brasiliensis (*AAU04569.1*), Triatoma infestans (*AY253830*),*Rho*dnius prolixus A (*EU250274*),* Rho*dnius prolixus B (*EU250275*), Drosophila melanogaster (*NP_476828.1*), Helicoverpa armigera* (ABF51015.1), *Anopheles stephensi (*BAC82382.1*), Aedes aegypti P* (XP_001647756.1).

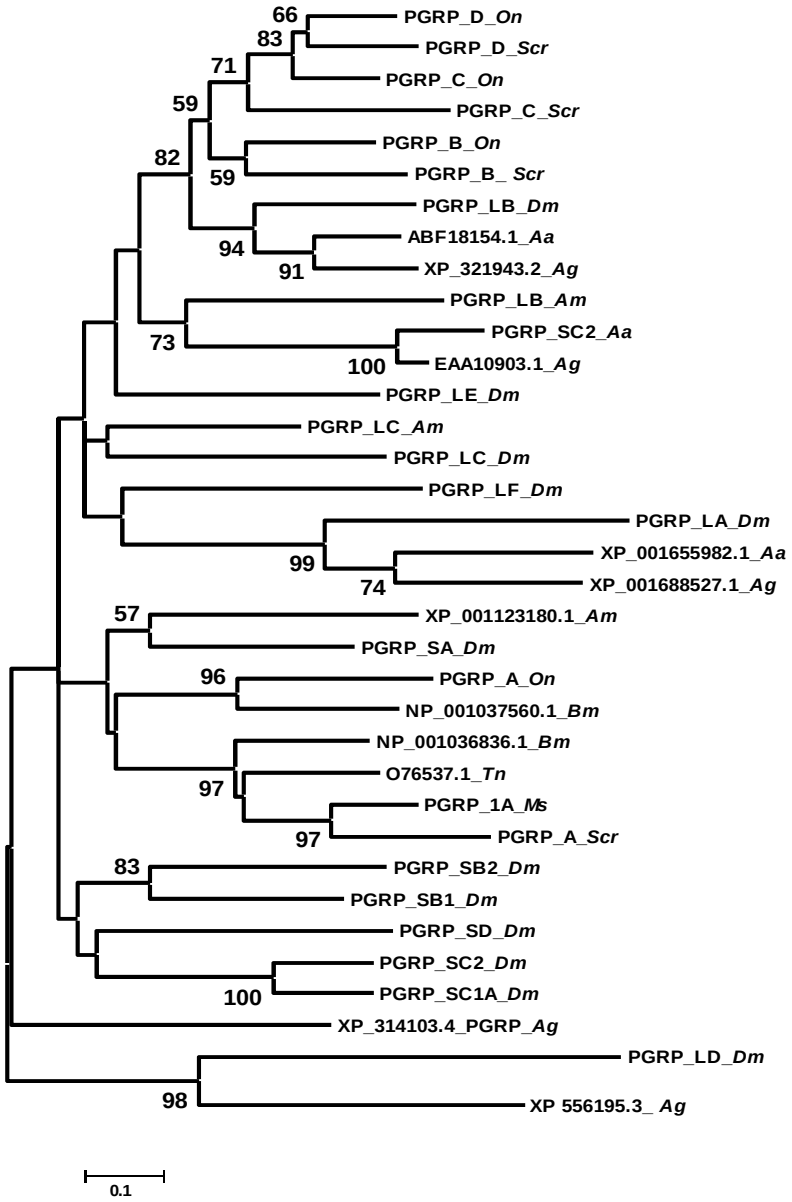

Figure 5.3 Neighbor-joining phylogenic tree constructed from 25 full length amino acid sequences of peptidoglycan recognition proteins (PGRPs) including a four sequences from *Ostrinia nubilalis.* Bootstrap percentage values are shown on the branches. GenBank accession numbers along with short names are shown for all the sequences. Sequences from the following insects were used in construction of the tree: *Bombyx mori (Bm); Helicoverpa armigera (Ha); Heliothis virescens (Hv); Manduca sexta (Ms); Trichoplusia ni (Tn); Samia cynthia ricini (Scr); Galleria mellonella (Gm); Hyphantria cunea (Hc); Aedes aegypti(Aa); Drosophila melanogaster (Dm); Anopheles gambiae (Ag); Apis mellifera* (Am).

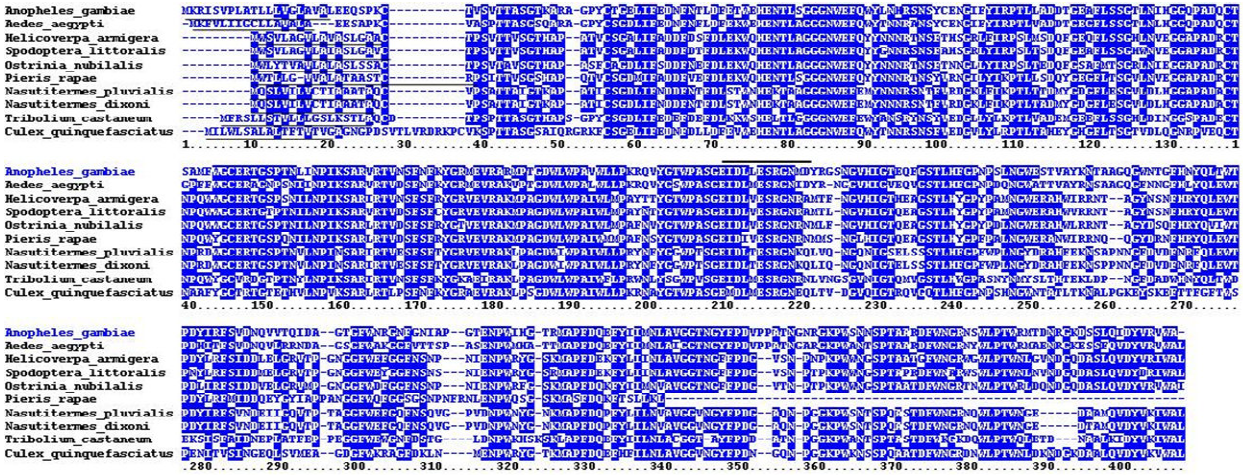

Figure 5.4 Multiple alignments of the amino acid sequences of *Ostrinia nubilalis* β-1-3 glucanse-1 gene with similar genes from other insects using CLUSTALW. The predicted signal peptide sequences for all the sequences are underline. The predicted active site for GH16 is indicated by the solid line above the alignment (Pauchet et al. 2009). Fully conserved residues are indicated by blue background. The sequences used in this analysis are: β-1-3 glucanse-1 from *Helicoverpa armigera* (GeneBank accsession no.ABU98621.1), *Spodoptera littoralis* (ACI32818.1), *Spodoptera frugiperda* (ABR28478.2), *Pieris rapae* (ACI32824.1), *Tribolium castaneum* (XP_970010.1); GNBP from *Nasutitermes pluvialis* (AAZ08500.1), *Nasutitermes dixoni* (AAZ08494.1), *Anopheles gambiae* (XP_312116.3), *Aedes aegypti* (XP_001659796.1); βGRP from *Culex quinquefasciatus* (XP_001845281.1).

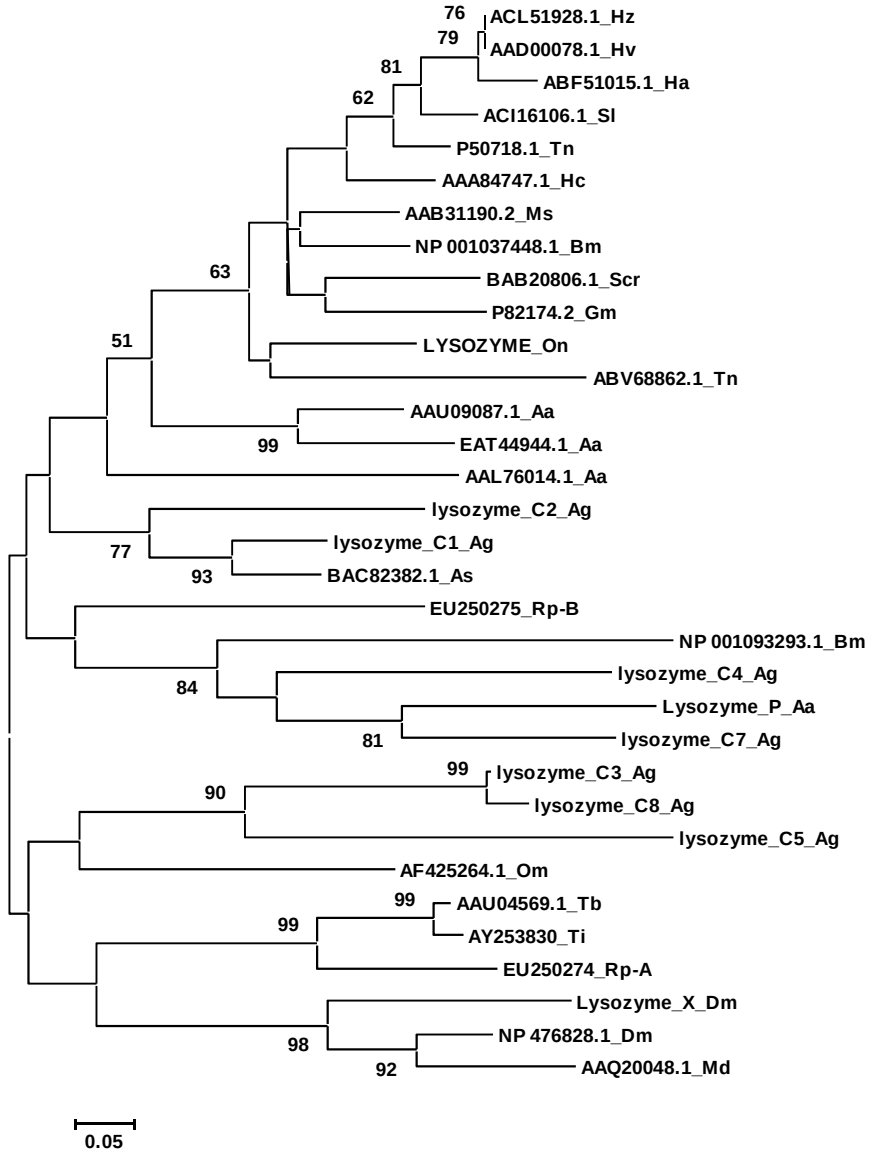

Figure 5.5 Neighbor-joining phylogenic tree constructed from 33 full length lysozyme sequences including a sequence from *Ostrinia nubilalis* (Lysozyme_*On*). Bootstrap percentage values are shown on the branches. GenBank accession numbers along with abbreviations for organism name are shown for all the sequences. Sequences from the following insects were used in construction of the tree: *Bombyx mori (Bm); Helicoverpa armigera (Ha); Spodoptera litura (Sl); Heliothis virescens (Hv); Manduca sexta (Ms); Trichoplusia ni (Tn); Samia cynthia ricini (Scr); Galleria mellonella (Gm); Hyphantria cunea (Hc); Aedes aegypti(Aa); Drosophila melanogaster (Dm); Anopheles gambiae (Ag); Musca Domestica (Md); Anopheles stephensi (As); Ornithodoros moubata (Om); Triatoma brasiliensis (Tb); Rhodnius prolixus (Rp); Triatoma infestans (Ti).*

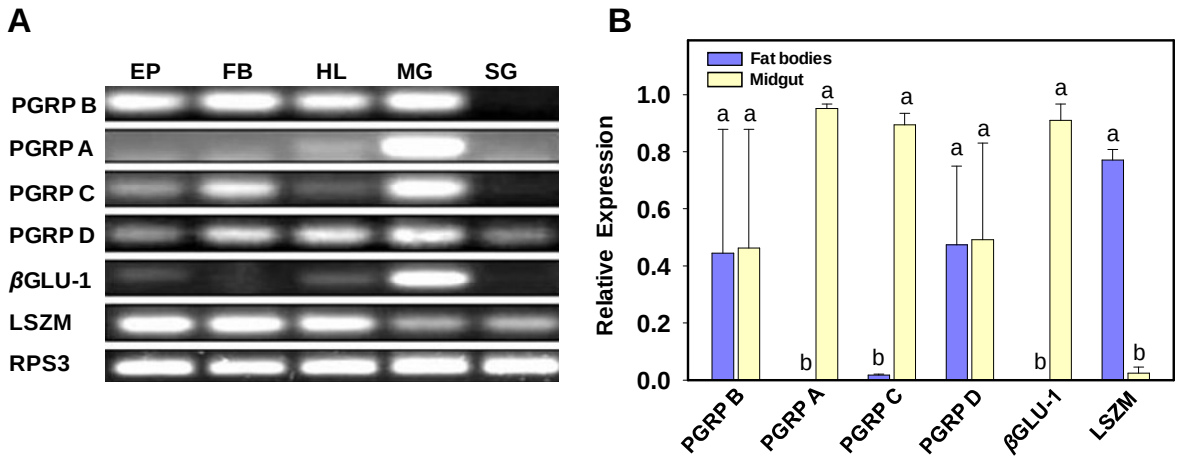

Figure 5.6 Expression patterns of six antibacterial response genes in the larval tissues of naive European corn borer. (A) Gene expression were determined in epidermis (EP), fatbodies (FB), haemolymph (HM), midgut (MG), salivary glands (SG) by RT PCR. The ribosomal S3 (*RPS3*) gene was used as a reference gene. (B) Gene expression determined by using realtime PCR in fatbodies and midgut. Ribosomal protein S3 (*RPS3*) gene was used as a reference gene to calculate the relative expression. Standard errors of the mean were determined from two biological replications and two technical replications. Different letters for the same gene represent significant difference at P value ≤ 0.05.

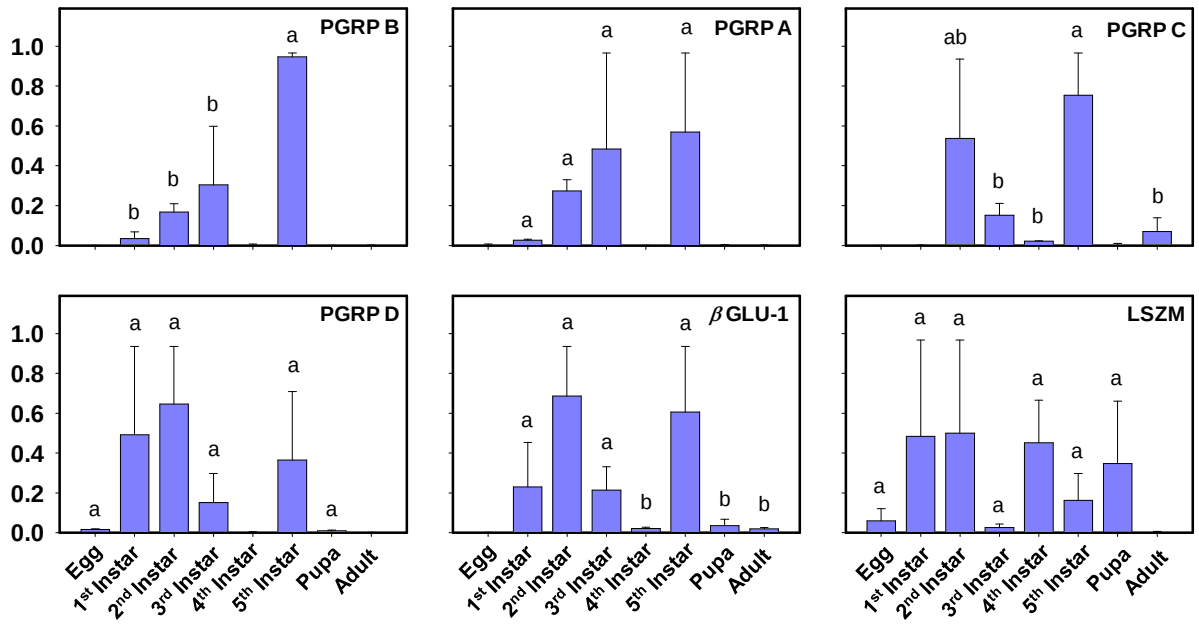

Figure 5.7 Expression pattern of of six antibacterial response genes in different developmental stages of naive European corn borer. Gene expressions were studied in all developmental stages including egg, first-, second-, third-, fourth-, and fifth-instar larvae, pupa, and adult. Ribosomal protein S3 (*RPS3*) gene was used as a reference gene to calculate the relative expression. Standard errors of the means were determined from two biological replications and two technical replications. Different letters for the same gene represent significant difference at P value ≤ 0.05.

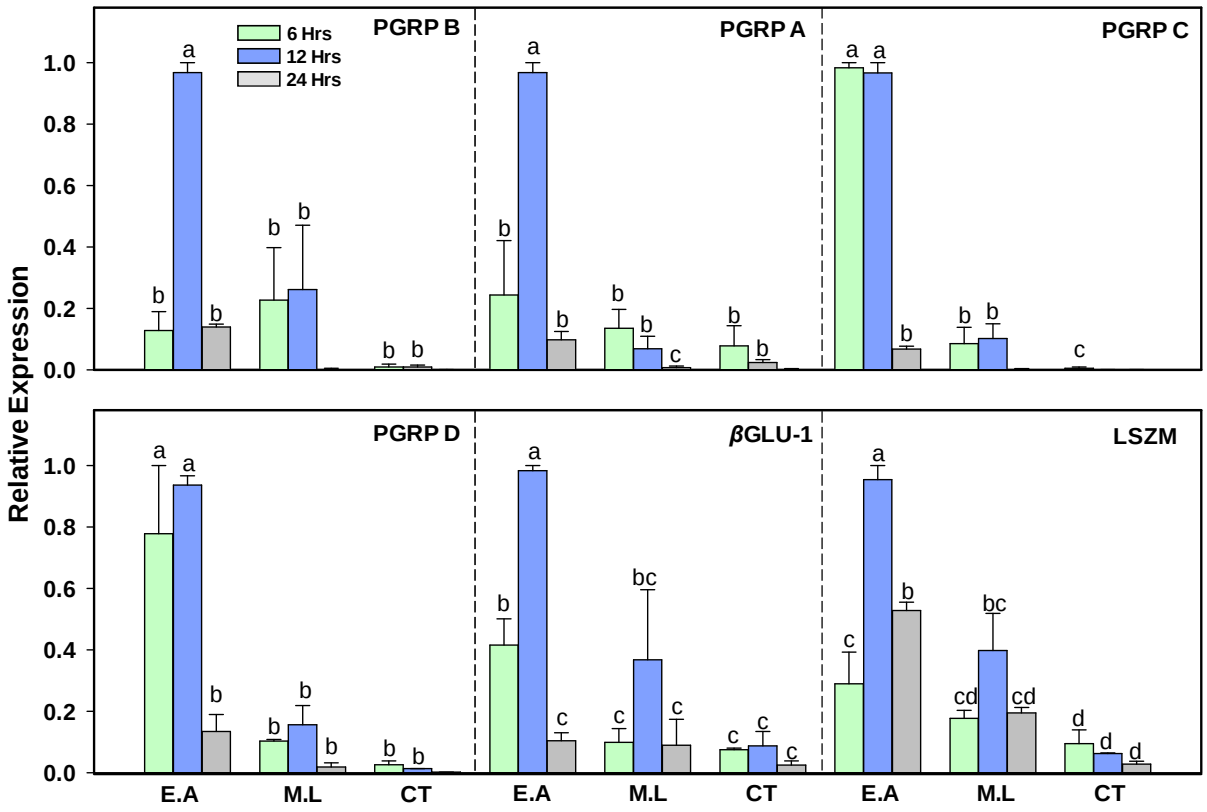

Figure 5.8 Expression profiles of four PGRPs, one βglu-1, and one lysozyme genes in fatbodies of fifth-instar larvae of European corn borer when exposed to Gram negative bacteria, *Enterobacter aerogenes* (E.A); Gram positive bacteria, *Micrococcus luteus* (M.L); and phosphate buffer saline (PBS) alone as control (CT). Relative gene expressions were determined after 6, 12, and 24 hrs of bacteria exposure. Ribosomal protein S3 (*RPS3*) gene was used as a reference gene to calculate the relative expression. Standard errors of the mean were determined from two biological replications and two technical replications. Different letters for represent significant difference at P value ≤ 0.05.

Table 5.1 Sequences of PCR primers used to profile gene expressions of six antibacterial response genes in European corn borer

Name	Forward primer	Reverse primer	Product size (bp)
PGRP B	**TTGCCGTCGGAAACTCAAC**	**AGTGTAGTCGGGCCTGATG**	**84**
PGRP A	**GTGAGTCTACTGAACAATCTACG**	**GATGGACCGCTGGTTGTAG**	**162**
PGRP C	**AAGACCTGCTAGACCTGAAAG**	**ATAATCATCATAAGTTGCATTCCC**	**77**
PGRP D	**ACATACAACTTTCCTTTCGTGAC**	**GTACTGGAGTGTGTAGAGGTAAG**	**82**
Lysozyme	**AAGATTAGCAGTCGTTGTGTTG**	**GCACTCTCATTCTCCACCAG**	**156**
***β*-1-3 glucanse-1**	**CCAGTGGTCGTCTGAACATC**	**AGGAGTCTACGGTGCGAAG**	**141**

CHAPTER 6 - Summary

In the recent years, a large amount of genomic information has been generated from various insect species and accumulated in public databases. However, such information is still limited on lepidopteran species, particularly agricultural pest species. There is an urgent need for genomic information on lepidopteran species due to their economic importance and biodiversity. The success of Bt (*Bacillus thuringiensis*) toxins in managing the insect pests has encouraged scientists to better understand molecular composition of insect gut and to identify new targets for novel toxins that can be used in insect pest management. My dissertation addresses the questions on the genomics of the larval gut of the European corn borer (ECB, *Ostrinia nubilalis*). The ECB is one of the major pests of corn in United States and western world. Knowledge of genes expressed in the ECB gut will lead to a better understanding of basic physiology of food digestion and their interactions with Bt toxins and pathogens. It may also lead to the discovery of new targets for which novel toxins can be designed for ECB pest management.

In Chapter 2, we established a large database of 15,000 ESTs from the gut of the fifth-instar larvae of ECB, which represents 2,895 unique sequences, including 1,738 singletons and 1,157 contigs. Analysis of unique sequences using BLASTx search revealed that 62.7% of them have significant matchs (E-value $\leq 10^{-3}$) with the sequences available in GenBank. To our knowledge, this database represents the largest gut-specific EST database from a lepidopteran pest and it will provide crucial information on the physiology of the larval gut of the ECB. In depth analysis of these ESTs revealed 52 candidate genes with potential roles in Bt toxicity and in Bt resistance. Furthermore, we showed differential expressions of 15 out of the 41 representative candidate genes between Cry1Ab-resistant and –susceptible strains of ECB. These results help us further narrow down the list of candidate genes that could be involved in Cry1Ab

resistance. These results will provide researchers with new insights into mechanisms of Bt resistance in ECB.

We have also used all the unique sequences identified in this study to develop ECB gut-specific microarray. This microarray can be used to analyze changes or differences in gene expression on a global basis between Cry1Ab-resistant and –susceptible strains of ECB as well in response to Bt protoxins/toxins. This will also allow us to analyze genetic differences that occur between Bt resistant and -susceptible strains of ECB. Our genomic information from the ECB could also serve as a valuable resource for identifying critical/vulnerable genes from the gut of ECB that would be useful physiological targets for new toxins that could be developed for use in pest management.

In Chapter 3, we analyzed aminopeptidase-like genes in Cry1Ab-resistant and –susceptible ECB strains and explored their role in Cry1Ab toxicity and resistance. It is well known that ECB can be effectively managed by using transgenic Bt corn. However, widespread use of transgenic Bt corn is expected to lead to the development of Bt resistance in the ECB. Indeed, several laboratory colonies of ECB have already developed resistance when exposed to Bt toxins. The mode of action of Bt toxin involves several steps and insects can develop resistance by changing the genes or their products at any of these steps. In ECB, two independent resistance mechanisms have been reported to occur: reduced protease level and modified cadherin receptors. However, studies on Cry1Ab ECB resistant strains have led to suggestions that resistance due to changes in the midgut-specific Bt toxin receptors may also involve other receptors in addition to the cadherin. The Bravo model of Bt toxicity in insects suggests that Cry toxins need two receptors to kill the insects, cadherin and GPI-anchored aminopeptidase N. Therefore, we identified and analyzed the aminopeptidase-like genes in Cry1Ab resistant and

susceptible ECB strains. The expression analysis for 10 aminopeptidase-like genes revealed that most of these genes expressed predominantly in the midgut tissues of all larval stages of ECB. There were no differences in the expression of these genes between Cry1Ab resistant and susceptible strains. This suggests that altered expression of these genes is unlikely to be responsible for resistance. However, there remains a possibility that there could be mutations in the nucleotide sequences of these genes that differed for resistant and susceptible larvae so this needed further investigation. Interestingly, we found several nucleotide differences in the region from 912 to 930 bp of the aminopeptidase-P like (*OnAPP*) gene. The change in the nucleotide sequence was similar for the two resistant strains we were studying: the changes lead to changes in two amino acids, Glu^{305} was changed to Lys^{305} and Arg^{307} was changes to Leu^{307}. We have not found any reports where *OnAPP*-like genes were implicated in Bt toxicity or resistance. This appears to be first report of an APP-like gene from insects with a predicted GPI-anchor signal peptide at the C-terminal being identified as being associated with Bt toxicity and Bt resistance. To gain better understanding of the role of *OnAPP*, we developed a feeding-based RNA interference for *OnAPP* for ECB larvae and achieved 38% reduction in the *OnAPP* transcript after 8 days. Furthermore, Bt bioassay using insects fed *OnAPP* and GFP dsRNA resulted in reduced susceptibility to Cry1Ab by 25% in *OnAPP* dsRNA fed larvae as compare to the control. Therefore, presence of the mutations in resistant larvae, presence of the GPI anchor, and reduced susceptibility of *OnAPP* dsRNA treated larvae, stronlgy suggest that this gene is a strong candidate for its role in the Cry1Ab toxicity and resistance in ECB.

In Chapter 4, we described a study on the functional analysis of a gut-specific chitinase-like gene from ECB larvae and showed that this gene was involved in chitin regulation in its peritrophic membrane (PM). Chitinases are large and diverse enzymes and have received much

attention from researchers in recent years due to their important biochemical functions in chitin metabolism. Chitinases are suggested to be involved in the degradation of chitin in the peritrophic membrane (PM) and cuticle. They are also potential targets for novel insect-specific pesticides for use in insect pest management. Therefore, we identified and analyzed the gut-specific chitinase-like gene (*OnCht*) in ECB. The sequence analysis of *OnCht* showed that this gene have all four conserved regions with one catalytic domain and no chitin binding domain. The expression of *OnCht* was only detected during larval feeding stages, and more than 97% of *OnCht* expression was found in the midgut. Within the midgut, *OnCht* has highest expression level in the anterior midgut (75.8 %) which suggests that *OnCht* may play an important role in regulating PM chitin content and assembly in the anterior part of the midgut. Furthermore, our results showed that transcript levels of *OnCht* and chitin synthase B (*OnCHS-B*) were affected by feeding. When larvae were not provided food, the *OnCht* gene expression decreased significantly and *OnCHS-B* expression increased significantly relative to larvae maintained on food. These changes occur rapidly and reversibly. Interestingly, we also found a negative correlation between *OnCht* gene expression and chitin content as well as a positive correlation between *OnCHS-B* gene expression and chitin content of the PM. Chitin contents increased significantly when larvae were maintained with no food as compared with the larvae maintained on food for the same period of time. As expected, the chitin contents decreased dramatically, when the starved larvae were allowed to feed. By using a feeding-based RNAi technique, we were able to reduce the *OnCht* transcript levels by 63-64% in the larvae fed a diet containing *OnCht* dsRNA as compared with the larvae fed a diet containing GFP dsRNA. Such a suppression of the *OnCht* transcript level in larvae fed *OnCht* dsRNA resulted in a significant increase of chitin content (26%) in the PM. This suggests that *OnCht* was involved in the regulation of the PM chitin in

ECB larvae, probably through a reduced rate of degradation of the chitin by this enzyme. More interestingly, the growth and development of these larvae were were reduced by 54% compared with larvae fed GFP dsRNA. This was most likely due to defective food assimilation. The decreased porosity of the PM and/or loss of compartmentalization may hinder the digestion. Thus, these results provided strong evidence for the first time that *OnCht* plays an essential role in regulating chitin content of the PM and that this affects larval growth, presumably by influencing food digestion, nutrient absorption or movement of digestive enzymes through the PM.

In Chapter 5, we identified and characterized six antibacterial response genes from the ECB larvae, including four peptidoglycan recognition proteins (PGRPs), one β-1-3 glucanse-1 (*β*glu-1), and one lysozyme. Tissue-specific analysis showed that all of the ECB antibacterial response genes except lysozyme have high mRNA levels in the gut tissues. This may be because gut is constantly being exposed to the microorganisms while feeding. All these genes showed expression during the ECB larval stage. None or low mRNA expression for these genes was detected in egg, pupa and adult. To obtain better evidence that these genes are really involved in the immune defense response, we challenged ECB larvae with Gram-positive bacteria (*Enterobacter aerogenes*) and –negative bacteria (*Micrococcus luteus*). The expression of all six antibacterial response genes in fatbodies was up-regulated when ECB larvae were challenged with Gram-positive bacteria (*E. aerogenes*), however only PGRP-C and lysozymes were induced when challended with gram-negative bacteria (*M. luteus*). This difference in response may be due to specificity of the peptidoglycan (PGN), as Gram-negative bacteria have DAP-type PGN and most Gram-positive bacteria have Lys-type PGN. This is the first study to characterize

antibacterial response genes in the ECB larvae, which may lead to better understanding of the immune defense response in ECB.

These findings have several significant implications. In addition to these results, the establishment of the feeding-based RNA interference technique could potentially help us in delivering dsRNA orally to the ECB larvae for high throughput screening of effective genes to be targeted for insect pest management.