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Molecular Mechanisms of *Bacillus thuringiensis* Resistance in the Sugarcane Borer

Yunlong Yang

Louisiana State University and Agricultural and Mechanical College, yangyunlong521@yahoo.com.cn

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**MOLECULAR MECHANISMS OF *BACILLUS THURINGIENSIS*
RESISTANCE IN THE SUGARCANE BORER**

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Entomology

by
Yunlong Yang
B.S., Huazhong Agricultural University, 2004
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ABSTRACT

The sugarcane borer, *Diatraea saccharalis*, is a major target pest of transgenic corn expressing *Bacillus thuringiensis* (Bt) proteins in many areas of the U.S. mid-southern region. A Cry1Ab-resistant (Cry1Ab-RR) strain of *D. saccharalis* has been developed from a single two-parent family-line. To examine the molecular mechanisms of the Cry1Ab resistance in this insect strain, cDNAs of five types of potential candidate genes related to Bt resistance were sequenced using reverse transcriptase polymerase chain reaction (RT-PCR) and 5' rapid amplification of cDNA end (5' RACE). The Bt resistance candidate genes examined included those encoding three trypsins (DsTRYs), three chymotrypsins (DsCHYs), three aminopeptidases N (DsAPNs), one cadherin (DsCAD1), and three alkaline phosphatases (DsALPs). cDNA sequence of each gene and its expression levels were compared between a Cry1Ab-susceptible strain (Cry1Ab-SS) and the Cry1Ab-RR at different larval growth stages. The cDNA sequences of these genes were identical between Cry1Ab-SS and -RR strains. Gene expression levels of the trypsins, chymotrypsins, and alkaline phosphatases were similar between the two strains. There were also no significant differences in total enzymatic activity of trypsins, chymotrypsins, and alkaline phosphatases between Cry1Ab-SS and -RR. However, the gene expression levels of the three DsAPNs and the DsCAD1 in Cry1Ab-RR were significantly lower than those of the Cry1Ab-SS. RNA interference (RNAi) was employed to knock-down the three DsAPNs and the DsCAD1 in the Cry1Ab-SS strain by oral droplet feeding to neonates. Down-regulation of expression of these four genes by RNAi was correlated with the decrease in susceptibility to Cry1Ab toxin. Silencing each of the three DsAPNs in *D. saccharalis in vivo* by RNAi resulted in a decrease of total APN activity. In addition, the total specific APN activities from Cry1Ab-RR larvae were significantly lower than those of the Cry1Ab-SS strain. These results suggest that reduction in

expression of the three DsAPNs and DsCAD1 is functionally associated with the Cry1Ab resistance in *D. saccharalis*.

CHAPTER 1

INTRODUCTION

1.1 *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) is an aerobic, gram-positive bacterium that was initially isolated in Japan by Ishiwata and formally described by Berliner in 1915 (Schnepf et al., 1998). It produces different kinds of crystal inclusions during sporulation. These crystal inclusions are composed of one or various crystal (Cry) and cytolitic (Cyt) toxins which are also called δ -endotoxins or insecticidal crystal proteins. Both Cry and Cyt proteins are parasporal inclusion proteins from Bt, except that Cyt proteins exhibit hemolytic activity (Schnepf et al., 1998). Some of these proteins are highly toxic to certain insects, but they are harmless to most other organisms including vertebrates and beneficial insects (Gould, 1998; Christou et al., 2006). Cry proteins have been used as bio-insecticides for many years (Schnepf et al., 1998). To date, more than 560 Cry protein have been sequenced and are classified into at least 68 groups (Cry1-Cry68) according to their amino acid sequence similarities (Crickmore et al., 2010). In addition, 35 Cyt genes from 3 groups (Cyt1-Cyt3) have also been sequenced and identified (Crickmore et al., 2010). In addition to the Cry and Cyt proteins, Bt also produces vegetative insecticidal proteins (Vips) during its vegetative growth stages. To date, at least 90 Vip genes have been sequenced (Yu et al., 1997; Crickmore et al., 2010). Both Cry and Vip genes have been successfully bioengineered into crop plants for insect pest control (Christou et al., 2006).

1.2 Mode of Action

The mode of action of Bt toxins to kill insects is still not fully understood. Two different hypotheses have been proposed for the mode of action: one relies on osmotic lysis by pore formation (Bravo et al., 2004), and the other relates to a signaling cascade that promotes cell

death (Zhang et al., 2006). These two models might not exclude each other (Jurat-Fuentes and Adang, 2006). Most published studies support the pore-formation model. This model involves ingestion of the crystalline inclusions by insect larvae and solubilization of the inclusions to release Cry protoxins in insect guts. The Cry protoxins are then cleaved by insect midgut proteinases (e.g., trypsins, chymotrypsins) to form active Cry toxins. The activated Cry protein then binds to specific receptors at the midgut epithelium (Zhuang et al., 2002; Bravo et al., 2007). It is proposed that the cadherin, aminopeptidase N (APN), and alkaline phosphatase (ALP) proteins are involved in Bt toxin binding by interacting sequentially with different toxin structures (Pigott and Ellar, 2007). The monomeric toxin first binds to primary receptors (e.g., cadherin-like proteins) to induce further proteolytic processing and oligomerization of the protein (Gómez et al., 2002). The oligomeric protein then binds to secondary receptors (e.g., APNs, ALPs, ATP-binding cassette (ABC) transporter proteins) that drive the proteins into the membrane microdomains to cause pore formation (Bravo et al., 2004; Gahan et al., 2010). The formation of the insertion pores results in osmotic imbalance of the membrane epithelium leading to swelling of the intestine cells and ultimately the death of the insect (Knowles and Dow, 1993). Detailed mechanisms of the pore formation in the membrane and death after pore formation are still unclear.

1.3 Transgenic Bt Plants

Bt is a valuable source of insecticidal proteins for use in conventional sprayable formulations as one of the most promising alternative to synthetic insecticides (Nester et al., 2002). Compared to traditional chemical insecticides, Bt microbial insecticides exhibit many advantages. Bt microbial sprays are highly effective to the target pests and very safe to most non-target organisms in the environment (e.g., natural enemies of insects, pollinators, fish, mammals)

(Gill et al., 1992). However, there are some limitations for using Bt as a microbial insecticide. These limitations include environmental inactivation of the proteins under unfavorable weather conditions and poor coverage (Nester et al., 2002). For example, control efficacy of foliar sprays of Bt insecticides is usually low against pests hiding inside plant parts such as stalk borers (Perlak et al., 1998; Nester et al., 2002).

Bt Cry genes were first engineered into tobacco plants for insect pest control in 1987 (Vaeck et al., 1987). Since then, Bt genes have been transferred into many plant species including two major economic crops, corn, *Zea mays* L. (Koziel et al., 1993) and cotton, *Gossypium* spp. (Flint et al., 1995). The first generation of transgenic Bt corn and cotton hybrids (e.g., YieldGard[®] corn and Bollgard[®] cotton) expressed only a single Cry protein and thus had a relatively narrow target spectrum. Lepidopteran species are the primary insects targeted by the first generation Bt plants (Perlak et al., 1991; Koziel et al., 1993; US EPA, 2002; 2004; 2007a). Later Bt corn expressing Cry3Bb1 or Cry34/35Ab1 were developed for managing below-ground corn rootworms including the northern corn rootworm, *Diabrotica barberi*, western corn rootworm, *Diabrotica virgifera virgifera*, and Mexican corn rootworm, *Diabrotica virgifera zea* (US EPA, 2005a; 2005b). Recently, pyramided Bt plants (i.e., 2nd generation of Bt crops) that express two or more Bt proteins for a target pest have become commercially available. For example, Bollgard II[®] cotton, which contains pyramided genes of Cry1Ac and Cry2Ab, and YieldGard VT Pro[™] corn that expresses Cry1A.105 and Cry2Ab2 have been used to control lepidopteran pests (Monsanto, 2007). Other common pyramided/stacked Bt crop technologies include VT Triple Pro[™] and SmartStax[™] corn (Monsanto, 2010).

Since first commercialized in 1996, transgenic Bt crops (e.g., corn and cotton) have been adopted rapidly worldwide. In 2010, ~58.6 mha of Bt crops were planted in 25 countries

worldwide (James, 2010). This included 39.0 mha of Bt corn planted in 16 countries and 19.6 mha of Bt cotton planted in 13 countries. In 2010, the United States planted 22.4 mha of Bt corn, which accounted for 63% of its total corn area, and 3.2 mha of Bt cotton, which accounted for 73% of its total cotton area (NASS, 2010). Most Bt crop growers have recognized great economic and environmental benefits offered by Bt crops (James, 2010). For example, in the US, Illinois, Iowa, Minnesota, Nebraska, and Wisconsin of the United States, the economic benefits of Bt corn for area-wide suppression of the European corn borer, *Ostrinia nubilalis*, were estimated to be \$ 6.9 billion during the 14-year period from 1996 to 2009 (Hutchison et al., 2010).

1.4 Sugarcane Borer and Its Damage on Corn

Sugarcane borer, *Diatraea saccharalis*, was first introduced into Louisiana in 1855, and then spread to the other warm Gulf Coast regions. *D. saccharalis* also occurs throughout the Caribbean, Central America, and the warmer portions of South America (Reagan, 2001; Capinera, 2001). Although it is a key pest of sugarcane, *Saccharum officinarum*, *D. saccharalis* has a wide range of hosts. It also infests many other species in the family Gramineae such as corn; rice, *Oryza sativa/Oryza glaberrima*; and sorghum, *Sorghum* spp. (Reagan, 2001; Capinera, 2001; Braga et al., 2003). Recently, *D. saccharalis* has expanded its geographic range and become the dominant corn stalk borer pest in many areas of the U.S. mid-southern region, especially in Louisiana and the Gulf Coastal area of Texas (Castro et al., 2004; Huang and Leonard, 2008). Larval *D. saccharalis* usually injure corn in two ways. Early in the season they feed on the young developing tissue of the whorl. Light damage may cause series of holes across the leaf blade, while extensive damage can kill the growing point of the plant and make plant growth stunted. Later in the season the larvae descend to and burrow in the corn stalk. Large larvae

tunnel through the stalk, causing the plant to be prone to breakage. Second generation larvae can also burrow into corn ears (Rodriguez-del-Bosque et al., 1990).

1.5 Bt Resistance

Widespread use of Bt toxins and planting of Bt crops place strong selection pressure on the pest populations, which could result in resistance development (Tabashnik, 1994; Gould, 1998; Ferré and Van Rie, 2002; Bravo and Soberón, 2008). Laboratory-selected resistance to Bt toxins has been found in many insects (Ferré and Van Rie, 2002; Bravo and Soberón, 2008), such as Indianmeal moth, *Plodia interpunctella* (McGaughey, 1985), tobacco budworm, *Heliothis virescens* (Gould et al., 1992), diamondback moth, *Plutella xylostella* (Tabashnik, 1994), beet armyworm, *Spodoptera exigua* (Moar et al., 1995), European corn borer, *Ostrinia nubilalis* (Huang et al., 1999; Siqueira et al., 2004), pink bollworm, *Pectinophora gossypiella* (Tabashnik et al., 2004) and *Helicoverpa armigera* (Akhrst et al., 2003; Gunning et al., 2005; Xu et al., 2005). In field conditions, three lepidopteran insect pests have developed resistance to formulated Bt microbial insecticide sprays, which include *P. interpunctella* (McGaughey, 1985), *P. xylostella* (Tabashnik, 1994), and *Trichoplusia ni* (Janmaat and Myers, 2003). More importantly, field resistance to commercial Bt crops that lead to field control failures or reduced efficacy have been documented in three cases. The first case is the resistance of fall armyworm, *Spodoptera frugiperda*, to Cry1F corn in Puerto Rico (US EPA, 2007b). The second case is the resistance of an African stem borer, *Busseola fusca*, to Cry1Ab corn (e.g., YieldGard[®] corn) in South Africa (van Rensburg, 2007). The third case is the resistance of *P. gossypiella* to Cry1Ac cotton in India (Tabashnik and Carrière, 2010; Dhurua and Gujar, 2011).

1.6 Bt Resistance Management

To delay resistance, a mandatory “high dose/refuge” insecticide resistance management (IRM) strategy has been implemented for planting Bt crops in the United States and several other countries (US EPA-SAP, 1998; Bourguet et al., 2003). This strategy involves to plant “high dose” Bt plants that can kill $\geq 95\%$ heterozygotes for Bt resistance. The “high/dose refuge” strategy also requires Bt crop growers to plant a specified proportion of their crop to a non-Bt variety of the crop to serve as a refuge for hosting susceptible insects. Bt-susceptible insects should emerge from refuge areas and mate with the rare potentially resistant homozygous individuals that might emerge from the Bt crop so that most offspring will be heterozygous and thus be killed by the “high dose” Bt plants. Therefore resistance allele frequencies in field populations should remain low for long period of time. The 15 years of success of transgenic Bt crops in managing four major corn and cotton pests, *O. nubilalis*, *Diatraea grandiosella*, *H. virescens*, and *P. gossypiella* in North America without any signs of resistance is believed to be resulted from a successful implementation of the “high-dose/refuge” IRM strategy (Huang et al., unpublished).

In addition to the “high dose/refuge” IRM strategy, a gene-pyramiding strategy has been also used for Bt resistance management. This strategy relies on development of transgenic plants that express two or more dissimilar Bt proteins for targeting the same group of insect pests. Mathematical modeling indicates that pyramiding two or more Bt genes with different insecticidal mechanisms into one plant for controlling the same target species should delay resistance development and can be used as a useful supplement for Bt crop IRM (Roush, 1998; Zhao et al., 2003). For example, genes encoding for Cry1A.105 and Cry2Ab2 proteins have been transferred into corn plants for controlling above-ground lepidopteran corn pests (Monsanto,

2007). The resulting Bt event, MON 89034, has been incorporated into several Bt corn technologies including Genuity VT Triple Pro™ and SmartStax™ (Monsanto, 2010). Such pyramided Bt corn technologies have been commercially available since the 2010 planting season in the U.S. and Canada (Monsanto, 2010). A study from Ghimire et al. (2011) demonstrated that MON 89034 provided complete control of a Cry1Ab-resistant strain of *D. saccharalis*, which was highly resistant to Cry1Ab corn plants.

1.7 Mechanism of Bt Resistance

Knowledge of Bt resistance mechanism is essential in understanding Bt resistance evolution and developing effective IRM strategies for Bt crops. For this reason, Bt resistance mechanism has been discussed as one of the hottest topic in the agricultural science during the past 20 years. Several mechanisms of insect resistance to Bt toxins have been proposed (Gill et al., 1992; Ferré and Van Rie, 2002; Bravo and Soberón, 2008). These mechanisms include 1) reduction in Bt proteins binding of the midgut receptors; 2) reduction in activation of protoxins by midgut proteinases; 3) increase in detoxification of activated Bt toxins; and 4) increase in immune response (Ma et al., 2005; Rahman et al., 2007; Hernández-Martínez et al., 2010) or enhanced esterase sequestration (Gunning et al., 2005).

Mechanisms of Bt resistances in corn stalk borers have been poorly understood because of the lack of highly resistant strains to work on. Recently, a Cry1Ab-resistant strain of *D. saccharalis* has been isolated from a single two-parent family-line by screening 213 F₂ family-lines collected from non-Bt corn fields in northwest Louisiana during 2004 (Huang et al., 2007a). Several studies have been conducted to characterize this Bt resistance in *D. saccharalis*. The Cry1Ab-resistant strain of *D. saccharalis* was able to survive and complete entire larval development (neonatal to pupal stage) on commercial transgenic corn plants expressing the

Cry1Ab protein (Huang et al., 2007a; Wu et al., 2007). Laboratory bioassay confirmed that the survival of this *D. saccharalis* strain on commercial Bt corn plants was due to Cry1Ab resistance (Huang et al., 2007b). Meridic diet treated with purified trypsin-activated Cry1Ab toxin at 32 µg/g did not cause significant mortality to the resistant neonates. The Cry1Ab resistant strain of *D. saccharalis* also showed a significant resistance level to purified Cry1Ac and Cry1Aa toxins, and a low resistance level to Cry1A.105, but it was not resistant to the Cry2Ab2 protein (Wu et al., 2009). The availability of this highly Bt-resistant strain of *D. saccharalis* provides an excellent model for studying resistance mechanisms to Bt corn in stalk boring pests. Beside the resistance to Cry1Ab corn recently reported in *B. fusca* in South Africa (van Rensburg, 2007), this *D. saccharalis* resistant strain is the only highly resistant strain to Cry1Ab corn documented among all corn stalk boring pests worldwide. To elucidate the molecular mechanism(s) of the Cry1Ab resistance in *D. saccharalis*, five types of Bt resistance candidate genes were investigated in this study.

1.7.1 Trypsins and Chymotrypsins

Serine proteinases are common luminal midgut digestive enzymes of dietary proteins in many insect species including Lepidoptera (Terra and Ferreira, 1994). Among the insect serine proteinases, midgut trypsin and chymotrypsin have been most extensively studied due to their roles in hydrolyzing ingested proteins into peptides (Terra and Ferreira, 1994). In addition, these midgut proteinases are also involved in the solubilization and activation of Bt protoxins (Gill et al., 1992). Following ingestion by susceptible insects, serine proteinases in the midguts of lepidopteran species can activate Cry protoxins by cleaving highly basic residues like Arg and Lys (Milne and Kaplan, 1993; Martínez-Ramírez and Real, 1996).

Midgut proteinases may be involved in Bt resistance in two different ways: 1) reduction in activation of Bt protoxins to active toxins by reduced proteinase activity and 2) increase in detoxification of activated Bt toxins due to up-regulation of midgut proteinases. For example, a Bt resistance in a strain of *P. interpunctella* was found to be associated with a significant reduction in midgut trypsin and chymotrypsin activity (Oppert et al., 1996; 1997; Herrero et al., 2001; Candas et al., 2003). Zhu et al. (2000) also reported that expression of two trypsin genes in this Bt-resistant strain of *P. interpunctella* were reduced significantly compared to that of the susceptible larvae. Similarly, Dipel[®]-resistance in *O. nubilalis* was also found to be associated with reductions in midgut trypsin-like activity (Huang et al., 1999). Later, Li et al. (2004; 2005) further demonstrated that a significantly reduced mRNA level of a trypsin gene was related to the Dipel[®]-resistance in *O. nubilalis*. A study also showed that larvae of a Bt-resistant strain of *H. virescens* had a significantly lower trypsin activity compared to its susceptible counterparts, while another Bt resistant strain had completely lost the chymotrypsin activity (Karumbaiah et al., 2007).

In contrast, up-regulation of midgut proteinases, in some cases, has also been found to be associated with Bt resistance. For example, studies have shown that midgut extracts from a Bt-resistant strain of *H. virescens* degraded activated Cry1Ab toxin faster than those from a susceptible strain *in vitro* because of up-regulation of midgut proteinases (Forcada et al., 1996; 1999). Another early study also reported that enhanced serine proteinase activities could increase degradation of Cry1C toxin in *Spodoptera littoralis* (Keller et al., 1996). Shao et al. (1998) also observed that excessive degradation of Cry1A toxins by the midgut juice was responsible for a low sensitivity of *H. armigera* to these toxins. In addition, Bt resistance in Colorado potato beetle, *Leptinotarsa decemlineata*, was also associated with elevated midgut proteinase activity

(Loseva et al., 2002). Objective 1 of my project was designed to examine the enzymatic activity, cDNA sequences, and gene expression of midgut trypsins and chymotrypsins of Cry1Ab-susceptible and -resistant strains of *D. saccharalis*. Data generated from the objective 1 were used to determine if changes in midgut proteinases were associated with the Cry1Ab resistance in *D. saccharalis*.

1.7.2 Aminopeptidases N

APNs play a prominent role in proteolysis in insects, as shown by the significant reduction of azocasein proteolysis by adding a specific APN inhibitor (Ortego et al., 1996). The presence of multiple APNs in lepidopteran larvae has also been considered to be important for digestion of peptides with various N-terminal residues (Hua et al., 1998; Emmerling et al., 2001). To date, five classes of APN isoforms have been identified from lepidopteran species (Herrero et al., 2005; Pigott and Ellar, 2007).

In Lepidoptera, midgut APNs are proposed to be involved in the pathogenesis of Bt toxins as receptors of the toxins (Bravo et al., 2004). It has been documented that APN is a functional receptor to Cry1A in *Manduca sexta* (Gill and Ellar, 2002). Rajagopal et al. (2002) also found that silencing of APN of *S. litura* by RNA interference (RNAi) resulted in tolerance of larvae to Cry1C. In addition, the functional relevance of APNs to Cry toxins has been demonstrated in *S. litura* and *H. armigera* by gene silencing via RNAi (Rajagopal et al., 2003; Sivakumar et al., 2007). For dipteran species, glycosylphosphatidylinositol (GPI)-anchored APN has been identified as Bt binding proteins in *Anopheles quadrimaculatus* (Abdullah et al., 2006), *Anopheles gambiae* (Zhang et al., 2008), and *Aedes aegypti* (Chen et al., 2009a; Likitvivanavong et al., 2011).

To date, two cases of Bt resistance have been found to be associated with the changes of midgut APNs. A Cry1Ac resistance strain of *S. exigua* was related to lack of the mRNA transcript encoding a GPI-anchored APN1 (Herrero et al., 2005). Another study showed that a deletion mutation of the gene encoding APN1 in *H. armigera* was genetically linked to Cry1Ac susceptibility (Zhang et al., 2009). Objective 2 of this project examined the activity of total APN, cDNA sequences and gene expressions of three midgut APN genes of Cry1Ab-susceptible and -resistant strains of *D. saccharalis*. In addition, RNAi-mediated gene silencing was employed to assess functional linkage of the three APN genes with Cry1Ab susceptibility in *D. saccharalis*. The goal of this study was to determine if the Cry1Ab resistance in *D. saccharalis* was associated with any changes in gene structure/expression of APNs in the midgut.

1.7.3 Cadherins

Cadherins represent a large and diverse family of glycoproteins which exist in both invertebrates and vertebrates. They are essential for numbers of cellular processes, such as cell recognition/signaling/communication, maintenance of cell structure, morphogenesis, angiogenesis, and possibly even neurotransmission (Angst et al., 2001; Wheelock and Johnson, 2003). Insect cadherins that can bind to Cry toxins belong to a subset of the cadherin protein family. All insect cadherins have an ectodomain formed by 11-12 cadherin repeats (CR), a transmembrane (TM) domain and an intracellular domain (Bel and Escriche, 2006).

Cadherins are believed to be a primary binding receptor to Cry proteins in Lepidoptera, Diptera, and Coleoptera (Bravo et al., 2011). The binding to cadherins in the midgut has been proposed to induce a conformational change in Cry1A toxins that facilitates the formation of a pre-pore toxin oligomer and finally lead to osmotic imbalance of the insect gut (Gómez et al., 2002). Cadherin proteins from at least six lepidopteran species have been found to bind to Cry1A

toxins. These species include *M. sexta* (Keeton et al., 1997), *Bombyx mori* (Nagamatsu et al., 1998), *H. virescens* (Gahan et al., 2001), *P. gossypiella* (Morin et al., 2003), *H. armigera* (Xu et al., 2005), and *O. nubilalis* (Flannagan et al., 2005). In Diptera, cadherin proteins have been identified as Bt binding receptors in *A. aegypti* and *A. gambiae* (Hua et al., 2008; 2009; Chen et al., 2009b). For coleopteran species, cadherin proteins have been documented to function as Bt toxin binding receptors in *Tenebrio molitor* (Fabrick et al., 2009) and *D. virgifera virgifera* (Park et al., 2009).

A mutation resulting in premature stop codon in the cadherin gene was first shown to lead to resistance to Cry1A toxins in a laboratory-selected strain of *H. virescens* (Gahan et al., 2001). Later, changes in three cadherin alleles were found to be responsible for in a field-isolated Cry1Ac-resistance in *P. gossypiella* (Morin et al., 2003). Xu et al. (2005) also reported that a deletion of a cadherin gene was associated with a high level of Cry1Ac resistance in a laboratory-selected *H. armigera* strain. In addition, studies have shown that single amino acid mutations in the toxin-binding region of a cadherin gene in *H. virescens* affected its toxin binding ability to Cry1A toxins (Xie et al., 2005). However, it has not been conclusively shown that all mutations in cadherins associated with Bt resistance are occurred in the toxin-binding regions. For example, one amino acid substitution in a cadherin gene of *H. armigera* which was believed to cause the Cry1Ac resistance was located between the Cry1Ac-binding region and the TM domain (Zhao et al., 2010). It is possible that changes in cadherin proteins could alter secondary structures of the proteins and then affect other downstream post-toxin-binding steps (Zhao et al., 2010). Objective 3 of this project analyzed the cDNA sequences and expressions of a cadherin of Cry1Ab-susceptible and -resistant strains of *D. saccharalis*. Functional studies of the cadherin gene were also performed using the RNAi technology. The goal of this study was to

determine if the Cry1Ab resistance in *D. saccharalis* was associated with any changes in gene structure/expression of the cadherin gene in the midgut.

1.7.4 Alkaline Phosphatases

ALPs (EC 3.1.3.1) are mainly localized in microvilli of columnar cells and the midgut epithelium cells of insects. Insect ALPs have been proposed to function in active absorption of metabolites and transport processes as well as to participate in cell adhesion and differentiation in some cases (Eguchi, 1995). ALPs can be divided into two groups, soluble and membrane-bound ALPs (Eguchi, 1995; Itoh et al., 1991; 1999). Both ALPs are found in larval midgut epithelium cells. The soluble ALPs are localized only in the apical region of the midgut, while membrane-bound ALPs are particularly restricted in the brush border membrane of columnar cells from the middle and posterior midgut (Azuma and Eguchi, 1989).

In the pore formation model, membrane-bound ALPs have been identified as Cry1A secondary binding receptors in midguts of *M. sexta* (McNall and Adang, 2003) and *H. virescens* (Jurat-Fuentes and Adang, 2004; 2006). The critical role of midgut ALPs in Bt mode of action has also been documented in several dipteran and coleopteran species. For example, a GPI-anchored ALP has been identified from *A. aegypti* midgut, which is shown to interact with Cry11Aa and Cry4Ba toxins in the Bt-resistant mosquito (Fernández et al., 2006; Moonsom et al., 2007). Martins et al. (2010) also found that two ALP proteins (62 and 65 kDa) in the midgut of the cotton boll weevil, *Anthonomus grandis*, can bind to Cry1Ba6 toxin.

Studies have shown that Cry1Ac resistance in a strain of *H. virescens* was associated with reduction in gene expression levels of two membrane-bound ALPs in the midgut (Jurat-Fuentes and Adang, 2004; 2006; Jurat-Fuentes et al., 2011). Reduced levels of membrane-bound ALP homologues were also detected for a laboratory-selected Cry1Ac-resistant strain of *H. armigera*

and field-collected Cry1Fa-resistant strains of *S. frugiperda* (Jurat-Fuentes et al., 2011). In *M. sexta*, ALP seems to play a more important role in Cry1Ab toxicity than APN, since ALP is produced at higher levels at the first instars with lower levels of expression in the late larval stages (Arenas et al., 2010). The expression profile of ALP correlates with the sensitivity of *M. sexta* larvae to Cry1Ab because younger larvae are more sensitive to the toxin. Objective 4 of this project assessed the enzymatic activity of total ALP, and cDNA sequences and gene expressions of three midgut ALP genes of Cry1Ab-susceptible and -resistant strains of *D. saccharalis*. The goal of this study was to determine if the Cry1Ab resistance in *D. saccharalis* was associated with any changes in gene structure/expression of the three ALPs in the midgut.

1.8 Objectives

The specific objectives of this project include:

- I. Characterization and comparison of trypsins and chymotrypsins between Cry1Ab-susceptible and -resistant strains of sugarcane borer, *Diatraea saccharalis*;
- II. Molecular characterization and RNA interference of three midgut aminopeptidase N isozymes from *Bacillus thuringiensis*-susceptible and -resistant strains of sugarcane borer, *Diatraea saccharalis*;
- III. Identification and functional analysis of a cadherin gene from *Bacillus thuringiensis*-susceptible and -resistant strains of sugarcane borer, *Diatraea saccharalis*;
- IV. Molecular identification and functional analyses of three alkaline phosphatase genes from *Bacillus thuringiensis*-susceptible and -resistant strains of sugarcane borer, *Diatraea saccharalis*.

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CHAPTER 2

CHARACTERIZATION AND COMPARISON OF TRYPSINS AND CHYMOTRYPSINS BETWEEN CRY1AB-SUSCEPTIBLE AND -RESISTANT STRAINS OF SUGARCANE BORER, *DIATRAEA SACCHARALIS*

2.1 Introduction

Serine proteinases are common luminal midgut digestive enzymes of dietary proteins in many insect species including Lepidoptera (Terra and Ferreira, 1994). Among the insect serine proteinases, midgut trypsins and chymotrypsins have been most extensively studied due to their roles in hydrolyzing ingested proteins into peptides (Terra and Ferreira, 1994). In addition, these midgut proteinases are also involved in the solubilization and activation of Bt protoxins (Gill et al., 1992). Following ingestion by susceptible insects, serine proteinases in the midguts of lepidopteran species can activate Cry protoxins by cleaving highly basic residues like Arg and Lys (Milne and Kaplan, 1993; Martínez-Ramírez and Real, 1996; Li et al., 2004; 2005). After a sequential enzymatic digestion, ~600 amino acids residues from the C-terminus and the first 28 N-terminal residues are removed from the Cry protoxins and thus the protoxins become activated toxins (Lightwood et al., 2000). The midgut proteinases may also play a concurrent role in the hydrolytic degradation via cleavage of aromatic residues (Trp, Tyr, and Phe) and inactivate the toxic protein subsequently (Gill et al., 1992; Oppert, 1999; Miranda et al., 2001).

Alteration of midgut proteinases has been documented to be responsible for development of Bt resistance in some insect species. In some insects, Bt resistance can be caused by reduced proteinase activity, which could lead to decrease activation of Bt protoxins to real toxins (Oppert et al., 1996; 1997; Herrero et al., 2001; Huang et al., 1999, Li et al., 2004; 2005). In contrast, resistance can be also due to up-regulation of midgut proteinases, which results in increased toxin inactivation (Forcada et al., 1996; 1999).

Sugarcane borer, *Diatraea saccharalis*, is a major corn borer pest in the U.S. mid-southern region (Castro et al., 2004; Porter et al., 2005; Huang et al., 2009). Because of the recent expansion of *D. saccharalis* in this region, it has been listed as a target pest of *Bacillus thuringiensis* (Bt) corn in the U. S. since 2005 (US EPA, 2005a; 2005b). A Bt-resistant strain of *D. saccharalis* was isolated from a population in northeast Louisiana. This Bt resistant strain has exhibited >100-fold resistance to Cry1Ab protein compared to its susceptible counterpart (Huang et al., 2007b). Larvae of the resistant strain were capable of completing their entire development (neonates-pupae) on commercial corn hybrids expressing the Cry1Ab protein (Huang et al., 2007a). The objective of this study was to investigate the potential roles of midgut trypsin and chymotrypsin in the Cry1Ab-resistant strain of *D. saccharalis*.

2.2 Materials and Methods

2.2.1 Sources of Cry1Ab-Susceptible and -Resistant *D. saccharalis*

Two strains of *D. saccharalis* were used in this study. A Cry1Ab-susceptible (Cry1Ab-SS) strain was established using larvae collected from corn fields near Winnsboro in northeastern Louisiana during 2004. A Cry1Ab-resistant (Cry1Ab-RR) strain was generated from a single two-parent family-line collected from the same location as the Cry1Ab-SS insects (Huang et al., 2007a). The Cry1Ab-RR insects can complete larval development on Cry1Ab corn and showed >100-fold resistance to the purified Cry1Ab protein (Huang et al., 2007b). The Cry1Ab-RR strain had been backcrossed four times with those of Cry1Ab-SS and the F₂ generations of the backcrosses were reselected on Cry1Ab corn leaf tissue. The backcrossed and reselected Cry1Ab-RR strain was used in the current study.

2.2.2 Assaying Total Midgut Trypsin and Chymotrypsin Activity

Larval midguts of three instars (3rd, 4th, and 5th) were obtained by holding the thorax with one forceps and pulling the gut out from the cut-off terminal abdomen segments with another forceps as described in Zhu et al. (2003). A single midgut was homogenized in a centrifuge tube containing 100 μ l cold 0.1M Tris-HCl buffer (pH 8.0) with a motorized micro tissue grinder. The enzyme extracts were then centrifuged and the supernatant of each homogenate was transferred to an individual tube using a method similar to that described in Oppert et al. (1994). Supernatants of enzyme extracts were collected and protein concentration quantified using the Bradford method (Bradford, 1976) with BSA as the standard.

N- α -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and N-Succinyl-(Ala)₂-Pro-Phe-*p*-nitroanilide (SAAPFpNA) were used as substrates to measure total activities of trypsins and chymotrypsins, respectively. The universal buffer of pH 8.5 (Frugoni, 1957) was used in assaying both trypsins and chymotrypsins. BAPNA or SAAPFpNA solution was prepared by diluting 50 mg/ml stock in dimethyl sulfoxide (DMSO) with the buffer to obtain a final concentration of 0.5 mg/ml. Activity of trypsins or chymotrypsins was determined by addition of 45 μ l of diluted BAPNA or SAAPFpNA solution and measured using a spectrophotometer (ELx808iu; Bio-Tek Instruments, Winooski, VT) at a 15-second interval for 15 min. Absorbance was measured at 405 nm and 37 °C. Kinetic curves were generated using the KC4 program (Ver. 2.7, Bio-Tek), and the mean *V* (velocity) was calculated as the increase in milliabsorbance over 15 min. Three negative controls, 1) enzyme without substrate, 2) substrate without enzyme, and 3) buffer only, were included to monitor background readings in each assay. For each combination of insect strain, instar, and proteinase, activity was measured from eight reactions (replications) and two readings (sub-samples) for each reaction. Specific proteinase activities

were presented as means and standard errors of the mean (\pm SEM). Student's t-test was used to determine differences in proteinase activity between the Cry1Ab-SS and -RR strains at each instar.

2.2.3 Cloning Full-Length cDNAs of Midgut Trypsins and Chymotrypsins

Five total RNA samples of each insect strains were used for cloning the full-length cDNAs of midgut trypsin and chymotrypsin. Larval midguts of three instars (3rd and 5th) were obtained using the same procedure as described in section 2.2.2. Each total RNA sample was isolated from three midguts of 3rd instars using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. First strand cDNAs from both strains were synthesized from the total RNAs with an oligo-dT primer using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). cDNA libraries of the Cry1Ab-SS and -RR strains of *D. saccharalis* were constructed and sequenced by Dr. Yu Cheng Zhu (USDA-ARS, Stoneville, MS). Based on the results of Blastx-NR similarity search of GenBank nucleotide database by Dr. Yu Cheng Zhu, I sorted out 595 trypsin contigs and 102 chymotrypsin contigs. A total of 17 trypsin-like and 16 chymotrypsin-like partial cDNAs were assembled from these contigs. Based on a microarray analysis of expression of multiple genes from the cDNA libraries (Guo and Zhu, unpublished data), two potential candidate genes for Bt resistance were selected for each of the two proteinases. These genes were named as DsTRY1 and DsTRY2 for the trypsin-like genes, and DsCHY1 and DsCHY2 for the chymotrypsin-like cDNAs.

To obtain full length cDNAs of DsTRYs and DsCHYs, PCRs were conducted to amplify cDNA fragments with four forward specific primers (DsTRY1F0, DsTRY2F0, DsCHY1F0, and DsCHY2F0) (Table 2.1) and four reverse specific primers (DsTRY1R0, DsTRY2R0,

Table 2.1. Sequences of primers used in cDNA cloning and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for characterization of three trypsin and three chymotrypsin genes from Cry1Ab-susceptible and -resistant strains of *Diatraea saccharalis*.

Specific primer for PCR	DsTRY1F0	5'-GCGGACATGTGGGCATTTATAT-3'
	DsTRY1R0	5'-TGAGCTCAATGTGGGACTG-3'
	DsTRY2F0	5'-CCATCATGCGTGTATTAATTGCTC-3'
	DsTRY2R0	5'-GGCACATATGTATGTAGTGTTTTTACTG-3'
	DsCHY1F0	5'-CGTAGAAGACAATGATGGCGG-3'
	DsCHY1R0	5'-TCCATTCTACTCTTCTTTTTCAAGAGC-3'
	DsCHY2F0	5'-GGTGACTTCGTAACATGAAAACCA-3'
	DsCHY2R0	5'-GATAGGTCGTTTTAAAGATGCTGATGG-3'
Specific primer for qRT-PCR	rtDsTRY1F0	5'-GCTTCGTAGACAGATACAACCC-3'
	rtDsTRY1R0	5'-CGCCATAACTGCTATATCGCT-3'
	rtDsTRY2F0	5'-GACCATTATTACCAACAGAGCCG-3'
	rtDsTRY2R0	5'-GGGTTGTATTGTGGATGATTGATGA-3'
	rtDsCHY1F0	5'-CCGAGATACATAGAATTTCAAGGTGG-3'
	rtDsCHY1R0	5'-CGTTCAGAGATCATCAGTACG-3'
	rtDsCHY2F0	5'-TGTTGGTGAAGCAGTTATCGC-3'
	rtDsCHY2R0	5'-CAAATGTGACTCGGTTGAATGAAG-3'
	Ds18SF1	5'-CAAATGTCTGCCTTATCAACTTTC-3'
	Ds18SR1	5'-GCCTTCCTTGGATGTGGTAG-3'

DsCHY1R0, and DsCHY2R0) (Table 2.1). The specific forward and reverse primers were designed to flank the 3'- and 5'-ends of the entire coding region of cDNA fragments. To eliminate any potential error created by Taq DNA polymerases, Platinum High Fidelity Taq DNA polymerase (Invitrogen, Carlsbad, CA) was used to amplify DNAs from RT-cDNAs from each of the Cry1Ab-SS and -RR strains. PCR-amplified DNA fragments (801 to 947 bp) from each strain were purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA) and sequenced from both directions by using an automated sequencer (ABI 3730XL). Several potential nucleic acid substitutions were found in the sequences of DsTRY1 and DsCHY2 for both Cry1Ab-SS and -RR strains. To determine if these potential nucleic acid substitutions were consistent in the two insect strains and correlated to the Cry1Ab resistance, full-length cDNAs of the DsTRY1 and DsCHY2 were sequenced four more times with RNA samples extracted from

midguts of different larval samples. The PCR and RT-PCR procedures for the repeated sequencings were the same as described above.

2.2.4 Identification and Phylogenetic Analyses of Trypsin and Chymotrypsin Genes

Homology of trypsins and chymotrypsins among lepidopteran species was analyzed using the NCBI/BLAST database and the ClustalW (Thompson et al., 1994; gap weight = 8, gap length weight = 2). Presence of a signal peptide was determined with the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>). Data of deduced protein sequences were analyzed using the ExPASy proteomics tools of the ExPASy molecular biology server of Swiss Institute of Bioinformatics (<http://www.expasy.ch/tools/>). Localization of the active sites of trypsins and chymotrypsins was predicted at Myhits server (http://myhits.isb-sib.ch/cgi-bin/motif_scan) (Zhu et al., 2000a; 2000b).

The molecular evolutionary genetics analysis (MEGA) software (Ver. 4.1; Tamura et al., 2007) was used to draw bootstrap neighbour-joining phylogenetic trees (1000 replicates) derived from multiple sequence analysis (ClustalW). The ClustalW analysis was based on 50 trypsin and 35 chymotrypsin sequences from lepidopteran species that were available from the GenBank. Sequences that do not contain conserved sequences for trypsin or chymotrypsin active-site residues were not included in the ClustalW analysis. In addition, those trypsin and chymotrypsin sequences from GenBank that are not related to digestion were also excluded from the analysis.

2.2.5 Transcriptional Levels of the Selected Trypsin and Chymotrypsin Genes from Cry1Ab-SS and -RR Strains of *D. saccharalis*

Two quantitative RT-PCRs (qRT-PCRs) were conducted to compare the transcriptional levels of the selected DsTRYs and DsCHYs between the Cry1Ab-SS and -RR strains of *D. saccharalis* as described in Yang et al. (2010). Gene expression levels were examined for 3rd and

5th instars of each insect strains. For each treatment combination of insect strain and instar, three guts were pooled as one sample and total RNA was extracted from the pooled samples using Trizol reagent (Invitrogen, Carlsbad, CA). Total RNAs were treated with DNaseI (2 μ l of 1 mg/ml, Boehringer Mannheim GmbH, Germany) at 37 °C for 1 h and quantified via Nanodrop spectrophotometer. Total RNAs were then diluted to the equal concentration (10 ng/ μ l). After elimination of possible trace amounts of DNA contamination, total RNAs were added to the first round qRT-PCRs with the 18S rRNA specific primers Ds18SF1 and Ds18SR1 (Table 2.1). Specific primers for DsTRY1, DsTRY2, DsCHY1, and DsCHY2 (Table 2.1) (amplicon length =164, 156, 190, and 156 bp, respectively) were then designed and used in the second round qRT-PCR reactions. The qRT-PCRs were performed in a 25 μ l reaction volume using iScript One-Step RT-PCR Kit with SYBR green kit (Bio-Rad, Hercules, CA) in a thermal cycler PTC200 with attached Chrome4 detector (Bio-Rad, Hercules, CA). The qRT-PCR procedure was programmed and data output were collected through software OpticonMonitor3 (Bio-Rad, Hercules, CA). There were five replications for each combination of insect strain, instar, and gene. Gene expression levels were presented as means and standard errors of the mean (\pm SEM). Student's t-test was used to determine treatment differences between the two insect strains for each gene and instar.

2.3 Results

2.3.1 Specific Activities of Total Trypsin and Total Chymotrypsin of Cry1Ab-SS and -RR Strains of *D. saccharalis*

Specific activities of total trypsins and total chymotrypsins were not significantly different between the Cry1Ab-SS and -RR strains of *D. saccharalis* for all three instars tested ($P = 0.19, 0.84, \text{ and } 0.34$ for 3rd, 4th, and 5th instars, respectively) (Fig. 2.1A). There appeared to

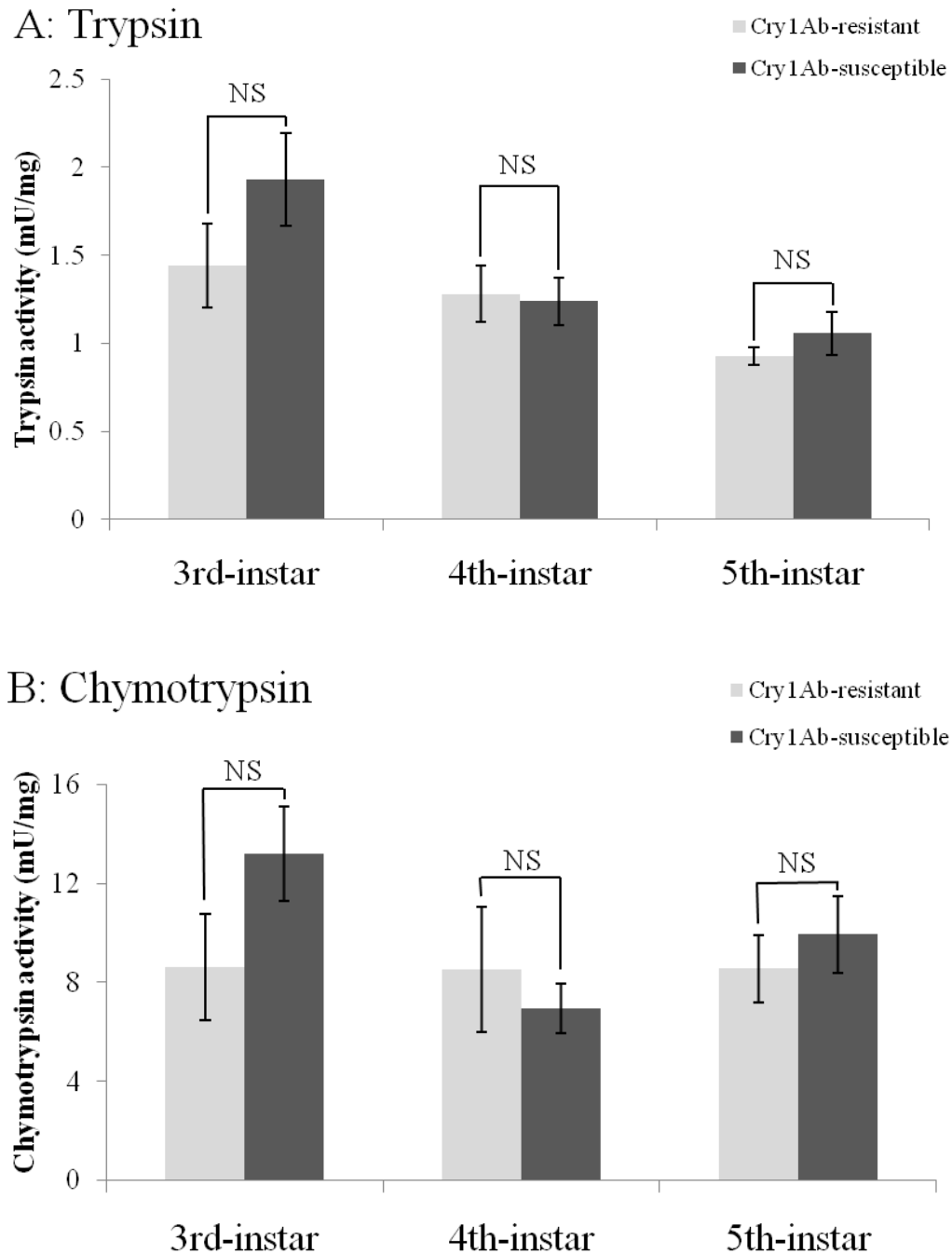


Figure 2.1. Total trypsin (A) and chymotrypsin (B) activities of whole gut tissue of different larval instars from Cry1Ab-susceptible and -resistant strains of *D. saccharalis*. Trypsin/chymotrypsin activities were tested by measuring the formation of chromophore *p*-nitroaniline at 405 nm. One unit of enzyme activity was defined by hydrolysis of 1 μ mol of substrate in 1 min. Bars represent the means and standard errors of eight gut samples from a total of eight different larvae. NS indicates no significant difference ($P > 0.05$, Student's *t*-test).

be a trend of decrease in specific trypsin activity as the midguts were collected from later instars for both strains. Similarly, the specific chymotrypsin activities were also not significantly different between the Cry1Ab-SS and -RR strains ($P = 0.13, 0.54,$ and 0.52 for 3rd, 4th, and 5th instars, respectively) (Fig. 2.1B). Unlike trypsin activities, the total chymotrypsin activities of Cry1Ab-SS and -RR strains were similar at different larval development stages.

2.3.2 Trypsin and Chymotrypsin cDNAs from Cry1Ab-SS and -RR Strains of *D. saccharalis*

Five single nucleotide polymorphisms (SNPs) from DsTRY1 ($A^{252} \rightarrow G^{252}, C^{349} \rightarrow T^{349}, C^{437} \rightarrow T^{437}, A^{725} \rightarrow G^{725},$ and $C^{744} \rightarrow T^{744}$) and two from DsCHY2 ($C^{330} \rightarrow T^{330}$ and $A^{343} \rightarrow G^{343}$) were found in the cDNAs of trypsin and chymotrypsin. Repeated cDNA cloning and sequencing of DsTRY1 and DsCHY2 from four additional insect samples showed that these SNPs existed among individuals within an insect strain for both Cry1Ab-SS and -RR. The result of the repeated sequencings suggested that these SNPs were not associated with Cry1Ab resistance. Because of the existing SNPs, for the subsequent sequence analysis and alignment, each of the DsTRY1 and DsCHY2 were redefined into two genes, respectively. The two genes for DsTRY1 were named as DsTRY1a and DsTRY1b and these for DsCHY2 were termed DsCHY2a and DsCHY2b. Besides these SNPs, there were no differences in the cDNA sequences of the three DsTRYs and the three DsCHYs between Cry1Ab-SS and -RR.

All cDNA sequences of the three trypsin and three chymotrypsin proteins identified from *D. saccharalis* were confirmed by homology search of GenBank via the National Center for Biotechnology Information using the Blastx protocol (Altschul et al., 1997). cDNAs of DsTRY1a and DsTRY1b coded 295 amino acid trypsin precursors with a similar molecular weight (Mw) of ~32 kDa with various isoelectric points (pIs), 9.08 for DsTRY1a and 8.93 for

DsTRY1b. DsTRY2 coded a 257 amino acid protein with Mw of 28 kDa and pI 8.74. All three deduced trypsin precursors contained a 17-residue signal peptide and three conserved trypsin active site residues, His^{69/75}, Asp^{114/125}, and Ser^{213/222}, which form the catalytic triad in serine proteinases (Kraut, 1977; Zhu et al., 2000a) (Fig. 2.2A). The three mature trypsins from *D. saccharalis* had a conserved IVGG N-terminus (Fig. 2.2A), 16-19 positively charged residues, and 12-13 negatively charged residues. Six cysteine residues for disulfide bridges were also included in the three trypsin precursors (Fig. 2.2A). Other important residues, including Asp^{207/216}, Gly^{230/240}, and Gly^{240/250}, which define the substrate binding pocket, were also conserved in the all trypsin-like proteins encoded by the three DsTRYs. Two and three S/T-xx-D/E sequence motifs for casein kinase (CK) II phosphorylation sites were predicted from mature trypsins deduced from DsTRY1(a/b) and DsTRY2, respectively.

Comparing to the three DsTRYs, DsCHY1 had a longer open reading frame (ORF) and encoded a 309 amino acid chymotrypsin precursor with 34 kDa Mw and 5.53 pI. DsCHY2a and DsCHY2b encoded a 288 amino acid protein with ~31 kDa Mw and pI of 7.08. The three chymotrypsin cDNAs from *D. saccharalis* were predicted to consist of a 17-residue signal peptide and three chymotrypsin active site residues (Fig. 2.2B). Mature chymotrypsin DsCHY1 and DsCHY2(a/b) also had a conserved IVG(A)G N-terminus (Fig. 2.2B), 17-21 positively charged residues, and 17-31 negatively charged residues (Zhu et al., 1997). Three conserved active site residues, His^{84/96}, Asp^{130/143}, and Ser^{232/240}, as well as six cysteine residues were also included in the three DsCHYs (Fig. 2.2B). The Asp^{226/234} was replaced by either a Gly or Ser residue in all the chymotrypsin-like proteins. Three and two S/T-xx-D/E sequence motifs for casein kinase II phosphorylation sites were predicted for deduced proteins of DsCHY1 and DsCHY2(a/b).

A

DsTRY1a	<u>MWAFISIFTFVYCLFQAVCSAAPQLESVVSNTR</u> IVGG TPTTIERYPYAVQVNMN-----	55
DsTRY1b	<u>MWAFISIFTFVYCLFQAVCSAAPQLESVVSNTR</u> IVGG TPTTIERYPYAVQVNMN-----	55
DsTRY2	<u>MRVLIALLG-----VLALVA</u> <u>AE</u> P--K----NPQR IVGG SLTTINQY ^{69/75} PDMVSL ^{114/125} LWSPWGSG	49
	His ^{69/75}	
DsTRY1a	NRLVCGGTLSR [▲] TCVLSAAH <u>CFVDRYNPTPNPSHYSARV</u> GATRRDSGGSTYRTRAIIVHE	115
DsTRY1b	NRLVCGGTLSR [▲] TCVLSAAH <u>CFVDRYNPTPNPSHYSARV</u> GATRRDSGGSTYRTRAIIVHE	115
DsTRY2	HNQNCGGTII [▲] TNRAVL [▲] TAAH <u>CTIN</u> -----DHPFMWQSRVGS [▲] TFANS [▲] GGV [▲] YATSQII [▲] NHP	104
	Asp ^{114/125}	
DsTRY1a	RHNFITQDS <u>DI</u> AVMALRNSVRLSN-TVALAQLPRQGLVVPDNATLIHIGWGALWVNGPLA	174
DsTRY1b	RYNFITQDS <u>DI</u> AVMALRNSVRLSN-TVALAQLPRQGLVVPDNAMLIHIGWGALWVNGPLA	174
DsTRY2	QYNPTIYDN <u>DI</u> AILRTVNP [▲] IFVNNLIQQASIASGTYILADNQQV [▲] VWATGWGROYSGGPPS	164
	Ser ^{213/222}	
DsTRY1a	QELNHVTIQVNH [▲] IACARQYAMIQLTVTTNMCAG-LPSGGADACQGD <u>SGG</u> PLIASNGLV	233
DsTRY1b	QELNHVTIQVNH [▲] IACARQYAMIQLTVTTNMCAG-LPSGGADACQGD <u>SGG</u> PLIASNGLV	233
DsTRY2	EQLRHVQI [▲] WTVNQALCRARYAEVRLTV [▲] DNMLCSGWL [▲] DVGG [▲] RDQCQGD <u>SGG</u> PLYH-NRVV	223
DsTRY1a	VGVTSWGNCGHPLFP [▲] GV SARVSNF [▲] SNWIDLQVARYNASPTLSSVNVLVLMASLLASVAV	293
DsTRY1b	VGVTSWGNCGHPLFP [▲] GV SARVSNF [▲] SNWIDLQVARYNASPTLSSVNVLVLMASLLASVAV	293
DsTRY2	VGVCSWGYGCAQARFP [▲] GVNARVSR [▲] FTSWIQSNAQ-----	257
DsTRY1a	LK	295
DsTRY1b	LK	295
DsTRY2	--	257

B

DsCHY1	--MMAAGYILGVLP [▲] GVGLAQAGPTIR--KG-EPAIIEDVRAG----S--R IVAG WPAAAYE	49
DsCHY2a	<u>MKTIAIFV</u> VVAVALAQANVLPYTSIRGYLENSIKLADQIREREERSLSQR IVGG SPSEEG	60
DsCHY2b	<u>MKTIAIFV</u> VVAVALAQANVLPYTSIRGYLENSIKLADQIREREERSLSQR IVGG SPSEEG	60
	His ^{84/96}	
DsCHY1	QIPYQISLR--LIGNTGGVSSCGGSIIH [▲] HNWVITAAHCVANRHTFVIRYGLTNLTRPEIV	107
DsCHY2a	QFPYQAGLLSDIIG-LQNGVCGGSLV [▲] SANRVITAAHCWFDGVHQAWR--VTVVLGSALL	117
DsCHY2b	QFPYQAGLLSDIIG-LQNGVCGGSLV [▲] SANRVITAAHCWFDGVHQAWR--VTVVLGSTLL	117
	Asp ^{130/143}	
DsCHY1	LETSHKYIHP--RYIEFQGGVQTD <u>DI</u> ALLGVNQHPYTRLIQPSRLQNSGRKDQSYLGV	164
DsCHY2a	FSGGQRF [▲] LSSSVITHAEWQPLLVRH <u>DV</u> AMIRLPSNAISNTVAPIALPTGDLLNQNFVGE	177
DsCHY2b	FSGGQRF [▲] LSSSVITHAEWQPLLVRH <u>DV</u> AMIRLPSNAISNTVAPIALPTGDLLNQNFVGE	177
DsCHY1	RLVVS [▲] GYGR [▲] TDDLWNGGVASEILLWTHLRGITNEECREWYNSSV [▲] IQAQTVC [▲] AEY [▲] NHTS	224
DsCHY2a	AVIASGFGLTSDGAN- <u>ISGNQFLSHVNLN</u> VISNTICRISFP--IFIQPSHICTSGIGGV-	233
DsCHY2b	AVIASGFGLTSDGAN- <u>ISGNQFLSHVNLN</u> VISNTICRISFP--IFIQPSHICTSGIGGV-	233
	Ser ^{232/240}	
DsCHY1	QSSCQGD <u>SGG</u> PLTVVNTDGKTTMIGIVSFGSSAGCNSPFPSAYVRPDHYHDWFLEVTGMS	284
DsCHY2a	-GTCQGD <u>SGG</u> PLV [▲] IIRNN-RPILIGITSFGSPLGCE [▲] SVM [▲] PAAYARVTEYVRFIHQHL---	288
DsCHY2b	-GTCQGD <u>SGG</u> PLV [▲] IIRNN-RPILIGITSFGSPLGCE [▲] SVM [▲] PAAYARVTEYVRFIHQHL---	288
DsCHY1	FDWNSDDILPPNPEVS [▲] AE [▲] EEALEKEE	309
DsCHY2a	-----	288
DsCHY2b	-----	288

Figure 2.2. Deduced amino acid sequences of three trypsin (A) and three chymotrypsin (B) isoforms from *Diatraea saccharalis* aligned using ClustalW. Residues identical to the consensus sequence of the three trypsin/chymotrypsin sequences are shaded. The predicted signal peptide sequences are single-underlined. Trypsin active site residues (His^{69/75}, Asp^{114/125}, and Ser^{213/222}) and chymotrypsin active site residues (His^{84/96}, Asp^{130/143}, and Ser^{232/240}) are double-underlined. N-terminal residues IVGG/IVG(A)G are in bold. Cysteines corresponding to the sites of the predicted disulfide bridges are marked with dark triangle at the bottom. Specificity determinant/binding pocket residues are indicated using gray triangle at the bottom. Hyphens represent sequence alignment gaps.

2.3.3 Sequence Comparison and Phylogenetic Analysis of Trypsin and Chymotrypsin cDNAs

Based on the sequences from the current study and those available in the GenBank, a total of 53 trypsin and 38 chymotrypsin sequences from various lepidopteran species were aligned and used to construct phylogenetic trees (Fig. 2.3). Sequence analysis of DsTRY1(a/b) and DsTRY2 cDNA fragments indicated 39% identity, suggesting that these cDNAs were derived from two different trypsin-like genes. DsTRY1(a/b) and DsTRY2 of *D. saccharalis* were located in separated groups of the phylogenetic tree and shared low amino acid sequence identity with the trypsins from other lepidopteran species (Fig. 2.3A). Similarly, cDNA fragments of DsCHY1 and DsCHY2(a/b) demonstrated a 40% identity. DsCHY2(a/b) were also located on an independent branch which was distant from other chymotrypsin-like proteinases. In contrast, DsCHY1 is highly similar to and is in the same cluster of the two chymotrypsin genes in *Antheraea assama* and *Helicoverpa punctigera* (Fig. 2.3B).

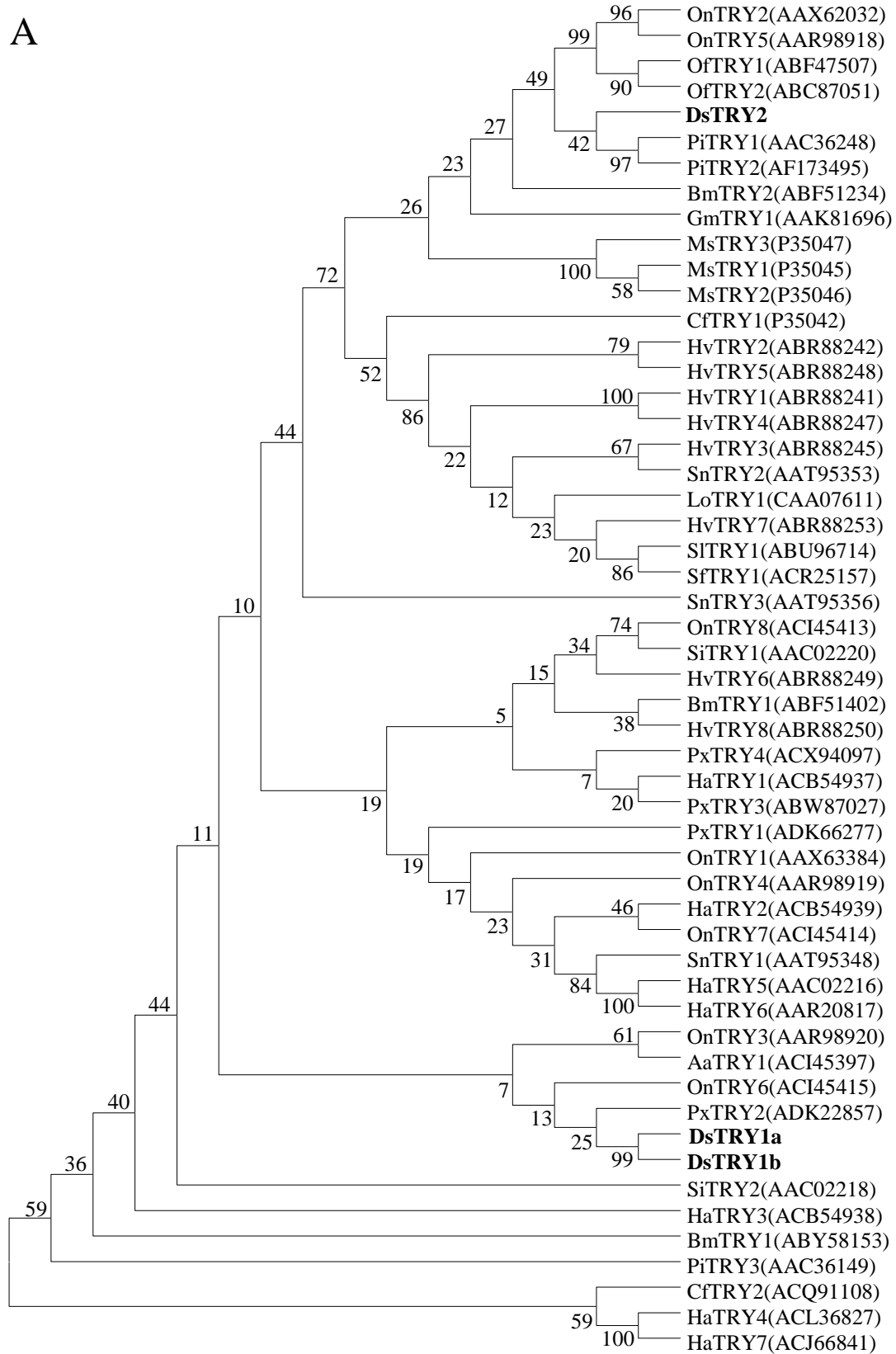
2.3.4 Transcriptional Levels of DsTRYs and DsCHYs from Cry1Ab-SS and -RR Strains of *D. saccharalis*

There was a trend that the gene expression levels of both DsTRY1(a/b) and DsTRY2 for the Cry1Ab-RR strain were somewhat (8%-37%) greater than those of the Cry1Ab-SS for both instars tested (Fig. 2.4A). However, the differences in the expression levels of DsTRY1(a/b) were not statistically significant ($P = 0.21$ for 3rd instar and $P = 0.15$ for 5th instar). Similarly, the differences in the gene expression levels of DsTRY2 were also not statistically significant ($P = 0.68$ for 3rd instar and $P = 0.16$ for 5th instar) (Fig. 2.4B). Transcript levels of DsTRY2 were ~8-fold more abundant than that of DsTRY1(a/b) for both insect strains and both instars.

There was a down-regulating trend (31%-44%) for gene expression levels of DsCHY1 but an up-regulating trend for DsCHY2(a/b) transcription (23%-54%) in the Cry1Ab-RR strain

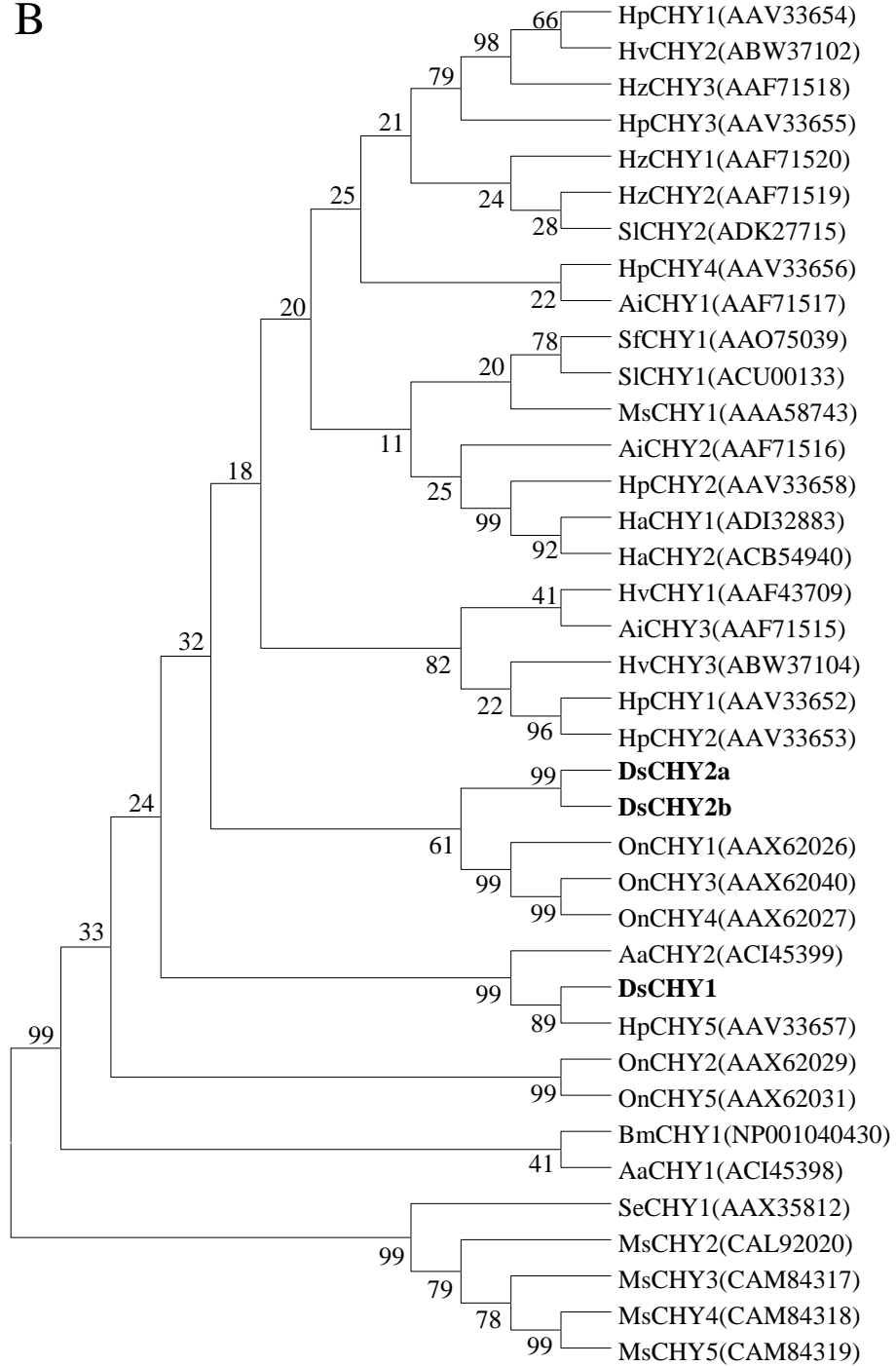
Figure 2.3. Phylogenetic trees obtained by using complete protein sequences with GenBank accession numbers of selected 53 trypsins (A) and 38 chymotrypsins (B) from lepidopteran species including *Diatraea saccharalis*. ClustalW was used to generate a basic sequence alignment to search for homology among protein sequences. After the general alignment was performed, an unrooted phylogenetic tree was generated and displayed by MEGA 4.1 (Tamura et al., 2007). Bootstrap values, expressed as percentages of 500 replications, are shown at branch points.

A



(fig. cont'd)

B



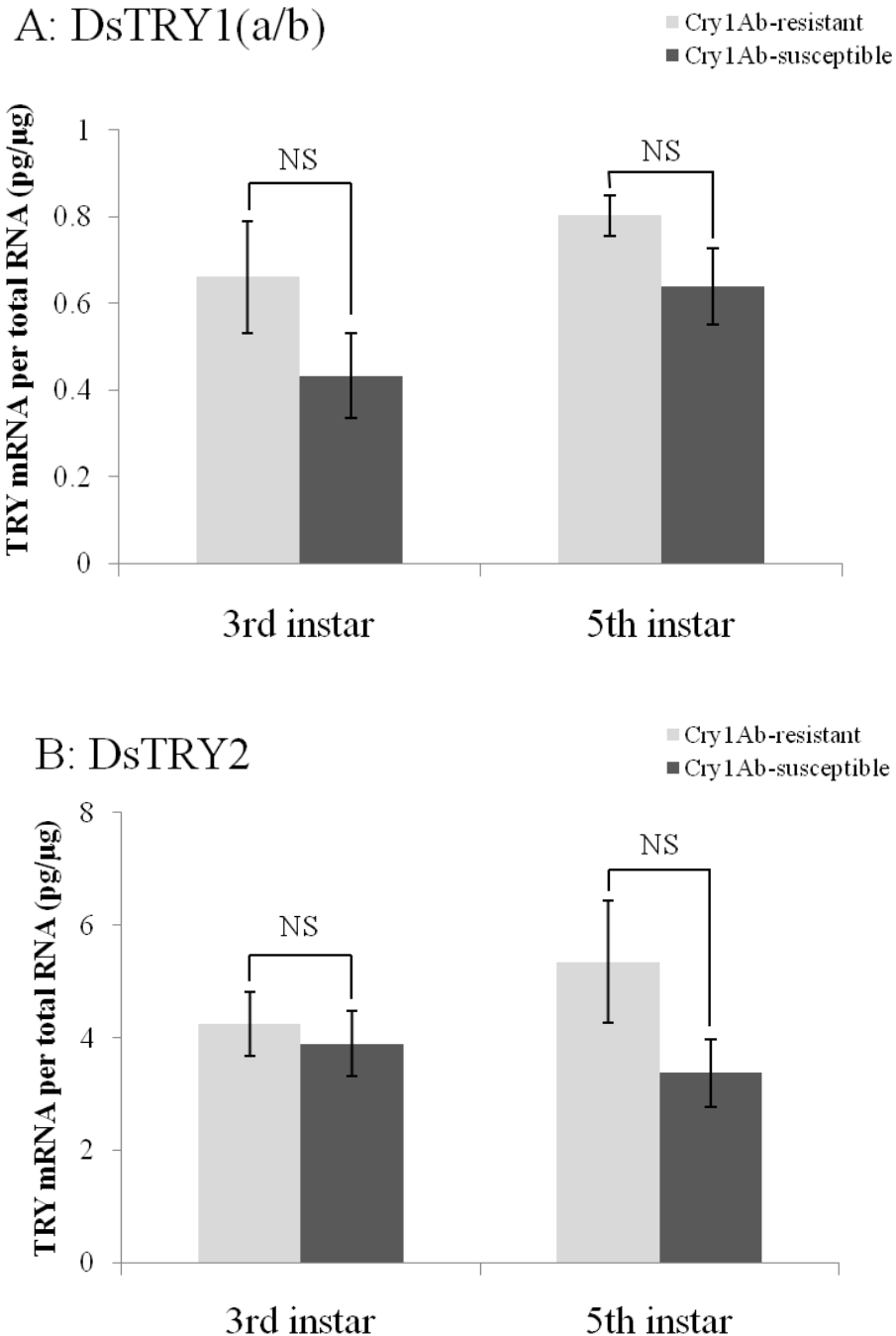


Figure 2.4. Expression levels of three trypsin genes (A: DsTRY1(a/b), B: DsTRY2) in 3rd and 5th instars of the Cry1Ab-susceptible and -resistant strains of *Diatraea saccharalis*. Absolute transcript abundance (trypsin mRNA per total RNA (pg/µg)) was determined using qRT-PCR with SYBR green. Bars represent the means and standard errors of five total RNA samples that each contains a pool of total RNAs from three larvae. NS indicates no significant differences between Cry1Ab-susceptible and -resistant strains ($P > 0.05$, Student's t-test).

comparing to those of the Cry1Ab-SS for both instars tested (Fig. 2.5). As observed for the DsTRYs, however, there were no significant differences ($P = 0.067-0.22$) in the expression of both DsCHY1 and DsCHY2(a/b) between the Cry1Ab-SS and -RR strains at each larval stage. In addition, the three chymotrypsin genes of the two insect strains showed a similar transcriptional level between 3rd and 5th instars (Fig. 2.5).

2.4 Discussion

Results of the current study showed that besides a few random SNPs, there were no differences in the cDNA sequences of the three trypsins and the three chymotrypsins between Cry1Ab-SS and -RR strains of *D. saccharalis*. In the deduced sequences of most mature trypsin- and chymotrypsin-like proteinases, there were some conserved structural features including: 1) the amino acid residues which are considered to determine trypsin or chymotrypsin substrate specificities; 2) the conserved histidine and serine catalytic sites; 3) the catalytic triad; and 4) six cysteine residues. These conserved features are located at conserved positions of cDNAs (Kraut, 1977; Wang et al., 1995; Peterson et al., 1994; 1995; Zhu et al., 1997). Deduced proteins of all DsTRYs and DsCHYs in *D. saccharalis* contained a typical secretion signal peptide consisting of 17 amino acids at their N-termini (Fig. 2.1). Between the predicted signal peptides and the mature enzyme sequences, all deduced proteins in *D. saccharalis* contained three putative trypsin/chymotrypsin active site residues. In addition to the three pairs of conserved cysteines which form disulfide bonds in DsTRYs and DCHYs proteins, four and two additional cysteine residues were found in DsTRY1(a/b) and DsTRY2 proteins, respectively (Fig. 2.1). It has been speculated that such free cysteine residues might be responsible for vulnerabilities of serine proteinases to some thio-reacting inhibitors (Gatehouse et al., 1997; Zhu et al., 2003; 2005). All

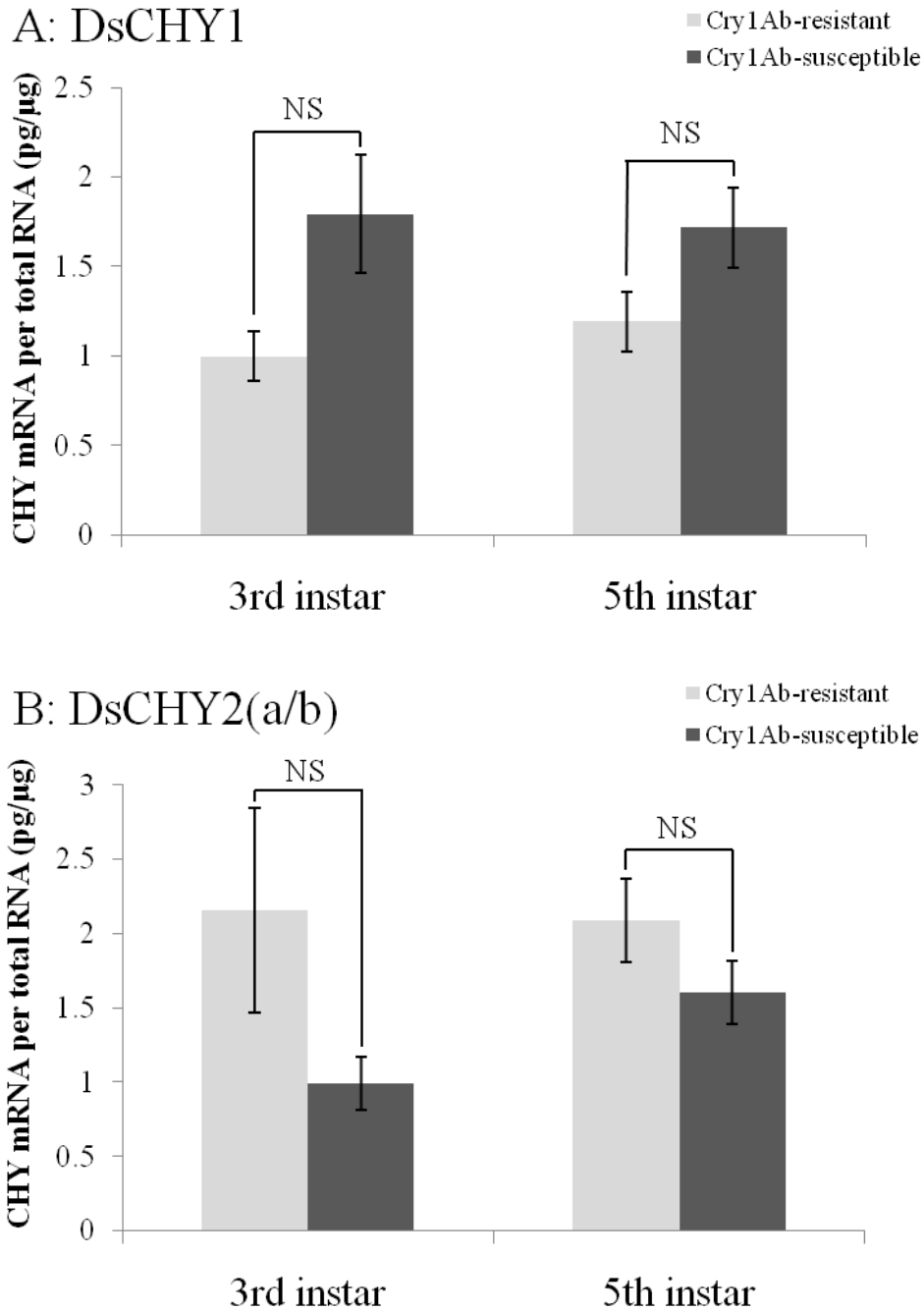


Figure 2.5. Expression levels of three chymotrypsin genes (A: DsCHY1, B: DsCHY2(a/b)) in 3rd and 5th instars of the Cry1Ab-susceptible and -resistant strains of *D. saccharalis*. Absolute transcript abundance (chymotrypsin mRNA per total RNA (pg/µg)) was determined using qRT-PCR with SYBR green. Bars represent the means and standard errors of five total RNA samples that each contains a pool of total RNAs from three larvae. NS indicates no significant differences between Cry1Ab-SS and -RR strains ($P > 0.05$, Student's t-test).

these features suggested that these cDNAs of the three DsTRYs and the three DsCHYs from *D. saccharalis* encode active trypsins and chymotrypsins, respectively.

Multi-trypsin and -chymotrypsin families may have evolved to provide a multi-protein digestion mechanism which should be more efficient for multi protein source (Bown et al., 1997). Such type of digestive system could also provide an adaptive advantage for phytophagous insects which feed on plants containing many proteinase inhibitors (Reeck et al., 1999). It was suggested that ancestors of lepidopterans may have had only one trypsin gene, while some species may have gained extra copies by subsequent gene duplications (Wang et al., 1995). In the current study, sequences of six cDNAs (DsTRY1a/b, DsTRY2, DsCHY1, and DsCHY2a/b) encoding full-length trypsin and chymotrypsin proteinases were compared within and between the Cry1Ab-susceptible and -resistant strains of *D. saccharalis*. A total of seven nucleotide/amino acid differences (SNP) were found in the six cDNAs within individuals of *D. saccharalis*. The replacements of Met¹⁵⁸ by Thr¹⁵⁸ and Gly²⁴² by Glu²⁴² in the predicted protein sequences were determined as nonpolar-to-polar substitutions between DsTRY1a and DsTRY1b among individuals for both insect strains. In contrast, there were only two substitutions of amino acids (nonpolar-to-polar or polar-to-nonpolar) between the predicted protein sequences of DsCHY2a and DsCHY2b among individuals for both insect strains. These substitutions of polar-to-nonpolar amino acid could have effects on molecular structures of proteins and their stability and catalytic activity. However, this variation of the deduced protein sequences of DsTRY1(a/b) and DsCHY2(a/b) occurred in both strains and thus apparently did not correlate to the Cry1Ab resistance in *D. saccharalis*. Studies have shown that SNPs are common in cDNA sequences of trypsins and chymotrypsins. For example, Zhu et al. (2005) cloned 15 trypsins and chymotrypsins that belonged to five serine proteinase groups of *Mayetiola destructor*. SNPs

were found in trypsins or chymotrypsins in all five groups. Another study with *O. nubilalis* identified 10-32 SNPs in three trypsins and two chymotrypsins (Coates et al., 2006).

A study has shown that midgut extracts from a Bt-resistant strain of *H. virescens* degraded Cry1Ab faster than those from a susceptible strain *in vitro* because of up-regulation of proteinases (Forcada et al., 1996; 1999). Shao et al. (1998) also reported that excessive degradation of Cry1A toxins by the midgut juice was responsible for a low sensitivity of *Helicoverpa armigera* to these toxins. Similarly, studies showed that enhanced serine protease activities could increase Cry1C toxin degradation in *Spodoptera littoralis* (Keller et al., 1996). In contrast, in some cases, reduced midgut proteinase activities can be associated with Bt resistance. For example, a Dipel[®]-resistance in *O. nubilalis* was found to be associated with reductions in midgut soluble trypsin-like activity (Huang et al., 1999). Later, Li et al. (2004; 2005) further demonstrated that a significantly reduced mRNA level of a trypsin gene existed in the Dipel[®]-resistant *O. nubilalis*. Zhu et al. (2000b) also reported that expression of two trypsin genes was reduced significantly in a Bt-resistant strain of *P. interpunctella* comparing to those of the susceptible larvae. The resistance in this strain was also correlated to a significant reduction in the total trypsin activity (Oppert et al., 1996; 1997; Herrero et al., 2001). In addition, Bt resistance in two coleopterans, *Melolontha melolontha* and *Leptinotarsa decemlineata*, was also associated with alterations in the midgut proteinase activity (Loseva et al., 2002; Wagner et al., 2002).

Transgenic Bt corn (e.g., YieldGard[®]) usually expresses truncated Cry proteins instead of full-length protoxins (Koziel et al., 1993). If the truncated proteins are the activated form, the mutated trypsins or chymotrypsins responsible for the protoxins activation cannot protect the insect from the pre-activated Bt toxins (Koziel et al., 1993). Studies have showed that the

Cry1Ab-RR larvae of *D. saccharalis* can survive on high concentrations of trypsin-activated Cry1Ab toxin (Huang et al., 2007a) as well as on commercial Cry1Ab corn hybrids (YieldGard®) (Huang et al., 2007b). Thus the Cry1Ab resistance in *D. saccharalis* was unlikely related to decreased activities of serine proteinases. The current study showed, beside a few random SNPs, there were no differences between the cDNA sequences of the three trypsins and three chymotrypsins between the Cry1Ab-SS and -RR strains of *D. saccharalis*. The expression levels of DsTRY1(a/b), DsTRY2, and DsCHY2(a/b) genes in the Cry1Ab-RR strain were somewhat higher compared to the Cry1Ab-SS larvae although the differences were not significant. Whereas DsCHY1 gene expression was lower in Cry1Ab-RR than in the Cry1Ab-SS strain but the difference was not significant at $\alpha = 0.05$. In addition, no significant differences were found in the total enzymatic activity of trypsin and chymotrypsin between the susceptible and resistant insects. These results suggested that the Cry1Ab resistance in *D. saccharalis* was not likely associated with alteration of total proteinase activities of trypsins and chymotrypsins. However, the results of this study cannot exclude the potential associations of the Cry1Ab resistance with other unidentified trypsins and chymotrypsins in *D. saccharalis*, which may involve in the mechanism of increased Cry1A toxins detoxification by up-regulated proteinases.

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CHAPTER 3

MOLECULAR CHARACTERIZATION AND RNA INTERFERENCE OF THREE MIDGUT AMINOPEPTIDASE N ISOZYMES FROM *BACILLUS THURINGIENSIS*-SUSCEPTIBLE AND -RESISTANT STRAINS OF SUGARCANE BORER, *DIATRAEA SACCHARALIS* *

3.1 Introduction

Bacillus thuringiensis (Bt) is a gram-positive bacterium that produces parasporal crystalline inclusions consisting of crystal proteins known as endotoxins or Cry proteins. The Cry proteins are selectively entomocidal to the larvae of various insect orders and nematodes (Pigott and Ellar, 2007). Several Bt strains have been used as biopesticides to control some important pests of agricultural crops and public health pests (Gould, 1998). Since first being commercialized in 1996, transgenic corn plants expressing Cry1Ab toxin have been the most widely grown Bt corn for controlling corn stalk boring pests in the United States and several other countries (NASS, 2009; James, 2008). The sugarcane borer, *Diatraea saccharalis*, is a major corn pest and a primary target of Bt corn in the mid-south region of United States (Castro et al., 2004; Huang et al., 2006). Recently, a resistance allele to Cry1Ab protein in Bt corn was identified from *D. saccharalis* (Huang et al., 2007a). This Bt-resistant strain completed its entire larval development (from neonate to pupa stages) on commercial Cry1Ab corn hybrids. Molecular resistance mechanisms of Bt resistance in corn stalk boring pests are poorly understood because of the lack of a highly resistant insect strain for study. The Cry1Ab-resistant strain of *D. saccharalis* provides unique opportunities to explore the mechanisms of Bt resistance in corn borer species.

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The mode of action of Bt toxins to kill insects is not fully understood. Two different hypotheses have been proposed for the mode of action: one relies on osmotic lysis by pore formation (Bravo et al., 2004), and the other relates to a signaling cascade that promotes cell death (Zhang et al., 2006). These two models might not exclude each other (Jurat-Fuentes and Adang, 2006). Compared to the evidence from only one study with an insect cell line for the signal transduction model, most published studies support the pore-formation model. The pore-formation model involved ingestion of the crystalline inclusions by insect larvae and solubilization of the inclusions to release Cry protoxins in insect guts. The Cry protoxins are then cleaved by insect midgut proteinases (e.g., trypsins, chymotrypsins) to form active Bt toxins. The activated Cry protein then binds to specific receptors at the midgut epithelium (Bravo et al., 2007). It is proposed that the cadherin-like and aminopeptidase N (APN) proteins are involved in Bt toxin binding by interacting sequentially with different toxin structures (Pigott and Ellar, 2007). The toxin monomeric structure first binds to the cadherin-like proteins (primary receptors) to induce further proteolytic processing and oligomerization of the protein (Gómez et al., 2002). The oligomeric protein then binds to APNs (secondary receptors) that drive the proteins into the membrane microdomains to cause pore formation (Bravo et al., 2004). The formation of the insertion pores results in osmotic imbalance of the membrane epithelium leading to swelling of the intestine cells and finally the death of the insect (Knowles and Dow, 1993). Detailed mechanisms of the pore formation in the membrane and death after pore formation are still unclear.

Theoretically, any changes in insect gut physiology and/or biochemistry that affect one or more steps in the mode of action of Bt toxins could interfere with toxicity and confer resistance. Early studies with several other lepidopteran species showed that membrane receptors including

APNs, cadherins, alkaline phosphatases, and glycolipids in the midgut can function as critical binding receptors to Bt toxins and thus are involved in Bt resistance (Denolf et al., 1997; Yaoi et al., 1999; Gahan et al., 2001; Knight et al., 1994; Oltean et al., 1999; Bravo et al., 2004; Jurat-Fuentes and Adang, 2006; Griffiths et al., 2005). In the three target species of Bt cotton, *Heliothis virescens*, *Pectinophora gossypiella*, and *Helicoverpa armigera*, Bt resistance has been found to be caused by mutations in a toxin-binding 12-cadherin-domain protein expressed in the larval midgut membrane. The mutations of the cadherin genes interrupt the primary sequence of the protein and prevent its normal localizations in the membrane (Heckel et al., 2007). Bt resistance in *H. virescens* could also be associated with other midgut receptor molecules (Jurat-Fuentes and Adang, 2004) and changes in midgut proteinase activities (Forcada et al., 1996). For example, additional mutations linked to resistance in the YHD2 strain of *H. virescens* significantly affected the production of glycosylphosphatidylinositol (GPI)-anchored alkaline phosphatase (Jurat-Fuentes and Adang, 2004). Bt resistance mechanisms have also been investigated for *P. interpunctella*. Resistance in some strains of the insect was due to changes in binding affinity (Ferré and Van Rie, 2002; Herrero et al., 2001; Baxter et al., 2005), while in some strains, resistance was associated with changes in midgut proteinase activity (Oppert et al., 1997; Karumbaiah et al., 2007). Several studies have shown that other mechanisms or multiple mechanisms could also play an important role in the Bt resistance evolution (Jurat-Fuentes et al., 2002, 2003; Gunning et al., 2005; Jurat-Fuentes and Adang, 2006; Ma et al., 2005; Mahbubur Rahman et al., 2007).

The objective of this study was to determine if Cry1Ab resistance in *D. saccharalis* is associated with changes in gene structure/expression of APNs in the midgut. To achieve this goal, full length cDNAs of three APN genes were cloned and sequenced from both Cry1Ab-

susceptible (Cry1Ab-SS) and -resistant (Cry1Ab-RR) strains of *D. saccharalis*. Quantitative RT-PCR (qRT-PCR) was then performed to examine expression levels of the three APN genes. Finally, RNA interference (RNAi)-mediated gene silencing was employed to assess functional linkage of the three APN genes with Cry1Ab susceptibility in *D. saccharalis*. Our results showed that a reduced expression of the three APNs in the Cry1Ab-RR strain is likely associated with the Cry1Ab resistance in *D. saccharalis*.

3.2 Materials and Methods

3.2.1 Insect Strains

A Cry1Ab-SS strain of *D. saccharalis* was developed from a collection of larvae in a corn field near Winnsboro in Franklin Parish, Louisiana, during 2004 (Huang et al., 2007a). A Cry1Ab-RR strain of *D. saccharalis* was isolated from a single two-parent family-line collected from the same location and was shown to carry a major resistance allele by using an F₂ screen (Huang et al., 2007a). These Bt-resistant insects completed larval development on commercial Bt maize hybrids expressing the Cry1Ab protein (Huang et al., 2007a) and demonstrated a significant resistance level (ca. 100-fold) to purified trypsin-activated Cry1Ab toxin (Huang et al., 2007b). Individuals of the Cry1Ab-RR strain had been backcrossed three times with those of the Cry1Ab-SS strain and re-selected for Bt resistance with Cry1Ab corn leaf tissue in the F₂ generation of the backcross. The backcrossed re-selected resistant strain was used in the current study.

3.2.2 Gut Tissue Preparation and Total Aminopeptidase N Activity Assays

Third, 4th, and 5th instars of *D. saccharalis* reared on a meridic diet were chilled on ice and dissected in cold 0.1M Tris-HCl buffer (pH 8.0). To avoid leakage of gut contents, the anterior and posterior ends of the gut were held tightly with forceps and the gut was detached

from the larva. Each gut was homogenized with 50 μ l 0.1M Tris-HCl buffer (pH 8.0) and centrifuged at 10,000g for 5 min at 4 $^{\circ}$ C. The supernatant of each homogenate was transferred to an individual tube and used as an enzyme source for APN activity analysis.

The protein quantity of the homogenate was determined using the Bradford method (Bradford, 1976) with Coomassie Plus (Bradford) Assay Kit (Pierce, Rockford, IL) and bovine serum albumin (BSA) as standards. After protein concentration determination, each homogenate was diluted to 1 mg/ml. The diluted homogenate was used for measurement of total APN activity using leucine-*p*-nitroanilide (LpNA) (Sigma, St. Louis, MO) as a chromogenic substrate (Valaitis et al., 1999). Five microliters of gut homogenate were suspended in 95 μ l of prewarmed (37 $^{\circ}$ C) 0.1M Tris-HCl buffer (pH 8.0) containing 0.8 mM LpNA. The enzymatic reaction was immediately monitored for the optical absorbance increase at wave-length 405 nm at 37 $^{\circ}$ C for 15 min in the ELx808iu microtiter plate reader (Bio-Tek, Winooski, VT). The mean velocity (Mean *V*) was calculated as increase of OD₄₀₅ per min in the linear portion of initial velocity of the enzymatic reaction using the KC4 program (Ver. 3.6, Bio-Tek, Winooski, VT). One unit of the enzyme activity was defined as the amount of enzyme that hydrolyzed one μ mol of substrate to chromogenic product per min. Specific APN activity calculations were performed based on the extinction coefficient (9.9 mM⁻¹ cm⁻¹) for *p*-nitroanilide (Hafkenschied, 1984). For each combination of insect strain and instar, APN activity was estimated from 8 reactions (replications). Specific APN activities were presented as means and standard errors of the mean (\pm SEM). Student's t-test was used to determine differences in APN activity between the two insect strains for each instar.

3.2.3 Cloning Full-Length cDNAs of Three DsAPNs

For cloning full-length APN cDNAs, cDNA libraries of Cry1Ab-SS and -RR strains of *D. saccharalis* were constructed, sequenced, and BLASTed by Dr. Yu Cheng Zhu (USDA-ARS, Stoneville, MS). The contigs from both libraries that matched the characters of APN gene were then assembled into partial cDNAs of three APN genes, named DsAPN1, DsAPN2, and DsAPN3. Larvae of the Cry1Ab-SS and -RR strains were dissected to obtain guts. The gut tissue was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA was precipitated in isopropanol according to the manufacturer's protocols. To eliminate genomic DNA contamination, total RNA was treated with 2 μ l (1 mg/ml) DNaseI (Boehringer Mannheim GmbH, Germany) at 37 $^{\circ}$ C for 1 h. RNA concentration was determined using NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Five micrograms of RNA were used as a template for cDNA synthesis using an oligo-dT primer in a reverse transcription (RT) reaction with SuperScript First Strand cDNA Synthesis System (Invitrogen, Carlsbad, CA). Following cDNA synthesis, template RNA was removed by adding 1 μ l (2 U) RNase H.

To determine the major part of the APN cDNA fragments, five degenerate forward primers were designed based on the five conserved regions of the three APN cDNAs previously identified in nine other lepidopteran species (Table 3.1). The amino acid sequences of the five conserved regions were EI(V)VM(I)HCND and MRGFYRSWY for DsAPN1, TSQLQPTFA for DsAPN2, and YRLPT(N)TT and WMAT(S)TQFQA for DsAPN3. Three specific reverse primers, named DsAPN1R1, DsAPN2R1, and DsAPN3R1 (Table 3.1), were also designed based on the three APN sequences identified from the cDNA libraries. For each RT-PCR amplification, one degenerate forward primer and one specific reverse primer were used to amplify a fragment with expected size. Then, three specific forward primers, named DsAPN1F1, DsAPN2F1, and

Table 3.1. Sequences of primers used in cDNA cloning, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and double-stranded RNA (dsRNA) synthesis for characterization of three midgut aminopeptidase N genes from Cry1Ab-susceptible and -resistant strains of *Diatraea saccharalis*.

Gene Identity	Primer Name	Primer Sequence (5'-3')	
DsAPN1	degenerate primer (RT-PCR)	dgDsAPN1F1 dgDsAPN1F2	GARATHGNTNHTNCAYTGAAAYGA ATGMGNGGNTTYTAYMGNWSNTGGTA
	specific primer (RT-PCR and 5'RACE)	DsAPN1F0 DsAPN1F1 DsAPN1R0 DsAPN1R1 DsAPN1R2 DsAPN1R3	AAAGTGACATGATGGCGTACC TCTCACTACTGCTCTCAACTC CAAAATACTAAAAACATTAAGCTTTATTAATAAAAACTCTAA AGAGGTGTAGCGATACTTCC TGTTCACTAGCAAGGTCTTCATTC GGTATTATGGTCAGAGTACCAACTC
	specific primer (Quantitative RT-PCR)	rtDsAPN1F1 rtDsAPN1R1	TTGCCTCATCTGAACAGAACTTG GGTATTATGGTCAGAGTACCAACTC
	specific primer ^a (dsRNA synthesis)	iDsAPN1F1 iDsAPN1R1	TAATACGACTCACTATAGGGGAGTTGGTACTCTGAC CATAATACC TAATACGACTCACTATAGGGCTCTGTAGGTTAAGAGTCCCCA
DsAPN2	degenerate primer (RT-PCR)	dgDsAPN2F1	ACNWSNCARYTNCARCCNACNTTYGC
	specific primer (RT-PCR and 5'RACE)	DsAPN2F0 DsAPN2F1 DsAPN2R0 DsAPN2R1 DsAPN2R2 DsAPN2R3	TTAGTGTTCCGGGTACACA GCTATAAGGGAGAACATCGC GAATTTGAAATATTTTTATTCTGCTTATAATTGGTT CAAAGAAAGACAGAAGCACTA CCCTTGAACATTTGGTTGTATCG CTTGAATACTGCTTTGATTGCTGG
	specific primer (Quantitative RT-PCR)	rtDsAPN2F1 rtDsAPN2R1	ACAGACTTGGTACTTCCAGAATG TTGGTAAGCGACAGCGTTAAAT
	specific primer ^a (dsRNA synthesis)	iDsAPN2F1 iDsAPN2R1	TAATACGACTCACTATAGGGACAGACTTGGTACTTCCAGAATG TAATACGACTCACTATAGGGAAAGTGAACGGTCGGTCAAATATC
DsAPN3	degenerate primer (RT-PCR)	dgDsAPN3F1 dgDsAPN3F2	TAYMGNNTCCNAMNACNAC TRBATGGCNWSNACNCARTTYCARGC
	specific primer (RT-PCR and 5'RACE)	DsAPN3F0 DsAPN2F1 DsAPN3R0 DsAPN3R1 DsAPN3R2 DsAPN3R3	TTGTTTGCGAAGAAGTTTGGGA ATTACAATGGGCTCAGGAAAAC CATTCAAAACCATGAACTACCCTTG ACACCTGTTGGAAATTCATC CTGGAGTAGTGGTGACAATAGG TGAAACTTGGCTCATCATAACAGG
	specific primer (Quantitative RT-PCR)	rtDsAPN3F1 rtDsAPN3R1	ACTCTCTATCGAGTTAATGCACCA TGAAACTTGGCTCATCATAACAGG
	specific primer ^a (dsRNA synthesis)	iDsAPN3F1 iDsAPN3R1	TAATACGACTCACTATAGGGCCTGTTATGATGAGCCAAGTTTCA TAATACGACTCACTATAGGGCATCGTACATCAAGTAAGCTTCTCT
specific primer (Internal standard for quantitative RT-PCR)	Ds18SF1 Ds18SR1	CAAATGTCTGCCTTATCAACTTTC GCCTTCCTTGGATGTGGTAG	

^a dsRNA template forward and reverse primers additionally had T7 RNA polymerase recognition sites (5'-TAATACGACTCACTATAGGG-3') appended to their 5' and 3' ends, respectively.

DsAPN3F1 (Table 3.1), were designed based on their sequences cloned from the cDNA libraries. These three primers were used with oligo-dT primer in PCRs to amplify the 3'-end cDNAs of DsAPN1, DsAPN2, and DsAPN3, respectively. Finally, the 5' RACE system (Invitrogen, Carlsbad, CA) was used to clone the 5'-end of the three APN cDNAs. For 5' RACE, RT reactions were first performed using the specific reverse primers DsAPN1R1, DsAPN2R1, and DsAPN3R1 mentioned above. Based on the cDNA sequences of the three APNs generated above, two specific reverse primers for each APN gene (Table 3.1) were designed and used in semi-nested amplifications with a forward abridged anchor primer from 5'-RACE kit. The 5'-end of the cDNAs were isolated and C-tailed, and then were cloned into a pGEM-T vector. Finally, plasmid DNA was prepared and sequenced from both directions on the ABI 3730XL sequencer to confirm the full coding sequences for the three APN proteins.

To obtain error-proof full-length cDNAs of the three APNs, total RNA was extracted from Cry1Ab-SS and -RR strains for RT-cDNA synthesis as described above. PCR reactions were performed for flanking the 5' and 3' non-coding regions of the three DsAPNs by using a pair of specific forward and reverse primers for each DsAPN (Table 3.1). Additionally, a thermal-stable proof-reading Platinum High Fidelity Taq DNA polymerase (Invitrogen, Carlsbad, CA) was used to reamplify the near full-length cDNAs of the three APNs from the RT-cDNAs. The resolved near full-length cDNA fragments (~3.3 kb for DsAPN1, ~2.8 kb for DsAPN2, and ~3.0 kb for DsAPN3) were purified from a low melting point agarose gel and then A-tailed for 30 min at 72 °C using the nucleotide adenine and Taq DNA polymerase. The PCR products were then cloned into a pGEM-T vector (Promega, Madison, WI), and the resolved clones were sequenced from both directions as described above.

3.2.4 Nucleotide and Amino Acid Sequence Analysis of Three DsAPNs

The sequence-similarity analyses were performed through the National Center for Biotechnology Information (Bethesda, MD) (<http://www.ncbi.nlm.nih.gov/>). Molecular weight calculations and pI prediction of mature proteins were conducted with the ExPASy proteomics tools on the website (<http://www.expasy.ch/tools/>) of the Swiss Institute of Bioinformatics. The ISREC-ProfileScan server (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>) was used to analyze data of deduced protein sequences. ClustalW (Thompson et al., 1994; gap weight = 8, gap length weight = 2) was employed in sequence comparison. The molecular evolutionary genetics analysis (MEGA) software (Ver. 4.1; Tamura et al., 2007) was used to conduct multiple-sequence alignment and phylogenetic analysis based on the APN cDNA sequences of lepidopteran species that had been deposited in the GenBank nucleotide database.

3.2.5 Quantitative RT-PCR to Determine Transcript Levels of Three APNs

Total RNAs of 3rd and 5th instars from Cry1Ab-SS and -RR strains of *D. saccharalis* were extracted and treated with DNaseI as described above. Concentrations of total RNAs were measured with a NanoDrop spectrophotometer using the same methods as mentioned before. For each treatment replication, total RNA from three guts was pooled as one sample into a centrifuge tube. Each RNA sample was diluted to two concentrations (1 and 10 ng/μl). The qRT-PCR assays were performed in a 25 μl reaction volume using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) in a Thermal Cycler PTC200 with Chrome4 detector attached (Bio-Rad, Hercules, CA). qRT-PCR procedures included 50 °C for 10 min (cDNA synthesis), 95 °C for 5 min (iScript Reverse transcriptase inactivation), and 40 cycles (95 °C for 10 s, and 55 °C to 60 °C for 30 s) for data collection. To check amplification specificity, a melting curve was established from 55 °C to 95 °C with an increment of 0.5 °C for 10 s for each step

(Bautista et al., 2009). All PCR reactions and data output were controlled through Opticon Monitor 3 (Bio-Rad, Hercules, CA).

To obtain the absolute quantities of each gene, two qRT-PCRs were performed for the candidate genes (Bustin, 2000). To standardize sample RNA concentration, ribosomal 18S gene was used in the first qRT-PCR as internal standard to estimate RNA concentration for each sample. One pair of primers, Ds18SF1 and Ds18SR1 (Table 3.1), designed based on the 18S gene from *D. saccharalis* were used. Full RT-cDNA of 18S gene was prepared and used as internal standard in the first qRT-PCR. Concentrations of internal standards were determined with the NanoDrop spectrophotometer mentioned above. Serial dilutions of the 18S internal standard (0.5, 5, 50, 500, and 5000pg/ μ l) plus a negative control that contained all compounds of qRT-PCR except the 18S cDNA were used to draw a standard curve in the first qRT-PCR. Five microliters of RNA template (1 ng/ μ l) or 5 μ l of internal standard was added to the reaction. After the first qRT-PCR, the threshold cycle (C_t) value for each dilution was plotted against the log of its quantity. Absolute quantities of all RT-cDNA were calculated from the standard curve and all initial RNA samples were standardized for the second qRT-PCR.

For the second qRT-PCR, three primer pairs (Table 3.1) specific for DsAPN1, DsAPN2, and DsAPN3 (replicon length = 173, 159, and 160 bp, respectively) were designed based on the above sequenced full-length of the APN cDNAs. Three cDNA fragments of 1222, 1149, and 1735-bp from DsAPN1, DsAPN2, and DsAPN3 were amplified and used as internal standards for the second qRT-PCRs, respectively. Serial dilutions of each internal standard (0.001, 0.01, 0.1, 1, and 10pg/ μ l) were prepared to establish a standard curve as described above. Five microliters of standardized RNA template (10 ng/ μ l) or 5 μ l of internal standard solution was added to each qRT-PCR reaction. A negative control as described in the first qRT-PCR was also

included in the reactions. The reactions were conducted using the primer pairs for DsAPN1, DsAPN2, and DsAPN3 as described above, respectively. After the second qRT-PCR, absolute quantities of RT-cDNA of the target gene of each sample were calculated from the internal standard curve and compared between Cry1Ab-SS and -RR strains at specific instars. There were five replications for each treatment combination of insect strain and instar. Data on the gene expression levels were presented as means and standard errors of the mean (\pm SEM). Student's t-tests were used to analyze the data to determine treatment difference for each instar.

3.2.6 RNAi to Suppress the Gene Expression of Three APNs

Double-stranded RNAs (dsRNAs) corresponding to position 546 to 1057 of DsAPN1, position 1120 to 1576 of DsAPN2, and position 608 to 1087 of DsAPN3 were synthesized using a method that was able to eliminate the cloning step (Schepers, 2005; Nunes and Simões, 2009). After pairwise alignments among the three APNs, a nearly unique region was selected from each APN for synthesis of the corresponding dsRNA to minimize any off-target effects. The primers used to produce cDNAs with T7 promoter sequences were designed based on the nucleotide sequences of the three DsAPN cDNAs obtained above. Templates for *in vitro* transcription were produced using primers with T7 RNA polymerase recognition sequences (5'-TAATACGACTCACTATAGGG-3') appended onto both 5'- and 3'-ends. The PCR products were examined on agarose gels prior to *in vitro* transcription to verify the single bands and the expected sizes. Resulting amplicons, excluding the fused T7 promoters, were 512, 457, and 480 bp in length, for DsAPN1, DsAPN2, and DsAPN3, respectively. After purification with Minelute purification kit (Qiagen, Valencia, CA), dsRNA was produced following the protocol described in the MEGAScript RNAi kit (Ambion, Austin, TX). After incubation at 37 °C for 4 h, dsRNA was prepared in an elution solution (ES) (10 mM Tris-Cl, pH 7, 1 mM EDTA) provided in the

RNAi kit. Finally, dsRNA were quantified using Nanodrop spectrophotometer and stored at -80 °C until used (Bautista et al., 2009).

Oral delivery (Turner et al., 2006; Bautista et al., 2009) was apparently more suitable for our purpose because this technique is not invasive (no mortality) and the larvae could be subjected to subsequent bioassays. Third instars of Cry1Ab-SS strain of *D. saccharalis* were individually placed in 1 oz plastic cups (Solo, Highland Park, IL) and starved for 12 h. The starved-larvae were then droplet-fed with 0.5 µl of the ES (control) or 0.5 µl ES containing 250 ng of target gene dsRNA (RNAi-treated) using a 0.1-2.5 µl pipette (Eppendorf, Hamburg, Germany) (Bautista et al., 2009). dsRNA delivery was also validated by feeding larvae with blue-colored ES+dsRNA (1% vol.) and photographing with a Nikon Coolpix 5700 digital camera connected to a Nikon SMZ1000 stereomicroscope (Turner et al., 2006; Zhou et al., 2008). The droplet-fed larvae were then individually placed in wells of 96-well plates for 3 h before being transferred to the individual cup containing 10 g meridic diet. Total RNA and homogenates' supernatant of guts were isolated from larvae at 24 h postfeeding (Bautista et al., 2009) using the methods described above.

Gene expression of the three DsAPNs of the RNAi-treated and control larvae was determined by qRT-PCR as described above. RNAi knockdown analysis was conducted by comparing the gene expression levels between the RNAi-treated and control insects. Our dsRNA design described above resulted in no more than 14 identical continuous nucleic acids between the dsRNA of an APN and the full-length cDNAs of the other two APNs. Because of the nearly unique dsRNA used for each APN, any off-target effects of dsRNAs among the three APNs were minimized. To reduce the overall costs, changes in gene expression after RNAi by one dsRNA was measured only for the APN targeted by that dsRNA. There were four replications for each

treatment and each replicate contained a pool of total RNAs from 3 larvae (Bautista et al., 2009). Student's t-tests were used to analyze the data to determine RNAi treatment and control. In addition, total APN activity of RNAi-treated and control larvae was determined individually with the same procedures mentioned above. There were 8 insects (replications) for each treatment in the APN activity analysis. Data on total APN activity were presented as means and standard errors of the mean (\pm SEM). One-way ANOVA with Bonferroni post hoc tests was used to determine treatment difference.

Neonates (< 24 h) of Cry1Ab-SS strain of *D. saccharalis* were droplet-fed with 0.1 μ l of ES containing 50 ng of target gene dsRNA (RNAi-treated) or ES only (control) as described above. The droplet-fed neonates were then individually placed in wells of 128-cell trays (C-D international, Pitman, NJ) for 3 h. The bioassays with Cry1Ab toxin were conducted using a similar method as described in Huang et al. (2007b). The RNAi-treated and control neonates were released on a meridic diet containing Cry1Ab toxin (16 μ g g⁻¹ diet) or diet only (blank control). A previous study showed that the Cry1Ab concentration used in the bioassays caused approximately 50% larval mortality of the Cry1Ab-SS strain after 7 days (Huang et al., 2007b). The bioassay trays were held in an environmental chamber maintained at 28 °C, 50% RH, and a photoperiod of 16:8 (L:D) h. Larval mortalities were examined after 7 days. Mortality was corrected based on the mortality observed on the blank control using Abbott's method (1925) (Huang et al., 2006). Each RNAi-treated or control treatment was replicated three times with 48 larvae in each replication. The mortalities were presented as means and standard errors of the mean (\pm SEM). One-way ANOVA with Bonferroni post hoc tests were performed to determine treatment differences.

3.3 Results

3.3.1 Total APN Activity Profiling of Cry1Ab-SS and -RR Strains of *D. saccharalis*

Total APN activity was significantly different ($P = 0.0019$ for 3rd instar, $P = 0.0022$ for 4th instar, and $P = 0.0089$ for 5th instar) between the two insect strains across all the three instars tested. Compared to the Cry1Ab-SS strain, a 36.8% reduction in total APN activity was observed from 3rd instars of Cry1Ab-RR strain (Fig. 3.1). The total APN activity for 4th and 5th instars of Cry1Ab-RR strain was 21.2 and 22.1% lower than that of the Cry1Ab-SS strain, respectively.

3.3.2 cDNAs Coding for Three APNs of *D. saccharalis*

Eighteen expressed sequence tags (ESTs) of APN genes were found including a polyadenylation signal in the 3'-UTR from the libraries. The selected ESTs were assembled into

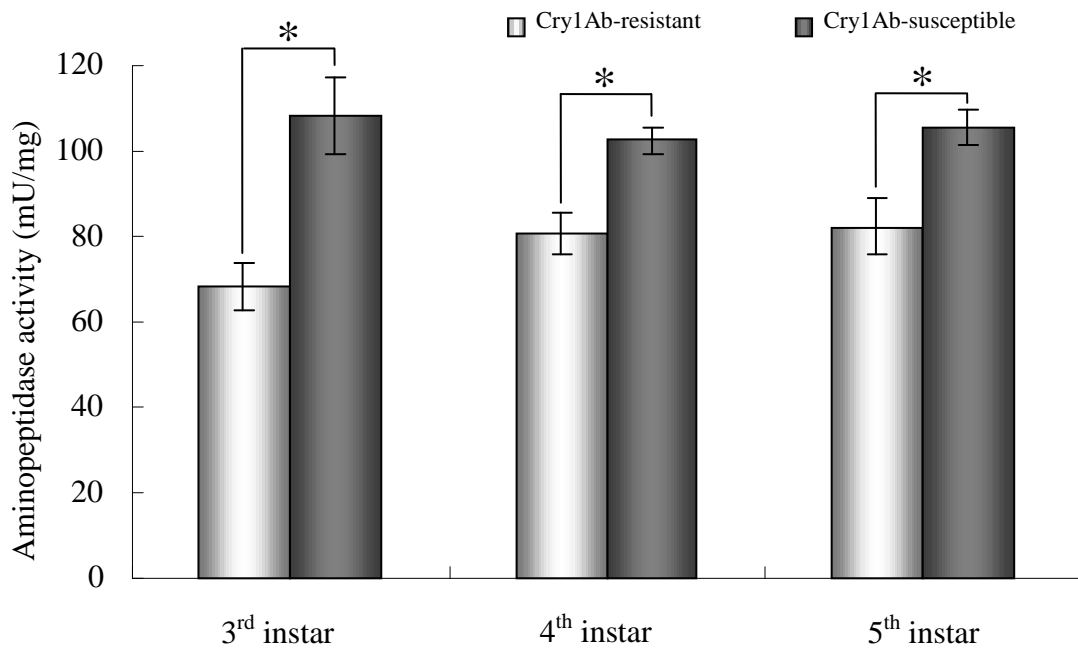


Figure 3.1. Total aminopeptidase N activity of whole gut tissue of different larval instars from Cry1Ab-susceptible and -resistant strains of *Diatraea saccharalis*. One unit of enzyme activity was defined by hydrolysis of 1 μ mol of substrate in 1 min. Bars represent the means and standard errors of 8 gut samples from a total of 8 different larvae. Asterisk indicates significant differences ($P < 0.05$, Student's t-test).

cDNA fragments of three APNs of *D. saccharalis* (GenBank accession nos. DsAPN1: HM231316; DsAPN2: HM231317; DsAPN3: HM231318). Pairwise alignment showed that Cry1Ab-SS and -RR had identical DsAPN1, DsAPN2, or DsAPN3. No deletion or single site mutation existed between the two strains. The three cDNA sequences ranged from 3320 to 3510 bp in length, each of which contained a full length open reading frame (ORF). At the 3' end of the three cDNA sequences, a polyadenylation signal sequence AATAAA in DsAPN1, DsAPN2, and DsAPN3 was apparent at the position 15-18 bp upstream of the polyA tails. Homology search of GenBank using Blastx revealed that the three DsAPNs of *D. saccharalis* shared 59-70% sequence identity with the corresponding APNs from *Ostrinia furnacalis*, *Bombyx mori*, and *Manduca sexta*.

Lengths of the ORFs were 3354 in DsAPN1, 2823 in DsAPN2, and 3039 bp in DsAPN3. Each ORF coded for the APNs with the predicted molecular weights of 127, 106, and 116 kDa, respectively. Based on the deduced protein sequences of the three APNs, a signal peptide at the N-termini and a GPI anchor signal at the C-termini were predicted using the SignalP 3.0 (Bendtsen et al., 2004) and big-PI Predictor (Eisenhaber et al., 1999) online servers (Fig. 3.2). The presence of the signal peptide and GPI anchor signal was consistent with the known APNs from other insect species. Excluding the signal peptides and the C-terminal propeptides, the molecular weights of the mature DsAPN1, DsAPN2, and DsAPN3 proteins were 122, 102, and 111 kDa, respectively. All three APNs of *D. saccharalis* contained the gluzincin aminopeptidase motif GAMENWG and the zinc binding/gluzincin motif HEX₂HX₁₈E 29 bp downstream (Fig. 3.2). These motifs also existed in the APNs that had been identified from other lepidopteran larvae (Adang, 2004).

Figure 3.2. Deduced amino acid sequences of aminopeptidase N isoforms of DsAPN1, DsAPN2, and DsAPN3 from *Diatraea saccharalis* aligned by ClustalW (GenBank accession nos. DsAPN1: HM231316; DsAPN2: HM231317; DsAPN3: HM231318). Residues identical to the consensus sequence of the three APN sequences are shaded. The predicted signal peptide and the C-terminal propeptide sequences are underlined. Gluzincin aminopeptidase motif GAMENWG and zinc binding/gluzincin motif HEX₂HX₁₈E are marked in boxes.

DsAPN1 MMAYRWLSLSVVTLLQALFLSPLVVPDKQWDDFDVHPLQTAEEEWEQYNE LLRN --- 56
DAPN2 -----MYLVLLGALLGSVASSTIPVVEPRNEDLAA-----MYN----- 33
DAPN3 -MAT--LGLIVLATSCFAVHAFFESP RP YRNTIFLDEKLEGEVFEDVDVSFKDITLYNTVI 57

DsAPN1 SDYRLPRTTVPDHYVLSLTPYFEHTD VNRAFTFDGKVKINIRATTEGVNEILMHCNDLTV 116
DAPN2 ----LPRETTPTFYDVTLYLDPDNEE----YFYGNVSI RIVPN-IATNVIVLHAMEMSI 83
DAPN3 NPFRLPPTTRPQHYNVEWIIDMEK-----LWFSGSVDIELYATQPNVNEIVIHAHDLNI 111

DsAPN1 KSVTVQYTDSSNNETKNIASSEQNLCQMPYSFLRISTTEY-LQOVVTVYEVEMEFTGHLQS 175
DAPN2 ENIEVLTNTNNIINTNNNIYLSHELATDDTHLLRIHLAET-LIESRIYILNINIVGQYAT 142
DAPN3 TSLSLRQGGTTPMYQEYFLQP----QYHFLYVALTNGSLDYNAINPIIYTLSEIEFNAPLRT 167

DsAPN1 NMRGFYRSWYSDHNTTR-RWMATTQFQPGHARQAFPCYDEPGFKATFDITINREPDFSPT 234
DAPN2 NMFGIYVSNYVENG IQQ--KLITSQLQPTFARRAFPCYDEPAIKAVFKTTIVSPASYTVV 200
DAPN3 DMYGIYRSWFRNTPTEEP RWMAS TQFQATAARFAFPCYDEPSFKATFDINI AH PQNYT-S 226

DsAPN1 ISNMPIKDTSNELVPGRVS---ETFHTTPTRTSTYLLAFIVSHYEVVASK-----NDEER 285
DAPN2 RTNMPEINNSTDPDGNVRIH---E-FQDTEIMSTYLLAYLVSNFEHVSNE---ENPIYRV 252
DAPN3 WSCTRLRITSTPYVVDNETIYSLDQYRTPVMSTYLLALIVA EYDTKELFGPHPQTNEEVL 286

DsAPN1 PFRIYARNNAGTGG--DWSLKIGIDLLRAMEEYTCQIPYTMADNMDMKQAAIPDFS AAGAM 343
DAPN2 PFKVFSRPGTKENA--EFAMDFGQKNMVKLEBYTEFDYVFP---KLDKVAVPDFA AAGAM 306
DAPN3 KYEVIGRS GAMERGGQGNFSFDIGQELLSEMSHINLDFFSVHQYLKMTQAAIPDFG AAGAM 346

DsAPN1 ENWGLLT YREALI LYDPQNSNHFYKQRVANIVSHEVAHMMWFGNLVTCAWWDNLWLN EGF 403
DAPN2 ENWGLVVYREVALLVTEGVTTATRONIGRIICHENMHMMWFGNEVSPYSWTYTWLN EGF 366
DAPN3 ENWGLLT YREAYLMYDENHTNSYFKQLIAYILSHEIAHMMWFGNLVTCDDWWDALWLN EGF 406

DsAPN1 RFYQYYLTASVAPELGYETRFIVEQFEQAMSADSVDTAHALTNQAVSDPITVSAHFSSIT 463
DAPN2 NFEFNFATDLVLP EWRMDQFVIN-MQNVFQSDAVISINEMTHP-VFTPSQILGTENAVA 424
DAPN3 RYQYYLTHWVAPEMGLATRFITEQIHTSLLSDSADFPHPLTNP GVGSPASVSAMFSTLS 466

DsAPN1 YARGACILRMTEHLLSHTTFVKGLRKYQLQDRSYDVAEPHDLFKALDDAALEDGVLNDY-D 522
DAPN2 YQKSGSVIRMIQHFMTPELERQGLVHYIKTRMQRKAAQPADLYRSLQHVLQNSNHSIPF-- 482
DAPN3 YNKGAAVIRMT EHLMGFQNHHLGLRRYIISNRQFQATARPIDLFE DLQVSAVETGAIADYGP 526

DsAPN1 GITIDQYFRSWEKAGHPLLTVNI DQATGEMTIKQARWERSSSGESIHFSLWDIPITWTRA 582
DAPN2 --SIESILTRWTTGGFPVLNVTRSSAAANSLVFQ QERYLTD RSLSSPDRWHVPINVVLN 540
DAPN3 DFNIVDYK TWT EQGGHPVLNVAVDHQTGNMTITQRRFNINTGYSTANSHWIIPVTFATA 586

DsAPN1 DDPDFLNLKPSQFMRTESTVIQR---GTTGNEWVFNKQASGFYRINYDNTN WALITQAL 639
DAPN2 DNPDFSDTKPDGIVSITATSIDVPGLSGAEWYIVNKQQTGYRVNYDEANWRALTOVL 600
DAPN3 SNPDEFNTKPTHIRISLTLINR---GTIGDEWYIFNKQQTGYRVNYDDYTWDLIVMQ 643

DsAPN1 R-SDVHVIHEYNRAQIIDDLFNEGRATVMPYDRVFNIISFLEFEDQYAPWINAITGFNFI 698
DAPN2 A-NNHNVIHVLNRAQIIDD SFNLARNGRLSYAHFPQIATYLVNEEDYIPWASANA AISYL 659
DAPN3 RGPNR TD IHEYNRAQIVNDVFQFARSGLM TYTRAFNILSFLEHEDA YAPWVAAITGFNWI 703

DsAPN1 IRRLAHDTPNLKRLQEVIIAFSKTITARLG YGEIENEPFMDGLLRMYVMQFLCNIGDQQC 758
DAPN2 DVVLSG-TEVYELFQRFLELTA PYSYERLTFNFPSEEHVTPY YRNIILDLNCRYGNQC 718
DAPN3 NNRVIG-TPLEEPLNALFRSWAVNIMSDLSYEP EIPGESFMRSYLRYQLA PVMCRIGHPC 762

DsAPN1 INVGKERFTMWRSG-NVHIPANMRPWVYCVGLREGTAEDFNFFW GKYLNE DLASEQVVM 817
DAPN2 VNTSMQLLQVQVTC--STLNPD IQTLVYCSGLRGGNAANFDFLWGLYTETTDSSAQAIL 776
DAPN3 LSAASSQFSA LRNAPFTEVAVDSRNWVYCNGLRQGLFSQYEF LKNRFLNHVYTEKIQIL 822

DsAPN1 QAAGCTTDQNSLTVFLDAIVAE EEEIVRPQDLTTALNSAVTRNEVNTLRVFEWLKNNIDKT 877
DAPN2 NALGCTSN EKRTPYLRQ MIDENSLVREQDRHTI AVATINS GPENMEYALDFIENFPSI 836
DAPN3 MVLGCTEDATA LRQFMDYIVDDNFVIRRDYNTAFNSAVTGNENNTDIIFQYIGENLDRV 882

DsAPN1 AAKFG---SITTPLSYI APRLLTQSDIDRFESWLQENEDRIGPTAFATGSSGVNNVRNAL 934
DAPN2 QPNVQGLTGT TNILNALARRLTTTSHLQKINAFATSHANIFTAGELASIAAIRENIAASV 896
DAPN3 ANAFG---SVV IPLSSVAARLRTPADVTRFRNWAVENRGALG-MNFQQVFNA AETSLSAL 938

DsAPN1 IWSDLRIPEIVKFL ENGYVEDDIENNTTTTETTTTETTTTETTTTETTTTETTTT 994
DAPN2 SWNDQNLN NVESWINDY Y-----Y 916
DAPN3 QWAQEN TADFNNYFTG---NVE-----LVTS TPAPIVTTTPVT 974

DsAPN1 DTTTTTEAPTEAPTEAPTEAPTEAPTEATETTTTETTTTETTTTETTTTETTTTETTTT 1054
DAPN2 GSASAMT----- 923
DAPN3 ATVA AIS-----AP-----TP----- 986

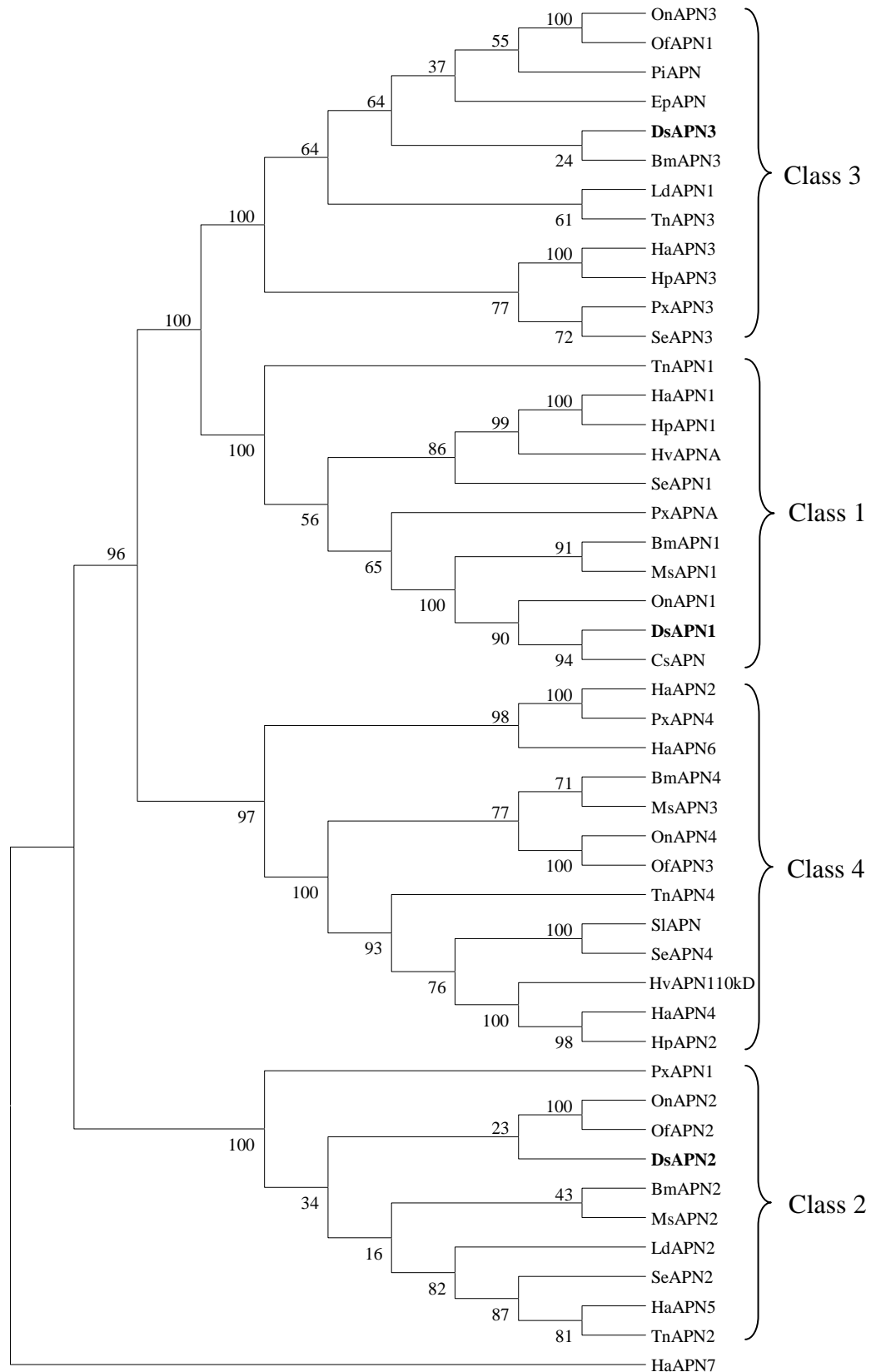
DsAPN1 TTTTEAPTEAPTEAPTEAPTEAPTEAPTEAPTEAPTEAPTEAPTEAPTEAPTEAPTEAPTE 1114
DAPN2 -----ASIVVLLSFFVTLFNQ----- 939
DAPN3 -----DLE-----DSANTS FISILTIISALLIK----- 1009

DsAPN1 FFN 1117
DAPN2 L- 940
DAPN3 YIF 1012

Glycosylation is generally regarded as an important determinant of Cry1A-binding (Pigott and Ellar, 2007). Similarly, the three APNs from *D. saccharalis* had the predicted N-glycosylation sites as identified from other lepidopterans by NetNGlyc 1.0 Server (Gupta et al., 2004). Results of this study showed that DsAPN1 had three N-glycosylation sites at Asn128, Asn189, and Asn960, while DsAPN2 had five N-glycosylation sites at Asn60, Asn209, Asn477, Asn501, and Asn720. In DsAPN3, there were eight N-glycosylation sites at Asn110, Asn142, Asn223, Asn242, Asn303, Asn556, Asn647, and Asn866. Additionally, O-glycosylation sites were also predicted in the regions close to the C-termini of the three APNs of *D. saccharalis* using the NetOGlyc 3.1 server (Julenius et al., 2005). In DsAPN1, there were 76 putative O-glycosylation sites ranged from Thr963 to Thr1063 and all of them were threonines. DsAPN3 contained eight putative O-glycosylation sites at Thr969, Thr970, Thr971, Thr974, Thr976, Ser981, Thr984 and Thr985; whereas, there was only one predicted O-glycosylation site in the N-terminal region for DsAPN2 that was at Thr734.

A phylogenetic tree was generated based on the information available among lepidopteran species, in which 47 APN genes have been identified from 16 lepidopteran species. Based on the sequence similarity analysis, these APN genes could be classified into four classes (Fig. 3.3). Class 1 contained APN1s/APNAs from 11 species. Class 2 mainly contained APN2s from 8 species as well as an APN1 from *Plutella xylostella* and an APN5 from *H. armigera*. Class 3 and 4 mostly consisted of APN3s and APN4s, respectively. One APN (HaAPN7 from *H. armigera*), which was categorized in the class 5 in Herrero et al. (2005), is apparently different from others because it had only 17.5-21.8% sequence identity with other 46 APNs. Therefore, classification of HaAPN7 was not resolved in our phylogenetic analysis of 47 APNs. The phylogenetic analysis revealed that the DsAPN1, DsAPN2 and DsAPN3 of *D. saccharalis*

Figure 3.3. A phylogenetic tree generated by ClustalW alignment of aminopeptidase N amino acid sequences from lepidopteran species using MEGA. Bootstrap values, expressed as percentages of 500 replications, are shown at branch points. In addition to the three *Diatraea saccharalis* APNs, other lepidopteran APN sequences used in the analysis included: *Ostrinia nubilalis* APN1 (OnAPN1), APN2 (OnAPN2), APN3 (OnAPN3), and APN4 (OnAPN4) (GenBank accession nos. ABL01481, ABL01482, ABL01483, and ABL01484); *Ostrinia furnacalis* APN1 (OfAPN1), APN2 (OfAPN2), and APN3 (OfAPN3) (GenBank accession nos. ABQ51393, ACF34999, and ACF34998); *Spodoptera exigua* APN1 (SeAPN1), APN2 (SeAPN2), APN3 (SeAPN3), and APN4 (SeAPN4) (GenBank accession nos. AAP44964, AAP44965, AAP44966, and AAP44967); *Trichoplusia ni* APN1 (TnAPN1), APN2 (TnAPN2), APN3 (TnAPN3), and APN4 (TnAPN4) (GenBank accession nos. AAX39863, AAX39864, AAX39865, and AAX39866); *Plutella xylostella* APNA (PxAPNA), APN1 (PxAPN1), APN3 (PxAPN3), and APN4 (PxAPN4) (GenBank accession nos. AAB70755, CAA66467, AAF01259, and CAA10950); *Bombyx mori* APN1 (BmAPN1), APN2 (BmAPN2), APN3 (BmAPN3), and APN4 (BmAPN4) (GenBank accession nos. AAC33301, BAA32140, AAL83943, and BAA33715); *Helicoverpa armigera* APN1 (HaAPN1), APN2 (HaAPN2), APN3 (HaAPN3), APN4 (HaAPN4), APN5 (HaAPN5), APN6 (HaAPN6), and APN7 (HaAPN7) (GenBank accession nos. AAK85538, AAK85539, AAN04900, AAN04899, ACB54941, ACA35025, and ACA35024); *Helicoverpa puntigera* APN1 (HpAPN1), APN2 (HpAPN2), and APN3 (HpAPN3) (GenBank accession nos. AAF37558, AAF37559, and AAF37560); *Heliothis virescens* 110 kDa APN (HvAPN110kD) and APNA(HvAPNA) (GenBank accession nos. AAK58066 and AAF08254); *Epiphyas postvittana* APN (EpAPN) (GenBank accession no. AAF99701); *Lymantria dispar* APN1 (LdAPN1) and APN2 (LdAPN2) (GenBank accession nos. AAD31183 and AAD31184); *Plodia interpunctella* APN (PiAPN) (GenBank accession no. AAC36148); *Manduca sexta* APN1 (MsAPN1), APN2 (MsAPN2), and APN3 (MsAPN3) (GenBank accession nos. CAA61452, CAA66466, and AAM18718); *Spodoptera litura* APN (SlAPN) (GenBank accession no. AAK69605); *Chilo suppressalis* APN (CsAPN) (GenBank accession no. ABC69855).



belonged to the classes 1, 2, and 3 respectively. The three DsAPNs of *D. saccharalis* closely related to *Chilo suppressalis*, *P. xylostella*, and *Epiphyas postvittana* counterparts (Fig. 3.3).

Other closely related species included *Ostrinia nubilalis* and *O. furnacalis*.

3.3.3 Expression of Three APN Genes of Cry1Ab-SS and -RR Strains of *D. saccharalis*

Transcription profiling for the three APN genes (DsAPN1, DsAPN2, and DsAPN3) of *D. saccharalis* was analyzed for 3rd and 5th instars. Expression levels of mRNA of Cry1Ab-RR strain were significantly down-regulated compared to the same aged larvae of Cry1Ab-SS strain for all three APNs and for the two instars ($P = 0.0021$ and 0.0047 for DsAPN1 at 3rd and 5th instars, $P = 0.0104$ and 0.0049 for DsAPN2 at 3rd and 5th instars, and $P = 0.0027$ and 0.0087 for DsAPN3 at 3rd and 5th instars, respectively) (Fig. 3.4). Compared to 5th instars of Cry1Ab-SS strain, mRNA levels of 5th instars of Cry1Ab-RR strain were reduced by 67.9% for DsAPN1, 67.1% for DsAPN2, and 62.5% for DsAPN3, while they were reduced by 31.3, 26.7, and 38.2% for the 3rd instars, respectively. Of the three APNs, mRNA for DsAPN3 was the most abundant, while mRNA for DsAPN2 expressed the least. It was apparent that mRNA levels of the 3rd instars were greater than those of the 5th instars for both insect strains (Fig. 3.4).

3.3.4 Validation of dsRNA Acquisition in Larvae of *D. saccharalis*

To validate the acquisition of dsRNA, ten 3rd instars of *D. saccharalis* were orally fed with dsRNAs and blue food color (Fig. 3.5). This approach depended on volunteer feeding for dsRNA acquisition. Within 3 h, blue coloration was detected throughout the digestive tracts in all assayed individuals, which could be observed through the dorsal cuticle (Fig. 3.5). After 3h, blue coloration was observed in dissected larvae guts over their entire lengths. This was consistent with a previous feeding study showing dsRNA acquisition through the gut of the termite

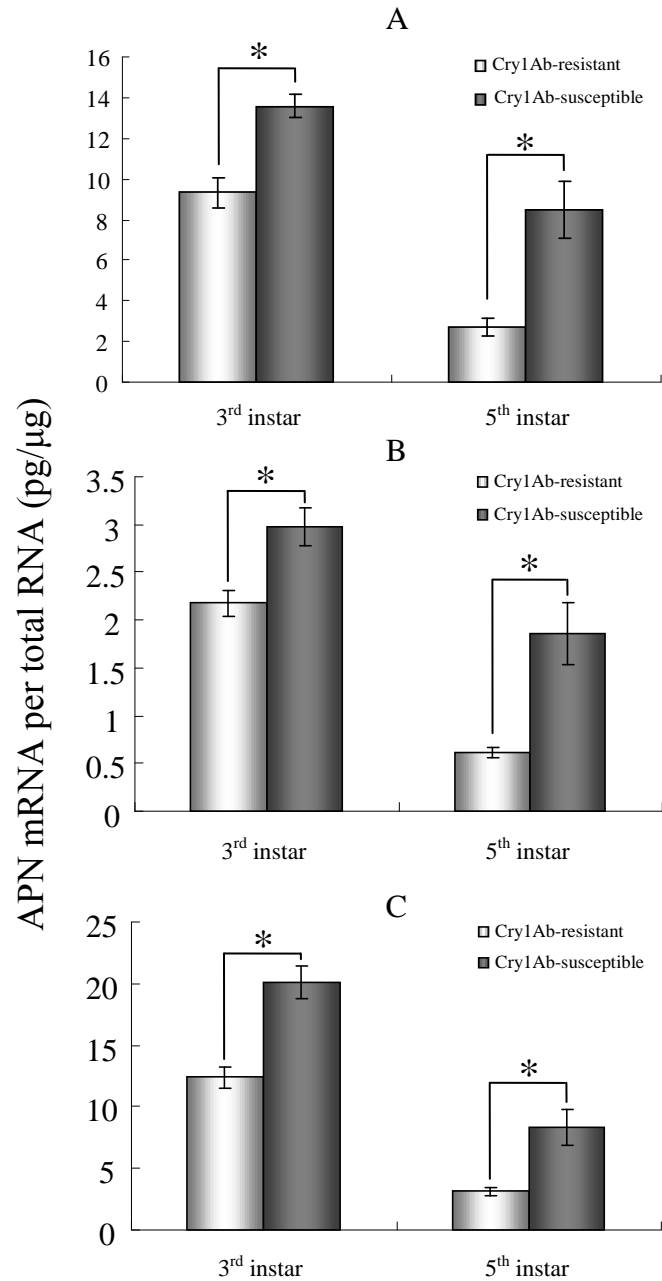


Figure 3.4. Gene expression levels of three aminopeptidase N genes (A: DsAPN1, B: DsAPN2, and C: DsAPN3) in 3rd and 5th instars of the Cry1Ab-susceptible and -resistant strains of *Diatraea saccharalis*. Absolute transcript abundance (APN mRNA per total RNA (pg/μg)) was determined using qRT-PCR and SYBR green. Bars represent the means and standard errors of 5 total RNA samples that each contains a pool of total RNAs from 3 larvae. Asterisk indicates significant differences between Cry1Ab-susceptible and -resistant strains (Student's t-test, $P < 0.05$).

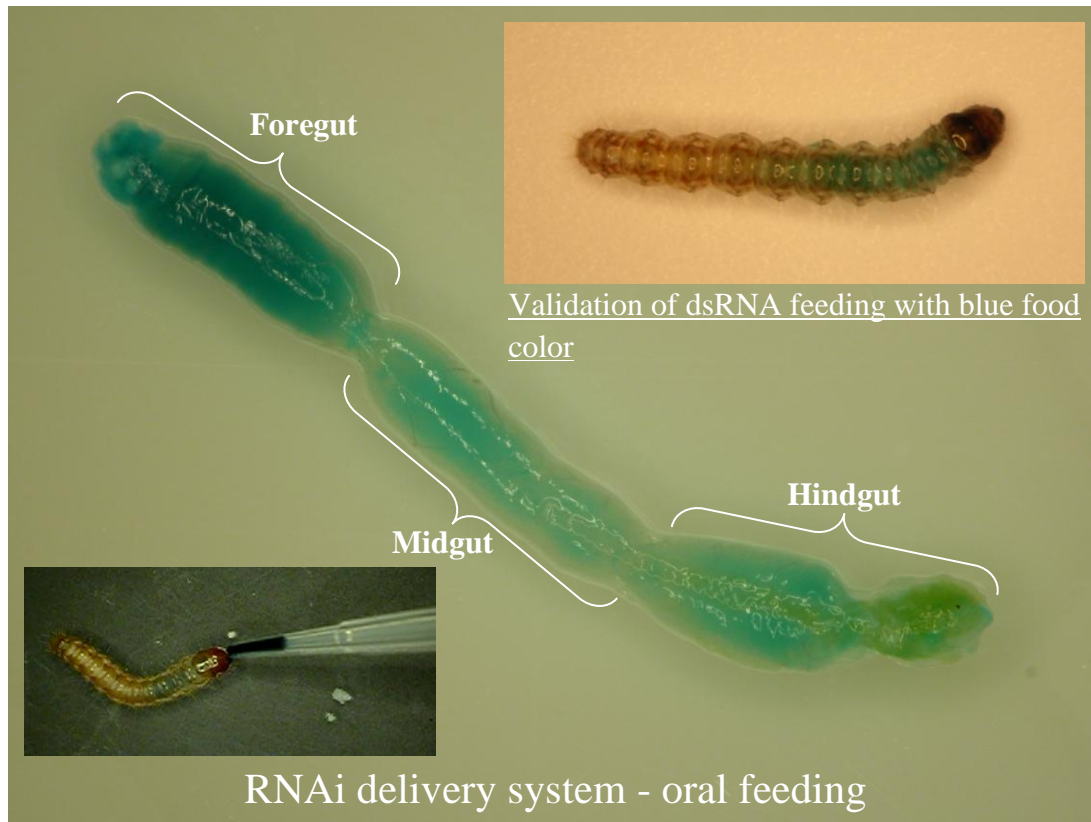


Figure 3.5. Oral dsRNA feeding to larvae of *Diatraea saccharalis*. Top right: one example of 3rd instar after 3 h of dsRNA + blue food color oral feeding. The prominent gut was stained blue, which was visible through the cuticle. Center: a dissected larval gut after dsRNA + blue food color feeding. The three gut regions are highlighted: foregut (top), midgut (middle) and hindgut (right). Bottom left: a picture representing the oral feeding assay with dsRNA + blue food color through an Eppendorf pipette.

Reticulitermes flavipes (Zhou et al., 2008). The results of this study showed that *D. saccharalis* larvae could rapidly acquire dsRNA through oral feeding using a pipette.

3.3.5 Knockdown DsAPN1, DsAPN2, and DsAPN3 by RNAi

Varied levels of gene knockdown by RNAi were observed for the three APNs of *D. saccharalis*. qRT-PCR analyses showed that gene expression of DsAPN1, DsAPN2, and DsAPN3 in 3rd instars 24 h after feeding 250 ng dsRNA/larva was reduced by 37, 25, and 44%, respectively (Fig. 3.6). The reduction for DsAPN1 and DsAPN3 was statistically significant ($P = 0.0005$ for DsAPN1 and $P = 0.0096$ for DsAPN3), but it was not significant for DsAPN2 ($P = 0.112$) (Fig. 3.6).

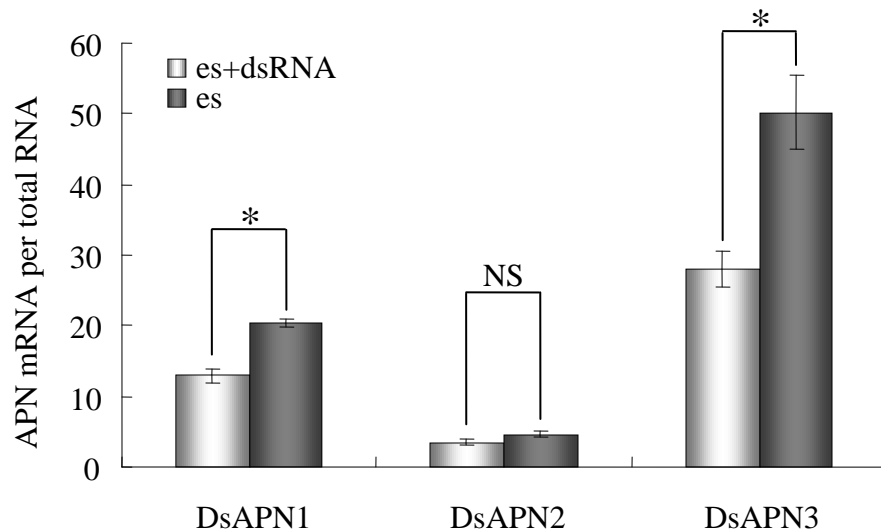


Figure 3.6. Transcriptional levels of the three DsAPNs after dsRNA feeding with 3rd instars from Cry1Ab-susceptible strain of *Diatraea saccharalis*. DsAPN1, DsAPN2, and DsAPN3 genes were treated with 250 ng dsRNA via droplet feeding. With elution solution (ES)-fed larvae as a control group, significant reduction of mRNA level was detected in RNAi-treated larvae for DsAPN1 and DsAPN3. However, there was trend of reduction of transcriptional level observed in RNAi-treated larvae for DsAPN2. Bars represent the means and standard errors of 4 total RNA samples that each contained a pool of total RNAs from 3 larvae. Asterisk indicates significant difference ($P < 0.05$, Student's t-test) and NS indicates no significant difference ($P = 0.112$, Student's t-test).

3.3.6 Effects of RNAi on Total APN Activities

Total APN activity of 3rd instars 24 h after dsRNA feeding was significantly lower than that of controls for all three DsAPNs ($F = 715.68$; $df = 3, 28$; $P < 0.001$) (Fig. 3.7). The reductions in total APN activity of the RNAi-treated larvae compared to the control were 37.1% for DsAPN1-RNAi, 31.2% for DsAPN2-RNAi, and 11.6% for DsAPN3-RNAi. The reduction for DsAPN1-RNAi was significantly greater than those for DsAPN2-RNAi and DsAPN3-RNAi ($P < 0.05$). There was also a significant difference in total APN activity between the larvae treated with DsAPN2-RNAi and DsAPN3-RNAi ($P < 0.05$) (Fig. 3.7).

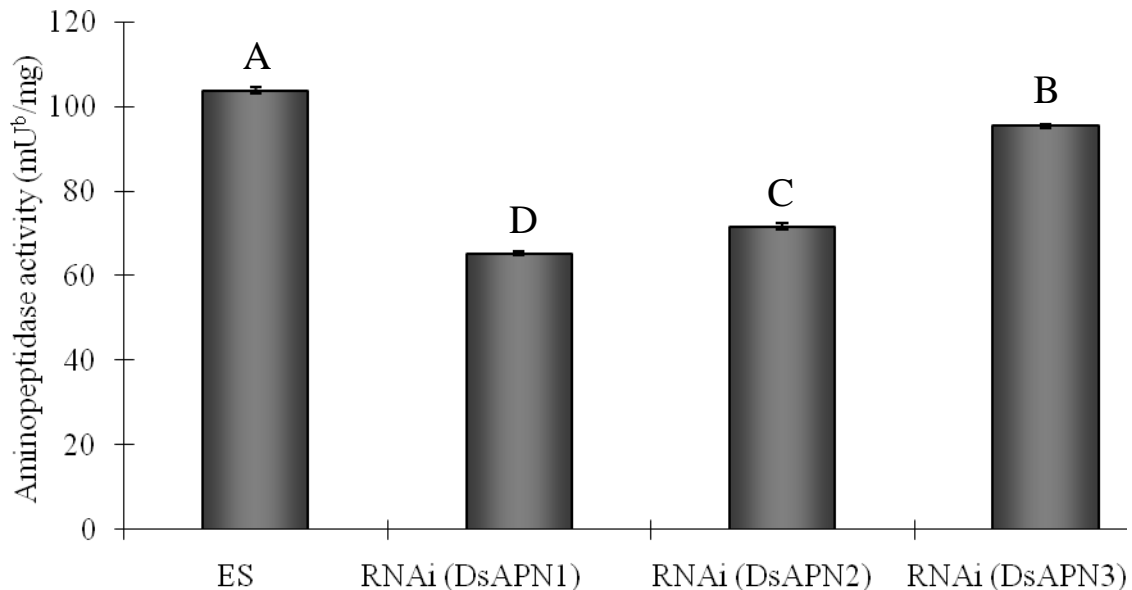


Figure 3.7. Total aminopeptidase N activity of whole gut^a tissue of 3rd instars from Cry1Ab-susceptible strain of *Diatraea saccharalis* after RNAi for the three DsAPNs. DsAPN1, DsAPN2, and DsAPN3 genes were silenced by a 250 ng dsRNA droplet feeding. Total APN activities of the three RNAi treatments decreased significantly compared to that of the control (ES). Bars represent the means and standard errors of 8 total gut samples, each of which contains one larva. Duplicate reads as technical replicates were collected and averaged for each sample. Different letters indicate significant differences (one-way ANOVA, $F = 715.68$, Bonferroni post hoc test, $P < 0.001$).

^a Whole gut including gut tissue plus gut contents in the lumen.

^b One unit of enzyme activity was defined by hydrolysis of 1 μ mol of substrate in 1 min.

3.3.7 Effects of RNAi on Larval Susceptibility to Cry1Ab Toxin

Mortalities of RNAi-treated larvae fed on diet containing 16 µg Cry1Ab/g diet were significantly ($F = 27.38$; $df = 3, 8$; $P = 0.0001$) less than those of ES-treated control larvae for all three APNs (Fig. 3.8). Larval mortality of ES-treated control larvae at the Cry1Ab concentration of 16 µg/g diet was 43.8%, while it was only 13-14% for RNAi-treated insects. There were no significant differences in larval mortality among the three RNAi treatments ($P > 0.05$).

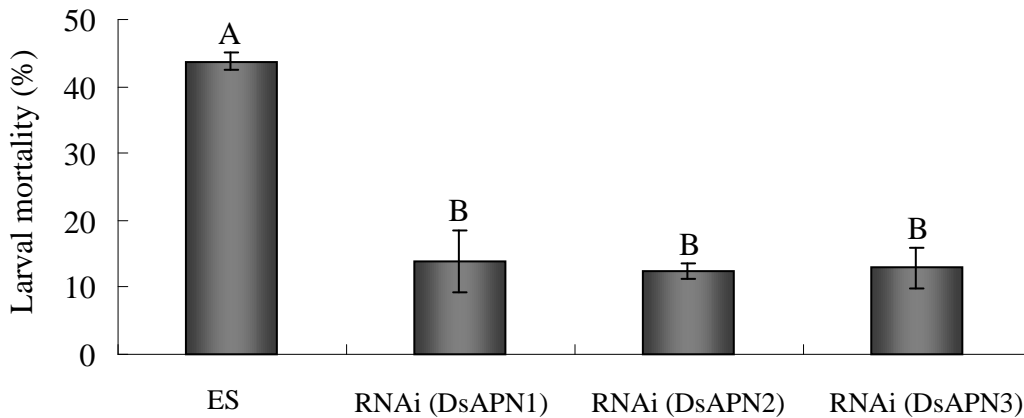


Figure 3.8. Larval mortality of larvae from Cry1Ab-susceptible strain of *Diatraea saccharalis* after RNAi for the three DsAPNs. The RNAi-treated neonates were droplet fed 0.1 µl elution solution (ES) containing 50 ng dsRNA for DsAPN1, DsAPN2, or DsAPN3 while the non-treated neonates were fed ES only before released for bioassays. Mortality of RNAi-treated larvae decreased significantly for all three DsAPNs after 7 days exposure to diet containing 16 µg Cry1Ab per gram diet. The bioassays were conducted in three independent experiments and each treatment/control contained 48 neonates. Bars shown are the means of three experiments with their standard errors. Different letter indicates significant differences (one-way ANOVA, $F = 27.38$, Bonferroni post hoc test, $P = 0.0001$).

3.4 Discussion

APNs play a prominent role in proteolysis in Lepidoptera, as shown by the significant reduction of azocasein proteolysis by adding a specific APN inhibitor (Ortega et al., 1996). Thus it is not surprising that APNs are so strongly expressed in larval gut. As APNs perform the physiological function of protein digestion, studies of insect APN activities and their gene

expression have so far been focused on the insect midgut. *D. saccharalis* is a generalist herbivore with a broad host range (Castro et al., 2004). Therefore, it is expected that the digestive proteases in *D. saccharalis* are composed of numerous types that are presumably different from each other in susceptibility to protease inhibitors from host plants and substrate specificity. The presence of multiple APNs in lepidopteran larvae has also been considered to be important for digestion of peptides with various N-terminal residues (Hua et al., 1998; Emmerling et al., 2001). To date, at least four classes of APN isoforms have been identified from lepidopteran species (Herrero et al., 2005; Pigott and Ellar, 2007). Cloning of more diverse APNs, such as HaAPN7 from *H. armigera* (Angelucci et al., 2008), indicated that additional classes of APNs might exist. Based on our phylogenetic analysis, classes 1 to 4 correspond with four of the lepidopteran clades reported in *B. mori*, *P. xylostella*, *H. armigera* and *Trichoplusia ni* (Nakanishi et al., 2002; Wang et al., 2005). In this study, three APN isoforms were found in *D. saccharalis* by screening approximately 12,500 clones from both Cry1Ab-SS and -RR cDNA libraries. Due to the limitation of cDNA library capacity, other potential APN isoforms could exist in *D. saccharalis*.

As observed in other lepidopteran species (Emmerling et al., 2001; Simpson et al. 2008; Angelucci et al., 2008), mRNA expression of APNs in *D. saccharalis* varied in different life stages. The higher expression level of APNs in earlier instars of *D. saccharalis* detected in the current study may be a result of adaptation to the protein source of the diet and increase enzyme levels in response to the form of the available protein. Results from our study also showed that the levels of gene expression levels varied for the three APNs in *D. saccharalis*. The expression of DsAPN3 was approximately 7-fold greater than that of DsAPN2. Studies have shown that expression of APNs of classes 1, 2, 3, and 4 in *H. armigera*, was approximately 1000-fold greater than that of class 5 APN (Angelucci et al., 2008). Emmerling et al. (2001) also reported

that expression of HpAPN3 was the lowest among the three APNs in *Helicoverpa punctigera*. Similarly in *Epiphyas postvittana*, expression levels of the five APNs identified were considerably different: expression of EpAPN4 was the highest whereas that of EpAPN5 was the lowest (Simpson et al., 2008).

In lepidopterans, midgut APNs are proposed to be involved in the pathogenesis of Bt toxins as receptors of the toxins (Bravo et al., 2004). It has been documented that APN is a functional receptor to Cry1A in *M. sexta* (Gill and Ellar, 2002). In some insect species only a single APN was found to be involved in binding a single Bt toxin (Luo et al., 1997; Valaitis et al., 2001; Pigott and Ellar, 2007). Our results showed all three APNs identified in the present study appeared to be associated with Bt toxicity in *D. saccharalis*. Additional studies on Bt binding have been proposed to confirm if all three APNs involve in Cry1Ab binding in *D. saccharalis*. Bt binding associated with multiple APN genes in an insect has also been reported in several other insect species (Pigott and Ellar, 2007). For example, four out of the seven APNs identified in *H. armigera* were involved in Cry1Ac-binding (Angelucci et al., 2008). Similarly, at least three APNs were associated with Cry1Ac binding in *H. virescens* (Banks et al., 2001) and four APNs could bind to the exogenous non-denatured Cry1Aa protein in *P. xylostella* (Nakanishi et al., 2002). The functional relevance of APNs to Bt Cry toxins was also demonstrated in *Spodoptera litura* and *H. armigera* by using a gene silencing strategy with RNAi (Rajagopal et al., 2003; Sivakumar et al., 2007). Rajagopal et al. (2002) reported that silencing of APN of *S. litura* by RNAi resulted in tolerance of larvae to Cry1C. Among those species, deletion mutation of APN1 in *H. armigera* was genetically linked to Cry1Ac susceptibility (Zhang et al., 2009). Similarly, a Cry1Ac resistance strain of *S. exigua* was found to be associated with the lack of the mRNA transcript encoding a GPI-anchored APN1 (Herrero et al., 2005). Results of the current study

showed that cDNA sequences of the three APN genes were identical between the Cry1Ab-SS and -RR strains of *D. saccharalis*. However, total APN activity and transcriptional expression levels of the three APNs of the Cry1Ab-RR larvae were significantly reduced compared to the Cry1Ab-SS. It appeared that a cumulative effect of the reduced expression of all APNs might contribute to the Cry1Ab resistance in *D. saccharalis*.

In the current study, functional genomics analysis of the three APNs was also conducted using RNAi technology to validate the association of Cry1Ab susceptibility with the three APNs in *D. saccharalis*. Recent functional genomics research has demonstrated that RNAi is an achievable approach in studying insect resistance (Rajagopal et al., 2002; Sivakumar et al., 2007; Bautista et al., 2009). Rajagopal et al. (2002) reported that an injection of dsRNA silenced an APN gene in the midgut of *S. litura* larvae, but a dsRNA feeding attempt was not successful. Non-invasive oral delivery of dsRNA has recently become a more attractive method in RNAi study although it might be less effective compared to dsRNA injection procedure (Araujo et al., 2006). The oral droplet feeding procedure for dsRNA delivery to larvae of *D. saccharalis* was proven to be successful in the current study. It was believed that this delivery method can minimize undesirable effects caused by larvae manipulations compared to the injection procedures (Bautista et al., 2009). Our studies demonstrated that larvae of *D. saccharalis* could rapidly acquire dsRNA through the non-invasive feeding under natural conditions. In addition, studies have showed that most lepidopteran gut is a hostile environment for RNAs because of the alkaline pH and numerous RNases (Terra and Ferreira, 1994). Therefore, not all lepidopteran species are suitable for RNAi initiated with dsRNA feeding due to variation in the midgut environment. We measured the pH values of gut contents from Cry1Ab-SS and Cry1Ab-RR larvae of *D. saccharalis* reared on non-Bt diet and found that the midgut pH in both strains was

8.9 (Yang, unpublished data), which was well within the pH range reported in other Lepidoptera (Berenbaum, 1980). The significant suppression of the three APNs after RNAi indicated that dsRNA appeared to remain sufficiently intact in the midguts of *D. saccharalis*, suggesting that the dsRNA droplet feeding method with an optimized dose at appropriate insect stages could circumvent such disadvantages. Furthermore, the RNAi effects may vary in different species, instars, and even among individuals within a same species regardless of delivery methods (Geldhof et al., 2006). To reduce variability in individuals' responses to RNAi, a relatively high dose of dsRNA (250 ng for each 3rd instar and 50 ng for each neonate) (Kamath et al., 2000; Bautista et al., 2009) was used in the current study and samples were pooled for qRT-PCR. Although the reduction in expression (25%) of APN2 by dsRNA was not statistically significant at $\alpha = 0.05$ level, results of the present study generally showed that expressions of the three APN genes were all down-regulated after RNAi.

Knocking-down one gene at a time may not cause obvious changes in susceptibility in *D. saccharalis*. To overcome this limitation, we used qRT-PCR to verify the suppression of the target gene and *in vivo* bioassay to examine the changes in Cry1Ab susceptibility in RNAi-treated insects. The bioassays showed that silencing of any one of the three APNs in *D. saccharalis in vivo* by RNAi resulted in a decrease in Cry1Ab susceptibility. The present study also showed that larval mortality of *D. saccharalis* on Cry1Ab-treated diet could be significantly reduced by only a partial suppression of any of the three APNs. However, statistical correlation between the changes in transcript levels and the relative Cry1Ab susceptibility after RNAi could not be established due to the similar response (e.g., 25-44% reduction in gene expression versus 29.8-30.8% reduction in larval mortality) of the larvae to the three dsRNAs. A previous study also showed that 3rd instars of *D. saccharalis* were much more tolerant to Cry1Ab toxin than

neonates (Huang et al., 2006). Measurement of 3rd instar mortality of *D. saccharalis* on Cry1Ab-treated diet could not be performed because the amount of Cry1Ab toxin needed for bioassays that could cause a significant mortality to 3rd instars was unaffordable. Therefore, determination of Cry1Ab susceptibility was conducted only for neonates of *D. saccharalis*, while 3rd instars were used in examination of gene expression. Correspondingly, different dsRNA concentrations (50 ng for a neonate and 250 ng for a 3rd instar) were applied in treating neonates for assaying Cry1Ab susceptibility and 3rd instars for analysis of gene expressions and APN activity. The use of different insect life stages and dsRNA concentrations might also make it difficult in analyzing the correlation between reductions in expression levels of APNs in 3rd instars and larval mortality in neonates. In addition, the present study did not include a test to examine if RNAi of other genes non-related to Cry1Ab toxicity affects larval mortality. However, data from parallel experiments clearly showed that the significantly down-regulating expressions of the three APN genes by RNAi were corresponding to the significant reductions in the specific APN activity. These results strongly suggest that reduction in expression of the three APNs was functionally associated with the Cry1Ab resistance in *D. saccharalis*. A previous study suggested that Cry1Ab resistance in this strain of *D. saccharalis* was likely inherited as one or a few tightly linked incompletely recessive gene(s) (Wu et al., 2009a). If the single gene inheritance is true, it is possible that an upstream regulatory gene for the three APN genes may exist in *D. saccharalis*. As determined from some P450-mediated resistance instances, changes in the upstream regulatory gene (mutation, deletion, etc.) could result in down-regulated expressions of its downstream genes which may result the resistance to pesticides (ffrench-Constant et al., 2006). Otherwise, these three APN genes could be tightly linked in *D. saccharalis* as the counterparts observed in *B. mori*. It has been documented from SilkDB in China

(<http://silkworm.genomics.org.cn/>) that at least three APNs in *B. mori* are located sequentially and closely linked to each other. Molecular studies have shown that Bt resistance in insects could be also associated with other mechanisms. Studies to examine other possible Bt resistance mechanisms in *D. saccharalis* are currently undergoing.

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CHAPTER 4

IDENTIFICATION AND FUNCTIONAL ANALYSIS OF A CADHERIN GENE FROM *BACILLUS THURINGIENSIS*-SUSCEPTIBLE AND -RESISTANT STRAINS OF SUGARCANE BORER, *DIATRAEA SACCHARALIS*

4.1 Introduction

A soil-borne bacterium, *Bacillus thuringiensis* (Bt), can produce crystal inclusions composing of one or various entomopathogenic Cry proteins (also known as δ -endotoxins) (Schnepf et al., 1998; de Maagd et al., 2003). Due to the toxicity to some insects, Cry proteins have been used to control many agricultural insect pests and disease vectors including Lepidoptera, Coleoptera, and Diptera (Schnepf et al., 1998; Pigott and Ellar, 2007). In addition, transgenic plants including corn and cotton which express Cry proteins have been planted in the United States and many other countries to control phytophagous insect pests (Van Rie, 2000). Transgenic Bt corn for controlling corn insect pests (e.g., stalk borers, rootworms) is the most widely grown Bt crops in U.S. and the whole world (NASS, 2010; James, 2010). The sugarcane borer, *Diatraea saccharalis*, is a major corn pest and a primary target of Bt corn in the mid-south region of United States (Castro et al., 2004; Huang et al., 2006). Recently, a Cry1Ab resistant strain of *D. saccharalis* has been established using the F₂ screening method (Huang et al., 2007a). This Bt-resistant strain is able to complete entire larval development on commercial Cry1Ab corn plants and is an ideal subject for studying Bt resistance mechanisms in corn stalk boring species.

Effects of Bt Cry protein as toxins involve a multistage process. Among those, binding of the activated toxin is a prerequisite for subsequent toxin activation, toxin aggregation, and pore formation (Bravo et al., 2005; Pigott and Ellar, 2007). Cadherins are believed to be a primary binding receptor to Cry proteins. The binding to cadherins in the midgut has been proposed to

induce a conformational change in Cry1A toxins that facilitates the formation of a pre-pore toxin oligomer and finally lead to osmotic imbalance of the insect gut (Gómez et al., 2002). Genetic and molecular studies have revealed that some regions of cadherins in *Bombyx mori*, *M. sexta*, and *Heliothis virescens* were involved in binding to Cry1Aa, Cry1Ab, and Cry1Ac toxins (Nagamatsu et al., 1999; Hua et al., 2004; Gahan et al., 2001). Mutation, resulting in premature stop codon in the cadherin gene, was first proven to lead to resistance to Cry1A toxins in a laboratory-selected strain of *H. virescens* (Gahan et al., 2001). Three cadherin alleles in a field-isolated resistant strain of *Pectinophora gossypiella* were also linked with the resistance to the Cry1Ac protein in transgenic Bt cotton (Morin et al., 2003). Recently, Xu et al. (2005) also reported that the disruption of a cadherin gene was associated with a high level of resistance to Cry1Ac in a laboratory-selected *Helicoverpa armigera* strain. In addition, a critical toxin-binding site in cadherin gene was also identified in *H. virescens* and point mutations at this site affected its toxin binding ability to Cry1A toxins (Xie et al., 2005).

Given that mutations in cadherins are associated with resistance to Cry1A in several Lepidoptera, any changes on cadherin(s) could also lead to the Cry1Ab resistance in *D. saccharalis*. The objective of this study was to determine if Cry1Ab resistance in *D. saccharalis* is associated with changes in gene structure/expression of cadherin(s) in the midgut.

4.2 Materials and Methods

4.2.1 Insect Strains

A Cry1Ab-resistant strain (Cry1Ab-RR) of *D. saccharalis* was selected from a single two-parent family-line from a collection in a corn field near Winnsboro in Franklin Parish, Louisiana, during 2004 (Huang et al., 2007a). The Cry1Ab-RR strain was shown to carry a major resistance allele identified using an F₂ screen method. It has demonstrated a significant resistance

level (ca. 100-fold) to purified trypsin-activated Cry1Ab toxin (Huang et al., 2007b). Individuals of the Cry1Ab-RR strain could complete larval development on commercial Bt corn hybrids expressing the Cry1Ab protein. A Cry1Ab-susceptible strain (Cry1Ab-SS) of *D. saccharalis* was also developed from the same location. Cry1Ab-RR strain had been backcrossed with the Cry1Ab-SS strain and re-selected on Cry1Ab corn leaf tissue for 3 times before it was used in this study.

4.2.2 Full-Length cDNA Cloning of a Cadherin Gene from *D. saccharalis*

cDNA libraries of Cry1Ab-SS and -RR strains of *D. saccharalis* were constructed, sequenced, and BLASTed by Dr. Yu Cheng Zhu (USDA-ARS, Stoneville, MS). To clone full-length cadherin cDNAs, results from BlastX similarity searching (Altschul et al., 1997) of both the cDNA libraries were sorted. Three contigs that matched the characters of other cadherin genes available from the Genbank were identified in the libraries of both Cry1Ab-SS and -RR strains of *D. saccharalis*. Sequences of the three contigs partially overlapped each other and thus they belonged to only one gene. These three contigs were then assembled into incomplete cDNA of one cadherin gene, named DsCAD1. Cloning of the full-length cDNA of DsCAD1 from *D. saccharalis* was achieved using the similar procedures as described in Zhu et al. (2004) with some modifications mentioned previously in (Chapter 3). Partial cadherin cDNA sequences from the midgut cDNA libraries of *D. saccharalis* were first assembled using SeqMan module of the Lasergene (DNAStar, Madison, WI). To obtain full length cDNA of DsCAD1, three larval guts from each of the Cry1Ab-SS and -RR strains were homogenized and total RNA was precipitated according to the manufacturer's protocols. The concentration of total RNA was determined using NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The SuperScript First Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA) was used in a reverse transcription (RT)

reaction with 5 µg of total RNA and an oligo-dT primer for cDNA synthesis. The template RNA was removed by adding 1 µl (2 U) RNase H after cDNA synthesis.

To determine the major cDNA sequence of DsCAD1, four degenerate forward primers (Table 4.1) were designed based on the conserved regions of 10 cadherin cDNAs previously identified in other lepidopteran species and deposited in the Genbank (Footnote of Table 4.1 for Genbank accession nos.). The according amino acid sequences of the four conserved regions were ITQRQDYE, LINWNDE, ATDIDGP, and DEDGLHAG. One specific reverse primer, DsCAD1R0 (Table 4.1), was also designed based on the partial DsCAD1 sequence from the cDNA libraries. In each PCR reaction, a fragment of expected size was amplified using one degenerate forward and one degenerate/specific reverse primers. The 3'-end of the cDNA was amplified with a specific forward primers, named DsCAD1F2 (Table 4.1), along with the oligo-dT primer, while the 5'-end of the cDNA was obtained by using the 5' RACE system (Invitrogen, Carlsbad, CA). To synthesize 5'-RACE cDNA, reverse transcriptase polymerase chain reaction (RT-PCR) was first performed using the specific reverse primer DsCAD1R1. Based on the cDNA sequence obtained above, two specific reverse primers (Table 4.1) were used in the semi-nested amplifications with a forward abridged anchor primer from 5'-RACE kit. The 5'-end of the cDNA was isolated and C-tailed, and then was cloned into a pGEM-T vector. Finally, plasmid DNA was sequenced using an ABI 3730XL DNA analyzer at the Genomic Center (USDA-ARS, Stoneville, MS) to confirm the full coding sequences of DsCAD1.

To obtain error-proof full-length cDNA, a thermal-stable proof-reading Platinum High Fidelity Taq DNA polymerase (Invitrogen, Carlsbad, CA) was used the PCR reactions. Total RNA extracted from Cry1Ab-SS and -RR strains were used for synthesizing RT-cDNA. Two specific primers were designed to flank the 5' and 3' non-coding regions. By using 3 pairs

Table 4.1. Sequences of primers used in cDNA cloning, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and double-stranded RNA (dsRNA) synthesis for characterization of a midgut cadherin gene from Cry1Ab-susceptible and -resistant strains of *Diatraea saccharalis*.

Purpose of use	Primer Name	Primer Sequence (5'-3')
Degenerate primer* for PCR	dgDsCAD1F1 dgDsCAD1R2 dgDsCAD1F2 dgDsCAD1R3 dgDsCAD1F3 dgDsCAD1R4 dgDsCAD1F4	ATHACNCARMGNCARGAYTAYGA TCRTCRTTCCARTTDATNAR YTNATHAAAYTGGAAYGAYGA GGNCCRTCDATRTCNGTNGC GCNACNGAYATHGAYGGNCC CCNGCNTSNARNCCRTCNTSRTC GAYSANGAYGGNYTNSANGCNGG
Specific primer for 5'RACE	DsCAD1R3	GTGCTGATCTCTCCGTCTCT
Specific primer for PCR cloning	DsCAD1F0 DsCAD1R1 DsCAD1F1 DsCAD1R2 DsCAD1F2 DsCAD1R0	CTTCTTATGATAACAATCGCCTAACAAAA GTCTTCCAGATGTATACCAATTGTG TGC GTATTGACGAGGAGAGT CATTAGTAATAGTGAAGTGTTTCGATGGC CGTGCGATGGCAAACAATC CAGTGATATATTTTGTGCTTATAGCGC
Specific primer for qRT-PCR	Ds18SF1 Ds18SR1 rtDsCAD1F1 rtDsCAD1R1 DsCAD1F0 DsCAD1R4	CAAATGTCTGCCTTATCAACTTTC GCCTTCCTTGGATGTGGTAG CTGGTGTCACGTTGGTAATAGTAAA GTGCTGATCTCTCCGTCTCT CTTCTTATGATAACAATCGCCTAACAAAA GAATATCTCCACCCATCGAGG
Specific primer for dsRNA synthesis**	iDsCAD1F1 iDsCAD1R1	TAATACGACTCACTATAGGGCTGGTGTCACGTTGGTAATAGTAAA TAATACGACTCACTATAGGGGAATATCTCCACCCATCGAGG

* Lepidopteran species with Genbank accession numbers of 10 cadherin sequences for degenerate primers used in PCRs are as followed: *Heliothis virescens* (AAK85198), *Helicoverpa armigera* (AAM69351), *Lymantria dispar* (AAL26896), *Manduca sexta* (AAM21151), *Bombyx mori* (BAA99406), *Pectinophora gossypiella* (AAP30715), *Ostrinia nubilalis_A1* (AAT37678), *Ostrinia nubilalis_M1* (ACK37449), *Ostrinia furnacalis* (ABL10442), *Chilo suppressalis* (ABG91735).

** dsRNA template forward and reverse primers have T7 RNA polymerase recognition sites (5'-TAATACGACTCACTATAGGG-3') appended to their 5' and 3' ends, respectively.

of primers, DsCAD1F0+DsCAD1R1, DsCAD1F1+DsCAD1R2, and DsCAD1F2+DsCAD1R0 (Table 4.1), three overlapping fragments of DsCAD1 were re-amplified. The cDNA fragments from the PCR reactions purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA) and sequenced from both directions as described above.

4.2.3 Nucleotide and Amino Acid Sequence Analysis of DsCAD1 from *D. saccharalis*

Presence of a signal peptide at the N-terminus of the deduced protein sequence was determined using the SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP>), while molecular weight and protein isoelectric points were predicted using the ExPASy Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html) (Bendtsen et al., 2004). Analysis of deduced protein sequences was conducted in the Myhits server (http://myhits.isbsib.ch/cgi-bin/motif_scan). Sequence-similarity analyses were performed using BLAST through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) (Altschul et al., 1997). Sequence comparisons were conducted using the ClustalW (gap weight = 8, gap length weight = 2) as described by Thompson et al. (1994). The molecular evolutionary genetics analysis (MEGA) (Ver. 4.1; Tamura et al., 2007) was used to perform multiple-sequence alignments and to examine phylogenetic relations based on the cadherin amino acid sequences of other lepidopteran species available in the GenBank (<http://www.ncbi.nlm.nih.gov/genbank>).

4.2.4 Gene Expression Profiles of DsCAD1 by Quantitative RT-PCR

Total RNAs from 3rd and 5th instars of the Cry1Ab-SS and -RR strains of *D. saccharalis* were extracted as described above. Total RNAs then were treated with 2 µl DNaseI (1 mg/ml) (Boehringer Mannheim GmbH, Germany) at 37 °C for 1 h to reduce any potential genomic DNA contaminations. For each treatment replication, three guts were pooled as one sample and total RNA was extracted from the pooled samples. Concentration of total RNAs was measured with

the NanoDrop spectrophotometer as mentioned above and adjusted to 1 and 10 ng/ μ l. The iScript One-Step RT-PCR Kit with SYBR green (Bio-Rad, Hercules, CA) was used in a 25 μ l reaction system for qRT-PCR as described in (Chapter 3) for examining the gene expression of the APNs of *D. saccharalis*. For each combination of instar and insect strain, there were three replications in the qRT-PCR analysis.

To obtain the absolute quantities of DsCAD1 mRNA, two qRT-PCRs were performed as described in (Chapter 3). Briefly, the ribosomal 18S gene was used to estimate RNA concentration for each sample. One pair of primers, Ds18SF1 and Ds18SR1 (Table 4.1), were designed based on the house-keeping 18S gene of *D. saccharalis*. Full-length cDNA of 18S gene was prepared and used as internal standard in the first qRT-PCR. Serial dilutions (0.5, 5, 50, 500, and 5000 pg/ μ l) of the 18S internal standard and a negative control without the 18S cDNA were used to establish a standard curve after the qRT-PCR. To determine RNA sample concentrations, five microliters of RNA template (1 ng/ μ l) or internal standard was introduced into the qRT-PCR reaction. The threshold cycle (C_t) value for each dilution was then plotted against the log of the cDNA quantity. The absolute quantities of all RT-cDNA were calculated from the standard curve. All initial RNA samples were standardized and then used in the second qRT-PCR.

For the second qRT-PCR, one specific primer pair (replicon length = 145) was designed and a fragment of 929-bp cDNA of DsCAD1 was amplified as internal standard. Serial dilutions of internal standard (0.001, 0.01, 0.1, 1, and 10 pg/ μ l) were used to establish a standard curve as described above. Five microliters of the internal standard solution or standardized RNA template (10 ng/ μ l) were added into each reaction along with a negative control that contains everything except the RNA template. Based on the internal standard curve of the second qRT-PCR, absolute quantities of RT-cDNA of DsCAD1 for a specific instar were compared between Cry1Ab-SS

and -RR strains. Three replications were used for each treatment combination of insect strain and instar. The transcript levels were presented as means and standard errors of the mean (\pm SEM). Student's t-tests were used to determine treatment difference for each instar (SAS Institute, 2008).

4.2.5 RNA Interference of DsCAD1

Double-stranded RNA (dsRNA) that corresponded to position 488 to 888 of DsCAD1 was synthesized using primers fused with T7 promoter sequence that eliminates the sub-cloning step to a pGEM-T vector as the DNA template (Schepers, 2005; Nunes and Simões, 2009). In synthesis of the dsRNA, the T7 promoter sequence (5'-TAATACGACTCACTATAGGG-3') was appended onto both 5'- and 3'-ends of the designed primers. After verified on agarose gel, the PCR products were used as template *in vitro* transcription. Resulted template was 401 bp in length excluding the fused T7 promoters. After purified with Minelute kit (Qiagen, Valencia, CA), dsRNA was transcribed and diluted in an elution solution (ES) (10 mM Tris-Cl, pH 7, 1 mM EDTA) using MEGAScript RNAi kit (Ambion, Austin, TX) based on the manufacturer's protocol. Finally, dsRNA was quantified using Nanodrop spectrophotometer as described for analyzing the three DsAPNs of *D. saccharalis* in (Chapter 3).

Compared with micro-injection, non-invasive oral delivery of dsRNA has recently become a more attractive method in RNAi study although it might be less effective than dsRNA micro-injection (Rajagopal et al., 2002; Araujo et al., 2006; Baum et al., 2007). To analyze gene expression of DsCAD1 after RNAi, oral delivery of dsRNA (Turner et al., 2006; Bautista et al., 2009) was applied as described in (Chapter 3) for RNAi of the three APNs. To increase RNAi effectiveness and reduce variability in individuals' responses, a relatively high dose of dsRNA (250 ng) for each 3rd instar larva was used for dsRNA feeding (Bautista et al., 2009). Briefly, 3rd

instars from Cry1Ab-SS strain of *D. saccharalis* were droplet-fed with 0.5 μl of the ES (control) or 0.5 μl ES containing 250 ng dsRNA (RNAi-treated). The droplet-fed larvae were transferred to the individual cups each containing 1 g of the meridic diet and held in an environmental chamber maintained at 28 $^{\circ}\text{C}$, 50% RH, and a photoperiod of 16:8 (L:D) h. After droplet-feeding for 24 h, larvae were dissected to obtain total RNA. The DsCAD1 transcript levels of the RNAi-treated and non-treated control larvae were determined by two qRT-PCRs as described above. Three replications for each treatment were used and each replicate contained a pool of total RNAs from three larval guts. The transcript levels of RNAi-treated and control larvae were analyzed using Student's t-tests to determine treatment differences at $\alpha = 0.05$ level (SAS Institute, 2008).

In addition, Cry1Ab susceptibilities of dsRNA treated and non-treated control larvae of *D. saccharalis* were assayed using a standard method of diet incorporating Bt toxin described in Huang et al. (2007b). Based on a previous study by Huang et al. (2007b), the Cry1Ab at the concentration of 16 $\mu\text{g g}^{-1}$ diet caused ~50% larval mortality to the Cry1Ab-SS strain at 7 days. This concentration of Cry1Ab (16 $\mu\text{g g}^{-1}$ diet), therefore, was used for the current bioassay. To examine larval susceptibility to Cry1Ab toxin after RNAi, ES only or 0.1 μl of ES containing 50 ng dsRNA was droplet-fed to neonates (< 24 h) of the Cry1Ab-SS strain. The droplet-fed neonates were placed in cells of 128-cell trays (C-D international, Pitman, NJ) individually and starved for 3 h before they were used for bioassays. In the bioassays, approximately one gram of regular diet (non-treated control) or diet treated with purified trypsin-activated Cry1Ab toxin at the concentration of 16 $\mu\text{g g}^{-1}$ was dispensed into each cell of the 128-cell C-D International trays using 20-ml syringes (Becton, Dickinson and Company, Franklin Lakes, NJ). One dsRNA-treated and starved larva was then placed on the diet surface of each cell. The bioassay trays

were held in an environmental chamber maintained at 28 °C, 50% RH, and a photoperiod of 16:8 (L:D) h. Larval mortality was checked after 7 days. The 7-d mortality data were corrected based on the mortality observed on the control diet using the Abbott's method (Abbott 1925). There were three replications for each treatment with 48 larvae in each replicate. The corrected mortalities were presented as means and standard errors of the mean (\pm SEM). Student's t-test (SAS Institute, 2008) was performed to determine treatment differences at $\alpha = 0.05$ level.

4.3 Results

4.3.1 Characterization and Analysis of DsCAD1 cDNA and Putative Protein from Cry1Ab-SS and -RR Strains of *D. saccharalis*

Three partially overlapping expressed sequence tags from one cadherin gene were found in the cDNA libraries of both Cry1Ab-SS and -RR strains of *D. saccharalis*. The three selected ESTs were assembled into one cadherin cDNA fragments, named DsCAD1. The DsCAD1 included a polyadenylation signal in the 3'-UTR.

Pairwise alignment showed that cDNA sequences of the DsCAD1 gene in the Cry1Ab-SS and -RR strains of *D. saccharalis* are identical. No deletions or insertions and no base substitutions were detected between the DsCAD1 cDNAs from two insect strains. Full-length cDNA of 5304 bp of the DsCAD1 was cloned and characterized from *D. saccharalis*. The cDNA sequence had an open reading frame of 5157 bp encoding a 1718 amino acid putative cadherin-like glycoprotein. At the 3' end of the cDNA sequence, two polyA signal sequences (AATAAA) were apparent at the positions 15 bp and 169 bp upstream of the polyA tail, respectively. The putative pI for the cadherin protein was 4.38 and the estimated molecular weight (MW) was 193 kDa. Based on sequence analysis of the predicted protein from full-length DsCAD1 cDNA, the cadherin-like protein contained a transmembrane (TM) region of 23 amino acids (Fig. 4.1). The

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1  MMEVKYLAAL LLLALVASVK TQVVDHCAFV VAIKRPPTPE LPEFDFDGLS WEDRQLLPPP
61  VREEVCMGLY QRQLNVGQOI IYMEEEIEGD VIIAKLNYQG NATPTIGVPEL IQGYFDMLGA
121 EIRRIPELDN YWYLVITQRQ DYETQMMQLY QFMIRIPGET VQAGVTLVIV NIDDMPIIH
181 ALEPCVPEL  GDARLTECVY  EVTDRDGEIS TRFMTFEVLS DRNDNEIFYM KGEYNSADWQ
241 TMSMTVGITR ELNFETKRLH SFTVVAKDSL PNNDTVSMVLV RVENVHRPE RWVEIFAVQQ
301 FDEKTKQNFV VRAIDADIEI DKRIDYYLIT DEEDDFFSIE ENYDELGGAT LFAVIDRDK
361 LERELFRLTI VAHKYDNMSL ATEANVIIIV NDINDQRPKP LRKEYHIEIE EETPVTLNFG
421 PDFGFHDRDL GENARYTVEL RDVSEPGAAS AFAVSEDEGY QRQTFIMSTI NHAMLDYVEP
481 EFQNVLELV ATDKNNSEFV GVARLYIDLI NWNDELPIFG ETAYSVSFNE TVEKDFVGT
541 VLATDRDIDD TVVHSLMGNA GDLLRIDEES GNIYVKIDDA FDYHRQNILF VQVRADDTLI
601 TLDRGTHTTT TQLVIHLEDV NNTPTLRLP RASESVEENV PEGYVVEDNL RATDPDTAH
661 LVFEINWDES WATKQGRPTH VDEYYGCVAI KTEYVDGNTG SAIGIIEVRQ IRPDVTIDFE
721 EFEVLYLSIR VRDINTVYGD EDDEAIFTVN IIDMNDNPII FSEGSLEQSM RVREMSATGT
781 VIGSLLATDI DGPLYNNVHY SIKPINETPD GLVKIDLISG QLTVDADAET DADVPPRYHL
841 YYEVTASDRC LEEECPPDCN HFNTGYIAI EIIDTNKFP EERTDLFRTV VPMENETSG
901 YEIEQIFASD LDRDELYHQV RYQINYAWNP RLRAPFDIDL DTGMLFVNYT TDEVLDLDRG
961 EPTHITIFSL IDNFFTADG  RRNQSTTIVE IVLLDVNDNA PELPEGLSWS ISEAIPEGEL
1021 VGDIVFAPDI DEPGTNSRV GYSVLNMTTD RDIIILPILFD TYLIEHDEGN RARLRTQAL
1081 RGYWGDHMIH IKAFDHGVEP MHSMDWYPVE IREYNPHDPV FVFPFPGATI RFARERAMAN
1141 NLLITADGNV LERLSATDED GLHAGRVTFS IFGDASAIEH FTITNDGENS ATLLLSKVPD
1201 DNVMEFQITI RATDGGTEPH SRHTDSTIRA LFTVTLGPEI FNQESITVPF TEGPIGLEES
1261 HQLPLAKDEK NYGCTDDCND IFYRIAGGTT SEYFHLDEVS NRTLARELD QEQRTHITV
1321 IATSNSPTAS GTPLDGTTLT VTVNVIEENP RPIFERKLYT AGISVLDTIQ RELLTVOARH
1381 SLGDTITYAI DMDSMEMGSS LNAVAGSAFL LHPSTGVLT LNMQPTANMHG MFQFDVTATD
1441 TANGTGRAQV QIFLISSQNR VVFVFDNSLD EVVKYSDFIT DIFSAAFEMT CNIDQVLEAS
1501 DDQGIATDDK TEVRAHFIRD NVPVPADEIE ELGRDTQLLH QIQLWLNEHS LVLQDLSTGE
1561 GSVADNTAQI TVYVLAALSA LLAFLCIVIL ITFCKTRAL NRRMKAMSLT KFGSVDSGLN
1621 RMGITPGTNK HTAEGSNPIY NETLKAPDFD ALSDASNDS LIGIEDLPQF GNDFPPGDN
1681 NSLQIGMGDQ DTVSTHRNMF GFKTSPFSPE FTNKNFGR

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Figure 4.1. Deduced amino acid sequence of a cadherin-like protein from *Diatraea saccharalis*. The putative signal peptide sequences are underlined and the transmembrane (TM) spanning regions are boxed. Full-black arrows denote the predicted putative N-glycosylation sites. Hollow arrow denotes the putative amidation site and hollow circle represents the leucine zipper. CR1-CR11 and MPR are cadherin repeats and the membrane-proximal region, respectively. The bolded sequence at the C-terminal sequence represents the intracellular domain.

extracellular domain comprised a signal sequence (SP) of 21 amino acid residues, 11 cadherin repeats (CR1-CR11), and a membrane-proximal region (MPR) (Fig. 4.1). Seven putative N-glycosylation sites, an amidation site, and a leucine zipper motif were identified in the protein sequence. The protein's intracellular domain was composed of 124 amino acid residues (Fig. 4.1). All above sequences/domains match the structural characteristics of cadherin genes from other lepidopteran insects that have been investigated (Nagamatsu et al., 1999; Hua et al., 2004; Flannagan et al., 2005).

A phylogenetic tree was generated based on the alignment with 15 cadherins identified as putative Cry protein receptors from 13 other lepidopteran species (Fig. 4.2). The phylogenetic analysis indicated that the DsCAD1 in *D. saccharalis* is most closely related to six cadherins from *Chilo suppressalis* (Family Pyralidae) (ABG91735), *Ostrinia funarcalis* (Family Crambidae) (ABL10442), *Ostrinia nubilalis* (Family Crambidae) (AAT37678), and *P. gossypiella* (Family Gelechiidae) (AAU25884, AAU25882, and AAP30715) (Fig. 4.2). While DsCAD1 is more distant from the cadherin genes identified from the rest eight species (Fig. 4.2).

4.3.2 mRNA Expression Levels of DsCAD1 from Cry1Ab-SS and -RR Strains of *D. saccharalis*

Expression levels of mRNA of the DsCAD1 gene in the Cry1Ab-RR strain were significantly down-regulated compared to the same aged larvae of Cry1Ab-SS strain for both 3rd and 5th instars (Student's t-tests, $P = 0.0118$ for 3rd instar and $P = 0.0298$ for 5th instar). Compared to 3rd instars of the Cry1Ab-SS strain, mRNA level of DsCAD1 in the 3rd instars of Cry1Ab-RR strain were reduced by 57.6%, while it was lowered by 29.3% for the 5th instars (Fig. 4.3).

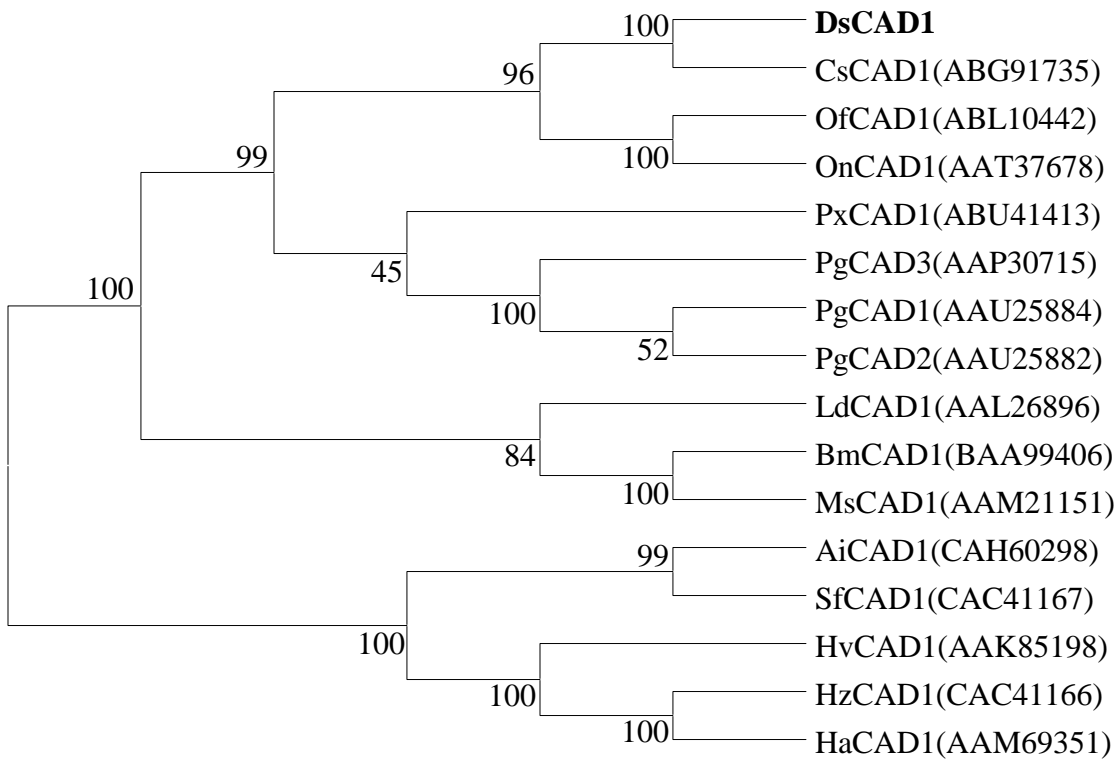


Figure 4.2. A phylogenetic tree generated by ClustalW alignments of cadherin amino acid sequences from lepidopteran species using MEGA. Bootstrap values, expressed as percentages of 500 replications, are shown at branch points. GenBank accession numbers are displayed within the tree. The first two letters of cadherins stand: Ai, *Agrotis ipsilon*; Bm, *Bombyx mori*; Cs, *Chilo suppressalis*; Ds, *Diatraea saccharalis*; Ha, *Helicoverpa armigera*; Hv, *Heliothis virescens*; Hz, *Helicoverpa zea*; Ld, *Lymantria dispar*; Ms, *Manduca sexta*; Of, *Ostrinia funarcalis*; On, *Ostrinia nubilalis*; Pg, *Pectinophora gossypiella*; Px, *Plutella xylostella*; Sf, *Spodoptera frugiperda*.

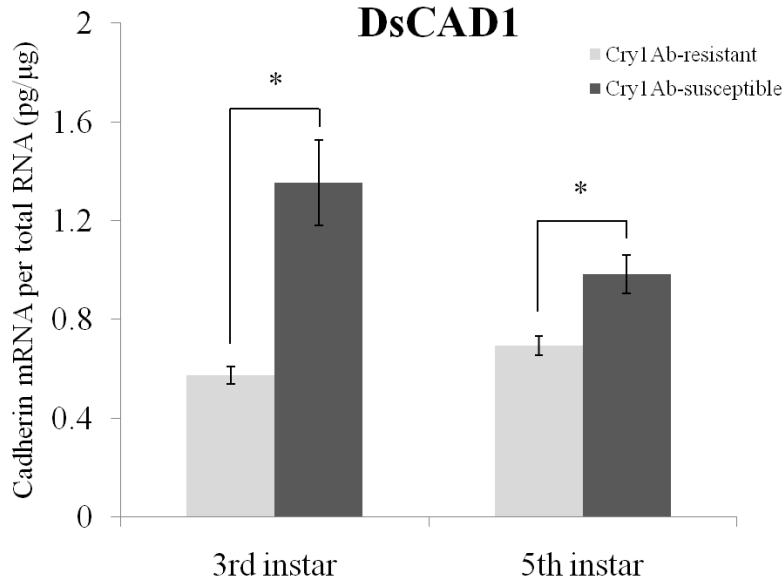


Figure 4.3. Gene expression levels of DsCAD1 in 3rd and 5th instars of the Cry1Ab-susceptible and -resistant strains of *Diatraea saccharalis*. Absolute transcript abundance (cadherin mRNA per total RNA (pg/μg)) was determined using SYBR green and qRT-PCR. Bars represent the means and standard errors of three total RNA samples. Each sample contains a pool of total RNAs from three larvae. Asterisk indicates significant differences in the gene expression levels between Cry1Ab-susceptible and -resistant strains (Student's t-test, $P < 0.05$).

4.3.3 mRNA Expression Levels of DsCAD1 after RNAi and Its Effect on Larval Susceptibility of *D. saccharalis* to Cry1Ab Toxin

Successful acquisition of DsCAD1-dsRNA in the 3rd instars of *D. saccharalis* was validated by direct observation and digital documentation using Nikon Coolpix 5700 digital camera connected to a Nikon SMZ1000 stereomicroscope as described in (Chapter 3). Knock-down of DsCAD1 gene by RNAi was observed in 3rd instars of *D. saccharalis*. qRT-PCR analysis showed that the transcriptional level of the DsCAD1 gene in 3rd instars was reduced by 52.2% after RNAi treatment (250 ng dsRNA/larva) for 24 h (Fig. 4.4). The difference was statistically significant ($P = 0.0004$).

Larval mortality of ES-treated control larvae at 16 μg Cry1Ab/g diet was 43.1%, while it was only 23.7% for RNAi-treated insects (Fig. 4.5). Mortalities of RNAi-treated Cry1Ab-SS

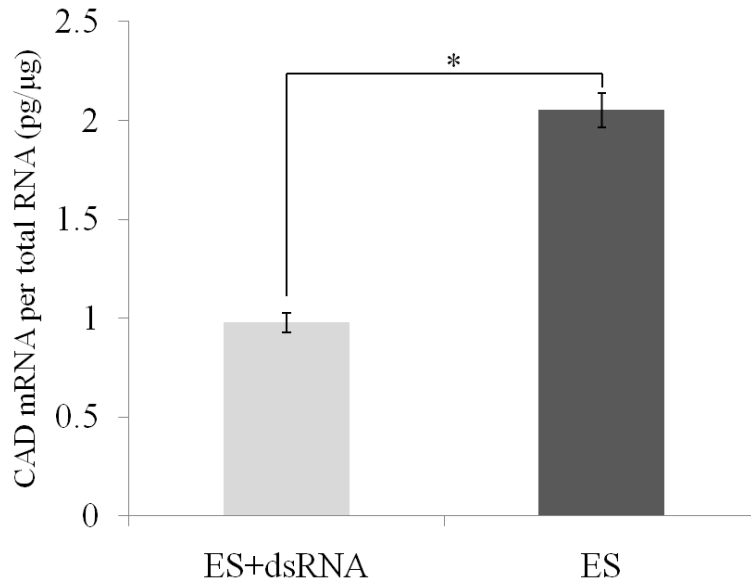


Figure 4.4. Transcriptional levels of DsCAD1 after dsRNA feeding to 3rd instars of the Cry1Ab-susceptible strain of *Diatraea saccharalis*. Bars represent the means and standard errors of three total RNA samples. Each sample included a pool of total RNAs from three larvae. Asterisk indicates significant difference ($P < 0.05$, Student's t-test).

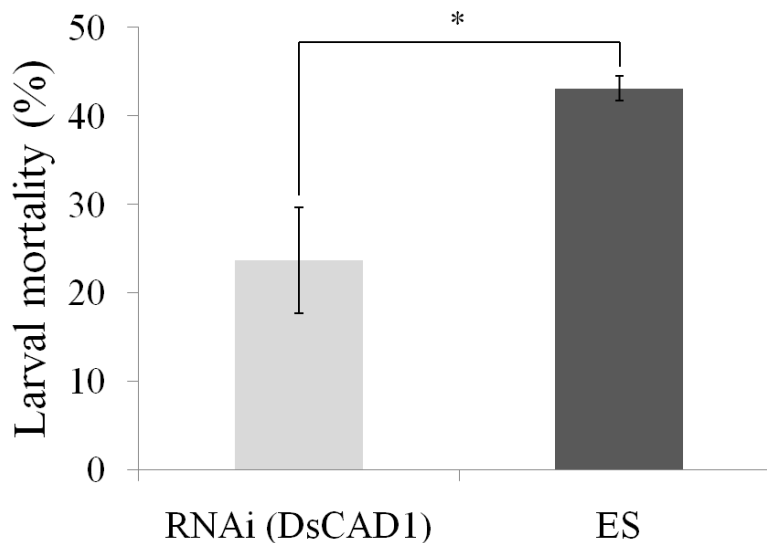


Figure 4.5. Larval mortality of the Cry1Ab-susceptible strain of *Diatraea saccharalis* after RNAi for DsCAD1. The RNAi-treated neonates were droplet-fed 0.1 µl elution solution (ES) containing 50 ng cadherin dsRNA, while the non-treated neonates were fed ES only before released for bioassays. The bioassays were conducted in three independent replications and each replicate for a treatment contained 48 neonates. Bars represent the means and standard errors of the mean. Asterisk indicates significant difference ($P < 0.05$, Student's t-test).

neonates of *D. saccharalis* fed on diet containing 16 µg Cry1Ab g⁻¹ diet were significantly less than those of ES-treated control larvae ($P = 0.0343$) (Fig. 4.5).

4.4 Discussion

Cadherins from animals constitute a large family (more than 6 families) of transmembrane glycoproteins responsible for essential cellular processes such as cell adhesion or maintenance of the integrity of selective cell-cell interactions (Nollet et al., 2000). Unlike the classical cadherins, lepidopteran cadherin-like proteins are primarily located in columnar cell apical membranes of midgut, of which they are more abundant in the anterior than in the posterior part (Gahan et al., 2001). Although the specific functions of the cadherin-like proteins in insects have not been fully understood, their important role in Bt toxicity has attracted a great interest in studying Bt resistance evolution in a few insects (Nagamatsu et al., 1999; Gahan et al., 2001; Hua et al., 2004).

The cadherin-like protein identified from *D. saccharalis* in this study shows a relatively high similarity and identity to other members of the cadherin super-family in lepidopteran insects. Eleven cadherin repeats (CRs) are present in the *D. saccharalis* cadherin and seven N-glycosylation sites are distributed along the extracellular domains. Glycosylation is generally regarded as an important determinant of Cry1A-binding (Pigott and Ellar, 2007). The relatively high similarity to other lepidopteran cadherin-like proteins indicates that the cadherin-like protein from *D. saccharalis* may share structures, functions, and consequently specificity for Cry1A toxins. Several previous studies have been conducted to determine and characterize the binding region for lepidopteran cadherin-like proteins (Nagamatsu et al., 1999; Gómez et al., 2002). For example, Hua et al. (2004) found that the cadherin domain 12 is critical for Cry1Ab binding in *M. sexta*. The current study showed that the putative Bt toxin binding regions (CR9-

CR11) in *D. saccharalis* are highly similar in amino acid sequence to those identified as in other lepidopteran cadherin-like proteins (Nagamatsu et al., 1999; Hua et al., 2004; Flannagan et al., 2005). Based on the cadherin sequences available from the Genbank, all lepidopteran species that have been investigated contain one dominantly expressed cadherin with the exception of *P. gossypiella*, which has three cadherins. However, the three cadherin genes from *P. gossypiella* shared 97% identity, which strongly suggested that they are different variants of one cadherin gene. Therefore, it is likely that only one cadherin exists in *D. saccharalis*, but multiple cadherins are also possible.

The current study showed that although cDNA sequences of the DsCAD1 gene were identical between the Cry1Ab-SS and -RR strains of *D. saccharalis*, the expression levels of DsCAD1 in both the 3rd and 5th instars of the Cry1Ab-RR strain were significantly reduced compared to those same age larvae of the Cry1Ab-SS strain. Functional studies with the RNAi in addition demonstrated that DsCAD1 is involved in the toxicity of Cry1Ab toxin in *D. saccharalis*. These results suggest that the reduced expression of DsCAD1 is associated with the Cry1Ab resistance in *D. saccharalis*. It should be noted that there were differences in the expression levels of DsCAD1 between the two insect life stages tested in this study. Similar differences were also observed in other lepidopteran species like *P. gossypiella* (Carrière et al., 2009).

Previous studies have shown that Cry1A resistance in several lepidopteran species was associated with mutations of the cadherin genes resulting in either deletions (Xu et al., 2005; Yang et al., 2007; Bel et al., 2009) or premature stop codons (Gahan et al., 2001; Morin et al., 2003; Bel et al., 2009). For examples, mutation of a cadherin-like protein caused by a retrotransposon insertion was linked to high levels of Cry1Ac resistance in *H. virescens* (Gahan

et al., 2001). Later, Xu et al. (2005) also found that a deletion in a cadherin gene was genetically-linked with high levels of Cry1Ac resistance in a laboratory-selected strain of *H. armigera*. Xie et al. (2005) in addition reported that single amino acid mutations in the toxin-binding region of the cadherin protein in *H. virescens* caused substantial decrease in toxin binding (Xie et al., 2005). These results indicated that such single amino acid mutations and deletions can lead to high levels of Bt resistance in the insects. However, it has not been conclusively shown that all mutations in cadherins associated with Bt resistance were in the toxin-binding regions of cadherin protein. For example, one amino acid substitution in a cadherin gene of *H. armigera* occurred between the Cry1Ac-binding region and the TM domain (Zhao et al., 2010). It is possible that changes in the cadherin protein could alter secondary structures of the protein and then affect other downstream post-toxin-binding steps. The identical cDNA sequences of the DsCAD1 gene between the Cry1Ab-SS and -RR strains, while difference in the gene expression, suggested that other mechanism(s) rather than changes in DsCAD1 structure could be associated with the Cry1Ab resistance in *D. saccharalis*. Further examination of transcription and translation factors may reveal underlying mechanisms of altered cadherin gene expression and Cry1Ab resistance in *D. saccharalis*.

Up to date, at least four groups of Bt toxin receptors have been identified in insects: cadherin-like proteins, GPI-anchored aminopeptidases N (APNs), alkaline phosphatases (ALPs) and glycolipids (Pigott and Ellar, 2007). Results of Chapter 3 also showed that reduced expression of three APNs could be associated with the Cry1Ab resistance in *D. saccharalis*. As observed for DsCAD1 in the current study, the cDNA sequences of the three APNs were also identical between the Cry1Ab-SS and -RR strains. Genetic studies suggested that the Cry1Ab resistance in this strain of *D. saccharalis* was most likely controlled by one or a few tightly

linked gene(s) (Wu et al., 2009). If the single-gene resistance model is true, the resistance in this Cry1Ab-RR strain of *D. saccharalis* would likely be caused by changes in a regulatory gene that controls the expression of more than one Bt binding receptors, including cadherins and APNs. Such resistance mechanisms have been identified in the P450- and GST-mediated insecticide-resistance in some insect species (ffrench-Constant et al., 2006; Che-Mendoza et al., 2009). For instance, an insertion into upstream of a detoxifying P450 gene, *Cyp6g1*, resulting in increases of *Cyp6g1* expression was associated with the DDT resistance in *Drosophila melanogaster* (ffrench-Constant et al., 2006). Similarly in mosquitoes (e.g., *Anopheles gambiae*, *Aedes aegypti*), elevated GST activity in several insecticide-resistant strains was mostly due to regulatory gene changes that increases the GST transcription (Che-Mendoza et al., 2009). In this type of mutation, changes in some upstream regulatory element gene(s) such as insertions and deletions may also cause down-regulated expressions of its downstream genes (e.g., cadherin and APNs). Various mutation types can lead to changes in gene expression. These mutations can occur either in cis-elements or in trans-factors. Mutations in cis-elements include disruption or deletion of URE genes whose function is to enhance or repress gene expression, while in trans-factor that can bind to the cis-elements mentioned above (Feyereisen, 1995). Otherwise, the DsCAD1 and these APN genes might be tightly linked in *D. saccharalis*.

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CHAPTER 5

MOLECULAR IDENTIFICATION AND FUNCTIONAL ANALYSES OF THREE ALKALINE PHOSPHATASE GENES FROM *BACILLUS THURINGIENSIS*-SUSCEPTIBLE AND -RESISTANT STRAINS OF SUGARCANE BORER, *DIATRAEA SACCHARALIS*

5.1 Introduction

Cry proteins are produced as crystalline inclusions by a spore-forming bacterium, *Bacillus thuringiensis* (Bt), during the sporulation phase of growth (Feitelson et al., 1992; Bravo et al., 2005). To date, > 560 Cry genes have been sequenced. These Cry genes are classified into at least 68 groups (Cry1-Cry68) according to their amino acid sequence similarities (Crickmore et al., 1998; 2010). One of the membrane-bound alkaline phosphatases (ALPs) in the midgut brush border membrane of *Heliothis virescens* has been identified as a Cry1Ac-binding protein (Jurat-Fuentes and Adang, 2004). Studies have shown that Cry1Ac resistance in a strain of *H. virescens* was associated with reduction in gene expression levels of an ALP in the midgut (Jurat-Fuentes and Adang, 2004). The critical role of midgut ALPs in Bt mode of action has been also documented in several other insect species. For example, a GPI-anchored ALP has been identified from *Aedes aegypti* midgut, which is shown to interact with Cry11Aa and Cry4Ba toxins in Bt-resistant mosquito (Fernandez et al., 2006; Moonsom et al., 2007). Martins et al. (2010) also found that two ALP proteins (62 and 65 kDa) in the midgut of the cotton boll weevil, *Anthonomus grandis*, can bind to Cry1Ba6 toxin.

The objective of this study is to clone full-length cDNAs of midgut alkaline phosphatase and to determine if changes in the cDNA structures/expression of these genes are associated with the Cry1Ab resistance in sugarcane borer, *Diatraea saccharalis*, a major target pest of Bt maize in the U.S. mid-south region (Castro et al., 2004; Huang et al., 2009).

5.2 Materials and Methods

5.2.1 Insect Sources

A Cry1Ab-resistant strain (Cry1Ab-RR) of *D. saccharalis* was originated from a single two-parent family-line using an F₂ screen (Huang et al., 2007b). A Cry1Ab-susceptible strain (Cry1Ab-SS) was established from larvae collected in corn fields near Winnsboro, LA during 2004. The Cry1Ab-RR strain has shown >100 fold resistance to the purified Cry1Ab toxin and can complete entire larval stages on commercial Cry1Ab corn hybrids (Huang et al. 2007a). The Cry1Ab-RR strain had been backcrossed and reselected for Bt resistance on Cry1Ab corn leaf tissue for three times before it was used for this study.

5.2.2 Midgut Homogenate Preparation and Enzymatic Activity of Alkaline Phosphatases of *D. saccharalis*

Three instars (3rd, 4th, and 5th) of the Cry1Ab-SS and -RR strains of *D. saccharalis* were carefully dissected on ice to obtain the midgut without other tissues attached. The whole midguts included the gut tissue and the inside contents as described in (Chapter 2). A single midgut was homogenized in a centrifuge tube containing 100 µl cold 0.1M Tris-HCl buffer (pH 8.0) using a motorized micro tissue grinder. Midgut homogenates were clarified by centrifugation for 5 min at 10,000g at 4 °C. Supernatants were collected and protein concentrations were quantified using Coomassie Plus Assay Kit (Pierce, Rockford, IL) with BSA as standard (Bradford, 1976). The quantified supernatants of midgut homogenates were stored at -80 °C as the enzyme source for the study.

Specific ALP enzymatic activities of midgut homogenates were measured with *p*-nitrophenyl phosphate disodium (*p*NPP) (Sigma, St. Louis, MO) as the substrate using the method as described in Jurat-Fuentes and Adang (2004). Diluted proteins (1 mg/ml) were mixed

with 0.1 M Tris-HCl buffer (pH 8.0) with *p*NPP (1.25 mM at final concentration). Enzymatic activities were measured by monitoring changes of the A405-value for 15 min with reading interval of 15 s at 37 °C in a microplate reader ELx808iu (Bio-Tek, Winooski, VT). ALP activity was defined as the amount of enzyme producing 1 μmol of chromogenic product *p*-nitrophenol (*p*-NP) min⁻¹ mg of protein⁻¹ at 37 °C. The extinction coefficient of *p*-NP (17.8 mM⁻¹ cm⁻¹) (Sigma, St. Louis, MO) was used for calculation of the specific ALP activities for both the Cry1Ab-SS and -RR strains. For each combination of insect strain and instar, ALP activity was measured from eight reactions (replications) and two readings (sub-samples) for each reaction. Specific ALP activities were presented as means and standard errors of the mean (±SEM). Student's t-test (SAS Institute, 2008) was used to determine differences in ALP activity between the Cry1Ab-SS and -RR strains at each instar.

5.2.3 Cloning Full-Length cDNAs of Three DsALPs of *D. saccharalis*

cDNA libraries were constructed and sequenced by Dr. Yu Cheng Zhu (USDA-ARS, Stoneville, MS), using the SMART™ cDNA Library Construction kit (Clontech, Mountain View, CA) and manufacturer's procedures with certain modifications (Zhu et al., 2011). To obtain alkaline phosphatase cDNAs, Blastx-NR similarity search of GenBank database (Altschul et al., 1997) identified 30 putative ALP cDNA contigs from 12,500 *D. saccharalis* midgut expressed sequence tags (ESTs). These cDNA contigs were assembled using SeqMan module of the Lasergene (DNASar, Madison, WI). The assembly resulted in partial cDNAs of three ALP genes and their corresponding 3'-ends with polyA tails. These three ALP genes were named DsALP1, DsALP2, and DsALP3.

Full-length cDNAs of three DsALPs from *D. saccharalis* were cloned using the similar procedures as described for cloning the aminopeptidase N cDNAs of this species in (Chapter 3).

Briefly, total RNA of each insect strain was extracted from 15 midguts of 3rd instars (three guts per tube) with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The total RNAs were reverse-transcribed with SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA) to obtain the corresponding cDNAs. The cDNAs were used as the templates in the subsequent PCR amplifications. To obtain full-length cDNAs of the three DsALPs, specific primers (Table 5.1) were designed based the cDNA sequences from the libraries. These primers were then used to amplify cDNA fragments in PCR reactions with an initial denaturation step at 94 °C for 3 min, 35 cycles running each with 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and an additional step of 10 min at 72 °C for extension. For full length cDNA cloning, the 5' RACE protocol was performed according to the instructions of the 5' RACE system (Invitrogen, Carlsbad, CA). After 5'RACE, three specific primer pairs, DsALP1F0 and DsALP1R0, DsALP2F0 and DsALP2R0, DsALP3F0 and DsALP3R0 (Table 5.1), were designed to flank the 3'- and 5'-UTR-ends to amplify entire coding region for the three corresponding DsALPs. After PCR reactions, the expected amplicons were purified on gel, ligated to pGEM-T vector (Promega, Madison, WI), transformed into OneShot TOP10 competent cells (Invitrogen, Carlsbad, CA), and sequenced from both directions as described for cloning the DsAPN cDNAs in (Chapter 3).

5.2.4 Identification and Phylogenetic Analysis of Three DsALP Genes from *D. saccharalis*

Homology of the three DsALPs of *D. saccharalis* was compared with ALPs identified from other species retrieved using GenBank similarity search of NCBI/BLASTX-NR database. ClustalW (Thompson et al., 1994; gap weight = 8, gap length weight = 2) was applied for multiple sequence alignment. Presence of a signal peptide was tested in the SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>). Two GPI modification site prediction servers

Table 5.1. Sequences of primers used in cDNA cloning and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for characterization of three midgut alkaline phosphatase genes from Cry1Ab-susceptible and -resistant strains of *Diatraea saccharalis*.

Purpose of use	Primer Name	Primer Sequence (5'-3')
Specific primer for cDNA cloning	DsALP1F0 DsALP1R0 DsALP2F0 DsALP2R0 DsALP3F0 DsALP3R0	TATAGATTGTCGCTCCGTCTCG CGTTATAAAGTATTATACTTATATTTATCTAAACAAGGTACG AGAGCAATTGACTTGCTTGTGT GGTGGAGTGACATATATTTTGTAGTTTATTTAC ACCGGCAGTATCAATCATGAATT CACTCGGTTTCTCTGTCTACAG
Specific primer for qRT-PCR	Ds18SF1 Ds18SR1 rtDsALP1F0 rtDsALP1R0 rtDsALP2F0 rtDsALP2R0 rtDsALP3F0 rtDsALP3R0	CAAATGTCTGCCTTATCAACTTTC GCCTTCCTTGGATGTGGTAG GAAGAAGGGCAGTTAGGATTAAGG TACTCAGGTGGTGATTGCATATTC CGTTTACTCTATCGCAGCATGG TTCAGCAGTGTGGGCATATG CTGATTCCGCTTGTAGTGCTA GCAGCTAGTGATTTAAGCCTGG
Specific primer for internal standards in qRT-PCR	rtDsALP1F1 rtDsALP1R1 rtDsALP2F1 rtDsALP2R1 rtDsALP3F1 rtDsALP3R1	CCGTATAACACATGCGTCTCC GCATGATCCGCAGTCACCAC AGAGCAATTGACTTGCTTGTGT TTGGATCGTCTCGTCCAATG ACCGGCAGTATCAATCATGAATT ACTGCTTGGCAAGAAACCAT

(PredGPI: <http://gpcr2.biocomp.unibo.it/gpipe/pred.htm> and GPI-SOM: <http://gpi.unibe.ch/>) were used to predict the GPI-anchor signal sequence and GPI anchoring sites. Molecular weight and isoelectric points of the predicted proteins were predicted using the ExPASy Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html). Presence of N- and O-glycosylation on the predicted protein sequences was tested using the NetNGlyc 1.0 and NetOGlyc 3.1 servers (<http://www.cbs.dtu.dk/services>), respectively. The localization of the active phosphatase site was predicted by using the Myhits server (http://myhits.isbsib.ch/cgi-bin/motif_scan). Based on the ALP cDNA sequences of *D. saccharalis* and those from other insect species available in GenBank, phylogenetic analysis was conducted using the molecular evolutionary genetics analysis (MEGA) software (Ver. 4.1; Tamura et al., 2007).

5.2.5 Expression of Three DsALPs of Cry1Ab-SS and -RR Strains of *D. saccharalis*

Quantitative RT-PCRs (qRT-PCRs) were conducted to determine the transcriptional levels of the three DsALPs of Cry1Ab-SS and -RR strains of *D. saccharalis* using the similar procedures as described in the examination of the DsAPN expressions of this insect in (Chapter 3). Shortly, midguts from 3rd and 5th instars of both Cry1Ab-SS and -RR strains of *D. saccharalis* were dissected and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). To eliminate possible trace amounts of genomic DNA contamination, total RNA samples were treated with 2 μ l (1 mg/ml) DNaseI (Boehringer Mannheim GmbH, Germany) at 37 °C for 1 h. To obtain absolute quantities, total RNAs were initially standardized using 18S rRNA specific primers (Ds18SF1 and Ds18SR1) in the first qRT-PCR. Specific primers for DsALP1, DsALP2, and DsALP3 (Table 5.1) (replicon length =143, 112, and 139 bp, respectively) were designed based on the cDNA sequences of the three DsALPs and used in the second qRT-PCR reactions. The cycling conditions for both qRT-PCRs were as follows: 50 °C for 10 min, 95 °C for 5 min,

and 40 cycles (95 °C for 10 s, and 55 °C to 60 °C for 30 s) for data collection coupled with a melting curve established from 55 °C to 95 °C with an increment of 0.5 °C for 10 s for each step. For each combination of instar and insect strain, there were three replications in the qRT-PCR analysis. The transcript levels were presented as means and standard errors of the mean (\pm SEM). Student's t-tests were used to determine treatment difference for each instar (SAS Institute, 2008).

5.3 Results

5.3.1 Alkaline Phosphatase Activity of Cry1Ab-SS and -RR Strains of *D. saccharalis*

The ALP activities were 0.351, 0.490, and 0.691 mU/mg protein for 3rd, 4th, and 5th instars from Cry1Ab-SS strain. For Cry1Ab-RR strain, the ALP activities were 0.450, 0.454, and 0.656 mU/mg protein for larvae from 3rd to 5th instar. There appeared to be a trend of increase in ALP activity as *D. saccharalis* larvae aged from 3rd to 5th instars for both the Cry1Ab-SS and -RR strains (Fig. 5.1). However, the ALP activities were not significantly different between the two insect strains at each instar for all the three instars tested ($P = 0.21$ for 3rd instar, $P = 0.66$ for 4th instar, and $P = 0.74$ for 5th instar) (Fig. 5.1).

5.3.2 Cloning and Analysis of Three ALP cDNAs and Putative Enzymes in Cry1Ab-SS and -RR Strains of *D. saccharalis*

By using RT-PCRs and 5' RACE, full-length cDNAs encoding the three ALPs from midguts of *D. saccharalis* larvae were cloned from both Cry1Ab-SS and -RR strains. These clones were named DsALP1, DsALP2, and DsALP3, respectively. ClustalW pairwise alignment showed that the cDNA sequences of each of the three DsALPs were identical between the Cry1Ab-SS and -RR strains. No single site nucleotide substitutions, insertions and deletions were observed in the three DsALPs between the two strains.

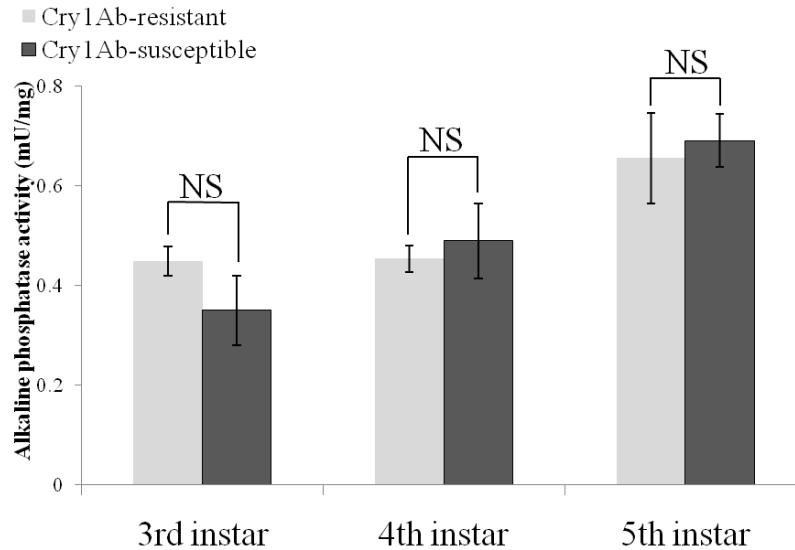


Figure 5.1. Total alkaline phosphatase (ALP) activity of whole midgut (gut tissue plus gut contents in the lumen) at different instars of Cry1Ab-susceptible and -resistant strains of *Diatraea saccharalis*. ALP activities were tested by measuring the formation of chromogenic product based on the free *p*-nitrophenol absorbance at 405 nm. One unit of enzyme activity was defined by hydrolysis of 1 μ mol of substrate in 1 min. Bars represent the means and standard errors of eight gut samples from a total of eight different larvae. NS indicates no significant difference ($P > 0.05$, Student's *t*-test).

The three ALPs in *D. saccharalis* displayed a low amino acid identity (~28.8%) among each other, indicating that they may represent different variants. The open reading frames (ORFs) of the three DsALPs were 1647, 1551, and 1554 bp, encoding a predicted protein of 548, 516, and 517 amino acid residues, respectively. The predicted molecular weights were 60.4 kDa for DsALP1, 57.0 kDa for DsALP2, and 57.2 kDa for DsALP3. Their isoelectric points were 5.59, 5.24, and 5.55, respectively, for the three DsALPs. Hydrophobic signal sequences (17, 16, and 17 amino acids, respectively) were found in the N-terminal region of the three DsALPs from *D. saccharalis* (Fig. 5.2), which was consistent with the known ALPs from other insect species. The GPI-anchor signal sequences observed in the ALPs of most other insect species were highly possibly existed in the C-terminal regions of DsALP1 and DsALP3 in *D. saccharalis* (Fig. 5.2). This suggested that DsALP1 and DsALP3 were possible membrane-bound ALPs with GPI

DsALP1	<u>MLSAQILVLFVLSVALADRYHPDAKGLGKLNARTGVGPSAAEKTAQYWYNEALNGINKR</u>	60
DsALP2	<u>MLRIFFLITLVHFSFERDVYHGSRGGGSEMND</u> -----GERQPSYWHEEARAAIEAR	52
DsALP3	<u>MNSVFVVLAVLLSTLSLR</u> -----TDKQFWKDLAKQEELEEA	36
DsALP1	QARLQDDK-VARNTIMFLGDGMSVPTLAAARTLLGQRQGLAGEEAELSFESFPTVGLAKT	119
DsALP2	EARRKETYGVARNIIMFLGDGMSVPTLAAARALLGQRAGRTGEESQLSFEAFPTVGLSKT	112
DsALP3	LHTKWNLH-TAKNVIMFIGDGMGPNTVTATRMYKG-----GESHRLAYEKFPHMGLLKT	89
DsALP1	YCVDHQ <u>IA</u> <u>DSACTAT</u> AYLCGVKNNYGTLGVTGEVSRNDCPASENTNNQLESIAAWALKNG	179
DsALP2	YCVDAQV <u>ADSACSAT</u> AYLCGAKANYGTIGVTPAVPRHHCHAATEPNDHVYSIAAWALKAG	172
DsALP3	YSADKM <u>V</u> <u>PDSACSAT</u> ALFSGVKNNKDTIGVDASVKRGDCERSLLPEARLKSLAAEALDAG	149
DsALP1	KDVGIVTTTTRITHASPSGAYARTANRNWEHNG-QVQEANFDPTRCP---DIAHQLIHTEP	235
DsALP2	KDAGIVTTTTRVTHASPAQTYAHTAERDWEADMISECPIGDESVPQQDIAAQLVHNYP	232
DsALP3	KSAGFVTTMRVTHATPGPLYAHSADRSWECD-----FMPAGTEHCK---DIARQLVEDEP	202
DsALP1	GKSFVKVILGGGRNFIPNSFTDEEGQLGLRDTQORNLINEWRNDKNARN <u>LSNAYIWNRDQL</u>	295
DsALP2	GNQFKVILGGGRREFIPKDFVDEEGAFGRREDNRNLIEEWRVDKNLR <u>NVTHEYVWNRDGL</u>	292
DsALP3	GKNLNVILGGGRNGFLPSSAANAWS--CARNDSRNLIEDYEKDKKDRGLKYSIVS <u>SNKTEL</u>	260
DsALP1	MANMQSPPEYLLGLFESDHLQYNLEADKTTEPTLTELTEIAIRSLNRNEKGFFLFVEGGR	355
DsALP2	MALSSSPPEYLLGLFESDHSMQYNMQSDKTTEPTLAEELTEIAIKSLSRNENGFFLFVEGGR	352
DsALP3	ETFDTDNTDYLLGIFADGHLSEYEQ <u>RNETDMPSLS</u> DMVAAAIAKVLKNDNGYFLMVEGGN	320
DsALP1	IDHAHHDNYVHLALDETIELSKAVERSMGLLPEDDTLIVVTADHAHVMAFNQYTORGGDI	415
DsALP2	IDHAHHDNYPRALDETIQFSEAVARAAELPEDESLIVVTSDHAVMTLNGYTSRGADI	412
DsALP3	IDMAHHRGWAKVAIDESAEEMEEAVKVALEMIDEENTLFIVTSDHTHTLSINGYPERGSNI	380
DsALP1	LGPSDSVDSNDIRYKTIISYTNG-PG---AREQIDG-----VRPDVFEDEDNAGDTRWRAHA	466
DsALP2	LGPAGQLGDDSVPYMTLSYTNG-PG---FRPHVSH-----GVRDDLTKYNYEALEFRYAA	463
DsALP3	FDIAGASTHDGINYTTLSTGGPDSFHFYVHTDDNNETTVGRRDPSKENTNDMLYNQVA	440
DsALP1	DVPLGSETHGGDDVAVFARGPHHHMFAGLYEQSQIPLHMAYAGCFDTISENPPHCNTAHA	526
DsALP2	EVPLDSETHGGDDVAVFARGPHHHMFSGVYEQSQIPLHMAYTACIG---EGKTVCK-----	516
DsALP3	GIRLIENSHGGDVAIYAKGPHAHLFHNVHEQHYYVYHAVMYAMTHS---STEHYYSGGCA	497
DsALP1	LRSPLLTVLFIVVVSLVRTLFR	548
DsALP2	-----	516
DsALP3	LFASVIMLTSIFMINIILTF--	517

Figure 5.2. Deduced amino acid sequences of alkaline phosphatase isoforms from DsALP1, DsALP2, and DsALP3 in *Diatraea saccharalis* aligned by ClustalW. Residues identical to the consensus sequence of the three ALP sequences are shaded. The predicted signal peptide sequences are single-underlined. Potential N-glycosylation sites are double-underlined. Alkaline phosphatase active domains are indicated inside the rectangle. Predicted GPI anchor sites are indicated by triangles.

anchor but DsALP2 was a possible soluble ALP. All three DsALPs contained a predicted phosphatase domain (I(V)A(P)DS*ACT(S)AT) with S* being the enzymatic active site in the highly conserved protein sequence regions. One potential N-glycosylation site (²⁸³NLSN²⁸⁶ and ²⁸⁰NVTH²⁸³, and ²⁵⁶NKTE²⁵⁹, respectively) was found in each of the three DsALP proteins. Another potential N-glycosylation site was also observed at ²⁸⁷NETD²⁹⁰ in DsALP3 (Fig. 5.2). No potential O-glycosylation sites were detected in any of three DsALPs from *D. saccharalis*.

5.3.3 Phylogenetic Analysis of Three DsALPs from *D. saccharalis*

Compared to 39 ALPs from 8 other insect species that have been reported in the GenBank (accession numbers in Fig. 5.3), the three DsALPs of *D. saccharalis* were located in three separated groups and all shared with a low sequence identity (Fig. 5.3). Amino acid sequences of DsALP1 and DsALP2 of *D. saccharalis* were highly similar to those membrane-bound ALPs from four lepidopteran species, *Bombyx mori*, *Bombyx mandarina*, *H. virescens*, and *Helicoverpa armigera* (Fig. 5.3). Phylogenetic tree also revealed that DsALP3 had a high homology with the DmALP2 from *Drosophila melanogaster*, while it was less similar to the ALPs from other insect species.

5.3.4 Transcriptional Levels of Three DsALP Genes of the Cry1Ab-SS and -RR Strains of *D. saccharalis*

Of the three DsALPs from *D. saccharalis*, transcripts for DsALP1 were the most abundant, while mRNA for DsALP2 expressed the least (Fig. 5.4). There was a trend that the gene expression levels of the three DsALPs of the Cry1Ab-RR were somewhat (15%-48%) greater than those of the Cry1Ab-SS strain for both instars tested. However, the differences were not statistically significant for each of the three DsALPs and for both instars (at 3rd instar, $P =$

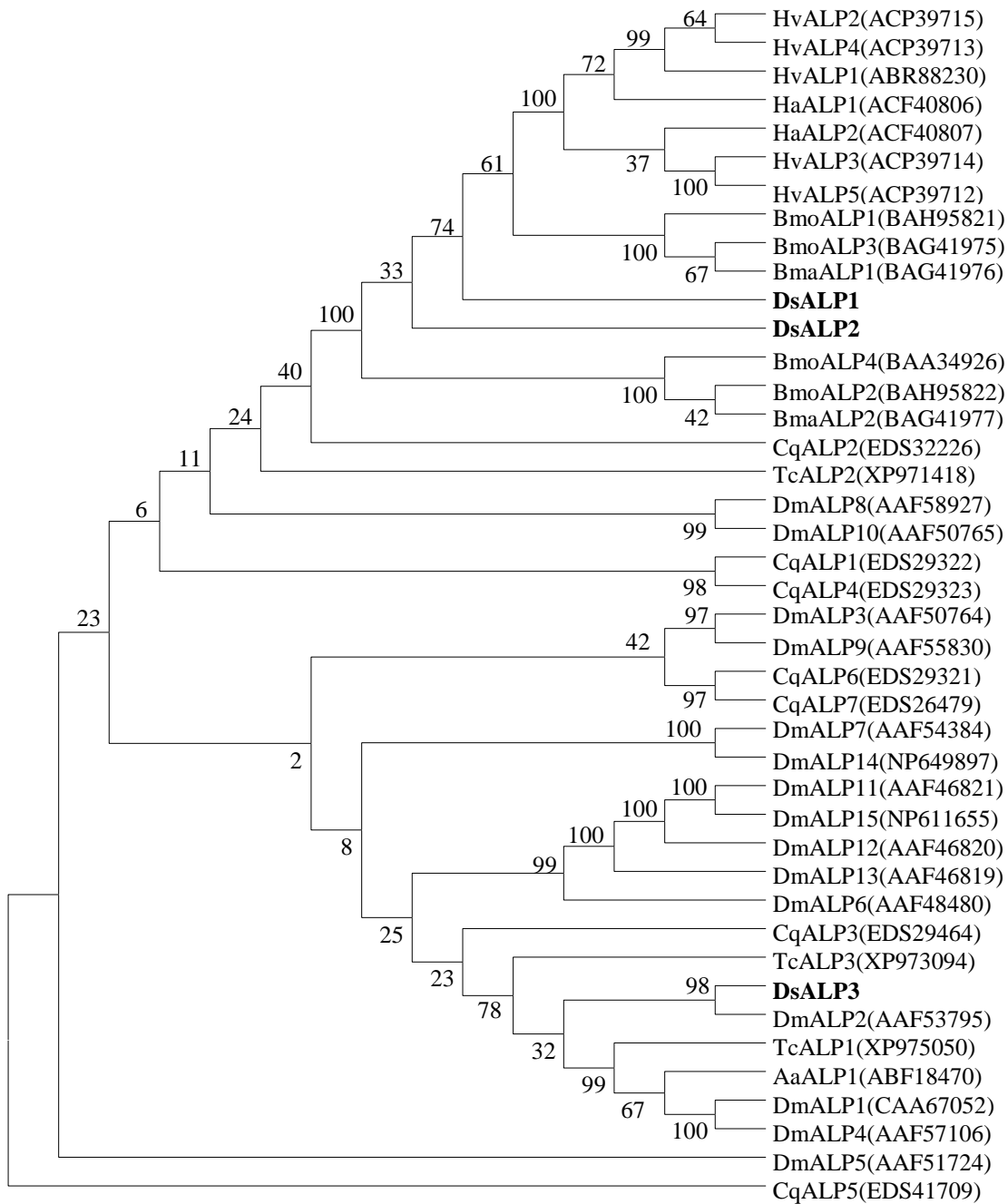


Figure 5.3. Phylogenetic tree to show relationship between DsALPs and ALPs from other insect species using complete protein sequences with Genbank accession numbers of selected and predicted alkaline phosphatases from 42 insect species. ClustalW and MEGA 4.1 (Ver. 4.1; Tamura et al., 2007) were used to generate a basic sequence alignment and an unrooted phylogenetic tree. GenBank accession numbers are shown in parentheses except the three DsALPs. Bootstrap values, expressed as percentages of 500 replications, are shown at branch points.

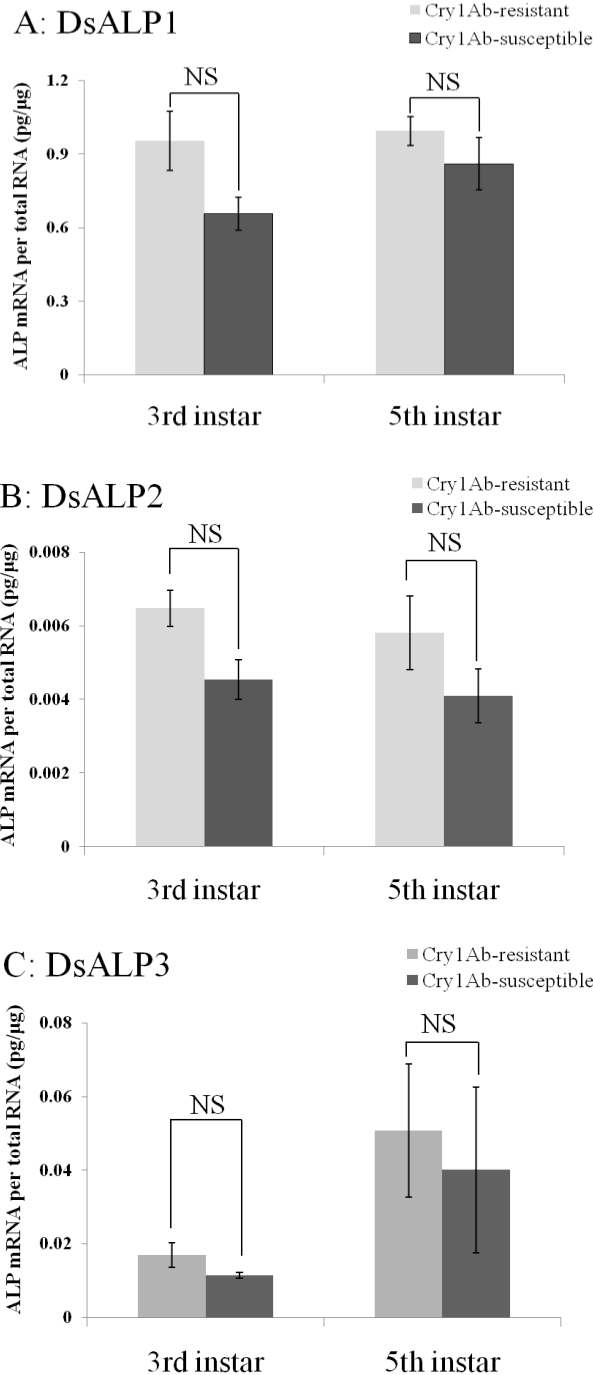


Figure 5.4. Gene expression levels of three alkaline phosphatase genes (A: DsALP1, B: DsALP2, and C: DsALP3) of 3rd and 5th instars of Cry1Ab-susceptible and -resistant strains of *Diatraea saccharalis*. Absolute transcript abundance (ALP mRNA per total RNA (pg/μg)) was determined using qRT-PCR with SYBR green. Bars represent the means and standard errors of three total RNA samples each containing a pool of total RNAs from three larvae. NS indicates no significant differences between Cry1Ab-susceptible and -resistant strains ($P > 0.05$, Student's t-test).

0.11, 0.057, and 0.12 for the three DsALPs, respectively; at 5th instar, $P = 0.32, 0.22, \text{ and } 0.72$ for the three DsALPs, respectively). It appeared that the expression levels of DsALP1 and DsALP3 of the 5th instar larvae were somewhat greater than those of 3rd instars for both insect strains, while such differences were not observed for the DsALP2 (Fig. 5.4).

5.4 Discussion

ALPs (EC 3.1.3.1) are mainly localized in microvilli of columnar cells and the midgut epithelium cells of insects (Wolfersberger, 1984; Eguchi, 1995). Insect ALPs have been proposed to function in active absorption of metabolites and transport processes as well as to participate in cell adhesion and differentiation in some cases (Eguchi, 1995; Chang et al., 1993). ALPs can be divided into two groups, soluble and membrane-bound ALPs (Eguchi, 1995; Itoh et al., 1991; 1999). Both are found in larval midgut epithelium cells. The soluble ALPs are localized only in the apical region of the midgut, while membrane-bound ALPs are particularly restricted in the brush border membrane of columnar cells from the middle and posterior midgut (Eguchi et al., 1972; Azuma and Eguchi, 1989). The two ALP groups are believed to have different functions *in vivo* due to the differences in enzymatic activity and the structure of sugar side chain (Okada et al., 1989).

Membrane-bound ALPs in the midgut are thought to act as Cry toxin binding proteins in lepidopteran, dipteran, and coleopteran insect larvae (Jurat-Fuentes and Adang, 2004; Fernandez et al., 2006; Martins et al., 2010). For example, McNall and Adang (2003) reported that a 65-kDa GPI-anchored ALP in *Manduca sexta* was identified to bind Cry1Ac protein. A recent study by Arenas et al. (2010) also suggested that both GPI-anchored APN and ALP play important roles as functional receptors for Cry1Ab in *M. sexta*. Similarly in *H. virescens*, a 68-kDa GPI-anchored ALP was also demonstrated as a Cry1Ac toxin receptor (Jurat-Fuentes and Adang,

2004). More recently, Ning et al. (2010) identified two ALPs from *H. armigera* with putative roles as receptors for Cry1Ac toxin. For dipteran species, a study by Fernandez et al. (2006) showed that a membrane-bound ALP from *A. aegypti*, Aa-mALP1, could interact with mosquito-larvicidal Cry11Aa and also interact weakly with toxin Cry4Ba. Beside the Aa-mALP1, another 58-kDa GPI-anchored membrane-bound ALP protein was found to be responsible for mediating Cry4Ba toxicity in *A. aegypti* (Dechklar et al., 2011). In a coleopteran species, *A. grandis*, the Cry1Ba6 protein showed its toxicity to the larvae and binding affinity to two GPI-anchored proteins from brush border membrane vesicles with ALP activity (Martins et al., 2010).

In this study, cDNAs encoding three ALPs (DsALP1, DsALP2, and DsALP3) were isolated from larval midguts of Cry1Ab-SS and -RR strains of *D. saccharalis*. No nucleic or amino acid changes existed in the three DsALPs between the two strains. Based on the lepidopteran ALP sequences available in the GenBank (Fig. 5.3), seven out of the 13 ALPs identified in four lepidopteran species have a predicted GPI-anchoring site in their deduced protein sequences. Among those, all seven ALPs with a GPI-anchoring site were identified to be membrane-bound ALPs (Itoh et al., 1999; Perera et al., 2009; Ning et al., 2010). The GPI sequence signature of DsALP1 and DsALP3 identified in *D. saccharalis* suggests that these two DsALPs may likely be membrane-bound ALPs and thus may be functional receptors of Cry toxins. The DsALP2 without the possible GPI-anchoring site may likely be a soluble ALP. However, the phylogenetic analysis revealed that DsALP1 and DsALP2 were grouped with GPI-anchored ALPs that are considered as Bt toxin binding receptors for *B. mori*, *B. mandarina*, *H. virescens*, and *H. armigera* (Itoh et al., 1999; Perera et al., 2009; Ning et al., 2010).

Previous reports have demonstrated that interactions between Cry1Ac toxin and ALPs could lead to decreased enzymatic activity in *M. sexta* (Sangadala et al., 1994). In addition, Jurat-

Fuentes and Adang (2004) showed reduced expression levels of an ALP were associated with Cry resistance in *H. virescens*. Results of the current study showed that the cDNA sequences of the three DsALPs in *D. saccharalis* were identical between the Cry1Ab-SS and -RR strains. In addition, there were also no significant differences in the specific ALP activities between the susceptible and resistant larvae in all three instars tested. However, the expression levels of the three ALP genes of Cry1Ab-RR were higher than those of Cry1Ab-SS strain for the two larval stages examined although the differences were not statistically significant. These results suggested that three DsALPs may not be associated with the Cry1Ab resistance in *D. saccharalis*.

Genetic studies showed that the Cry1Ab resistance in this *D. saccharalis* strain was most likely controlled by one or a few tightly linked gene(s) (Wu et al., 2009). My current and previous studies showed that the cDNAs sequences of trypsins, chymotrypsins, APNs, ALPs, and cadherin genes that have been identified in *D. saccharalis* were identical between the Cry1Ab-SS and -RR strains. The gene expression levels of the trypsins, chymotrypsins, and ALPs were also similar between the two strains. However expression of the DsCAD1 and the three DsAPNs in the Cry1Ab-RR strain was significantly reduced compared to that of the Cry1Ab-SS strain. These results suggest that the Cry1Ab resistance in *D. saccharalis* is likely associated with reductions in gene expressions of the DsCAD1 as well as the three DsAPNs instead of changes in the cDNA sequences of these genes. If the single-gene resistance model suggested by Wu et al. (2009) is true, the Cry1Ab resistance in *D. saccharalis* would likely be caused by a mutated regulatory gene that controls the expression of more than one Bt toxin binding receptors, including the cadherin(s) and DsAPNs identified in other studies. Such type of resistance mechanism has not been reported in Bt resistance. However, mutations in regulatory

genes have been found to be associated with some insecticide resistance in several insect species (French-Constant et al., 2006; Che-Mendoza et al., 2009). For example, mutations in a cis-acting regulatory element are involved in a glutathione S-transferases (GST) over-expression in a DDT (1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane)-resistant strain of *Anopheles gambiae* (Ding et al., 2005). Similarly in *A. aegypti*, a mutation disrupts a trans-acting repressor allowing the over-expression of GST that leads to the DDT resistance for this insect (Grant and Hammock, 1992). A previous study also showed that a transposon insertion into the upstream of the *Cyp6g1* gene enhancers causes over-transcription of the *Cyp6g1*, which results in *Cyp6g1*-mediated cross-resistance to DDT and neonicotinoid imidacloprid in *D. melanogaster* (Daborn et al., 2001). Future studies, such as genome sequencing and genetic mapping by AFLP markers, are needed to validate if such a mutated regulatory gene is responsible for the changes in the gene expressions of DsCAD1 and the three DsAPNs in the Cry1Ab resistance in *D. saccharalis*.

5.5 References

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Recently, sugarcane borer, *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae), has become a common corn pest in many areas of the U.S. mid-southern region. Transgenic *Bacillus thuringiensis* (Bt) corn has been used to manage corn stalk borers including *D. saccharalis* since it was commercialized in 1999 in this region. In 2004, a strain of *D. saccharalis* (Cry1Ab-RR) that expressed a high level of Cry1Ab resistance was isolated from a field population collected from northeast Louisiana. In the most commonly proposed Bt mode of action, Cry protoxins ingested by insect are first cleaved by midgut proteinases (e.g., trypsins, chymotrypsins) to form activated toxins. The activated Cry protein then binds to primary receptors (e.g., cadherins) at the midgut epithelium resulting in further proteolytic cleavage of Bt toxins into smaller oligomers. The toxin oligomers then bind to secondary receptors (e.g., glycosylphosphatidylinositol (GPI)-anchored aminopeptidase N or alkaline phosphatase) on the midgut membrane. The secondary bindings result in insertion of toxin oligomers into the lipid rafts, where they form pores that cause cells to burst, resulting in insect death. Theoretically, disruption of any step of the toxin action pathway could cause insect resistance.

Three different types of Bt resistance mechanisms have been documented. These mechanisms include 1) changes in Bt binding receptor proteins in the midgut that lead to reduce in Bt binding; 2) reduced midgut proteinase activity that results in decrease in Bt protoxin activation; 3) up-regulated expression of proteinases which can cause increase in Bt toxin degradation; and 4) increase in immune response or enhanced esterase sequestration. The major goal of this study is to elucidate the mechanism(s) of the Cry1Ab resistance in *D. saccharalis*. To achieve this goal, studies were performed to compare the cDNA sequences, gene expressions,

and enzymatic activities (except cadherins) of five common types of Bt resistance candidates between Cry1Ab-susceptible (Cry1Ab-SS) and Cry1Ab-RR strains. These Bt resistance candidate genes included three trypsins (DsTRYs), three chymotrypsins (DsCHYs), three aminopeptidases N (DsAPNs), one cadherin (DsCAD1), and three alkaline phosphatases (DsALPs). cDNAs of these candidate genes were cloned and sequenced using reverse transcriptase polymerase chain reaction (RT-PCR), PCR, and 5' rapid amplification of cDNA end (5' RACE). Quantitative RT-PCR was used to determine the transcriptional levels of each gene. RNA interference (RNAi) was employed using dsRNA oral droplet feeding for the three DsAPNs and DsCAD1 of the Cry1Ab-SS strain. Enzymatic activity of total midgut trypsins, chymotrypsins, and aminopeptidases N, and alkaline phosphatases were also determined for both insect strains.

Beside a few random SNPs which were found in individuals within each of the insect strain, cDNA sequences of the three trypsin and three chymotrypsin genes were the same between Cry1Ab-SS and -RR strains of *D. saccharalis*. cDNA sequences of the three DsALPs in *D. saccharalis* were also identical between the Cry1Ab-SS and -RR strains. Quantitative RT-PCRs showed that the transcriptional levels of the three DsTRYs, three DsCHYs, and three DsALPs in Cry1Ab-RR were not significantly different compared to those in the Cry1Ab-SS strain. There were also no significant differences in specific activities of total trypsins, chymotrypsins, or alkaline phosphatases between the two strains. The results suggest that the three trypsins, the three chymotrypsins or the three alkaline phosphatases are not associated with the Cry1Ab resistance in *D. saccharalis*.

Similarly, cDNA sequences of the three DsAPNs and DsCAD1 genes were identical between the Cry1Ab-SS and -RR strains. However, the gene expressions of the three DsAPNs

and DsCAD1 from Cry1Ab-RR larvae were significantly lower than those of the Cry1Ab-SS strain. In addition, total APN activity of the Cry1Ab-RR larvae was significantly lower than that of the Cry1Ab-SS strain. RNA interference (RNAi) study also showed that down-regulating expression of each of the three DsAPN and DsCAD1 genes by RNAi was correlated with the decrease in susceptibility of *D. saccharalis* to Cry1Ab. In addition, gene expression of each of the three DsAPNs as well as total APN activity after RNAi was significantly reduced compared to the non-RNAi-treated control insects. The results demonstrate that reduction in expression of the three DsAPNs and DsCAD1 is associated with the Cry1Ab resistance in *D. saccharalis*.

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VITA

Yunlong Yang was born in Wuhan, Hubei Province, China, on September, 1981, the son of Xiantong Yang and Yalan Li. Mr. Yang was married to Dongli Guan and they have a lovely son, Wallace Yang, seven month old. After completing his degree at Wuhan No.2 High School, Wuhan, Hubei, in 2000, Mr. Yang entered Huazhong Agricultural University, Wuhan, Hubei, China, and received the degree of Bachelor of Science (Biotechnology) in July, 2004. After graduation, Mr. Yang enrolled into the graduate program of biochemistry and molecular biology at Huazhong Agricultural University from 2004 to 2006. From August, 2006, Mr. Yang enrolled into graduate studies under the co-supervision of Dr. Fangneng Huang in the Department of Entomology at Louisiana State University and Agricultural and Mechanical College and Dr. Yu Cheng Zhu in the United State Department of Agriculture, Agricultural Research Service at Stoneville, Mississippi. He currently is a doctoral candidate in the Department of Entomology.