RESISTANCE TO *BACILLUS THURINGIENSIS* TOXIN CRY2AB IN THE CABBAGE LOOPER, *TRICHOPLUSIA NI*

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RESISTANCE TO *BACILLUS THURINGIESIS* TOXIN CRY2AB IN THE CABBAGE LOOPER, *TRICHOPLUSIA NI*

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Development of insect resistance to *Bacillus thuringiensis* (Bt) toxins threatens the long-term success of transgenic crops expressing the Bt toxins. However, current understanding of Bt resistance evolved in agricultural situations is very limited. The goal of this dissertation research was to understand the greenhouse-evolved resistance to Bt toxin Cry2Ab in a *Trichoplusia ni* strain (Glen-BGII) with dual resistance to both Cry1Ac and Cry2Ab. Diet overlay bioassays with Cry2Ab determined that the Glen-BGII strain is highly resistant to Cry2Ab, with a resistance ratio > 1,492-fold. Characterization of the inheritance of Cry2Ab resistance revealed that the resistance is incompletely recessive, monogenic, autosomal, and with no maternal effects. Investigation on the genetic association between the resistance to Cry1Ac and Cry2Ab in the Glen-BGII strain demonstrated that Cry2Ab resistance is not genetically associated with Cry1Ac resistance.

The Cry2Ab-resistance trait in the Glen-BGII strain was introgressed into an inbred susceptible laboratory strain (Cornell-SS), and the subsequent introgression strain (Glen-Cry2Ab-BCS8) and the Cornell-SS strain were used for comparative analysis to identify the biochemical changes that might be associated with the resistance trait. Biochemical analysis included assays of caseinolytic activity and zymogram profiles of

midgut proteases, activities of serine proteases (trypsin, chymotrypsin, and elastase), activity and zymogram profile of midgut esterases, activity of midgut aminopeptidase, activity and protein abundance of midgut alkaline phosphatase, and melanization activity of hemolymph plasma. No biochemical changes were observed to be associated with the resistance. In addition, an *in vitro* proteolytic digestion assay showed that Cry2Ab was similarly digested by midgut fluid from the susceptible and resistant larvae.

Genetic linkage analysis of Cry2Ab resistance with the genes coding for putative midgut receptors for Cry toxins indicated that Cry2Ab resistance is not genetically linked with the cadherin, aminopeptidase N, or alkaline phosphatase genes. The lack of genetic linkage between Cry2Ab resistance and the ATP-binding cassette transporter ABCC2 gene locus indicated that Cry2Ab resistance is not genetically linked with the ABCC2 gene locus.

Comparative quantitative proteomic analysis of larval midgut brush border membrane vesicle proteins identified 1,462 proteins and 951 were quantitatively analyzed between the susceptible Cornell-SS and resistant Glen-Cry2Ab-BCS8 strains. Two midgut BBMV proteins were putatively found significantly different in quantity between the susceptible and resistant larvae, but no significant difference was detected by quantitative real-time RT-PCR analysis at the transcript level of the two genes between the two strains. Further functional analysis of these two candidate proteins is required to confirm and understand their potential involvement in Cry2Ab resistance in *T. ni.*

BIOGRAPHICAL SKETCH

Xiaozhao Song was born in Deqing County, Zhejiang Province, China in 1983. She received her Bachelor of Science degree in Biological Sciences from Shanghai Ocean University (called Shanghai Fisheries University back then) in 2005. She spent the next two years studying the ecology of benthic algal community in Northern Michigan with Dr. Rex Lowe, and obtained her Master of Science degree from Bowling Green State University in Ohio. In 2007, she began to pursue her doctorate degree in Entomology at Cornell University under the supervision of Dr. Ping Wang. Her dissertation research was on insect resistance to *Bacillus thuringiensis* toxins.

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CHAPTER 1

REVIEW OF THE LITERATURE AND STATEMENT OF THE PROBLEM

Introduction

The soil bacterium *Bacillus thuringiensis* (Bt) produces environmentally benign and target specific insecticidal proteins, and has been used for insect pest control for more than 70 years (Schnepf et al., 1998; Bravo et al., 2011). Genetically modified crops expressing Bt toxins, known as Bt-crops, have dramatically increased the efficacy of Bt applications and have been increasingly adopted worldwide for insect pest management since their first commercialization in 1996 (James, 2013). However, the long-term success of Bt-crops is threatened by the development of insect resistance to Bt toxins. To date, insect resistance or reduced susceptibility to Bt-crops in the field has been reported in at least one coleopteran and six lepidopteran pests (Van Rensburg, 2007; Tabashink et al., 2009; Bagla, 2010; Downes et al., 2010; Storer et al., 2010; Gassmann et al., 2011; Zhang H, et al., 2012a). Thus, for long-term sustainable application of Bt-based biopesticides and Bt-crops in modern agriculture, it is imperative to develop and employ Bt resistance management programs whose success requires a thorough understanding of Bt resistance evolved in insects and the underlying mechanisms.

Toxin pyramiding, or stacking, which involves simultaneous expression of two or more toxins with alternative modes of action or different toxin binding sites, is one of the resistance management tactics to delay the evolution of insect resistance to Bt-crops

(Roush, 1998; Zhao et al., 2003). Bt Cry1A toxins have been the major toxins used in Bt-crops, primarily Bt-cotton and Bt-maize, for lepidopteran pest control since Bt-crops became commercially available in 1996. Bt Cry2Ab, due to its lack of shared binding sites with Cry1A toxins in insects (English et al., 1994; Morse et al., 2001; Luo et al., 2007; Hernandex-Rodriguez et al., 2008), have been pyramided with Cry1A toxins in the second generation Bt-crops. For instance, Bollgard II cotton variaties expressing Cry1Ac and Cry2Ab have been commercially available to target lepidopteran pests since 2002. Although a few studies on resistance to Cry2Ab have been reported in *Helicoverpa punctigera* and *Helicoverpa zea* (Tabashnik et al., 2009a; Downes et al., 2010), there is very limited knowledge on resistance to Cry2A. This dissertation focuses on the biology, genetics and molecular basis of resistance to Bt toxin Cry2Ab in the cabbage looper, *Trichoplusia ni*.

Bt and its application

Bt is a rod-shaped, aerobic, spore-forming, gram-positive soil bacterium. Bt was first isolated in 1901 by a Japanese biologist, S. Ishiwatari, who identified the bacterium as the causal agent of a disease of silkworms (Ishiwata, 1901). In 1911, Bt was rediscovered and named by a German scientist Ernst Berliner when he isolated a related strain from dead Mediterranean flour moth larvae that he found in a flour mill in the German state of Thuringia (Berliner, 1915).

During bacterial sporulation, Bt produces δ-endotoxins known as crystal (Cry) and cytolitic (Cyt) proteins. To date, more than 700 Cry sequences in 73 classes (Cry1-

Cry73) have been described, as well as 38 Cyt sequences in 3 classes (Cyt1-Cyt3) (Crickmore et al., 2014). Bt proteins are classified based on their amino acid sequence similarity (Figure 1.1, Figure 1.2). The Cry proteins are selectively toxic to different insect orders such as Lepidoptera, Coleoptera, Hymenoptera and Diptera, and different species (Hofte and Whiteley, 1989; Crickmore et al., 1998; Bravo et al., 2007), whereas the Cyt proteins are mostly active against Diptera with a few exceptions of Cyt proteins being active against Coleoptera (Federici and Bauer, 1998; Guerchicoff et al., 2001). Table 1.1 presents the target insect spectrum of major Cry and Cyt proteins. In addition to Cry and Cyt proteins, vegetative insecticidal proteins (Vips) are secreted from the cell as non-crystal-forming proteins by some Bt strains during the vegetative growth phase before sporulation (Estruch et al., 1996; de Maagd et al., 2003).

The discovery of the insecticidal properties of Bt led to the development of application of Bt for insect pest control, and the initial attempts to use Bt for insect control started in the late 1920s (Sanchis, 2011). In 1938, the first commercial Bt product, Sporeine, was developed and used for insect control in France. However, it was not until 1961 that Sporeine was registered as a pesticide in the United States (Sanahuja et al., 2011). Thuricide was the first commercial Bt product that became available for field testing in the United States in 1958. In the 1980s, commercial interest in Bt grew rapidly when alternatives to synthetic insecticides were sought. Despite their potent and specific insecticidal activities, the practical use of topical Bt sprays is limited by their susceptibility to proteases in leaf exudates, vulnerability to inclement weather conditions, and lack of effectiveness against certain cryptic pests inside the stalk or near the root (Kaur, 2000).

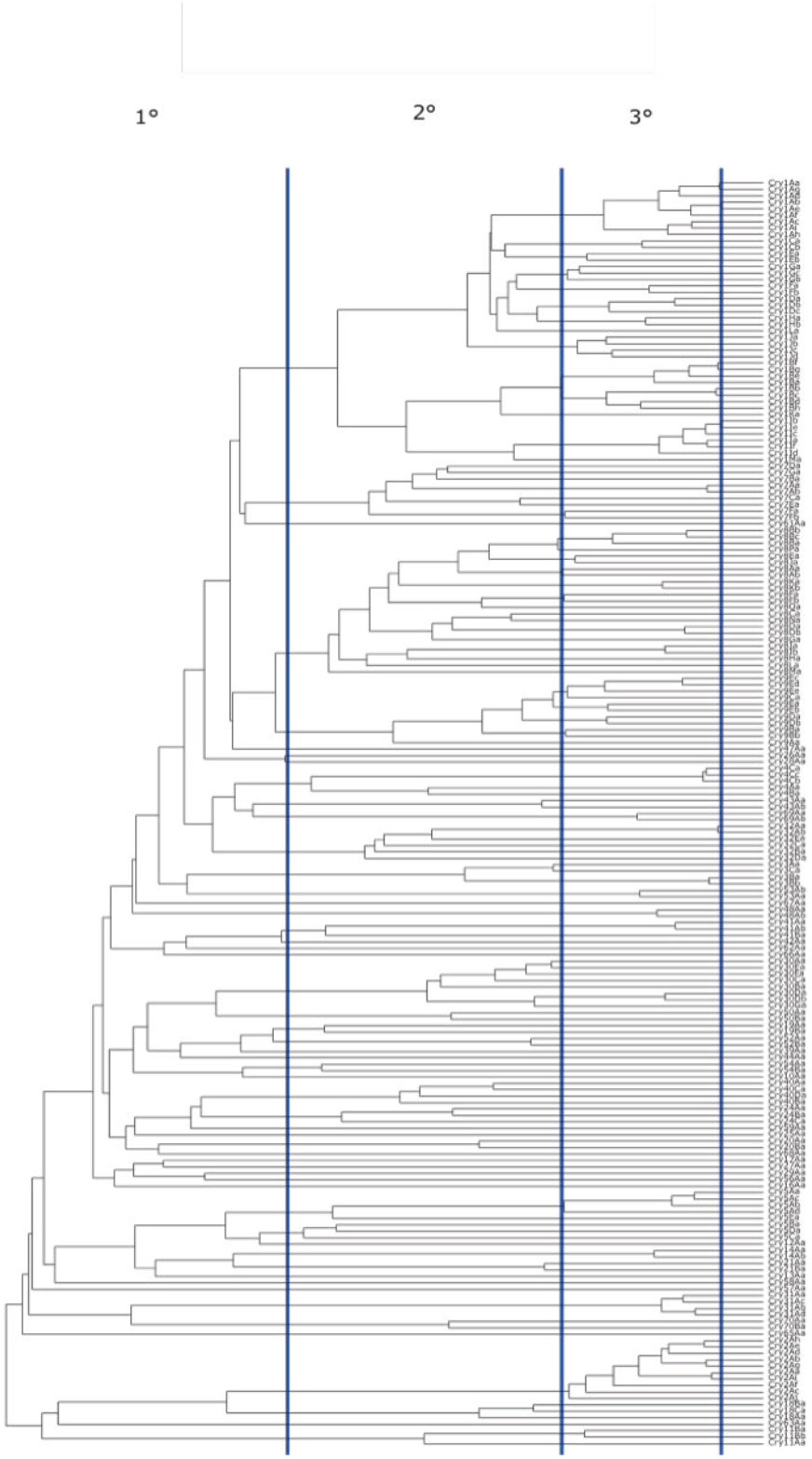

Figure 1.1. Phylogenetic trees representing three-domain Cry proteins. The vertical bars demarcate the four nomenclature ranks (Crickmore, 2014).

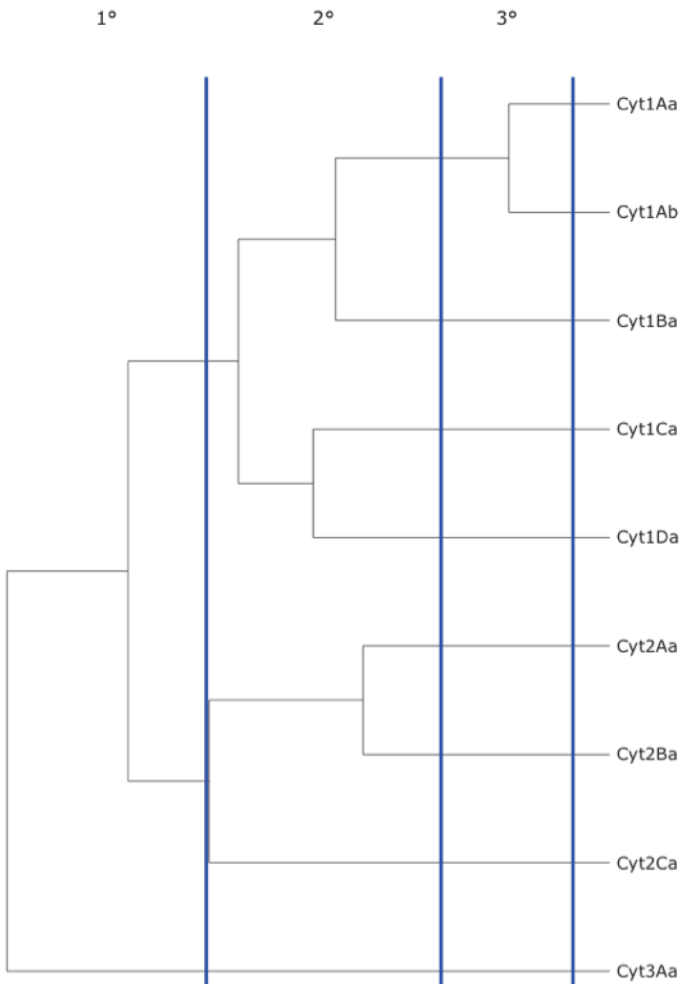

Figure 1.2. Dendrogram of Cyt proteins. The vertical bars demarcate the four nomenclature ranks (Crickmore, 2014).

Table 1.1. Target insect spectrum of major Cry and Cyt proteins.

Bt toxin class	Primary target spectrum
Cry1	Lepidoptera
Cry2	Lepidoptera
Cry3	Coleoptera
Cry4	*Diptera* (mainly, mosquitoes and blackflies)
Cyt1	*Diptera* (mainly, mosquitoes and blackflies)

Introduction of the Cry genes from Bt var. *kurstaki* into tobacco and tomato plants in the mid-1980s opened a new era of pest management using biotechnology (Barton et al., 1987; Fischhoff et al., 1987; Vaeck et al., 1987). In 1995, the US Environmental Protection Agency (EPA) approved the first registration of Bt-crops (potato, corn and cotton crops) with commercially viable expression of Bt toxin genes. Since then, Bt-crops, mainly Bt-corn and Bt-cotton, have become increasingly adopted in agriculture, and are now grown widely across the globe. In 2012, a record 170.3 million hectares of Bt-crops were planted worldwide, at an annual growth rate of 6% (James, 2013). The Bt toxin genes that are engineered in commercial Bt-cotton include those encoding Cry1Ac, Cry1Ab, Cry2Ab, Cry1F, and Vip3Aa, providing crop protection from a number of economically important lepidopteran pests, including *Heliothis virescens*, *Helicoverpa zea*, *Helicoverpa armigera*, *Pectinophora gossypiella*, and *Spodoptera frugiperda* (ISAAA, 2012). As with cotton, Bt-corn hybrids harbor Bt toxin genes encoding varying proteins (e.g., Cry1Ab, Cry3Aa, Cry3Bb1, Cry1F, Cry34Ab1/Cry35Ab1) and are active to their target pests, such as *Ostrinia nubilalis*, *Diatraea grandiosella*, *Diatraea saccharalis*, and *H. zea* (Head and Greenplate, 2012). Table 1.2 and Table 1.3 list the commercially approved Bt-cotton and Bt-corn events, respectively. Benefits of Bt-crops include enhanced efficacy in insect pest control, reduced usage of synthetic chemical insecticides, conservation of beneficial natural enemies, and increased yields and farmer profits (Qaim and Zilberman, 2003; Cattaneo et al., 2006; Hutchison et al., 2010).

Table 1.2. Commercially approved Bt-cotton events (Head and Greenplate, 2012)

Event	Bt proteins expressed	Targets	Provider
DAS-24236-5	Cry1F	Lepidoptera	Dow Agro Sciences
DAS-21023-5	Cry1Ac	Lepidoptera	Dow Agro Sciences
COT 102	Vip3A	Lepidoptera	Syngenta
COT 67B	Cry1Ab	Lepidoptera	Syngenta
MON 15985	Cry1Ac and Cry2Ab	Lepidoptera	Monsanto
MON 531	Cry1Ac	Lepidoptera	Monsanto
sGK321	Cry1A and cowpea trypsin inhibitor	Lepidoptera	Chinese Acad Sci
GK12	Cry1Ac/Cry1Ab fusion	Lepidoptera	Chinese Acad Sci
BNLA-601	Cry1Ac	Lepidoptera	CICR (Indian Govt)
Event 1	Cry1Ac	Lepidoptera	JK Agrigenetics Ltd. (India)
GFM	Cry1Ac/Cry1Ab fusion	Lepidoptera	Nath Seeds (India)
MLS-9124	unknown protein	Lepidoptera	Metahelix Life Sciences (India)
Silver Six	unknown proteins	Lepidoptera	Myanmar Govt

Table 1.3. Commercially approved Bt-corn events

Event	Bt proteins expressed	Targets	Provider
MIR604	Cry3Aa	Diabrotica spp	Syngenta
MIR162	Vip3A	Lepidoptera	Syngenta
MON 810	Cry1Ab	Lepidoptera	Monsanto
MON863; MON 88017	Cry3Bb1	Diabrotica spp	Monsanto
MON 89034	Cry1A105 and Cry2Ab2	Lepidoptera	Monsanto
TC 1507	Cry1F	Lepidoptera	Dow Agro Sciences
DAS 59122	Cry34Ab1 and Cry35Ab1	Diabrotica spp	Dow Agro Sciences

Bt resistance and resistance management

Despite the success of Bt-based biopesticides and Bt-crops, their long-term sustainable applications in agriculture is threatened by development of Bt resistance in target insect populations (Tabashnik et al., 1994; Gould, 1998; Carriere et al., 2010). Insect populations may evolve resistance to the insecticidal Bt Cry toxins, following prolonged exposure to the widespread use of the Bt-based biopesticides and Bt-crops.

Laboratory-selected resistance to Bt toxins was first found in the indianmeal moth, *Plodia interpunctella* (McGaughey, 1985). Since then, a number of insect species have developed Bt resistance by laboratory selections, including *H. virescens*, *O. nubilalis*, *P. gossypiella*, *H. armigera*, and *Spodoptera exigua* (Ferre and Van Rie, 2002; Bravo and Soberon, 2008). The most common type of Bt resistance is "Mode 1," which is characterized by a high level of resistance (>500-fold) to one or more Cry1A toxins, recessive inheritance, reduced binding of one or more Cry1A toxins to the midgut brush border membrane and little or no cross-resistance to Cry1C toxin (Tabashnik et al., 1998). "Mode 1" type resistance has been reported in at least one strain of the following insect species: *P. xylostella*, *H. virescens*, *P. interpunctella*, *P. gossypiella*, *H. armigera*, and *T. ni* (Tabashnik et al., 1998; Tabashnik et al., 2002; Morin et al., 2003; Kain et al., 2004).

The first case of field-evolved resistance to formulations of Bt sprays was reported in populations of diamondback moth, *Plutella xylostella*, in Hawaii and Florida in the USA (Tabashnik et al., 1990; Shelton et al., 1993). *T. ni* is another species that has been found to have developed Bt resistance in agricultural settings. *T. ni* developed

resistance to Dipel, a Bt-based biopesticide, following its intensive use in commercial greenhouses (Janmaat and Myers, 2003). *T. ni* is a member of the Noctuidae family of Lepidoptera, and is one of the most damaging lepidoteran pests on cabbage and other cole crops (Shelton et al., 1982; Liu et al., 1999; Hines and Hutchison, 2001). As an alternative to common synthetic insecticides, Bt-based biopesticides have been effective in the control of *T. ni* in the field and commercial greenhouses (Cao et al., 1999; Liu, 1999; Lundgren et al., 2002; Cho et al., 2001; Janmaat and Myers, 2003). Following intensive exposure to Bt spray formulations, populations of *T. ni* showed resistance to Dipel, a product of *B. thuringiensis* subs. *kurstaki* (Btk), in commercial greenhouses in British Columbia, Canada (Janmaat and Myers, 2003).

More recently, resistance to Bt-crops has been reported in field populations of at least seven insect pest species (Van Rensburg, 2007; Tabashink et al., 2009; Bagla, 2010; Downes et al., 2010; Storer et al., 2010; Gassmann et al., 2011; Zhang H, et al., 2012a). In some populations of *S. frugiperda* in Puerto Rico, an extremely high level of field-evolved resistance to Cry1F corn was observed in 2006, leading to the withdrawal of Cry1F corn from the marketplace (Storer et al., 2010). Field-evolved resistance in a population of maize stalk borer *Busseola fusca* to Bt-maize producing Cry1Ab in South Africa was first reported in 2007 (Van Rensburg, 2007). Later, a second population of *B. fusca* resistant to Bt-maize was found in South Africa, an area 60km from the site of former resistant population of the same species (Kruger et al., 2009). Also, populations of the western rootworm, *Diabrotica virgifera virgifera*, in the United States exhibited resistance to Cry3Bb1 corn (Gassmann et al., 2011). Resistance to Bt toxins expressed in Bt-corn can happen in field populations of insects, jeopardizing the effectiveness of Bt-

corn in the field. Cases of resistance or reduced susceptibility to Bt-cotton were reported in target insect species, including *P. gossypiella* to Cry1Ac cotton in China and India (Wan et al., 2012; Bagla, 2010), *H. zea* to Cry1Ac and Cry2Ab cotton in the southeastern United States (Tabashnik et al., 2008, 2009a), *H. armigera* to Cry1Ac cotton in northern China (Zhang el al., 2011), and *H. punctigera* to Cry2Ab toxin which is a major toxin in second generation Bt-cotton in Australia (Downes et al., 2010).

Most reports of field-evolved insect resistance to Bt-crops involve resistance to Cry1A toxins, whereas only two insect species have evolved resistance to Cry2A toxins (Tabashnik et al., 2009a; Downes et al., 2010). Tabashnik et al. (2009a) found evidence for reduced susceptibility to Cry2Ab in field populations of *H. zea* in the southeastern United States by analyzing the dose-response data of 82 *H. zea* populations to Cry2Ab from diet incorporation assays (Ali and Luttrell, 2007). The percentage of field-derived populations of *H. zea* with a resistance ratio >10 and an LC_{50} value greater than a diagnostic concentration increased from 0% in 2002 to 50% in 2005 (Tabashnik et al., 2009a). In addition, substantial decreases in susceptibility of field populations of *H. zea* in Arkansas to a discriminating dose of Cry2Ab were observed in 2010 when compared to the previous four years (Jackson et al., 2011). Nevertheless, Cry2Ab resistance in *H. zea* remains controversial and needs more empirical data to validate (Tabashnik et al., 2013). In Australia, field populations of *H. punctigera* displayed significant increases in the frequency of Cry2Ab-resistant alleles since the adoption of Bt-cotton expressing Cry1Ac and Cry2Ab, although the emergence of field-evolved Cry2Ab resistance has not caused field control failures to date (Downes et al., 2010a).

Insect resistance management programs have been designed and implemented to slow down the selection for Bt resistance and thereby sustain the effectiveness of Bt-based biopesticides and Bt-crops in agriculture. High dose/refuge and gene pyramiding are the major tactics in resistance management. Other resistance management approaches involves engineering Bt-crops with novel or modified Cry toxins, monitoring the development of resistance by regular assessment of target pest populations, developing remedial action plans, and promoting communications between growers and researchers (Bravo and Soberon, 2008; Head and Greenplate, 2012).

The high dose/refuge strategy is the most widely adopted approach to delay resistance to Bt-crops and has been mandated in the USA, Australia, Brazil and elsewhere (Gould, 1998; Tabashnik et al., 2008). This approach requires the planting of non-Bt crops as refuge adjacent to Bt-crops that express high concentrations of Cry toxins. The non-Bt crop refuges harbor susceptible insects that mate with the rare homozygous resistant insects from Bt-crop planting areas, and the heterozygous F_1 progeny die on Bt-crops expressing high dose of toxins (Gould, 1998; Carriere and Tabashnik, 2001). This strategy works for recessive resistance, and is most effective in delaying the development of insect resistance with the following conditions: abundant refuges of non-Bt crops near Bt-crops, low initial frequency of resistance alleles, and fitness costs and incomplete resistance (Gould, 1998; Carriere and Tabashnik, 2001).

Gene pyramiding involves concurrent expression of two or more toxins with different modes of action, for example, the toxins have different target binding sites, in genetically modified crops, and it also has been effective in delaying the development of resistance to Bt-crops (Roush, 1998; Zhao et al., 2003). The multiple toxins stacked in

pyramided Bt-crops usually have different target binding sites in insects. In this case, it requires insect populations to evolve multiple resistance mechanisms to lose susceptibility to pyramided Bt-crops, significantly hindering resistance development. Pyramided Bt-crops are known as second generation Bt-crops, as compared to the first generation Bt-crops producing single toxins. Besides delayed development of resistance to Bt-crops, the benefits of pyramided Bt-crops also include broadened insecticidal spectrum and increased efficacy of Bt-crops for less susceptible insect species (Roush, 1998; Ferre et al, 2008). Some examples of pyramided Bt-crops are Bollgard II cotton (Monsanto) expressing Cry1Ac and Cry2Ab toxins, SmartStax corn (Monsanto and Dow Agro Sciences) containing a total number of six Cry genes (Cry34Ab, Cry35Ab, Cry3Bb, Cry1A.1.05, Cry2Ab and Cry1F), and the most recently launched Agrisure Duracade corn (Syngenta) expressing a chimera of modified Cry3A and Cry1Ab genes.

Inheritance of Bt resistance

Most cases of Bt resistance selected either in the laboratory or in the field are incompletely recessive, autosomally inherited with no maternal effects, and controlled by a single major resistance locus (Ferré and Van Rie, 2002; Kain et al, 2004; Pereira et al., 2008). Exceptions to this pattern include the incompletely dominant resistance in a Dipel-resistant strain of *O. nubilalis* (Huang et al., 1999), polygenic resistance to Cry1Ab in two laboratory selected strains of *O. nubilalis* (Alves et al. 2006), sex-linked resistance to Cry1Ac in strains of *P. xylostella* in Malaysia (Sayyed et al., 2000), and resistance to

Cry1C with an obvious paternal effect in a colony of *Spodoptera littoralis* (Chaufaux et al., 1997).

Mode of action of Bt toxins

A thorough understanding of how insects develop Bt resistance is required for successful resistance management programs. To identify the genetic and molecular basis of Bt resistance, it is necessary to understand the mode of action of Bt toxins.

Studies and literature on the mode of action of Bt toxins have been mainly on Bt Cry proteins. Currently, there are two major models proposed for the mode of action of Bt Cry toxins, the pore-formation model (Bravo et al., 2013) and the signal transduction model (Zhang, et al., 2006). According to both models, the pathogenic pathway of Cry toxins comprises multiple sequential steps that eventually result in insect mortality. Both models share the same first steps, including ingestion of pro-toxins by a susceptible insect, solubilization and consequential release of pro-toxins by an alkaline environment in the insect gut, activation of protoxins to active forms by midgut digestive proteases, and specific recognition and binding of active toxins to primary receptors (cadherin-like proteins) located in the microvilli of the midgut epithelium (Gill et al., 1992). The succeeding steps of the two models diverge. The pore-forming model postulates oligomerization of toxin monomers to form structures capable of binding to secondary receptors (i.e., aminopeptidase N and membrane-bound alkaline phosphatase), insertion of toxin oligomer into lipid raft membranes and formation of pores, followed by cell lysis and eventual death of the insect (Pigott and Ellar, 2007; Bravo and Soberon, 2007). By

contrast the signal transduction model proposes that binding of the Cry toxin to the cadherin receptor triggers a signaling pathway involving activation of G protein and adenylyl cyclase, increased cAMP levels, and activation of protein kinase A, which in turn leads to cell lysis and death (Zhang et al., 2006). Although the specific mode of action of Bt Cry toxins remains to be better understood, it is generally accepted that the interaction between Cry toxins and host midgut receptors is crucial for the toxicity of Cry toxins. This interaction is specific to Bt Cry toxins and host insect receptors, and is considered to be the underlying mechanism for the highly selective toxicity of different Cry toxins.

The multistep pathway of Bt Cry toxin activation and binding after ingestion by insects, as illustrated in Figure 1.3, determines toxicity. Therefore alteration of any of these steps may lead to development of resistance to Cry toxins. Multiple Bt resistance mechanisms have been identified in laboratory selected Bt-resistant lepidopterans, indicating that insects have the potential to develop resistance to Bt toxins in very diverse and complex ways (Ferre and Van Rie, 2002; Griffitts and Aroian, 2005; Heckel et al., 2007; Pardo-Lopez et al., 2013).

Mechanism of Bt resistance

Alteration of Bt toxin binding to midgut receptors

Loss or reduction of toxin binding in the midgut epithelium has been reported to be the most common mechanism underlying high level insect resistance to Bt Cry toxins (Ferré and Van Rie, 2002; Carcia et al., 2010; Jurat-Fuentes et al.). Several studies have

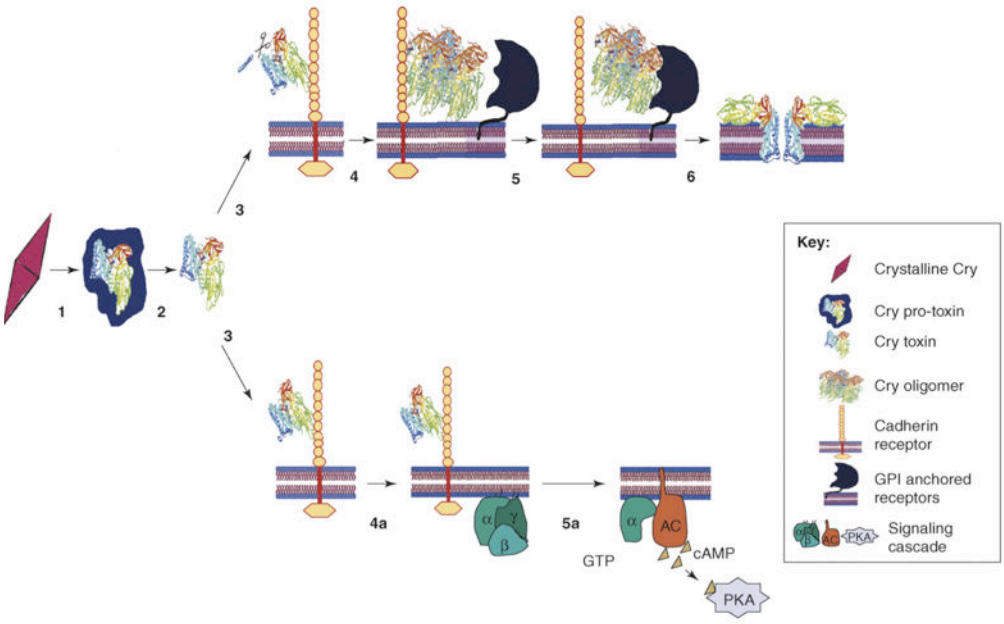

Figure 1.3. Schematic representation of the mode of action of Cry toxins in Lepidoptera. Two different mechanisms can be distinguished: the pore-formation model (top) and the signal tranduction model (bottom), which both include similar steps for toxin solubilization in midgut lumen (1), activation by midgut proteases (2), and binding to a midgut receptor cadherin (3). In the pore-formation model (top), step 3 induces the cleavage of helix α-1 and triggers toxin oligomerization (4). The toxin oligomer then binds to ALP or APN, which are anchored by glycosylphosphatidylinosito anchor in the membrane (5). Finally, the toxin inserts itself into the membrane, forming a pore that kills the insect cells (6). In the signal transduction model (bottom), the interaction of the toxin with the cadherin receptor triggers an intracellular cascade pathway that is mediated by activation of protein G (4a), which, in a subsequent step (5a), activates adenylyl cyclase. This signal then increases the levels of cyclic adenosine monophosphate, which activates protein kinase A and leads to cell death.

examined mutations in the genes coding for midgut receptors, and they are reviewed below.

Cadherin, and the glycosylphosphatidylinositol (GPI)-anchored proteins aminopeptidase N (APN) and membrane-bound alkaline phosphatase (mALP) are the major known midgut receptors for Bt Cry toxins, especially for Cry1A toxins (Piggot and Ellar, 2007). Knowledge of mutations in Bt toxin receptor genes will not only shed light on how insect species evolve resistance to Bt toxins but also facilitate development of successful DNA-based monitoring methods for resistance management (Morin, 2003; Gahan et al., 2007), as well as provides insight into toxin mechanism of action.

Cadherins are a superfamily of proteins that are involved in biological processes including cell adhesion, morphogenesis, cytoskeletal organization, and cell sorting/migration (Angst et al., 2001). In Lepidoptera, caherin-like proteins localized on the apical membrane of midgut columnar epithelial cells have been extensively studied as Cry1A receptors. The well-characterized cadherin-like proteins serving as Cry1A receptors in lepidopterans include BT-R_1 in *Manduca sexta* (Vadlamudi et al., 1995), BtR175 in *Bombyx mori* (Nagamatsu et al., 1998), HevCaLP in *H. virescens* (Jurat-Fuentes et al., 2004).

Gahan et al. (2001) reported the first evidence of cadherin mutation-mediated resistance to Bt toxins. In their study, a 2.3-kb insertion disrupting the full-length cadherin-like protein sequence was shown to be tightly linked to the high level of Cry1Ac resistance in a laboratory-selected strain of *H. virescens*. This 2.3-kb insert had a long terminal repeat-type retrotransposon, introducing a stop codon that truncated the cadherin protein prior to the transmembrane domain (Gahan et al., 2001). Deletion mutations were

later found in three alleles of a cadherin gene in a Cry1Ac-resistant strain of *P. gossypiella* (Morin et al., 2003). Genetic linkage analysis showed that those mutant alleles co-segregated with resistance to Cry1Ac, providing evidence that mutations in the cadherin-coding gene were genetically linked to resistance. Similarly, a deletion of a cadherin gene, resulting in a premature stop codon, was genetically associated with high levels of Cry1Ac resistance in *H. armigera* (Xu et al., 2005). Additional resistance alleles of the cadherin gene resulted from an insertion, a deletion or a point mutation, were identified in the field populations of *H. armigera* in China (Zhao et al., 2010; Zhang H, et al., 2012b).

The APN family is a class of enzymes that catalyze the proteolytic cleavage of peptides from the amino terminus. Together with endopeptidases and carboxypeptidases, APNs play a significant role in the dietary protein digestion in the midgut of lepidopteran larvae (Wang et al., 2005). The GPI-anchored APNs function as receptors for Bt toxins.

Bt resistance is associated with mutations in APN genes. Zhang et al. (2009) detected a 22-amino-acid deletion mutation of the APN1 gene (HaAPN1-BtR) in a Cry1Ac-resistant strain of *H. armigera*, and the recombinant HaAPN1-BtR protein did not bind to Cry1Ac (Zhang et al., 2009). However, genetic association of the deletion mutation in the APN1 gene with Cry1Ac resistance is lacking. Altered APN expression is also associated with resistance to Cry1 toxins. Kiewsiri and Wang (2011) demonstrated that down-regulation of APN1 was genetically associated with Cry1Ac resistance in *T. ni*, whereas the APN genes showed no genetic linkage with the resistance. In another study, Northern blot analysis of the APN1-4 genes revealed a complete suppression in APN1 expression in a Cry1Ca-resistant *S. exigua* strain (Herrero et al., 2005). However, the

genetic association between the observed lack of APN1 expression and Cry1Ca resistance is unknown (Herrrero et al., 2005).

Unlike soluble ALP, mALPs are restricted in the insect midgut brush border membrane of columnar cells (Eguchi, 1995) and belong to the GPI-anchored protein class. Their roles in lepidopterans as midgut receptors for Cry toxins have been established in *H. virescens* (Jurat-Fuentes and Adang, 2004) and *M. sexta* (McNall and Adang, 2003; Arenas et al., 2010). Reduced mALP levels have been reported in strains of Cry-resistant *H. virescens*, *H. armigera*, *S. frugiperda*, and *H. zea* (Jurat-Fuentes and Adang, 2004; Jurat-Fuentes et al., 2011; Caccia et al., 2012). Jurat-Fuentes and Adang (2004) described the identification of a 68-kDa glycoprotein as a mALP in *H. virescens*, and detected reduced level of the mALP in a Cry1Ac-resistant strain of the insect. Down-regulation of mALP, as elucidated by reductions in enzyme activity, protein abundance, and transcript level, were detected in the brush border membrane vesicle (BBMV) proteomes of three Cry1Ac/Cry2Aa-resistant *H. virescens* strains (Jurat-Fuentes et al., 2011). A similar pattern in mALP expression was observed in BBMV proteins from the Cry1Ac-resistant larvae of *H. armigera* and Cry1Fa-resistant larvae of *S. frugiperda* (Jurat-Fuentes et al., 2011). In addition, enzymatic activities of mALP was reduced 3-fold in BBMVs from Cry1Ac-resistant *H. zea* larvae compared to the susceptible larvae (Caccia et al., 2012). However, there are no reports on mutations in the mALP gene to confer resistance to Bt Cry toxins.

Besides mutations in known Bt toxin receptor genes, mutations in the gene coding for an ATP-binding cassette transporter, ABCC2, is also genetically linked to “mode 1” type resistance. In a Cry1Ac-resistant strain of *H. virescens*, a 22-base deletion in the

ABCC2 gene abolishing expression of ABCC2 was genetically linked to Cry1Ac resistance and was correlated with loss of toxin binding to the midgut membranes (Gahan et al., 2010). Similarly, in a Cry1Ac-resistant strain of *P. xylostell*a, loss of expression of ABCC2, due to a 10-amino-acid-residue deletion in one of the transmembrane helixes, was genetically linked with Cry1Ac resistance (Baxter et al., 2011). Genetic linkage of Cry1Ac resistance with the ABCC2 gene was also found in Cry1Ac-resistant *T. ni* (Baxter et al., 2011). In a Cry1Ab- resistant strain of *B. mori*, the resistance was genetically associated with a single tyrosine insertion in the outer loop of a transmembrane domain of the ABCC2 protein (Atsumi et al., 2012). The identification of resistance-conferring mutations of the ABCC2 gene of different species suggests that ABCC2 plays a pivotal role in toxin mode of action (Gahan et al., 2010; Heckel, 2012; Bravo et al., 2013).

Altered proteolytic processing of Bt toxins

Proteolytic processing of Bt toxins by midgut proteinases may confer Bt resistance by either hindering the activation of protoxins to their active forms with reduced proteinase activities (Oppert et al., 1996, 1997; Herrero et al., 2001; Li et al., 2004; Karumbaiah et al., 2007) or accelerating the degradation of toxins with increased proteinase activities (Forcada et al., 1996; Keller et al., 1996; Shao et al., 1998; Loseva et al., 2002; Wagner et al., 2002). Midgut extracts from a strain of *P. interpunctella* resistant to Bt subsp. *entomocidus* had lower proteolytic capacity in activating Cry1Ac protoxins than extracts from the susceptible larvae (Oppert et al., 1996). A subsequent study showed that resistance to Cry1Ac in the resistant *P. interpunctella* strain was genetically

linked to the lack of a major gut trypsin-like proteinase (Oppert et al., 1997). This resistance mechanism was further manifested in resistance to Cry1Ab in the same colony (Herrero et al., 2001). Similarly, proteolytic digestion of Cry1Ab protoxin by soluble proteinase extracts of a resistant *O. nubilalis* strain was about 20-30% slower than by the extract from its susceptible counterpart (Li et al., 2004). In addition, comparative analysis of proteinase activities between three Bt-resistant strains of *H. virescens* and a susceptible strain revealed that differences in trypsin-like and chymotrypsin-like proteinase activity may be involved in Bt resistance (Karumbaiah et al., 2007).

Resistance may also be associated with faster degradation and elimination by midgut proteinases. Forcada et al (1996) reported that a Bt-resistant strain of *H. virescens* activated Cry1Ab protoxin more slowly and degraded Cry1Ab toxin faster than a susceptible strain *in vitro* using crude extracts from the two strains. In another example, reduced susceptibility to Cry1C in *S. littoralis* larvae was correlated to increased degradation of the toxin by changes of the specific activity of gut proteinases (Keller et al., 1996). Other examples of proteinacious degradation-mediated resistance to Bt toxins have been reported in strains of *H. armigera* (Shao et al., 1998) and Colorado potato beelte, *Leptinotarsa decemlineata* (Loseva et al., 2002), European cockchafer, *Melolontha melolontha* (Wagner et al., 2002). For most of the cases above, the levels of resistance were relatively low (<100-fold resistance).

Increased carboxylesterase sequestration

Studies of numerous insects and acarids repeatedly implicate carboxylesterase, a class of serine hydrolase, in metabolic resistance to chemical insecticides such as

organophosphates and synthetic pyrethroids (Wheelock et al., 2005). So far only one case of Bt resistance is thought to be caused by overproduction of a non-specific carboxylesterase to sequester Bt toxins (Gunning et al., 2005). In this study, a concentration-dependent inhibition of total carboxylesterase activity by Cry1Ac toxin was detected in the larval homogenates of *H. armigera* larvae, and the number and activity of the carboxylestease isozymes revealed by zymograms were increased in the Cry1Ac-resistant strain as compared to the susceptible strain. However, there is no evidence of genetic linkage of any of the carboxylesterase isozymes to Bt resistance (Heckel et al., 2007).

Elevated immune response

Elevated immune response, as measured in the forms of melanization reaction in the larval cell-free hemolymph, was correlated to increased tolerance to Bt formulations in a laboratory colony of flour moth, *Ephestia kuehniella*. Such increases in Bt tolerance was induced by feeding the larvae with a sub-lethal dose of the Bt formulation, and can be transmitted into the next generation by a maternal effect (Rahman et al., 2004). The same research group later provided evidences that the induced Bt tolerance in the *E. kuehniella* strain was not caused by increased level of hemolymph melanization, but by an increased production of lipophorin (a pro-coagulant) in the gut lumen to form aggregates around Bt toxins (Rahman et al., 2007). Immune-mediated Bt tolerance mechanism is also manifested in *H. armigera* (Ma et al., 2005). In this example, the observed Cry1Ac resistance in a *H. armigera* strain relied on an elevated immune response and the inducible immune protein was identified as a glycoprotein called

hexamerin, serving as a pro-coagulant to interact with Cry1Ac in the gut lumen. Another example of immune-mediated Bt tolerance can be found in the *S. exigua* strains that exhibited a higher level of tolerance to the commercial Bt product XenTari when having an increased immune status by carrying a higher bacterial load in the gut (Hernández-Martínez et al., 2010).

Research Objectives and experimental approaches

Bt Cry2A toxins are important in the resistant management strategy of pyramiding *Cry1A* and *Cry2A* genes in the same crop, but knowledge of the insect resistance to Cry2A toxins is very limited. Elucidation of the molecular and biochemical basis of Cry2A resistance is crucial not only for understanding the mode of action of Cry2A toxin but also obtaining knowledge to prevent or delay insect resistance to pyramided Bt-crops. Thus, this dissertation was to investigate the resistance to Cry2Ab in a *T. ni* strain (Glen-BGII) that had developed resistance to Cry2Ab (unpublished data, Ping Wang). The research objectives in this dissertation were to: 1) investigate the inheritance of Cry2Ab resistance in the Glen-BGII strain of *T. ni* and generate a Cry2Ab-resistant strain nearly isogenic to a susceptible laboratory strain (Cornell-SS); 2) analyze the biochemical characteristics to identify differences that may be associated with Cry2Ab resistance; 3) examine the genetic linkage between Cry2Ab resistance and putative Cry toxin receptor genes; 4) compare the midgut brush border membrane proteins from the Cry2Ab-resistant introgression strain and its near-isogenic Cornell-SS

strain to identify differentially expressed proteins that may be associated with Cry2Ab resistance.

A combination of different approaches were used to accomplish these objectives. Diet overlay bioassays were performed to investigate the inheritance of Cry2Ab resistance by analyzing the response of the parental Glen-BGII and Cornell-SS strains, their F_1 progenies, as well as the backcross progenies from the crosses between F_1s and the parental Glen-BGII strain, to Cry2Ab toxin. The genetic association between Cry1Ac resistance and Cry2Ab resistance was examined by examining the segregation of the two resistance traits in the Glen-BGII strain of *T. ni*. The genetic linkage of Cry1Ac resistance with Cry2Ab resistance was also tested by analyzing the observed and expected mortalities of the backcross progenies from crosses between heterozygous F_1s and the Glen-BGII strain to single toxin or multiple toxins at their diagnostic doses. A complementation test involving two parental strains, the Glen-BGII strain and a Cry1Ac-resistant strain (Glen-Cry1Ac-BCS, Wang et al., 2007), was conducted to study the Cry1Ac resistance in those two strains. The Cry2Ab resistance trait in the Glen-BGII strain was introgressed into the susceptible Cornell-SS strain to generate a Cry2Ab-resistant strain (Glen-Cry2Ab-BCS8) nearly isogenic to the Cornell-SS strain for further biochemical analysis.

Midgut biochemical characteristics were comparatively analyzed using the Glen-Cry2Ab-BCS8 and Cornell-SS strains to identify biochemical alterations associated with Cry2Ab resistance. The tested midgut biochemical characteristics included caseinolytic protease and serine protease (trypsin, chymotrypsin and elastase) activities, proteolytic processing of Cry2Ab by midgut fluid, aminopeptidase and ALP activities, esterase

activities, and melanization activities of hemolymph plasma. Genetic linkage analysis of Cry2Ab resistance with genes coding for putative midgut receptors (cadherin, mALP, and APN1-6) was also performed. Finally, midgut BBMV proteins from the Glen-Cry2Ab-BCS8 and Cornell-SS strains were comparatively studied by a liquid-based 2D-LC-MS/MS proteomic approach using the isobaric Tandem Mass Tag labeling technique.

CHAPTER 2
GREENHOUSE-SELECTED RESISTANCE TO *BACILLUS THURINGIENSIS* TOXIN CRY2AB IN THE CABBAGE LOOPER, *TRICHOPLUSIA NI*

Introduction

Development of resistance to transgenic crops expressing toxins from the soil bacterium *Bacillus thuringiensis* (Bt) threatens the long-term success of the Bt-crops in insect pest control. Field-evolved (or field-selected) resistance is defined as a genetically based decrease in susceptibility of a population to a toxin caused by exposure of the populations to the toxin in the field (Tabashnik et al., 2009a). Despite that Bt-crops are effective against target pest populations since their first commercialization in 1996, resistance to Bt-crops has been reported in some field populations of at least seven species (Van Rensburg, 2007; Tabashink et al., 2009; Bagla, 2010; Downes et al., 2010a; Storer et al., 2010; Gassmann et al., 2011; Zhang H, et al., 2012a). Cry2A toxins have been increasingly used in conjunction with Cry1A toxins in pyramided Bt-crops to delay the evolution of insect resistance to Bt-crops (Roush, 1998; Zhao et al., 2003).

Since their commercialization in 2002, pyramided Bt-crops, especially the Bollgard II Bt-cotton expressing Cry1Ac and Cry2Ab, have been widely planted to target cotton pests (Tabashnik et al., 2009a). To date, insect resistance to Cry2A toxins expressed in Bt-crops in the field has been only reported in Australian field populations of *Helicoverpa punctigera* that showed a significant exponential increase in the frequency of the resistance alleles over a five-year period after planting of Bollgard II cotton containing Cry1Ac and Cry2Ab (Downes et al., 2010a). The observed Cry2Ab

resistance, however, did not lead to field failures (i.e., complete inability to control a pest outbreak), and the efficacy of Bollgard II cotton was not compromised (Downes et al., 2010a). Additionally, lethal concentrations of Cry2Ab (LC_{50}) of the field-collected populations of *Helicoverpa zea* in the southeastern United States increased during the 2002-2005 period (Ali and Luttrell, 2007), which was interpreted that Cry2Ab resistance may have occurred in the Bt-crop planting area where those testing populations were collected (Tabashnik et al., 2009a). However, resistance to Cry2Ab in *H. zea* is controversial, as empirical data on frequencies of the resistance trait of field *H. zea* populations as compared to the baselines before the introduction of Bt-cotton expressing the toxin has not yet been reported (Tabashnik et al., 2013). Thus, little is known about the underlying mechanism of insect resistance to Cry2Ab evolved in agricultural settings. To identify the mechanism of Cry2Ab resistance, it is a prerequisite to acquire knowledge on the genetic basis of the resistance.

In this study, the genetic inheritance of the Cry2Ab resistance and its genetic association with Cry1Ac resistance were examined in a Bt-resistant strain of the cabbage looper, *Trichoplusia ni*. The results from this study demonstrated that Cry2Ab resistance in *T. ni* is monogenic, incomplete recessive, autosomally inherited without maternal effects, and genetically independent from Cry1Ac resistance.

Materials and methods

Insects and Bt toxins. The susceptible strain (Cornell-SS) used herein was a laboratory inbred colony of *T. ni* that had been maintained without exposure to Bt toxins

for over 30 years (Kain et al., 2004). The Bt-resistant strain of *T. ni*, Glen-BGII, was isolated from a Dipel-resistant strain (Janmaat et al., 2004) by selections with Bollgard II cotton foliage expressing Cry1Ac and Cry2Ab. The Dipel-resistant strain of *T. ni* (Glen-Dipel) was originally collected in 2001 from commercial greenhouses in British Columbia, Canada (Janmaat and Myers, 2003). The Glen-BGII strain survived on Bollgard II cotton plants, and had high level resistance to both Cry1Ac and Cry2Ab (unpublished observation, P. Wang). In addition, a Cry1Ac-resistanct strain of *T. ni*, Glen-Cry1Ac-BCS (Wang et al., 2007), was used in this study to analyze the resistance to Cry1Ac in the Glen-BGII strain. *T. ni* larvae were reared on a wheat germ-based artificial diet at 27°C with 50% humidity and a photoperiod of 16 h of light and 8 h of darkness. Adults were maintained under the same conditions as those of larvae and provided with 10% sucrose solution.

Cry1Ac protoxin was produced by the *B. thuringiensis* subsp. *kurstaki* strain HD-73, and prepared as described by Kain et al. (2004). Cry2Ab toxin was obtained from the Monsanto Company (St. Louis, MO).

Characterization of inheritance of Cry2Ab resistance in the Glen-BGII strain of *T. ni*. Crosses were carried out with adults emerging from pupae whose sexes were visually identided as described by Shorey et al. (1962). Reciprocal crosses were made between the Glen-BGII and the Cornell-SS strains to produce F_1 progeny. A backcross was made between the F_1 progeny and the Glen-BGII strain to prepare a backcross family. There were about 100 pupae of each sex included in each of the crosses. Diet overlay bioassays (Zhao et al., 2002; Kain et al., 2004) were used to determine the response of the neonates from the two parental strains (Glen-BGII and Cornell-SS), their

F_1, as well as the backcross family to Cry2Ab. For bioassays, an aliquot of 0.2 mL toxin solution was overlayed on the diet surface of a 30-mL clear plastic cup filled with 5mL of high wheat germ diet. Ten neonates were transferred to each cup with a lid covered, and maintained under the insect rearing conditions as described above. Five replications (a total of 50 neonates) were included for each of the five or six toxin concentrations. Treatment without toxin was used as blank control. Larval mortalities were recorded after seven days, and the percent mortalities were corrected using the Abbott's formula (Abbott, 1925). LC_{50} values and their 95% fiducial limits were calculated by probit analysis using the statistic program POLO (LeOra Software, 1987). Difference between two strains was considered significant if their LC_{50} values had no overlapping 95% fiducial limits. Resistance ratios were calculated by dividing LC_{50} of a given family by LC_{50} of the susceptible Cornell-SS strain assayed under the same condition. The degree of dominance (D) for resistance was calculated based on the method described by Stone (1968),

$$D = \frac{2 \log LC_{50}^{F1} - \log LC_{50}^{RR} - \log LC_{50}^{SS}}{\log LC_{50}^{RR} - \log LC_{50}^{SS}}$$

where LC_{50}^{F1} was the LC_{50} for the F_1 progeny, LC_{50}^{RR} was the LC_{50} for the Glen-BGII strain, and LC_{50}^{SS} was the LC_{50} for the Cornell-SS strain.

Isolation of Cry1Ac and Cry2Ab resistance from the Glen-BGII strain of *T. ni*. The Glen-BGII strain was crossed with the Cornell-SS strain, and their F_1 progeny was backcrossed with Cornell-SS to generate a backcross family, followed by crossing of the backcross family again with Cornell-SS. The subsequent progenies were intracrossed to produce a population (Glen-Cry1Ac-Cry2Ab) in which the frequencies of resistant

alleles for Cry1Ac and Cry2Ab, respectively, in the population were expected to be 12.5%, according to Mendel's independent assortment. Each of the reciprocal crosses involved mass crossing among 100 male adults and 100 female adults. The predicted percentage of homozygotes for Cry1AC resistance or Cry2Ab resistance in the Glen-Cry1Ac-Cry2Ab population was 1.56%. Discriminating doses of Cry1Ac (2 mL of 100 μg/mL Cry1Ac spread on the diet surface of ca. 50 cm^2) and Cry2Ab (2mL of 440 μg/mL Cry2Ab spread on the diet surface of ca. 50 cm^2) were used to select the Glen-Cry1Ac-Cry2Ab population for Cry1Ac resistance and Cry2Ab resistance, respectively. The doses used herein were confirmed to kill 100% of homozygous susceptible larvae and heterozygotes for Cry1Ac resistance or Cry2Ab resistance. The resulting strains selected with Cry1Ac and Cry2Ab, respectively, were designated as Cry1Ac-SEL and Cry2Ab-SEL, respectively.

Determination of cross-resistance between Cry1Ac and Cry2Ab in *T. ni*. The levels of resistance to Cry1Ac and resistance to Cry2Ab in the Cry1Ac-SEL and Cry2Ab-SEL populations were determined using the diet overlay bioassays as described above. The Cornell-SS strain was also bioassayed in parallel, as susceptible control.

Complementation analysis of the Cry1Ac resistance gene in the Glen-BGII strain with the Glen-Cry1Ac-BCS strain of *T. ni*. A complementation test was conducted to examine whether Cry1Ac resistance in the dual resistant Glen-BGII strain was conferred by the same gene as in the Glen-Cry1Ac-BCS strain (Wang et al., 2007). Specifically, the Cry1Ac-SEL strain was crossed with the Glen-Cry1Ac-BCS strain to produce F_1 progeny. The F_1 progeny, along with the two parental strains and the Cornell-

SS strain, were assayed for their susceptibility to Cry1Ac using the diet overlay assaying method described above.

Analysis of genetic linkage between Cry1Ac and Cry2Ab resistance in *T. ni*.

The genetic linkage between Cry1Ac resistance and Cry2Ab resistance in *T. ni* was examined by generation of a backcross family from the Cornell-SS and Glen-BGII strains followed by susceptibility assays as shown in Figure 2.1. Briefly, females from the Glen-BGII strain were crossed with Cornell-SS males and their F_1 females were crossed with males from the Glen-BGII strain to generate a backcross population. The neonates from the backcross population were divided into three treatment groups. In the first treatment group (Group I), neonates were transferred to diet overlayed with toxin Cry1Ac at its discriminating dose (3 μg/cm^2 diet surface) and the larvae that reached second instar after 4 days were counted as resistant individuals and were transferred to diet overlayed with Cry2Ab at its discriminating dose (6 μg/cm^2 diet surface) to monitor larval survivorship. In the second treatment group (Group II), neonates were transferred to diet overlayed with a mixture of Cry1Ac + Cry2Ab at 3 μg/cm^2 and 6 μg/cm^2, respectively. In the third treatment group (Group III), neonates were treated with Cry2Ab at 6 μg/cm^2 and the survivors after four 4 days were then exposed to Cry1Ac at 3 μg/cm^2. Larvae that did not reach second instar after 4 days feeding on the Cry toxins would eventually die during larval stage. For each treatment group, a total of 150 or 200 larvae were included, and survival rates were scored.

The null hypothesis for data analysis was that the Cry1Ac resistance conferred by the resistance gene R^{1Ac} and Cry2Ab resistance conferred by the resistance gene R^{2Ab} would independently assort (Figure 2.1). As resistance to Cry1Ac is recessive and

monogenic (Kain et al., 2004), it was expected that in the treatment group I, those carrying two copies of R^{1Ac} would survive the discriminating does of Cry1Ac. Thus, with the genotypes of $R^{1Ac}R^{1Ac}R^{2Ab}R^{2Ab}$ and $R^{1Ac}R^{1Ac}R^{2Ab}S^{2Ab}$ in this group would survive Cry1Ac treatment and the expected percentage of those Cry1Ac survivors was 50% (Figure 2.1). Among those Cry1Ac survivors, only the ones with two copies of R^{2Ab} would survive Cry2Ab treatment, as Cry2Ab resistance is also recessive and monogenic. Therefore, only those with the genotype of $R^{1Ac}R^{1Ac}R^{2Ab}R^{2Ab}$ survived Cry2Ab, and they were expected to account for 50% of the Cry1Ac survivors (Figure 2.1). As for treatment group II, it was expected those homozygotes for both R^{1Ac} and R^{2Ab} (i.e., $R^{1Ac}R^{1Ac}R^{2Ab}R^{2Ab}$) survived the discriminating doses of Cry1Ac and Cry2Ab simultaneously, and the expected percentage of those two toxin survivors was 25% (Figure 2.1). Similarly, for treatment group III, those with two copies of R^{2Ab} ($R^{1Ac}R^{1Ac}R^{2Ab}R^{2Ab}$ and $R^{1Ac}S^{1Ac}R^{2Ab}R^{2Ab}$, 50% of the whole group) would survive exposure to Cry2Ab and among the Cry2Ab survivors, only those homozygotes for both R^{1Ac} and R^{2Ab} ($R^{1Ac}R^{1Ac}R^{2Ab}R^{2Ab}$, 50% of the Cry2Ab survivors) would survive Cry1Ac at its discriminating dose (Figure 2.1). The hypothesis that there was no genetic linkage between Cry1Ac and Cry2Ab resistance in *T. ni* was evaluated using a *chi*-square test.

Results

Level of Cry2Ab resistance and inheritance of the resistance in the Glen-BGII strain of *T. ni*. Figure 2.2 and Table 2.1 summarize the results of diet overlay bioassays for the Cornell-SS and Glen-BGII strains of *T. ni*, their F_1s, and the backcross

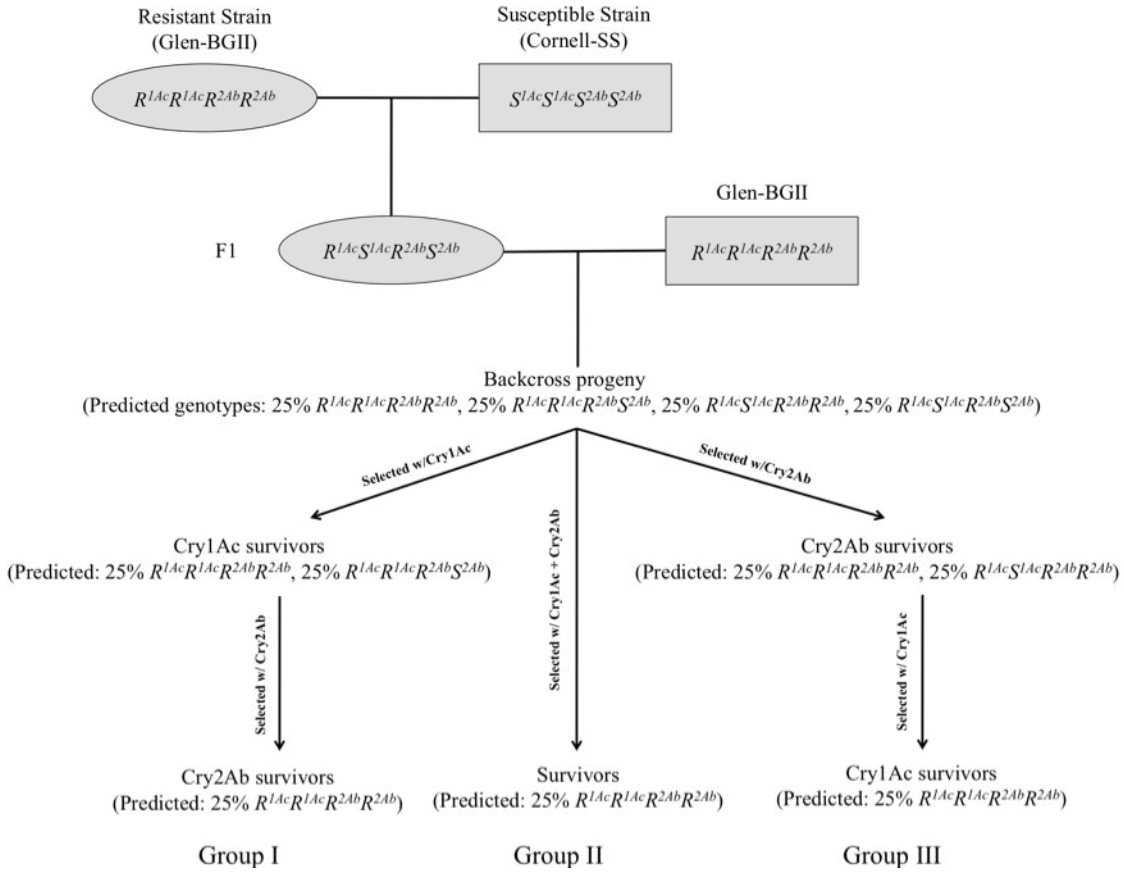

Figure 2.1. Experimental design for the genetic linkage analysis between Cry1Ac resistance and Cry2Ab resistance in the Glen-BGII strain of *T. ni*. R^{1Ac} refers to the allele conferring Cry1Ac resistance while S^{1Ac} refers to the wild type allele of R^{1Ac}; R^{2Ab} refers to the allele conferring Cry2Ab resistance while S^{2Ab} refers to the wild type allele of R^{2Ab}.

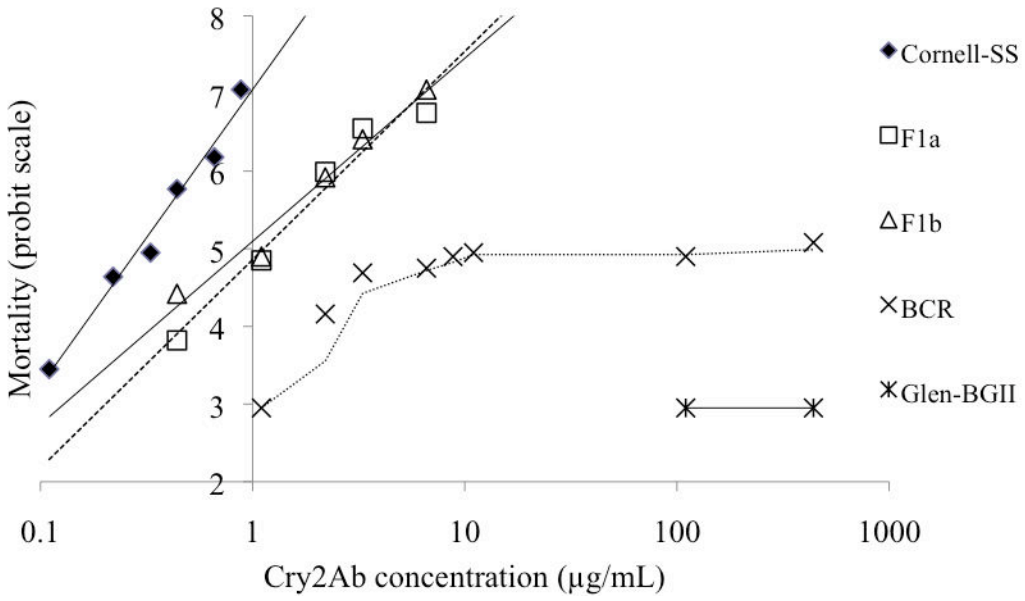

Figure 2.2. Dose-response curves for *T. ni* neonates from the resistant (Glen-BGII) and susceptible (Cornell-SS) strains, F_1 progeny of the reciprocal crosses [F_1a (Cornell-SS♂ x Glen-BGII♀) and F_1b (Cornell-SS♀ x Glen-BGII♂)], and backcross progeny [BCR (pooled F_1 x Glen-BGII)] to Cry2Ab.

Table 2.1. Inheritance of Cry2Ab resistance in the Glen-BGII strain of *T. ni*.

Strain	n	Slope (SE)	LC_{50} (95% CI)[a]	χ^2 (df)	RR[b]	D[c]
Cornell-SS (SS)	350	3.69 (0.37)	0.295 (0.262-0.329)	3.31 (4)	1.0	
Glen-BGII (RR)	200		> 440[d]		>1,492	
F1a (SS♂ × RR♀)	300	2.71 (0.29)	1.16 (0.961-1.38)	2.46 (3)	3.9	-1< D <-0.72
F1b (RR♂× SS♀)	300	2.18 (0.26)	0.946 (0.737-1.18)	3.09 (3)	3.2	-1< D <-0.76

[a]LC_{50} (95% confidence interval), values expressed in micrograms of toxin per milliliter (μg/mL).
[b]Resistance ratio calculated by dividing LC_{50} of a given strain with LC_{50} of the Cornell-SS strain.
[c]Degree of dominance calculated using the formula described by Stone (1968).
[d]No mortality was observed when larvae of the Glen-BGII strain exposed to 440 μg/mL Cry2Ab.

progeny. Diet overlay bioassays gave an LC_{50} value for Cry2Ab in the Cornell-SS strain of 0.295 μg/mL (Table 2.1). By contrast, the highest dose of Cry2Ab (440 μg/mL) caused only low mortality (10%-20%) in the Glen-BGII strain, indicating that the Glen-BGII strain is highly resistant to Cry2Ab, with a resistance ratio of >1,492-fold (Table 2.1). The resistance ratios for the F_1 progeny from the reciprocal crosses between the Glen-BGII and Cornell-SS strains were determined to be 3.9 and 3.2, respectively (Table 2.1). The dose-mortality response curves for the F_1 from the reciprocal crosses slightly shifted from that of the susceptible strain (Figure 2.2), and the slopes for the dose-response curves were 2.71 (±0.29) and 2.18 (±0.26), respectively (Table 2.1). The degree of dominance (D) of the resistance calculated from the F_1 progeny in the reciprocal crosses was < -0.72 and < -0.76, respectively, indicating incomplete recessive resistance. The LC_{50} values of the F_1 progenies from the two reciprocal crosses between the resistant and susceptible parents were not statistically different (Table 2.1), suggesting that the Cry2Ab resistance is autosomally inherited without maternal effects. The dose-mortality response curve of the progeny from the backcross between the F_1 and the resistant strain displayed a plateau at 50% mortality (Figure 2.2), which was in agreement with the typical monogenic inheritance (Tsukamoto, 1963). Therefore, the Cry2Ab resistance in the Glen-BGII strain of *T. ni* is conferred by a single major resistance locus.

The Cry1Ac and Cry2Ab resistance traits in the Glen-BGII strain of *T. ni* are genetically independent. Consecutive crosses of the Glen-BGII strain with the susceptible strain, followed by intracrossing, allowed segregation of the Cry1Ac and Cry2Ab resistance genes in the Glen-Cry1Ac-Cry2Ab population. The percentages of survivors from the Glen-Cry1Ac-Cry2Ab population on Cry1Ac and Cry2Ab selections

at discriminating doses were 0.47% and 0.43%, respectively. The predicted percentage of homozygotes for Cry1Ac resistance or Cry2Ab resistance in this population was 1.56% each, higher than those survival rates observed. Thus, the doses used in the selections were stringent enough to eliminate the susceptible alleles. The two resulting populations, Cry1Ac-SEL and Cry2Ab-SEL, showed distinctively different responses to Cry1Ac and Cry2Ab. The Cry1Ac-SEL strain showed 1,221-fold resistance to Cry1Ac but only 2.9-fold resistance to Cry2Ab (Table 2.2). The Cry2Ab-SEL strain showed high level resistance to Cry2Ab (>515-fold) but low level resistance to Cry1Ac (7.9-fold). Therefore, the Cry1Ac resistance and Cry2Ab resistance traits in the Glen-BGII strain reside at different loci.

Cry1Ac resistance in the Glen-BGII strain is conferred by the same gene as that in the Glen-Cry1Ac-BCS strain. The F_1 progeny from the Cry1Ac-SEL and Glen-Cry1Ac-BCS strains showed 2,174-fold resistance to Cry1Ac, comparable to the two parental lines with 1,221- and 5,395-fold resistance, respectively (Table 2.3). Therefore, the results from this complementation test confirmed that the two strains, Cry1Ac-SEL (isolated from Glen-BGII) and Glen-Cry2Ab-BCS, share the same mechanism of Cry1Ac resistance.

Cry1Ac resistance and Cry2Ab resistance are genetically independent in *T. ni*. Backcross progeny were generated from the cross between F_1 females and the resistant males, and their responses to Cry1Ac and Cry2Ab were examined as illustrated in Figure 2.1. Among the 150 larvae used in the treatment group I, 73 (49%) survived exposure to Cry1Ac, statistically not different from the expected 50% survival (*chi*-square test, p=0.744, Table 2.4). Among the 73 Cry1Ac-surviving larvae, 41 (56%)

Table 2.2. Cross-resistance between Cry1Ac and Cry2Ab in *T. ni*.

Strain	Toxin					
	Cry1Ac			Cry2Ab		
	Slope (SE)	LC_{50} (95% CI)[a]	RR[b]	Slope (SE)	LC_{50} (95% CI)	RR
Cornell-SS	3.47 (0.34)	0.0860 (0.0747-0.0974)		4.45 (0.45)	0.854 (0.778-0.933)	
Cry1Ac-SEL	3.03 (0.32)	105 (92.7-120)	1,221	3.09 (0.31)	3.38 (2.88-3.92)	2.9
Cry2Ab-SEL	2.74 (0.26)	0.683 (0.587-0.793)	7.9		>440	>515

[a]LC_{50} (95% confidence interval), values expressed in micrograms of toxin per milliliter.
[b]Resistance ratio calculated by dividing LC_{50} of a given strain with LC_{50} of the Cornell-SS strain.

Table 2.3. Complementation test for Cry1Ac resistance in the Cry1Ac-SEL and Glen-Cry1Ac-BCS strains of *T. ni*.

Strain	n	Slope (SE)	LC_{50} (95% CI)[a]	χ^2 (df)	RR[b]
Cornell-SS	350	3.47 (0.34)	0.0860 (0.0747-0.0974)	4.82 (4)	
Cry1Ac-SEL	350	3.03 (0.32)	105 (92.7-120)	2.20 (4)	1,221
Glen-Cry1Ac-BCS	300	3.55 (0.37)	464 (401-532)	1.19 (3)	5,395
F1[c]	350	2.97 (0.29)	187 (163-213)	6.97 (4)	2,174

[a]LC_{50} (95% confidence interval), values expressed in micrograms of toxin per milliliter.
[b]Resisance ratio calculated by dividing LC_{50} of a given strain with LC_{50} of the Cornell-SS strain.
[c]pooled progeny from reciprocal crosses between the Cry1Ac-SEL and the Glen-Cry1Ac-BCS strain.

Table 2.4. Genetic linkage analysis between Cry1Ac resistance and Cry2Ab resistance in *T.ni*

	Treatment group[a]	No. of larvae	Observed No. (%) of survivors	Expected No. (%) of survivors	p-value
Group I	Total (treated w/ Cry1Ac)	150	73 (49%)	75 (50%)[b]	0.744
	Cry1Ac survivors (treated w/ Cry2Ab)	73	41 (56%)	37 (50%)[c]	0.292
Group II	Total (treated w/ Cry1Ac and Cry2Ab)	150	35 (23%)	38 (25%)[d]	0.637
Group III	Total (treated w/ Cry2Ab)	200	92 (36%)	100 (50%)[e]	0.258
	Cry2Ab survivors (treated w/ Cry1Ac)	92	44 (48%)	46 (50%)[f]	0.677

[a] A discriminating dose of Cry1Ac at 3 μg/cm^2 was used for treatment of backcross progeny with Cry1Ac; a discriminating dose of Cry2Ab at 6 μg/cm^2 was used for treatment of backcross progeny with Cry2Ab.
[b] Expected No. (%) of survivors were calculated based on the fact that Cry1Ac resistance was recessive and monogenic in *T. ni*.
[e] Expected No. (%) of survivors were calculated based on the fact that Cry2Ab resistance was recessive and monogenic in *T. ni*.
[c,d,f] Expected No. (%) of survivors were calculated under the above assumptions, as well as under the assumption that Cry1Ac and Cry2Ab resistance were not genetically linked.

survived on the subsequent treatment with Cry2Ab (Table 2.4), which was not statistically different from the expected 50% survival under the assumption that there is no genetic linkage between Cry1Ac resistance and Cry2Ab resistance in *T. ni* (*chi*-square test, $p = 0.292$, Table 2.4). Similarly, 92 (46%) of 200 backcross progeny in the treatment group III survived on diet treated with Cry2Ab, and 44 (48%) of the Cry2Ab survivors were resistant to Cry1Ac (Table 2.4). In the treatment group II, 35 of 150 (23%) backcross progeny survived on diet treated with a mixture of Cry1Ac and Cry2Ab, statistically not different from the predicted 25% survival of the backcross progeny to the two toxins (*chi*-square test, p=0.637, Table 2.4). Therefore, there is no genetic linkage between Cry1Ac resistance and Cry2Ab resistance in *T. ni*.

Discussion

The Bt foliar insecticide Dipel contains a mixture of Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, and Cry2Ab, and spores. Therefore, the greenhouse-derived Dipel-resistant strain (Glen-Dipel) had been selected with multiple Cry toxins. The Glen-BGII strain, resulting from selection of the Glen-Dipel strain with Bollgard II cotton plants containing Cry1Ac and Cry2Ab, exhibited a high level resistance to Cry1Ac and Cry2Ab. The bioassay data showed that the Cry2Ab resistance in the Glen-BGII strain of *T. ni* is incompletely recessive, autosomally inherited with no maternal effects, and monogenic. This mode of inheritance of Cry2Ab resistance was similar to that of the "mode 1" type resistance for Cry1Ac resistance in lepidopterans (Tabashink et al., 1998; Ferre and Van Rie, 2002), and typical to Cry2Ab resistance in the resistant strains of *Helicoverpa*

armigera (Mahon et al., 2007), *P. punctigera* (Downes et al., 2010b), and *Pectinophora gossypiella* (Tabashnik et al., 2009b).

Cross-resistance between Cry1A and Cry2A toxins has been examined in eight lepidopteran species in 21 experiments, the majority (15 out of 21) of which were tests for cross-resistance to Cry2A toxins in lepidopteran populations being selected for Cry1A resistance (Brevault et al., 2013). Studies on cross-resistance to Cry1A toxins after selections with Cry2A toxins has been conducted in five strains of four lepidopterans (i.e., *Heliothis virescens*, *P. gossypiella*, *H. armigera*, and *H. punctigera*) (Jurat-Fuentes et al., 2003; Mahaon et al., 2007; Tabashnik et al., 2009b; Downes et al., 2010b). Among those 21 experiments, low to moderate levels of cross-resistance were detected in strains of *H. virescens* (Gould et al., 1992, 1995; Jurat-Fuentes et al., 2003), *P. gossypiella* (Tabashnik et al., 2009b), and *H. zea* (Burd et al., 2000) under laboratory selection conditions. Cross-resistance between Cry1Ac and Cry2Aa in a resistant strain of *H. virescens* (CP73) was suggested as the additive effects of multiple minor resistance genes, as there was no evidence in genetic linkage mapping for a major resistance gene to confer the Cry2Aa resistance (Gahan et al., 2005). Cross-resistance between Cry1Ac and Cry2Aa in a different resistant strain of *H. virescens* (KCBhyb) might be conferred by at least two different mechanisms, according to the observation that binding of Cry1Aa was reduced in the resistant strain when compared to the susceptible insects while Cry1Aa and Cry2Aa did not share binding sites in the insect (Jurat-Fuentes et al., 2003).

The result that detectable cross-resistance between the two toxins *in T. ni* was minimal is not unexpected, given that Cry1Ac and Cry2Ab have substantially different amino acid sequences and binding sites for the two toxins in insect midguts are known to

be different (English et al., 1994; Morse et al., 2001; Luo et al., 2007; Hernandez-Rodriguez et al., 2008). The general lack of cross-resistance between Cry1A and Cry2A toxins has also been reported in 13 strains of six lepidopteran species (reviewed in Brevault et al., 2013), supporting the resistance management strategy of pyramiding Cry1A and Cry2A toxins in second generation Bt-crops to delay resistance development.

The genetic linkage analysis between Cry1Ac resistance and Cry2Ab resistance in the present study showed that the two types of resistance in the Glen-BGII strain were genetically independent and controlled by two different single major loci. The complementation test confirmed that the same resistance gene confers Cry1Ac resistance in the Cry1Ac-SEL strain and the Glen-Cry1Ac-BCS strain, in which the molecular genetics of Cry1Ac resistance has been studied (Wang et al., 2007; Tiewsiri and Wang, 2011; Zhang X, et al., 2012). So far, knowledge of the mechanisms of resistance to Cry2A toxins in insects is very limited. In a *H. virescens* strain resistant to both Cry1Ac and Cry2Aa, resistance to Cry2Aa was not linked to genes encoding cadherin or ABCC2, the two loci implicated in Cry1Ac resistance (Gahan et al., 2005). A recent study on binding of Cry2Ab to the midgut brush border membrane vesicles (BBMVs) from two Cry2Ab-resistant *Helicoverpa* species determined that the midgut BBMVs from the resistant larvae had reduced binding to the toxin, suggesting altered toxin binding sites in the resistant larvae (Caccia et al., 2010). Nevertheless, the underlying biochemistry and molecular genetics of Cry2Aa resistance in *H. virescens* or Cry2Ab resistance in *H. armigera* and *H. punctigera* remain to be revealed.

In conclusion, the Glen-BGII strain of *T. ni* has developed high level Cry2Ab resistance, which is incomplete recessive, autosomally inherited without effects, and

monogenic. The mechanism of Cry2Ab resistance in *T. ni* is different from that for Cry1Ac resistance.

CHAPTER 3

BIOCHEMCIAL CHARACTERIZATION OF RESISTANCE TO *BACILLUS THURINGIENSIS* TOXIN CRY2AB IN A GREENHOUSE-DERIVED STRAIN OF THE CABBAGE LOOPER, *TRICHOPLUSIA NI*

Introduction

The Bt Cry toxin intoxication pathway consists of a series of consecutive events involving toxin solubilization, activation (if Cry protoxin is ingested), passage through insect midgut peritrophic membrane, interaction with specific binding sites on the midgut brush border membrane, and eventually cell lysis killing insects (Pardo-Lopez et al., 2013). Resistance to Bt Cry toxins could arise if any of these steps is altered in insects. Insect resistance to Bt toxins has been well documented in literature and different Bt resistance mechanisms have been reported in Bt-resistant populations of lepidopterans (Ferre and Van Rie, 2002; Heckel et al., 2007; Pardo-Lopez et al., 2013).

Cry2A toxins are the major Cry toxins used in current Bt-crops. Understanding the mechanisms of Cry2A resistance is essential for the long-term efficacy of Bt-crops. To date, knowledge of the biochemical and molecular genetics of insect resistance to Cry2A toxins is very limited. In one of the very few reports on Cry2Ab resistance, midgut brusher border membrane vesicles (BBMVs) from Cry2Ab-resistant larvae of *Helicoverpa armigera* and *Helicoverpa punctigera* showed greatly reduced binding to Cry2Ab, suggesting that the resistance might be conferred by altered binding sites

(Caccia et al., 2010). However, information on biochemical processes potentially associated with Cry2Ab resistance in insects is very limited.

To identify the mechanism of greenhouse-derived Cry2Ab resistance in *T. ni*, I compared the biochemical processes potentially involved in Cry2Ab activation and inactivation in Cry2Ab-resistant and -susceptible strains of *T. ni*. Results from this study indicated that greenhouse-evolved Cry2Ab resistance in *T. ni* is not associated with alterations of midgut protease activities, specific proteolytic digestion of the toxin Cry2Ab, midgut aminopeptidase and alkaline phosphatase (ALP) activities, midgut esterase activities, or the melanization activities of hemolymph plasma.

Materials and methods

Insects. The susceptible *T. ni* strain (Cornell-SS) was an inbred colony that had been maintained in the laboratory for more than 30 years without exposure to Bt products (Kain et al., 2004). The Bt-resistant strain of *T. ni* (Glen-BGII) was established from a Dipel-resistant population that was originally collected from commercial production greenhouses in British Columbia, Canada (Kain et al., 2004). The Glen-BGII strain was described and its inheritance of resistance to Cry2Ab was characterized in Chapter 2. The insect rearing conditions were described by Kain et al. (2004).

Generation of a Cry2Ab-resistant *T. ni* introgression strain nearly isogenic to the laboratory susceptible strain. The Glen-BGII strain was used to introgress its Cry2Ab-resistance gene to the susceptible Cornell-SS strain (Figure 3.1). The Glen-BGII strain was crossed with the Cornell-SS strain, and their F_1 progenies were backcrossed

Figure 3.1. Schematic diagram of the crosses performed to introgress the Cry2Ab-resistance gene in the Glen-BGII strain to the Cornell-SS strain of *T. ni*.

with Cornell-SS for two additional generations. The backcross progenies were allowed to intra-breed to generate a backcross population and the backcross population was then selected with Cry2Ab. The Cry2Ab selection was performed by feeding neonates on Cry2Ab-overlayed artificial diet (2 mL of 440 μg/mL Cry2Ab spread on diet surface of ca. 50 cm^2). The survivors after 7 days on Cry2Ab were transferred to fresh diet without toxin. The resulting Cry2Ab colony from the backcross population that had been backcrossed with the susceptible strain twice was designated Glen-Cry2Ab-BCS2. The Glen-Cry2Ab-BCS2 strain was crossed with the Cornell-SS strain for two additional consecutive generations, followed by intra-breeding and Cry2Ab selection as described above, to generate the Cry2Ab-resistant backcross strain designated Glen-Cry2Ab-BCS4. Further backcrosses for two generations of the Glen-Cry2Ab-BCS4 strain with the Cornell-SS strain, followed by intra-breeding and Cry2Ab selection as described above, led to the generation of the Cry2Ab-resistant backcross strain Glen-Cry2Ab-BCS6. Two more generations of backcrosses with Cornell-SS, followed by Cry2Ab selection, generated the final backcross strain Glen-Cry2Ab-BCS8.

The frequencies of the Glen-BGII-originated cadherin gene allele in the backcross strains were determined to assess the introgression rates in the process of generation of the backcross *T. ni* strains. A genomic DNA fragment of the cadherin gene was amplified from individuals of the backcross strains Glen-Cry2Ab-BCS6 and Glen-Cry2Ab-BCS8 by PCR, followed by DNA sequencing to determine the allele types, as described by Zhang X, et al. (2012). Genomic DNA was prepared from fifth-instar larvae using a rapid DNA isolation method (Kikkert et al., 2006). Briefly, a small piece of tissue excised from a larva or adult was ground in 20 μL lysis buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl,

2.5 mM $MgCl_2$, 0.45% (vol/vol) Nonidet P-40, 0.45% (vol/vol) Tween 20, 0.01% (wt/vol) gelatin, 60 μg/mL protease K], and the homogenates were incubated at 65°C for 15 min, followed by heating at 95°C for 30 min to inactivate protease K. The cadherin genomic DNA fragment (Genbank accession no. JF303656) was amplified by PCR using primers 5'-GCGCTGCTGGGCTTCCTGT-3' and 5'-CGCTTTGATGGTCTCGTTC-3' as described by Zhang X, et al. (2012). Each 25-μL PCR amplification reaction contained 0.5 μL of genomic DNA lysate, 0.2 μM of each primer, 0.2 μM of dNTPs, 2.5 μL of 10x PCR buffer (New England Biolabs), and 1 U of Taq polymerase (New England Biolabs). PCRs were performed for 40 cycles of 30 s at 94°C, 30 s at 55°C, and 40 s at 72°C followed by a final extension at 72°C for 10 min. For DNA sequencing, the PCR product was examined by agarose gel electrophoresis, and the DNA fragment was recovered from the gel using the QIAquick gel extraction kit (Qiagen), according to the manufacturer's protocol. The purified PCR products were sequenced at the Biotechnology Resource Center of Cornell University (Ithaca, NY). Sequence data was analyzed using the Lasergene software package (DNAStar, Madison, WI).

The level of Cry2Ab resistance in the Glen-Cry2Ab-BCS8 strain was determined using the neonate bioassay protocol as described in Chapter 2. Fifth-instar larvae from the Glen-Cry2Ab-BCS8 line were also assayed for their resistance to Cry2Ab. Briefly, 10 fourth-instar larvae close to molting into fifth-instar from the Glen-Cry2Ab-BCS8 and the Cornell-SS strains, respectively, were isolated in wells of a 24-well plate without feeding for 8 hours to obtain synchronized early fifth-instar larvae prior to toxin treatment. The larvae were given fresh artificial diet containing 880 μg toxin/g diet.

Mortality was monitored over a period of 12 days to examine the susceptibility of the fifth-instar larvae to Cry2Ab.

Midgut protease activity assays. Midguts were isolated from mid-fifth-instar larvae by dissection and midgut protease activities were assayed to determine whether the activities in the Glen-Cry2Ab-BCS8 larvae were different from those in the Cornell-SS larvae. Mid-fifth-instar larvae were immobilized on ice for five minutes, and dissected to obtain midguts containing intact gut contents. The midguts were then individually weighed and stored at -20˚C for no more than two weeks before assayed. Midgut homogenate (10% wt/vol) was prepared by homogenizing a midgut with 9 times the tissue weight of deionized water in a 1.5 mL microcentrifuge tube.

Total midgut protease activity was determined using azocasein (Sigma, St. Louis, MO) as the substrate (Broadway, 1997; Wang et al., 2007). A 20-µL aliquot of midgut homogenate was mixed with 150 µL of 1% azocasein in 50 mM $NaHCO_3$-Na_2CO_3 buffer (pH 10) and incubated at 28˚C for 2 h. The reaction was stopped by addition of 170 µL of 10% trichloroacetic acid. The mixture was incubated at room temperature for 1 h and then centrifuged at 16,000 x g for 10 min. One hundred microliters of the supernatant containing digested azocasein products was mixed with an equal volume of 1 M NaOH, and the optical absorbance at 450 nm was measured in a microplate reader (Dynext Technologies, Chantilly, VA).

Activities of midgut serine proteases, including elastase, trypsin, and chymotrypsin, were assayed by spectrophotometrically monitoring the release of *p*-nitroaniline from their respective substrates in the enzymatic reactions (Wang et al., 2007). For midgut elastase activity assays, 10 µL of midgut homogenate was mixed with

490 µL of 50 mM $NaHCO_3$-Na_2CO_3 (pH 10) and 10 µL of 55 mM *N*-succinyl-$_L$-ananyl-$_L$-alanyl-$_L$-alanine-*p*-nitroanilide (Sigma), and the reaction was recorded for its optical absorbance increase at 410 nm at 28˚C for 60 min in a spectrophotometer attached with a temperature-controlled cell positioner (Shimadzu Corporation). For midgut trypsin activity assays, 3 µL of midgut homogenate was mixed with 3 mL of 50 mM $NaHCO_3$-Na_2CO_3 (pH 10) containing 1mM Na-benzoyl-$_L$-arginine *p*-nitroanilide (Sigma). The enzymatic reactions at 28˚C were immediately monitored by recording optical absorbance at 405 nm for 60 min. Midgut chymotrypsin activity assay was similar to the method used for the trypsin assays except that 3 µL of 10-fold-diluted midgut homogenate was used for the assays and 1 mM *N*-succinyl-Ala-Ala-Pro-phenylalanine *p*-nitroanilide (Sigma) was used as the substrate. Five replicates, with each comprising of midgut sample from a single larva from the Glen-Cry2Ab-BCS8 or Cornell-SS strain, were included in all the protease assays described above. The enzymatic activity for each sample was calculated using the data points in the linear portion of the initial reaction curves, and enzyme activities for samples from the two strains were subjected to statistical significance analysis.

Zymogram analysis of midgut proteases. The midgut proteases were profiled by zymogram analysis as described by Wang et al. (2007). One microliter of 10% midgut homogenate was solubilized in SDS-PAGE sample buffer without addition of a reducing agent and boiling, and the proteins were separated by SDS-PAGE with a 10% gel. After electrophoresis, the gel was washed in 2% Triton X-100 for 15 min, followed by incubation with 2% casein (Sigma) in 50 mM Tris-HCl (pH 8) for 3 h at room

temperature. The midgut protease activities were visualized by Coomassie blue staining of the gel after a brief rinse of the gel with deionized water.

Proteolytic processing of Cry2Ab toxin with *T. ni* larval midgut fluid. The digestion of Cry2Ab toxin by larval midgut digestive fluid was performed according to the procedures described by Wang et al. (2007). Briefly, larval midgut fluid was collected as regurgitant from mid-fifth-instar larvae by stimulation of the larval mouth parts. Ten microliters of 44 µg/mL Cry2Ab was incubated with 10 µL of midgut fluid prepared in a 10-fold serial dilution in 50 mM CAPS (pH 11) for 60 min at 26 ˚C. The proteolysis of Cry2Ab was terminated by heating samples in SDS-PAGE sample buffer at 100˚C for 5 min and subjected to SDS-PAGE analysis, followed by Commassie blue staining.

Midgut aminopeptidase and ALP analysis. Midgut aminopeptidase activity was determined by mixing 20 µL midgut homogenate in 2 mL of 0.1 M Tris-HCl (pH 8.6) containing 1 mM leucine *p*-nitroanilide (Sigma), followed by monitoring of the increase of optical absorbance at 405 nm at 26˚C for 5 min (Wang et al., 2005). For midgut ALP activity assays, 10 µL of midgut homogenate was mixed with 2 mL of ALP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 1.2 mM *p*-nitrophenyl phosphate disodium (Sigma). ALP activity was measured as changes of optical absorbance at 405 nm at 26 ˚C for 10 min.

Midgut ALP in the midgut homogenate was also examined after separation by SDS-PAGE as described by Jurat-Fuentes and Adang (2004) and by Western blot analysis using antibodies specific to *T. ni* ALP. Ten microliters of midgut homogenate was solubilized in SDS-PAGE sample buffer without boiling and the proteins were separated on a 10% SDS-PAGE gel. After electrophoresis, the proteins on the gel were

transferred to Immobilon-P membrane (Millipore, Billerica, MA) by electrotransfer. The membrane was then washed with ALP buffer for 15 min at room temperature, followed by addition of 330 μg/mL of Nitro Blue tetrazolium (NBT) and 165 μg/mL of 5-bromo-4-chloroindol-3-indolyl phosphate (BCIP) to the buffer to visualize the ALP activity on the membrane. For Western blot analysis of the midgut ALP, proteins from the midgut homogenate were boilded in SDS-PAGE sample buffer and separated by 10% SDS-PAGE, and then transferred onto Immobilon-P membrane. The membrane was blocked with 3% BSA in PBS and then incubated with an antiserum specific to the *T. ni* membrane-bound ALP at 4˚C overnight, followed by incubation with the ALP-conjugated secondary antibody after several washes of the membrane with PBS. Colorimetric detection of ALP activity associated with the secondary antibody was achieved by incubating the membrane with the NBT-BCIP substrate as described above.

Midgut esterase analysis. Midgut esterase activities from the Glen-Cry2Ab-BCS8 and Cornell-SS larvae were compared by a total activity assay and isozyme profile analysis (Wang et al., 2007). Midguts were dissected without loss of its content and homogenized in 0.1% Triton X-100 to make 10% (wt/vol) midgut homogenate. The midgut homogenate was clarified by centrifugation at 16,000 x g for 10 min, and the supernatant was used for esterase activity and isozyme profile analysis. For esterase activity assay, 5 μL of midgut extract was mixed in 3 mL of 0.1 M sodium phosphate buffer (pH 6.5) containing 2.5 mg/mL of Fast Blue RR and 0.1 mg/mL of α-naphthyl acetate (Sigma), and incubated at 26˚C. Changes of optical absorbance at 450 nm in 5 min were recorded to determine the enzymatic activity. For midgut esterase isozyme analysis, 10% native PAGE was performed to separate the proteins from 10 μL of midgut

extract. The gel was washed twice with 0.1 M sodium phosphate buffer (pH 6.5) for 10 min, and then incubated at room temperature in 0.1 M sodium phosphate buffer (pH 6.5) containing 0.75 mg/mL of Fast Blue RR and 0.2 mg/mL of α-naphthyl acetate to colorimetrically detect the esterase activity in the gel. The colorimetric reaction was stopped by fixation of the gel in 5% acetic acid and 10% methanol when the activities of esterase isozymes were visualized.

Hemolymph melanization assays. Hemolymph plasma melanization assays were performed to compare the immune response of the Glen-Cry2Ab-BCS8 and Cornell-SS larvae according to the method described by Wang et al. (2007). Twenty five microliters of larval hemolymph was collected from a cut proleg of a mid-fifth-instar larva and immediately mixed with 125 μL PBS on ice, followed by centrifugation at 16,000 x g for 4 min to separate the plasma from the hemocytes. One hundred microliters of the plasma preparation was mixed with 100 μL PBS, and transferred to a 96-well microliter plate, and the optical density at 490 nm was recorded for 180 min with a microplate reader (BioTek).

Results

Introgression of the Cry2Ab resistance trait from the Glen-BGII strain to the susceptible laboratory Cornell-SS strain.

Based on toxin overlay bioassays with neonates, the Glen-Cry2Ab-BCS8 strain exhibited the high level resistance (> 1,492-fold) similar to the parental Glen-BGII strain (Chapter 2). Furthermore, the bioassay using fifth-instar larvae with Cry2Ab-incorporated diet (880 μg toxin/g diet) showed that the 10 larvae from the Cornell-SS

strain died within 10 days following toxin feeding. By contrast, no mortality was observed for the 10 larvae of the Glen-Cry2Ab-BCS8 strain, indicating that fifth-instar larvae of the Glen-Cry2Ab-BCS8 strain were highly resistant to Cry2Ab.

Genotyping of the cadherin gene alleles in four individuals from the Cornell-SS and the Glen-BGII strains, respectively, showed that the cadherin gene in both strains were homozygous. The allele in the Cornell-SS strain (cad^C) was identical to that reported by Zhang X, et al. (2012), and the allele in the Glen-BGII strain was named cad^G (Figure 3.2). Genotyping of 30 individuals from the introgression strain Glen-Cry2Ab-BCS6 showed that 24 individuals had the homozygous genotype $cad^C cad^C$ and six individuals had the heterozygous genotype $cad^G cad^C$. Therefore, the frequency of the cad^C introgressed from the Cornell-SS strain in the Glen-Cry2Ab-BCS6 strain was 90%. For the 10 individuals from the Glen-Cry2Ab-BCS8 strain, six individuals had the genotype $cad^C cad^C$, two had the genotype $cad^G cad^C$, and the rest two were homozygous with the genotype $cad^G cad^G$, indicating that the frequency of the cad^C allele from the Cornell-SS strain in the Glen-Cry2Ab-BCS8 strain was 70%. The reason for the reduction in the frequency of the cad^C allele from 90% in the Glen-Cry2Ab-BCS6 strain to 70% in the Glen-Cry2Ab-BCS8 strain as determined was unclear. It is possible that it is due to the small sample size, as only 10 individuals were analyzed for the Glen-Cry2Ab-BCS8 strain.

Midgut protease activities. The total caseinolytic protease activity in the midgut homogenates from the Glen-Cry2Ab-BCS8 larvae was not statistically different from those in the susceptible larvae (Figure 3.3). The enzymatic activities of serine proteases,

GCGCTGCTGGGCTTCCTGTGCGTGCTGCTACTCATCACCTTCATAGTCAG *cad*^{C}
GCGCTGCTGGGCTTCCTGTGCGTGCTGCTACTCATCACCTTCATCGTCAG *cad*^{G}

GACTAGAGCgtgagtatctgatgcgatccggaggacaatacattcagaat *cad*^{C}
GACTAGAGCgtgagtatctgatgcgatccggaggacaaaacattcagaat *cad*^{G}

ttagctttcttatttttaaattaatggttctccaaacgaatttgtctct *cad*^{C}
taggctttcttatttttaaattaatggttctccaaacgaatttgtctct *cad*^{G}

ttggcatggaggctttactgaaatacaaatcatatgcacataaaattaac *cad*^{C}
ttggcatggaggctttactgaaattcaaatcatatgcacataaaattaac *cad*^{G}

cccgacttggacaaattcattgattgattcaccataaaaaatttagctaa *cad*^{C}
cccgatttggacaaattcattgattgattcaccataaaaaatttagctaa *cad*^{G}

cttctttaatgctattttcaattgaaatcaaatatagtaataattgtttt *cad*^{C}
cttctttaatgctattttcaattgaaatcaaatatagtaataattgattt *cad*^{G}

ctcatgcaatgttacaatattgtcaactgtaattatcttccctttctcag *cad*^{C}
ctcatgcaatgttacaatattgtccactgtaattatcttccctttctcag *cad*^{G}

GCTCAACCGTCGTCTGGAAGCCCTATCCATGACTAAAGGCGGCTCAGTGG *cad*^{C}
GCTCAACCGTCGTCTGGAAGCCCTATCCATGACTAAAGGCGGCTCAGTGG *cad*^{G}

ACTCTGGACTGAACCGCGTGGGTCTGGCCGCGCCCGGGACTAACAAGCAC *cad*^{C}
ACTCCGGGCTGAACCGCGTGGGTCTGGCCGCGCCCGGGACCAACAAGCAC *cad*^{G}

GCCGTCGAGGGTTCCAACCCCATCTGGAACGAGACCATCAAAGCG *cad*^{C}
GCCGTCGAGGGCTCCAACCCCATCTGGAACGAGACCATCAAAGCG *cad*^{G}

Figure 3.2. Nucleotide sequence of the PCR amplified genomic DNA fragment of the cadherin gene. The cadherin gene alleles from the Cornell-SS strain and the Glen-BGII strain are named *cad*^{C} and *cad*^{G}, respectively. Lowercase sequence indicates the intron sequence. Polymorphic nucleotides are shaded.

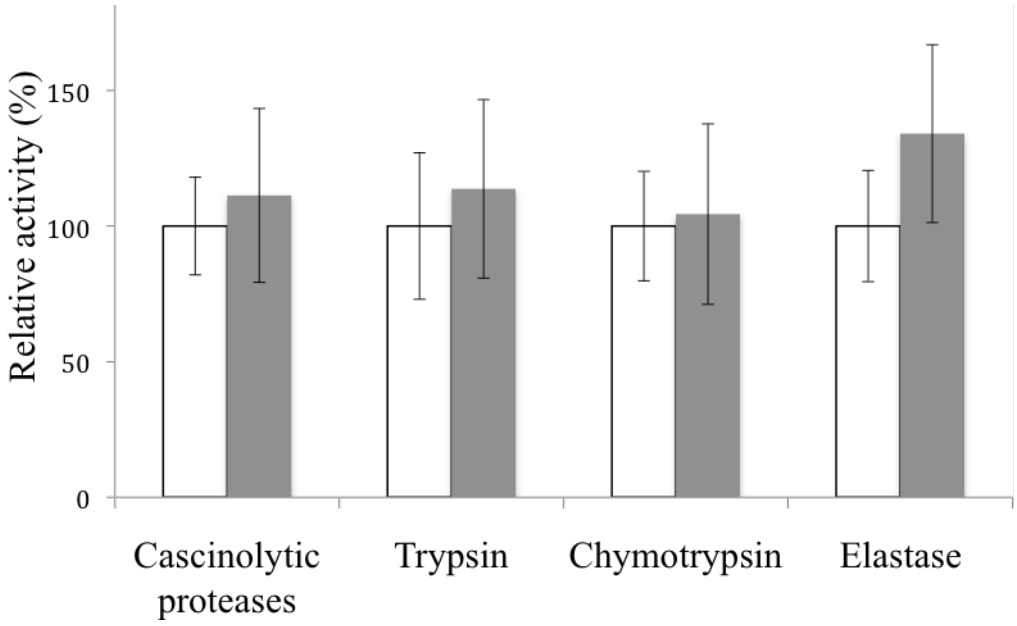

Figure 3.3. Midgut digestive protease activities from *T. ni* larvae of the Cornell-SS (open bars) and Glen-Cry2Ab-BCS8 (grey bars) strains. Protease activities are presented as relative activities with the susceptible strain having 100% activities. Error bars indicate the 95% confidence intervals for the means from five replications.

including elastase, trypsin, and chymotrypsin, in the midgut extracts from larvae of both strains were statistically the same (Figure 3.3).

Zymogram analysis of midgut proteases. Zymogram analysis of midgut protease activities showed that midgut proteases could be separated into multiple bands with activity towards casein (Figure 3.4). The zymograms of the midgut homogenates of the resistant and susceptible larvae exhibited a consistent profile among different individuals. There was intrastrain heterogeneity shown as varying relative intensity of two protease activity bands (arrowed in Figure 3.4). However, the variation of these two protease bands was not strain specific. Overall, zymogram analysis demonstrated that there is no significant difference between the susceptible and resistant larvae in midgut protease activity and composition.

Proteolytic processing of Cry2Ab with midgut digestive fluid. Cry2Ab toxin was susceptible to digestion by midgut fluid from both the resistant and susceptible larvae (Figure 3.5). The extent of Cry2Ab toxin (61 kDa) degradation increased with increase of the midgut fluid used in the digestion (Figure 3.5). The Cry2Ab toxin was completely degraded after incubation with 10% gut fluid (Figure 3.5, lane 3 and 8). However, the extent and patterns of proteolytic processing of Cry2Ab toxin at different concentrations of midgut digestive fluid from the Glen-Cry2Ab-BCS8 and Cornell-SS larvae were not different.

Midgut aminopeptidase and ALP analysis. The aminopeptidase activity in the midgut homogenates from the resistant strain was not statistically different from that of the susceptible strain (Figure 3.6). As for ALP, the spectrophotometrical activity assays showed that midgut homogenate from the resistant and susceptible strains had similar

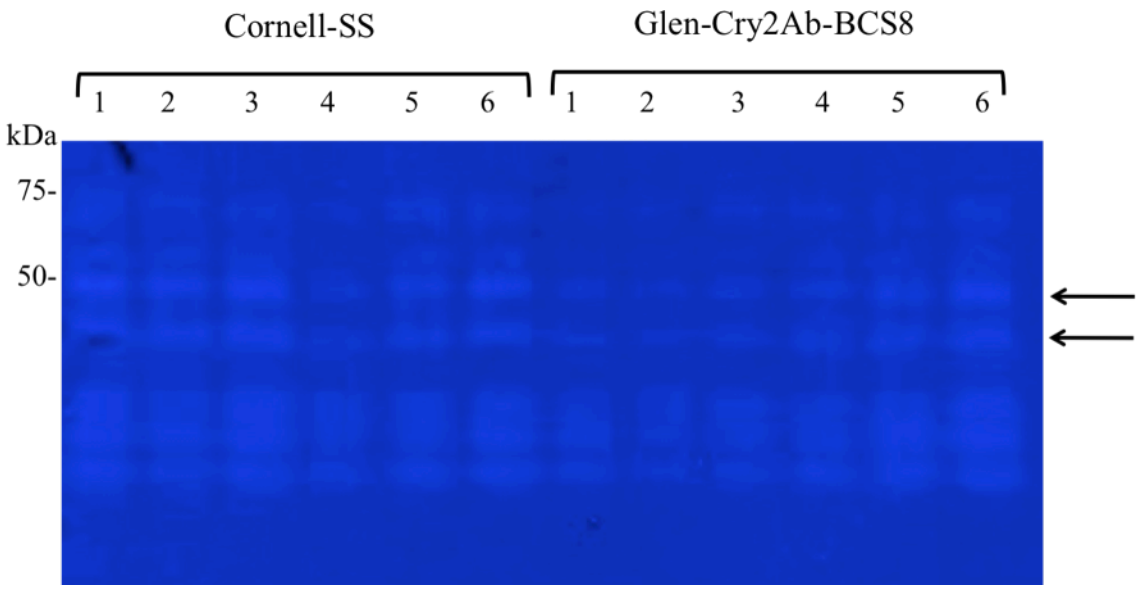

Figure 3.4. Zymogram analysis of larval midgut digestive proteases from the Cornell-SS and Glen-Cry2Ab-BCS8 strains of *T. ni*. Six individuals were included for each strain in the analysis. Arrows indicate the two protease activity bands showing intrastrain heterogeneity.

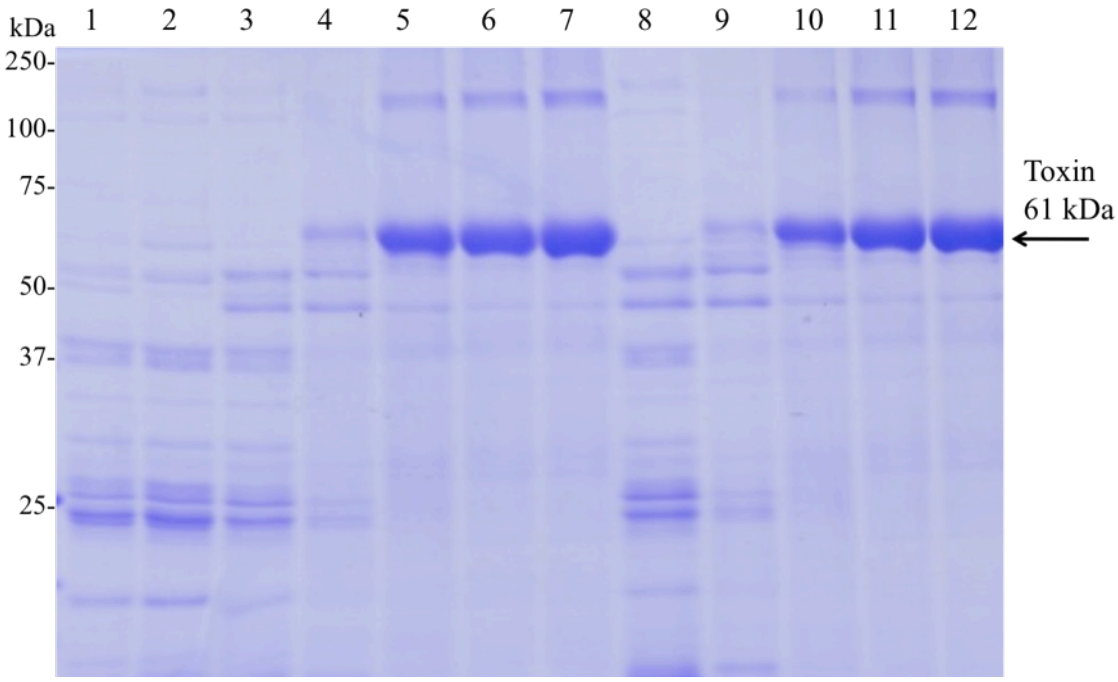

Figure 3.5. SDS-PAGE gel showing in vitro proteolysis of Cry2Ab toxin by diluted *T. ni* larval midgut fluid from the Cornell-SS (SS) and Glen-Cry2Ab-BCS8 (RR) strains. Lanes: 1 and 2, 10% larval midgut fluid from SS and RR strains, respectively; 3 to 6, Cry2Ab incubated with 10%, 1%, 0.1%, 0.01% midgut fluid, respectively, from SS larvae; 8 to 11, Cry2Ab incubated with 10%, 1%, 0.1%, 0.01% midgut fluid, respectively, from the RR larvae; 7 and 12, Cry2Ab toxin control.

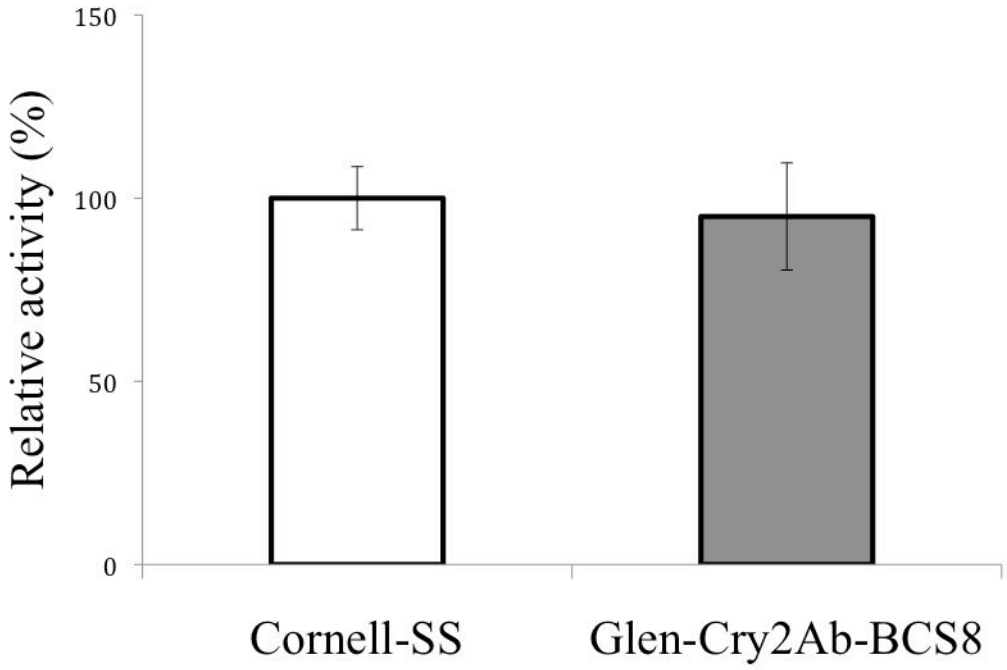

Figure 3.6. Midgut aminopeptidase activities from *T. ni* larvae of the Cornell-SS and Glen-Cry2Ab-BCS8 strains. Protease activities are presented as relative activities with the susceptible strain having 100% activities. Error bars indicate the 95% confidence intervals for the means from five replications.

ALP activities (Figure 3.7, A). In addition, midgut ALP activity was visualized as one major band and two minor bands in the 75 to 100 kDa region by native SDS-PAGE separation, and the

enzymatic activity exhibited slight intrastrain heterogeneity among the individuals in the two strains (Figure 3.7, B). However, there was no significant difference in midgut ALP activity between the two strains. In the Western blot analysis of the mALP, the antiserum specific to the membrane-bound ALP detected a 68-kDa band in the midgut homogenate, and the variation in intensity of this band among different larvae of the two strains showed no correlation with resistance (Figure 3.7, C).

Midgut esterase analysis. Midgut homogenates from the resistant and susceptible larvae did not differ significantly in α-naphthyl acetate hydrolyzing activities(Figure 3.8, A). Zymogram analysis identified a total of six major esterase isozyme bands in the midgut homogenates from both of the resistant and susceptible larvae (Figure 3.8, B). Although the levels of esterase isozymes showed intrastrain variations, there was no difference between the two strains.

Melanization activity of hemolymph plasma in vitro. Analysis of the melanization activity of hemolymph plasma showed that the melanization activities from the Cry2Ab-resistant and -susceptible larvae were relatively low, similar to the levels of activity detected in the Cry1Ac-resistant Glen-Cry1Ac-BCS strain of *T. ni* (Wang et al., 2007). More importantly, the melanization activity in hemolymph plasma was not statistically different between the susceptible and resistant larvae under the experimental conditions (Figure 3.9).

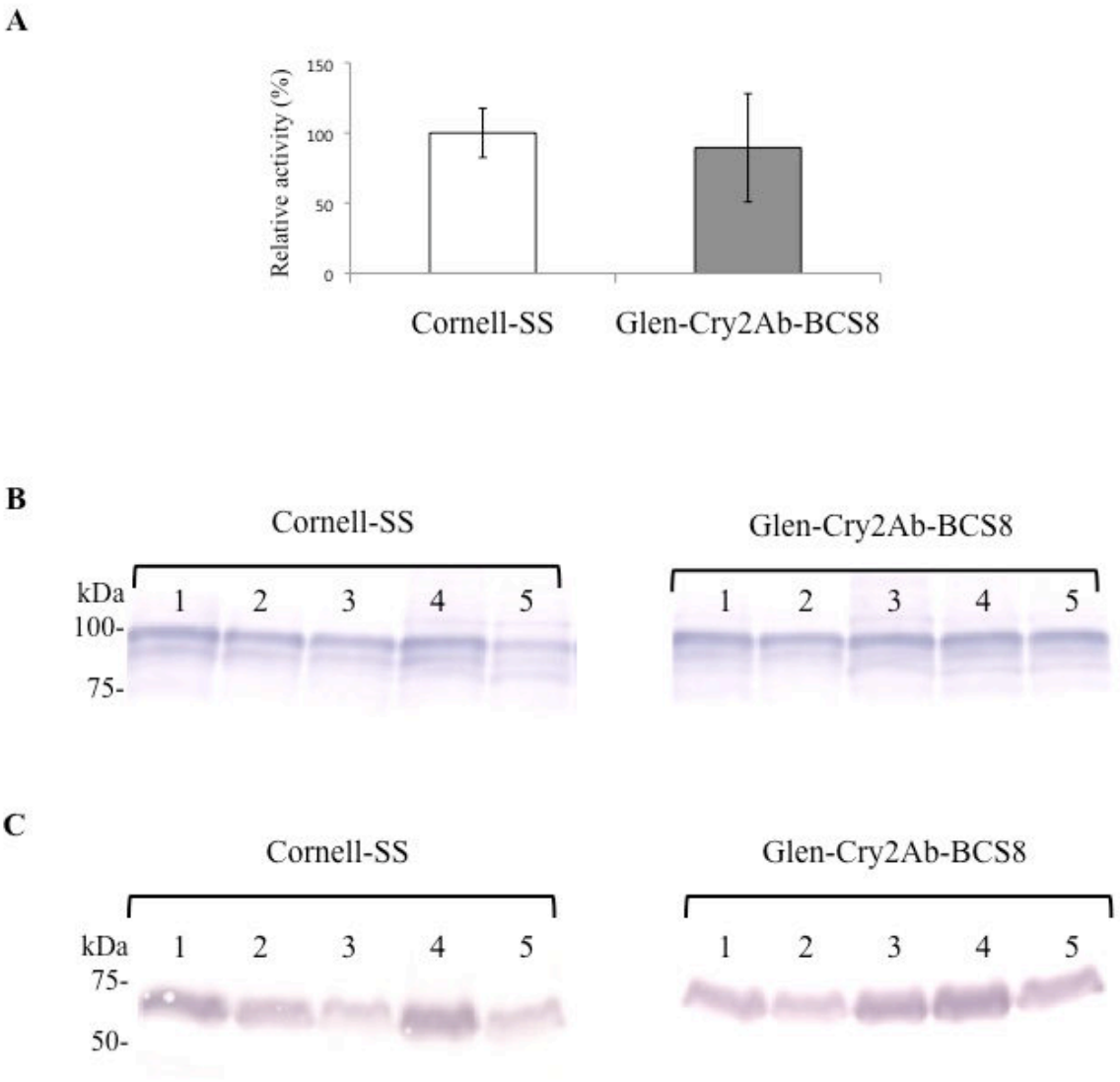

Figure 3.7. Midgut ALP analysis. A) ALP activities from *T. ni* larvae of the Cornell-SS and Glen-Cry2Ab-BCS8 strains. ALP activities are presented as relative activities with the susceptible strain having 100% activities. Error bars indicate the 95% confidence intervals for the means from five replications. B) Enzymatic activity blots showing ALP activities from the larval midguts of the Cornell-SS and Glen-Cry2Ab-BCS8 strains. Five individual larvae were analyzed for each strain. C) Western blot analysis showing membrane-bound ALP abundance from *T. ni* larvae of the Glen-SS and Glen-Cry2Ab-BCS8 strains. Five individual larvae were analyzed for each strain.

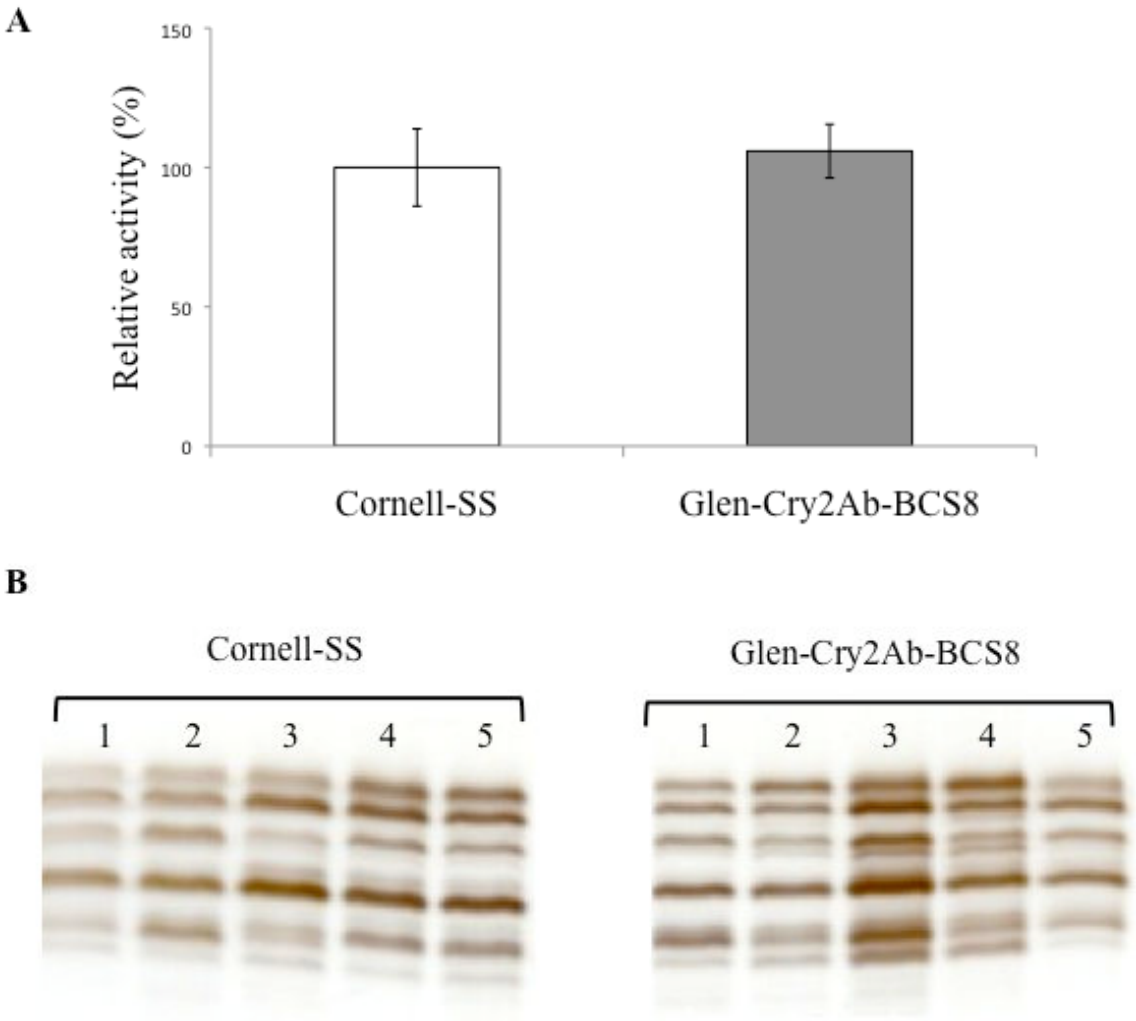

Figure 3.8. Midgut esterase analysis. A) Esterase activities from *T. ni* larvae of the Cornell-SS and Glen-Cry2Ab-BCS8 strains. Protease activities are presented as relative activities with the susceptible strain having 100% activities. Error bars indicate the 95% confidence intervals for the means from five replications. B) Esterase isozyme profiles of *T. ni* larvae from the Cornell-SS and Glen-Cry2Ab-BCS8 strains. Each lane is the homogenate of an individual larva.

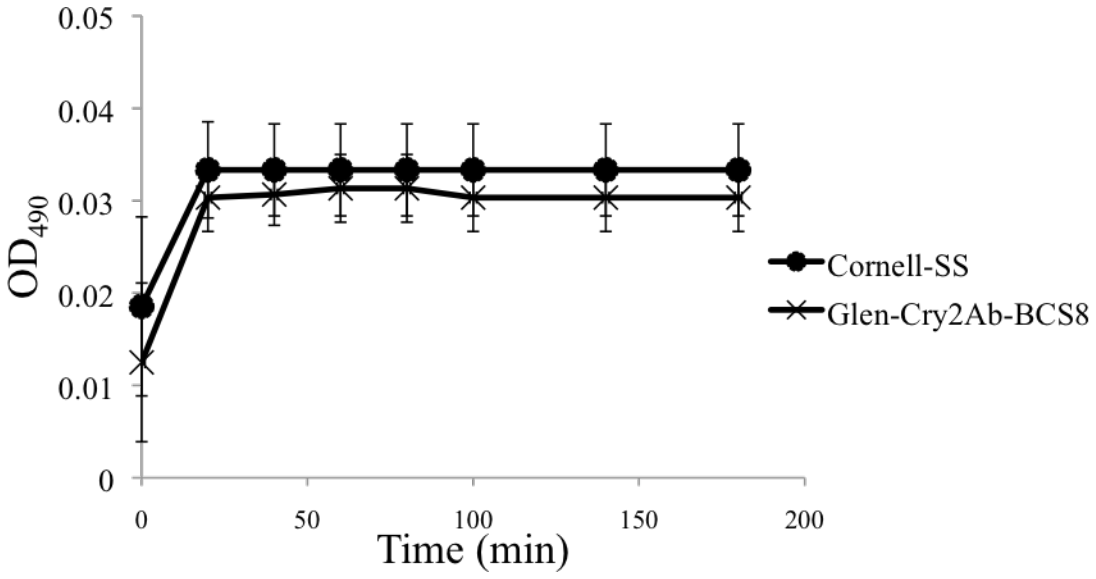

Figure 3.9. In vitro melanization activity of hemolymph plasma from the Cornell-SS and Glen-Cry2Ab-BCS8 larvae of *T. ni*. Error bars indicate 95% confidence intervals of the means from three replications.

Discussion

Identification of resistance mechanisms by comparative analysis of Bt-resistant and -susceptible populations can be confounded if the two populations do not share the same genetic background. To reduce the non-resistance related variations between the Bt-resistant and -susceptible strains, in the present study, the Cry2Ab resistance trait in the Glen-BGII strain was introgressed into the laboratory susceptible Cornell-SS strain to generate the near-isogenic introgression strain Glen-Cry2Ab-BCS8. As expected, the Glen-Cry2Ab-BCS8 strain was highly resistance to Cry2Ab.

Alteration of midgut digestive proteases could confer Bt resistance by either hindering the activation of protoxins (Oppert et al., 1997; Herrero et al., 2001; Li et al., 2004; Karumbaiah et al., 2007) or accelerating the degradation of toxins (Forcada et al., 1996; Keller et al., 1996; Shao et al., 1998; Loseva et al., 2002; Wager et al., 2002). The Glen-Cry2Ab-BCS8 strain and the laboratory susceptible strain of *T. ni* did not differ significantly in midgut digestive proteases, suggesting that Cry2Ab resistance in the greenhouse-derived resistant strain is not caused by changes in those proteases. The naturally-occurring Bt Cry endotoxins are protoxins that require an alkaline environment for solubilization after ingestion by insects (Gill et al., 1992). These Bt Cry protoxins also require midgut digestive proteases, mainly serine proteases in lepidopteran larvae, for activation into active forms in the midgut lumen (Gill et al., 1992). In contrast, Bt Cry toxins expressed in most transgenic Bt-crops are already in their active forms prior to ingestion by insect larvae (http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad2/2-id_health.pdf). Thus, the use of active Bt toxins in transgenic crops removes the abovementioned steps whose alteration can confer resistance. However, it is still possible

that alteration of midgut digestive proteases causes differential proteolysis of the active toxins in the plants. In this study, differential proteolysis of Cry2Ab was not found in the resistant larvae of *T. ni*.

Aminopeptidase and membrane-bound ALP may serve as receptors for Bt Cry toxins (Pigott and Ellar, 2007). However, whether Cry2A toxins bind to aminopeptidase or membrane-bound ALP in insects remains unknown. Regarding aminopeptidase, Northern blot analysis revealed a lack of expression of aminopeptidase N1 (APN1) in Cry1C-resistant *Spodoptera exigua* (Herrero et al., 2005). In Cry1Ac-resistant *T. ni* larvae, the APN1 was significantly down-regulated at both protein and transcriptional levels, and the down-regulation of APN1 expression was genetically linked to the resistant trait (Tiewsiri and Wang, 2011). In terms of ALP, reduced levels of midgut membrane-bound ALP have been observed in Bt-resistant *Heliothis virescens*, *Helicoverpa armigera* and *Spodoptera frugiperda* (Jurat-Fuentes et al., 2011). In addition, there was a 10-fold increase in the specific ALP activity within the midgut lumen of Cry1Ac-resistant *Helicoverpa zea* larvae when compared to that of the susceptible larvae, and the luminal ALP was suggested to bind Cry1Ac to prevent binding of the toxin to the midgut epithelial membrane (Caccia et al., 2012). In this study, the enzymatic activity assays showed that specific aminopeptidase and ALP activities were statistically the same in the midgut samples from the resistant and susceptible strains of *T. ni*, and Western blots detected no significant difference in the protein abundance of membrane-bound ALP for the larvae from the two strains. However, it remained unknown whether the individual midgut aminopeptidases were altered in the resistant strain, as aminopeptidases are a multigene family of proteins in the midgut.

Thus, there was possibility that one or some of the aminopeptidases might be associated with Cry2Ab resistance. Further research was conducted to exam whether genes encoding midgut aminopeptidase Ns and membrane-bound ALP are genetically linked to Cry2Ab resistance in *T. ni* (Chapter 4).

As altered binding of Cry toxins to the midgut receptors resulted from modification of receptors is the most common mechanism for high-level Cry1A resistance (Ferre and Van Rie, 2002; Bravo et al., 2011), information about receptor binding of Cry2A could possibly shed light on the mechanisms of resistance to Cry2 toxins. Recently, reduced binding of Cry2Ab toxin to midgut BBMV proteins in the Cry2Ab-resistant *H. armigera* and *H. punctigera* larvae was proposed to be the mechanism of Cry2Ab resistance in these species (Caccia et al., 2010). Therefore, future studies are required to determine whether the mechanism of Cry2Ab resistance in the Glen-Cry2Ab-BCS8 strain of *T. ni* is reduced binding.

Overexpression of esterase has been reported in a Cry1Ac-resistant *H. armigera* strain (Gunning et al., 2005). However, activities and isozyme profiles of midgut esterase between the resistant and susceptible samples were similar in *T. ni*. Furthermore, insect tolerance to Bt Cry toxins could be associated with heightened immune response (Rahman et al., 2004, 2007; Ma et al., 2005). Elevated immune response, as determined by plasma hemolymph melanization activity, was not observed in the Cry2Ab-resistant strain of *T. ni.*

In conclusion, the results from this study indicate that the Cry2Ab-resistant *T. ni* strain Glen-Cry2Ab-BCS8 is not different from the susceptible Cornell-SS strain in midgut protease activities, proteolytic digestion of Cry2Ab, midgut aminopeptidase and

ALP activities, midgut esterase activities, as well as melanization activities of hemolymph plasma. To further understand the biochemical basis of Cry2Ab resistance in the greenhouse-derived resistant strain of *T. ni*, comparative analysis of additional biochemical components will be required, including qualitative and quantitative binding of Cry2Ab to midgut brush border membrane vesicles.

CHAPTER 4

LINKAGE ANALYSIS OF PUTATIVE CRY TOXIN RECEPTOR GENES WITH CRY2AB RESISTANCE IN THE CABBAGE LOOPER, *TRICHOPLUSIA NI*

Introduction

Binding of Cry toxins to insect midgut epithelial receptors is a crucial determinant of toxin specificity (Gill et al., 1992; Bravo et al., 2013). Loss or reduction of toxin binding resulting from alteration of toxin receptors often leads to high-level insect resistance to Bt Cry toxins (Ferre et al., 1991; Ferre and Van Rie, 2002; Caccia et al., 2010; Jurat-Fuentes et al., 2011). Cadherin, aminopeptidase N (APN), and membrane-bound alkaline phosphatase (mALP) in the midgut are the major Cry toxin receptors that have been identified and characterized in insects (Piggot and Ellar, 2007). More recently, a sequential toxin binding model suggested by Bravo et al. (2007) has been extended by implicating an ATP-binding cassette (ABC) transporter, ABCC2, in pore insertion (Gahan et al., 2010; Heckel, 2012; Bravo et al., 2013).

Midgut cadherin serves as a receptor for Cry1A toxins in lepidopterans (Vadlamudi et al., 1995; Nagamatsu et al., 1998; Jurat-Fuentens et al., 2004), and mutations of the cadherin gene have been found to be genetically linked to the mode 1 type resistance in laboratory-selected populations of *Heliothis virescens*, *Pectinophora gossypiella*, and *Helicoverpa armigera* (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005). The linkage of cadherin mutations with Bt resistance has been proposed as a basis

for DNA-based molecular detection techniques for monitoring development of resistance in the field (Tabashnik et al., 2006; Gahan et al., 2007).

The glycosylphosphatidylinositol (GPI) -anchored APN and ALP are also implicated as toxin receptors that play a significant role for toxicity by mediating initial low-affinity interactions with Cry toxin monomers and subsequent high-affinity interactions with toxin oligomers, which induce pore formation in the midgut epithelial cell membrane and eventual cell lysis (Pardo-Lopez et al., 2013). Mutations in the APN1 gene and the aminopeptidase-P gene have been reported in a Cry1Ac-resistant strain of *H. armigera* and a Cry1Ab-resistant strain of *Ostrinia nubilalis* (Zhang et al., 2009; Khajuria et al., 2011). Furthermore, reduction of APN and ALP at the expression level is associated with Bt resistance in insect strains of *Spodoptera exigua*, *H. virescens*, and *Trichoplusia ni* (Herrero et al., 2005; Jurat-Fuentes et al., 2011; Tiewsiri and Wang, 2011).

By contract, the role of the abovementioned Cry1 toxin receptors in Cry2 toxin action and resistance is not known in any insects. In this study, I performed genetic linkage analyses to examine the genetic linkage of the genes coding for these known toxin receptors (i.e., cadherin, mALP, and APN1-6) with Cry2Ab resistance in *T. ni*. Results from this study revealed that the Cry2Ab resistance in *T. ni* is not genetically linked to the putative Cry1 receptor genes examined in this study.

Materials and methods

Insects. A highly inbred colony of *T. ni* (Cornell-SS) that had been maintained for over 30 years in the laboratory without exposure to Bt products was used as the susceptible strain (Kain et al., 2004). The Cry2Ab-resistant strain used herein was established from Bt-resistant populations that developed resistance to Bt in commercial greenhouses and referred to as Glen-BGII (Chapter 2). *T. ni* larvae were reared on a wheat germ-based artificial diet at 27°C with 50% humidity and a 16h:8h (light:dark) photoperiod. Adults were maintained under the same conditions as those of larvae and flourished on 10% sucrose solution.

Cry2Ab toxin. Cry2Ab toxin was obtained from Monsanto Company (St. Louis, MO) and stored in 50 mM CAPS (pH 11) with 2 mM DTT at -20°C.

Genotyping of Cry toxin receptor genes by polymerase chain reaction (PCR) amplification and sequencing of polymorphic fragments of the genes. For the genes coding for the toxin binding receptors (i.e., cadherin, mALP, APN1-6), primers were designed based on their cDNA sequences, and used for PCR amplification of a genomic DNA fragment of each receptor gene to identify polymorphic sites to be used for genotyping the receptor gene alleles in the *T. ni* individuals (Table 4.1).

For genotyping, genomic DNA was prepared from fifth-instar larvae or adults using a rapid DNA isolation method as described in Chapter 3. PCRs were performed to amplify the genomic DNA fragments using the genomic DNA lysates as templates with the specific primers described in Table 4.1. PCR amplification reactions were prepared and performed as described in Chapter 3. For DNA sequencing, 5 μL of the PCR product

Table 4.1. Primers used for PCR amplification of the genomic DNA fragments of the cadherin, membrane-bound alkaline phosphatase and aminopeptidase N genes (*cadherin*, *malp*, and *apn1-6*) for analysis of genetic linkage of Cry2Ab resistance with the toxin receptor genes.

Gene	cDNA sequence (Genbank accession no.)	Primers
cadherin	JF303656	5'-GCGCTGCTGGGCTTCCTGT-3' (F) 5'-CGCTTTGATGGTCTCGTTC-3' (R)
malp	JF825967	5'-CACAACTGCGAGTCATCCAT-3' (F) 5'-GTTCTCCCAGTTGCGGTTCG-3' (R)
apn1	AY836579	5'-GGCCTTCCCTTGCTACGATG-3' (F) 5'-GGGCTTCTCTGTATGTCAAAAGA-3' (R)
apn2	AY836580	5'-CCACTTTCCTGGACCTACACTTG-3' (F) 5'-TGGGGTTCACACTGATAACGG-3' (R)
apn3	AY836581	5'-GGCGCTAACCCTGACTTCTCTA-3' (F) 5'-AATTGGAAGACATCGTTGAC-3' (R)
apn4	AY836582	5'-GTCCTTGACTTCTTGGGCAG-3' (F) 5'-CCGAGGTTGCAAGCGAAGT-3' (R)
apn5	JF303657	5'-GCTGAGAAACAGGTTCTTGCAT-3' (F) 5'-ACGGCATGGCTCACACAG-3' (R)
apn6	JF303658	5'-CGATTTTTCCTAAGTTCGTCTGC-3' (F) 5'-CAAAATATGGATTGGTGAGGG-3' (R)

was incubated with 0.5 U of shrimp alkaline phosphatase and 1.25 U of exonuclease I (USB Biochemicals, Cleveland, OH) at 37°C for 60 min, followed by incubation at 90°C for 10 min to inactivate the shrimp alkaline phosphatase and exonuclease I. Sequencing reactions were carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) following the instruction provided by the manufacturer. After the sequencing reactions, unincorporated dye terminators were removed by Sephadex G-50 columns in a 96-well plate. The purified DNA sequencing reaction products were subjected for sequence reading at the Biotechnology Resource Center of Cornell University (Ithaca, NY). Sequence data was analyzed using the Lasergene software package (DNAStar, Madison, WI).

Genetic linkage analysis of Cry toxin receptor genes with Cry2Ab resistance in *T. ni*. A susceptible male (Cornell-SS) was crossed with a Cry2Ab-resistant female (Glen-BGII) to generate F_1 progeny (Figure 4.1). After egg laying, the adults were subjected to genotyping the toxin receptor genes as described above, to determine the alleles in the susceptible and resistant parents. As crossing over between homologous chromosomes does not occur in female lepidopterans, a F_1 female was crossed with a resistant male (Glen-BGII) to generate backcross progeny for toxin selection to examine the genetic association between the toxin binding receptor genes and the Cry2Ab resistance (Figure 4.1). The resistant male used to generate the backcross family was also subjected to genotyping to determine its allele type of the receptor genes. A total number of 120 larvae from the backcross family were reared on an artificial diet overlayed with a discriminating dose of Cry2Ab (i.e., 2 mL of 440 μg/mL toxin spread on the diet surface of ca. 50 cm^2) to eliminate susceptible heterozygous individuals. After 7 days, the

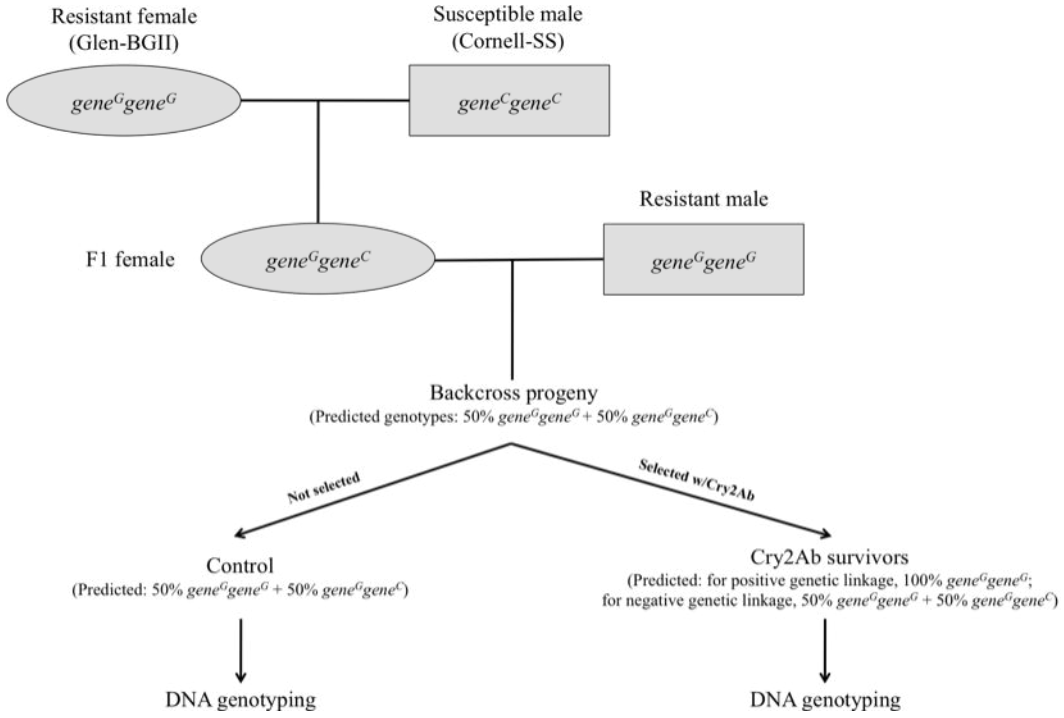

Figure 4.1. Experimental design for the genetic linkage analysis of Cry2Ab resistance with the Cry toxin receptor genes in *T. ni*. For each tested gene, the allele from the susceptible strain is named $gene^C$, and the allele from the resistant strain is named $gene^G$. The prediction on genotypes for the backcross progeny is made based on random assortment of the gene alleles.

survivors on Cry2Ab treated diet were transferred to fresh new diet without toxin and reared to fifth instar. Forty larvae from the backcross family reared to fifth instar on diet without Cry2Ab treatment were used as the non-selection control group. Sixteen Cry2Ab-selected larvae and 16 larvae from the non-selection group were randomly chose for genotyping the receptor gene alleles as described above, to examine the genetic association of the toxin binding receptor genes with the Cry2Ab resistance by analyzing the segregation of the gene alleles with the resistance trait.

Results

Identification of toxin receptor gene allelic markers by genomic DNA PCR fragment sequencing. Allelic variations were identified in the cadherin, mALP, APN1-6 genes between the susceptible and resistant strains. For the cadherin gene, the 495 bp PCR fragment, covering the cDNA sequence (Genbank accession no. JF303656) from nucleotide position 4911 to 5114 with an intron of 291 bp, harbored 12 single nucleotide polymorphisms (SNPs) between the allele from the susceptible strain, named cad^C, and the allele from the resistant strain, named cad^G (Figure 4.2). For the mALP gene, the PCR fragment covered the cDNA sequence from nucleotide position 457 to 618 with an intron of 343 bp (Figure 4.3). Three SNPs and a 11-nucleotide deletion were identified between the two ALP alleles, alp^C and alp^G, from the susceptible and resistant strains (Figure 4.3). For the APN1-6 genes, the susceptible and resistant strains also carried different alleles, apn^C and apn^G. The nucleotide differences between the alleles are detailed in Figure 4.4 through Figure 4.9 for the six APN genes.

GCGCTGCTGGGCTTCCTGTGCGTGCTGCTACTCATCACCTTCATAGTCAG *cad*^{C}
GCGCTGCTGGGCTTCCTGTGCGTGCTGCTACTCATCACCTTCATCGTCAG *cad*^{G}

GACTAGAGCgtgagtatctgatgcgatccggaggacaatacattcagaat *cad*^{C}
GACTAGAGCgtgagtatctgatgcgatccggaggacaaaacattcagaat *cad*^{G}

ttagctttcttatttttaaattaatggttctccaaacgaatttgtctct *cad*^{C}
taggctttcttatttttaaattaatggttctccaaacgaatttgtctct *cad*^{G}

ttggcatggaggctttactgaaatacaaatcatatgcacataaaattaac *cad*^{C}
ttggcatggaggctttactgaaattcaaatcatatgcacataaaattaac *cad*^{G}

cccgacttggacaaattcattgattgattcaccataaaaaatttagctaa *cad*^{C}
cccgatttggacaaattcattgattgattcaccataaaaaatttagctaa *cad*^{G}

cttctttaatgctattttcaattgaaatcaaatatagtaataattgtttt *cad*^{C}
cttctttaatgctattttcaattgaaatcaaatatagtaataattgattt *cad*^{G}

ctcatgcaatgttacaatattgtcaactgtaattatcttccctttctcag *cad*^{C}
ctcatgcaatgttacaatattgtccactgtaattatcttccctttctcag *cad*^{G}

GCTCAACCGTCGTCTGGAAGCCCTATCCATGACTAAAGGCGGCTCAGTGG *cad*^{C}
GCTCAACCGTCGTCTGGAAGCCCTATCCATGACTAAAGGCGGCTCAGTGG *cad*^{G}

ACTCTGGACTGAACCGCGTGGGTCTGGCCGCGCCCGGGACTAACAAGCAC *cad*^{C}
ACTCCGGGCTGAACCGCGTGGGTCTGGCCGCGCCCGGGACCAACAAGCAC *cad*^{G}

GCCGTCGAGGGTTCCAACCCCATCTGGAACGAGACCATCAAAGCG *cad*^{C}
GCCGTCGAGGGCTCCAACCCCATCTGGAACGAGACCATCAAAGCG *cad*^{G}

Figure 4.2. Nucleotide sequence of the PCR amplified genomic DNA fragment of the cadherin gene. The cadherin gene alleles from the Cornell-SS strain and the Glen-BGII strain are named *cad*^{C} and *cad*^{G}, respectively. Lowercase sequences indicate the intron sequences. The 5'-GT and 3'-AG splice sites in introns are underlined. Polymorphic nucleotides are shaded.

CACAACTGCGAGTCATCCATCGACACCGCCCGCCACGTGGAGTCTATCGC *malp*^{C}
CACAACTGCGAGTCATCCATCGACACCGCCCGCCACGTGGAGTCTATCGC *malp*^{G}

GGAGTGGGCGCTCGCCGACGGCAGAGATGCTGgtcagtttccacacaaac *malp*^{C}
TGAGTGGGCGCTCGCCGACGGCAGAGATGCTGgtcagtttccacacaaac *malp*^{G}

tctaaaattgacaactttccttaaaactcaaaattgtttctccttgtata *malp*^{C}
tctaaaattgacaactttccttaaaactcaaaattgtttctccttgtata *malp*^{G}

ttacgaatatatcatattttaaaatcctccctgtcgatatgatatggata *malp*^{C}
ttacgaatatatcatattttaaaatcctccctgtcgatatgat------- *malp*^{G}

tgatcatacacctaagggaaatcaaactacgcctaggaagcttattttta *malp*^{C}
----catacacctaaggaaaatcaaactacgcctaggaagcttattttta *malp*^{G}

ccacaataaatagaaactacactacttcgtgaatgttatgtgaaaaatac *malp*^{C}
ccacaataaatagaaactacactacttcgtgaatgttatgtgaaaaatac *malp*^{G}

taatataattattaaatgtgaaattgtcacttcaaatttacaccggcatt *malp*^{C}
taatataataattaaatgtgaaattgtcacttcaaatttacaccggcatt *malp*^{G}

aggaagtcggagacagtcgggttgcccttgtcaaattaatcgttgattgt *malp*^{C}
aggaagtcggagacagtcgggttgcccttgtcaaattaatcgttgattgt *malp*^{G}

ttaatatttttcgttgggtcgcagGTATTGTGACAACAACCCGCATTAC *malp*^{C}
ttaatatttttcgttgggtcgcagGTATTGTGACAACAACCCGCATTAC *malp*^{G}

TCACGCGTCTCCAGCCGGCGTGTTCGCCAAGACGGCGAACCGCAACTGGG *malp*^{C}
TCACGCGTCTCCAGCCGGCGTGTTCGCCAAGACGGCGAACCGCAACTGGG *malp*^{G}

AGAAC *malp*^{C}
AGAAC *malp*^{G}

Figure 4.3. Nucleotide sequence of the PCR amplified genomic DNA fragment of the mALP gene. The mALP gene alleles from the Cornell-SS strain and the Glen-BGII strain are named *malp*^{C} and *malp*^{G}, respectively. Lowercase sequences indicate the intron sequences. The 5'-GT and 3'-AG splice sites in introns are underlined. Polymorphic nucleotides are shaded. Dashes show absence of nucleotides.

Figure 4.4. Nucleotide sequence of the PCR amplified genomic DNA fragment of the APN1 gene. The APN1 gene alleles from the susceptible strain and the resistant strain are named $apn1^{C}$ and $apn1^{G}$, respectively. Lowercase sequences indicate the intron sequences. The 5'-GT and 3'-AG splice sites in introns are underlined. Polymorphic nucleotides are shaded. Dashes show absence of nucleotides.

GGCCTTCCCTTGCTACGATGAACCGTCTTTCAAGGCCTTATTCGATATTA *apn1*^{C}
GGCCTTCCCTTGCTACGATGAACCGTCTTTCAAGGCCTTATTCGATATTA *apn1*^{G}

CCATCAAGCGGCTACCGGACTTCTCTGAAACCCTATCCAACATGCCAATC *apn1*^{C}
CCATCAAGCGGCTACCGGACTTCTCTGAAACCCTATCAAACATGCCAATC *apn1*^{G}

AAAACAAGGGGACCGCTgtaagctacttaaatacatttaaattgtaattt *apn1*^{C}
AAAACAAGGGGACCGCTgtaagctacttaaatacatttaaattgtaattt *apn1*^{G}

tgacaatatcaagataaattggcttttgatcgattatgtcaattggatca *apn1*^{C}
tgacaatatcaagataaattggcttttgatcgattatgtcaattggatca *apn1*^{G}

ttaaaatataaaatactcttaaaacaatatagcaagtgataggaatattt *apn1*^{C}
ttaaaatataaaatactcttaaaacaatatagcaagtgataggaatattt *apn1*^{G}

ttaatgtgtatgcaattaagcttgattcatttaatatcattttgacatt *apn1*^{C}
ttaatgttatgcaattaagcttgattcatttaatatcattttgacatt *apn1*^{G}

gaacgctaattgtaaaatatcatgaccccatttctgttacagCACTGATG *apn1*^{C}
-aacgctaattataaaatatcatgaccccatttctgttacagCACTGATG *apn1*^{G}

GTAGAATTGCTGAAACCTTCCACACTACTCCTAAAACCTCTACGTATTTG *apn1*^{C}
GTAGAATTGCTGAAACCTTCCACACTACTCCTAAAACCTCTACGTATTTG *apn1*^{G}

CTTGCGTTCATTGTTTCTCACTACAAGGAGGTTGCTACTGGCACCGACCT *apn1*^{C}
CTTGCGTTCATTGTTTCTCACTACAAGGAGGTTGCTACTGGCACCGACCT *apn1*^{G}

CAATAGACCCTTTAAGATCTATGCTCGTGACAATGCTAAACTCACCGGAG *apn1*^{C}
CAATAGACCCTTTAAGATCTATGCTCGTGACAATGCTAAACTCACCGGAG *apn1*^{G}

ATTGGTCTTTGGATATTGGTGAACGTCTTCTCGAAGAGATGGAGAAGATC *apn1*^{C}
ATTGGTCTTTGGATATTGGTGAACGTCTTCTCGAAGAGATGGAGAAGATC *apn1*^{G}

ACAGATGTTCCATACTACGGAATGGCTCTAAACATGGATATGAAACAGGC *apn1*^{C}
ACAGATGTTCCATACTACGGAATGGCTCTAAACATGGATATGAAACAGGC *apn1*^{G}

CGCCATCCCTGACTTTTCTGCAGGAGCTATGGAAAATTGGGGTCTTTTGA *apn1*^{C}
CGCCATCCCTGACTTTTCTGCAGGAGCTATGGAAAATTGGGGTCTTTTGA *apn1*^{G}

CATACAGAGAAGCCC *apn1*^{C}
CATACAGAGAAGCCC *apn1*^{G}

Figure 4.5. Nucleotide sequence of the PCR amplified genomic DNA fragment of the APN2 gene. The APN2 gene alleles from the susceptible strain and the resistant strain are named *apn2*^{C} and *apn2*^{G}, respectively. Lowercase sequences indicate the intron sequences. The 5'-GT and 3'-AG splice sites in introns are underlined. Polymorphic nucleotides are shaded. Dashes show absence of nucleotides.

Figure 4.6. Nucleotide sequence of the PCR amplified genomic DNA fragment of the APN3 gene. The APN3 gene alleles from the susceptible strain and the resistant strain are named *apn3*^{C} and *apn3*^{G}, respectively. Lowercase sequences indicate the intron sequences. The 5'-GT and 3'-AG splice sites in introns are underlined. Polymorphic nucleotides are shaded.

GGCGCTAACCCTGACTTCTCTAATACCAAACCCACTCATATCATGAGGAA *apn3*^{C}
GGCGCTAACCCTGACTTCTCTAACCCCAAACCCACTCATATCATGAGGAA *apn3*^{G}

GAGCAGCATCAAGATCAATCGCCAGGTGACTGGTGACCACTGGGTACTCT *apn3*^{C}
GAGCAGCATCAAGATCAATCGCCAGGTGACTGGTGACCACTGGGTACTCT *apn3*^{G}

TCAACACCCAACAAACTGgtaaatatgttacttttatttttgaaacaaga *apn3*^{C}
TCAACACCCAACAAACTGgtaaatatgttacttttatttttgaaacgtga *apn3*^{G}

agacaagtacctgtcaatatctatttgccttttgcacccctcagagatt *apn3*^{C}
agacaagtacctgtccatatctatttgcctttgtgcacccctcagagatt *apn3*^{G}

ttaatgactttatttttctgctgtctttgttttaatttttaaaactcgtg *apn3*^{C}
ttaatgactttatttttctgctgtctttgttttaatttttaaaacttgtg *apn3*^{G}

ggatttacatatttcggtaaactaattctctagcacatataccagataaa *apn3*^{C}
ggatttacatatttcggtaaactaattctctagcacatataccagataaa *apn3*^{G}

aatatcagtcagtcttcagaattatgaggacactaaacgttattgtgttc *apn3*^{C}
aatatcagtcagtcttcagaattatgaggacactaaacgttattgtgttc *apn3*^{G}

tgatttttccttatttttttttgttttttttatacatcccttgtatttgtc *apn3*^{C}
tgatttttccttaatttttttgttttttttatacatcccttgtatttgtc *apn3*^{G}

agGATTCTACCGCGTGAACTACGATGATTACACTTGGGATCTGATCATTC *apn3*^{C}
agGATTCTACCGCGTGAACTACGATGATTACACTTGGGATCTGATCATTC *apn3*^{G}

AGGCTCTGAGAGGCCCGGACAGGACTAAAATTCACGAGTACAACAAAGCA *apn3*^{C}
AGGCTCTGAGAGGCCCGGACAGGACTAAAATTCACGAGTACAACAAAGCA *apn3*^{G}

CAGgtaaataataagttactaataataataagttgatccatgaatgcatt *apn3*^{C}
CAGgtaaataataagttactaataataataagttgatccatgaatgcatt *apn3*^{G}

gtctgagtcgagatgtcttgtgcatgtgactttaatgtttgtgaaaccca *apn3*^{C}
gtctgagtcgagatgtcttgtgcatgtgactttaatgtttgtgaaaccca *apn3*^{G}

agcgacacgagaattaattttttattgcaaaactgataattgatttgat *apn3*^{C}
agcgacacgagaattattttttttattgcaaaactgataattgatttgat *apn3*^{G}

ttttgaaatcaagtcaaattattgacaagatttattaaaaattacccagt *apn3*^{C}
ttttgaaatcaagtcaaattattgacaagatttattaaaaattacccagt *apn3*^{G}

ctctttcgaaaacattttgtacctttttatttacattatttctgttttct *apn3*^{C}
ctctttcgaaaacattttgtacctttttatttacattatttctgttttct *apn3*^{G}

tttagATCGTCAACGATGTCTTCCAATT *apn3*^{C}
tttagATCGTCAACGATGTCTTCCAATT *apn3*^{G}

GTCCTTGACTTCTTGGGCAGCGAGACTGACTACTACGTATGGGCCGGTGC *apn4*^{C}
GTCCTTGACTTCTTGGGCAGCGAGACTGACTACTACGTATGGGCCGGTGC *apn4*^{G}

CCTCACCCAGCTGGACTGGATCCGCAGACGTCTGGAGCACATCCCTCAGG *apn4*^{C}
CCTCACCCAGCTGGACTGGATCCGCAGACGTCTGGAGCACATCCCTCAGG *apn4*^{G}

CTCACGAAGCTTTCACTgtgaatatttttttcaagttttgaacaaattgc *apn4*^{C}
CTCACGAAGCTTTCACTgtgagtatttttttcaagttttgaacaaattgc *apn4*^{G}

tacttgataatctgatcaatagaattctttgtttctatctaatagtgaaa *apn4*^{C}
tacttgataatctgatcaatagaattctttgtttctatctaatagtgaaa *apn4*^{G}

tgcttctacgagcaacaaaagaaacaatttaatttaccgtgacactgatc *apn4*^{C}
tgcttctacgagcaacaaaagaaacaatttaatttaccgtgacactgatc *apn4*^{G}

taaaattctctaacttgattgataatgcattgttccagAACTACCTTCTT *apn4*^{C}
taaaattctctaacttgattgataatgcattgttccagAACTACCTTCTT *apn4*^{G}

TCACTTATGAACGCCGTGATCAACCATCTTGGTTACAACGAGCTTGCTAC *apn4*^{C}
TCACTAATGAACGCCGTGATCAACCATCTTGGTTACAACGAGCTTGCTAC *apn4*^{G}

TGACTCAACCTCCACGATCCTGAACAGGATGCAAATCATGAACTTCGCTT *apn4*^{C}
TGACTCAACCTCCACGATCCTGAACAGGATGCAAATCATGAACTTCGCTT *apn4*^{G}

GCAACCTCGG *apn4*^{C}
GCAACCTCGG *apn4*^{G}

Figure 4.7. Nucleotide sequence of the PCR amplified genomic DNA fragment of the APN4 gene. The APN4 gene alleles from the susceptible strain and the resistant strain are named *apn4*^{C} and *apn4*^{G}, respectively. Lowercase sequences indicate the intron sequences. The 5'-GT and 3'-AG splice sites in introns are underlined. Polymorphic nucleotides are shaded.

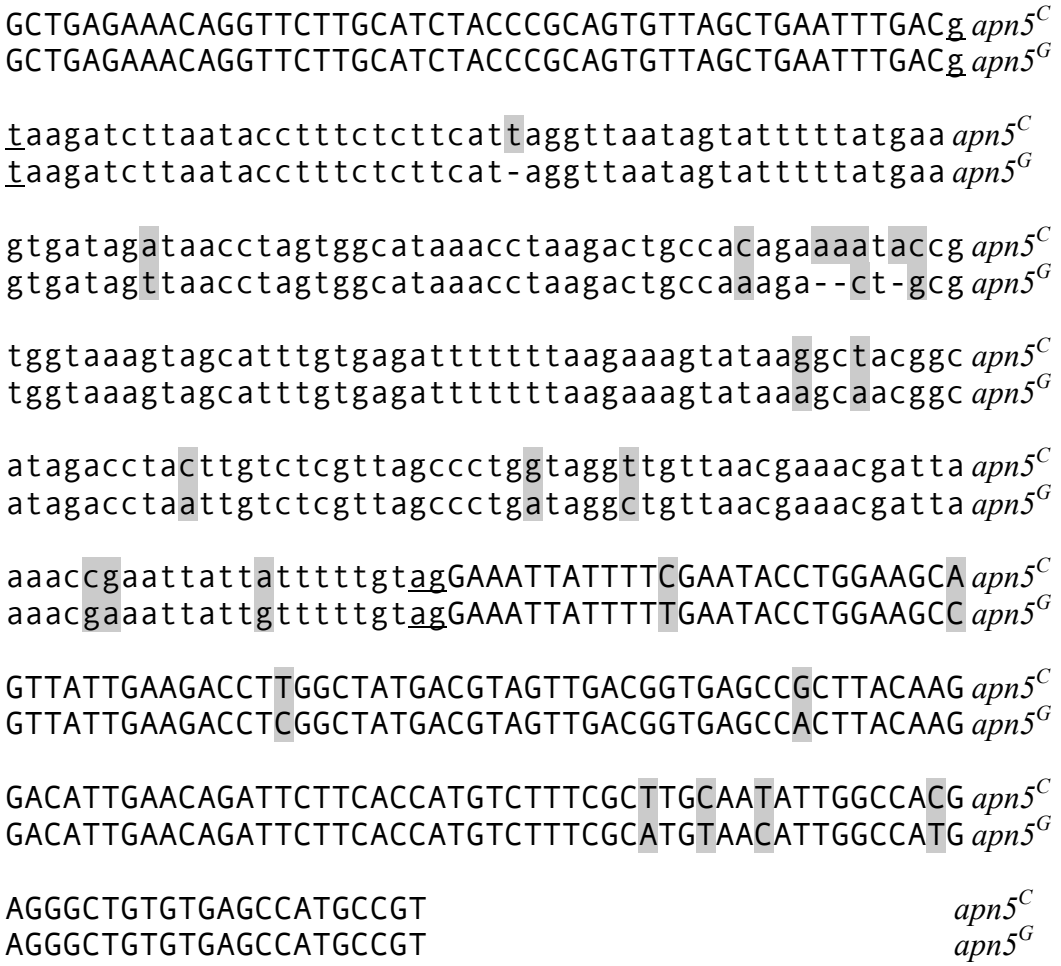

Figure 4.8. Nucleotide sequence of the PCR amplified genomic DNA fragment of the APN5 gene. The APN5 gene alleles from the susceptible strain and the resistant strain are named *apn5*^{C} and *apn5*^{G}, respectively. Lowercase sequences indicate the intron sequences. The 5'-GT and 3'-AG splice sites in introns are underlined. Polymorphic nucleotides are shaded. Dashes show absence of nucleotides.

CGATTTTTCCTAAGTTCGTCTGCTGCACCTACTGGTCAAATCTATCCAAT *apn6*^{C}
CGATTTTTCCTAAGTTCGTCTGCTGCACCTACTGGTCAAATCTACCCAAT *apn6*^{G}

TCCTATTACTTTCTCAACAAAAACAAACCCCAGCTTTTCAATCCTGAAGC *apn6*^{C}
TCCTATTACTTTCTCAACAAAAACAAACCCCAGCTTTTCAATCCTGAAGC *apn6*^{G}

CTTCTCATATAATGACGGGAGCGACTCTCACTATAAACAAAGCGGCTGTC *apn6*^{C}
CTTCTCATATAATGACGGGAGCGACTCTCACTATAAACAAAGCGGCTGTC *apn6*^{G}

GAAGAATGGGTGATATTTAATAATATGCAGCACGgtaagtattttattt *apn6*^{C}
GAAGAATGGGTGATATTTAATAATATGCAGCACGgtaagtattttattt *apn6*^{G}

tttgaggaacttcaatacatcttagttttccgaccaagcaaagattatga *apn6*^{C}
tttgacgaaccttcaatacatcttagttttccgaccaagcaaagattatga *apn6*^{G}

attcaattcgaattataaaatttttattaagtataagattaagataatg *apn6*^{C}
attcaattcgaattataaaatttttatattaagtataagattaagataatg *apn6*^{G}

caagatttattttctttagGTCACTACAGAGTTAACTATGATTCGAAAA *apn6*^{C}
caagatttattttctttagGTCACTACAGAGTTAACTATGATTCGAAAA *apn6*^{G}

CCTGGTCTTTGATTGCGGAAGCTTTGTTAGAGGAACCCTCACCAATCCAT *apn6*^{C}
CCTGGTCTTTGATTGCGGAAGCTTTGTTAGAGGAACCCTCACCAATCCAT *apn6*^{G}

ATTTTG *apn6*^{C}
ATTTTG *apn6*^{G}

Figure 4.9. Nucleotide sequence of the PCR amplified genomic DNA fragment of the APN6 gene. The APN6 gene alleles from the susceptible strain and the resistant strain are named *apn6*^{S} and *apn6*^{R}, respectively. Lowercase sequences indicate the intron sequences. The 5'-GT and 3'-AG splice sites in introns are underlined. Polymorphic nucleotides are shaded.

Association of the toxin receptor genes with Cry2Ab resistance in *T. ni*. The survival rate of the backcross progeny at the diagnostic does of the toxin was 45%, which was close to the expected survival of 50% for monogenic recessive inheritance of the resistance (p=0.27, *chi*-square test). For the cadherin gene, genotyping of the 16 larvae from the non-selection control group showed that 9 larvae had the genotype $cad^{G}cad^{G}$ and 7 larvae had the genotype $cad^{G}cad^{C}$, which statistically fitted the predicted ratio of 1:1 between the two genotypes for random assortment of the alleles (Table 4.2). Genotyping cadherin alleles of the 16 Cry2Ab-selected larvae of the backcross family showed that 10 larvae had the genotype $cad^{G}cad^{G}$ and 6 larvae had the genotype $cad^{G}cad^{C}$, not statistically different from the ratio of 1:1 between the two genotypes of the cadherin alleles. This result indicates that the cad^{G} allele from the resistant strain did not co-segregate with the Cry2Ab resistance trait, and therefore the cadherin gene was not genetically linked to the resistance. For the mALP gene, of the 16 larvae selected with Cry2Ab, 7 larvae had the homozygous genotype $alp^{G}alp^{G}$ whereas 9 larvae had the heterozygous genotype $alp^{G}alp^{C}$ (Table 4.2). Similarly, 9 larvae from the non-selection group had the homozygous genotype $alp^{G}alp^{G}$ and 7 larvae had the heterozygous genotype $alp^{G}alp^{C}$ (Table 4.2). For both the Cry2Ab treated and non-treated backcross groups, the number of homozygotes and heterzygotes for the ALP gene statistically fitted the predicted ratio of 1:1 for random assortment of the ALP alleles inherited from their parents, demonstrating that the Cry2Ab resistance was genetically independent of the alp^{G} allele from the resistant strain. As the APN1-6 genes are clustered in the same linkage group, it is not surprising that those 6 genes shared the same pattern of genetic association with Cry2Ab resistance. In the non-selection control group, 6 larvae had the

Table 4.2. Allele frequencies of the Cry toxin receptor genes (*cadherin*, *malp*, and *apn1-6*) in the backcross progeny with and without Cry2Ab treatment.

gene	Non-selected control (16 larvae)			Cry2Ab-selected (16 larvae)		
	Observed genotype	Predicted genotype	*p*-value	Observed genotype	Predicted genotype	*p*-value
cadherin	9 cad^Gcad^G, 7 cad^Gcad^C	8 cad^Gcad^G, 8 cad^Gcad^C	0.62	10 cad^Gcad^G, 6 cad^Gcad^C	8 cad^Gcad^G, 8 cad^Gcad^C	0.32
malp	9 $malp^Gmalp^G$, 7 $malp^Gmalp^C$	8 $malp^Gmalp^G$, 8 $malp^Gmalp^C$	0.62	7 $malp^Gmalp^G$, 9 $malp^Gmalp^C$	8 $malp^Gmalp^G$, 8 $malp^Gmalp^C$	0.62
apn1	6 $apn1^Gapn1^G$, 10 $apn1^Gapn1^C$	8 $apn1^Gapn1^G$, 8 $apn1^Gapn1^C$	0.32	4 $apn1^Gapn1^G$, 12 $apn1^Gapn1^C$	16 $apn1^Gapn1^G$, 0 $apn1^Gapn1^C$	0.0001
apn2	6 $apn2^Gapn2^G$, 10 $apn2^Gapn2^C$	8 $apn2^Gapn2^G$, 8 $apn2^Gapn2^C$	0.32	4 $apn2^Gapn2^G$, 12 $apn2^Gapn2^C$	16 $apn2^Gapn2^G$, 0 $apn2^Gapn2^C$	0.0001
apn3	6 $apn3^Gapn3^G$, 10 $apn3^Gapn3^C$	8 $apn3^Gapn3^G$, 8 $apn3^Gapn3^C$	0.32	4 $apn3^Gapn3^G$, 12 $apn3^Gapn3^C$	16 $apn3^Gapn3^G$, 0 $apn3^Gapn3^C$	0.0001
apn4	6 $apn4^Gapn4^G$, 10 $apn4^Gapn4^C$	8 $apn4^Gapn4^G$, 8 $apn4^Gapn4^C$	0.32	4 $apn4^Gapn4^G$, 12 $apn4^Gapn4^C$	16 $apn4^Gapn4^G$, 0 $apn4^Gapn4^C$	0.0001
apn5	6 $apn5^Gapn5^G$, 10 $apn5^Gapn5^C$	8 $apn5^Gapn5^G$, 8 $apn5^Gapn5^C$	0.32	4 $apn5^Gapn5^G$, 12 $apn5^Gapn5^C$	16 $apn5^Gapn5^G$, 0 $apn5^Gapn5^C$	0.0001
apn6	6 $apn6^Gapn6^G$, 10 $apn6^Gapn6^C$	8 $apn6^Gapn6^G$, 8 $apn6^Gapn6^C$	0.32	4 $apn6^Gapn6^G$, 12 $apn6^Gapn6^C$	16 $apn6^Gapn6^G$, 0 $apn6^Gapn6^C$	0.0001

Note: alleles with the superscript C are from the susceptible strain and alleles with the superscript G are from the Cry2Ab-resistant strain of *T. ni*. Statistical significance was tested by *chi*-square test.

genotype $apn^{G}apn^{G}$ and 8 larvae had the genotype $apn^{G}apn^{C}$, statistically the same as the predicted ratio of 1:1 (Table 4.2). For the Cry2Ab-selected larvae from the backcross family, 4 larvae were homozygotes with the genotype $apn^{G}apn^{G}$ whereas 12 larvae were heterzygotes with the genotype $apn^{G}apn^{C}$, which deviated from the predicted ratio of 1:1 between the two genotypes for random assortment of the two alleles of each APN gene (Table 4.2). However, the majority of the toxin surviving individuals (75%) were heterozygous genotype $apn^{G}apn^{C}$, the resistance to Cry2Ab was clearly independent of the *apn* alleles from the resistant strain (Table 4.2). Therefore, the APN genes were not genetically linked with the Cry2Ab resistance.

Discussion

The midgut brush border membrane proteins cadherin, APNs, and mALP are identified as receptors for Cry1A toxins (Pigott and Ellar, 2007). Cry2A toxins differ from Cry1A toxins in their amino acid sequences and the specificity of their high-affinity binding sites (English et al., 1994; Morse et al., 2001; Luo et al., 2007; Hernandez-Rodriguez et al., 2008). Specific receptors for Cry2A toxins have yet been identified in insects. Despite the general absence of shared binding sites between Cry1 and Cry2 toxins in midgut epithelia, there was still a possibility that resistance to Cry2A toxins could be associated with alterations in these major known midgut receptors for Cry1A toxins, as Cry2A toxins and Cry1A toxins may still bind to the same proteins but different binding sites (Ferre and Van Rie, 2002; Heckel et al., 2007; Pardo-Lopez et al., 2013). More recently, reduced levels of mALP were detected in the brush border

membrane vesicle proteins of the resistant strains of three lepidopteran species that had displayed diverse resistant phenotypes, including resistance to Cry2Aa in two strains of *H. virescens* (CXC and KCBhyb), so mALP could be potentially involved in resistance to Cry2A toxins (Jurat-Fuentes et al., 2011). The results from this study determined that the resistance to Cry2Ab in *T. ni* is not genetically linked with the tested eight midgut receptor genes. The lack of genetic linkage between the receptor genes and Cry2Ab resistance, however, does not exclude the possibility of *trans*-regulatory mechanisms conferring the resistance by altering expression of the receptor genes at transcript and/or protein levels. A *trans*-regulatory resistance mechanism has been identified in a resistant strain (Glen-Cry1Ac-BCS) of *T. ni*, in which the resistance is not genetically linked with the APN1 gene, but is associated with the down-regulation of the gene at the mRNA level (Tiewsiri and Wang, 2011). Therefore, further downstream analysis (i.e., quantitative mRNA and protein analysis, protein function analysis by toxin binding assay, and protein localization analysis) is required to determine whether those toxin receptors have possible involvement in Cry2Ab resistance in *T. ni*.

Mutations of the cadherin gene have been identified in cases of "Mode 1" type resistance in strains of *H. virescens*, *P. gossypiella*, and *H. armigera* (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005). However, the correlation between cadherin gene mutation and altered toxin binding requires further understanding. A Cry1Ac-selected strain of *P. gossypiella* (AZP-R) was associated with mutations of the cadherin gene. However, the cadherin gene mutations resulted in loss of the binding of Cry1Ab, but did not affect the binding of Cry1Ac to the midgut BBMV (Gonzalez-Cabrera et al., 2003). In another study, the absence of cadherin gene by retrotransposon-mediated mutation in

the Cry1A-resistant strain (YHD2-B) of *H. virescens* caused reduced binding of Cry1Aa to the midgut BBMV, but binding of Cry1Ab and Cry1Ac remained unchanged (Jurat-Fuentes et al., 2004). Regarding Cry2A resistance, reduced binding of Cry2Ab in the resistant strains of *H. armigera* and *H. punctigera* was observed, but it remains unclear whether the cadherin in the two *Helicoverpa* species was involved in Cry2Ab binding and whether the reduced binding was genetically linked to the resistance (Caccia et al., 2010). In this study, the results showed that the cadherin gene is not genetically linked to the Cry2Ab resistance in *T. ni*.

Several lines of evidence have suggested that Cry1 resistance might be associated with GPI-anchored APNs by gene mutations or transcriptional alteration of the APN genes (Herrero et al., 2005; Zhang et al., 2009; Yang et al., 2010; Khajuria et al., 2011; Kiewsiri and Wang, 2011). However, among those studies, no genetic linkage analysis was performed to confirm the correlation between Cry1 resistance and the mutant APN genes or differential transcript levels of APNs, except the most recent study in which genetic linkage was established between the reduced expression of the APN1 gene and Cry1Ac resistance in *T. ni* (Tiewsiri and Wang, 2011). Baxter et al. (2008) used a linkage mapping approach to test whether mutations in eight APN genes were genetically linked to Cry1Ac resistance in *Plutella xylostella*, and found that none of the APN genes was on the same chromosome as the resistance locus (Baxter et al., 2008). My results indicated that neither mutations of the APN1-6 genes nor altered larval midgut APN activity (Chapter 3) is associated with Cry2Ab resistance in *T. ni*.

The putative role of mALP as a Cry toxin receptor in resistance has been suggested from analysis of Cry1Ac resistance in *H. virescens*, *H. armigera* and

Helicoverpa zea, as well as Cry1Fa resistance in *Spodoptera. frugiperda* (Jurat-Fuentes and Adang, 2004; Jurat-Fuentes et al., 2011; Caccia et al., 2012). However, more research is required to ascertain the association between altered ALP levels and Cry1 resistance, as well as the specific role of ALP in conferring the resistance. In this study, Cry2Ab resistance was not genetically associated with the mALP gene. Furthermore, the resistance was not correlated with midgut ALP, as demonstrated by the similar ALP activities and protein abundance in the Cry2Ab-resistant and -susceptible strains of *T. ni* (Chapter 3).

Most recently, "forward genetics" approaches designed to map and clone novel Bt resistance genes in lepidopterans have led to the discovery that mutations of the ABCC2 gene is linked to "Mode 1" type resistance in *T. ni*, as well as in *H. virescens*, *P. xylostella*, and *Bombyx mori* (Gahan et al., 2010; Baxter et al., 2011; Atsumi et al., 2012). By contrast, the results in this study indicated that Cry2Ab resistance in *T. ni* is not genetically linked with the ABCC2 gene, as Cry2Ab resistance is genetically independent of the Cry1Ac resistance (Chapter 2) whereas the latter is genetically linked to the ABCC2 gene (Baxter et al., 2011).

CHAPTER 5

COMPARATIVE PROTEOMIC ANALYSIS OF BRUSH BORDER MEMBRANE VESICLE PROTEINS FROM CRY2AB-SUSCEPTIBLE AND -RESISTANT LARVAE OF THE CABBAGE LOOPER, *TRICHOPLUSIA NI*

Introduction

The high specificity of the insecticidal activity of Bt Cry toxins against insect species is mainly attributed to the specific interaction of the toxins with the receptors on the insect midgut epithelium (Piggot and Ellar, 2007; Pardo-Lopez et al., 2013). The Bt receptors have been studied using brush border membrane vesicles (BBMVs) prepared from the midgut epithelium. The best characterized are the cadherin-like receptors and the aminopeptidase N (APN) receptors (Piggot and Ellar, 2007). Alteration in the binding of Bt Cry toxins to midgut BBMVs is one major resistance mechanism for high level Bt resistance in insects (Ferre and Van Rie, 2002; Caccia et al., 2010; Jurat-Fuentes et al., 2011).

Comparative proteomic analysis of midgut BBMV proteins from Bt-resistant and -susceptible insect strains is a powerful approach to identify protein changes associated with Bt resistance (Candas et al., 2003; Jurat-Fuentes et al., 2011; Tiewsiri and Wang, 2011). Candas et al. (2003) used a proteomic approach involving gel-based two-dimensional electrophoresis (2DE) coupled with peptide mass fingerprinting to study the midgut BBMV proteins from a Bt-resistant strain of *Plodia interpunctella*, and found

changes of certain specific midgut proteins, indicating increased glutathione utilization, elevated oxidative metabolism, and differential maintenance of energy balance within the midgut epithelial cells, in the resistant strain. In another study, comparative analysis of midgut BBMV proteins from Cry-resistant strains of *Heliothis virescens*, *Helicoverpa armigera* and *Spodoptera frugiperda*, using a similar 2DE-based proteomic approach, detected reduced levels of membrane-bound alkaline phosphatase (mALP) in the resistant larvae (Jurat-Fuentes et al., 2011). Furthermore, quantitative proteomic analysis of midgut BBMV proteins from Cry1Ac-susceptible and -resistant *T. ni* larvae by isobaric tagging for relative and absolute quantitation (iTRAQ) identified that APN1 was significantly down-regulated whereas APN6 was significantly up-regulated in the resistant larvae compared to the susceptible larvae (Tiewsiri and Wang, 2011).

The mechanism of high level (>1,492-fold, Chapter 2) of greenhouse-derived Cry2Ab resistance in *T. ni* remains to be identified. In this study, the larval midgut BBMV proteins from the Cry2Ab-resistant and -susceptible *T. ni* strains were quantitatively analyzed using a liquid-based 2D-LC-MS/MS proteomic approach involving the isobaric Tandem Mass Tags (TMT) labeling technique, to identify the differentially expressed proteins between the two strains. Two proteins showed significantly altered abundance in the midgut BBMVs from the Cry2Ab-resistant larvae, but quantitative real-time RT-PCR (qRT-PCR) analysis of their corresponding genes showed that the transcript levels of the two genes did not differ between the two strains.

Materials and methods

Insects. The susceptible strain used herein was a highly inbred laboratory strain of *T. ni* (Cornell-SS) that had been maintained without exposure to Bt toxins for over 30 years (Kain et al., 2004). A Cry2Ab-resistant strain, referred to as Glen-Cry2Ab-BCS8, was established by introgressing the Cry2Ab resistance trait from a Bt-resistant population (Glen-BGII) to the Cornell-SS strain (Chapter 3). Both strains were maintained on a wheat germ-based artificial diet under the conditions as described by Kain et al. (2004).

Preparation of midgut BBMV proteins. Mid-fifth-instar larvae were dissected in ice-cold MET buffer (250 mM mannitol, 17mM Tris-HCl, 5mM EGTA, pH 7.5) to extract the midgut epithelium free of attached tissues and the peritrophic membrane with food content. The midgut tissue was used to prepare BBMVs, using the differential-magnesium-precipitation method developed by Wolfersberger et al. (1987). The midgut BBMV preparations were quantified by their protein contents using the Bradford method with BSA as standards (Bradford, 1976). The consistency of the BBMV preparations was examined by 10% SDS-PAGE, followed by Coomassie blue staining. The enrichment of brush border membranes in the BBMV preparations was examined by determination of the enrichment of the aminopeptidase activity in BBMV preparations compared with the enzyme activity in their initial midgut homogenates. Aminopeptidase activity was measured using leucine-ρ-nitroanilide (Sigma, St. Louis, MO) as the substrate as described in Chapter 3. The enrichment of the aminopeptidase activity was typically from five- to eight-fold.

Quantitative proteomic analysis of midgut BBMV proteins. Isobaric TMT tagging was used for relative quantitation of the BBMV proteins from the Cry2Ab-resistant and -susceptible larvae. Midgut BBMV proteins were solubilized in 6M urea. The solubilized proteins were subjected to tryptic digestion, and the tryptic peptides were labled using TMT reagents (Thermo Scientific, Waltham, MA) according to manufacturer's protocol performed by the Cornell Proteomics Facility, followed by 2D Nano LC-MS/MS analysis as described by Tiewsiri and Wang (2011). For each of the Cornell-SS and the Glen-Cry2Ab-BCS8 strains, three BBMV samples were independently prepared from three different generations of *T. ni* larvae, and used as three biological replicates to ensure adequate variation analysis for the comparative quantitative proteomic analysis. The three samples from the Cornell-SS strain were labeled with reporter ion tags 126, 127, and 128, respectively, and those from the Glen-Cry2Ab-BCS8 strain were labeled with reporter ion tags 129, 130, and 131, respectively. The MS/MS spectra collected from the analysis were processed with the software Proteome Discover 1.1 (Thermo Scientific), followed by database search using a *T. ni* cDNA sequence database including *T. ni* cDNA sequences downloaded from Genbank, and *T. ni* midgut transcriptome sequences (generated by Illumina RNA sequencing) and ESTs generated in house. For protein identification and quantitative analysis using the MS/MS data, the peptide mass tolerance value was set at 5 ppm, and the fragment mass tolerance value was set at 100 mmu. The significance threshold was chosen at 0.05, and only those peptides passing this filter were used for protein identification. Furthermore, identified proteins with at least two unique peptides were used for further relative quantification analysis. Intensities of the reporter ions from the TMT tags were used for

protein quantitation. For each identified protein, the 126 reporter ion intensity was used as the common denominator to calculate the relative quantity, given as a ratio (e.g., 127/126, 130/126), for each sample.

The quantitative variation of each identified protein was evaluated by examination of the 95% confidence interval (CI) of the $\log_2$ ratios of each protein calculated from the three biological replicates. Based on the biological variations observed in the two sets of samples (the susceptible and resistant strains), the cutoff value to determine the significance of quantitative difference between the proteins from the two strains was set such that 95% of the identified proteins showed their 95% CIs below this cutoff value. Proteins were identified as being significantly differentially expressed in the resistant strain when their relative quantity ($\log_2$ ratios) of the three biological replicates (i.e., $\log_2(129/126)$, $\log_2(130/126)$ and $\log_2(131/126)$) were all higher than the significance cutoff value as described above or all below the negative cutoff value.

Quantitative real-time RT-PCR analysis. The transcripts of two genes, *noxred* and *apn6*, coding for an NADP-dependent oxidoreductase and APN6, respectively, were analyzed by quantitative real-time PCR (qRT-PCR). Primers 5'-ACAGGTTGGTGCGACTACAGC-3' and 5'-ACCGACTCCGTAAGATGCTGAT-3' were used for *noxred*, and primers 5'-CATTCCATACGGAGAAAGTCTACC-3' AND 5'-GAAGTGAGTGGTGGCGTAAATC-3' were used for *apn6* analysis.

Midguts were dissected from mid-fifth instar larvae of *T. ni* in ice-cold PBS and individually stored in RNAlater solution (Life Technologies, Carlsbed, CA) at -20°C until used. Total RNA was extracted from the individual midgut using the RNeasy Mini Kit (Qiagen) coupled with an on-column digestion procedure with DNase to ensure

complete removal of residual genomic DNA contamination, according to the manufacturer's instructions. The isolated RNA was then used for cDNA synthesis with the ImProm-II reverse-transcription system (Promega, Madison WI) following the instructions provided by the manufacturer. The cDNA was mixed in iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) for real-time PCR analysis following the manufacturer's instruction. qRT-PCR reactions were performed with two technical replicates for each of the five individual larvae from each strain on a CFX96 Real-Time System C1000 Touch Thermal Cycler (Bio-Rad). Amplification was performed by an initial hot start at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. Relative gene expression was calculated using the ΔΔCT method as described in Livak and Schmigggen (2001). *T. ni* β-actin gene (Genbank accession no. JF303662) was used as an endogenous control with primers 5'-GTTGCTGCGTTGGTAGTAGACA-3' and 5'-TCCCAGTTGGTGACGATGC-3'.

Results

Differentially expressed proteins in the BBMVs from the Cry2Ab-resistant larvae were identified by TMT-based quantitative proteomic analysis. The BBMV preparations of the Cry2Ab-susceptible and -resistant larvae had highly similar protein profiles by SDS-PAGE analysis (Figure 5.1). Analysis of the BBMV proteins after trypsin digestion by 2D-nano-LC MS/MS identified a total of 1,462 proteins. The relative abundance of the identified proteins was estimated using the exponentially modified

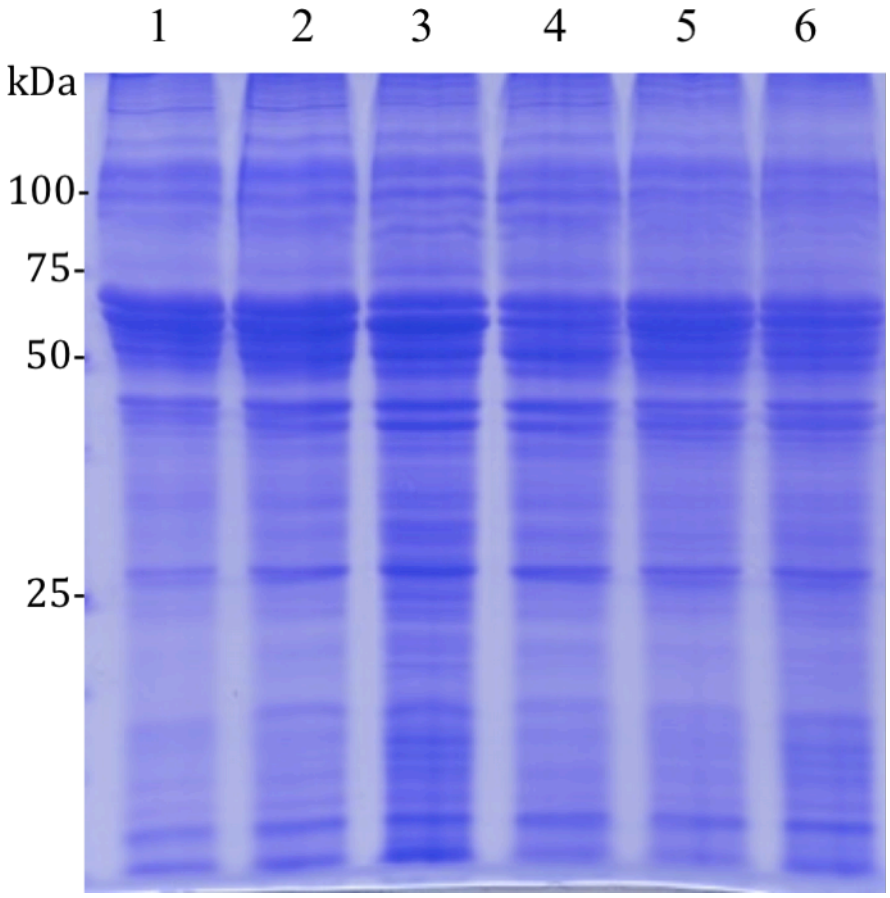

Figure 5.1 SDS-PAGE analysis of midgut BBMV proteins from Cry2Ab-resistant and -susceptible larvae. Lanes: 1-3, three biological replicates of the Cornell-SS strain; 4-6, three biological replicates of the Glen-Cry2Ab-BCS8 strain.

protein abundance index (emPAI) (Protein abundance (mol %) = emPAI of each protein/$\sum$(emPAI) × 100, Ishihama et al., 2005). The relative abundance of the identified proteins was in the range from 0.001% to 1.000%, except four proteins that showed an abundance value greater than 1.000% (Figure 5.2). Among the total proteins identified, 951 containing at least two unique peptides with the confidence level of at least 95% were used for further quantitative comparative analysis between the susceptible and the resistant strains.

With TMT tagging, the midgut BBMV proteins from the six samples were quantified in relative quantities from the MS/MS analysis. The biological variations of the two sets of samples (BBMVs prepared from the susceptible strain versus the resistant strain) were analyzed by 95% CIs of the $\log_2$ ratios of the three biological replicates of each strain. The significance cutoff point to cover the variations of 95% of the proteins was determined to be 2.44 (i.e., 95% of the BBMV proteins quantified from the TMT analysis ad their 95% CIs below 2.44, Figure 5.3). In other words, the midgut BBMV proteins from the resistant strain that had the $\log_2$ ratio being greater than 2.44 (or smaller than -2.44) in all the three replicates were considered to be significantly up-regulated (>2.44) or down-regulated (<-2.44). Data analysis showed that in the resistant strain, only one protein, an NADP-dependent oxidoreductase, showed its relative quantity of its three replicates ($\log_2$(129/126), $\log_2$(130/126) and $\log_2$(131/126)) greater than 2.44 (Table 5.1). Only one protein, APN6, from the resistant strain showed its relative quantity of its three replicates ($\log_2$(129/126), $\log_2$(130/126) and $\log_2$(131/126)) all smaller than -2.44 (Table 5.1). The average $\log_2$ ratios for the NADP-dependent oxidoreductase and APN6 in the resistant sample set were 4.61 and -3.21, respectively (Table 5.1). Therefore, in the

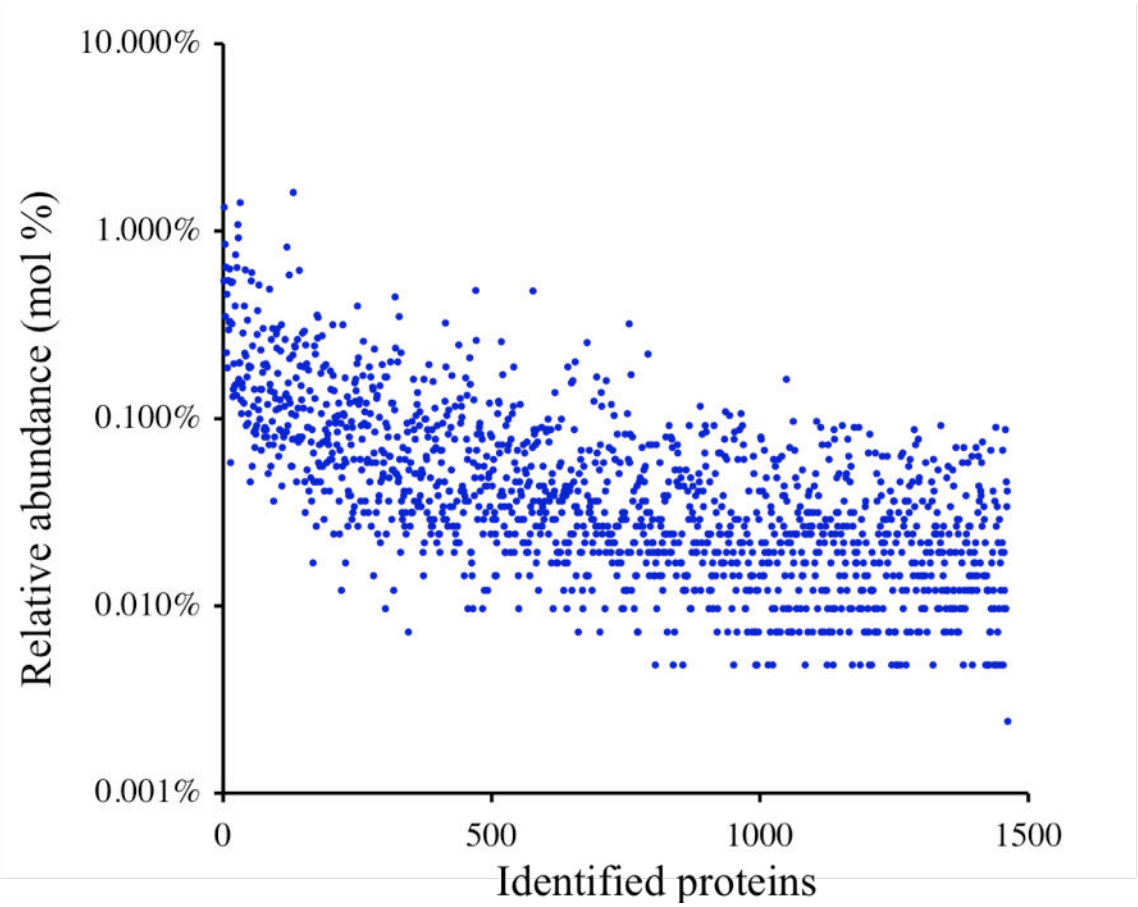

Figure 5.2. Relative abundance of the proteins identified using quantitative proteomic analysis of *T. ni* midgut BBMVs.

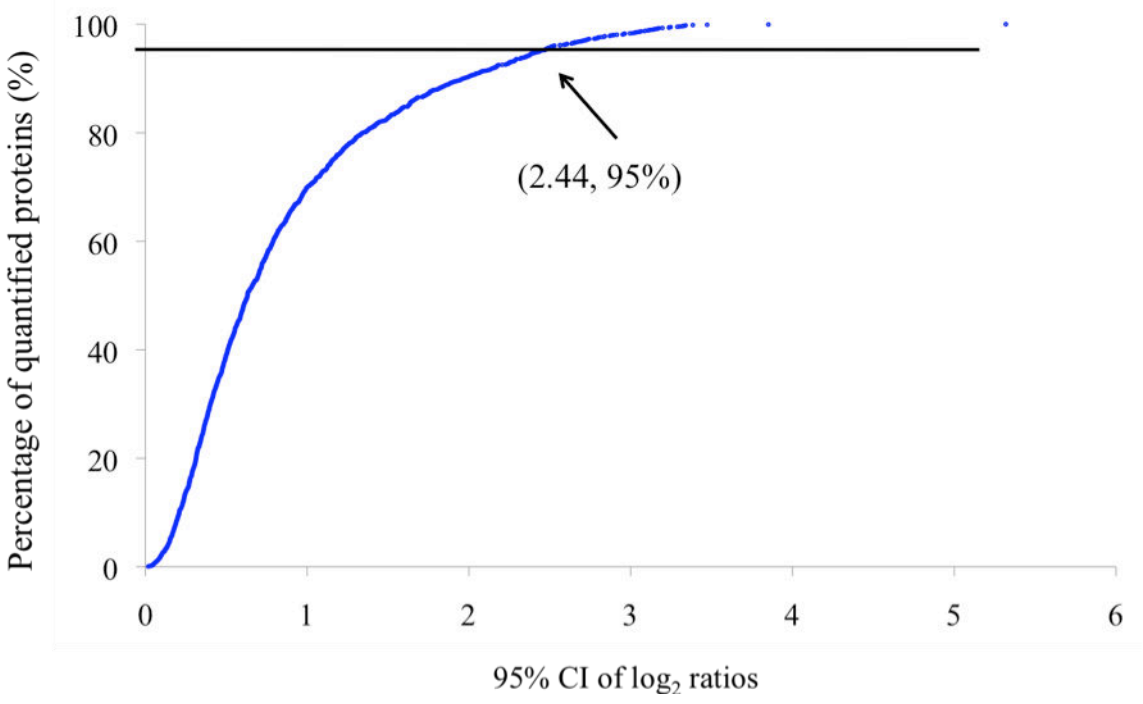

Figure 5.3. 95% confidence intervals (CIs) of the $\log_2$ ratios calculated for the quantified proteins from three biological replicates of the Glen-Cry2Ab-BCS8 and the Cornell-SS strains of *T. ni*. 95% of the proteins have their 95% CIs below 2.44.

Table 5.1. Relative quantities (log_2 ratios) of the two midgut BBMV proteins that showed significant difference in the Cry2Ab-resistant strain when compared to the Cry2Ab-susceptible strain of *T. ni*.

	$log_2$129/126	$log_2$130/126	$log_2$131/126	Mean
NADP-dependent oxidoreductase	4.87	4.73	4.24	4.61
APN6	-3.32	-3.49	-2.85	-3.21

resistant strain, the NADP-dependent oxidoreductase was determined to be 24.4-fold higher and APN6 was 10-fold lower than that in the susceptible strain.

The transcript levels of the genes coding for NADP-dependent oxidoreductase and APN6 were statistically the same in the Cry2Ab-susceptible and -resistant larvae. The qRT-PCR analysis showed that NADP-dependent oxidoreductase was not differentially expressed in the midgut of Cry2Ab-resistant larvae at the transcriptional level (Figure 5.4). In addition, the transcript level of *apn6* did not statistically differ between the resistant and susceptible strains (Figure 5.4).

Discussion

The results from this study showed that the midgut BBMV proteins from the Cornell-SS strain and its introgression Glen-Cry2Ab-BCS8 line were highly similar in relative abundance. The profiles of the BBMV proteins from the two strains by SDS-PAGE analysis were almost identical (Figure 5.1). The high level similarity in midgut BBMV protein composition between the two strains was further confirmed by the comparative quantitative proteomic analysis. Only two proteins, accounting for less than 0.15% of all the identified proteins, were significantly different between the two strains. The quantitative proteomic approach using TMT tags herein identify 1,462 proteins in the larval midgut BBMVs of *T. ni*, comparable to the numbers of *T. ni* BBMV proteins identified by the same approach using iTRAQ tags (Tiewsiri and Wang, 2011).

In this study, only two proteins of the 1,462 identified BBMV proteins, NADP-dependent oxidoreductase and APN6, were found to be significantly different in quantity

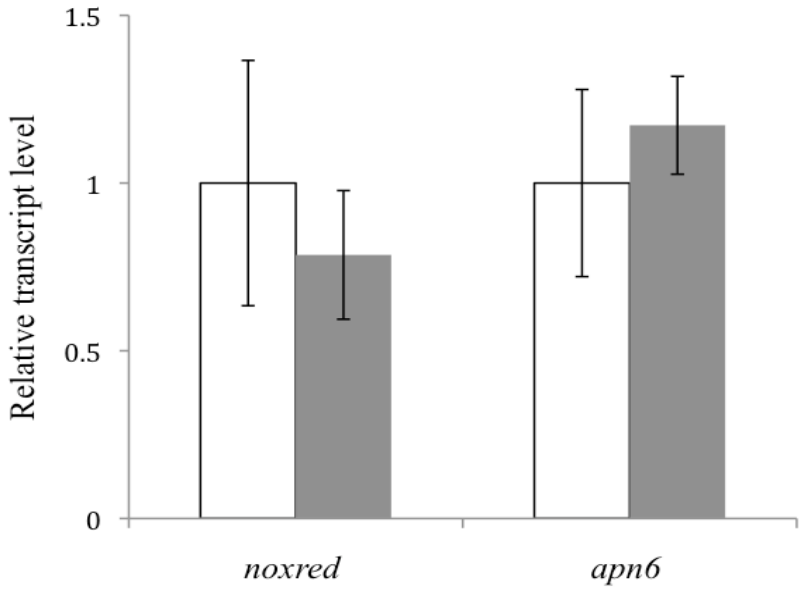

Figure 5.4. Relative transcript levels of the genes coding for NADP-dependent oxidoreductase and APN6 in the Cry2Ab-susceptible (open bars) and -resistant (grey bars) larvae of *T. ni* by qRT-PCR analysis. Error bars indicate SEMs from the analysis of five individuals.

in the resistant strain in comparison to the susceptible strain. The NADP-dependent oxidoreductase exhibited a pronounced increase (24.4-fold) in the Cry2Ab-resistant strain. The NADP-dependent oxidoreductase is a member of the enzyme family that use NADP as coenzyme to catalyze oxidoreduction reactions and plays an important role in oxidative metabolism. Thus, the overexpression of this oxidative metabolism related enzyme might indicate that the resistant larvae had elevated oxidative metabolism. A similar hypothesis was proposed by Candas et al. (2003) that the development of Bt resistance in *P. interpunctella* mostly likely involved enhanced oxidative metabolism and cellular stress responses, as reflected by the up-regulation of several relevant enzymes (i.e., GSH transferase, cytochrome c oxidase, NADH dehydrogenase, and phosphopyruvate hydratase) in the resistant larvae (Candas et al., 2003). Thus, an enhanced state of oxidative metabolism due to increased expression of certain relevant enzymes might be an adaptive response of insects that undergo certain physiological events to counteract with Bt and its insecticidal Cry toxins.

To gain more insight into the role of the NADP-dependent oxidoreductase in the Cry2Ab-resistant *T. ni* larvae, qRT-PCR was performed to compare the transcript levels of the corresponding gene in the larval midguts of the two strains. However, no significant difference in mRNA abundance of *noxred* was found between the resistant and susceptible larvae. The lack of correlation between protein abundance and transcript level requires confirmation and further investigation. The association of this specific protein alteration with Cry2Ab resistance in *T. ni* is not yet understood.

The role of APNs as Cry toxin receptors is documented (Pigott and Ellar, 2007) and alteration of APNs by gene mutations or differential expression has been reported in

several Cry1-resistant populations of lepidopterans, including *Spodoptera exigua*, *Helicoverpa armigera*, *Diatraea saccharalis*, *Ostrinia nubilalis*, and *T. ni* (Herrero et al., 2005; Zhang et al., 2009; Yang et al., 2010; Khajuria et al., 2011; Kiewsiri and Wang, 2011). Previous studies suggest that APN6 does not bind to Cry1Ac (Angelucci et al., 2008; Tiewsiri and Wang, 2011). However, it remains unknown whether APN6 is a Cry2Ab-binding protein. In the present study, the protein abundance of APN6 was significantly reduced in the resistant strain by 10-fold, when compared to the susceptible strain. However, qRT-PCR analysis revealed that the transcript level of *apn6* did not significantly differ between the resistant and susceptible strains of *T. ni*, indicating that alteration in protein abundance was not directly associated with differential transcription of the gene. In addition, the APN6 gene is not genetically linked to the Cry2Ab resistance in *T. ni* (Chapter 4). Therefore, it remains to be determined whether APN6 is functionally responsible for Cry2Ab resistance in *T. ni*.

In conclusion, the comparative quantitative proteomic analysis using TMT labeling coupled with LC-MS/MS in this study led to the identification of two proteins that had significant differential abundance in the BBMVs of the Cry2Ab-resistant larvae. Analysis of these putative resistance-associated proteins by qRT-PCR revealed that the resistance was not associated with these two genes at the transcriptional level. Further functional analysis of these proteins is required to understand their potential roles in Cry2Ab resistance, in order to gain knowledge for development of resistance management tactics to utilize Cry2 toxins.

CHAPTER 6

CONCLUSIONS

Concurrent expression of multiple Bt toxins with different binding sites in insects in Bt-crops, also refereed to as gene pyramiding, is one of the most effective biotechnological strategies to delay the development of insect resistance to Bt-crops. Cry2A toxins and Cry1A toxins are two major groups of toxins used in pyramided Bt-crops, as they do not share the same binding sites in insects. Currently, the number of cases of insect resistance to Cry2A toxins expressed in pyramided Bt-crops is very limited, so is the knowledge on how insects develop resistance to Cry2A toxins. The Cry2Ab resistance in a Bt-resistant strain (Glen-BGII) of *T. ni* provided the opportunity to study Cry2A resistance in insects. The Glen-BGII strain of *T. ni* was isolated by selections of a greenhouse-derived Dipel-resistant strain with Bollgard II cotton foliage expressing Cry1Ac and Cry2Ab, hence resistant to both Cry1Ac and Cry2Ab.

In order to understand the mechanism of Cry2Ab resistance in *T. ni*, the mode of inheritance of Cry2Ab resistance in the Glen-BGII strain was characterized. In addition, the genetic association between Cry1Ac resistance and Cry2Ab resistance was examined to determine whether the dual resistance to both Cry1Ac and Cry2Ab in the Glen-BGII strain was conferred by two independent mechanisms. My results, presented in Chapter 2, showed that the Glen-BGII strain of *T. ni* was highly resistant to Cry2Ab with a resistance ratio of >1,492-fold compared to a laboratory susceptible strain (Cornell-SS). The inheritance of Cry2Ab resistance in *T. ni* was autosomal, incompletely recessive, and monogenic. The Cry2Ab resistance was genetically independent from Cry1Ac resistance,

indicating that there were two independent mechanisms involved in the dual resistance to Cry1Ac and Cry2Ab, respectively, in the Glen-BGII strain. Furthermore, the Cry2Ab resistance trait was introgressed to the susceptible Cornell-SS strain in order to obtain an introgression strain of *T. ni* (Glen-Cry2Ab-BCS8) nearly isogenic to the Cornell-SS strain, for further comparative biochemical analysis of Cry2Ab-susceptible and -resistant *T. ni* on the same genetic background.

The second experimental objective of this dissertation aimed to identify biochemical alteration(s) associated with Cry2Ab resistance in *T. ni* by comparative biochemical analysis of *T. ni* larvae from the Cry2Ab-susceptible and -resistant strains on the same genetic background. My results, presented in Chapter 3, showed that the Cry2Ab-resistant and -susceptible strains were not significantly different in midgut proteases activities, specific proteolytic digestion of the toxin Cry2Ab, midgut aminopeptidase and ALP activities, midgut esterase activities, as well as melanization activities of hemolymph plasma. This indicates that Cry2Ab resistance in *T. ni* may not be associated with those biochemical characteristics examined. Further biochemical analysis focusing on toxin binding may be required to uncover the biochemical basis of Cry2Ab resistance.

The genetic linkage analysis of Cry2Ab resistance with genes encoding known Cry toxin receptors (i.e., cadherin, mALP and APN1-6), presented in Chapter 4, showed that these genes are not genetically linked to Cry2Ab resistance in *T. ni*. However, the Cry toxin receptors themselves may still play a role in conferring the resistance. Cry2Ab resistance could possibly be mediated by a regulatory factor altering the expression of these toxin receptors, or by post-transcriptional modifications of the receptors. Thus,

further quantification of expression levels of these receptor genes may provide insight into the mechanism of Cry2Ab resistance in *T. ni*.

As presented in Chapter 5, comparative quantitative proteomic analysis of midgut BBMVs from the Glen-Cry2Ab-BCS8 and the Cornell-SS strains by iTRAQ using the TMT labeling technique led to the identification of two proteins, an NADP-dependent oxidoreductase and APN6, that exhibited significantly altered protein abundance in the resistant strain. The NADP-dependent oxidoreducatase was up-regulated by 24.4-fold whereas APN6 was down-regulated by 10-fold in the BBMVs from the resistant larvae. However, quantitative RT-PCR analysis showed that the transcriptional levels of these two proteins in the resistant strain were not significantly different from those in the susceptible strain. Further studies are required to confirm the change of abundance of the putative NADP-dependent oxidoreductase and APN6 in Cry2Ab-resistant *T. ni* and to functionally examine these two putative proteins in the intoxication pathway of Cry2Ab to understand their association with Cry2Ab resistance in *T. ni*.

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