Effects of inducible tolerance to *Bacillus thuringiensis* on the egg transcriptomes and egg parasitism in *Helicoverpa armigera*

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Abstract

In the Australian cotton industry, toxins produced by the soil bacterium *Bacillus thuringiensis* (*Bt* toxins) are utilised to control two lepidopteran pests, *Helicoverpa armigera* (cotton bollworm) and *H. punctigera* (native budworm). *Bt* toxins kill insects by forming pores in the insect midgut, which leads to sepsis. The extensive use of *Bt* toxins, including in the form of transgenic crops, has put strong selection pressure on the pest insects in the field, which can lead to resistance. Understanding the resistance mechanism is essential for planning the resistance management strategy to prolong the effectiveness of the *Bt* toxins.

Previous studies have demonstrated that larvae of cotton bollworm can develop a low-level tolerance to *Bt* toxins after being exposed to a sub-lethal dose. This induced tolerance is associated with increased immune activity in the midgut and haemolymph. In addition, the induced tolerance and the increase in the immune activity can be transferred to the next generation via a maternal effect, and the level of tolerance can increase over generations of exposure. Interestingly, the characteristics of inducible tolerance are also found in a Cry1Ac-resistant strain of *H. armigera* known as the Bx strain (CSIRO, Narrabri, NSW). Even though many studies have reported immune responses against *Bt* toxins, the role of the immune system in facilitating inducible tolerance against *Bt* toxins and its transmission mechanism are still unclear. The primary aim of this study was to investigate the transmission mechanism of inducible *Bt* tolerance.

The effect of the maternal experience on the offspring's immune system (trans-generational immune priming; TGIP) has been demonstrated in several studies. Although there is speculation about the mechanisms of TGIP, such as the insertion of immune substances into eggs and changes in the DNA methylation state of the offspring's genome, the genes and metabolic pathways involved in the transmission mechanisms are still undefined. Given that immune components could be maternally transmitted via eggs,

together with the importance of egg parasitoids to integrated cotton pest management, it is important practically to also understand whether there is any negative effect of *Bt* tolerance/exposure on *H. armigera* eggs with regard to parasitisation.

There are two research questions in this study: 1) what genes are involved in the transmission mechanism of inducible *Bt* tolerance? and 2) what are effects of inducible tolerance on eggs and parasitism?

To address the first question, I investigated the gene expression profiles of eggs. First, two transcriptomic assemblies for eggs of *H. armigera* were generated by combined deep sequencing results from five strains of *H. armigera*: two Cry1Ac-susceptible, Cry1Ac-tolerant (low level *Bt* toxin selection), Cry1Ac-resistant (Bx strain, high level selection, highly resistant), and Cry2Ab-resistant strains. Then, the assemblies were used to compare gene expression profiles of eggs from susceptible and induced tolerant *H. armigera*. Four genes were identified, and confirmed by quantitative RT-PCR, to differentially express between eggs from tolerant and susceptible individuals. These genes are histone cluster 3 H2BB, translationally controlled tumor protein, receptor for activated C kinase, and glyceroldehyde-3-phosphate dehydrogenase. Currently, the roles of these genes in inducible *Bt* tolerance are still unclear. The changes in the expression of these genes could be a part of the mechanism of *Bt* tolerance, or a response to *Bt* exposure. Further investigations on the functions of these genes in inducible *Bt* tolerance are needed.

Since the Cry1Ac-resistant (Bx) strain also has the characteristics of inducible *Bt* tolerance, it is possible that the mechanism of inducible *Bt* tolerance in the Bx strain is the same as the tolerant strain. Interestingly, the four genes mentioned above that were expressed differently between susceptible and Cry1Ac-tolerant eggs (Waite strain) were not expressed differently between eggs of Cry1Ac-susceptible and Cry1Ac-resistant strains. On the other hand, four genes were expressed differentially between eggs of Cry1Ac-susceptible and Cry1Ac-resistant strains. They were pyruvate kinase, olfactory receptor 29,

transmembrane proteins 9, and proteasome 25 kDa. The functions of these genes in eggs and Cry1Ac-resistance are as yet uncharacterized, and need to be further investigated.

The differences in the sets of differentially expressed genes in eggs of Cry1Ac-tolerant and Cry1Ac-resistant strains suggested that the mechanisms of maternally transmitted tolerance/resistance might be different. It is possible that different mechanisms might be necessary to survive the different concentrations of *Bt* toxins that were encountered during the selection process. This might also indicate that there is more than one pathway that leads to the similar immune responses activated in response to *Bt* exposure.

I further investigated whether there was any effect of inducible tolerance on eggs of *H. armigera* and its suitability as a host for egg parasitism by *Trichogramma pretiosum*. Three key measurements were assessed: parasitism success, the number of wasps emerged per host egg, and the proportion of male and female offspring emerged per host egg. The results showed that there was no difference in parasitism success between susceptible and tolerant eggs. However, there was a significant increase in the number of emergent parasitoids, especially male offspring, in eggs laid by tolerant *H. armigera*. Further investigation of the size of host eggs indicated those from Cry1Ac tolerant *H. armigera* were larger than eggs from the susceptible population. The result also showed that the increase in the egg size was correlated with *Bt* exposure. These results confirm that maternally-transmitted *Bt* tolerance affects on the phenotype of the eggs from tolerant *H. armigera*, which consequently affects egg parasitoids. Interestingly, the differences in egg size is correlated with the differences in the egg gene expression profiles, although the link between these two differences remains unclear. However, the differences in egg size and the gene expression profiles did not appear to negatively affect parasitism rates of *T. pretiosum*. In fact, there were more wasps emerged from the larger eggs of tolerant insects compared to eggs of susceptible insects. In conclusion, no negative effect of inducible *Bt*

tolerance on the use of egg parasitoids in cotton pest management systems in terms of the number of wasp progeny produced has been detected.

In conclusion, I identified the genes that were differentially expressed between eggs of susceptible and inducible *Bt* tolerant *H. armigera*. However, the roles of these genes in the transmission mechanism of inducible *Bt* tolerance and in the insect immune system are still unclear, and need further investigation. In addition, inducible *Bt* tolerance or *Bt* exposure has an effect on the egg volume, but this does not have an adverse effect on egg parasitism. Further works should include functional studies on the expression of the genes identified in this study in the larval midgut, and their roles in the transmission mechanisms of inducible *Bt* tolerance.

Thesis Declaration

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Jutamat (Kay) Anantanawat

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Abbreviation list

ALP	Alkaline phosphatase
AMP	Anti-microbial peptide
APN	Aminopeptidase N
Apo III	Apolipophorin III
bp	base pair
Bt	*Bacillus thuringiensis*
C_Contig	Contigs generated from CLC assembler
CAD	Cadherin-like protein
Cry toxins	Crystal toxins
DEG	Differentially expressed gene
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM	Genetically modified
GO	Gene ontology
GST1	Gluthatione S-transferase I
H2BB	Histone cluster 3 H2B
hr	hour
kDa	kilo Daltons
LC	Lethal concentration
LPS	lipopolysaccharide
MAPK	Mitogen-activated protein kinase
Mb	Megabase pair
min	Minute
min.	Minimum
mM	millimolar
N_Contig	Contigs gneerated from Newbler assembler
NCBI	National centre for biotechnology information
ng	nanogram
nr	non-redundant
nt length	nucleotide length
OLC	Overlap-layout consensus
PAE	Phenoloxidase activating enzyme
PCR	Polymerase chain reaction
PiGV	*Plodia interpunctella* Granulosis Virus
PO	Phenoloxidase
PPO	Prophenoloxidase
PRP	Pattern recognition protein
qPCR	Qantitative Polymerase Chain Reaction (Quantitative PCR)
RACK	Receptors for activated C kinase
RNAi	RNA interference
RPKM	Reads Per Kilobase of exon model per Million Mapped read
RPS15	Ribosomal protein subunit 15
RR	Resistance ratio
RT	Reverse transcription
RT-PCR	Reverse transcript polymerase reaction

s	second
SEM	Standard error of the mean
sptz	spätzle
TCTP	Translationally controlled tumor protein
TGIP	Transgenerational immune priming
ul	microlitre
uM	micromolar

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Chapter 1

Introduction
& Literature Review

1.1 Introduction

Toxins from a soil bacterium, *Bacillus thuringiensis* (*Bt*), are widely used as pesticides throughout the world. Since its first commercial use in 1970, many studies have reported the development of resistance against *Bt* toxins. Recently, a unique resistant mechanism, known as inducible *Bt* tolerance, has been reported in three lepidopteran species: *Ephestia kuehniella* (flour moth), *Helicoverpa armigera* (cotton bollworm: Noctuidae) and *Plutella xylostella* (diamondback moth). The mechanism of the tolerance is unclear. However, it is correlated with an increase in immune activity, and the mode of inheritance shows a strong maternal effect. Understanding the mechanism of tolerance is crucial for designing pest management strategies which delay the onset of resistance for as long as possible, and hopefully prevent resistance development entirely.

This study aims to understand the mechanism of inducible tolerance, in particular, those elements arising through a maternal effect. The main question is: what genes are involved in the vertical transmission of inducible tolerance in the model species *Helicoverpa armigera*? Because there is a strong maternal effect, it is speculated that genes or proteins that are involved in inducible tolerance could be transferred to the next generation via the eggs, which are the focus. This study also investigates whether there is any change in egg phenotype, particularly the size of eggs, due to the effect of inducible tolerance, and whether the changes have an impact on egg parasitism.

In this chapter I broadly review the biology and use of *Bt* toxins and inducible tolerance to them. Details of the insect immune response against *B. thuringiensis* and its toxins will also be covered. Then, the possible effects of the immune related tolerance on parasitism will be discussed. Finally, the rationale and aims of the study are stated.

1.2 Literature review

1.2.1 Toxins of *Bacillus thuringiensis* (*Bt* toxins)

1.2.1.1 *Bt* toxin: General information

Bt toxins are produced by a Gram-positive soil bacterium, *Bacillus thuringiensis*. The protoxins, which are crystalline (Cry) proteins, are produced as a by-product during sporulation. *Bt* toxins are toxic to certain invertebrates including some insects and nematodes, but harmless to vertebrates (Griffitts & Aroian 2005). The toxins cause pore formation in cells of the insect midgut, and eventually the midgut contents leak into the haemocoel, causing sepsis and death. *Bt* toxins were first discovered in 1901 by a Japanese microbiologist (Ishiwata 1901), and fully characterised in 1915 (Berliner 1915). In general, Cry toxins can be divided into eight groups according to the affected species (Table 1.1). There are approximately four subtypes of *Bt* toxins, amounting to about 70 different Cry toxins (George & Crickmore 2012).

The efficiency of Cry toxins and their environmentally friendly features led to the development of *Bt* transgenic crops. In the 1980s, a gene that encoded a crystal toxin was inserted into tobacco to produce the first genetically modified (GM) plant that can produce *Bt* toxin (Vaeck et al. 1987). The success of experimental GM *Bt*-tobacco crops led to the commercial availability of other GM *Bt*-expressing crops such as cotton and maize. Almost 100% of commercial cotton grown in Australia are GM *Bt* crops (Cotton Australia n.d.), mainly targeting larvae of *Helicoverpa armigera* (cotton bollworm) and *H. punctigera* (native budworm), the major pests which damage flowers, leaves and most importantly, cotton bolls.

Table 1.1 Groups of invertebrates affected by different types of Cry toxins (Hofte & Whiteley 1989; Crickmore et al. 2012).

Invertebrate groups	Cry toxin
Lepidoptera	Cry1, Cry9, Cry15
Diptera	Cry4, Cry10, Cry11, Cry16, Cry17, Cry19, Cry20
Coleoptera	Cry3, Cry7, Cry8
Nematoda	Cry6
Hymenoptera	Cry22
Lepidoptera & Diptera	Cry2
Lepidoptera & Coleoptera	Cry1I
Nematoda & Hymenoptera	Cry5, Cry12, Cry13, Cry21

Most Cry toxins share three specific functional and structural domains named Domains I, II and III (Figure 1.1)(Li, Carroll & Ellar 1991). Domain I consists of seven alpha-helices arranged in such a way that one helix is surrounded by the other six (Li, Carroll & Ellar 1991). Domain II contains three anti-parallel β-strands, and Domain III contains two anti-parallel β-strands. Domains II and III are responsible for receptor binding whereas Domain I is believed to be inserted into the cell membrane during pore formation. Cry toxins are produced as protoxins, 130 kDa in molecular weight. The protoxin is activated by being proteolytically cleaved into a 65 kDa active form in the alkaline midgut of a susceptible insect before binding to any receptors. There are also other *Bt* toxins, known as Cyt toxins, that can enter cells without binding to any receptors (Pardo-López, Soberón & Bravo 2013). Unlike Cry toxins, the molecular structures of Cyt toxins only consist of two domains.

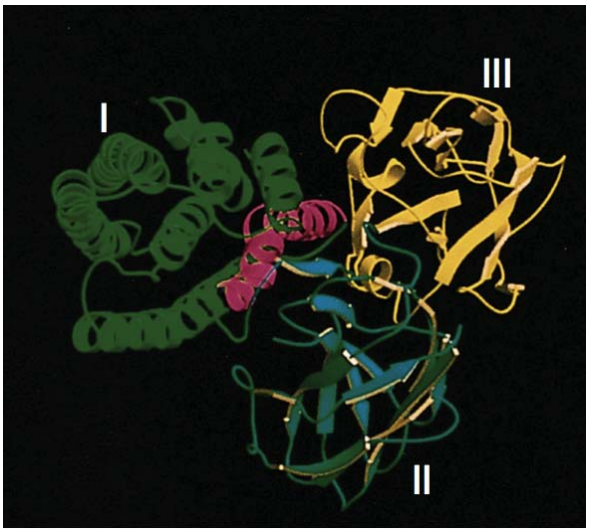

Figure 1.1 Structure of Cry1Aa toxin. Most Cry toxins contain three domains: Domain I (green), Domain II (cyan) and Domain III (yellow). Domain II and Domain III are responsible for receptor binding, whereas Domain I is inserted into the cell membrane during pore-formation, causing osmotic disruption of midgut cells. From Grochulski et al. (1995).

1.2.1.2 *Bt* toxins: Modes of action

Despite the discovery of the *Bt*-toxin structure and their intensive use worldwide, the exact molecular mode of action of *Bt* toxins is still unclear. Compared to the other Cry toxins used in agriculture, the mechanism of the Cry1A group is the most well studied (Pardo-López, Soberón & Bravo 2013). There are two steps involved in the lethal action of *Bt* toxins: 1) the pore formation in the midgut epithelial cells, and 2) the septicemia caused by the leaking of midgut material and enterobacteria into the haemocoel.

The process of pore-formation begins with the solubilisation of a crystal inclusion body releasing the 130 kDa Cry protoxin. First, the protoxin interacts with a midgut receptor such as aminopeptidase N (APN) or alkaline phosphatase (ALP) via Domain II and III (Figure 1.2) (Pardo-López, Soberón & Bravo 2013). The protoxin has a very low affinity with these receptors, but due to the abundance of APN and ALP, these toxin-receptor interactions lead to a high affinity binding between the protoxin and a cadherin-like protein (CAD). The binding of CAD then initiates the activation of the protoxin by allowing proteases to cleave the protoxin from the N-terminal region up to the helix-1 of Domain I, producing a 65 kDa active toxin. The active toxins then form an oligomeric structure, which binds to APN and ALP receptors with high affinity. This binding leads to the insertion of Domain I into the plasma membrane of midgut cells, and subsequent pore formation. Multiple pores in the gut cell membranes result in the non-selective entry of ions into the cells, leading to cell lysis.

Mode of action of Cry toxin

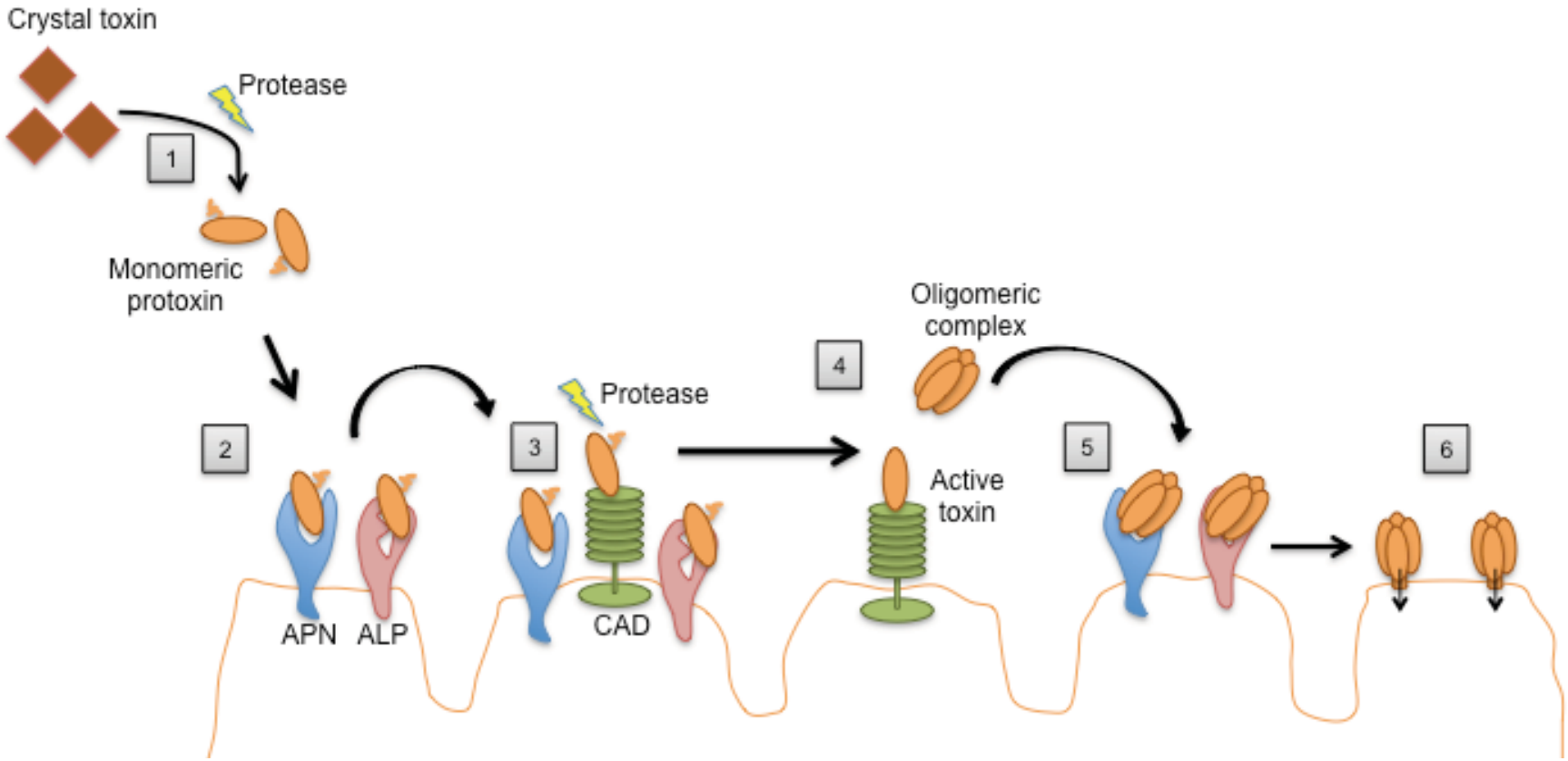

Figure 1.2 Mode of action of Cry toxins from *Bacillus thuringiensis*. (1) Ingested crystal inclusion bodies are solubilised by protease in the alkaline condition of the insect midgut, releasing 130 kDa monomeric protoxins. (2) The protoxins bind to midgut receptors such as aminonpeptidase N (APN) and alkaline phosphatase (ALP) in a low affinity manner. (3) The binding of protoxins to APN and ALP leads to the interaction of the bound protoxin to cadherin-like-proteins (CAD), which leads to (4), the proteolytic activation of the protoxins, producing a 65 kDa active toxin. (5) Active toxins form an oligomeric structure, which can be recognised by APN and AL and are bound with high affinity. (6) The binding of the oligomeric complex to APN and ALP allows the toxins to form a pore in the epithelial cell membrane. Adapted from Pardo-López, Soberón and Bravo (2013) (See animated version http://www.youtube.com/watch?v=1Ks11VoYYao).

Another mechanism of *Bt*-toxin pore formation has been proposed. Zhang et al. (2006) suggested that, instead of the physical pore-formation on the surface of the epithelial cell, the binding of the protoxin to CAD can trigger a G-protein mediated signal transduction pathway, which results in an oncotic cell death. With this mechanism, there is no need for proteolytic activation of the protoxins and the oligomerisation of the active toxins. However, other studies have shown that mutant Cry1Aa and Cry1Ab toxins, which cannot form a normal oligomeric structure but can still bind to CAD, fail to kill insects (Vachon et al. 2004; Girard et al. 2009). This indicates that binding of the toxin to CAD is not sufficient to form a pore in the epithelial cell membranes. Thus, the mechanism proposed by Zhang et al. (2006) has not has not been well supported.

The second part of the *Bt*-toxin action involves septicemia. A study using antibiotics has shown that an infection from the leaking of the bacteria into the haemocoel is a crucial part of *Bt*-toxin activity (Broderick, Raffa & Handelsman 2006). If a larva is fed with an antibiotic to kill midgut bacteria prior to being fed with *B. thuringiensis*, the percentage mortality decreases dramatically. However, if a larva is fed enterobacteria together with *B. thuringiensis* after being fed with an antibiotic, the percentage mortality of larva was recovered to normal. This indicates that *B. thuringiensis* needs enterobacteria to kill insects efficiently. Broderick et al. (2006) demonstrated that the Cry toxins produced by *B. thuringiensis*, not the live enterobacteria, are what is needed to kill the susceptible insect . Heat-killed transgenic *Escherichia coli* that expresses *Bt* toxins can also kill insects, again suggesting that it is not live *E. coli*, but the crystal toxins being produced, that cause mortality. This is also the basis of transgenic *Bt*-cottons. Crystal toxins generated by transgenic plants are sufficient to kill insects without the need for live *B. thuringiensis*.

However, a study by Raymond et al. (2009) showed *B. thuringiensis* itself can kill insects without the need for enterobacteria. They used a similar experimental procedure as Broderick. et al (2006) that involved antibiotic treatment and followed by feeding *B.*

thuringiensis. However, they showed that an antibiotic-resistant strain of *B. thuringiensis* could kill insects without the presence of enterobacteria. This suggested that *B. thuringiensis* can also cause septicemia. In the study by Broderick et al. (2006), the reason that *B. thuringiensis* did not kill larvae after they were fed antibiotics might be because the antibiotics killed *B. thuringiensis* as well.

In nature, it is likely that the mortality caused following consumption of *Bt* toxin involves both enterobacteria and *B. thuringiensis*. It is crucial to understand what causes septicemia in the haemolymph of infected larvae because the differences in the bacteria might activate different types of immune responses, as will be discussed later.

Interestingly, Broderick et al. (2010) proposed that immune responses generated by bacterial infection from *Bt* exposure might play a role in killing the insect. Their study showed that larvae can be killed after being administered peptidoglycan, a component of the Gram-negative bacterial cell wall, which was used in this case as an immune elicitor, together with *Bt* toxins and antibiotics. Finally, the study has also demonstrated that the percentage of mortality decreased when immune-suppressors such as antioxidants and eicosanoid inhibitors were administered together with *Bt* toxins and peptidoglycan.

1.2.1.3 *Bt* toxins: Resistance

This study focuses on inducible *Bt* tolerance, which is a state in which the insect can withstand increased concentrations of *Bt* toxins due to prior low dose exposure. However, these tolerant individuals do not possess mutations in the *Bt*-toxin receptors or enzymes that are commonly associated with *Bt* resistance.

There are many studies reporting the development of resistance to *Bt*-toxins in a range of insect species (Pardo-López, Soberón & Bravo 2013) (Table 1.2). At least 6 lepidopteran species have developed resistance against *Bt* toxins, including *Helicoverpa* spp. (Tabashnik & Carrière 2010). Three lepidopterans have displayed resistance against *Bt*

crops (Ali, Luttrell & Young 2006; Ali & Luttrell 2007; van Rensburg 2007; Tabashnik et al. 2009; Storer et al. 2010). The most common mechanisms for resistance are the disruption of the toxin action by changes in the *Bt*-toxin receptors, and changes in the enzymes that activate protoxins (Table 1.2). However, other mechanisms that reduce the toxicity of *Bt* toxins have also been reported, such as shedding of the APN receptors (Hernandez-Martinez et al. 2010), increased immune activity against *Bt* toxins (inducible *Bt* tolerance) (Rahman et al. 2004; Ma et al. 2005), and sequestration through binding of *Bt* toxins to non-*Bt* receptors such as esterases (Gunning et al. 2005) and glycolipids (Griffitts et al. 2005).

Mutations in the *Bt* receptors CAD, APN and ALP are very common, and are known as "Mode I" resistance. Any insect that develops this type of resistance usually has a very high resistance ratio (more than 200) (Tabashnik, Van Rensburg & Carrière 2009). CAD mutations are responsible for the high resistance to Cry1Ac toxins found in 3 lepidopteran species: *Pectinophora gossypiella* (Morin et al. 2003), *Heliothis virescens* (Gahan, Gould & Heckel 2001; Jurat-Fuentes et al. 2004) and *H. armigera* (Xu, Yu & Wu 2005). Two types of mutation reported in CADs are the insertion of a retrotransposon (Gahan, Gould & Heckel 2001; Jurat-Fuentes et al. 2004) and a deletion (Morin et al. 2003). The combination of both types has also been found in Cry1Ac-resistant *P. gossypiella* (Fabrick et al. 2011).

Similar mutations of the APN1 and ALP genes are responsible for resistance to *Bt* toxins. A deletion mutation in APN1 was found in a Cry1C-resistant strain of *Spodoptera exigua* (Herrero et al. 2005) and Cry1Ac-resistant *H. armigera* (Zhang et al. 2009). In addition, Hernandez-Martinez et al. (2010) reported the increase in shredded APN1 receptor in the midgut of a Xentari™ resistant strain of *S. exigua*. Xentari™ contains Cry1Aa, Cry1Ab, Cry1C, Cry1D, and Cry2Ab toxins. It is thought that an increase of free APN1 in the midgut could bind and sequester Cry1Ca toxin, preventing it from binding to APN located

on the surface of epithelial cell membranes. Mutations in the ALP gene are another mechanisms that have been reported to confer resistance to Cry1Ac in *H. virescens* and *H. armigera,* and to Cry1Fa in *S. frugiperda* (Jurat-Fuentes et al. 2011).

Recently, a mutation in ABC transporter has been found in Cry1Ac resistant strains of 3 lepidopteran species: *H. virescens*, *P. xylostella* and *Trichoplusia ni* (Gahan et al. 2010; Baxter et al. 2011). It is still unclear if the ABC transporter plays a role in *Bt*-toxin binding, or whether it is actually a *Bt*-toxin receptor. Interestingly, studies have shown that Cry1Ac-resistant *H. virescens* containing an ABC transporter mutation were more resistant than those with a CAD receptor mutation (Gahan et al. 2010; Baxter et al. 2011).

Table 1.2 Summary of *Bt* toxin resistant strains.

Species	Type of *Bt* toxins	Resistance mechanism	Reference
Aminopeptidase N and P			
Ostrinia nubilalis	Cry1Ab	Aminopeptidase P	Khajuria et al., 2011
Helicoverpa armigera; Spodoptera exigua	Cry1Ac; Cry1C	Mutation in APN1	Zhang et al., 2009; Herrero et al., 2005
*Trichoplusia ni**	Cry1Ac	Post translational modification of APN1	Tiewsiri et al., 2011, Janmaat et al., 2004
S. exigua	*Bt* formular Xentari$^{TM\ 1}$	Shredding of APN	Hernandez-Martinez et al., 2010
Alkaline phosphatase			
Heliothis virescens	Cry1Ac	Decrease in production of ALP	Jurat-Fuentes et al., 2002
H. armigera; H. virescense; Spodoptera frugiperda	Cry1Ac	Mutation in midgut membrane-bound ALP	Jurat-Fuentes et al., 2011
Cadherins			
Pectinophora gossypiella	Cry1Ac	Deletion mutation and present of retrotransporson in CAD	Fabrick et al., 2011
H. armigera; P. gossypiella	Cry1Ac	Deletion mutation in CAD	Xu et al., 2005; Morin et al., 2003
H. virescense	Cry1Ac	Retrotransposon in CAD	Jurat-Fuentes et al., 2004; Gahan et al., 2001
ABCC Transporter			
H. virescense; Plutella xylostella	Cry1Ac; Cry1A	Mutation in ABCC2	Gahan et al., 2010; Tabashnik et al., 1997

*P. xylostella; T. ni**	Cry1Ab, Cry1Ac	Mutation in ABCC2	Ferre et al 1991; Tabashnik et al., 1994; Mason et al., 1995 Escriche et al., 1995; Baxter et al., 2011; Wang et al., 2007
Enzymes involved in the protoxin activation			
P. interpunctella; *O. nubilalis*	Cry1Ac; Cry1Ab; *Bt* formular Dipel [2]	Decrease in protease activity	Oppert et al., 1997; Johnson et al., 1998; Li et al., 2004
Spodoptera littoralis	Cry1C	Overdigestion of the protoxin	Keller et al 1996
Others			
Ephestia kuehniella; H. armigera	*Bt* formula Syngenta[3]; Cry1Ac	Immune activity	Rahman et al., 2004; Ma et al., 2005
Caenorhabditis elegans	Cry5	Changes in postmodification of glycolipid receptors	Griffitt et al., 2005
H. armigera	Cry1Ac	Sequestering of toxins binding by the overproduction of esterases	Gunning et al., 2005
H. armigera	Cry1Aa, Cry2Ab	Sequestering of toxins binding by the overproduction of glycolipid	Ma et al., 2012

* *Trichoplusia ni* in these studies are the same population.

[1]Xentari™ contains Cry1Aa, Cry1Ab, Cry1C, Cry1D, and Cry2Ab.

[2]Dipel contains Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa

[3]Syngenta contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, bacterial proteins and spores.

APN = Aminopeptidase N; ALP = Alkaline phosphatase; CAD = Cadherin; ABCC2 = ABC transporter ABCC2

Differential post-translational modification of receptors can also lead to *Bt* resistance. A study of the model nematode, *Caenorhabditis elegans*, demonstrated that *Bt* resistance could develop due to mutations in the gene encoding glycosyltransferase, an enzyme that attaches galactose monomers to a glycolipid receptor required for *Bt*-toxin binding (Griffitts et al. 2005). The mutation in this enzyme resulted in a population of nematodes that was more than 500 fold resistant to Cry5B. This discovery suggested that there is a receptor that can bind to *Bt* toxins only after post-translational modification (Griffitts & Aroian 2005). Even though glycoplipids were not included as among the receptors for Cry toxins mentioned earlier, inhibition using a glycolipid-binding molecule has resulted in significant tolerance to *Bt* toxin. Ma et al. (2012) reported that the tolerance of *H. armigera* to Cry1Ac toxin can be increased using a glycolipid-binding protein, LEC-8, from *C. elegans*. This study has shown that LEC-8 could bind to Cry1Ac toxin, possibly sequestering the toxin and thereby preventing it from binding to a glycolipid receptor on gut epithelial cells.

Mutations in the enzymes essential for activating the protoxins have also been reported. A field population of *Plodia interpunctella* was selected under laboratory conditions over several generations of exposure, and was found to develop Cry1Ac resistance (Oppert et al. 1996). The resistance was caused by a reduction of serine protease activity in the midgut, resulting in under-digestion (and therefore reduced activation) of the protoxin. This was indicated by the size of the proteolytically cleaved toxins being larger than expected after exposure to the insect midgut. A resistant population of *H. virescens* also showed the same type of resistance due to an abnormality in protease profile in the midgut (Forcada et al. 1996).

2.1.3.1 Bt toxins: Inducible Bt tolerance

Low level *Bt* tolerance can develop independently of mutation-based mechanisms. A study of *E. kuehniella* by Rahman et al. (2004) found that an initially susceptible laboratory

population expressed increasing levels of *Bt* tolerance by continual inter-generational exposure to low-mortality concentrations of *Bt* toxins. Neonate larvae of *E. kuehniella* that were fed with a sub-lethal concentration of *Bt* toxins (LC_{10} of susceptible population) developed tolerance to higher concentrations of *Bt* toxin fed again in the later larval stages. In this case, *Bt*-tolerance development was not related to the rare *Bt*-resistant alleles reported in many other studies, as the tolerance developed within the exposure generation. In addition, it was not a high dose selection (Rahman et al. 2004). This inducible tolerance has also been shown in Cry1Ac-tolerant *H. armigera* (Rahman et al. 2011) and *P. xylostella* (Mahbub Rahman, Personal communication).

The inducible *Bt* tolerance was correlated with an increase in melanisation rate in haemolymph plasma and midgut extracts from toxin-exposed larvae (Rahman et al. 2004). The correlation with increased melanisation led to speculation that the immune system might be involved in inducible tolerance. However, many other aspects of immune response, such as the hydrolytic action of lysozymes, the antibiotic effects of antimicrobial peptides, and the activity and number of haemocytes, have not been assessed.

Intergenerational increases in inducible tolerance to *Bt* toxins are passed on from the induced generation to the offspring, and are reflected in increases in the melanisation activity of hemolymph (Rahman et al. 2004). In addition, the level of tolerance increases over generations in response to increasing doses of toxins exposure. Reciprocal crosses indicated that the offspring from tolerant mothers were more highly tolerant (as indicated by increasing LC values over time) compared to the offspring from susceptible mothers; however, offspring from tolerant father also showed some degree increased tolerance (Table 1.3). This result implied that the tolerant trait was partly transmitted from mothers to offspring, a phenomenon known as a maternal effect.

Table 1.3. Resistance ratios (RR) of larval populations exhibiting inducible Cry1Ac *Bt* tolerance in *H. armigera*.

Insect strains	LC_{50} (mg/ml)	RR	LC_{95} (mg/ml)	RR	LC_{99} (mg/ml)	RR
SxS	**0.035**	**1**	**0.0099**	**1**	**0.227**	**1**
SxT	**0.055**	**1.57**	**0.464**	**4.69**	**2.644**	**11.65**
TxS	**0.324**	**9.26**	**2.286**	**23.1**	**11.32**	**48.48**
TxT	**1.043**	**29.8**	**4.334**	**43.78**	**13.842**	**60.98**

The table shows the LC_{50}, LC_{90} and LC_{99} values (mg/ml *Bt* toxin formulation applied to food) for susceptible larval populations derived from tolerant parents (SxS), reciprocal crosses (SxT, TxS) and tolerant parents (TxT). The resistance ratio (RR) represents the ratio of the LC values for Cry1Ac-tolerant populations compared to those of the susceptible population. Note that the reciprocal cross involving a tolerant mother (TxS) produced larvae exhibiting a higher RR than those derived from a tolerant father (Rahman et al. 2011).

Interestingly, the increase in immune-activity in the haemolymph and in the midgut has also been found in a high dose selected strain of Cry1Ac-resistant *H. armigera* (Ma et al. 2005). This Cry1Ac-resistant strain, Bx, from CSIRO, Narrabri, NSW has a resistance ratio of 5,386, and can survive on GM *B*t cotton plants (Akhurst et al. 2003; Bird & Akhurst 2007). Genetic crosses showed that this resistance involves multiple genes of unknown identity and is autosomal semi-dominant (Akhurst et al. 2003). Research in our laboratory in 2005 demonstrated that, similar to induced tolerance, the midgut and the haemolymph of the Bx strain also showed an increase in phenoloxidase activity (Figure 1.3 and 4) (Ma et al. 2005). Some resistant larvae also contained melanin, a dark pigment, which is a product of melanisation, in the midgut (Figure 1.4). Even though research indicates that resistance in the Bx strain is autosomal and semi-dominant, the resistance ratio of offspring of resistant females crossed with susceptible males, was higher than that of the offspring from the reciprocal cross, indicating that a part of the resistance is also inherited via a maternal effect. The high level resistance of the Bx strain could therefore be derived through multiple genes and an associated increase in immune activity. It is also possible that the resistance mechanisms in the low-dose Cry1Ac tolerant and high-dose Cry1Ac resistant strains are similarly based in part on the shared features of an upregulated immune status and a maternally-biased vertical transmission.

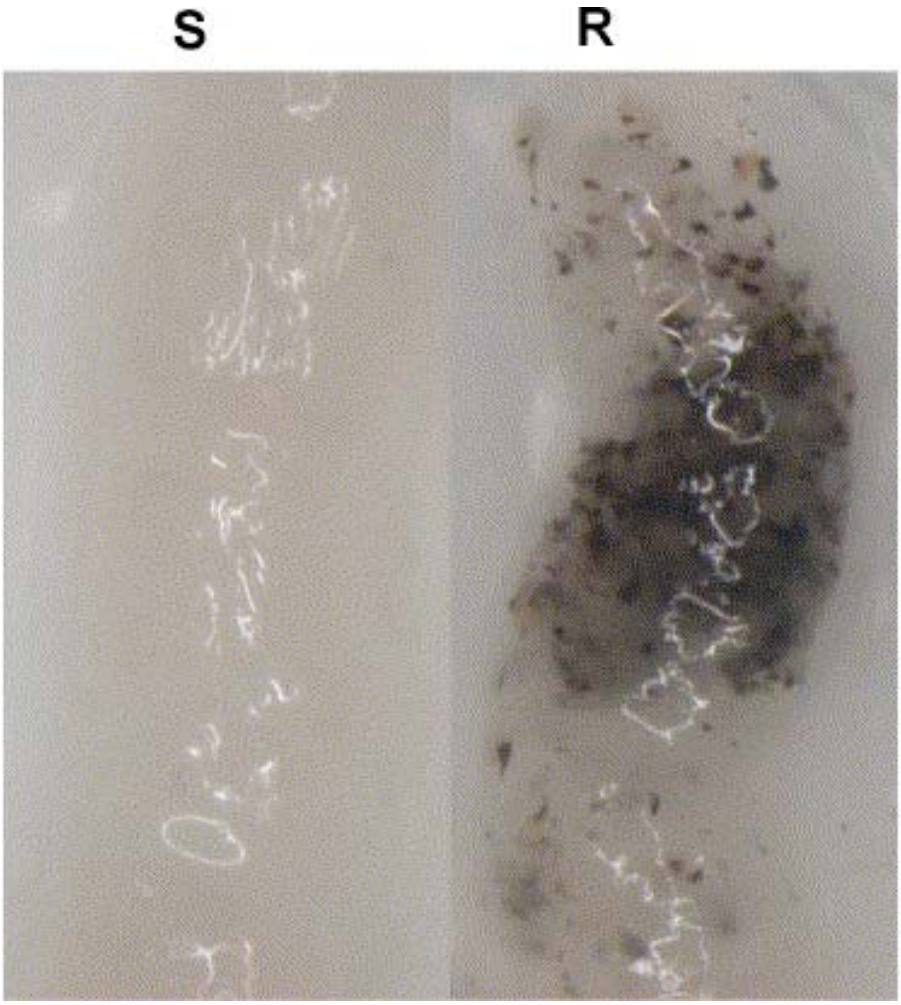

Figure 1.3 Increased melanisation is visible in the midgut of a high-dose selected Cry1Ac-resistant larva of *Helicoverpa armigera.* Increased melanisation is visible in the midgut of a representative Cry1Ac-resistant strain of *H. armigera* (Bx strain) larva (R) compared to the midgut of a susceptible (S) larva after feeding with Cry1Ac toxin (Ma et al. 2005). This indicates the immune-related melanisation cascade is more active

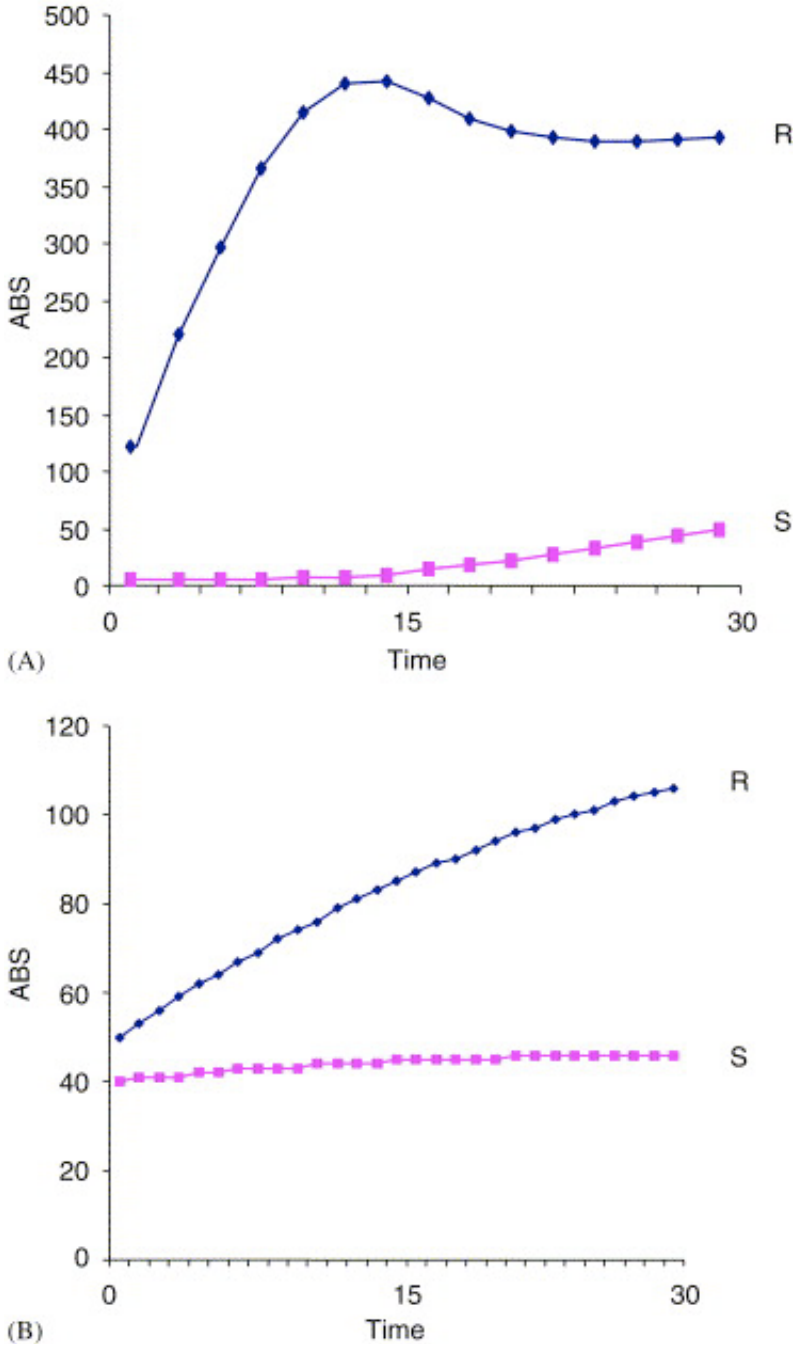

Figure 1.4 Melanisation reactions over 30 minutes as measured by optical absorbance at 490nm in the high-dose selected Cry1Ac-resistant strain of *H. armigera* (Bx strain). PPO activity in haemolymph (A) extracted from resistant individuals (blue line; R) is higher than in susceptible individuals (pink line; S). Similarly, PPO activity measured in midgut extracts (B) extracted from resistant individuals (blue line; R) is higher than in susceptible individuals (pink line; S). From Ma et al. (2005).

1.2.2 Insect immune response against *Bt* toxins

Insects display several immune responses against *Bt* toxins. The insect immune system can be activated by the bacterial cell wall component of *B. thuringiensis*, the process of pore-formation of the bacterial toxin, and enterobacteria that enter the haemocoel through the leaking midgut. There are two interlinked components of insect immunity to bacteria: cellular and cell-free immunity. Although there is no clear division between them, cellular immunity generally involves direct cellular interaction with invading entities such as phagocytosis and encapsulation by haemocytes, whereas a cell-free immune response involves cell-free aggregates, anti-microbial peptide production and other oxidative reactions in the haemolymph (Figure 1.5).

1.2.2.1 Cellular immune system

The insect cellular immune system is comprised of four groups of cells: midgut epithelial cells, cuticular epithelial cells, haemocytes and fat body cells (Schmidt et al. 2010). The main functions of cellular immunity are the recognition of pathogens, phagocytosis or encapsulation, and the secretion of antimicrobial peptides. Epithelial cells in the midgut and cuticle play a major role in the cellular immunity as they are naturally the first group of cells to encounter epithelial disruption, microorganisms and other foreign objects. These cells detect bacterial components via pattern recognition proteins (PRPs), which can bind to a specific molecular components on the exterior of a microorganism, such as peptidoglycan and lipopolysaccharide. When epithelial cells encounter microbes or sense cell ruptures, they release molecular signals into the haemocoel to alert other immune cells, such as haemocytes, fat body cells and cell-free immune factors, that gather at the tissue break where they act in defense against bacteria and wound healing (Schmidt et al. 2010). Haemocytes, in addition to their role in nutrient transport, function mainly in phagocytosis and encapsulation (Schmidt et al. 2010). Haemocytes engulf foreign objects if they are small in what is known as phagocytosis, or entrap an object by encapsulation, if it is too

large (Figure 1.5). Haemocytes can secrete anti-microbial peptides and other immune proteins as well, although not in large amounts. The role of fat body in immunity is mainly in generating and secreting anti-microbial peptides and other immune proteins into the haemolymph (Schmidt et al. 2010).

The cellular immune response against *Bt* toxins starts at the midgut epithelial cells. The disruption of epithelial cells by pore-formation activates the stress-response pathways JNK and p38 MAPK (Huffman et al. 2004; Cancino-Rodezno et al. 2010). These pathways are both a part of the mitogen-activated protein kinases (MAPK) family. This family of protein-serine/threonine kinases is involved in intracellular regulation in response to extracellular stimuli or physical stresses such as radiation, osmotic shock and ischemic injury. The activation of the MAPK pathway leads to several cellular responses including embryogenesis, cell differentiation, cell proliferation, immune responses and cell death. In terms of immunity, the p38 MAPK pathway is activated in response to inflammatory cytokines, whereas the JNK pathway is activated through several members of the immune-related Toll-like pathway. The activation of these two pathways leads to the production of anti-microbial peptides (Troemel et al. 2006), the activation of haemocytes and the production of other immune components such as prophenoloxidase (PPO).

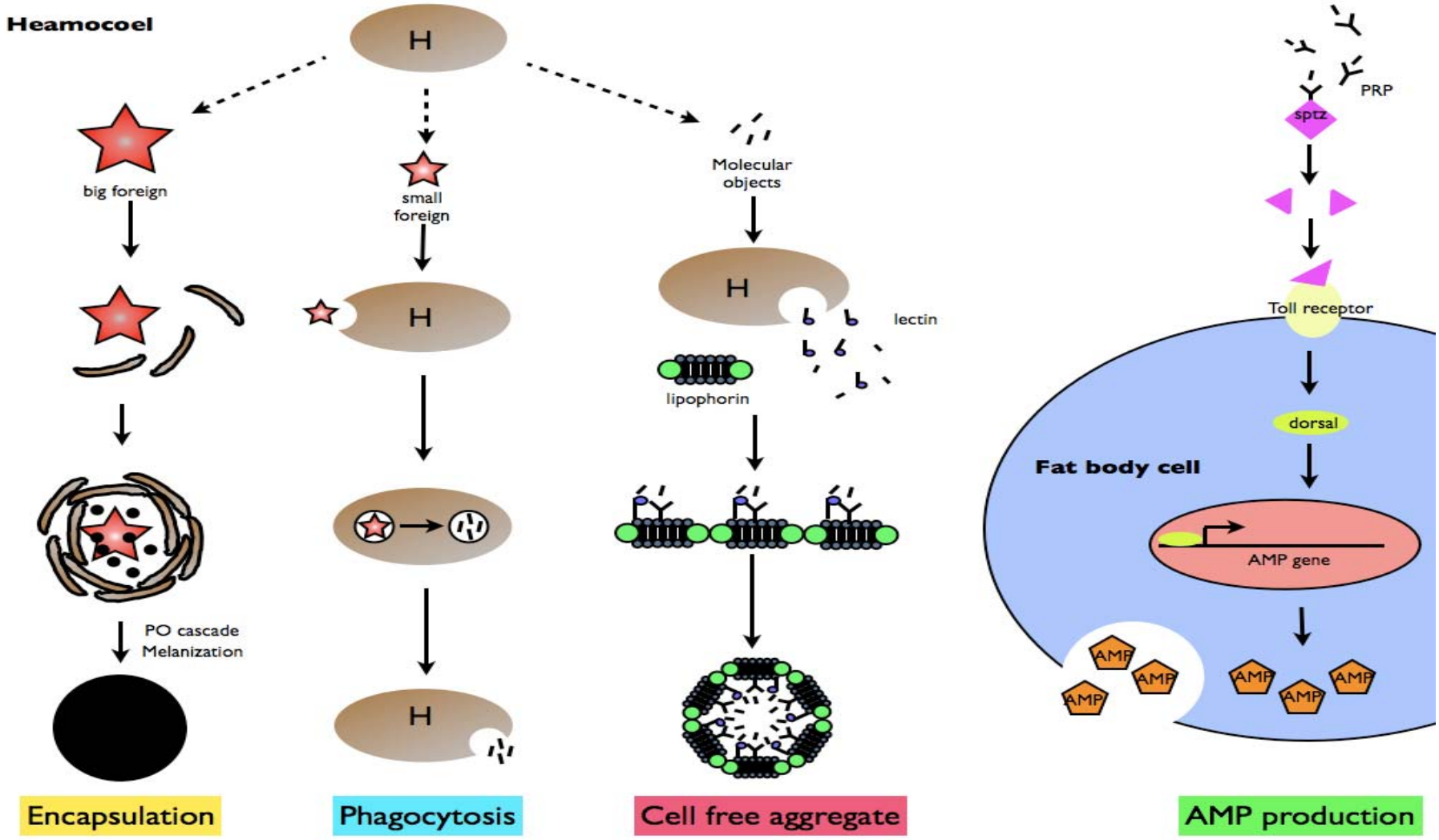
Heamocoel
H
big foreign
small foreign
Molecular objects
lectin
lipophorin
PO cascade
Melanization
Encapsulation
Phagocytosis
Cell free aggregate
PRP
sptz
Toll receptor
dorsal
Fat body cell
AMP gene
AMP
AMP production

Figure 1.5 Insect immune system. There are at least 4 broad mechanisms known to be activated in response to infection: encapsulation, phagocytosis, cell-free aggregation and the production of anti-microbial peptides (AMPs). Each mechanism gives a specific response to particular types of infection. Encapsulation involves the formation of a capsule by many haemocytes as a response to foreign objects that are too big to be engulfed by haemocytres, e.g., glass beads and parasitoid eggs. Phagocytosis is a response to bacterial infection. Haemocytes detect the presence of bacteria, engulf the bacterial cells, proteolytically attack the cells and secrete bacterial fragments into the haemocoel. In cell-free aggregation, pattern recognition proteins (PRPs) such as lectins, which are synthesised and secreted from haemocytes in response to the presence of microbial molecules, bind to the foreign molecules and form a complex. The complex is bound by lipophorin, and this results in changes in the adhesive properties of lipophorin. This allows the lipophorins to cross-link and form an aggregate. Aggregates and capsules function in the same way, which is to sequester the microbes or microbial molecules, and prevent them from damaging the insect tissues. AMP production occurs in fat body cells in response to infection. The presence of microbes is detected by PRPs, which then leads to the proteolytic activation of spätzle (sptz). Active sptz binds to Toll receptors in the fat body cells' membranes, resulting in activation of the transcription factor, dorsal. Dorsal enters the nucleus and activates the transcription of AMP-coding genes, which leads to synthesis of AMPs in the cytoplasm, and secretion of AMPs by fat body cells into the haemocoel.

Studies in *C. elegans* and *Manduca sexta* demonstrated the importance of the JNK and p38 MAPK pathways in the susceptibility of organisms to *Bt* toxins. A study using *C. elegans* has shown that mutations in members of the JNK and p38 MAPK pathways significantly increased the susceptibility of nematodes to Cry5B toxin (Huffman et al. 2004). An RNAi-based study involving *M. sexta* also reported that MAPK p38-silenced larvae became hypersensitive to Cry1Ab toxins (Cancino-Rodezno et al. 2010). In addition, it has been shown that these two pathways are activated in response to the disruption of epithelial cells because mutated Cry toxins that fail to form pores in the midgut membrane and also fail to activate these pathways (Cancino-Rodezno et al. 2010). The generality of this finding is further supported by a study done of *T. ni,* which showed that the immune system is not activated in response to *Bt* toxin in homozygous *Bt*-resistant individuals, but it is in susceptible heterozygous resistant larvae, possibly due to the lack of a *Bt* binding site (Ericsson et al. 2009).

The exact roles of the p38 MAPK and JNK pathways in the responses of circulating haemocytes to *Bt* toxin are still unknown. However, a study has shown that there is an increase in the number of phagocytic haemocytes and the encapsulation rate of the haemocytes in the heamocoel in response to the disruption of epithelial cells (Dubovskiy, Krukova & Glupov 2008). The number of haemocytes circulating the haemocoel is also reported to decrease in response to *Bt* toxin (Ericsson et al. 2009). However, it is unknown whether the lower number of circulated haemocytes in the haemolymph is due to the increase in the number of haemocytes engaged in phagocytosis and encapsulation.

Observations of responses by the cellular immune system to *Bt* toxins must be critically considered since they might not provide defense against the toxins. Insects that are pre-treated with antibiotics, and thus have no midgut microflora, have a higher mortality rate when they are exposed to *Bt* toxins mixed with fragmented peptidoglycan compared to insects treated by *Bt* toxins alone (Broderick, Raffa & Handelsman 2010). Furthermore,

larval survival increases when immune-suppressors such as antioxidants and eicosanoid inhibitors are given together with *Bt* toxins. These results suggest that, instead of defending against the toxins, the immune system induced by *Bt* exposure may instead increase mortality.

1.2.2.2 Cell-free immune system

The cell-free immune system is characterised by the action of anti-microbial peptides (AMPs) and the production of cell-free aggregates. AMPs are synthesised in fat body tissue after microbial challenge, before being secreted into the haemocoel (Figure 1.5). The production of AMPs is specific and depends on the type of microorganism encountered. For example, in *Drosophila melanogaster*, diptericin is produced in response to Gram-positive bacteria, whereas, drosomycin is produced in response to Gram-negative bacteria (Schmidt et al. 2010). Cell-free aggregation involves the recognition of microbial surface determinants, and the formation of an aggregate in which the microbe is detained, and possibly engulfed later by haemocytes (Figure 1.5). Even though it is known that lipoproteins and PRPs play an important role in the process, the biochemical cascades associated with cell-free aggregation were only demonstrated in *in vitro* (Schmidt et al. 2010).

The synthesis of AMPs is activated by two pathways: the Toll pathway and the Imd pathway (Figure 1.5). Different microorganisms activate different pathways; fungi and Gram-positive bacteria activate the Toll pathway, whereas Gram-negative bacteria activate the Imd pathway. The Toll pathway involves recognition of surface molecules of microorganisms such as bacterial cell wall components, LPS and peptidoglycan by PRPs. This recognition leads to protease activation, resulting in cleavage of the soluble molecule spätzle. Cleaved spätzle binds to the Toll receptor on the fat body cell, leading to the activation of the transcription factor, dorsal, which then enters the nucleus and triggers the transcription of AMP-coding genes, and the resultant production of AMPs (Figure 1.5).

The Imd pathway involves similar activation to the Toll pathway, but other pattern recognition proteins and transcription factors are subsequently activated.

Cell-free aggregations (Figure 1.5) involve the coagulation of lipid particles in response to an infection by a microorganism (Schmidt et al. 2010). The mechanism can be separated into four steps: 1) the recognition of microbial determinants by PRPs, 2) the binding of microbial molecules to the lipid moiety of lipophorin proteins, possibly via lectin-binding glycolipids, 3) changes to the adhesive property of lipophorin and 4) self-assembly of lipophorin-microorganism complexes into globular structures (Schmidt et al. 2010; Ma et al. 2012). The globular structures formed can inactivate the microbial cells, sequester foreign objects, inhibit both bacterial growth and spread of damaging toxins (Schmidt et al. 2010). These functions are mediated by oxidation reactions utilising reactive oxygen species. There is still uncertainty regarding what molecules are involved with the changes of lipophorin adhesion properties. Several studies have suggested that these molecules may include a peptidoglycan recognition protein, immulectins, glycolipids and apolipophorin III (ApoIII) (Schmidt et al. 2010; Ma et al. 2012).

After containing pathogens by encapsulation or cell-free aggregation, enclosed pathogens can be killed by quinone-like substances that are associated with melanisation. As mentioned earlier, melanisation is a fundamental metabolic cascade involved in wound healing and immune responses against foreign invaders (Figure 1.5 & 1.6). Melanisation involves the conversion of the zymogen PPO into phenoloxidase (PO) by phenoloxidase activating enzymes (PAE) (Kanost & Gorman 2008). PO converts tyrosine into quinone, which eventually becomes melanin. Melanin is toxic to microorganisms. Melanin is released by haemocytes during encapsulation or acts as a plasma chemical in cell-free aggregates.

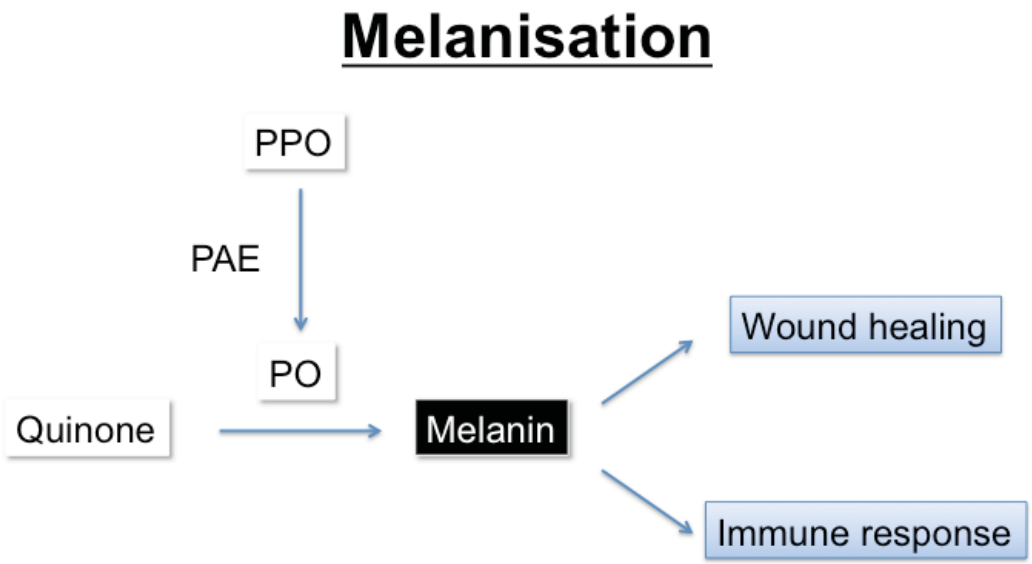

Figure 1.6 Melanisation cascade. The cascade is initiated by molecules from cell ruptures and bacterial components. Prophenoloxidase (PPO), a zymogen, is proteolytically activated by prophenoloxidase-activating-enzyme (PAE) to become an active enzyme phenoloxidase (PO). PO transforms quinone into melanin, a dark pigment, which is toxic to microorganisms. PO cascade also triggers other molecules in the wound healing system.

Several anti-microbial peptides and cell-free immune components increase in concentration in response to *Bt* challenge (Ma et al. 2005; Tamez-Guerra et al. 2008). Interestingly, AMPs produced in *T. ni* in response to *Bt* toxins are not a Gram-specific (Tamez-Guerra et al. 2008). This might be due to the mode of action of *Bt* toxins. The main mode of action that has been proposed is that *Bt* toxins cause leakage in the midgut of the infected larva, thus allowing the gut bacteria to enter the haemocoel, causing sepsis and death. If this is the case, then the production of anti-microbial peptides have to be active against both Gram-positive and Gram-negative bacteria. One of the anti-microbial peptides that has been shown to increase expression in response to *Bt* toxins and LPS is gloverin, which functions in inhibiting cell-membrane synthesis in bacteria (Tamez-Guerra et al. 2008; Kawaoka et al. 2008). The increase in gloverin expression in response to *Bt* toxins is probably a part of an immune defense as a study done in beet armyworm, *S. exigua*, has shown that the depletion of gloverin using RNAi results in an increase in susceptibility to *Bt* toxins (Hwang & Kim 2011).

The formation of cell-free aggregates has been proposed to be the mechanism of inducible *Bt* tolerance (Ma et al. 2005; Rahman et al. 2006). *In vitro* studies have demonstrated that lipophorin can form a cell-free aggregate with LPS and Cry1Ac toxin (Ma et al. 2005; Rahman et al. 2006). This binding is possibly initiated via an interaction between a lectin-binding site in Domain II of the *Bt* crystal toxin and a glycolipid, such as N-acetyl-galactosamine-binding lectin within lipophorin (Ma et al. 2005; Rahman et al. 2006; Ma et al. 2012). Binding might lead to the formation of aggregates, which sequester the toxins and prevent them from binding to the epithelial cells. Following this, the melanisation cascade can occur within an aggregate, thereby detoxifying the toxin molecule.

However, the role of melanization following cell-free aggregation in inducible *Bt* tolerance is still unclear. Rahman, Roberts and Schmidt (2007) demonstrated that inducible *Bt* tolerance is not affected by the inhibition of PO using tropolone. Thus, PO or the

melanisation process might not be involved in the mechanism of tolerance even though increased melanisation is correlated with it.

Apo III, a type of a storage-lipophorin, has been shown to be involved in the immune system by carrying immune components such as PPO, and PRPs. ApoIII is involved in activating more than one component of the insect immune system. It acts as a pathogen recognition protein (Whitten et al. 2004) to activate the production of AMP (Zdybicka-Barabas & Cytrynska) and participates in cellular immune reactions including stimulating phagocytosis and encapsulation (Wiesner et al. 1997; Whitten et al. 2004), and regulates the production of PO (Marmaras & Lampropoulou 2009). An increase in the expression of ApoIII can have a positive or negative effect on the concentration of PO (Halwani, Niven & Dunphy 2000; Zdybicka-Barabas & Cytrynska 2011). The expression of ApoIII has also been shown to increase in response to Cry3Ba *Bt* toxin in *Tribolium castaneum* (Contreras, Rausell & Dolores Real 2013). Unlike PO, depletion of ApoIII using RNAi affects the susceptibility of the *T. castaneum* against *Bt* toxins (Contreras, Rausell & Dolores Real 2013).

So far, there has been no study done in induced-tolerant insects to investigate whether agglutination of the toxins is part of the tolerance mechanism. As mentioned earlier, the immune system is activated via either binding of *Bt* toxins to receptors or damage of the epithelial cells from pore-formation. So, even though aggregate formation could prevent toxins from binding to the epithelial cells, the role activation of cell-free aggregation and other immune responses in induced tolerance remains unclear. In addition, only a single immune system indicator, PO activity, has been monitored in response to *Bt* toxins in the inducible tolerance model. The possible activity of other immune components is still unknown.

1.2.2.3 Within- and trans- generational immune priming

Even though an immune response against *Bt* toxins has been reported in many studies, the

immune correlation reported in inducible *Bt* tolerance is the only one that exhibits both within- and trans-generational immune priming (Rahman et al. 2004; Rahman et al. 2011). Immune priming is defined by the increase in the immune response against a specific pathogen following a low dose exposure to the pathogen or an immune elicitor such as LPS. This increase in the immune response resulting from immune priming can last for a week (Pham & Schneider 2008), thus protecting the primed organism against more serious infections. In inducible tolerance, a population of larvae that has been previously "primed" with a sub-lethal dose of toxin becomes more tolerant to a higher dose of *Bt* toxins compared to a non-primed larva (Rahman et al. 2004). As mentioned, the increase in tolerance is correlated with the increased PO activity in haemolymph. The offspring of the *Bt* primed larvae become innately tolerant and also possess the increased PO activity.

1.2.2.3.1 Within generational immune priming

In the animal kingdom, vertebrates have an immune system that allows the development of immune memory after being primed. This is known as an adaptive immune system. The adaptive immune system relies on special immune cells, such as B and T cells, to generate antibodies and memory cells after the first infection (the priming). These anti-bodies and memory cells are highly active and specific against the pathogen they encountered, and this allows a faster reaction towards subsequent infections by the same pathogen. The insect immune system does not have immune cells that are involved in the adaptive immune system (Pham & Schneider 2008) and does not exhibit the immune memory found in vertebrates.

In fact, it might not be advantageous developing an immune memory against pathogens for an insect since an insect's life span is much shorter than that of vertebrates. The chance that a particular individual will encounter a given pathogen multiple times before reproduction is low (Little et al. 2003; Sadd & Schmid-Hempel 2009; Zanchi et al. 2012). In addition, the immune system is costly as it has been shown that an increase in immunity

is usually associated with a trade off in other systems (Sadd & Schmid-Hempel 2009). So, it might be more efficient to invest just enough energy to produce a defense system that will defend against pathogens when encountered, and reserve the remaining metabolic resources for growth and reproduction. Nevertheless, many insects have shown that their immune system can be primed using a low dose of pathogen, and that priming allows them to become less susceptible to the later infection.

Many studies have shown that the insect immune system can be primed to generate a prolonged immune response using bacteria or LPS. A study of the bumblebee *Bombus terrestris*, found that infection with commensal gut protozoan, *Crithidia bombi*, doubled PPO activity in the haemolymph (Brown, Moret & Schmid-Hempel 2003). This increase in haemolymph PPO activity persisted up to 14 days post-infection, during which time bees were afforded a better protection against the same protozoan. In the field cricket *Gryllus campestris*, when male nymphs were injected with LPS, the haemolymph of adults had increased lysozyme and PPO concentrations (Jacot et al. 2005). Elevated PO activity in haemolymph increases the sensitivity of the immune system against infection and allows the insect to respond quicker if the infection becomes serious. Interestingly, in the Jacot et al. study of *G. campestris*, the amount of PPO was increased, but PO was not. The reason for this might be to prevent any damage to the cricket's body (e.g. autoimmune damage) in the absence of real bacterial infection (Pham & Schneider 2008).

The priming mechanism that induces prolonged immunity is still unclear. However, several studies have shown that the prolonged immunity in primed individuals is due to a persistent cell-free immune response, including the actions of anti-microbial peptides and immune proteins like apolipophorin (Wiesner et al. 1997; Moret & Siva-Jothy 2003). A study of immune responses by the wax moth, *Galleria mellonella*, has demonstrated that injection of either formalin-killed *Pseudomonas aeruginosa* or LPS provided protection against subsequent *P. aeruginosa* infection (Deverno, Aston & Chadwick 1983). The

protection lasted from 3 to 18 h post-priming. A subsequent transplantation study has shown that haemolymph from a primed donor insect collected during 3-40 hr post-priming can protect a recipient insect from the same infection (Deverno, Aston & Chadwick 1983). However, only haemocytes collected from the primed donor during 30 minutes to 4 hr post-priming can protect the recipient from the same infection. Wiesner et al. (1997) further investigated the haemolymph and isolated ApoIII from the hemolymph of *P. aeruginosa* challenged *G. mellanola*, and found that this molecule can activate the immune system. The function of ApoIII has been mentioned earlier in this chapter as being involved in activating the production of AMPs, stimulating phagocytosis and encapsulation of haemocytes, and regulating the production of PO (Wiesner et al. 1997; Whitten et al. 2004; Marmaras & Lampropoulou 2009; Zdybicka-Barabas & Cytrynska 2011).

Moret and Siva-Jothy (2003) induced prolonged-immunity against the entomopathogenic-fungus *Metorhizium anisopliae* in the mealworm, *Tenebrio molitor* by challenging the mealworm with LPS. They have found that *T. molitor* become more tolerant to the fungal infection, and this was due to the production of a long-lasting antimicrobial peptide. In addition, the level of protection relied on the amount of antimicrobial peptide produced (Moret & Siva-Jothy 2003).

Even though the role of haemocytes in prolonged immunity is still unclear, it is possible that haemocytes play a role in molecular signaling that activates the production of anti-microbial peptides in fat-body cells (Trenczek & Faye 1988).

1.2.2.3.2 Transgenerational immune priming

Transgenerational immune priming (TGIP) is the phenomenon where an individual's susceptibility to infection is affected by parental exposure to the same pathogen (Moret 2006). In TGIP, the offspring of the individuals that have been exposed to a particular pathogen usually show less susceptibility to that pathogen and an increase in the immune

response against it. TGIP plays a crucial role in increasing survival of offspring if the pathogen persists from the parental generation.

TGIP has been found in many species. For example, offspring of female *Daphnia magna* infected with a bacterium, *Pasteuria ramosa,* have a lower overall susceptibility to infection by the bacteria compared to offspring of non-infected mothers, especially if offspring are inoculated with the same bacterial strain as the parents (Little et al. 2003). Another study demonstrated that male offspring in colonies of the bumblebees (*B. terrestris*) have higher PO activity when female workers in the parental generation are injected with LPS (Moret & Schmid-Hempel 2001). Offspring from LPS-challenged female *T. molitor* also display a higher antimicrobial activity than offspring from non-challenged females (Moret 2006). Finally, *P. interpunctella* that had been exposed to low doses of *P. interpunctella* Granulosis Virus (*Pi*GV) showed both within- and trans-generational immune priming. The offspring of individuals that were exposed to *Pi*GV had a lower susceptibility to viral challenge compared to the offspring of non-exposed members of the parental generation (Tidbury, Pedersen & Boots 2011).

Even though transmission of immune status can be from both parents, there are indications that the immune ability of offspring coming from challenged mothers is better (Freitak, Heckel & Vogel 2009b; Zanchi et al. 2011). This maternal effect should benefit the population as a whole if the pathogen is likely to be persistent in the environment. Maternal effects relating to offspring immunity have been shown in model species in both vertebrates (Grindstaff, Brodie & Ketterson 2003) and invertebrates (Little et al. 2003; Sadd et al. 2005; Moret 2006; Zanchi et al. 2012). In inducible *Bt* tolerance, offspring from tolerant females are also more tolerant to *Bt* toxins compared to offspring from tolerant males (Rahman et al. 2004; Ma et al. 2005; Rahman et al. 2011), as shown in other studies of TGIP.

The increase in melanization activity in inducible *Bt* tolerance is likely to be a specific response to *Bt* toxins. Despite the role in defense against bacteria, the encapsulation and melanisation processes are usually the part of the immune response that defend against the eggs of endoparasitoids. But the increase in the PO activity in *Bt*-tolerant larval *E. kuehniella* did not provide protection from parasitism by *Venturia canescens* (Rahman, Roberts & Schmidt 2004). Thus, it seems that the increase in the PO activity might be a specific response targeting *Bt* toxin, not a more general response. Even though the increased immune response did not protect a *Bt*-tolerant larvae from larval parasitism, the continued growth of the tolerant larvae was less hindered by the development of the endoparasitoid than growth in susceptible parasitised larvae (Rahman, Roberts & Schmidt 2004). This suggests that the change in the immune system in the *Bt*-tolerant larvae had some effects in mitigating the metabolic costs of parasitism (Rahman, Roberts & Schmidt 2004).

The mechanisms associated with transmission of TGIP remain unclear. In vertebrates, it has been shown that immune status can be passed from mothers to offspring via antibodies (Grindstaff, Brodie & Ketterson 2003). Several studies involving invertebrates have indicated that there is a transference of immune substances from the mother to her eggs via coating on the egg surface or incorporation into the eggs (Eisner et al. 1996; Marchini et al. 1997; Zanchi et al. 2012). Zanchi et al. (2012) demonstrated that eggs from infected adult female *T. molitor* had a higher antimicrobial activity compared to eggs from non-infected mothers. The increase in antimicrobial activity in eggs was correlated with a decline in the antimicrobial activity in mother's haemolymph (Zanchi et al. 2012). This suggests that the immune substances may come directly from the mother's haemolymph.

The mechanism of maternal effect in TGIP might not be a simple transference of immune proteins from bacterially-challenged mothers into the egg. Freitak et al. (2009a) examined the transcription profiles of immune genes in bacterially-fed *T. ni*. They found that there

was no difference in the expression of the immune gene cytochrome C oxidase between bacterially-fed larvae and larvae fed on a diet free of bacteria within a generation. However, the expression of another immune gene, gluthation S-transferase I (GST1), was 9-fold lower in bacterially-fed larvae. On the other hand, offspring from *T. ni* fed with bacteria during their larval stage had increased expression of both cytochrome C oxidase and GST1 compared to the parental generation (Freitak, Heckel & Vogel 2009a). This suggests that the genes expressed in response to the infection in the larval stage of the parental generation can be different from the relevant genes in the offspring. It also indicates that the mechanism of transferal of immunity is not just a simple transfer of parental gene expression profiles, but rather builds expression of a potentially different set of immune genes.

Maternal transfer of immunity could reduce the cost of the immunity in offspring against the same pathogen. This can be done by mothers inserting additional immune components into their eggs, or they may transfer substances that trigger the offspring to generate better immunity. However, the production of PO or any immune substance is costly and usually accompanied with a trade-off in other aspects of physiological performance, such as longer developmental times and reduced body mass. Offspring from *Bt*-tolerant parents have the same developmental time and body mass as offspring from susceptible parents when both are reared on non-toxic food (Rahman et al. 2011). Thus there is no fitness cost associated with inducible tolerance. In addition, when *Bt*-tolerant offspring are reared on food that contains *Bt* toxins, even though they exhibit increased PO activity similar to their tolerant parents, they had reduced developmental penalties from the *Bt* ingestion. This supports the idea that maternal effect benefits the offspring in terms of providing immunity that has low metabolic cost. It also suggests that the transference of the immune status through a maternal effect might not be a simple transference of immune substances, but rather might involve complex changes to the offspring's gene expression profile in a way that maintains

the tolerance of their parents but at a reduced cost. It is possible that the immune response against *Bt* is different from the immune system priming process leading to immune memory and TGIP.

The mechanism of maternal effect in *Bt* tolerance and molecules involved are still unknown. The form of any directly transferred immune substances could be proteins like AMPs, or other small molecules. It is also possible that mothers can affect the regulation of offspring immune gene expression by transferring maternal mRNA transcripts into eggs, as is seen during the regulation of offspring genes in the early development of embryos. For example, mRNA transcripts of transcription factors *bicoid* and *nanos* are incorporated into eggs to control the activation of the pair-rule genes, which are responsible for anterior-posterior and dorsal-ventral patterning in the embryo (see review by Johnston and Nüsslein-Volhard (1992)). Finally, the transmission can be in a form of DNA methylation in the offspring's genome, which is also known to affect regulation of gene expression (Hunt et al. 2013).

1.2.3 Possible effect of inducible *Bt* tolerance on egg parasitoids

Parasitoids are insects that live on or in other invertebrates, and eventually kill their hosts. The parasitoid develops using the host's body as food. An adult female parasitoid lays eggs on or into various stages of their host, depending on the type of parasitoid. An egg parasitoid lays eggs within the host egg. Parasitoids are well known as "natural enemies" of many insects and can be used in integrated pest management programs to control them (Bates et al. 2005). The use of parasitoids together with GM Bt crop is a common practice as it can control the pesticide resistance population in the field (Schuler et al. 1999; Chilcutt & Tabashnik 1999; Schuler et al. 2004). Many species of parasitoid wasps attack *H. armigera*, such as *Trichogramma* spp., *Microplitis* spp. and *Netelia* spp.

There are two possible pathways the use of *Bt* toxins could impact on parasitoid

populations in the field: 1) a direct toxicity of *Bt* toxins on parasitoids, and 2) an indirect effect via their host. Since each type of Cry toxins acts specifically to a particular group of insect (Table 1.1), *Bt* toxins should not have a direct effect on parasitoids, and this has been confirmed by many studies (Wang et al. 2007; Wang et al. 2012). However, Steinbrecher (2004) reported a low parasitism success in the eggs of *H. armigera* that have survived exposure to GM *Bt* maize (expressing Cry1Ab). As Cry1 toxin itself does not affect Hymenoptera, Steinbrecher (2004) suggested that survivors of *Bt*-toxin exposure might be less nutritious as hosts. In this case, *Bt*-exposed larvae might have expended their energy and metabolic resources to defend themselves against the *Bt* toxins, leaving less resources for eggs (Steinbrecher 2004).

One of many factors that affect the success of parasitism is host immunity. Proteins on the surface of a parasitoid egg can act as an epitope and be recognised by the host's haemocytes. The recognition leads to haemocytes gathering around the egg, and eventually leads to encapsulation of the eggs (Figure 1.5). Some parasitoids overcome host immunity by expressing the proteins on the egg surface that prevent detection by the host's immune system, and some parasitoids inject venoms which can regulate host development or kill the host (see review by Schmidt, Theopold and Strand (2001)). In *Trichogramma* spp., which are egg parasitoids, adult females injects venom into host eggs during oviposition (Strand 1986; Jarjees & Merritt 2004). The venom kills the embryo, and this allows the parasitoid larva to develop using egg proteins and proteins from the dead embryo as food.

There is a concern that the increase in PO activity in induced *Bt*-tolerant larvae would lead to the increase in encapsulation rate of parasitoid eggs in the defense against parasitism . There has been only one study done on the effect of inducible tolerance to *Bt* toxin on parasitoids (Rahman, Roberts & Schmidt 2004). The study investigated the effect of induced tolerance to *Bt* toxins in *E. kuehniella* on the larval parasitoid *V. canescens,* and concluded that there was no effect on parasitism success. However, the study did show that

the wasps took longer to develop in the induced tolerant hosts than in the susceptible hosts, with the authors suggesting that this could be due to partial inhibition of parasitoid growth in the tolerant host. In smaller hosts, the adult wasps that emerged from *Bt*-tolerant larvae were significantly larger compared to the ones that emerged from susceptible hosts. However, this host-dependent size difference in the adult wasps was not observed in larger larvae (Rahman, Roberts & Schmidt 2004).

1.3 Research aims

This study focuses on potential TGIP in induced Cry1Ac-tolerant *H. armigera*. *H. armigera* is an important pest insect in Australian cotton industry, together with its sister species, *H. punctigera*. The pest management strategy used to control these pests include the use of the Bollgard II™ variety of transgenic cotton, which expresses both Cry1Ac and Cry2Ab toxins, insecticide sprays, cultivation and the use of biological control agents such as *Trichogramma pretiosum* (The Australian Cotton Industry Development & Delivery Team 2012). The development of Cry1Ac tolerance is a threat to the use of *Bt* technology. Even though the use of *Bt* in pest management has been a longstanding practice, there are still gaps in the knowledge of the mechanism of *Bt* toxicity and the resistance mechanisms in insects. The primary aim of this study is to improve understanding of the mechanism of immune-related Cry1Ac tolerance, which will help in strengthening the established resistance management strategy (The Australian Cotton Industry Development & Delivery Team 2012). Furthermore, findings of the study could be broadly applicable to development of tolerance in a range of lepidopteran pests.

I conclude that previous studies have not clearly indicated the transmission mechanism of inducible *Bt* tolerance in *H. armigera*. *Bt* toxins have complex modes of action, which involve more than one toxin structure and activate various immune pathways. This makes elucidation of the tolerance transmission mechanism challenging. In addition, the mechanism of TGIP is poorly understood in insects generally and could involve a number

of transmission mechanisms. In view of the strong maternal effect in the transmission of inducible *Bt* tolerance in *H. armigera*, changes in the egg stage of an induced tolerant strain is the main focus of this study.

Currently, microarray gene expression studies on the midgut and other tissues of tolerant larvae are undertaken to elucidate the tolerance mechanism in this stage (Rahman, M. personal communication).

The main focus of this study is to identify the genes that might be involved in the maternal transmission of the immune status with regard to *Bt* toxin. To do this, the gene expression profiles (or transcriptomes) of the eggs from susceptible and induced tolerant *H. armigera* were generated using 454 pyrosequencing. By comparing the differences between susceptible and tolerant egg transcriptomes, genes that are expressed differently between treatments can be identified for further study. An analysis of egg transcriptomes from susceptible and *Bt*-tolerant *H. armigera* are presented in detail in Chapter 2 of this thesis.

In Chapter 3, the transcriptome of eggs of a Cry1Ac-resistant strain was investigated. Even though this Cry1Ac-resistant population was selected with higher toxin doses and is highly resistant to Cry1Ac toxin, it has also been reported in Ma et al. (2005) to have some features similar to lower-level induced *Bt* tolerance. Thus, it is possible that the resistance mechanism in these strains is partially the same. In this chapter, I investigated whether genes which were expressed differently between eggs of susceptible and Cry1Ac-tolerant *H. armigera* (described in Chapter 2), were expressed in a similar pattern in the eggs of the Cry1Ac-resistant strain. I also identified which genes were expressed differently between Cry1Ac-resistant and susceptible strains, as these genes could play a role in the elements of Cry1Ac-resistance transferred through maternal effect. Through this work I addressed the possibility that there is more than one mechanism that can lead to the increase in the immune activity in the immune-related Bt tolerance or resistance.

Finally, in Chapter 4, the maternal effect of inducible tolerance on eggs was investigated. This study investigated changes in egg size and possible effects of a change in immune status in inducible tolerant insects on egg parasitism. I compared the size of eggs from susceptible and induced tolerant populations. Then, I compared parasitism of eggs by *T. pretiosum* from induced tolerant and susceptible laboratory cultures of *H. armigera.* Parameters related to parasitism success, including the number of eggs successfully parasitised and the number of progeny produced per host egg, were assessed.

Chapter 2

Comparison of egg gene expression profiles between Bt-toxin susceptible and induced-tolerant cotton bollworm, *Helicoverpa armigera* (Manuscript)

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Title: Comparison of egg gene expression profiles between *Bt* toxin susceptible and induced-tolerant cotton bollworm, *Helicoverpa armigera*

Short title: Egg gene profiles from *Bt* tolerant *Helicoverpa armigera*

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Abstract

Insecticidal toxins produced by the bacterium *Bacillus thuringiensis*, *Bt* toxins, are environmental-friendly insecticides that are used widely in primary production as sprays and engineered into plants. Previous studies have demonstrated inducible *Bt* tolerance, which is a tolerance induced in lepidopteran larvae exposed to low doses of *Bt* toxins. This tolerance is associated with an increase in phenoloxidase activity in larval haemolymph and in the midgut. In addition, offspring from tolerant parents become innately tolerant to *Bt* toxins, and the tolerance level increases over generations of larval exposure. Interestingly, induced tolerance is strongly inherited via a maternal effect, similar to trans-generational immune priming (TGIP) reported in other species. Despite many studies, the mechanism of TGIP remains unclear. The aim of the research reported herein was to identify genes that might be involved in TGIP associated with inducible tolerance to *Bt* toxins. The transcriptome of eggs of *Helicoverpa armigera* was generated using 454 deep sequencing. Gene expression profiles of eggs laid by susceptible and induced tolerant *H. armigera* were compared and qRT-PCR was used to confirm four genes with increased expression in tolerant insects: histone cluster 3 H2BB, translationally controlled tumor protein, receptor for activated C kinase, and glyceraldehyde-3-phosphate dehydrogenase. Some of these genes have previously been reported to be up-regulated in the eggs and larval midgut of other insect species in response to *Bt* exposure. This work provides the basis for more targeted experiments that examine the functional role of these genes in inducible tolerance to *Bt* toxins.

2.1 Introduction

Bt toxins are insecticidal crystal toxins occurring in a Gram positive soil bacterium, *Bacillus thuringiensis*. The protoxin produced by the bacterium is activated by proteases in the insect midgut, and subsequently triggers pore formation in midgut cells, which leads to death of the insect by septicemia (Pardo-López, Soberón & Bravo 2013). Components of the bacterial cell wall and the metabolism associated with pore-formation can induce immune responses in insects. For example, it is known that the peptidoglycan cell wall of gram positive bacteria can activate the Toll pathway, and that the pore-forming toxin can generate a cellular response against stress and cell invasion (Michel et al. 2001). In fact, several studies have shown that there are also immune responses against *Bt* toxin (Huffman et al. 2004; Rahman et al. 2004; Ma et al. 2005; Cancino-Rodezno et al. 2010; Ahmed 2013). These include activation of signaling pathways, such as MAPPK p38 and JNK, which are involved in the innate immune system (Huffman et al. 2004; Cancino-Rodezno et al. 2010). The activation of these pathways can lead to cascades that result in the production of antimicrobial peptides and other immune components such as the enzyme phenoloxidase (PO), which functions in a melanisation cascade (Kanost & Gorman 2008). These immune components also appear to regulate the susceptibility of an insect to *Bt* toxins. For instance, studies involving mutation and RNAi-depletion of these genes have demonstrated an increased susceptibility of lepidopteran larvae against *Bt* toxins (Huffman et al. 2004; Cancino-Rodezno et al. 2010; Hwang & Kim 2011). The immune response is known to be correlated with *Bt* toxin pathology, but it remains unclear how each of the immune components function directly or indirectly in protecting insects from the damage caused by *Bt* toxins.

Previous work showed that exposure to low doses of *Bt* toxins can generate an immune response in larvae of two lepidopterans: *Ephestia kuehniella* and *Helicoverpa armigera* (Rahman et al. 2004; Rahman et al. 2011). Specifically, an increase in PO activity in *Bt*-

exposed larvae was correlated with an increased tolerance against *Bt* toxins. Furthermore, insects that were pre-exposed to a low dose of *Bt* toxins were more tolerant to a higher dose of the same protein encountered subsequently. It was also apparent that there is trans-generational immune priming (TGIP; see Moret (2006)) in *Bt* exposed insects; the offspring of *Bt* tolerant females exhibit an innate tolerance and an increase in immune activity (measured by PO activity). The level of trans-generational tolerance associated with PO activity was shown to increase with the number of generations exposed to *Bt*. In addition the effect was much stronger when mothers were tolerant compared to when fathers were tolerant, which indicated a strong maternal effect to the vertical transmission of *Bt* tolerance.

A maternal effect is an epigenetic phenomenon where an individual's phenotype is affected by the environment experienced by its mother. In TGIP, the immune status of the offspring is affected by a priming process that occurs when the maternal generation is exposed to pathogens. This maternal effect should benefit the population as a whole if the selection pressure imposed by the pathogen is also imposed on the offspring. Maternal effects have been shown in many model species involving the immune system (Little et al. 2003; Rahman et al. 2004; Ma et al. 2005; Moret 2006). In vertebrates, the immune transfer acts through antibodies (Grindstaff, Brodie & Ketterson 2003) but the mechanisms of TGIP remain unclear in invertebrates.

Investigations into invertebrate TGIP mechanisms include studies into the protein and transcript composition of the eggs. Zanchi et al. (2011) showed that female *Tenebrio molitor* had increased haemolymph antimicrobial activity after being challenged with an immune elicitor, lipopolysaccharide (LPS), and that this increase was reflected in the eggs. The precise identities of the immune substances involved in antimicrobial activity against LPS were unknown, and it was not confirmed whether they are proteins or antibodies as is observed in vertebrates. However, Freitak, Heckel and Vogel (2009a) showed that

immune-gene expression profiles in bacterially primed mothers are different from their immune offspring. In addition, Rahman et al. (2011) showed that developmental penalties, such as slow growth and low body mass present in induced *Bt* tolerant *H. armigera* larvae, declined over generations. This suggests that any induced immune response in offspring costs less than that required to survive exposure in the parental generation. Thus, it is possible that a set of immune genes involved in TGIP might be different from genes activated in response to the initial bacterial challenge in the parental generation.

Given this background, we hypothesised that the gene expression profiles of eggs from tolerant female insects might be affected by their maternal exposure to *Bt*, and induced tolerance to *Bt* toxins. We reasoned that genes which are expressed differently in eggs from *Bt*-tolerant versus *Bt*-susceptible females could function in TGIP, and could be used as molecular markers of inducible *Bt* tolerance. To test this hypothesis, we used 454 deep sequencing, transcriptomic analyses and quantitative RT-PCR to compare gene expression in eggs of susceptible and Cry1Ac-tolerant cultures of *H. armigera*.

2.2 Materials and methods

2.2.1 Cry1Ac toxin

Cry1Ac toxin used for tolerance induction was prepared as a bulk crude suspension of Cry1Ac-producing *Bacillus thuringiensis* strain subsp. *krustaki* HD73, supplied by John L. Reichelt (Bacterial Fermentation, Ltd, Pty., Arundale, Queensland, Australia) as per Rahman et al., (2011). Briefly, the bacterial culture was centrifuged, supernatant was removed and the cell lysate containing Cry1Ac toxin was kept as a bacterial paste at -20˚C. When required, the stock solution was diluted with MQ water to make a working concentration of 9.23 mg/ ml total protein, and stored for short periods at 4°C.

2.2.2 *Helicoverpa armigera* culture

H. armigera used in this study were from two sources. One strain was a *Bt*-susceptible laboratory population kept at the University of Adelaide at the Waite campus (known

hereafter as "susceptible Waite strain") and its history was described in Rahman *et al.,* (2011). Briefly, it resulted from mating a long-term *Bt*-susceptible laboratory strain ANGR (Ma et al. 2005; Rahman et al. 2011) and a *Bt*-susceptible strain that originated more recently from a population near Narrabri, New South Wales, Australia; both of the original strains were sourced from CSIRO. The culture was maintained under controlled conditions (25 ± 1°C; with a photoperiod of 14:10 h (light/dark). Larvae were fed with artificial diet (8.13% soy flour, 3.75% wheat germ, 3.31% brewing yeast, 1.25% agar, 0.21% ascorbic acid, 0.21% methyl paraben and 0.11% sorbic acid, mixed in water). Adult moths were fed with 20% honey solution.

The Cry1Ac tolerant strain (Tolerant F_{30}) was developed from the susceptible Waite strain and was maintained since 2009 for 30 generations under laboratory conditions (Rahman et al. 2011) by rearing larvae on a low dose (0.1 mg/ml) of Cry1Ac toxin. The LC_{50} value of the susceptible population was 0.117 mg/ml total protein. The LC_{50} value of tolerant population used in this study was 1.023 mg/ml total protein (Resistance Ratio = 8.72).

The second group of *H. armigera* comprised three *H. armigera* strains from CSIRO: a *Bt*-susceptible laboratory strain known as GR established in the mid-1980's from populations near Narrabri and that was periodically supplemented with *Bt*-susceptible individuals from the field (Mahon et al. 2007), a Cry1Ac-resistant strain known as ISOC initiated from 10 females from a Cry1Ac resistant strain BX (Akhurst et al. 2003) that completed development on Cry1Ac cotton and was outcrossed to ANGR and then backcrossed to create a colony with a RR of 5,836 (see Bird and Akhurst (2007)), and a Cry2Ab resistant strain known as SP15 which was established from a single pair collected as eggs on corn near Griffith, New South Wales, in 2002 and was outcrossed to GR and then backcrossed to create a colony with a RR of at least 6,380 (Mahon et al. 2007). These strains had also been maintained under laboratory conditions.

2.2.3 454 deep sequencing

Forty mg of eggs from each strain of *H. armigera* were collected within 24 hours of being laid. Polyadenylated RNA was purified using oligo d(T) beads by the GenElute Direct mRNA Miniprep Kit (Sigma-Aldrich, USA) as per the manufacturer's directions. cDNA libraries were prepared using a cDNA Synthesis System Kit (Roche, USA) and cDNA Rapid Library Preparation Kit (Roche, USA) following the manufacturer protocols for GS Junior 454 sequencing. Each cDNA library was amplified using emulsion PCR (emPCR kit, Roche) and the libraries were then subjected to sequencing using a GS Junior Sequencer.

Four 454 sequencing runs were performed on five egg samples of *H. armigera*. cDNAs from the Waite *Bt*-susceptible and Cry1Ac tolerant strains were sequenced together (using MID adaptors RL13 and RL14; Integrated DNA Technologies; Table 2.1) while cDNA libraries from the CSIRO *Bt*-susceptible, Cry1Ac-resistant and Cry2Ab-resistant populations were sequenced individually.

2.2.4 Transcriptome assembly and gene ontology analysis

Data generated by deep sequencing was subjected to analysis using two discrete software packages, with commonly identified transcripts being used for annotation and expression analysis; the workflow used is shown in Figure 2.1. Raw reads were processed using Newbler GS Run Browser to remove any sequencing adaptors and poor quality regions (Figure 2.1). All reads were further assessed for base quality and further trimmed using CLC Genomic Workbench 6 (minimum PHRED quality score = 20; minimum length = 50 bp).

For *de novo* assembly of a reference transcriptome, trimmed reads of at least 100 bp in length were assembled twice for comparison using CLC Genomic Workbench 6 and Newbler 2.7. Contigs from CLC and Newbler assemblies were assigned C_Contig and N_Contig in their names respectively. Contigs generated from each assembly were

subjected to BLASTx searches of the GenBank non-redundant (nr) database hosted by the National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) with the E-value threshold < $1E^{-25}$ using BLAST2GO software (version 2.6.3; http://www.blast2go.org). Then, gene ontology mapping and annotation were performed on all assembled contigs using Mapping and Annotation tools in BLAST2GO software with default settings. Translated sequences were matched against InterPro databases using the InterProScan tool. All InterPro terms were merged with GO terms for wide functional annotation range coverage.

2.2.5 Transcript expression comparison

The contigs generated from the *de novo* assembly using CLC and Newbler software were used as two reference sets of transcripts for expression analysis. Quality trimmed reads (with no minimum length) from eggs laid by susceptible and tolerant insects were each mapped against the two *de novo* assemblies, using the associated expression analysis tools: CLC Genomic Workbench RNAseq function and Newbler Reference Mapper, respectively, both with default settings (Figure 2.1). The relative transcript levels were output as RPKM (Reads Per Kilobase of exon model per Million Mapped reads) values by CLC Genomic, software and as percentages of unique reads per total reads by Newbler software. Only transcripts that were annotated similarly in both assemblies were investigated further (Figure 2.1). Transcripts that had a greater than 10-fold expression value difference between treatments were considered further.

2.2.6 Quantitative PCR

To generate cDNA libraries for quantitative PCR (qPCR), cDNA synthesis was carried out using SuperScript III First Strand Synthesis SuperMix (Invitrogen, USA) using 119 ng of polyadenylated RNA (measured using NanoDrop 1000 Spectrophotometer, Thermo Scientific) that had been extracted for sequencing (see above). Briefly, each RT reaction contained 1 ul of 50 uM oligo(dT) primers, 1 ul of annealing buffer, 119 ng of total RNA,

and RNAse/DNAse-free water to top up the volume to 8 ul. Then, the primer was allowed to bind to the template by incubating the reaction in the thermal cycler at 65°C for 5 min, and quickly chilled on ice for 1 mins. Then, the 10 ul of first strand reaction mix and 2 ul of Superscript® III/RNaseOUT^{TM} Enzyme mix was added into the RT reaction (RNase/DNase-free water was added instead in the negative control (–RT)). The reaction was then incubated in the thermal cycler for 50 min at 50°C, followed by 85°C for 5 min to terminate the reaction. cDNA produced was stored at -20°C.

qPCR was performed using a Rotorgene 3000 series thermocycler (QIAGEN, USA). 10 ul qPCR reactions, conducted in technical duplicates, contained 5 ul of SensiMix Low-Rox mix (with heat activated DNA polymerase, ultra-pure dNTPs, 3mM $MgCl_2$, 5-carboxy-X-rhodamine (ROX) internal reference and SYBR green I; Bioline Australia), 0.3 uM forward and reverse primers (Table 2.1), and 14 ng of cDNA template.

Each qPCR cycle consisted of denaturation for 15 sec at 95°C, annealing for 20 seconds at 58°C, and extension for 15 seconds at 72°C, for a total of 35 cycles. The fluorescence intensity of SYBR green I was acquired at 72°C for each cycle. Melting curve analysis was performed immediately after completion of PCR by heating at 95°C for 5 sec, followed by cooling to 70°C for 5 sec and then continuous heating to 95°C at 1°C/sec under permanent fluorescence detection. This was used to verify that a single product had been formed. PCR conditions were optimised by varying cDNA and primer concentrations in order to obtain a single uniform peak without generating products in “no template” and “no RT” negative controls. Actin and ribosomal protein subunit 15 (RPS15; Table 2.1) were used as house keeping genes (Brun-Barale et al. 2010). PCR products were analysed for size and sequence using gel electrophoresis (1% agarose gel and Gelgreen dye), and subsequent sequence analysis (AGRF, Australia).

The data was analysed using Rotor-Gene 6000 Series software (version 1.7) (Corbett Life Sciences). The quantity of the gene of interest in the sample was expressed as a fold

change based on the quantity of internal reference genes, actin and RPS15, using the "Comparative Quantitation" tool provided. The software determined the Ct value (the number of cycles required for the fluorescent signal to deviate from the background level), and the amplification coefficient, which is calculated from the slope of the section of the curve in exponential phase between the Ct point and the peak. The quantity of the gene of interest is then calculated using the following equation, where Ct1 value was of the calibrator (i.e. a housekeeping gene), and Ct2 refers to the gene of interest.:

Quantity of gene of interest = mean amplification coefficient$^{Ct1-Ct2}$

Amplification coefficients for all genes were 1.7-1.8.

Table 2.1 Primers and MID tags used for quantitative PCR and deep sequencing

MID Adaptor		
RL13		AGACTCGACGT
RL 14		AGTACGAGAGT
Housekeeping genes		Sequence
Actin A3a (X97614)*	Forward	CTCGACTTCGAGCAGGAGAT
	Reverse	TTCCATACCCAGGAATGAGG
RPS15 (AY818611)*	Forward	CTGAGATGATCGGCCACTAC
	Reverse	TGTTGGTCAGCGACTACTT
Genes		
Histone cluster 3, H2Bb	Forward	CTCACAGTAAGAAGACCACTATGAGTAG
	Reverse	GCATCCCTTTCGTGGCTC
Translationally controlled tumor protein (TCTP)	Forward	CCTTCGGTGACAAGAAATCCTACAC
	Reverse	GCCATCAACATCTCTGTACTCCATC
Serine/threonine kinase 23	Forward	GCATGGGCTTTAAGAAATCA
	Reverse	ATTGCAGCCTCTGGAAAATA
Vacuolar V-type H(+)-ATPase B subunit	Forward	CTTGGCCAACGATCCTACTA
	Reverse	CCGTGTACATGTAACCTGGGAA
Abnormal wing disc-like protein	Forward	GTCAAATGCTTGGTGCTACA
	Reverse	CATCCGACGACTTCTTTTTC
Transcription factor btf3	Forward	CGAAGTTGCCGACTAGGTTA
	Reverse	CTATCACCGGTCATGGAGAG
Protein aurora borealis	Forward	TCTATGGGACTGAAGCAAGG
	Reverse	CGGAGCAGACTGATGATGAAGTAC
Putative receptor for activated C kinase	Forward	GTGGATGAGGTGAAGAATGG
	Reverse	CGTTCCTGTTTATTCGTTGG
Glyceraldehyde-3-phosphate dehydrogenase	Forward	CATCAAACAGAAGGTCAAGGAGG
	Reverse	TCGATGACACGGTTGGAGTA

*Housekeeping gene primers adopted from Brun-Barale et al. (2010).

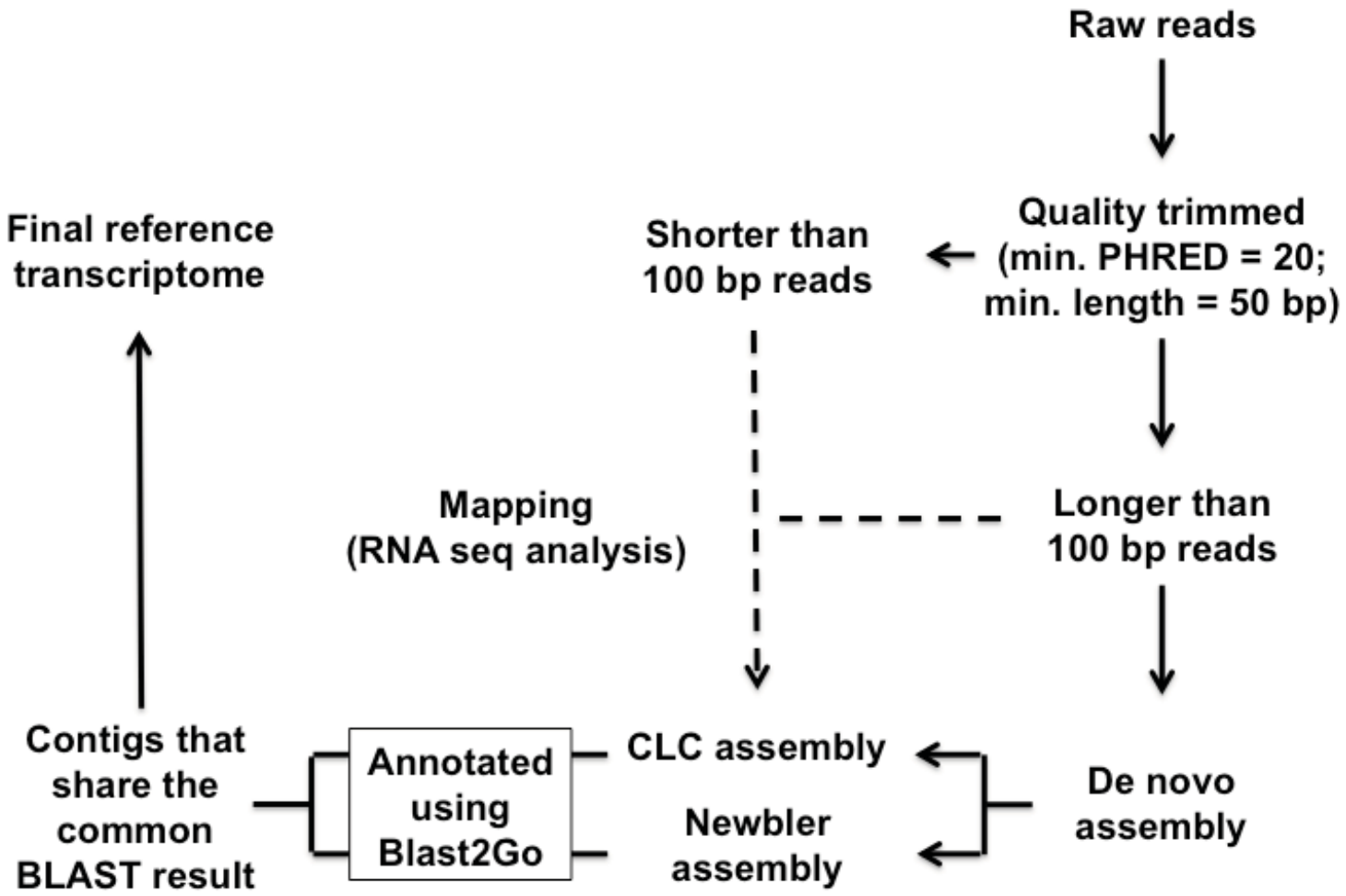
Raw reads
Quality trimmed
(min. PHRED = 20;
min. length = 50 bp)
Shorter than
100 bp reads
Final reference
transcriptome
Mapping
(RNA seq analysis)
Longer than
100 bp reads
Contigs that
share the
common
BLAST result
Annotated
using
Blast2Go
CLC assembly
Newbler
assembly
De novo
assembly

Figure 2.1 Flow chart of the deep sequencing analysis pipeline used in this study. The process of de novo assembly was represented in using the solid line, whereas the mapping process was represented using the dash line. Briefly, raw reads were subjected to trimming based on quality (min. PHRED score = 20), and length of the sequences (min. length = 50 bp). Then, all quality trimmed reads greater than 100 bp in length were used for de novo assembly using two different de novo assemblers: CLC Genomic workbench and Newbler assembler to generate CLC and Newbler assemblies respectively. All contigs generated from each assembler were subjected to BLASTx and annotated using Blast2GO software. Contigs that share the common BLAST result with the other assembly were chosen as a final reference transcriptome. The expression value of each gene was generated by mapping the quality trimmed reads to the assemblies generated from CLC Genomic Workbench and Newbler assemblers using their own mapping tools.

2.3 Result and Discussion

2.3.1 Roche 454 GS-Junior sequencing

We analysed the transcriptome of eggs from five strains of *H. armigera* using cDNA libraries prepared from poly-adenylated RNA from embryos of each strain that were deep sequenced using the 454 GS Junior platform. The material of interest was a *Bt*-susceptible and Cry1Ac-tolerant strain from the University of Adelaide, Waite Campus (hereafter Waite). We used *Bt*-susceptible, Cry1Ac-resistant, and Cry2Ab-resistant strains from CSIRO to generate transcriptome reference sets. Poly-adenylated RNA was prepared from embryos collected within 24 hrs after being laid. cDNA prepared from the two Waite strains was analysed on the same sequencing chip and discriminated using MID tags whereas the three CSIRO strains were each sequenced on individual chips. In brief, the total number of reads from all four runs was 394,630 (167.66 Mb; Table 2.2). After removing adaptor sequences and trimming poor quality bases (min. PHRED-score = 20, min. length = 100 bp), 394,611 separate reads (130.38 Mb) were used for *de novo* transcriptome assembly and RNA expression comparisons.

Each deep sequencing technique and assembly software package produces different biases in the data (Kumar & Blaxter 2010). Care is required to ensure that identified transcripts reflect actual biological, and are not artifacts of the sequencing or the analysis. Here, we utilised two *de novo* assemblers, CLC Genomic Workbench 6 and Newbler 2.7, and selected genes for further analysis that were consistent between them, with the aim of reducing the chance of analysing artificial transcripts (Figure 2.1). Even though these two assemblers employ different algorithms – the CLC Workbench uses the de Brujin graph approach (Miller, Koren & Sutton 2010), whereas Newbler 2.7 is designed for the relatively long 454 reads and uses an algorithm known as the overlap-layout consensus (OLC) strategy (Kumar & Blaxter 2010) – there is usually little difference in their output based on 454 data (Kumar & Blaxter 2010).

In our study, a reference transcriptome generated by CLC software contained 13,325 contigs (C_Contig) compared to only 5,786 contigs (N_Contig) generated using Newbler 2.7 (Table 2.3). This difference may reflect a number of factors, such as the assembly algorithms and assembly parameters. We found 537 and 25 contigs from the CLC and Newbler assemblies that could not be matched to raw reads used in contig assembly (Table 2.3), indicating that they might be artifacts, and thus, we discarded these sequences from further analyses.

To give a high degree of confidence in comparing gene expression of *H. armigera* strains, we focused on sequences that produced the same BLASTx results in both of the analyses. Some transcriptome information may have been lost during this process, but this conservative approach avoids false inclusion of genes into the *H. armigera* embryo transcriptome, particularly as there is no annotated *H. armigera* genome available as a reference.

The larger number of contigs in CLC assembly could possibly be due to a larger number of associated variant sequences produced by the lower threshold for mismatch within joined sequences, compared to the Newbler assembly, and the fact that the CLC algorithm does not recognise errors associated with homopolymer sequences present in 454 sequencing data. This is indicated by the slightly higher average number of sequences per BLAST match found in CLC assemblies (1.5 sequence per BLAST hit) compared to Newbler assembly (1.3 sequence per BLAST hit) (Table 2.3). However, it should be noted that the higher number of contigs could be true variants that were overlooked by the stringent Newbler parameters, or false variants generated due to the homopolymer errors.

The transcripts common to both assemblies were assigned Gene Ontology (GO) categories based on BLAST matches to proteins of known function. The distribution of the level 2 GO assignment for the *H. armigera* egg transcriptome was similar to larval transcriptomes of other lepidopterans such as *Plutella xylostella* and *Bombyx mori* (Etebari et al. 2011)

(Figure 2.2). The majority of sequences were assigned GO categories related to binding (70.6%) and catalytic functions (46.3%) (Figure 2.2).

To investigate the differences in the expression of immune genes between susceptible and induced tolerant eggs, sequences annotated with the gene ontology terms related to the innate immune system were selected. The gene ontology term (GO term or GO ID) describes the characteristics and functions of the genes (see Ashburner et al. (2000)). Each particular GO term is associated with a GO ID number. In our study, there are 57 non-redundant BLAST results with GO assignments involved with the innate immune system (GO:0002376). 19 sequences are hypothetical proteins. 38 genes were associated with immunity as shown in Table 2.4. However, these genes are not necessarily specific to the immune system, with many also considered to be involved in multiple pathways based on the other associated GO categories. For example, *dorsal* is known as a transcription factor activated by the Toll pathway and its activation leads to the production of antimicrobial peptides against gram-positive bacteria. However, dorsal is also a well known embryonic polarity gene and the Toll pathway is involved in dorsal-ventral patterning during embryonic development as well as immunity.

The top five species represented in the BLASTx searches were lepidopteran as expected (accounting for 98.3% of transcripts), which included *Helicoverpa armigera*, *Danaus plexippus*, *Papilio xuthus*, *Spodoptera frugiperda* and *Bombyx mori*. Transcript sequences that did not produce BLAST matches are possibly sequences spanning only untranslated mRNA regions or chimeric sequences derived from assembly errors. In addition, these sequences could also be novel genes unique to *H. armigera*.

2.3.2 Gene expression differences between susceptible and tolerant insects

To investigate whether there were differences in gene expression profiles between eggs produced by susceptible and tolerant parents, quality trimmed sequence data from eggs of

each were compared using CLC and Newbler software. This analysis was performed separately for each assembler using its own pipelines: CLC RNA-seq tool and Newbler GS Mapper (Figure 2.1). Since we were interested in the expression of known functional genes, we compared expression of the contigs producing the same BLAST result, regardless of whether two contigs were different isoforms of each other. Thus, for the presented analysis, we did not separate different gene isoforms (e.g. true single nucleotide polymorphisms or truncated versions). Putative transcripts of a given BLAST function, that differentially mapped to susceptible and tolerant contigs by a factor of at least 10, were chosen for further investigation. This amounted to 23 genes and 19 genes from the CLC and Newbler assemblies, respectively. However, we found that only 15 of these genes were common in both assemblies. Four sequences were hypothetical proteins, so 11 genes were chosen for further investigation (Table 2.5).

2.3.3 Quantitative PCR

Although we included more than 100 eggs per mRNA extraction to obtain an average representation off egg transcript expression, we could not perform enough replication for accurate statistical analysis on the deep sequencing data alone. For this reason, we also investigated the genes of interest using a robust quantitative method, qRT-PCR. We analysed the 10 genes that were expressed ≥ 10-fold differently by deep sequencing data analysis (Table 2.5). We compared transcript levels for these genes in susceptible versus tolerant *H. armigera* as a ratio of expression against two housekeeping genes: actin A3a and RPS15.

Only four genes showed at least two fold difference in expression levels between eggs of susceptible and tolerant *H. armigera* in qPCR: H2BB, TCTP, RACK-1 and GAPDH (Figure 2.3). Although relative expression levels of these 4 genes in the cDNA libraries used for deep sequencing showed the same trend by qPCR, the difference in expression levels indicated by qPCR were much less than indicated by the deep sequencing data

(Tables 2.5, 2.6 and Figure 2.3). For example, H2BB expression assessed from deep sequencing data indicated a level approximately 40-60 fold greater in tolerant eggs than susceptible eggs, but the qPCR result based on two different housekeeping genes showed only a four-fold increase in eggs produced by tolerant *H. armigera* (Figure 2.3). The magnitude of differences in H2BB expression as assessed by qPCR and deep sequencing data may reflect the preparation of the samples for 454 sequencing, including fragmentation and bead-based enrichment.

The four genes that clearly showed differential expression (H2BB, TCTP, RACK-1 and GAPDH) have been reported to be involved in transduction pathways activating the immune system. For example, TCTP functions to release histamines during allergic reactions in mammals (Bommer & Thiele 2004), while RACK-1 is a partner of phosphorylated protein C kinase, which is involved in many pathways including the JNK pathway, associated with activation of the innate immune response (Newton & Messing 2010). However, these two proteins are also involved in general stress responses (Bommer & Thiele 2004; Newton & Messing 2010). Thus, it is unclear whether the increase in the gene expression is in response to abiotic or biotic stress in this case.

The increased expression of H2BB, a variant of H2B type 3, found in eggs of *H. armigera* with induced tolerance to Cry1Ac, was consistent with a previous study on eggs of bacterial-exposed *Trichoplusia ni* (Freitak, Heckel & Vogel 2009b). Interestingly, H2B is also up regulated in response to heat stress in *Drosophila melanogaster* (Sanders 1981; Desrosiers & Tanguay 1986). As mentioned earlier, the immune and stress response pathway such as MAPK p38 and JNK were shown to be activated in response to *Bt* exposure (Huffman et al. 2004; Cancino-Rodezno et al. 2010), so it is possible that the increase in the expression of H2B might be involved with these two pathways. Histone proteins are relatively diverse, with a range of similar sub-types comprising histone families. They are modified by a range of enzymes to subsequently interact with an

organism's DNA to facilitate a range of processes (Hunt et al. 2013). Therefore, it is very difficult to separate potential roles in TGIP (tolerance development) and stress responses (symptoms of exposure), each of which could arise from epigenetic, maternally-biased transmission. Further experiments examining the expression of a range of histone proteins under various stresses would be useful in addressing this issue.

Interestingly, another proteomic study two genes displayed increased expression in of the midgut of *H. armigera* after being exposed to *Bt* toxins, V-type proton pumping ATPase and GAPDH (Yuan et al. 2010). This is similar to our findings, although our qPCR data showed that expression of V-type proton pumping ATPase was less than 2 fold higher in eggs from exposed parents (Table 2.5). It implies that it is possible that some changes in the gene expression in midgut in response to *Bt* toxin can be carried on into the eggs. However, the mechanism is still unclear.

GAPDH is a key enzyme in glycolysis, is generally thought to maintain a relatively constant expression level regardless of tissue or external stimuli, and has previously been used as a housekeeping gene for expression studies. However, GAPDH has been reported to express differently among different tissue types (Barber et al. 2005). For example, in humans, the expression of GAPDH in skeletal muscle can be 15-fold higher than in breast tissue. Again, the potential role, or symptomatic expression, of GAPDH in tolerance to *Bt* toxins remains unclear, as do links between expression of the genes in the midgut of the larvae, the resultant adults, and the eggs they produce, as a response to *Bt* exposure.

In conclusion, we have identified four genes that were differentially transcribed between eggs of susceptible and tolerant *H. armigera* populations. These genes could be directly related to the mechanism of *Bt* tolerance itself or may be involved in the mechanism of TGIP. Equally, they could merely be a symptomatic phenotype, a marker that the insect has been exposed to *Bt* toxins or a marker of a priming process (active defense mechanism). A combination of these scenarios is also possible. Further study should examine expression

of these genes in naïve and exposed larva, under a range of experimental conditions. Additionally, the effect of silencing these genes using RNAi at different life stages and exposure conditions should be examined. The role of epigenetic mechanisms in helping insects adapt to toxins and other environmental stresses is an emerging area of research, that is expected to have broad social, environmental and economic implications.

Table 2.2 Summary of 454 pyrosequencing data statistics for eggs from five strains of *H. armigera*

	Susceptible (Waite) SRR1013685*	Cry1Ac tolerant (Waite) SRR1015457	Susceptible (Narrabri) SRR1015458	Cry1Ac resistant (Narrabri) SRR1015459	Cry1Ac resistant (Narrabri) SRR1015461	Total
Raw reads	50,472	22,454	116,550	88,396	116,758	394,630
Raw bases	18,498,464	8,189,243	50,915,441	36,387,736	53,668,246	167,659,130
Average length before trimming	366.5	364.7	436.9	411.6	459.7	407.88
Trimmed reads	45,199	20,029	107,076	79,660	110,668	362,632
Trimmed bases	12,975,066	5,844,155	39,734,647	24,441,235	45,492,591	128,487,694
Average length for assembly	297.1	291.9	371.1	306.9	411.1	335.62

*Accession numbers of the raw reads on NCBI database

Table 2.3 Comparison of reference transcript sets generated from two assemblers: CLC Genomic Workbench 6 and Newbler 2.7.

Assembly	CLC Genomic Workbench 6	Newbler 2.7
Number of contigs	13,325	5,786
Total bases	10,866,999	5,816,378
Number of contigs (>= 1 kbp)	2,185	1,927
Total bases (in contigs >=1kbp)	5,062,264	3,282,254
Max contig length	10,347	10,340
Min contig length	200	91
Average contigs length	815.53	1005.25
N50	944	1134
Contigs without raw reads mapped	537	25
Blast results in each assembly		
Sequences produced BLAST hits (cut-off; E-value = E-25)	8,598 (64.5%)	4,355 (75.3%)
Sequences with no BLAST hit	4,727 (35.5%)	1,431 (24.7%)
Annotated sequences (cut off: GO weight = 5, Annotation cut off = 55, E-value > E-6)	5,417	3,484
Number of non-redundant BLAST hits	7,061	3,455
Blast results in common		
Sequences with BLAST results occurring in both assemblies	4,106	3,487
Number of non-redundant BLAST hits occurring in both assemblies	2,651	2,651

Table 2.4 A list of immune -related genes that were found expressed in the eggs from susceptible and inducible tolerant *H. armigera*

Accession no.	Protein	Species	Contig IDs	Nt length (bp)	E-value	Similarity (%)
BAM18063	26S proteasome non-ATPase regulartor subunit rpn 7	*P. xuthus*	N_Contig_01787; C_Contig_5854	1281; 1306	0; 0	96; 96
NP_001243977	Akirin protein	*B. mori*	N_Contig_01194; C_Contig_624	1978; 905	1.48E-77; 8.43E-82	84; 84
ABU94676	Alpha-tubulin	*X. cnigrum*	N_Contig_01528; C_Contig_151	1508; 1520	0; 0	100; 100
NP_001040336	ARP1 actin-related protein 1-like protein A	*B. mori*	N_Contig_03445; C_Contig_3674	754; 752	2.49E-151; 2.37-151	98; 98
ACI32826	Beta-1,3-glucan recognition protein 2a	*H. armigera*	N_Contig_01571; C_Contig_4969	1454; 1048	0; 0	98; 98
ACV86996	Cacophony, partial	*B. mori*	N_Contig_04631; C_Contig_5226	579; 889	3.1E-104; 3.9E-161	90; 91
AGB51151	CDT1	*B. mori*	N_Contig_01171; C_Contig_2879	2080; 2089	0; 0	86; 86
NP_001136443	Clathrin heavy chain	*B. mori*	N_Contig_01103; C_Contig_3326	2433; 405	0; 0	98; 98
EHJ77219	Cyclin D	*D. plexippus*	N_Contig_01277; C_Contig_4140	1826; 2391	9.5E-150; 2.8E-147	87; 87
AEO51736	Dorsal	*H. armigera*	N_Contig_02745; C_Contig_2809	893; 1215	0; 0	100; 98

N_Contigs and C_Contigs are contigs from Newbler and CLC assembly reference set.

Table 2.4 A list of immune -related genes that were found expressed in the eggs from susceptible and inducible tolerant *H. armigera* (cont.)

Accession no.	Protein	Species	Contig IDs	Nt length (bp)	E-value	Similarity (%)
BAM20653	Drumstick	*P. polytes*	N_Contig_04585; C_Contig_5583	584; 711	1.92E-49; 7.53E-49	100; 100
EHJ64394	Effete	*D. plexippus*	N_Contig_01306; C_Contig_45	1770; 1793	2.9E-99; 3.6E-99	100; 100
EHJ78851	Frizzled-2	*D. plexippus*	N_Contig_04055; C_Contig_3045	656; 844	1.19E-75; 1.12E-74	95; 95
ABU45982	G(alpha)q	*H. assulta*	N_Contig_01359; C_Contig_4529	1700; 1969	0; 0	100; 96
ADK55517	Heat shock protein 90 Cognate	*S. litura*	N_Contig_01123; C_Contig_1496	2359; 2490	0; 0	94; 94
EHB12217	Histone H4	*H. glaber*	N_Contig_04820; N_Contig_05666; C_Contig_248; C_Contig_247	552; 388; 348; 557	2.69E-49; 1.05E-49; 4.59E-50; 2.48E-49	100; 100; 100; 100
XP_001861658	Inosine-5'-monophosphate dehydrogenase	*C. quinquefasciatus*	N_Contig_01241; C_Contig_1473	1892; 2630	0; 0	91; 91
NP_001040383	Interleukin enhancer binding factor	*B. mori*	N_Contig_01741; C_Contig_1213	1308; 1368	7.15E-169; 0	95; 95

Table 2.4 A list of immune -related genes that were found expressed in the eggs from susceptible and inducible tolerant *H. armigera* (cont.)

Accession no.	Protein	Species	Contig IDs	Nt length (bp)	E-value	Similarity (%)
NP_001036922	MAP kinase-ERK kinase	*B. mori*	N_Contig_05044; C_Contig_3426	523; 425	4.73E-121; 7.01E-36	100; 98
NP_001139127	Oxygen resistance gene 1	*B. mori*	N_Contig_01635; C_Contig_8346	1400; 1653	0; 0	88; 88
EHJ70647	Poly	*D. plexippus*	N_Contig_03409; C_Contig_5477	761; 786	1.5E-86; 2.08E-86	73; 73
BAC02929	Proliferating cell nuclear antigen	*S. frugiperda*	N_Contig_01813; C_Contig_1362	1266; 2036	4.2E-173; 2.9E-169	99; 99
NP_001040459	Proteasome subunit alpha type 6-A	*B. mori*	N_Contig_02849; C_Contig_2811	869; 879	1.01E-146; 1.12E-146	95; 95
EHJ65913	Putative ETS-like protein pointed, isoform P1	*D. plexippus*	N_Contig_02889; C_Contig_8546	857; 863	2.22E-90; 2.38E-90	95; 95
EHJ76110	Putative fascin	*D. plexippus*	N_Contig_03320; C_Contig_1579	773; 809	5.2E-115; 9.2E-120	97; 97
EHJ73003	Putative fetal alzheimer antigen, falz	*D. plexippus*	N_Contig_01454; C_Contig_8855	1581; 2037	8.6E-166; 0	94; 94

N_Contigs and C_Contigs are contigs from Newbler and CLC assembly reference set.

Table 2.4 A list of immune -related genes that were found expressed in the eggs from susceptible and inducible tolerant *H. armigera* (cont.)

Accession no.	Protein	Species	Contig IDs	Nt length (bp)	E-value	Similarity (%)
CBH09282	Putative mitogen-activated protein kinase (MAPKK)	*H. melpomene*	N_Contig_03484; C_Contig_7735	747; 825	1.29E-76; 6.59E-91	96; 96
EHJ77267	Putative rac GTPase	*D. plexippus*	N_Contig_03014; C_Contig_1931	832; 892	3.2E-141; 6.8E-141	100; 100
EHJ71369	Putative ribosome biogenesis protein bop1	*D. plexippus*	N_Contig_02884; C_Contig_11533; C_Contig_11026	856; 879; 906	2.72E-67; 6.66E-67; 9.24E-78	
NP_001189459	Raf kinase, effector of Ras	*B. mori*	N_Contig_02448; C_Contig_2903	978; 424	0; 7.07E-72	92; 68
EHJ72273	Ras-like GTP-binding protein Rho1	*D. plexippus*	N_Contig_01235; C_Contig_1285	1907; 1566	3.3E-133; 1.1E-128	100; 100
NP_001037618	Ras-related GTP-binding protein Rab11	*B. mori*	N_Contig_01944; C_Contig_116	1194; 1937	3.03E-148; 1.21E-144	98; 98
EHJ65589	Ribosomal protein S6 kinase	*D. plexippus*	N_Contig_02343; C_Contig_3417	1019; 1068	1.29E-97; 7.9E-106	72; 73
AFR31806	Small G protein	*S. exigua*	N_Contig_01422; C_Contig_147	1607; 1664	5.9E-133; 1.1E-132	99; 99

Table 2.4 A list of immune -related genes that were found expressed in the eggs from susceptible and inducible tolerant *H. armigera* (cont.)

Accession no.	Protein	Species	Contig IDs	Nt length (bp)	E-value	Similarity (%)
EHJ75188	Small G protein ras	*D. plexippus*	N_Contig_01676; C_Contig_4294	1364; 1374	1.2E-111; 1.3E-111	94; 94
NP_001040215	Stathmin	*B. mori*	N_Contig_00397; N_Contig_00396; C_Contig_1173	3269; 3272; 1490	2.23E-173; 2.29E-173; 0	96; 96; 96
EAX04506	Ubiquitin B, isoform CRA_e	*H. sapiens*	N_Contig_05756; C_Contig_697; C_Contig_148	236; 303; 228	2.25E-48; 1.9E-64; 2.67E-46	100; 100; 100
EHJ63743	Ubiquitin-conjugating enzyme E2	*D. plexippus*	N_Contig_02775; C_Contig_1101	887; 1126	1.6E-112; 2.8E-111	100; 100

N_Contigs and C_Contigs are contigs from Newbler and CLC assembly reference set.

Table 2.5 Genes found to be expressed at least 10-fold higher in eggs from tolerant H. armigera compared to susceptible *H. armigera*, using deep sequencing data

Accession no.	Protein	Species	Contig IDs	Nt length (bp)	E-value	Sim. (%)	Fold differences*
EHJ66924	Putative histone cluster 3, H2bb	*D. plexippus*	N_Contig_00856; N_Contig_00857; N_Contig_00888; C_Contig_2370	542; 534; 501; 479	4.97E-50; 4.47E-50; 8.65E-54; 8.94E-49	88;88;89;91	41.21; 61.84
EHJ65991	Putative Serine/threonine-protein kinase 23	*D. plexippus*	N_Contig_01066; C_Contig_2058	2677; 1761	0; 2.17E-161	94; 93	29.77; 50.39
ADF45669	Translational controlled tumor protein	*H. armigera*	N_Contig_03283; C_Contig_2208	783; 787	3.74E-119; 3.86E-119	100; 100	24.04; 50.39
ADK94761	Vacuolar V-type H(+)-ATPase B subunit	*H. armigera*	N_Contig_01187; C_Contig_2899	2005; 2067	0; 0	100; 100	16.03; 20.61
AEC12435	Abnormal wing disc-like protein	*S. litura*	N_Contig_02076; C_Contig_228	1135; 790	6.36E-112; 1.04E-113	95; 95	16.03; 16.03
EHJ68971	Putative receptor for activated C kinase	*D. plexippus*	N_Contig_01441; C_Contig_1007	1594; 1722	0; 6.27E-166	91; 90	16.03; 13.74

Table 2.5 Genes found to be expressed at least 10-fold higher in eggs from tolerant *H. armigera* compared to susceptible *H. armigera*, using deep sequencing data (cont.)

Accession no.	Protein	Species	Contig IDs	Nt length (bp)	E-value	Sim. (%)	Fold differences*
BAM19296	Transcription factor btf3	*P. polytes*	N_Contig_01978; C_Contig_1121	1175; 1312	2.00E-84; 7.80E-84	98; 98	11.45; 13.74
EHJ66770	Protein aurora borealis	*D. plexippus*	N_Contig_01186; C_Contig_1966	2011; 2098	0; 1.84E-176	70; 70	13.74; 13.74
EHJ72081	Translation initiation factor eIF-2B subunit epsilon	*D. plexippus*	N_Contig_02429; C_Contig_2801	985; 990	4.47E-127; 2.25E-122	81; 81	11.45; 11.45
AEB26314	Glyceraldehyde-3-phosphate dehydrogenase	*H. armigera*	N_Contig_01826; C_Contig_389	1258; 1269	0; 0	99; 99	11.45; 11.45
EHJ66311	Putative striatin	*D. plexippus*	N_Contig_01831; N_Contig_03893; C_Contig_1564; C_Contig_2642	1255; 680; 1620; 1477	1.55E-86; 2.86E-108; 0; 7.75E-85	97; 86; 86; 97	0.094; 0.094

*For fold differences, the first value was calculated based on Newbler assembly whereas the second value was calculated based on CLC assembly. The fold difference = the gene expression value of eggs from tolerant *H. armigera*/the gene expression value of eggs from susceptible *H. armigera*.

Table 2.6 qRT-PCR validation of genes found to be differentially expressed between eggs from susceptible and Cry1Ac-tolerant *H. armigera* by 454 sequencing, as determined with the actin and RPS15 housekeeping genes.

Hit description	Fold difference in qPCR	
	Actin	RPS15
Putative histone cluster 3, H2bb	4.29	4.43
Putative serine/threonine- protein kinase 23	1.82	1.69
Translational controlled tumor protein	2.51	2.6
Vacuolar V-type H(+)-ATPase B subunit	1.29	1.21
Abnormal wind disc-like protein	1.46	1.36
Putative receptor for activated C kinase	2.76	2.57
Transcription factor btf3	1.33	1.24
Protein aurora borealis	1.58	1.7
Glyceraldehyde-3-phosphate dehydrogenase	3.12	2.91

Cellular component
Biological process
Molecular function
Newbler
CLC
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Cell
Macromolecular complex
Membrane
Organelle
Extracellular region
Synapse
Membrane-enclosed lumen
Cell junction
Cellular process
Metabolic process
Developmental process
Multicellular organismal process
Response to stimulus
Biological regulation
Localization
Signaling
Cellular component organization/biogenesis
Reproduction
Cell proliferation
Locomotion
Growth
Biological adhesion
Immune system process
Multi-organism process
Death
Rhythmic process
Pigmentation
Binding
Catalytic activity
Structural molecule activity
Nucleic acid binding transcription factor
Transporter activity
Enzyme regulator activity
Electron carrier activity
Molecular transducer activity
Receptor activity
Protein binding transcription factor activity

Figure 2.2 Graph of Level 2 Gene Ontology assignment of the final transcriptome data, generated from the sequences sharing the same BLAST results in Newbler and CLC assemblies.

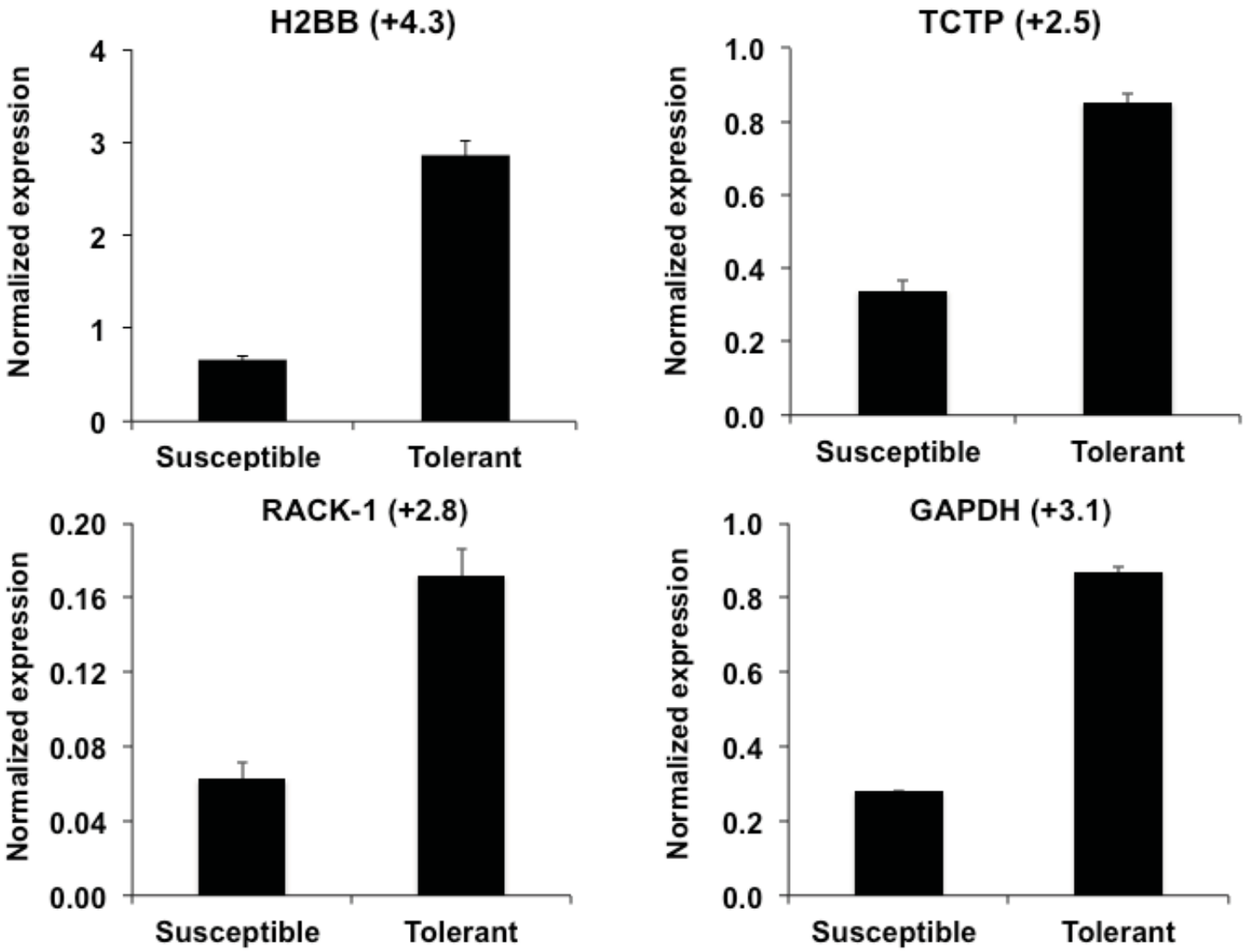
H2BB (+4.3)
Normalized expression
4
3
2
1
0
Susceptible
Tolerant
TCTP (+2.5)
Normalized expression
1.0
0.8
0.6
0.4
0.2
0.0
Susceptible
Tolerant
RACK-1 (+2.8)
Normalized expression
0.20
0.16
0.12
0.08
0.04
0.00
Susceptible
Tolerant
GAPDH (+3.1)
Normalized expression
1.0
0.8
0.6
0.4
0.2
0.0
Susceptible
Tolerant

Figure 2.3 qRT-PCR analysis of four selected H. armigera genes showing at least 2-fold differential expression between eggs produced by susceptible and tolerant populations: histone cluster 3 (H2BB), translationally controlled tumor proteins (TCTP), receptor for activated C kinase (RACK-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Error bars indicate standard error of means from two technical replicates. Fold changes are shown in brackets. The expression of these genes was normalised to the housekeeping gene, actin.

Chapter 3

Gene expression profile analysis of Cry1Ac-susceptible and Cry1Ac-resistant *Helicoverpa armigera*

3.1 Introduction

In the Australian cotton industry, toxins from *Bacillus thuringiensis* (*Bt* toxins) are widely used in transgenic cotton plants (GM *Bt* cotton) to control two main lepidopteran pests: *Helicoverpa armigera* and *Helicoverpa punctigera* (The Australian Cotton Industry Development & Delivery Team 2012). The toxins target the alkaline guts of larvae, form pores in midgut cells, and eventually kill the larvae by septicemia. The heavy usage of *Bt* toxins in agriculture strongly selects for pest insect populations in the field to overcome the effects of the toxin. This process eventually leads to the development of resistance populations in the field, a threat to *Bt* technology. In order to delay the development of resistance, a resistance management strategy, such as the use of refuge crops, trap crops, multiple insecticides, the regulation of planting windows and cultivation (pupae busting), has been employed. In addition, studies have been conducted to further understand the mechanism of resistance, which is crucial for planning the resistance management strategy (The Australian Cotton Industry Development & Delivery Team 2012).

The most common resistant mechanisms involve mutations in toxin receptors or enzymes that result in interruption of the functions of *Bt* toxins (Pardo-López, Soberón & Bravo 2013). A Cry1Ac-resistant strain, Bx, from CSIRO (Narrabri, NSW) has a resistance ratio of 5,386, and can survive on genetically modified cotton plants (Akhurst et al. 2003; Bird & Akhurst 2007). A high resistance ratio (>200) indicates that it is likely that there is a mutation that interrupts toxin action in the midgut (Tabashnik, Van Rensburg & Carrière 2009). However, a study using a toxin binding assay has shown that there was no loss in the toxin binding site in the midgut of *H. armigera* (Akhurst et al. 2003; Ma et al. 2005). Furthermore, in genetic analysis, researchers found no linkage group that can contribute more than 5% of the resistance in this strain (Karl Gordon, personal communication). Genetic crosses have shown that the resistance is the result of multiple genes and involves autosomal semi-dominance, unlike other known mutations that confer resistance which are

usually genetically recessive (Akhurst et al. 2003; Pardo-López, Soberón & Bravo 2013).

A study in our laboratory in 2005 demonstrated that a part of the resistance mechanism in this Cry1Ac resistant *H. armigera* could be a new mechanism which is related to the insect immune system (Ma et al. 2005). An earlier study of *Ephestia kuehniella* by our group demonstrated that susceptible larvae that have previously been exposed to a sub-lethal dose of *Bt* toxins (LC_{10}) became tolerant to a higher dose of *Bt* toxin in later instars (Rahman et al. 2004). This induced tolerance is correlated with an increase in phenoloxidase activity, an enzyme involved in the insect immune system, in hemolymph. Furthermore, the tolerance associated with the increase in the phenoloxidase activity can be transferred to the next generation via maternal effect. Interestingly, similar to induced tolerance, the midgut and the hemolymph of Cry1Ac-resistant *H. armigera*, mentioned earlier, also showed an increase in phenoloxidase activity (Ma et al. 2005). In addition, even though the resistance in *H. armigera* to Cry1Ac toxin is semi-dominant, the resistance of offspring from the resistant females was higher than the resistance of offspring from resistant males, indicating that a part of resistance is inherited via a maternal effect. The *Bt* tolerance developed in *E. kuehniella* showed many similarities to the Cry1Ac-resistant strain of *H. armigera*, but the Cry1Ac-resistant strain is highly resistance to Cry1Ac toxins (>200), whereas induced tolerant *E. kuehniella* showed a low tolerance to *Bt* toxins (<200). However, because of the similarities between these two strains, it is possible that they might partly share the same resistance mechanism .

The mechanism of resistance is still unclear. Ma et al., (2005) showed that the concentration of immune-related protein, hexamerin, was higher in this Cry1Ac-resistant strain compared to the susceptible strain. Hexamerin is a lipophorin. It functions as a lipid storage molecule, an immune protein and also a pro-coagulant (Scherfer et al. 2004). It has been shown to be able to bind to Cry1Ac toxin and form a coagulate (Ma et al. 2005). It was speculated that hexamerin can be expressed in the midgut of the Cry1Ac-resistant

strain, bind to Cry1Ac toxin and sequester the toxin by forming a coagulate. However, it is unclear whether up-regulated expression of hexamerin and/or other immune proteins are maternally transferred to the next generation.

A maternal effect is an epigenetic phenomenon where an individual's phenotype is affected by the environment experienced by its mother. Several studies have shown that the immune status of individuals can be affected by the pathogens experienced in the maternal generation (Little et al. 2003; Moret 2006; Zanchi et al. 2012). In vertebrates, the transfer of immunity via a maternal effect has been demonstrated to be via antibodies (Grindstaff, Brodie & Ketterson 2003). However, in invertebrates, the mechanisms of maternal transmission of immunity remain unclear.

This chapter focuses on identifying genes that might be involved in the transmission mechanism of immune-related resistance in the Cry1Ac-resistant (Bx) strain of *H. armigera*. The aim of this chapter is to investigate the gene expression profiles of Cry1Ac resistance strains that have been previously reported to have immune-related induced tolerance, using the same transcriptomic data reported in Chapter 2. Unlike induced tolerant *H. armigera*, the Cry1Ac-resistant strain was selected with a high dose of Cry1Ac toxin (LC_{70}, and then LC_{30}), and is highly resistant, even to GM *Bt* cotton, Ingard® (Monsanto, USA). In addition, the mechanism of resistance is partly due to mutations in the nucleotide sequences of multiple genes, not only the changes in expression of the immune genes. Because a part of tolerance is inherited via a maternal effect, it is possible that there is an overlap in differentially expressed genes between eggs from Cry1Ac-resistant and Cry1Ac-tolerant females. Here, we identified the genes that were expressed differently between Cry1Ac-susceptible and Cry1Ac-resistant strains. We also investigated changes in the immune genes expressed in these two types of eggs. In addition, we tested whether four genes found to have different express levels between susceptible and

Cry1Ac-tolerant *H. armigera* (Chapter 2) were expressed in the same pattern in the Cry1Ac resistant strain.

3.2 Method

Using the raw transcriptome data reported in Chapter 2, quality trimmed reads from Cry1Ac-susceptible and Cry1Ac-resistant strains were mapped to a reference assembly generated using CLC Genomic Workbench 6 and Newbler 2.7, using CLC RNA-seq tool and Newbler reference mapper, respectively (Chapter 2, Figure 2.1 flowchart). Both references were compared against the NCBI database using BLASTx, and were GO annotated using Blast2GO software as described in Chapter 2. As per the established workflow, only two or more contigs generated by two independent assemblers (CLC and Newbler), which shared the same BLAST result, were further investigated for expression values.

For RNA sequencing analysis, expression values for each gene were calculated as the number of reads per kilobase pair per million mapped reads (RPKM) in CLC Genomic Workbench 6, and as a percentage of reads per the total number of reads in Newbler. Expression values for contigs that were derived from the same gene (i.e. contigs that share the same BLAST result) were combined as an expression value of that gene. Genes that had at least a ten-fold difference in the expression values between Cry1Ac-susceptible and Cry1Ac-resistant strains, were categorised as possible differentially expressed genes (DEGs).

3.3 Results and Discussion

4,180 contigs from Newbler assemblies which has 3,395 non-redundant BLAST hits, and 7,908 contigs from CLC assemblies with 5,967 non-redundant (nr) BLAST results were found in Cry1Ac-susceptible and Cry1Ac-resistant eggs. Only 2,966 nr BLAST results are common between these two assemblies, which were used for further analysis.

The Cry1Ac-resistant strain was described by Ma et al., (2005). It has the immune-related induced tolerance, and part of the tolerance is maternally transmitted. Therefore we first investigated the immune genes found in the susceptible and resistant strains. From 2,966 nr BLAST results, 55 genes are annotated with GO term "innate immune system" (GO:0002376) and expressed in susceptible or Cry1Ac-resistant strains (Table 3.1). None of these immune genes were expressed differently between susceptible and Cry1Ac-resistant eggs. However, it is interesting that the transcripts of putative ribosome biogenesis bop1 (EHJ71396) were only detected in 9-10 reads in Cry1Ac-resistant eggs, but not any in susceptible eggs. This difference suggests that the expression of this gene functionally differs between susceptible and Cry1Ac-resistant eggs, but this requires further analysis. Ribosome biogenesis protein bop1 is an enzyme involved in the synthesis of ribosomes - a cellular activity which is highly active during cell growth and cell division, and which can also be activated as a part of a stress response (Deisenroth & Zhang 2010).

Next, we measured the expression of four genes (H2BB, TCTP, RACK-1 and GAPDH) that were previously shown to be expressed differently between eggs of Cry1Ac-susceptible and Cry1Ac-tolerant *H. armigera* (Chapter 2). In contrast to the previous comparison, there were no differences in the expression levels of these four genes between the susceptible and resistant strains. However, there were different sets of 21 contigs from CLC and 44 contigs from Newbler assemblies that were expressed 10 fold differently between the strains. We then compared BLAST results obtained for each of these sequences and found that only five contigs from each list shared the same non-redundant BLAST Hit between these two assemblies (Table 3.2). One of the contigs is a hypothetical protein (EHJ70627) with an unknown function. The other four genes are pyruvate kinase, olfactory receptor 29, proteasome 25 kDa subunit, and transmembrane protein (Table 3.2). It is not clear if there is a link between the higher expression of these four genes and Cry1Ac-resistance. These genes are not related to the immune system or pesticide

resistance, and their functions involve housekeeping metabolic pathways. For example, pyruvate kinase functions in turning phosphatepyruvate into pyruvate in glycolysis, which is a part of cellular respiration. Olfactory receptor 29 is a member of the olfactory receptor family, a transmembrane protein that functions as a chemosensor in olfactory epithelium cells in response to odors. However, olfactory receptors also function in response to other ligands in non-olfactory tissues. For example, in mammals, olfactory receptors expressed in kidney tissue are responsible for regulating the secretion of rennin, which leads to the regulation of blood pressure in response to gut microbiota (Pluznick et al. 2013). In addition, the olfactory proteins are also functionally involved in chemotaxis leading sperm to ova in mammals (Spehr et al. 2006). Proteasomes are multi-subunit proteolytic complexes involved in non-lysosomal protein degradation in the cytosol and nucleus of cells (Rivett 1993). Finally, transmembrane 9 superfamily member 2 protein (TM9SF2) is reported to be expressed in early endosomes. The protein contains nine transmembrane domains and it probably functions in transporting small molecules across membranes, or acts as an ion channel (Schimmöller et al. 1998).

In summary, even though Cry1Ac tolerant and Cry1Ac resistant strains both showed the increase in the immune activity associated with the increase level of tolerance to *Bt* toxins, and the trait was maternally transmitted, the gene expression profile of eggs from a high dose selected Cry1Ac-resistant strain of *H. armigera* was different from that of a low-dose selected Cry1Ac tolerant strain. It is possible that the difference in the levels of the selection pressure lead to the activation of different mechanisms that can lead to the same outcome, the observed increase in the immune activity. At the moment, it is unclear whether the immunity plays any common role in the development of both tolerance and resistance. Further analysis is needed to understand the function of these genes in the immune system.

Table 3.1 A list of immune-related genes that were found expressed in the eggs from susceptible and Cry1Ac-resistance *H. armigera*

Accesion no.	Proteins	Species	Contig ID	Nt. Length (bp)	E-value	% Sim.
ABU45982	G(alpha)q	*H. assulta*	N_Contig_01359; C_Contig_4529	1700; 1969	0; 0	100; 96
ABU94676	Alpha-tubulin	*X. cnigrum*	N_Contig_01528; C_Contig_151	1508; 1520	0; 0	100; 100
ACI32826	Beta-1,3-glucan recognition protein 2a	*H. armigera*	N_Contig_01571; C_Contig_4969	1454; 1048	0; 0	98; 98
ACV86996	Cacophony, partial	*B. mori*	N_Contig_04631; C_Contig_5226	579; 889	3.13E-104; 3.87E-161	90; 91
ADK55517	Heat shock protein 90 cognate	*S. litura*	N_Contig_01123; C_Contig_1496	2359; 2490	0; 0	94; 94
AEE62668	Unknown	*D. ponderosae*	N_Contig_01075; C_Contig_1302	2558; 2019	1.09E-82; 4.13E-84	88; 88
AEO51736	Dorsal	*H. armigera*	N_Contig_02745; C_Contig_2809	893; 1215	0; 0	100; 98
AFR31806	Small G protein	*S. exigua*	N_Contig_01422; C_Contig_147	1607; 1664	5.90E-133; 1.08E-132	99; 99
AGB51151	CDT1	*B. mori*	N_Contig_01171; C_Contig_2879	2080; 2089	0; 0	86; 86
BAC02929	Proliferating cell nuclear antigen	*S. frugiperda*	N_Contig_01813; C_Contig_1362	1266; 2036	4.15E-173; 2.88E-169	99; 99

Table 3.1 A list of immune-related genes that were found expressed in the eggs from susceptible and Cry1Ac-resistance *H. armigera* (cont.)

Accesion no.	Proteins	Species	Contig ID	Nt. Length (bp)	E-value	% Sim.
ABU45982	G(alpha)q	*H. assulta*	N_Contig_01359; C_Contig_4529	1700; 1969	0; 0	100; 96
ABU94676	Alpha-tubulin	*X. cnigrum*	N_Contig_01528; C_Contig_151	1508; 1520	0; 0	100; 100
ACI32826	Beta-1,3-glucan recognition protein 2a	*H. armigera*	N_Contig_01571; C_Contig_4969	1454; 1048	0; 0	98; 98
ACV86996	Cacophony, partial	*B. mori*	N_Contig_04631; C_Contig_5226	579; 889	3.13E-104; 3.87E-161	90; 91
ADK55517	Heat shock protein 90 cognate	*S. litura*	N_Contig_01123; C_Contig_1496	2359; 2490	0; 0	94; 94
AEE62668	Unknown	*D. ponderosae*	N_Contig_01075; C_Contig_1302	2558; 2019	1.09E-82; 4.13E-84	88; 88
AEO51736	Dorsal	*H. armigera*	N_Contig_02745; C_Contig_2809	893; 1215	0; 0	100; 98
AFR31806	Small G protein	*S. exigua*	N_Contig_01422; C_Contig_147	1607; 1664	5.90E-133; 1.08E-132	99; 99
AGB51151	CDT1	*B. mori*	N_Contig_01171; C_Contig_2879	2080; 2089	0; 0	86; 86
BAC02929	Proliferating cell nuclear antigen	*S. frugiperda*	N_Contig_01813; C_Contig_1362	1266; 2036	4.15E-173; 2.88E-169	99; 99

Table 3.1 A list of immune-related genes that were found expressed in the eggs from susceptible and Cry1Ac-resistance *H. armigera* (cont.)

Accesion no.	Proteins	Species	Contig ID	Nt. Length (bp)	E-value	% Sim.
EHJ65465	Hypothetical protein KGM_05646	*D. plexippus*	N_Contig_01431; N_Contig_01790; C_Contig_701	1599; 1280; 3027	3.87E-132; 4.85E-114; 0	98; 76; 85
EHJ65589	Ribosomal protein S6 kiinase	*D. plexippus*	N_Contig_02343; C_Contig_3417	1019; 1068	1.29E-97; 7.94E-106	72; 73
EHJ65913	Putative ETS-like protein pointed, isoform P1	*D. plexippus*	N_Contig_02889; C_Contig_8546	857; 863	2.22E-90; 2.38E-90	95; 95
EHJ67366	Hypothetical protein KGM_19207	*D. plexippus*	N_Contig_01839; C_Contig_254	1253; 2600	0; 0	95; 97
EHJ67415	Hypothetical protein KGM_12069	*D. plexippus*	N_Contig_01801; C_Contig_3546	1272; 2265	0; 0	99; 79
EHJ67986	Hypothetical protein KGM_08436	*D. plexippus*	N_Contig_01687; C_Contig_4907; C_Contig_8106	1351; 1369; 1311	2.50E-140; 1.45E-142; 0	75; 74; 99
EHJ68365	Hypothetical protein KGM_14915	*D. plexippus*	N_Contig_03334; C_Contig_10711	772; 940	2.11E-158; 8.92E-159	93; 94
EHJ69387	Hypothetical protein KGM_05975	*D. plexippus*	N_Contig_00642; N_Contig_02512; C_Contig_3165	1205; 958; 1361	1.92E-124; 3.23E-117; 2.74E-155	77; 95; 78
EHJ69388	Hypothetical protein KGM_05974	*D. plexippus*	N_Contig_03507; C_Contig_110	744; 921	2.65E-102; 7.70E-129	89; 91

Table 3.1 A list of immune-related genes that were found expressed in the eggs from susceptible and Cry1Ac-resistance *H. armigera* (cont.)

Accesion no.	Proteins	Species	Contig ID	Nt. Length (bp)	E-value	% Sim.
EHJ70633	Hypothetical protein KGM_15038	*D. plexippus*	N_Contig_02634; C_Contig_3620	923; 936	8.53E-165; 1.99E-169	95; 95
EHJ70647	Poly	*D. plexippus*	N_Contig_03409; C_Contig_5477	761; 786	1.50E-86; 2.08E-86	73; 73
EHJ71249	Hypothetical protein KGM_08605	*D. plexippus*	N_Contig_03723; C_Contig_10137	703; 739	2.86E-127; 2.92E-134	88; 89
EHJ71369	Putative ribosome biogenesis protein bop1	*D. plexippus*	N_Contig_02884; C_Contig_11026; C_Contig_11533	856; 906; 879	2.72E-67; 9.24E-78; 6.66E-67	79; 79; 87
EHJ72273	Ras-like GTP-binding protein Rho1	*D. plexippus*	N_Contig_01235; C_Contig_1285	1907; 1566	3.27E-133; 1.06E-128	100; 100
EHJ72442	Hypothetical protein KGM_09339	*D. plexippus*	N_Contig_00760; C_Contig_888	892; 1210	9.07E-60; 1.78E-58	82; 82
EHJ73003	Putative fetal alzheimer antigen, flaz	*D. plexippus*	N_Contig_01454; C_Contig_8855	1581; 2037	8.64E-166; 0	94; 94
EHJ75049	Hypothetical protein KGM_19145	*D. plexippus*	N_Contig_01127; C_Contig_3826	2339; 2921	6.31E-96; 2.20E-94	84; 84
EHJ75188	Small G protein ras	*D. plexippus*	N_Contig_01676; C_Contig_4294	1364; 1374	1.18E-111; 1.30E-111	94; 94
EHJ75874	Hypothetical protein KGM_06159	*D. plexippus*	N_Contig_00628; N_Contig_00629; C_Contig_4966	1693; 848; 1723	3.32E-127; 7.12E-114; 3.41E-127	97; 88; 97

Table 3.1 A list of immune-related genes that were found expressed in the eggs from susceptible and Cry1Ac-resistance *H. armigera* (cont.)

Accesion no.	Proteins	Species	Contig ID	Nt. Length (bp)	E-value	% Sim.
EHJ76110	Putative fascin	*D. plexippus*	N_Contig_03320; C_Contig_1579	773; 809	5.16E-115; 9.19E-120	97; 97
EHJ77219	Cyclin D	*D. plexippus*	N_Contig_01277; C_Contig_4140	1826; 2391	9.47E-150; 2.77E-147	87; 87
EHJ77267	Putative rac gtpase	*D. plexippus*	N_Contig_03014; C_Contig_1931	832; 892	3.19E-141; 6.82E-141	100; 100
EHJ78257	Hypothetical protein KGM_11959	*D. plexippus*	N_Contig_01776; C_Contig_1788	1290; 1485	0; 2.98E-142	98; 99
EHJ78283	Hypothetical protein KGM_17559	*D. plexippus*	N_Contig_02761; C_Contig_3734	889; 1435	4.04E-124; 2.26E-121	100; 100
EHJ78757	Hypothetical protein KGM_11850	*D. plexippus*	N_Contig_01048; C_Contig_24	2905; 2949	0; 0	86; 86
EHJ78851	Frizzled-2	*D. plexippus*	N_Contig_04055; C_Contig_3045	656; 844	1.19E-75; 1.12E-74	95; 95
EHJ78854	Hypothetical protein KGM_10325	*D. plexippus*	N_Contig_00383; N_Contig_00384; N_Contig_00385; C_Contig_810	1503; 1486; 1115; 2656	4.22E-95; 2.90E-94; 3.66E-99; 3.10E-90	76; 61; 73; 61
NP_001036922	MAP kinase-ERK kinase	*B. mori*	N_Contig_05044; C_Contig_3426	523; 425	4.73E-121; 7.01E-36	100; 98
NP_001037618	Ras-related GTP-binding protien Rab11	*B. mori*	N_Contig_01944; C_Contig_116	1194; 1937	3.03E-148; 1.21E-144	98; 98

Table 3.1 A list of immune-related genes that were found expressed in the eggs from susceptible and Cry1Ac-resistance *H. armigera* (cont.)

Accesion no.	Proteins	Species	Contig ID	Nt. Length (bp)	E-value	% Sim.
NP_001040215	Stathmin	*B. mori*	N_Contig_00396; N_Contig_00397; C_Contig_1173	3272; 3269; 1490	2.29E-173; 2.23E-173; 0	96; 96; 96
NP_001040336	ARP1 actin-related protein 1-like protein A	*B. mori*	N_Contig_03445; C_Contig_3674	754; 752	2.49E-151; 2.37E-151	98; 98
NP_001040383	Interleukin enhancer binding factor	*B. mori*	N_Contig_01741; C_Contig_1213	1308; 1368	7.15E-169; 0	95; 95
NP_001040459	Proteasome subunit alpha type 6-A	*B. mori*	N_Contig_02849; C_Contig_2811	869; 879	1.01E-146; 1.12E-146	95; 95
NP_001136443	Clathrin heavy chain	*B. mori*	N_Contig_01103; C_Contig_3326	2433; 405	0; 7.54E-51	98; 100
NP_001139127	Oxygen resistance gene 1	*B. mori*	N_Contig_01635; C_Contig_8346	1400; 1653	0; 0	88; 88
NP_001243977	Akirin protein	*B. mori*	N_Contig_01194; C_Contig_624	1978; 905	1.48E-77; 8.43E-82	84; 84
XP_001861658	Inosine-5'-monophosphate dehydrogenase	*C. quinquefasciatus*	N_Contig_01241; C_Contig_1473	1892; 2630	0; 0	91; 91

*N_Contigs and C_Contigs are from the Newbler and CLC assembly reference set.

Table 3.2 Genes that are expressed at least 10 fold higher in eggs of Cry1Ac-resistant *Helicoverpa armigera*, compared to eggs from Cry1Ac-susceptible *H. armigera*

Accession no.	Protein	Species	Contig ID*	Nt. (bp)	E-value	Sim %	Fold diff.**
BAM18144	Pyruvate kinase	*P. xuthus*	C_Contig_1626; N_Contig_01435	1751; 1599	0; 0	95; 95	39.22; 35.10
EHJ78030	Olfactory receptor 29	*D. plexippus*	C_Contig_3584; N_Contig_01981	1210; 1171	4.09E-169; 3.14E-169	85; 85	16.23; 16.85
EHJ74121	Proteasome 25 kDa subunit	*D. plexippus*	C_Contig_10767; N_Contig_03845	653; 687	1.57E-158; 8.81E-168	99; 99	13.52; 15.45
XP_624833	Predicted: transmembrane 9 superfamily member 2-like isoform 1	*A. mellifera*	C_Contig_3319; N_Contig_03235	865; 792	2.38E-100; 5.97E-99	79; 79	10.82; 11.23

*N_Contigs and C_Contigs are from the Newbler and CLC assembly reference set.

**For fold differences, the first value was calculated based on Newbler assembly whereas the second value was calculated based on CLC assembly. The fold difference is the gene expression value of eggs from Cry1Ac-tolerant *H. armigera*/the gene expression value of eggs from Cry1Ac-susceptible *H. armigera*.

Chapter 4

Effect of induced tolerance to *Bt* toxin on the egg size of *Helicoverpa armigera* and parasitism by *Trichogramma pretiosum*

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Title: Effect of induced tolerance to *Bt* toxin on the egg size of *Helicoverpa armigera* and parasitism by *Trichogramma pretiosum*

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Abstract

Insecticidal crystal toxins produced by *Bacillus thuringiensis* (*Bt*) have long been utilised for control of larvae of lepidopteran pests. The expression of *Bt* toxins is one of the most common traits expressed in genetically modified crops, particularly *Bt* cotton, which is utilised widely in Australian production systems to control noctuid pests. *Bt* cottons are utilised as part of an integrated suite of pest management measures, including the use of egg and larval parasitoids, and refuge plantings that do not contain the toxin genes. Larval *Helicoverpa armigera* can develop a form of *Bt* tolerance that is produced by exposure of to sub-lethal doses of *Bt*-toxin subclass Cry1Ac. Increasing levels of tolerance are produced over generations of larval exposure, which is not related to DNA sequence changes, and is largely maternally transmitted. The characteristic of maternal transmission, combined with the importance of egg parasitoids to cotton pest management, raised questions as to the effects of *Bt* tolerance/exposure on the egg size of *H. armigera* and on some key metrics of egg parasitism. In this study, we investigated the effect of inducible tolerance on eggs of *H. armigera* and parasitism by *Trichogramma pretiosum*. To do this, we first measured the volume of the eggs laid by susceptible and tolerant females. Then, we investigated the effect of inducible tolerance on egg parasitism by measuring parasitism success, the number of adult wasps emerged per host egg, and the proportion of male and female offspring emerged per host egg. The results showed that Cry1Ac tolerance was associated with increased egg volume. This effect was significant after just one generation of sub-lethal exposure. When tolerant *H. armigera* (F_{24} generation *Bt*-exposed) were freed from ongoing sub-lethal exposure, a corresponding decrease in egg volume was not detected. Although there was no difference in the percentage of eggs parasitised, there was a significant increase in the number of emergent parasitoids, especially male offspring, in eggs laid by tolerant *H. armigera*. These results confirm that maternally-transmitted *Bt*

tolerance is reflected in the phenotype of the eggs of tolerant offspring, which affects egg parasitism.

4.1 Introduction

Toxins extracted from various strains of the gram positive bacterium *Bacillus thuringiensis* (known as *Bt* toxins) have been widely used to control pest insect populations since 1977 (Schnepf et al. 1998). The bacterium was first identified as an agent causing insect disease by (Ishiwata 1901), and was more fully characterised by (Berliner 1915). During sporulation, *B. thuringiensis* produces a crystal protoxin (Cry toxin) which is toxic to selected groups of insects. After ingestion by a susceptible insect, Cry toxins are solubilised and proteolytically processed into activated toxins, which interact with midgut cell membrane proteins and form pores in epithelial cells (Pardo-López, Soberón & Bravo 2013). This allows gut contents to leak into the insect's haemolymph, which results in starvation, sepsis and death. There are four recognised subtypes of *Bt* toxins, producing about 70 types of Cry toxins (George & Crickmore 2012). Different types of Cry toxins kill specific groups of invertebrates including lepidopteran, coleopteran, dipteran and nematode species. This specificity, combined with being harmless to vertebrates, has seen the toxins become widely used in agriculture, including in organic farming (Zehnder et al. 2007). Economically important crops have been genetically modified to express *Bt* toxins, including cotton, maize (Gómez-Barbero, Berbel & Rodríguez-Cerezo 2008), tobacco, rice and tomato (George & Crickmore 2012). In Australia, almost 100% of commercial cotton crops are genetically modified varieties that express *Bt* toxins (Cotton Australia n.d.), mainly targeting larvae of the heliothine moths *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae).

There is a concern that pest insects can evolve resistance to *Bt* toxins because their use places selection pressure on pest populations. The mechanisms that confer resistance to *Bt* toxins generally relate to specific mutations within key genes. The products of these genes

activate the ingested protoxins (e.g. proteases) or subsequently interact with the toxins (e.g. cell receptor proteins) (Knight, Carroll & Ellar 2004; Gahan et al. 2005). Mutations that reduce toxin-receptor interactions generally produce high-levels of resistance relatively quickly, as they significantly reduce pore-formation and provide a huge selective advantage when exposed to Cry toxins. For example, mutations in *Bt* receptors, such as cadherin-like-receptors (Morin et al. 2003; Xu, Yu & Wu 2005; Yang et al. 2007; Zhang et al. 2012) and aminopeptidase N, can confer high levels of resistance (Guo et al. 2009).

Low level *Bt* tolerance can also develop independently of the mutation-based mechanisms of resistance. Studies of *Ephestia kuehniella* (Lepidoptera: Pyralidae) and *H. armigera* demonstrated that susceptible laboratory populations expressed *Bt* tolerance following continual inter-generational exposure to low concentrations of *Bt* toxin (Rahman et al. 2004; Ma et al. 2005). When neonate larvae were fed with a concentration of *Bt* toxins that normally kill 10-15% of the susceptible population, the surviving larvae subsequently exhibited tolerance to higher concentrations of *Bt* toxins in their later larval stages. These experiments suggested that the development of *Bt* tolerance was not related to the rare *Bt* resistance, as it was not a high dose selection and resistance alleles were not known to be present (Rahman et al. 2004). The novel expression of tolerance was correlated with an increased rate of melanisation in plasma and midgut extracts from toxin-exposed larvae (Ma et al. 2005). These studies also showed that the level of tolerance increased over generations of exposure. Reciprocal crosses between susceptible and tolerant populations indicated that the offspring from tolerant mothers were more highly tolerant compared to the offspring from susceptible mothers (Rahman et al. 2004; Ma et al. 2005). These results implied that the tolerance trait is transmitted mainly from mothers to offspring, a phenomenon known as a maternal effect. However, the mechanism of inducible tolerance to *Bt* toxins still remains unknown.

Hymenopteran parasitoids are widely used in pest management, and provide complementary control of insecticide resistant insects (Bates et al. 2005). *Trichogramma* spp. are egg parasitoids that are used in conjunction with GM crops to control *H. armigera* (Davies et al. 2011). A few studies have shown that there is no negative effect of *Bt* toxin exposure on parasitism by *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) of the eggs of hosts that survived *Bt* exposure (Wang et al. 2007; Wang et al. 2012) However, Steinbrecher (2004) found that parasitism success of *Trichogramma brassicae* was low in the eggs of *H. armigera* that survived exposure to *Bt* maize. It was not clear what the cause was. But it could be explained by many factors, such as a reduction in egg quality (e.g., size, nutritional value), parasitoid recognition by the host's immune system, or lower acceptance of host eggs. Steinbrecher suggested that it was due to low nutritional value.

A previous study investigating the effect of induced tolerance to *Bt* toxins in *E. kuehniella* on the larval parasitoid *Venturia canescens* (Hymenoptera: Ichneumonidae) indicated that there was no effect of induced tolerance on parasitism success (Rahman, Roberts & Schmidt 2004). However, the study did show that the wasps take longer to develop in induced tolerant hosts than in susceptible hosts, which the authors suggested could be due to partial inhibition of parasitoid growth in the tolerant host. Interestingly, the adult wasps that emerged from small tolerant larvae (young; 10 mg) were larger compared to the ones that emerged from susceptible hosts. However, this host-dependent size difference in the adult wasps disappeared in large larvae (older; 50 mg) (Rahman, Roberts & Schmidt 2004).

Here, we investigate the effects of inducible tolerance to *Bt* toxins on eggs of *H. armigera*, and on their parasitism by *Trichogramma pretiosum*. The maternal effect associated with the vertical transmission of *Bt* tolerance suggested that it may affect the size and quality of eggs, which could affect egg parasitoids like *T. pretiosum.* We compared parasitism of eggs from induced tolerant and susceptible laboratory cultures of *H. armigera.* To do this,

we assessed parameters relating to parasitism success, including the number of eggs being successfully parasitised and the number of progeny produced per host egg.

4.2 Materials and Methods

4.2.1 Cry1Ac toxin suspension

A bulk crude toxin suspension (bacterial lysate containing Cry1Ac toxin) produced from *Bacillus thuringiensis* strain *kurstaki* HD73 was supplied by John L. Reichelt (Bacterial Fermentaion, Ltd, Pty., Arundale, Queensland, Australia), and was prepared and assessed as described by Rahman et al. (2011). The suspension was stored at −20°C and was thawed before dilution with sterile MQ water to produce a 20 mg/ml protein suspension, which was stored for short periods at 4°C prior to use.

4.2.2 Susceptible and *Bt*-tolerant cultures of *Helicoverpa armigera*

The culture of *H. armigera* used in this study was descended from the culture as described in Rahman et al. (2011). The susceptible culture of *H. armigera* was established by combining moths from the ANGR strain (Ma et al. 2005) and a field strain collected in Queensland, Australia (provided by CSIRO, Narrabri, New South Wales, Australia). The cultures were maintained at 25 ± 1°C with a photoperiod of 14:10 h [light/dark]. Larvae were fed in 45 well plastic trays, with one larva per well containing an artificial diet (2 mL per well). The diet was composed of 8.13% soy flour, 3.75% wheat germ, 3.31% brewing yeast, 1.25% agar, 0.21% ascorbic acid, 0.21% methyl paraben and 0.11% sorbic acid, mixed in water. Adult moths were held in a 5 l container that had its lid cut to allow light to enter. A sheet of polyester polar fleece (325 mm x 230 mm) was used to line the top of the container for deposition and subsequent collection of eggs. Adult moths were fed with a 20 g/l honey solution on a cotton wick.

A Cry1Ac tolerant strain of *H. armigera* was produced in 2009 (Rahman et al. 2011) by keeping the susceptible insects on a diet surface overlaid by a low (sub-lethal) dose of

Cry1Ac toxin (0.1 mg/ml crude suspension), through the larval stages. During establishment of the population, bioassays were performed every five generations to confirm and measure the level of tolerance. The established tolerant population is presented in this paper was "tolerant F_{24}", indicating that it was in its 25^{th} generation of exposure. Using the crude bacterial suspension, the LC_{50} of the susceptible population used here was 0.117 mg/ml, whereas LC_{50} of the tolerant population was 1.023 mg/ml (Resistance Ratio = 8.72).

4.2.3 Culture of *Trichogramma pretiosum*

Trichogramma pretiosum was purchased from Bugs for Bugs (Queensland, Australia). A culture of *T. pretiosum* was maintained in an incubator at 25 ± 1°C with a photoperiod of 14:10 h [light/dark]. Eggs of *H. armigera* and honey were provided every day to maintain the culture. Wasps used in all experiments were confirmed to have mated based on the presence of female offspring.

4.2.4 Egg volume of *Helicoverpa armigera*

For each experiment, 40-50 adult moths were kept in 5 l containers for several days as described above. This allowed adult moths to mate and start laying fertilised eggs. When adult moths started to lay fertile eggs (eggs that turned yellow with a brown band after 24 h), they were collected to use in experiments. For all experiments, eggs were collected by replacing polar fleece sheets 24 h prior to collection. Thus, all eggs were collected at ≤ 24 hours of age. Eggs were then randomly chosen, and separated by cutting the polar fleece sheet around the eggs. They were then placed on an adhesive paper strip (Post-it® note, 3M, St. Paul, Minnesota, USA) for further experiments.

To investigate whether there is an effect of the size of the host eggs on parasitism, the volume of eggs of *H. armigera* was measured. For each treatment, eggs of *H. armigera* (n=30) were collected and individually photographed using a dissecting microscope with

an attached camera (Olympus SZX12; 900 X). The maximum egg height and diameter were measured using image analysis software *analySIS* FIVE (Olympus, Japan). The volume of the eggs was calculated using the formula for a truncated sphere, $V=22/7*d^2*h/3$, where d and h represent egg diameter and height, respectively.

We investigated whether the volume of the eggs laid by susceptible females was different from those laid by tolerant F_{24} females by comparing their egg volumes. Then, the relationship between *Bt* exposure and the difference in the egg volume was investigated using *Bt* induced susceptible eggs. For this investigation, susceptible larval *H. armigera* were induced by feeding them with a diet on which a bacterial suspension containing 50 µl of 0.03 mg/ml Cry1Ac toxin was spread on the surface. The eggs and resultant progeny of these induced insects are referred to as "induced tolerant F_1". The egg volumes of the F_1 generation of adults with and without *Bt* induction (control) were compared. In addition, we investigated whether removing the repeated toxin exposure from already tolerant insects would lead to a change in the mean volume of individual eggs they subsequently laid. To do this, induced tolerant F_{24} larvae were fed either with or without *Bt* toxin spread on their diet as 0.1 mg/ml Cry1Ac bacterial lysate. Eggs and resultant progeny from this treatment are referred to as "offspring of tolerant F_{24} with no *Bt* toxin", and the control group for this treatment is "offspring of tolerant F_{24} with *Bt* toxin". Differences in mean egg volumes were analysed using T-tests.

4.2.5 Effect of induced tolerance on successful egg parasitism

An experiment was conducted to investigate whether there was any effect of induced *Bt* tolerance on egg parasitism. Host eggs were collected and placed on an adhesive paper strip as described earlier (0.5 cm x 2.5 cm), two eggs per strip. Each paper strip was placed into a clear size 00 gelatine capsule. One female *T. pretiosum* was introduced into each capsule from the wasp culture (i.e. mated and previously exposed to eggs) and observed under a microscope until oviposition into both eggs was observed. The female wasp was

then removed and host eggs were maintained in the capsules under normal culture conditions (see above) until wasp progeny emerged. Three parameters were compared between susceptible and induced tolerant cultures. These parameters were 1) the percentage parasitism, 2) the number of wasp progeny to emerge per egg, and 3) the proportion of male progeny.

Two experiments were conducted to measure the effect of egg parasitism. Firstly, parasitism success was determined by the number of dark eggs 5 days after parasitoid oviposition, which indicates wasp development. Four replicates of 20 eggs per treatment, susceptible and induced tolerant F_{24}, were parasitised as mentioned above. Parasitism success was calculated as a percentage of dark eggs from the total number of eggs. The mean value of all replicates were calculated and analysed using a T-test.

Secondly, the number of wasps emerging from eggs of susceptible and tolerant populations was observed over two generations. There were 159 parasitised susceptible, and 93 parasitised tolerant (F_{23}), eggs in the first generation, and 44 parasitised susceptible, and 176 parasitised tolerant (F_{24}), eggs in the second generation. The number of male and female *T. pretiosum* emerging from each parasitised egg was recorded. The variation between generations was analysed using two-way ANOVA. The total number of wasps per egg from both generations were analysed using the Mann-Whitney U test. The proportion of males to emerge per egg was calculated and analysed using a T-test.

4.2.6 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.0d for Mac OSX (GraphPad Software, San Diego California USA).

4.3 Results

4.3.1 Volume of eggs from susceptible and tolerant (F_{24}) *Helicoverpa armigera*

The mean volume of eggs from induced tolerant females was significantly larger than those produced by susceptible females (t = 14.42, df=58, p<0.0001; Figure 4.1). This suggests that one of the effects of the induced tolerance to *Bt*-toxins has been to increase the volume of eggs.

4.3.2 Effect of one generation of sub-lethal *Bt*-exposure on egg volume

Eggs from the population of *H. armigera* that had been induced with a sub-lethal dose of Cry1Ac toxin had significantly larger volume in the following generation compared to eggs from the same strain of susceptible insects that were not exposed to the toxin (t=8.294, df=58, p<0.0001; Figure 4.2A). This indicates that exposure of susceptible *H. armigera* to a sub-lethal dose of *Bt* toxin for just one generation produces a significant increase in egg volume. However, the egg volume of tolerant F_1 females was significantly smaller than the eggs of the tolerant population exposed to *Bt* toxin over 25 generations (t = 9.318; df=58; p<0.0001; Shown in Figures 4.1 and 4.2A). Thus, the results suggested that the more generations over which insects are exposed to sub-lethal *Bt* toxin, the larger will be the volume of the eggs.

Cry1Ac tolerant *H. armigera* larval populations were normally maintained on toxin-treated food, using sub-lethal doses. Given that one generation of sub-lethal exposure was reflected in increased egg size, we decided to test whether one generation of non-exposure of induced tolerant F_{24} larva would lead to a significant decrease in volume of eggs produced by the resultant adult females. However, the volume of eggs from induced tolerant F_{24} larva that had been fed with untreated food did not significantly decrease compared to offspring of inducible tolerant F_{24} insects from the normal exposed culture (t=1.338, df=58, p = 0.1860; Figure 4.2B).

4.3.3 Effect of induced tolerance on egg parasitism

There was no difference in the proportion of eggs from susceptible or tolerant *H. armigera* population in which the wasp was able to produce at least one offspring (overall percentage parasitism ± SEM in susceptible eggs = 60.00 ± 6.46, overall percentage parasitism in tolerant eggs = 73.75 ± 12.48, t=0.9789, df=6, p=0.3655). However, significantly more *Trichogramma* emerged from each egg laid by tolerant mothers compared to eggs from the susceptible population (Figure 4.3; Mann-Whitney test; U=18230; p<0.0001). This occurred in both of the generations assessed and there was no difference in the number of wasps emerging between the generations for each population (Two-way ANOVA; F=2.473; p=0.1165).

4.4 Discussion

The aim of this study was to investigate possible effects of induced tolerance to *Bt* toxins in *H. armigera* on egg size and parasitism by the biological control agent *T. pretiosum*. Questions about such effects arose from previous data showing that induced tolerance was epigenetically transmitted with a strong maternal component to the transmission (Rahman et al. 2004; Ma et al. 2005). In addition, another study has reported low parasitism success in eggs from hosts that survive on genetically modified *Bt* maize (Steinbrecher 2004). Thus, it seemed plausible that effects of induced tolerance could be reflected in the eggs laid by surviving adult females, and that these effects could further be reflected in parasitism success of *T. pretiosum*. Because current integrated pest management in cotton aims to limit the development of genetic resistance, rather than the induced tolerance we are investigating, and relies in part on augmented control provided by *T. pretiosum*, such effects are important to understand in order to refine management of *H. armigera* in Australian cotton production. Additionally, there may be implications for many pest management systems in which ongoing sub-lethal toxin exposure of pest insect populations is occurring.

We found that the number of wasps emerging from eggs of tolerant *H. armigera* was greater than from eggs of susceptible hosts that were not exposed to *Bt* toxin. The difference in the outcome from the previous study of Steinbrecher (2004), where parasitism success was lower in hosts that were exposed to *Bt* toxin, might be because of the higher *Bt* dosage that was used previously. In our study, *H. armigera* larvae were exposed with a sub-lethal dose of bacterial lysate containing *Bt* toxin, whereas in Steinbrecher's study, the population was a selected by the genetically modified (GM) *Bt* Maize which is expected to kill 100% of susceptible insects. However, there was no verification that the population that survived *Bt* toxin exposure in that study was genetically resistant to *Bt* toxins. It is speculated that the effect of GM *Bt* maize might be greater than the effect of low dose *Bt* exposure in such a way that females surviving GM *Bt* maize might produce low quality eggs that are possibly less nutritious for parasitoid development. This could explain the low parasitism success in Steinbrecher's study (2004).

We also found that production of both male and female offspring by *T. pretiosum* from tolerant hosts increased. It is intuitive that the increase in the number of parasitoid offspring is beneficial and could be explained by the larger size of the host eggs. Although we did not assess how many parasitoid eggs were laid into each egg, it is plausible that female *T. pretiosum*, which are known to assess egg size and make decisions on the number and sex of oviposited eggs, laid more eggs into the larger eggs of tolerant *H. armigera* (Klomp & Teerink 1962).

The increase in egg volume in tolerant F_{24} was related to *Bt* exposure. However, there was no change in the egg volume in of the offspring of tolerant F_{24} reared on non-toxic food. We suspect that the reason for F_{24} tolerant *H. armigera* eggs remaining the same size after one generation free of toxin exposure was likely due to any change being small in comparison to the level of accumulated increase in egg size from 25 generations of

exposure. It is of course also possible that the effect cannot be reversed through sudden non-exposure.

The mechanism causing the increased egg size in *Bt* tolerant *H. armigera* is unknown. There are many interacting variables associated with egg production which make identification of the cause of a change in size complicated. It is unclear if increased egg size is a symptom of the induced tolerance with a significant increase occurring in the exposed generation, or it is in some way associated with the tolerance mechanism. Interestingly, inducible tolerance to *Bt*-toxin in larvae is correlated with an increase of lipophorin, a molecule functioning in lipid storage and as a pro-coagulant (Ma et al. 2005; Rahman et al. 2006; Rahman, Roberts & Schmidt 2007). Lipophorin is one of the main proteins in eggs (Gullan & Cranston 2010). It is possible that the increase in egg size might be due to an increased amount of lipophorin in the egg.

Future studies should investigate the differences in protein contents of eggs and the gene expression in eggs using molecular techniques such as proteomic gels, mass spectrophotometry or even transcriptome deep sequencing. These techniques should allow us to observe the gene expression profiles, and possibly allow us to identify which genes are expressed differently between susceptible and tolerant eggs. Such genes could potentially be a part of the mechanism of inducible *Bt* tolerance.

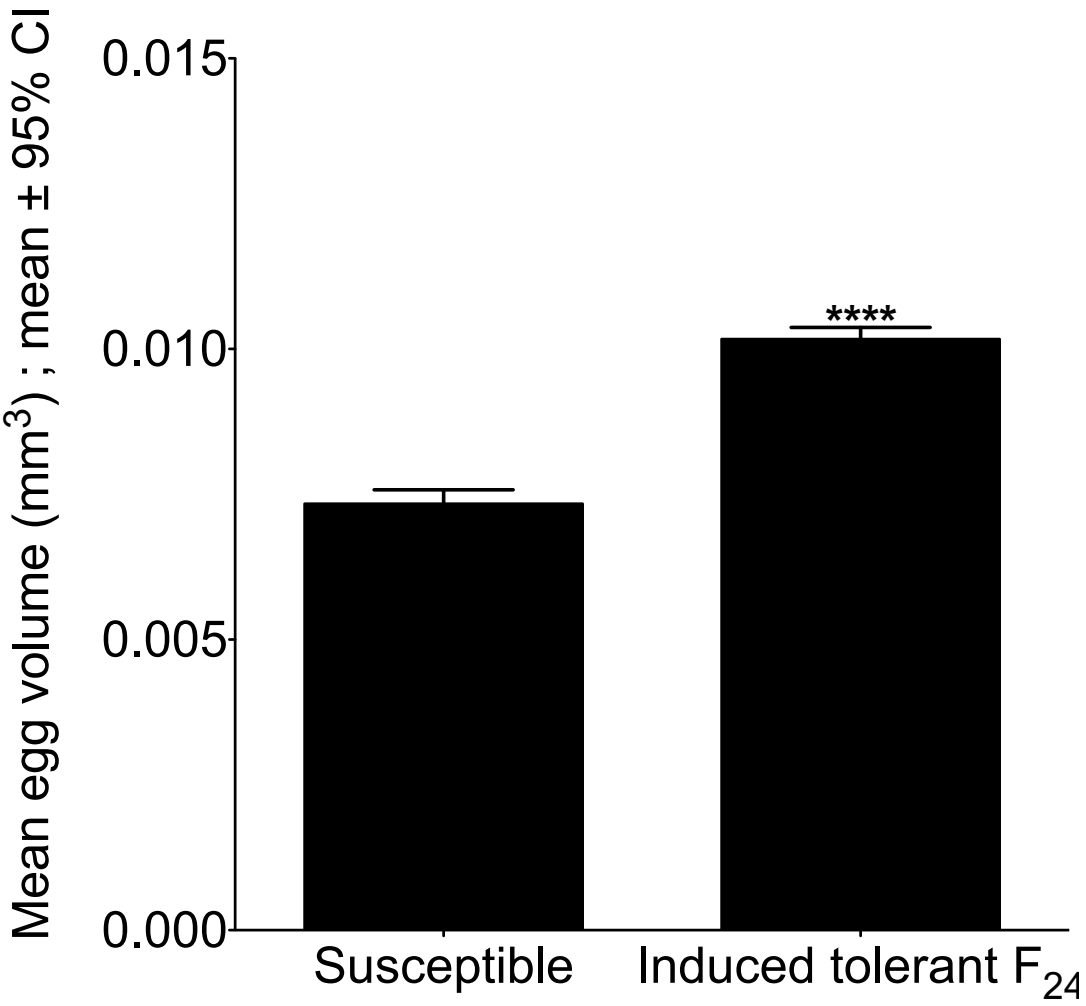

Figure 4.1 Difference in egg volume of susceptible and tolerant *H. armigera*. The mean volume of individual eggs laid by induced tolerant (F_{24}) female *Helicoverpa armigera* (n=30) was significantly larger than those laid by the susceptible strain (n=30).

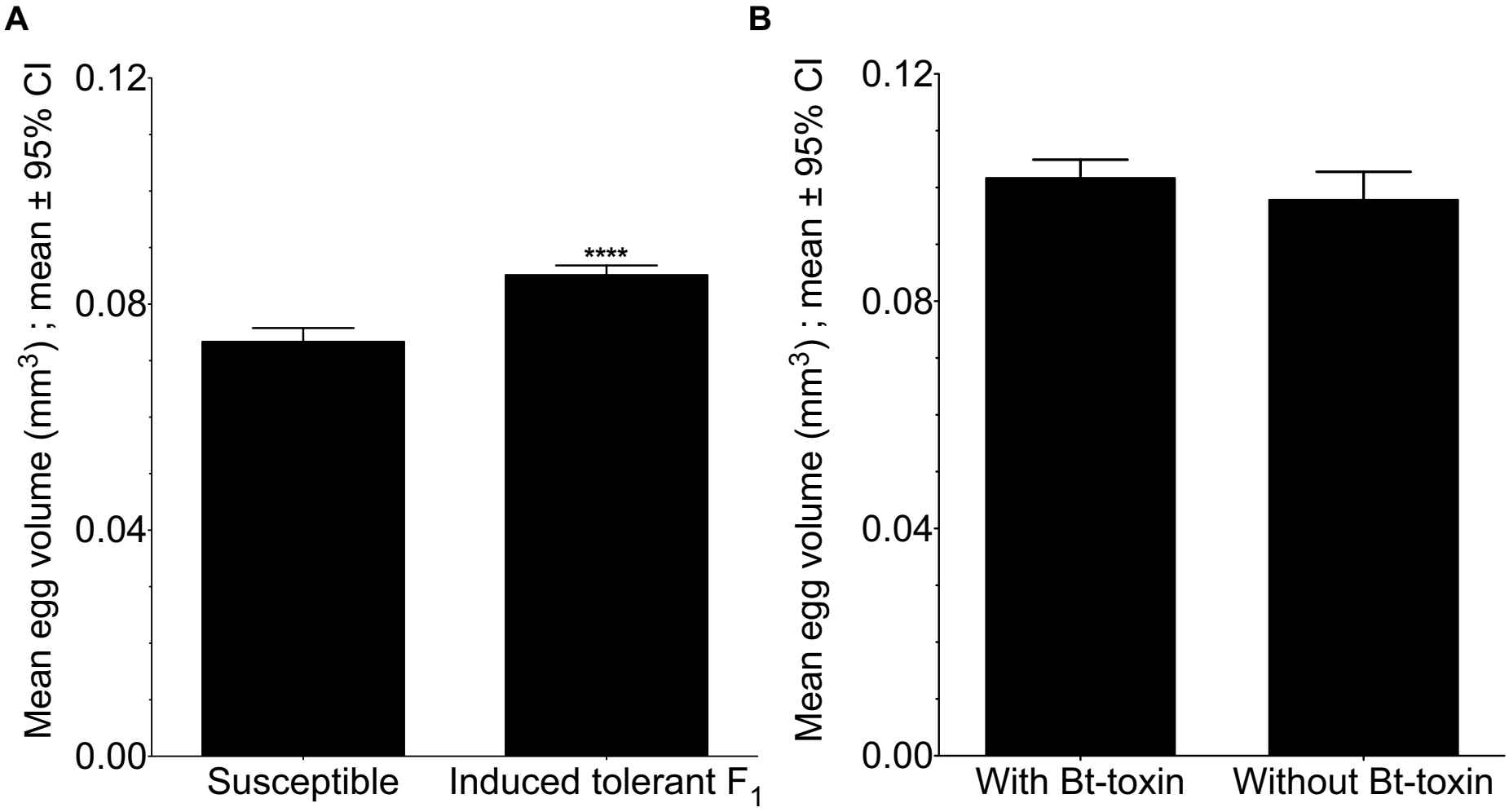
A
B
Mean egg volume (mm3) ; mean ± 95% CI
0.12
0.08
0.04
0.00
****
Susceptible
Induced tolerant F1
With Bt-toxin
Without Bt-toxin

Figure 4.2 Effect of one generation of sub-lethal *Bt*-toxin exposure (or removal of exposure) on *H. armigera* egg volume. (A) Susceptible *H. armigera* larvae were induced using a sub-lethal dose of *Bt* toxin for one generation ("induced tolerant F_1") and produced eggs of a significantly larger volume compared to eggs for the same population ("susceptible") that were not exposed (non-induced). (B) However, the egg volume of the offspring from tolerant F_{24} parent feeding on a diet with and without *Bt* toxin was not different.

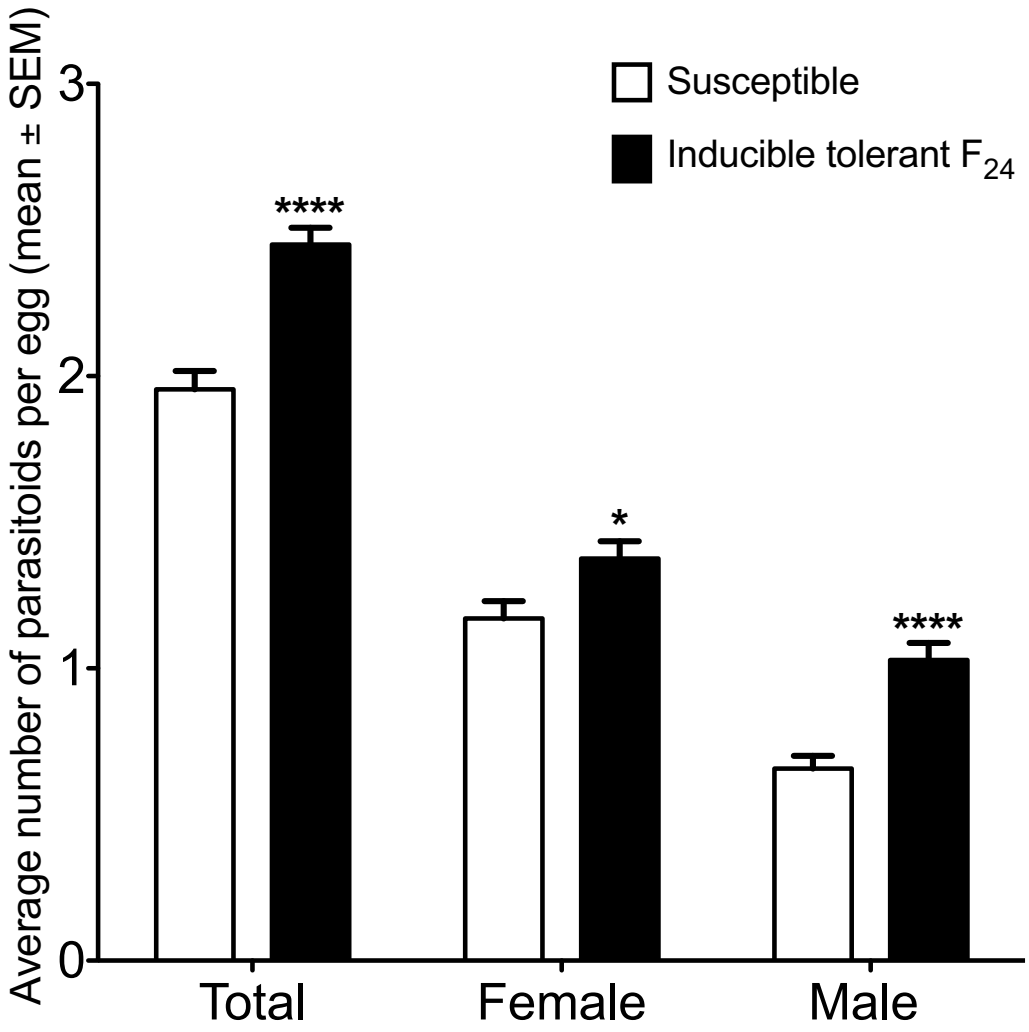

Figure 4.3 Effect of inducible tolerance on egg parasitism. Over two generations, the average of the total number of wasps that emerged per egg from induced tolerant mothers was significantly greater than from eggs of susceptible moths. The number of both females and males emerged are higher in tolerant eggs.

Chapter 5

General discussion

5.1 Synthesis

The goal of this study was to improve the understanding of the mechanism of transgenerational immune priming in inducible Bt tolerance by investigating egg transcriptomes of susceptible and inducible tolerant insects. The rationale was that if the up-regulated immune gene responses correlated with *Bt* toxin exposure and induced tolerance are maternally transmitted, then this should be reflected in the eggs of tolerant insects, as has been shown in other studies that involve maternal effects in immunity (Freitak, Heckel & Vogel 2009; Zanchi et al. 2011). The hypothesis being tested was that the gene expression profiles, and possibly the immune status, of eggs of tolerant and susceptible insects would be different. Genes that were expressed differently between eggs from these two strains might have a role in the mechanism of inducible *Bt* tolerance. In addition, as egg parasitism is one of the important controlling factors in integrated pest management in cotton farming (Schuler et al. 1999; Chilcutt & Tabashnik 1999; Schuler et al. 2004), I investigated effects of inducible *Bt* tolerance on egg parasitism. Thus, two main research questions were: 1) which genes are involved in TGIP? and 2) do the effects of *Bt* toxin-related TGIP affect the suitability of the eggs as hosts for an egg parasitoid?

To answer the first research question, egg transcriptomes of two strains of *H. armigera* were investigated: susceptible (Waite; control for Cry1Ac-tolerant strain) and Cry1Ac-tolerant (Waite; low concentration *Bt* selection). Interestingly, there were no differences in the expression of known immune genes between these two strains. In contrast, the genes that were expressed significantly higher in the tolerant strains were not directly involved with the immune system, but are more related to stress response pathways. The roles of these genes in inducible tolerance are still unclear.

In addition to the tolerant strain, additional two egg transcriptomes of *H. armigera* were investigated: susceptible (CSIRO, NSW; control for Cry1Ac-ressistant (Bx) strain) and Cry1Ac-resistant strain (CSIRO, NSW; high concentration *Bt* selection as described by

Akhurst et al. (2003)). This Bx strain, even though highly resistant to *Bt* toxins, showed the characteristics of inducible tolerance, including an increase in immune activity (PPO activity) in haemolymph and in the midgut, and the vertical transmission of the tolerance shows a maternal effect, suggesting that the mechanism of immune-related tolerance might be the same for populations under low and high dose *Bt*-toxin selection (Ma et al. 2005). The transcriptomic analyses conducted showed that, similar to the tolerant strain, there were no differences in the expression of known immune genes between susceptible and resistant strains. However, genes that were up-regulated in eggs of the Bx strain were different from those that were up-regulated in eggs of tolerant insects. This suggests that the mechanisms of immune-related tolerance in these two strains might be different. Different mechanisms might be necessary to survive the levels of Cry1Ac toxin used in the selection process, which were also proportional to the corresponding levels of *Bt* toxins able to be consumed by the selected populations. In addition, the resistance in the Cry1Ac-resistant strain has a genetic component involved as reported in previous studies (Akhurst et al. 2003; Ma et al. 2005). The offspring of the resistant males are less resistant than the offspring of the resistant females, but they are not as susceptible as the susceptible strain.

Interestingly, genes that were found to express differently between susceptible and Cry1Ac resistance/tolerance strains were not necessarily immune-related, but possibly stress-related. Even so, the changes in the expression of these genes might improve the plasticity of the immune response in the offspring, which leads to a more effective response upon *Bt* exposure. It is also possible that the changes in the expression of these genes might not be a part of the inducible *Bt* tolerance mechanism at all, but a symptom of being exposed to *Bt* toxins. This requires further investigation to determine the functions of these genes.

Many immune genes that were expressed in the eggs also function in cell cycle regulation and embryogenesis. Several studies have shown that a developmental penalty and fitness cost have been associated with increased immune activity (Moret & Schmid-Hempel 2000;

McKean et al. 2008; Stahlschmidt et al. 2013). This is because the cost of the immunity is high, and therefore, there was less energy directed toward growth and development. In addition, since the same genes involved in development are also involved in the immune system, it is conceivable that there are limited resources available to produce an increased requirement for proteins to accommodate both systems.

The differences in the gene expression profiles of eggs from susceptible and tolerant insects were correlated with a difference in the egg size. The result of this study has shown that the expression of four genes, histone H2BB, translationally controlled tumor protein, glyceraldehyde 3-phosphate dehydrogenase and receptors for activated C kinase, was higher in tolerant eggs than susceptible eggs. Tolerant eggs also have a larger volume than susceptible eggs. However, the link between gene expression differences and the egg volume is presently unclear. In addition, it is also unclear whether the increase in egg volume is a part of the mechanism of inducible tolerance or simply a marker of *Bt* toxin exposure.

Even though the gene expression profiles of susceptible and tolerant eggs were different, possible changes in the immune status of the tolerant eggs appeared to have no effect on egg parasitism. Instead, the increase in egg size correlated with *Bt* exposure is potentially a positive effect in that an increased number of parasitoids emerged. This study has neither demonstrated whether egg parasitoids have a preference for either susceptible or tolerant eggs, nor investigated the relative fitness of the resultant offspring. Nevertheless, the result indicates that the use of parasitoids in controlling the tolerant population will be as efficient as those attacking susceptible eggs, if not more so.

5.2 Future study

There is still a lot of gaps in understanding inducible *Bt* tolerance and associated TGIP. This includes the priming process in inducible tolerance, the immune genes that are effectors in the tolerance, and the process of how the transference of up-regulated

immunity to the next generation is achieved. This includes studying the gene expression profiles of the midgut and proteomic study of the haemolymph components of tolerant insects. During the course of this study, microarray analysis on the midgut of inducible *Bt* tolerance larvae and carcasses has been initiated by Dr Mahbub Rahman, which should provide further functional clues to the expression of tolerance to *Bt* toxins..

The mechanisms of maternal transmission in TGIP are still unclear. This study investigated the gene expression profiles of eggs in the expectation that there would be a difference in gene expression in the eggs that would be associated with *Bt* tolerance in larvae of the next generation. Other aspects that still need to be investigated including the proteomic profiles of susceptible and tolerant strains, and their microbiota which has recently been investigated to determine whether it is involved in tolerance to *B. thuringiensis* (Hernandez-Martinez et al. 2010).

This study focused on the gene expression profiles of the eggs. Thus, proteins or other molecules that are possibly associated with the transmission or expression of *Bt* tolerance and incorporated into the eggs by the female parent could not be detected in this study. Further investigation of the proteomic profiles are needed to characterise the proteomic profiles of the eggs of susceptible and tolerant strains.

Further functional studies are needed to understand the roles of genes reported in this study (e.g. H2BB, TCTP) in inducible tolerance, in both the priming and transmission processes. This includes characterisation of proteins *in silico* by predicting their functions based on the conserved motif, and identifying the pathways and functions of the proteins by manipulating the expression of the proteins using the UAS-GAL system and RNA interference (RNAi) (Cancino-Rodezno et al. 2010; Zhu 2013). In addition, other immune components that have been reported to be up-regulated in response to *Bt* exposure need to be further investigated to determine how they relate to inducible tolerance.

5.3 Significance of this study

Even though cotton bollworm was used as a model pest species, the implications of this study are likely to be relevant to other lepidopteran pests that are controlled by *Bt* toxins in pest management, and to the broader field of insect immunity. In terms of pest management, this study has demonstrated that the maternal effect of inducible tolerance has no negative effect on the use of egg parasitoids as an integrated control measure. In addition, this study has identified differently expressed genes in eggs of insects displaying induced *Bt*-tolerance. The expression levels of these genes could possibly be used as biological markers of induced tolerant strains. Furthermore, these genes could also be a potential targets for novel pest control methods such as RNAi (Zhang, Li & Miao 2013; Li et al. 2013).

Besides the implications for pest management, the findings of this study also contribute to the knowledge of invertebrate immune system and the trans-generational immune priming. In addition, since the insect immune system is also used in the medical field as a model for an innate immune system (Seabra & Bhogal 2009; Chambers & Schneider 2012; Kaito, Yoshikai & Sekimizu 2012), understanding the insect immune system will also contribute greatly to the broader understanding of innate immune systems.

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