Australian Government
Cotton Research and Development Corporation

FINAL REPORT 2013

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number: **UA1101**

Project Title: The use of biological control agents in resistance management of Helicoverpa

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Part 3 – Final Report

(The points below are to be used as a guideline when completing your final report.)

Background

1. Outline the background to the project.

In the Australian cotton industry, toxins produced by the soil bacterium *Bacillus thuringiensis* (*Bt* toxins) are utilized to control lepidopteran pests: *Helicoverpa armigera* (cotton bollworm) and *H. punctigera*. *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 a strong selection pressure on the pest insects in the field, leading to the development of resistant strains. 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 (Rahman et al. 2004; Rahman et al. 2011). This induced tolerance is associated with increased immune activity in the midgut and hemolymph. The induced tolerance and the increase in the immune activity can be transferred to the next generation mainly via maternal effect, and the level of tolerance can increase over generations of exposure. In addition, highly-selected Cry1Ac-resistant strain also exhibits the same feature as low dose selected inducible tolerance *H. armigera* (Akhurst et al. 2003; Ma et al. 2005). The development of Cry1Ac tolerance is a threat to the use of Bt technology. Even though many studies have reported the increased immune response against *Bt* toxins, the role of the immune system in facilitating inducible tolerance against *Bt* toxins is unclear. Understanding the mechanism of inducible tolerance will help strengthening the established resistant management strategy.

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 on the mechanism 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 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 to also understand whether there is any negative effect of *Bt* tolerance/exposure on *H. armigera* eggs with regard to parasitisation. A study done on egg parasitism by *Trichogramma brassicae* has also demonstrated that the parasitism success is greatly reduced in eggs from *H. armigera* survived from GM Bt maize (Steinbrecher 2004). Thus, the primary aim of this study was to investigate the TGIP mechanism of inducible *Bt* tolerance. The study also investigated the effect of inducible tolerance on key metrics of egg parasitism by the parasitoid wasp, *Trichogramma pretiosum*.

In this study, we compared the gene expression profiles of eggs from susceptible, Cry1Ac-tolerant and Cry1Ac-resistant *H. armigera*. We also investigated the parasitism success of Trichogramma wasp on eggs of susceptible and Cry1Ac-tolerant insects by measuring the number of eggs being successfully parasitized, and the number of progeny produced.

Objectives

2. List the project objectives and the extent to which these have been achieved.

   Objectives of the project were originally listed as:

   1. The abundance of immune proteins, such as prophenoloxidase and the pro-coagulant lipophorin in whole mounts of ovaries and eggs will be examined using confocal microscopy. Analyse the immune status of oocytes, eggs and neonates from induced and non-induced insects by examining the expression of Helicoverpa immune genes using microarray technology (if time and costs permit).
   2. Determine the reproductive success of egg parasitoids on eggs from induced/non-induced and susceptible/resistant parents.
   3. Determine the productive success of egg parasitoids in eggs from reciprocal genetic crosses using induced resistant and susceptible insects.

   The project objectives were significantly altered during the course of the project. This resulted in alterations to Milestones, which were detailed in the May and November reports submitted throughout the project.

   There were two main reasons for the altered objectives. Firstly, Prof. Otto Schmidt who was the project conceiver, Principal Supervisor of the PhD student (Kay Anantanawat) funded by the grant, and originally the Principal Researcher, passed away within the first year of the project. This meant that Assoc. Prof. Michael Keller took over as Principal Researcher and Principal Supervisor, and Dr Richard Glatz (then at SARDI) took over day-to-day supervision of the project and reporting duties. Because the backgrounds of Keller and Glatz were quite different to that of Prof. Schmidt, a different approach was needed to achieve the broad objectives of the project.

   Secondly, Dr Glatz was able to access recently acquired 454 Junior deep-sequencing technology through SARDI in a cost-effective manner. This allowed the project to assess expression of a large proportion of the genes in eggs of various cotton bollworm strains, instead of undertaking time-consuming protein-based analyses of products of just a few immune genes (Objective 1). This allowed us to do a much more rigorous assessment of the key Bt-tolerant strains, however, we only assessed egg-parasitoid wasp success in tolerant versus susceptible eggs (Objective 2). We did not assess wasp success in eggs of insects from reciprocal crosses (Objective 3) which was seen as more peripheral to the overall aim of assessing immune gene expression, and wasp success, in eggs of induced tolerant insects.

   Therefore, by examining many immune genes using deep sequencing we exceeded the original aim of Objective 1 by identifying four new genes that were expressed significantly higher in eggs of induced tolerant insects. We met the aim of Objective 2 by showing that certain attributes potentially relating to egg-parasitoid wasp fitness, were affected by exposure of cotton bollworm larvae to sub-lethal concentrations of Bt-toxin (e.g. egg size). Oabjective 3 was effectively replaced with an expanded Objective 1.

Methods

3. Detail the methodology and justify the methodology used. Include any discoveries in methods that may benefit other related research.

3.1 Toxins and insect cultures used in this study

3.1.1 Cry1Ac toxin used in the project

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.

3.1.2 *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 & 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.

3.1.3 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.

3.2.1 Gene expression profiles analysis

3.2.1.1 454 deep sequencing

To investigate the gene expression profiles of eggs from Cry1Ac-susceptible, Cry1Ac-tolerant and Cry1Ac-resistant insects, 40 mg of eggs from each strain mentioned above 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 1) while cDNA libraries from the CSIRO *Bt*-susceptible, Cry1Ac-resistant and Cry2Ab-resistant populations were sequenced individually.

Table 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., (Brun-Barale et al. 2010).

3.2.1.2 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 1. Raw reads were processed using Newbler GS Run Browser to remove any sequencing adaptors and poor quality regions (Figure 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).

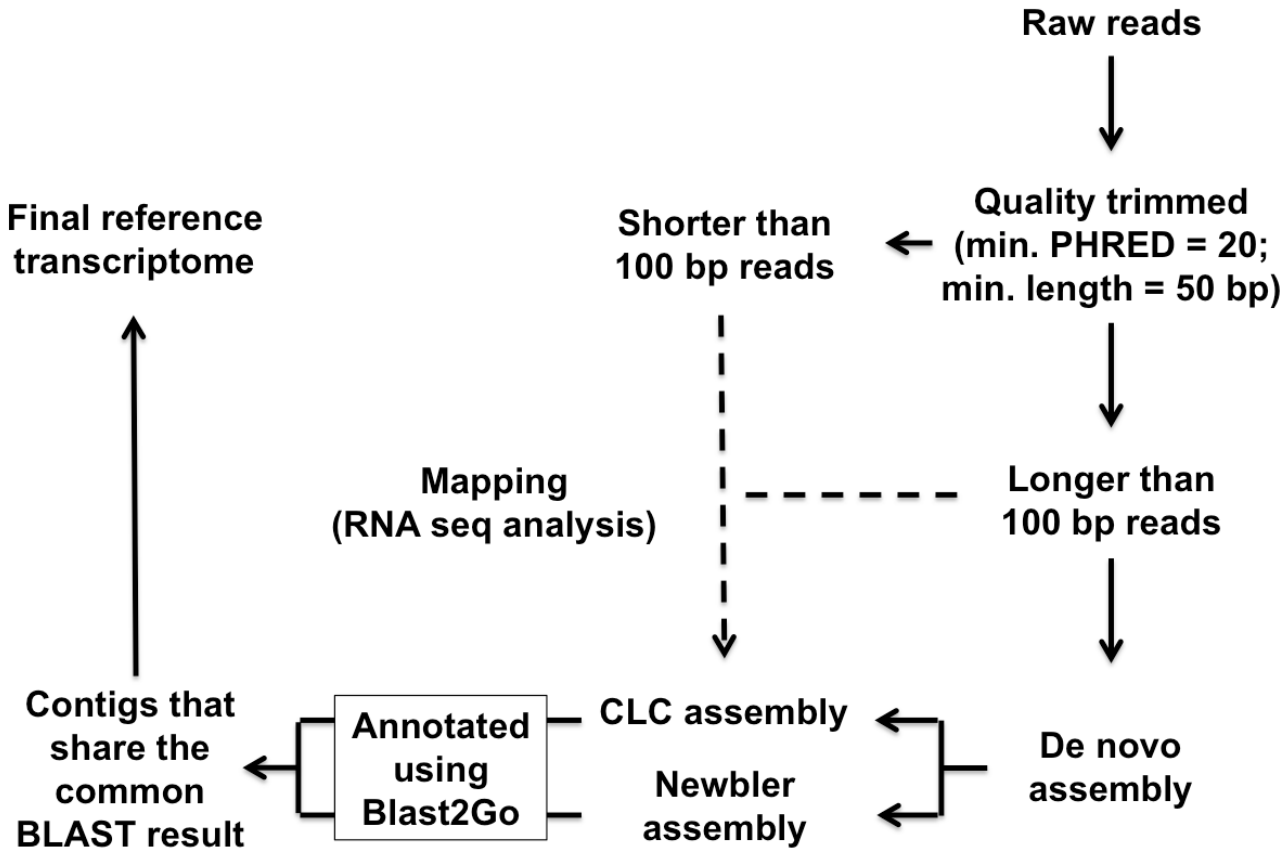

Figure 1. Flow chart of the deep sequencing analysis pipeline used in this study. The process of *de novo* assembly was represented in using the solid line, whereas the mapping process was represented using the dash line. Briefly, raw reads were subjected to trimming based on quality (min. PHRED score = 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.

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.

3.2.1.3 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 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 1). Transcripts that had a greater than 10-fold expression value difference between treatments were considered further.

3.2.1.4 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, andRNAse/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™ 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.

Total RNA was 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 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 optimized 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 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 analyzed 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.:

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

Amplification coefficients for all genes were 1.7-1.8.

3.2.2 Investigating effects of inducible tolerance on eggs of *Helicoverpa armigera* and its parasitism by *Trichogramma pretiosum*

3.2.2.1 Measuring egg volume of H. 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 fertilized eggs. When adult moths started to lay fertile eggs (eggs that turned yellow with a brown band on the top after 24 h), eggs 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 being fed 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 larvae 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.

3.2.2.2 Effect of induced tolerance on successful egg parasitism

To investigate whether there was any effect of induced *Bt* tolerance on egg parasitism, 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. For egg parasitism, 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.

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.

3.2.2.3 Statistical analysis

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

Results

4. Detail and discuss the results for each objective including the statistical analysis of results.

4.1 Gene expression profiles analysis

4.1.1 Roche 454 GS-Junior sequencing

To investigate the differences in gene expression profiles of eggs from susceptible, Cry1Ac-tolerant and Cry1Ac-resistant insects, we analyzed 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 analyzed 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). 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.

Table 2. Summary of 454 pyrosequencing data statistics for eggs from five strains of *H. armigera*

	Susceptible (Waite)	Cry1Ac tolerant (Waite)	Susceptible (Narrabri)	Cry1Ac resistant (Narrabri)	Cry1Ac resistant (Narrabri)	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

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 utilized 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 analyzing artificial transcripts (Figure 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 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 3), indicating that they might be artifacts, and thus, we discarded these sequences from further analyses.

Table 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

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 recognize errors associated with homopolymer sequences present in 454 sequencing data. This is indicated by the slightly 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 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 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*.

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). The majority of sequences were assigned GO categories related to binding (70.6%) and catalytic functions (46.3%) (Figure 2).

4.1.2 Gene expression differences between susceptible and tolerant insects

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 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.

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 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 5).

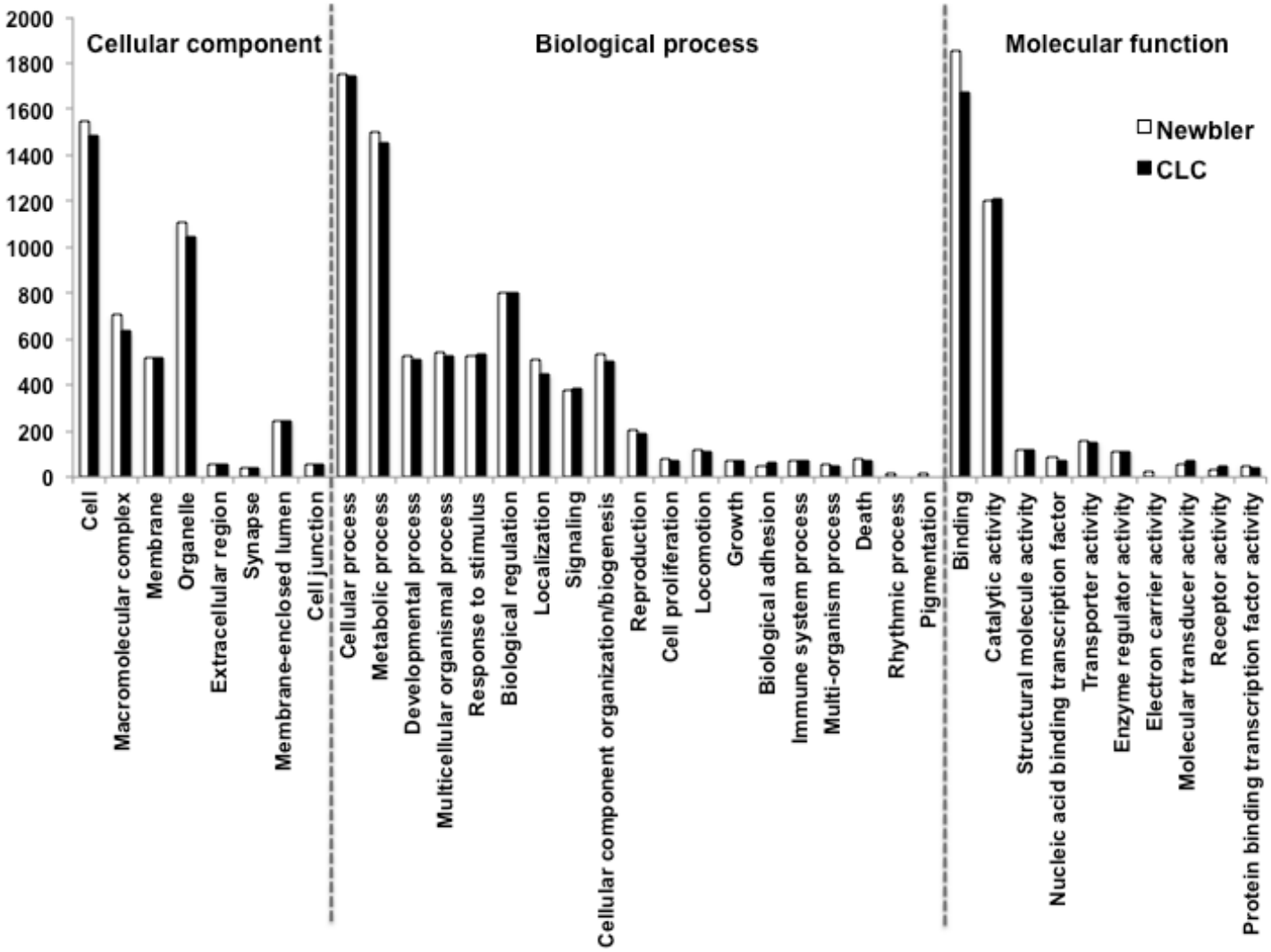

Figure 2. Graph of Level 2 Gene Ontology assignment of the final transcriptome data, generated from the sequences sharing the same BLAST results in Newbler and CLC assemblies.

Table 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	Sim (%)
BAM18063	26S proteasome non-ATPase regulator 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

Accession no.	Protein	Species	Contig IDs	Nt length (bp)	E-value	Sim (%)
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
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
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,	*D. plexippus*	N_Contig_01454; C_Contig_8855	1581; 2037	8.6E-166; 0	94; 94

Accession no.	Protein	Species	Contig IDs	Nt length (bp)	E-value	Sim (%)
	falz					
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
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.

Given the correlation between up regulated larval immunity and inducible *Bt* tolerance, it was surprising that only one gene, ubiquitin-conjugating enzyme e2, had a GO assignment associated with immunity (GO:0002376). However, this protein is also associated with embryonic development or egg hatching (GO:0009792) which are functions that are perhaps more relevant to an egg transcriptome.

4.1.3 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 16recognize the 10 genes that were expressed ≥ 10-fold differently by deep sequencing data analysis (Table 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 4). 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 5, 6 and Figure 3). For example, 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 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 2009). 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.

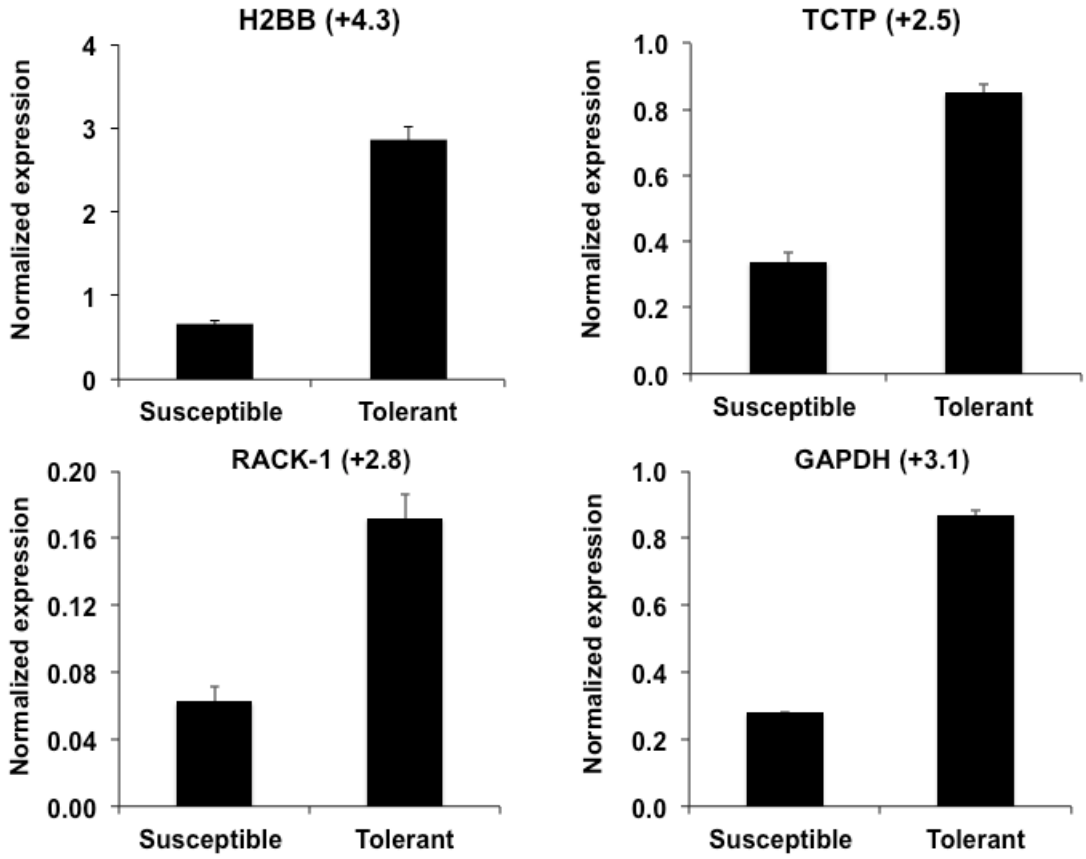

Figure 3. qRT-PCR analysis of four selected *H. armigera* genes showing at least 2-fold differential expression between eggs produced by susceptible and tolerant populations: histone cluster 3 (H2BB), translationally controlled tumor proteins (TCTP), receptor for activated C kinase (RACK-1) and glyceraldehyde-3-phosphate 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 normalized to the housekeeping gene, actin.

Table 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

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 5). This is intriguing as a gene implicated in the midgut response of exposed insects, is also upregulated in the egg (developing embryo) of these insects. This implies that some changes in midgut gene expression in response to *Bt* toxin may be reflected in the eggs, however, the mechanism is unclear. The midgut of Lepidopteran insects is highly characterized as an immune barrier to orally-ingested toxins and microorganisms, and the link between toxin-associated changes in midgut gene expression changes, and epigenetic effects on developing offspring, deserves further investigation.

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.

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

The aim of this part of the study was to investigate the gene expression profiles of the Bx (Cry1Ac resistant) strain that was previously reported to have immune-related induced tolerance, using the same transcriptomic data. Unlike induced Cry1Ac-tolerant *H. armigera*, this resistant strain was selected with a high dose of Cry1Ac (an initial dose of LC_{70}, and then subsequent rearing on LC_{30} ; see (Akhurst et al. 2003), and is highly resistant as discussed, even surviving on GM *Bt* cotton. In addition, the mechanism of resistance consisted of the associated changes in nucleotide sequences in multiple genes (Akhurst et al. 2003), and changes in expression of some immune proteins (Ma et al. 2005). Because a part of tolerance is inherited via maternal effect, it is possible that there is an overlap in genes that are differentially expressed between eggs from Cry1Ac-resistant and Cry1Ac-tolerant populations.

4,180 contigs from the Newbler assembly which gave 3,395 non-redundant (nr) BLAST hits, and 7,908 contigs from CLC assembly giving 5,967 nr BLAST hits, were sequenced from Cry1Ac-susceptible and Cry1Ac-resistant egg populations. 2,966 nr BLAST results were common to these two assemblies, and therefore, were used for further analyses.

Since the Bx resistant strain has been described in Ma et al. (2005) as having the immune-

related induced tolerance, and that part of the tolerance was shown to be maternally transmitted, we first investigated the immune genes found in susceptible and resistant strains. From 2,966 nr BLAST results, 55 genes were annotated with the GO term "innate immune system" (GO:0002376) and expressed in both strains (Table 7). None of the immune genes were expressed differently in eggs of the two strains. However, it is interesting that transcripts of putative ribosome biogenesis protein (bop1: GenBank Accession Number EHJ71396) were only detected in eggs of the resistant strain, where there were 9 reads and 10 reads mapped to this gene using Newbler and CLC respectively. This difference indicated that the expression of bop1 could be at least 10 fold higher in eggs of the resistant strain, however, because there were no reads detected in eggs of susceptible mothers, we are hesitant to conclude that it was differentially expressed as it cannot be statistically validated with the sequencing depth achieved. However, it is unlikely that no bop1 reads would be sequenced in the susceptible strain if the expression was indeed similar. Interestingly, no reads from susceptible and Cry1Ac-tolerant eggs were mapped to Bop1. However, it could be because the depth of the sequencing in these two samples were small (~ 50,000 reads for susceptible and 20,000 reads for Cry1Ac-tolerant strains) (Table 2). Ribosome biogenesis protein is an enzyme involved in the synthesis of ribosomes – a cellular activity which is high during cell growth and division, and which can also be activated as a part of stress response (Deisenroth & Zhang 2010).

Table 7 A list of immune-related genes (determined by GO analysis) that were found to be expressed in the eggs fr susceptible and Cry1Ac-resistant *Helicoverpa armigera* populations.

GenBank Accession No.	**Proteins**	**Species**	**Contig ID***	**Nt. $ Length (bp)**	**E-value**	**S**
ABU45982	G(alpha)q	*H. assulta*	N_Contig_01359; C_Contig_4529	1700; 1969	0; 0	1 9
ABU94676	Alpha-tubulin	*X. cnigrum*	N_Contig_01528; C_Contig_151	1508; 1520	0; 0	1 1
ACI32826	Beta-1,3-glucan recognition protein 2a	*H. armigera*	N_Contig_01571; C_Contig_4969	1454; 1048	0; 0	9 9
ACV86996	Cacophony, partial	*B. mori*	N_Contig_04631; C_Contig_5226	579; 889	3.13E-104; 3.87E-161	9 9
ADK55517	Heat shock protein 90 cognate	*S. litura*	N_Contig_01123; C_Contig_1496	2359; 2490	0; 0	9 9
AEE62668	Unknown	*D. ponderosae*	N_Contig_01075; C_Contig_1302	2558; 2019	1.09E-82; 4.13E-84	8 8
AEO51736	Dorsal	*H. armigera*	N_Contig_02745; C_Contig_2809	893; 1215	0; 0	1 9
AFR31806	Small G protein	*S. exigua*	N_Contig_01422; C_Contig_147	1607; 1664	5.90E-133; 1.08E-132	9 9

GenBank Accession No.	Proteins	Species	Contig ID*	Nt. $ Length (bp)	E-value	% Sim.
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
BAM18063	26s proteasome non-ATPase regulatory subunit rpn7	*P. xuthus*	N_Contig_01787; C_Contig_5854	1281; 1306	0; 0	96; 96
BAM20653	Drumstick	*P. polytes*	N_Contig_04585; C_Contig_5583	584; 711	1.92E-49; 7.53E-49	100; 100
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
EAX04506	Ubiquitin B, isoform CRA_e	*H. sapiens*	N_Contig_05756; C_Contig_148; C_Contig_697	236; 228; 303	2.52E-48; 2.67E-46; 1.90E-64	100; 100; 100
EHB12217	Histone H4	*H. glaber*	N_Contig_04820; N_Contig_05666; C_Contig_247; C_Contig_248	552; 388; 557; 348	2.69E-49; 1.05E-49; 2.48E-49; 4.59E-50	100; 100; 100; 100
EHJ63743	Ubiquitin-conjugating enzyme E2	*D. plexippus*	N_Contig_02775; C_Contig_1101	887; 1126	1.64E-112; 2.80E-111	100; 100
EHJ64010	Hypothetical protein KGM_07791	*D. plexippus*	N_Contig_02162; C_Contig_5485	1097; 1238	1.97E-135; 0	82; 86
EHJ64394	Effete	*D. plexippus*	N_Contig_01306; C_Contig_45	1770; 1793	2.93E-99; 3.63E-99	100; 100
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 kinase	*D. plexippus*	N_Contig_02343; C_Contig_3417	1019; 1068	1.29E-97; 7.94E-106	72; 73

GenBank Accession No.	Proteins	Species	Contig ID*	Nt. $ Length (bp)	E-value	S
EHJ65913	Putative ETS-like protein pointed, isoform P1	*D. plexippus*	N_Contig_02889; C_Contig_8546	857; 863	2.22E-90; 2.38E-90	9 9
EHJ67366	Hypothetical protein KGM_19207	*D. plexippus*	N_Contig_01839; C_Contig_254	1253; 2600	0; 0	9 9
EHJ67415	Hypothetical protein KGM_12069	*D. plexippus*	N_Contig_01801; C_Contig_3546	1272; 2265	0; 0	9 7
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	7 7 9
EHJ68365	Hypothetical protein KGM_14915	*D. plexippus*	N_Contig_03334; C_Contig_10711	772; 940	2.11E-158; 8.92E-159	9 9
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	7 9 7
EHJ69388	Hypothetical protein KGM_05974	*D. plexippus*	N_Contig_03507; C_Contig_110	744; 921	2.65E-102; 7.70E-129	8 9
EHJ70633	Hypothetical protein KGM_15038	*D. plexippus*	N_Contig_02634; C_Contig_3620	923; 936	8.53E-165; 1.99E-169	9 9
EHJ70647	Poly	*D. plexippus*	N_Contig_03409; C_Contig_5477	761; 786	1.50E-86; 2.08E-86	7 7
EHJ71249	Hypothetical protein KGM_08605	*D. plexippus*	N_Contig_03723; C_Contig_10137	703; 739	2.86E-127; 2.92E-134	8 8
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	7 7 8
EHJ72273	Ras-like GTP-binding protein Rho1	*D. plexippus*	N_Contig_01235; C_Contig_1285	1907; 1566	3.27E-133; 1.06E-128	1 1

GenBank Accession No.	Proteins	Species	Contig ID*	Nt. $ Length (bp)	E-value	% Sim.
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
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

GenBank Accession No.	Proteins	Species	Contig ID*	Nt. $ Length (bp)	E-value	S
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	7 6 7 6
NP_001036922	MAP kinase-ERK kinase	*B. mori*	N_Contig_05044; C_Contig_3426	523; 425	4.73E-121; 7.01E-36	1 9
NP_001037618	Ras-related GTP-binding protein Rab11	*B. mori*	N_Contig_01944; C_Contig_116	1194; 1937	3.03E-148; 1.21E-144	9 9
NP_001040215	Stathmin	*B. mori*	N_Contig_00396; N_Contig_00397; C_Contig_1173	3272; 3269; 1490	2.29E-173; 2.23E-173; 0	9 9 9
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	9 9
NP_001040383	Interleukin enhancer binding factor	*B. mori*	N_Contig_01741; C_Contig_1213	1308; 1368	7.15E-169; 0	9 9
NP_001040459	Proteasome subunit alpha type 6-A	*B. mori*	N_Contig_02849; C_Contig_2811	869; 879	1.01E-146; 1.12E-146	9 9
NP_001136443	Clathrin heavy chain	*B. mori*	N_Contig_01103; C_Contig_3326	2433; 405	0; 7.54E-51	9 1
NP_001139127	Oxygen resistance gene 1	*B. mori*	N_Contig_01635; C_Contig_8346	1400; 1653	0; 0	8 8
NP_001243977	Akirin protein	*B. mori*	N_Contig_01194; C_Contig_624	1978; 905	1.48E-77; 8.43E-82	8 8
XP_001861658	Inosine-5'-monophosphate dehydrogenase	*C. quinquefasciatus*	N_Contig_01241; C_Contig_1473	1892; 2630	0; 0	9 9

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

$The nucleotide length of the contigs

We then measured the expression of four genes that were previously shown to be expressed differently between eggs of Cry1Ac-susceptible and Cry1Ac induced-tolerant *H. armigera*, which are H2BB, TCTP, RACK-1 and GAPDH. In contrast to the previous comparison, there was no difference in the expression of these four genes between eggs of Cry1Ac-susceptible and Bx strains. Despite this, there were a different set of 21 contigs from CLC assembly, and 44 contigs from Newbler assembly, that were differentially expressed (>10 fold) between eggs of the two strains. We then compared BLAST results for each of these sequences and found that only five contigs shared the same nr BLAST Hit between these two assemblies (Table 8). One of the contigs is a hypothetical protein (GenBank Accession Number EHJ70627) of unknown function. The other four genes are pyruvate kinase, olfactory receptor 29, proteasome 25 kDa subunit, and transmembrane 9 superfamily member 2 protein (TM9SF2) (Table 8).

The link between higher expression of these four genes, and Cry1Ac-resistance, is unclear as they have not previously been directly related to the immune system or pesticide resistance, and their functions relate to "housekeeping" metabolic pathways. For example, pyruvate kinase functions in turning phosphoenolpyruvate into pyruvate during glycolysis, which is a part of cellular respiration. Olfactory receptor 29 is a member of the olfactory receptor (OR) family, which are proteins with seven transmembrane domains and which function as chemosensors in the olfactory neurons in response to odorants (Glatz & Bailey-Hill 2011). However, ORs also function in response to other ligands in non-olfactory tissues. For example, in mammals, ORs 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, an OR has been 24ecognize24zed in mammalian sperm and functions in chemotaxis allowing sperm to orient to an ovum (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, TM9SF2 is reported to express in early endosomes. As its name suggest, the protein contains nine transmembrane domains and probably functions in transporting small molecules within a cell, or acts as an ion channel (Schimmöller et al. 1998).

Table 8. 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*.

[$]The nucleotide length of the contigs

In summary, even though Cry1Ac-tolerant and Cry1Ac-resistant strains both displayed increased immune activity compared to susceptible insects, and the tolerance/resistance were both maternally transmitted, the gene expression profile of eggs from a the high dose selected Bx strain of *H. armigera* was different from that of the low-dose selected Cry1Ac-tolerant strain. Presumably, the selection for tolerance and (non-mutational) resistance involves (at least in part) selecting for relative expression levels of a complex range of associated genes. Thus, it is possible that the difference in the level of the selection pressure experienced by the resistant and tolerant populations, leads to the 25ecognize expression of genes regulating different immune mechanisms that can lead to the same outcome, an increase in *Bt*-related immune activity. Currently, it is still unclear whether upregulated immunity plays any role in the development of both tolerance and resistance, or if it is simply correlated as a "symptom" of exposure. Further analyses are needed to understand the function of these genes in the immune system and any potential role they play in overcoming the toxic effects of *Bt* exposure. Such analyses could include expression analysis of these genes in naïve individuals upon exposure to toxin and/or examination of immune/tolerance phenotypes associated with silencing of these genes.

4.2 Investigating effects of inducible tolerance on eggs of *Helicoverpa armigera* and its parasitism by *Trichogramma pretiosum*

The aim of this study was to investigate possible effects of induced tolerance to *Bt* toxins in *H. armigera* on egg 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 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 sublethal toxin exposure of pest insect populations is occurring.

We found that the mean volume of eggs from induced tolerant females was significantly larger than those produced by susceptible females (Figure 4; $t = 14.42$; df=58; $p<0.0001$). This suggests that one of the effects of the induced tolerance to *Bt*-toxins has been to increase the volume of eggs.

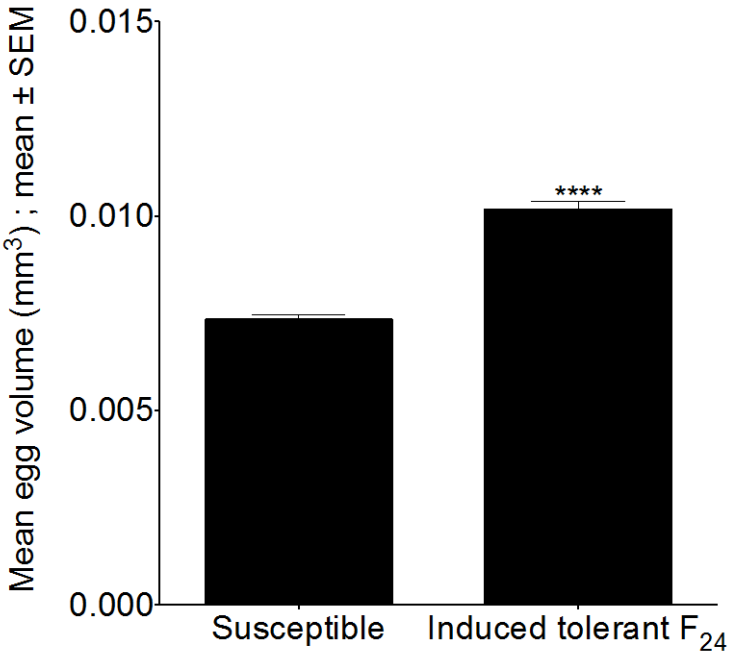

Figure 4. Difference in egg volume of susceptible and tolerant *H. armigera*. The mean volume of individual eggs laid by induced tolerant (F_{24}) female *Helicoverpa armigera* (n=30) was significantly larger than those laid by the susceptible strain (n=30) (T-test; t = 14.42; df=58; p<0.0001).

Eggs from the susceptible population of *H. armigera* that had been induced with a sub-lethal dose of Cry1Ac toxin had significantly larger volume compared to eggs from the same strain of susceptible insects that were not exposed (Figure 5A; t=8.294; df=58; p<0.0001). 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 was significantly lower than the egg volume of the tolerant population exposed over 25 generations (t = 9.318; df=58; p<0.0001; data not shown). Thus, the result suggested that the more generations over which insects were exposed to sub-lethal *Bt* toxin, the larger 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 (Figure 5B; t=1.338; df=58; p = 0.1860).

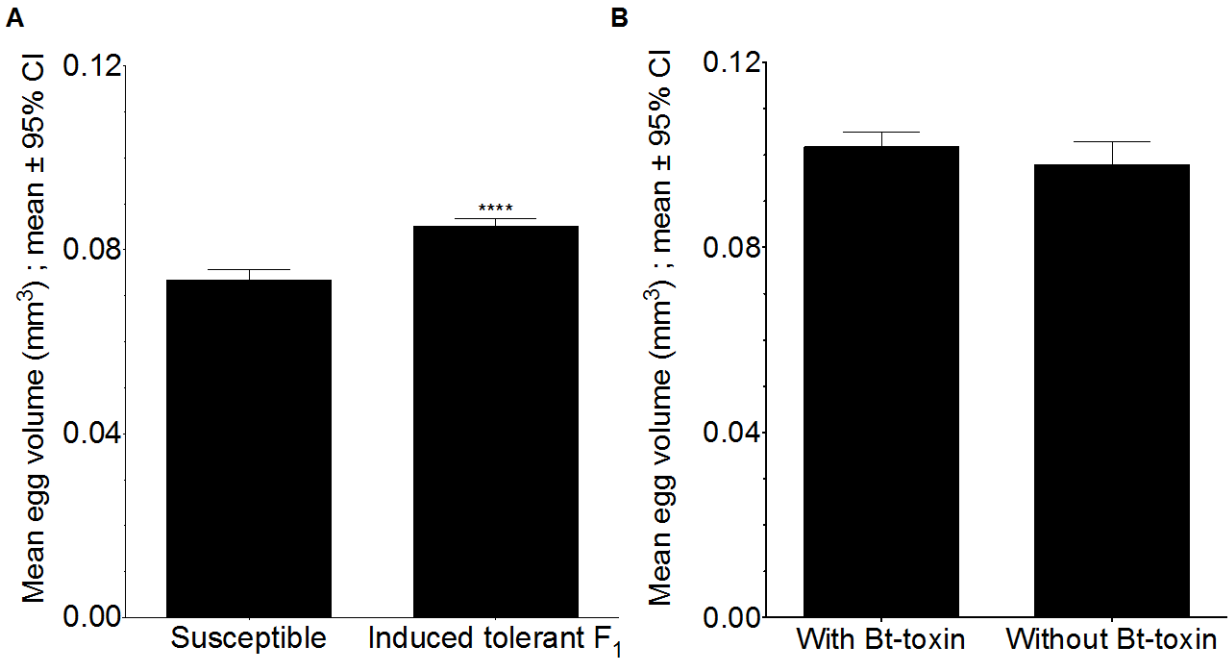

Figure 5. Effect of one generation of sub-lethal *Bt*-toxin exposure (or removal of exposure) on *H. armigera* egg volume. (A) Susceptible *H. armigera* larvae were induced using a sub-lethal dose of *Bt* toxin for one generation ("induced tolerant F_1") and produced eggs of a significantly higher volume compared to eggs for the same population ("susceptible") that were not exposed (non-induced) (T-test; t=8.294; df=58; p<0.0001). (B) However, the egg volume of the offspring from tolerant F_{24} parent feeding with and without *Bt* toxin are not different (t=1.338; df=58; p = 0.1860).

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.

It is hard to confidently speculate on the mechanism producing the increased egg size using these data as there are many interacting variables associated with egg production. In addition, 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 the increase of lipophorin in the egg.

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, although this was much less variable for the susceptible population (t=0.9789; df=6; p=0.3655; data not shown). However, significantly more *Trichogramma* emerged from each egg laid by tolerant mothers compared to eggs from the susceptible population (Figure 6; 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).

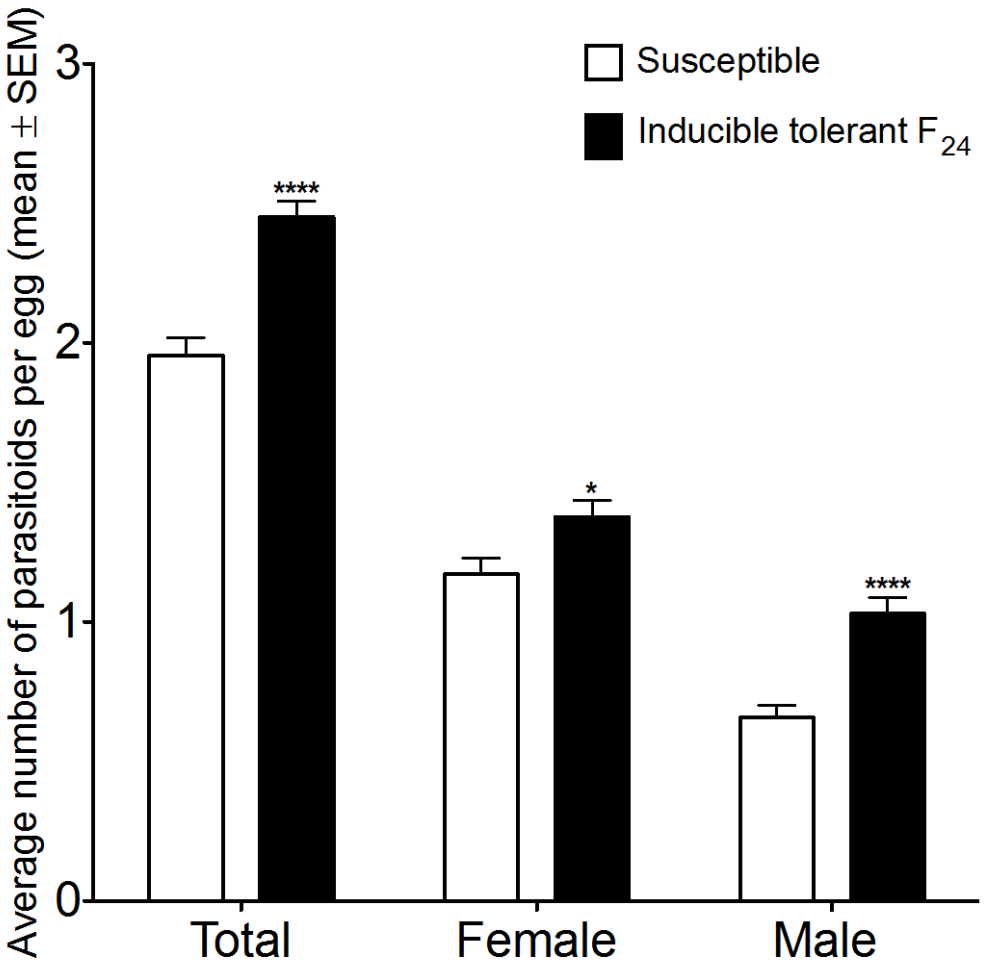

Figure 6. Effect of inducible tolerance on egg parasitism. Over two generations, the average of the total number of wasps emerged per egg from induced tolerant mothers was significantly greater than from eggs of susceptible moths ($N_{Susceptible}$ = 202; $N_{Tolerant}$ = 276; Mann-Whitney test, U = 18230; $p<0.0001$). The number of both females and males emerged are higher in tolerant eggs (female U = 240981, $p<0.05$; male U = 22019, $p<0.0001$).

We found that the number of wasps emerging from eggs of tolerant *H. armigera* was greater than from eggs of susceptible *H. armigera* 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. 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 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 *Bt* resistant. It is speculated that the effect of *Bt* GM maize might be greater than the effect of low dose *Bt* exposure in such a way that females surviving *Bt* GM maize might produce low quality eggs that are possibly less nutritious for parasitoid development. This could explain the low parasitism success in Steinbrechers study.

We also found that increase in production of offspring by *T. pretiosum* from tolerant hosts occurred for both male and female offspring. 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).

Future studies should investigate the difference in protein contents and the gene expression 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.

Outcomes

5. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

There were originally 3 planned science outcomes proposed in the project application. As discussed in part 2 (Objectives) the project methodology was improved and the focus was changed from an analysis of several pre-identified immune-proteins to a much broader assessment of gene expression related to inducible tolerance.

The relationship between the project outputs and planned science outcomes and discussed below:

Science Output 1: *Inducible tolerance mechanisms are the focus of investigations using new approaches, such as micro-array and proteomics technologies. The fact that inducible processes can be observed with Cry2Ab from transgenic maize extracts containing the mature toxin only, suggest that induction is toxin-mediated and not due to other bacterial elicitors present in Bt-formulations.*

Due to the availability of 454 deep sequencing technology, we utilised it in preference to the micro-array and proteomics mentioned originally. This has several advantages and allowed us to analyse presence and relative amount of large numbers of genes in eggs from induced Cry1Ac-tolerant and susceptible cotton bollworm. Thus, we were able to detect at least 4 genes that appear to be expressed at greater levels in tolerant larvae. These genes were: Histone H2B, translationally controlled tumor protein, glyceraldehyde-3-phosphate dehydrogenase and receptors for activated C kinase. Note that 454 deep sequencing technique measures the gene expression profiles by measuring transcripts, not the actual proteins. Thus, this technique will not be able to the detect proteins in eggs that might have changed in quantity due to the maternal input.

Science Output 2: *If toxin-mediated inducible mechanisms are responsible for the elevated immune and metabolic status, the absence of toxin receptors in resistant mutant insects are expected to preclude induction processes. If experimentally demonstrated, this is a major finding as it indicates a role of the toxin in regulatory induction processes.*

While we still cannot completely rule out some form of unexpected genetic mutation, we were unable to detect any sequence differences in important genes, in induced Cry1Ac-tolerant insects. All of our data still indicates that exposure to Bt-toxin is responsible for induction of the observed tolerance. However, we did find that certain genes were expressed more highly in tolerant insects (see Science Output 1 above).

Science Output 3: *If inducible tolerance can be transmitted to offspring by a maternal effect, one of the possible mechanisms of epigenetic transmission is the inclusion of immune proteins into the growing oocyte in immune-induced females. In the developing embryo these immune components in the remaining yolk are surrounded by the primordial gut, which contains mesenchymal tissues differentiating into fat body and hemocytes. It is therefore possible that maternal immune components of*

the 'rest-yolk' elicit an immune response in the developing immune tissues of embryos and neonates. We want to test this hypothesis by inducing susceptible and resistant larvae of Helicoverpa *using Bt-formulations or transgenic maize extracts and analyse the reproductive success of the egg parasitoid* Trichogramma *spec. on eggs from induced and non-induced susceptible/resistant larvae.*

Project outputs have directly addressed this predicted outcome. We tested the effect of induced Cry1Ac-tolerance on parasitism of eggs of tolerant insects by the biocontrol wasp *Trichogramma pretiosum.* We did find that eggs of tolerant bollworm were larger and this appeared to be responsible for the emergence of more wasps. Intuitively, this seems to be a positive effect on the biocontrol agent, however, we have no direct evidence for this. We consider that there is no adverse effects on parasitism, indicating that the presence of induced tolerant bollworm is not likely to effect the additional control provided by *T. pretiosum.*

Additionally, there was one predicted industry outcome, similar to Science Output 3:
The toxin-mediated induction of tolerance and its transmission to offspring by a maternal effect has been shown to affect egg parasitism, where eggs from induced susceptible parents are lower quality hosts for the parasitoids than eggs from non-induced parents. If resistant insects lack the capacity to be induced by the toxin, we can expect the host qualities of eggs from induced resistant parents to be equivalent to eggs from non-induced susceptible parents. In other words, under conditions of continuous exposure to the toxin (such as in transgenic Bt-crops), egg parasitoids may prefer or may reproduce better in eggs from resistant parents compared to susceptible parents. This can be tested under laboratory conditions and if confirmed, the outcome will enhance the sustainable use of Bt-technologies.

As discussed above, we found that inducible tolerance increases the egg size of *H. armigera,* and thus, subsequently increases the number of wasp emerging per egg. Based on this study, there appears to be negative effect of inducible tolerance on egg parasitism by the *Trichogramma* wasp.

6. Please describe any:-
   a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);
   b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and
   c) required changes to the Intellectual Property register.

n/a

Conclusion

7. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?

This study examined two main questions:

1) Because there is a strong maternal component to the transmission of inducible Bt tolerance, can we see any genes expressed differently in eggs of tolerant and susceptible insects?

Our work suggests that there are some differences between eggs of susceptible and tolerant *H. armigera*. Four genes in particular, were expressed at higher levels in eggs of tolerant mothers. Even though the roles of these genes in inducible tolerance are still unclear, they are potentially part of the mechanism of inducible tolerance, and may be an adaptation of exposure to stress. There is no direct impact of these findings on the cotton industry but they highlight the need to continue to be judicious with the use of Bt-cotton technologies and off-target/inefficient exposure. Further functional studies will help address their role, and this can lead to the development of tolerance reduction strategies.

Take home message: *when exposed to sub-lethal concentrations of Cry1Ac Bt toxin over generations, cotton bollworm will increase their tolerance to subsequent exposures and do not require genetic mutations but are likely rather being selected for altered expression levels of a range of genes required for their adaptation.*

2) Does inducible tolerance effect egg parasitism by the biological control wasp, *Trichogramma pretiosum*?

We have shown that even though the gene expression profiles between eggs of susceptible and Cry1Ac-tolerant insects were different, there was no negative effect the quality of eggs as a host for the wasp.

Take home message: *although sub-lethal exposure to Bt-toxin may produce insects with increased Bt tolerance, this is not likely to lessen the control achieved by* T. pretiosum*.*

Extension Opportunities

8. Detail a plan for the activities or other steps that may be taken:
   (a) to further develop or to exploit the project technology.
   (b) for the future presentation and dissemination of the project outcomes.
   (c) for future research.

There are still a lot of gaps in the knowledge regarding inducible *Bt* tolerance and associated trans-generational immune priming. 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. 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. In addition, other immune components that have been reported to be up-regulated in response to *Bt* exposure need to be measured in detail, in how they relate to inducible tolerance.

It would be useful to undertake gene-expression studies of different stages of the exposed developing larvae (tolerant vs susceptible) and to examine real-time changes in expression upon exposure to toxin, and removal from toxin exposure, in susceptible and tolerant insects

Investigations are required into whether inducible tolerance can be developed against non-microorganism-based pesticides such as synthetic and plant-based pesticides. The results from such a study would help elucidate the mechanism used by larval insects to recognize *Bt* toxins, and activate the immune system to such a degree as to provide an increased ability to withstand the lethal effects of the toxin in subsequent generations.

Findings from the project will soon be published in scientific journals, providing new information for researchers in the area of cotton management and insecticide resistance. Findings will be presented at science conferences in the future and at industry events.

8. A. List the publications arising from the research project and/or a publication plan.
   (NB: Where possible, please provide a copy of any publication/s)

   Two manuscripts have been submitted and will be provided to the CRDC upon acceptance.

   1) Anantanawat, K. Glatz, R. and Keller, M. A. Effect of induced tolerance to *Bt* toxin on the egg size of *Helioverpa armigera* and egg parasitism by *Trichogramma pretiosum*. **Submitted to *Physiological Entomology*.**

2) Anantanawat, K. Hill, K. Cooper, T. Downes, S. Keller, M. and Glatz, R. Comparison of egg gene expression profiles between Bt toxin susceptible and induced-tolerant cotton bollworm, *Helicoverpa armigera*. **In revision for *PLOS One*.**

B. Have you developed any online resources and what is the website address?

N/A

Part 4 – Final Report Executive Summary

Provide a one page Summary of your research that is not commercial in confidence, and that can be published on the World Wide Web. Explain the main outcomes of the research and provide contact details for more information. It is important that the Executive Summary highlights concisely the key outputs from the project and, when they are adopted, what this will mean to the cotton industry.

This study focused on the mechanism of immune-related inducible Bt-tolerance in the cotton bollworm (*Helicoverpa armigera).* Bollworm is still the most damaging pest insect to the Australian cotton industry, together with its sister species, *H. punctigera*. Inducible tolerance occurs when bollworm larvae are exposed to sub-lethal doses of Bt-toxin, and tolerance level can increase over generations of exposure. The current bollworm management strategy includes both the use of GM cotton and the use of biological control agents such as *Trichogramma* wasps. The development of Cry1Ac tolerance could reduce both the commercial efficiency and useful lifetime of *Bt* technology.

Studies have shown that the tolerance is correlated with the increase in the insect immunity after exposure to Bt toxins. In addition, the tolerance and the status of the immunity are transmitted via maternal effect. This unusual mode of inheritance led to speculation that inducible tolerance might have an effect on eggs of cotton bollworm, and subsequently have an effect on egg parasitism.

The primary aim of this study was to improve understanding of the mechanism of immune-related Cry1Ac-tolerance, to help in strengthening the established resistance management strategy, Furthermore, findings could be broadly applicable to development of tolerance to a range of challenges by insect pests.

We examined gene expression changes in eggs of Cry1Ac-tolerant and although we found no mutations, we confirmed 4 genes that were more highly expressed in eggs of tolerant insects. These genes include histone H2B, translationally controlled tumor proteins, glyceraldehyde-3-phosphate dehydrogenase and receptors for activated C kinase. Currently, the roles of these genes in inducible tolerance are still unclear and further functional studies are needed to understand their role. This suggests that tolerance is reflected in the eggs and is likely resulting from changed levels of expression of a range of responsible genes. Thus, non-target and inefficient toxin exposure should be minimised by industry management practices to avoid reduce tolerance development.

We also found that eggs of Cry1Ac-tolerant insects were larger, a phenomenon that appeared to produce more *Trichogramma* wasps. Therefore, although tolerance may develop in the field under conditions of sub-lethal exposure, these same conditions are not likely to impact negatively on the subsidiary control provided by parasitoid wasps that attack bollworm eggs.

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