Exploring the Midgut Transcriptome and Brush Border Membrane Vesicle Proteome of the Rice Stem Borer, *Chilo suppressalis* (Walker)

Weihua Ma1,2*, Zan Zhang3*, Chuahua Peng4, Xiaoping Wang2, Fei Li2*, Yongjun Lin1*

1 National Key Laboratory of Crop Genetic Improvement and National Centre of Plant Gene Research, Huazhong Agricultural University, Wuhan, China, 2 Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory (Huazhong Agricultural University), Wuhan, China, 3 Department of Entomology, Nanjing Agricultural University, Key Laboratory of Monitoring and Management of Plant Diseases and Pests, Ministry of Agriculture, Nanjing, China, 4 Hubei Plant Protection Station, Wuhan, China

**Abstract**

The rice stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae), is one of the most detrimental pests affecting rice crops. The use of *Bacillus thuringiensis* (Bt) toxins has been explored as a means to control this pest, but the potential for *C. suppressalis* to develop resistance to Bt toxins makes this approach problematic. Few *C. suppressalis* gene sequences are known, which makes in-depth study of gene function difficult. Herein, we sequenced the midgut transcriptome of the rice stem borer. In total, 37,040 contigs were obtained, with a mean size of 497 bp. As expected, the transcripts of *C. suppressalis* shared high similarity with arthropod genes. Gene ontology and KEGG analysis were used to classify the gene functions in *C. suppressalis*. Using the midgut transcriptome data, we conducted a proteome analysis to identify proteins expressed abundantly in the brush border membrane vesicles (BBMV). Of the 100 top abundant proteins that were excised and subjected to mass spectrometry analysis, 74 share high similarity with known proteins. Among these proteins, Western blot analysis showed that Aminopeptidase N and EH domain-containing protein have the binding activities with Bt-toxin Cry1Ac. These data provide invaluable information about the gene sequences of *C. suppressalis* and the proteins that bind with Cry1Ac.

**Citation:** Ma W, Zhang Z, Peng C, Wang X, Li F, et al. (2012) Exploring the Midgut Transcriptome and Brush Border Membrane Vesicle Proteome of the Rice Stem Borer, *Chilo suppressalis* (Walker). PLoS ONE 7(5): e38151. doi:10.1371/journal.pone.0038151

**Editor:** Alfredo Herrera-Estrella, Cinvestav, Mexico

**Received** August 26, 2011; **Accepted** April 30, 2012; **Published** May 29, 2012

**Copyright:** © 2012 Ma et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by the National Special Transgenic Project from the Chinese Ministry of Agriculture (grant No. 2008ZX08001-001; 2009ZX08011-007B; 2011ZX08012-004, http://www.agri.gov.cn/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding received for this study.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: yongjunlin@mail.hzau.edu.cn (YL); lifei@njau.edu.cn (FL)

† These authors contributed equally to this work.

**Introduction**

The rice stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae), is one of the most important rice pests. Various methods have been used to try to control *C. suppressalis*, including cultural practices, pheromone traps, planting resistant rice varieties, and insecticides. Among these methods, farmers still prefer to use insecticides because of their high efficiency [1]. However, widespread insecticide use poses potential threats to the environment and food safety. Thus, an alternative strategy for controlling the rice stem borer is needed. One promising possibility is the use of the toxic protein produced by the bacterium *Bacillus thuringiensis* (Bt). Bt toxins are considered to be safe and have been widely used in transgenic plants to control lepidopteran pests such as *Helicoverpa armigera* [2–5]. Although this tactic is successful in controlling rice stem borer at present, the ability of target pests to develop resistance poses a challenge. Better understanding of the interactions between Bt toxins and the receptors in the midgut of pests is very important for monitoring resistance and for pest management [6–11].

Despite the economic importance of the damage caused by *C. suppressalis*, genetic information about this species is scarce. Few *C. suppressalis* gene sequences are known: Currently, only 192 annotated genes and 147 protein sequences are available in the NCBI database. The lack of gene sequences hinders in-depth study of gene function in *C. suppressalis*. The midgut of insect pests is an important organ because it participates in digestion, detoxification, and nutrient intake. It is also the place where Bt toxins interact with insect receptors. Therefore, exploring the midgut transcriptome and proteome of *C. suppressalis* will provide invaluable information about its gene sequences and promote investigations of plant-insect interactions.

To gain insight into the complexity of the midgut transcriptome and proteome of *C. suppressalis* and to identify genes and proteins related to digestion and detoxification, we conducted high-throughput Illumina sequencing of the midgut transcriptome of *C. suppressalis* and two-dimensional protein electrophoresis in combination with mass spectrometry of the brush border membrane vesicle (BBMV) proteome. This analysis dramatically increases the number of known genes and proteins of midgut and enhances the understanding of the *C. suppressalis* midgut.
Results and Discussion

Illumina sequencing

The C. suppressalis midgut cDNA sample was prepared and sequenced using the Illumina sequencing platform. After cleaning and quality checks, we obtained 39 million 90-bp-long reads, which were submitted to the SRA database with accession number SRA050703.2. After they were assembled using Trinity [12] software, a total of 37,040 contigs were obtained (Table 1). The mean contig size was 497 bp, with lengths ranging from 201 to 9,744 bp. The size distribution of these contigs is shown in Figure S1.

Annotation of predicted proteins

Among the midgut transcripts, 15,446 (41%) showed significant similarity (E-value <1e-5) to known proteins in the NCBI database (Table 2). The majority of the transcripts with similar sequences in the database (35%) matched to arthropod proteins. The remaining midgut transcripts were similar to proteins of non-insect eukaryotes (3%) and bacteria (3%). A total of 132 sequences in the database (Table 2). The majority of the transcripts with similar similarity (E-value, d = 138; e = 1564; f = 435; g = 550; h = 22320. doi:10.1371/journal.pone.0038151.g001

Table 2. Summary of BLASTX search of the Chilo suppressalis midgut sequences.

| Non-significant matches | 21,594 |
|-------------------------|--------|
| Significant matches     | 15,446 |
| Archaea                 | 1      |
| Arthropoda              | 12,935 |
| Bacteria                | 1,224  |
| Other eukaryotes        | 1,154  |
| Viruses                 | 132    |
| Total                   | 37,040 |

Figure 1. Summary of the comparisons analysis. Chilo suppressalis transcriptomic sequences were used to compare with protein sequences from the draft genomes of Anopheles gambiae, Drosophila melanogaster, and Tribolium castaneum conducted using the BLASTX algorithm program revealed high sequence similarity (38%, 13,968 out of 37,040) to the T. castaneum genome (Figure 1). The analysis also showed 33% and 34% similarity to the genomes of D. melanogaster and A. gambiae, respectively. A total of 11,419 sequences were shared among all four insect species. About 60% of sequences (22,320 out of 37,040) did not show BLASTX similarity, implying that they represented un-translated regions, non-conserved regions, or proteins novel to C. suppressalis (Figure 1).

Comparative analysis

Comparison of the derived C. suppressalis midgut transcripts with protein sequences in the draft genomes of Drosophila melanogaster, Anopheles gambiae, and Tribolium castaneum conducted using the BLASTX algorithm program revealed high sequence similarity (38%, 13,968 out of 37,040) to the T. castaneum genome (Figure 1). The analysis also showed 33% and 34% similarity to the genomes of D. melanogaster and A. gambiae, respectively. A total of 11,419 sequences were shared among all four insect species. About 60% of sequences (22,320 out of 37,040) did not show BLASTX similarity, implying that they represented un-translated regions, non-conserved regions, or proteins novel to C. suppressalis (Figure 1).

COG classification

To further evaluate the completeness of our transcriptome library and the effectiveness of our annotation process, we searched the annotated sequences for the genes involved in COG classification. In total, out of 15,446 hits, 5,824 sequences have a COG classification (Figure 2). Among the 25 COG categories, the cluster for “general function prediction” represents the largest group (1,646, 28%), followed by “replication, recombination and repair” (704, 12%) and “translation, ribosomal structure and biogenesis” (584, 10%). The categories of “extracellular structures” (1, 0.01717%) and “nuclear structure” (2, 0.03434%) represent the smallest groups (Figure 2). Similar observations were reported by Wang et al. [13] for the transcriptome of the whitefly Bemisia tabaci. To identify the biological pathways that are active in the C. suppressalis midgut, we mapped the 15,446 annotated sequences to the reference canonical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) [14]. In total, we assigned 12,358 sequences to 218 KEGG pathways (Table S1). Among these sequences, 1,252 belong to metabolic pathways, followed by 373 sequences in spliceosome pathway and 246 sequences in purine metabolism.

Table 1. Summary for the Chilo suppressalis midgut transcriptome.

| Total number of reads | 39,400,002 |
|-----------------------|------------|
| Total base pairs (bp) | 3,546,000,360 |
| Total number of contigs | 37,040 |
| Mean length of contigs (bp) | 497 |
| Sequences with E-value <10^-5 | 15,446 |
| GC percentage | 42% |

doi:10.1371/journal.pone.0038151.t001

doi:10.1371/journal.pone.0038151.t002

doi:10.1371/journal.pone.0038151.s001

doi:10.1371/journal.pone.0038151.s002

Protein domains

A Pfam domain search yielded 11,895 domains within the 37,040 contigs that have significant hits in the NCBI nr database (Table 3). Among the identified Pfam domains, zinc finger C2H2 type (Znf–C2H2), which is the most common DNA-binding motif [15], was the most abundant, existing in 542 transcripts (5%) from the C. suppressalis midgut. The WD G-beta repeat was the second most abundant domain in the C. suppressalis midgut sequences (Table 3). The domains related to reverse transcriptase, protein kinase, sugar transporters, carboxyl esterase, and trypsin also were abundant in these contigs (Table 3, Table S2). These results are consistent with a previous analysis of Pfam domains in Agrilus planipennis midgut [16], which may indicate the existence of similar gene profiles in the midgut of insects.
Genes of interest

In this study, we were interested in identifying genes involved in detoxification and the antioxidant response, Bt Cry1A toxin binding, digestion, immunity and defense, metabolism, remodeling and peritrophic membrane biosynthesis, (Table 4). Of particular interest are proteins that bind the Bt-toxin Cry1A, which are known to be cadherin-like proteins [17–22] and glycosylphosphatidylinositol (GPI)-anchored proteins (aminopeptidase N, APN, or alkaline phosphatase, ALP) [23–33]. We identified 16 cadherin-like proteins, 27 APNs, and 11 ALP proteins in the *C. suppressalis* midgut.

Exploring the BBMV proteome of *C. suppressalis*

The most abundant proteins in the BBMV of *C. suppressalis* were also investigated in this study. Figure 3 shows a representative two-dimensional (2D) electrophoresis gel revealing the profile of BBMV proteins from *C. suppressalis*. Among the detected protein spots, 100 abundant proteins that were present on replicated gels were excised and subjected to mass spectrometry analysis. The spectra obtained from MALDI-TOF/TOF were searched for in a local database (translated transcriptome data) and the NCBInr database, and a total of 74 selected proteins had significant results (Table S3). The abundant proteins identified by this proteome analysis are consistent with the transcriptome data. The RNA transcripts encoding these proteins are also abundant in the transcriptome of the *C. suppressalis* midgut.

The annotations of the identified proteins from the UniProt knowledge base (http://www.expasy.org/sprot/) were used to categorize the proteins based on their molecular function in terms of information obtained from the GO database (http://www.geneontology.org/) (Table S3). The identified proteins were functionally categorized as follows: binding proteins, structural maintenance, metabolism and electron transport processes, translation factors, molecular transducers, transcription factors, antioxidant and redox processes, and digestion (Table S3). The proteins identified in this study are in good agreement with other proteomic analyses of the lepidopteran midgut [34–38], which indicates that a common protein complex is shared among lepidopteran insects.

Western blot

To verify the accuracy of our transcriptome and proteome sequences, we chose the Bt-toxin Cry1Ac binding protein and actin as the molecule markers. Western blot analysis was used to
detect the proteins that bind Bt toxins in the midgut of C. suppressalis. As expected, the biotin-labeled Cry1Ac showed significant binding activity with spots 3, 48, 73, and 74 (Figures 3 and 4, Table S3). Using the MALDI-TOF/TOF technique, spot 3 and 48 were identified as two isoforms of APN. Spot 74 was identified as EH domain-containing protein 1 (Table S3). Using the MALDI-TOF/TOF technique, spot 3 and 48 were identified as two isoforms of APN. Spot 74 was identified as EH domain-containing protein 1, but spot 73 had no significant match (Table S3). These results confirmed that APN binds with the Cry1Ac toxin [39]. To the best of our knowledge, this is the first report that EH domain-containing protein 1 interacts with Bt toxins, and this result should be confirmed by further study. Unexpectedly, cadherin was not identified as a potential binding protein for Cry1Ac, which was reported previously [39]. We reasoned that the absence of cadherin in our proteome might be due to the limitation of 2D electrophoresis that is not suitable for separating proteins larger than ~200 kDa. Actin is found in almost all eukaryotic cells and is widely used as a molecule marker in many kinds of molecular experiments. Spot 82 was identified as actin and showed strong binding activity to the mouse anti-beta-actin monoclonal antibody (Sigma) (Figures 3 and 5, Table S3).

### Materials and Methods

#### Insect rearing and midgut sample preparation

The C. suppressalis strain was collected from the farm at the Huazhong Agricultural University, Wuhan, Hubei, China in 2010. This strain was reared in plastic containers (15 cm diameter × 8 cm high) on an artificial diet at 28±1°C under a 16 h photoperiod and >80% relative humidity. For total RNA and protein preparation, midguts were dissected from fifth instar larvae and cleaned in ice-cold 0.7% NaCl solution, immediately frozen in liquid nitrogen, and then stored at −80°C until use. The midgut content was removed before processing.

#### RNA isolation and library preparation for transcriptome analysis

Total RNA was isolated using the SV total RNA isolation system (Promega) according to the manufacturer’s protocol. To obtain complete gene expression information, the RNA sample from the midgut was used for transcriptome analysis. Poly(A) + RNA was purified using oligo(dT) magnetic beads and fragmented into short sequences at 94°C for 5 min. The cleaved poly(A) + RNA was transcribed, followed by synthesis of the second-strand cDNA. After the end repair and ligation of adaptors, the products were amplified by PCR and purified using the QIAquick PCR Purification Kit.

The cDNA library was sequenced on the Illumina sequencing platform (GAII). The raw reads from the images were generated using Solexa GA pipeline 1.6.

#### Bioinformatics data analysis

The high quality reads were assembled into contigs using Trinity software [12]. All contigs were used as the queries to search a local protein database containing all of the protein sequences of the nr database with the BLASTX algorithm. COG classification was analyzed using annotated contigs to search the COG database with the cutoff 10−3. For KEGG pathway analysis, all contigs was analyzed using annotated contigs to search the COG database with default parameters. For comparative genomics analysis, the annotated contigs were used to BLASTP against known genes in three insect species: Drosophila melanogaster, Anopheles gambiae, and Tribolium castenaeum.

#### BBMV extraction and 2D SDS-PAGE Gel Analysis

The BBMV were prepared according to a published method [40]. Briefly, midguts were homogenized in ice-cold Tris/Mannitol buffer (300 mM mannitol, 5 mM EGTA, 17 mM Tris-HCl, pH 7.5) containing a cocktail of protease inhibitors (Roche) and 24 mM MgCl$_2$. The homogenate was centrifuged at 2500 g for 15 min, and the supernatant was centrifuged at 30,000 g for 30 min. The pellet was resuspended in Tris/CHAPS buffer (150 mM NaCl, 5 mM EGTA, 1 mM PMSF, 20 mM Tris-HCl, 1% CHAPS) containing a cocktail of protease inhibitors (Roche).

BBMV proteins were processed using the 2D-Clean-up Kit (Bio-Rad) following the manufacturer’s instructions. These proteins were resuspended in a solubilization buffer containing 2 M thiourea, 7 M urea, 4% CHAPS, 65 mM DTT, and 0.2% Bio-lyte ampholytes (pH 3–10, Bio-Rad). Final BBMV samples were centrifuged at 12,000 g, 4°C for 5 min to remove any insoluble material. Isoelectric focusing (IEF) was performed on

### Table 4. Selection of genes of interest related to the larval midgut physiological functions.

| Total number of contigs (nr annotation) |   |
|----------------------------------------|---|
| **Detox** related:                     |   |
| Cytochrome P450                         | 63 |
| Glutathione-S-transferase               | 14 |
| Carboxylesterase                       | 16 |
| Superoxide dismutase                   |  5 |
| *Bacillus thuringiensis* Cry1A toxins binding partners: |   |
| Cadherin-like                          |  16|
| Aminopeptidase N                       |  27|
| Alkaline phosphatase                   |  11|
| **Digestion:**                         |   |
| Serine proteinase all types             | 14 |
| Cysteine proteinase all types           |  7 |
| Carboxypeptidase all types              | 46 |
| Aminopeptidase all types                | 33 |
| Dipeptidyl-peptidase                   |  5 |
| α-amylace                              |  10|
| α-glucosidase (malate)                 |  2 |
| β-glucosidase                          |  11|
| Lipase                                 |  51|
| **Immunity-related and defence against**|   |
| β-1,3-glucan recognition protein       | 12 |
| β-1,3-glucanase                        |  1 |
| Peptidoglycan recognition protein      |  4 |
| Immuno- and C-type lectins             | 20 |
| Defensin-like                          |  1 |
| Lysozyme                               | 11 |
| Serine protease inhibitor (Serpin)      | 39 |
| Transferrin                            |  7 |
| **Peritrophic membrane biosynthesis, metabolization and remodelling:** |   |
| Chitin synthase                        |  3 |
| Chitinase                              | 11 |
| Chitin deacetylase                     | 10 |
| Peritrophin-like, Mucin-like            | 19 |

DOI:10.1371/journal.pone.0038151.t004
a Protean IEF Cell (Bio-Rad). Midgut BBMV proteins were applied to 11 cm IPG strips at pH 3–10 (Bio-Rad) for separation. Equilibrated strips were overlaid onto a 10% SDS-polyacrylamide gel for the second dimension separation. Gels were visualized by silver staining [41].

Mass spectrometric analysis and database searching

Stained protein spots were excised from the gel. Before MS/MS analyses, mixtures of proteolytic peptides were desalted using C18 ziptips. Samples were analyzed in the positive ion mode using a 4800 Plus MALDI-TOF/TOF TM Analyzer (Applied Biosystems), and MS/MS of the 10 most intense ions (with S/N >50 per digest) was analyzed using automated data acquisition. Spectra

Figure 3. Representative example of 2D-gel of *Chilo suppressalis* larval midgut BBMV. BBMV were separated by 2D-gel electrophoresis followed by silver staining. Positions of molecular size markers (kDa) are indicated on the side of gel. Abundant protein spots were excised (numbers 1–100), digested, and subjected to mass spectrometry analysis for identification. Spot numbers correspond to the proteins listed in Table S3.

doi:10.1371/journal.pone.0038151.g003

Figure 4. Blot analysis of Cry1Ac binding proteins in *Chilo suppressalis* midgut. Proteins were separated by 2D-gel electrophoresis. Arrows denote positions of the Cry1Ac binding proteins. To detect Cry1Ac binding proteins, filters were probed with biotin-Cry1Ac. Positions of molecular size markers (kDa) are indicated on the side of gel.

doi:10.1371/journal.pone.0038151.g004
were processed and batch analyzed in the “Combined (MS + MS/MS)” mode using the Applied Biosystems GPS Explorer software. The data were then used to search our local database of *C. suppressalis* midgut transcriptome sequences. MALDI-TOF/TOF data searches were also performed in the nr database (translated transcriptome sequences) using the MASCOT program. The search parameters used were as follows: “metazoa” was used for “taxonomy” and “trypsin” was used for “enzyme of specificity strict”; fixed modifications of carbamidomethyl (C); peptide tolerance of 100 ppm; fragment mass tolerance of 0.8 Da; peptide charge of 1+; and monoisotopic. Only significant hits (p < 0.05) were accepted. Manual de novo sequence searching against the NCBI-BLASTP database, e-values, and frequency of matches to a specific protein were also the primary criteria for these determinations.

Western and ligand blotting

Separated BBMV were transferred onto NC membranes at 15 V constant voltage for 1 h using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell system (Bio-Rad). Membranes were blocked with 5% skimmed milk in PBST (137 mM NaCl, 2 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4, 0.05% Tween-20) for 2 h. Following this incubation, the membranes were washed with three changes of PBST for 10 min each.

For detection of Cry1Ac binding proteins, the pure activated Cry1Ac toxins (EnviroLogix Inc.) were first marked with biotin (Roche). The blocked membranes were incubated with biotinylated Cry1Ac (1:5000) for 1 h at room temperature. After washing, membranes were incubated for 1 h with a 1:5000 dilution of streptavidin-alkaline phosphatase conjugate (Sigma). Binding proteins were visualized using the BCIP/NBT Kit (Sigma) following the manufacturer’s instructions.

For detection of actin, blocked filters were probed with a 1:1000 dilution of mouse anti-beta-actin monoclonal antibody (Sigma). Goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma) was used as the secondary antibody. Membranes were then developed using the BCIP®/NBT Kit (Sigma) following the manufacturer’s instructions. No endogenous alkaline phosphatase activity was detected when probing blots of BBMV proteins with streptavidin-AP or under direct exposure to NBT-BCIP. All blots were repeated in triplicate to ensure reproducibility of results.

Supporting Information

Figure S1 Size distribution of assembled contigs.
(TIF)

Table S1 KEGG summary of *Chilo suppressalis* midgut sequences.
(XLS)

Table S2 Pfam domain search of *Chilo suppressalis* midgut sequences.
(XLS)

Table S3 Proteins identified by MADIL-TOF/TOF and de novo sequence analysis.
(DOC)

Acknowledgments

We thank the technical assistance of Chunyan Guo. Lanzhi Han, Institute of Plant Protection, China Academy of Agricultural Sciences, assisted with insects indoor rearing and dissection of *C. suppressalis* larvae. We are grateful to Zhihui Zhu for comments on the manuscript. Our thanks also go to the two anonymous reviewers for their thoughtful comments that improved the paper.

Author Contributions

Conceived and designed the experiments: WM FL YL. Performed the experiments: WM. Analyzed the data: WM ZZ FL YL. Contributed reagents/materials/analysis tools: CP XW ZZ. Wrote the paper: WM FL.

References

1. Cheng X, Chang C, Dai S (2010) Responses of striped stem borer, *Chilo suppressalis* (Lepidoptera: Pyralidae), from Taiwan to a range of insecticides. Pest Manag Sci 66: 762–766.

2. Federici BA, Park H, Bideshi DK (2010) Overview of the basic biology of *Bacillus thuringiensis* with emphasis on genetic engineering of bacterial larvicides for mosquito control. Open Toxino J 3: 83–100.

3. Rodríguez-Almazán C, Zavala LE, Muñoz-Garay C, Jiménez-Juárez N, Pacheco S, et al. (2009) Dominant negative mutants of *Bacillus thuringiensis*
Cry1Ab toxin function as anti-toxins: Demonstration of the role of oligomerization in toxicity. PLoS ONE 4: e5545. doi:10.1371/journal.pone.0005545.

4. Sena JAD, Hernández-Rodríguez CS, Ferré J (2009) Interaction of Bacillus thuringiensis Cry1 and Vip3A proteins with Spodoptera frugiperda midgut binding sites. Appl Environ Microbiol 75(7): 2236–2237.

5. Ebokh M, Solera M, Kyanamaya S, Mvanga ROM, Odango B, et al. (2010) Toxicity of seven Bacillus thuringiensis cry proteins against Cylas puncticollis and Cylas brunnus (Coccinellidae) using a novel artificial diet. J Econ Entomol 103(4): 1495–1502.

6. Tabashnik RE, Umithan GC, Masson I, Crowder DW, Li X, et al. (2009) Asymmetrical cross-resistance between Bacillus thuringiensis toxins Cry1Ac and Cry2Ab in pink bollworm. Proc Natl Acad Sci USA 106: 11889–11894.

7. Tabashnik RE, Gassmann AJ, Crowder DW, Carrier Y (2008) Insect resistance to Bt crops: Evidence versus theory. Nat Biotechnol 26: 199–202.

8. Bagla P (2010) Hardy cotton-munching pests are latest blow to GM crops. Science 327: 1439.

9. Liu FY, Xu ZP, Zhu YC, Huang FN, Wang YH, et al. (2010) Evidence of field-evolved resistance to Cry1Ac-expressing Bt cotton in Helicoverpa armigera (Lepidoptera: Noctuidae) in northern China. Pest Manag Sci 66: 155–161.

10. Downes S, Mahon RJ, Rosster I, Kauter G, Leven T, et al. (2010a) Adaptive management of pest resistance by Helicoverpa species (Noctidae) in Australia to the Cry2Ab Bt toxin in Bollgard II® cotton. Evol Appl 3: 574–584.

11. Downes S, Parker T, Mahon R (2010b) Incipient resistance of Helicoverpa armigera to the Cry2Ab Bt toxin in Bollgard II® cotton. PLoS ONE 5: e12567.

12. Grubherr MG, Haas BJ, Yassour M, Levin JZ, et al. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011 May 15; 29(7): 644–652.

13. Wang X, Luan L, Li J, Bao Y, Zhang C, et al. (2010) De novo characterization of a whirligig transcriptome and analysis of its gene expression during development. BMC Genomics 11: 400.

14. Kaneshia M, Goto S, Kawashima S, Okuno Y, Hattori M (2004) The KEGG resource for deciphering the genome. Nucleic Acids Res 32: D277–280.

15. Brayer KJ, Segal DJ (2008) Keep your fingers off my DNA: protein-protein interactions mediated by C2H2 zinc finger domains. Cell Biochem Biophys 50: 31–46.

16. Pauchet Y, Wilkinson P, Vogel H, Nelson DR, Reynolds RE, et al. (2010) Pyrosequencing the Manduca sexta larval midgut transcriptome: messages for digestion, detoxification and defense. Insect Mol Biol 19(1): 61–75.

17. Pauchet Y, Muck A, Svatol A, Heckel DG, Preis S (2008) Mapping the larval midgut human proteome of Helicoverpa armigera, a generalist herbivorous insect. J Proteome Res 7(4): 1629–1639.

18. Pauchet Y, Muck A, Svatol A, Heckel DG (2009) Chromatographic and electrophoretic resolution of proteins and protein complexes from the larval midgut microvilli of Manduca sexta. Insect Biochem Mol Biol 39: 467–474.

19. Yao H, Xiang C, Chen L, Guo A, He F, et al. (2009) Identification of the proteome of the midgut of silkworm, Bombyx mori L., by multidimensional liquid chromatography (MDLC) LTQ-Orbitrap MS. Biosci Rep 29: 363–373.

20. Liu J, Zheng S, Liu L, Li L, Feng Q (2010) Protein profiles of the midgut of Spodoptera litura larvae at the sixth instar feeding stage by shotgun ESMIS approach. J Proteome Res 9: 2117–2147.

21. Yu H, Chen H, Zhang Y, Wu K, Liang G, et al. (2010) Gene cloning and expression of aminopeptidase N and cadherin from midgut of the rice stem borer, Chilo suppressalis. Insect Science 17: 393–399.

22. Woltersberger MG, Luthy P, Maurer A, Parenti P, Sacchi VF, et al. (1987) Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (Pieris brassicae). Comp Biochem Physiol 86A: 301–308.

23. Yan JN, Wait R, Berkelman T, Harry RA, Westbrook JA, et al. (2000) A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. Electrophoresis 21(17): 3666–3672.