CHAPTER 3

The Cotton-Insect Interactive Transcriptome

Molecular Elements Involved in Plant-Insect Interactions

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INTRODUCTION

Insects are the most diverse and adapted species of animals on our planet. Insects can live in all habitats of the earth, from the ocean floor to the peak of a mountain (Imms 1964) and play a significant role in plant and animal life. Insects have been classified as predators, anti-biological insect-pests, pollinators or producers of valuable products (Offor et al. 2014). In addition, they are used for pharmacological purposes such as anti-venoms or antibodies (Moreno and Giralt 2015). Among all known insects, only fifty percent of them are pests, but a significant few pose a serious threat to agriculture and food security (Offor et al. 2014).

Cotton (*Gossypium* sp.) is susceptible to a wide range of insect pests. There are different types of pests that are harmful to cotton, such as cotton bollworm (*Helicoverpa armigera*), plant bugs (*Miridae* sp.), stink bugs (*Halyomorpha halys*), aphids (*Aphis gossypii* Glover), thrips (*Thysanoptera* sp.) and spider mites (*Tetranychidae*). Cotton bollworm, specifically, the pink bollworm (*Pectinophora gossypiella*) brings more damage to crop production by damaging the bolls (Xiong et al. 2015; Tassone et al. 2016), and *Lygus lineolaris* (*Palisot de Beauvois*) is a polyphagous, phytophagous insect pest in cotton and other important crops (Showmaker 2016).

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The chewing insect boll weevil larvae (*Anthonomus grandis*) and phloem feeding insect *Bemisia tabaci* (whitefly) (Artico et al. 2014; Li et al. 2016) cause the loss of diverse cotton products.

Although researchers have elucidated many aspects of insect-host interactions and developed effective tools against the insect pests including genetic engineering of *Bacillus thuringiensis* (or Bt) Cry toxin gene and using RNA interference of vitally important insect genes (Abdurakhmonov et al. 2016), many aspects of molecular mechanisms of insect resistance in cotton require a more in-depth study with application of new generation of "omics" tools such as transcriptome analysis. In past years, to better understand molecular mechanisms in cotton plant-insect interactions, the transcriptome of insect venom and midgut extracts as well as olfactory systems were sequenced together with plant damaged tissues. Recent research revealed that most transcriptome related research studies have been dedicated to the analysis of cotton bollworm *Helicoverpa armigera*. This insect is one of the widely-spread pests feeding on hundreds of varieties of plant species (Liu et al. 2014), and causes large-scale damage to agricultural production. Various species of bollworm cause some of the greatest yield losses in Africa annually even in Bt-cotton plantations (Rangarirai et al. 2015; Liu et al. 2014). Hence, transcriptome analysis is a research priority directed not only to solve the problems resulting by a pest such as *Helicoverpa armigera* but also to understand major insect resistance pathways in a cash crop such as cotton.

Transcriptome Analysis of Insect-Insecticide Interactions

In conventional farming, most of the cotton pests were controlled by using insecticides. Insecticides differ by their targets, for example, ovicides target insect eggs, while larvicides target larvae. However, during the last decade, the usage of insecticides did not prove to be an efficient strategy. Efficiency of insecticides over time can be evaluated by resistance developed by insects. Even though the expression Cry1Ac gene from *Bacillus thuringiensis* in transgenic cotton cultivars led to significantly lower usage of pesticides for many years (Qiu et al. 2015; Cao et al. 2016), in subsequent years, several pests including green mirid bugs (GMB), *Apolygus lucorum* and *Adelphocoris suturalis* Jakovlev, have increased their resistance to Bt toxin. Therefore, *de novo* transcriptome assembly and gene expression analyses have been implemented to understand the molecular, genetic, biochemical and physiological mechanisms of toxin resistance of these insects during different developmental stages (Cao et al. 2016; Tian et al. 2015). To identify target genes, the transcriptome of all developmental stages of the insect pest *A. grandis* was analyzed and several key insect genes (e.g., chitin synthase 1) have been characterized (Firmino et al. 2013).

The pink cotton bollworm *Pectinophora gossypiella* is another model organism for insect responses to Bt toxins, but the molecular mechanism of its tolerance was studied to a lesser extent. Using *de novo* transcriptome assembly for the midgut of *P. gossypiella*, 46,458 transcripts have been derived from 39,874 unigenes ("Unigene" is the NCBI transcriptome database). The transcriptome data presented those relevant midgut proteins critical for xenobiotic detoxification, nutrient digestion and allocation, as well as for the discovery of protein receptors important for Bt intoxication (Tassone et al. 2016).

Interactions between secondary metabolites of cotton and *H. armigera* insect resistance was unclear before Tao (2012) who studied enzyme cytochrome P450 monooxygenases (P450s) in cotton bollworm that increases detoxification of gossypol (Tao et al. 2012). Subsequently, comparative detoxification enzymes of *H. assulta* and *H. armigera*, as well as transcriptome of *Aphis gossypii* Glover were investigated (Li et al. 2013a,b). Five gossypol-induced P450s genes contributed to cotton bollworm tolerance to deltamethrin insecticide. When one of the genes CYP9A14 was knocked down by plant-mediated RNA interference (RNAi), the larvae exhibited more sensitivity to the insecticide (Tao et al. 2012). For increasing adaptation to very different feeding sources, *A. grandis* was able to produce transcripts encoding proteins involved in catalytic processes of macromolecules. Besides, these proteins are involved in detoxification mechanisms such as expression of p450 genes, glutathione-S-transferase, and carboxylesterases (Salvador et al. 2014).

Effects of systemic insecticides, thiamethoxam and spirotetramat, belonging to the class of neonicotinoids widely used against *A. gossypii* Glover were also investigated using Illumina-Solexa sequencing technology (Bentley et al. 2008). A total of 22,569,311 and 21,317,732 clean reads (sequences "cleaned" of vector and adaptor sequences) were obtained from the thiamethoxam-resistant strain (ThR) and susceptible strain (SS) transcriptomes, as well as total of 22,430,522 and 21,317,732 clean reads from RS (resistant strain) and SS cDNA libraries. Studies revealed that unigenes significantly changed in both ThR and RS libraries compared to the both SS strains (Pan et al. 2015a,b). Transcriptomic sequences were used to analyze the involvement of potential heat shock protein (Hsp) homologs of *Latrodectus hesperus* (the western black widow spider or western widow) proteins in thermal stress response transcriptome analysis. Results showed the up regulation of Hsp70, Hsp40, and two small Hsps in the heat-challenged adults of *L. hesperus*. These results helped in understanding the fundamental mechanisms of abiotic stress response genes and their role in thermotolerance (Hull et al. 2013).

Heat challenged expression levels of ribosomal proteins, heat shock protein 70 (Hsp70), ATP synthase, ecdysteroid UDP-glucosyltransferase and esterase in aphids were up-regulated significantly in the ThR and SR strains compared to the SS strains. The decreased expression of genes encoding cuticle proteins, salivary glue proteins, and energy ATP synthase, and cytochrome c oxidase, fibroin heavy chain was observed (Pan et al. 2015a,b). A nicotinic acetylcholine receptor *(nAChR)* α subunit was down-regulated in the ThR strain (Pan et al. 2015a). Among the differentially expressed genes (DEGs) for cytochrome P450, *6A2* was the only up-regulated gene in the SR strain (Pan et al. 2015b). These data illustrated that genetic changes in *nAChR* genes and up-regulated ribosomal proteins, ecdysteroid UDP-glucosyltransferase, cytochrome c oxidase, esterase and peroxidase may confer the resistance of cotton aphids to thiamethoxam (Pan et al. 2015a). Subsequently, an over-expression of *CYP6A2* gene associated with spirotetramat resistance and cross-resistance in the resistant strain of *Aphis gossypii* Glover was achieved. Suppression of CYP6A2 transcripts by RNAi significantly increased the sensitivity of the resistant aphid to spirotetramat (Peng et al. 2016).

The increased quantity of secondary metabolites, namely, natural insecticides in plants is associated with abiotic stresses such as treatment with NaCl. The increase in levels of gossypol, flavonoids and tannic acid in plants was linked to reduced aphid population when cotton plant was treated with 50–200 mM NaCl. Compared to non-treated samples, a salt-treated aphid transcriptome analysis showed higher expressions of genes including fatty acid and lipid biosynthesis, carbohydrate and amino acid metabolism, energy metabolism and few others responsive for cell motility pathway (Wang et al. 2015). qRT-PCR showed a high expression of transcripts for *CYP6A14*, *CYP6A13*, *CYP303A1*, *NADH* and fatty acid synthase genes. In contrast, *CYP307A1* and two ecdysone-induced protein genes were down regulated. The study also showed expression of genes related to growth and development of aphids being highly expressed by enhanced secondary metabolism in cotton under salt stress. The involvement of aphid gene *CYP307A1* in ecdysone synthesis showed its positive correlation with the population dynamics (Wang et al. 2015).

Transcriptome Analysis of Olfactory Genes in Diverse Insect Species

Olfaction is a significantly essential factor in the life cycle of insects. Insects sense the smell through their antennae, the transcriptome of which can serve as a good model to understand the olfaction mechanisms in insects.

In *Adelphocoris suturalis,* a plant bug that is one of the most serious insect pests of Bt cotton in China, two soluble protein compositions, namely, odorant binding proteins (OBPs) and chemosensory proteins (CSPs) participate in the initial biochemical recognition steps in semi-chemical perception and insect olfactory signal transduction (Gu et al. 2013; Cui et al. 2016). Transcriptome of three asexual developmental stages (wingless spring and summer morphs and winged adults) of *Aphis gossypii* Glover have been compared and characterized. The number and length of introns observed were much higher in general and this appears to be a unique feature of Aphid OBP and CSP genes. On the other hand, higher abundance of CSP transcripts than OBP is another unique feature in aphids (Gu et al. 2013). Different transcripts were expressed in male and female species. For instance, 4 OBPs (AsutOBP1, 4, 5 and 9) and 1 CSP (AsutCSP1) were expressed at higher levels in male than in female antennae in *A. suturalis* (Cui et al. 2016).

Many candidate chemosensory genes were identified by sequencing female- and male-antennae transcriptomes of *Chrysopa pallens*, *Spodoptera littoralis* and *Chrysoperla sinica* including OBPs, CSPs, odorant receptors (ORs), and ionotropic receptors (IRs) (Li et al. 2013c; Poivet et al. 2013; Li et al. 2015b). Existence of three types of chemosensory receptors including ORs, gustatory receptors (GRs) and IRs in insects were identified by investigating expansion of a bitter taste receptor family in a polyphagous insect herbivore.

One hundred and ninety-seven novel GR genes were also identified from the polyphagous pest *Helicoverpa armigera* (Liu et al. 2014; Xu et al. 2016). These receptors play vital roles in sensing chemical signals, sex-specific or developmental stage-specific chemosensory behaviors that guide insect behaviors. Using transcriptome sequencing, trinity RNA-seq assemblies and extensive manual curation, 60 candidate ORs, 10 GRs and 21 IRs have been identified in *H. armigera* (Liu et al. 2014). Further, 83 and 68 transcripts related to olfaction have been identified *H. armigera* and *H. assulta*. Moreover, more than a thousand transcripts of digestive enzymes were identified in the same insect species. Comparative analysis showed that detoxification enzymes, e.g., P450, carboxypeptidase, and ATPase were higher in *H. assulta* than in *H. armigera*. These detoxification enzymes would help them to increase the food detoxification and utilization efficiency (Li et al. 2013b).

As in other organisms, innate immunity is important for defense of *H. armigera* from invading pathogens. Both fat body and hemocytes are important organs involved in the immune response (Xiong et al. 2015). *De novo* sequencing and transcriptome analysis of endoparasitoid

Aenasius arizonensis showed the involvement of venom glands in host developmental arrest, disrupting the host immune system, and host paralysis (Shaina et al. 2016). After pathogen infections, it was observed that the immunotranscriptome of *H. armigera* larvae and the related gene expression have changed. Some immunity-related genes were activated in fungus and bacterium-challenged fat bodies, while others were suppressed in the fungus, *Beauveria bassiana,* challenged by hemocytes. More immunity-related genes were induced by the fat bodies than those by hemocytes (Xiong et al. 2015). To observe the involvement of gene families in immunity between fat bodies and antennae, Legeai's study (2014) was very important in *Spodoptera frugiperda*. Sf_TR2012b transcriptome enabled researchers to explore the spatial and temporal expression of genes and to observe that some olfactory receptors are expressed in antennae and palps but also in other non-related tissues such as fat bodies (Legeai et al. 2014). The naturally occurring ecdysteroid hormone which controls the ecdysis (moulting) and metamorphosis of insects, 20-hydroxyecdysone (20E), influences innate immunity and induced the expression of the immunity-related genes in many holometabolous insects as well as in the cotton bollworm. This has been proved by transcriptome analysis of peptidoglycan-challenged fat body of cotton bollworm. Results showed that antibacterial activities were enhanced, mRNA levels of pattern recognition receptors and antimicrobial peptides significantly increased during the wandering or pupal stage of the insect life cycle (Wang et al. 2014).

Transcript Involvement in Pheromone Biosynthesis

Pheromones are produced in metathoracic scent glands (MTGs) in *A. suturalis* Jakovlev and play an important role in survival and population propagation of the insect. There was very little information about the molecular basis of the pheromone biosynthesis of the insects till recently (Luo et al. 2014). It is essential to clarify the involvement of genes in the production of pheromone components. Two similar sex pheromone components Z9-16:Ald and Z11-16:Ald have been found in both the sibling species cotton bollworm (*H. armigera*) and oriental tobacco budworms (*H. assulta*). Differences in sex pheromone component can lead to reproductive isolation and it is genetically controlled. To investigate how the ratios of the pheromone components are differently regulated in the two species, cDNA libraries have been sequenced from the pheromone glands of *H. armigera* and *H. assulta*. The research highlighted the involvement of some of the transcripts among the identified ones in the sex pheromone biosynthesis pathways (Li et al. 2015a). qRT-PCR results

demonstrated higher expression levels of *Asdelta9-DES*, *AsFAR*, *AsAOX*, *Ascarboxylesterase*, *AsNT-ES* and *AsATFs* genes in the releasing period of sex pheromones in female *A. suturalis* Jakovlev. Thus, many potential pheromone biosynthetic pathway genes were identified (Luo et al. 2014).

Transcriptome Analysis Under Herbivore Stresses

Insect herbivores destroy world's major crops and thus affect global economic growth. High yielding crop varieties have been developed for the past three decades by novel breeding techniques (Kerin 1994). In the process of artificial selection, suitable crops are largely selected by breeders for increased agricultural productivity and human consumption. In examining the genetic bottlenecks of the process, an important parameter continues to be the ability of every plant species to have its own mechanism to protect itself from herbivore attacks. The involvement of many genes in the biotic stress responses such as a mitogen-activated protein kinase (MAPK), transcription factors (WRKY and ERF protein domains) and signaling by ethylene (ET) and jasmonic acid (JA) hormones have been identified in *Anthonomus grandis* using transcriptome, differentially-expressed gene (DEG) sequence analysis and virus-induced gene silencing (VIGS) (Artico et al. 2014; Li et al. 2016). MAPK-WRKY-JA functions and ET pathways were suppressed by virus-induced gene silencing (VIGS) in *Bemisia tabaci* (Li et al. 2016). This results in the release of elevated levels of volatiles which can serve as a chemical signal. In response to the insect herbivore, plants directly and indirectly produce volatile organic compounds. However, the molecular basis for defense response during insect herbivory trigger in cotton plant and how the defensive compounds are manipulated as part of biological processes have not been well studied. Huang et al. (2015) showed the transcriptome changes and volatile characteristics of cotton plants in response to cotton bollworm (CBW). Around two thousand transcripts showed different expressions after CBW infestation. Cluster analysis indicated that CBW-induced genes play important roles in CBW-induced defenses (Huang et al. 2015).

Acyclic terpenes and the shikimate pathway product indole are biosynthesized *de novo* following insect damage and experiments demonstrated that the application of caterpillar oral secretions increased the production and release several volatiles that are synthesized *de novo* in response to insect feeding. The role of plants in mediating the interaction between herbivores and natural enemies of herbivores was demonstrated to be a critically dynamic phenomenon (Pare et al. 1997).

To better control the insects, a subunit of mitochondrial complex I NDUFV2 that catalyzes NADH dehydrogenation in respiratory chain was suppressed by double-stranded RNA (dsRNA). When cotton bollworm larvae were fed with transgenic cotton tissue, expression of NDUFV2 dsRNA caused mortality up to 80%, and no larvae survived. Comparative transcriptome analysis showed a repression of Dopa decarboxylase genes (Wu et al. 2016). At the same time, the role of insect predators that feed on insect-pests was examined. One of the insect predators *Arma chinensis*, effectively used to control several insect-pests including Colorado potato beetle, cotton bollworm, and mirid bugs, was fed with artificial diet (pig liver and tuna) to better understand the impact of such diets. Transcriptome analyses demonstrated the differential expressions of thousands of genes between pupae-fed and diet-fed *A. chinensis* which can be efficiently used for the reduction of the expected damage by insect-pests (Zou et al. 2013). These novel transcriptome approaches as vital applications in insect research should be very helpful to shed light on details of the molecular signatures and their interactions during insect-plant interaction.

Conclusions

This brief overview of research studies of the past decade (Table 1) highlighted herein reveals that the new generation "omics" approach such as transcriptome analysis has helped to better understand the molecular mechanisms of insect resistance in a globally important cash crop such as cotton. In particular, the transcriptome analyses for insecticide treatment, olfaction, and pheromone production processes in insects as well as research involving herbivore stresses in cotton has revealed novel information on key genes and molecular signatures that are essential to understand resistance mechanisms to help develop novel insect resistant cotton cultivars.

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Table 1. Recent studied cotton-insect transcriptome analyses.

No	Targeted genes and proteins	Used methods	Insect name	References
1	*Asdelta9-DES*, *AsFAR*, *AsAOX*, *Ascarboxylesterase*, *AsNT-ES* and *AsATFs*	qRT-PCR	*Adelphocoris suturalis* Jakovlev	Luo (2014)
2	Development pathways, hormone biosynthesis, sex differences and wing formation	Quantitative real-time PCR	*Adelphocoris suturalis* Jakovlev	Tian (2015)
3	Odorant binding proteins (OBPs) and chemosensory proteins (CSPs)	Transcriptome	*Adelphocoris suturalis* Jakovlev	Cui (2016)
4	Calreticulin, Serine Protease Precursor and Arginine kinase proteins	*De novo* sequencing and transcriptome analysis	*Aenasius arizonensis*	Shaina (2016)
5	Mitogen-activated protein kinase (MAPK), transcription factors (WRKY and ERF) and signaling by ethylene (ET) and jasmonic acid (JA) hormones	Transcriptome, DEG sequence	*Anthonomus grandis*	Artico (2014)
6	Chitin synthase 1	RNA interference	*Anthonomus grandis*	Firmino (2013)
7	P450	Pyrosequencing	*Anthonomus grandis*	Salvador (2014)
8	Odorant binding proteins (OBPs) and chemosensory proteins	RNA-seq analyses	*Aphis gossypii* Glover	Gu (2013)
9	*nAChR*	Transcriptome	*Aphis gossypii* Glover	Pan (2015a)
10	Cuticle proteins, salivary glue protein, fibroin heavy chain, energy ATP synthase, and cytochrome c oxidase	Transcriptome	*Aphis gossypii* Glover	Pan (2015b)
11	*CYP6A14*, *CYP6A13*, *CYP303A1*, CYP307A1, *NADH* dehydrogenase and fatty acid synthase	qRT-PCR	*Aphis gossypii* Glover	Wang (2015)
12	CYP6A2	RNA interference	*Aphis gossypii* Glover	Peng (2016)
13	Cry1Ac	Overexpression	*Apolygus lucorum*	Cao (2016)
14	Dopa decarboxylase	dsRNA	*Apolygus lucorum*	Wu (2016)
15	Thousands of genes	Transcriptome	*Arma chinensis*	Zou (2013)

16	Mitogen-activated protein kinase (MAPK), transcription factors (WRKY and ERF) and signaling by ethylene (ET) and jasmonic acid (JA) hormones	Virus-induced gene silencing (VIGS)	*Bemisia tabaci (whitefly)*	Li(2016)
17	Chemosensory	Transcriptome	*Chrysopa pallens*	Li (2013c)
18	Chemosensory	Transcriptome	*Chrysoperla sinica*	Li (2015b)
19	CYP9A14	RNA interference	*Helicoverpa armigera*	Tao 2012
20	Detoxification enzyme, e.g., P450, carboxypeptidase, and ATPase	Transcriptome	*Helicoverpa armigera*	Li (2013b)
21	Gustatory receptor	RNA-seq analyses	*Helicoverpa armigera*	Liu (2014)
22	20-hydroxyecdysone (20E)	Transcriptome	*Helicoverpa armigera*	Wang (2014)
23	Cry1Ac	Overexpression	*Helicoverpa armigera*	Qiu (2015)
24	PGRP-SA1, Serpin1, Toll-14, and Spz2	RNA-seq analyses	*Helicoverpa armigera*	Xiong (2015)
25	Jasmonic acid	Cluster analysis	*Helicoverpa armigera*	Huang (2015)
26	HarmGR35, HarmGR50 and HarmGR195	Feeding	*Helicoverpa armigera*	Xu (2016)
27	Detoxification enzymes	High-throughput sequencing	*Helicoverpa armigera* and *Helicoverpa assulta*	Li (2013a)
28	Pheromone components Z9-16:Ald and Z11-16:Ald	Semi-quantitative RT-PCR, qRT-PCR	*Helicoverpa armigera* and *Helicoverpa assulta*	Li(2015a)
29	Heat shock protein (Hsp) homologs	Pyrosequencing, *de novo* assamble	*Lygus hesperus*	Hull (2013)
30	Salivary gland proteins	Sialotranscriptome	*Lygus lineolaris*	Showmaker (2016)
31	BT-toxin	*de novo* transcriptome	*Pectinophora gossypiella*	Tassone (2016)
32	The acyclic terpenes (E,E)-[alpha]-farnesene, (E)-[beta]-farnesene, (E)-[beta]-ocimene, linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, and (E/E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	Biosynthesized *de novo*	*Spodoptera exigua Hubner*	Pare (1997)
33	Sf_TR2012b	Reference transcriptome	*Spodoptera frugiperda*	Legeai (2014)
34	Chemosensory	Transcriptome	*Spodoptera littoralis*	Poivet (2013)

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