Identification of Genes Potentially Responsible for extra-Oral Digestion and Overcoming Plant Defense from Salivary Glands of the Tarnished Plant Bug (Hemiptera: Miridae) Using cDNA Sequencing

Yu-Cheng Zhu,1,2 Jianxiu Yao1 and Randall Luttrell1

1USDA-ARS Southern Insect Management Research Unit, Stoneville, MS 38776, USA, and 2Corresponding author e-mail: yc.zhu@ars.usda.gov

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Abstract

Saliva is known to play a crucial role in tarnished plant bug (TPB, Lygus lineolaris [Palisot de Beauvois]) feeding. By facilitating the piercing, the enzyme-rich saliva may be used for extra-oral digestion and for overcoming plant defense before the plant fluids are ingested by TPBs. To identify salivary gland genes, mRNA was extracted from salivary glands and cDNA library clones were sequenced. A de novo-assembling of 7,000 Sanger sequences revealed 666 high-quality unique cDNAs with an average size of 624 bp, in which the identities of 347 cDNAs were determined using Blast2GO. Kyoto Encyclopedia of Genes and Genomes analysis indicated that these genes participate in eighteen metabolic pathways. Identifications of large number of enzyme genes in TPB salivary glands evidenced functions for extra-oral digestion and feeding damage mechanism, including 45 polygalacturonase, two α-amylase, one glucosidase, one glycan enzyme, one aminopeptidase, four lipase, and many serine protease cDNAs. The presence of multiple transcripts, multigene members, and high abundance of cell wall degradation enzymes (polygalacturonases) indicated that the enzyme-rich saliva may cause damage to plants by breaking down plant cell walls to make nutrients available for feeding. We also identified genes potentially involved in insect adaptation and detoxifying xenobiotics that may allow insects to overcome plant defense responses, including four glutathione S-transferases, three esterases, one cytochrome P450, and several serine proteases. The gene profiles of TPB salivary glands revealed in this study provides a foundation for further understanding and potential development of novel enzymatic inhibitors, or other RNAi approaches that may interrupt or minimize TPB feeding damage.

Key words: Lygus lineolaris, TPB, saliva, salivary gland, cDNA, polygalacturonases, extra-oral digestion, detoxification

During the past few years, the widespread implementation of transgenic plants has caused a pest status shift from chewing insects to piercing-sucking insects on row crops, such as the tarnished plant bug (TPB, Lygus lineolaris [Palisot de Beauvois]) and stink bugs [Acrosternum hilare (Say), Nezara viridula (L.), and Euschistus servus (Say)] (Greene et al. 1999; Lu et al. 2008, 2010). The economic importance of TPB has become prominent in recent years due to its fast population increase coupled with the development of insecticide resistance (Snodgrass and Scott 2000; Zhu et al. 2004; Zhu et al. 2012). TPB is capable of adapting to different ecosystems, and it has a wide range of host plant species, including cotton, alfalfa, fruits, nuts, and vegetables (Young 1986). TPBs cause direct damage by feeding on plant tissues, typically on plant parts with high rates of cell division, including buds, flowers, and maturing fruit. TPB feed by sucking sap from plants via piercing-sucking mouthparts and simultaneously inject enzyme-containing saliva (digestive enzymes) into the feeding site to aid in the breakdown of plant tissues (Wheeler 2001). TBP nymphal and adult feeding causes damage to the terminal growth area thereby reducing plant growth and causing yield loss (Layton 2000). Symptoms of damage include yellowed, dried, ragged, and discolored or dropped leaves that appear 1–2 weeks after feeding injury, and aborted flower buds can occur. The typical feeding symptoms on cotton include deep lessons or warts within externally evident dark lesions (Musser et al. 2009).

In addition to performing extra-oral digestion (Cohen 1998), saliva is also secreted to suppress and detoxify plant defense responses. It is well known that the hemipteran insects with piercing-sucking mouthparts are able to actively suppress plant defense responses during feeding by injecting saliva into a host plant. This prevents plant wound response to the saliva components (Tjallingii 2006).
The diverse range of salivary components is known to play a crucial role in the successful feeding of a number of different phytophagous insects. For example, aphids (Nasonovia ribisnigri, Aphis gossypii, and Myzus persicae) use their styles to penetrate plant epidermis cell walls and membrane, and inject saliva for extra-oral digestion before ingesting the cell contents. Insect saliva contains proteins with diverse activities to facilitate feeding, and function as effectors to induce or suppress plant defense responses. Aphid (Megalura vicicae) saliva contains calcium binding protein that prevents calcium-dependent signaling pathways in phloem sieve tubes and suppress sieve element occlusion, which is a common mechanism in plants to prevent loss of phloem sap upon injury (Will et al. 2007, 2009). Polygalacturonase (PG) secreted by Lygus bugs enzymatically digest plant tissue structures for subsequent ingestion (Celorio-Mancera et al. 2009). The caterpillar of Helioverpa zea secretes glucose oxidase into plant cells to suppress the production of nicotine which might be responsible for the resistance development to Nicotiana tabacum (Musser et al. 2002). Therefore, knowledge of salivary secretions is crucial to understand how insects interact with their host plants.

The tools for identification of effectors and functional characterization have been well-developed recently using diverse and fast sequencing techniques. Potential effectors that modulate plant defenses have been identified in the saliva of an aphid (M. persicae), a whitefly (Bemisia tabaci), a leafhopper (Empoasca fabae), and a planthopper (Nilaparvata lugens) (Harmel et al. 2008; DeLay et al. 2012; Su et al. 2012; Ji et al. 2013). In pea aphid (Acyrthosiphon pisum), the identification of candidate genes from salivary glands made it possible to silence gene expression of salivary glands and alter aphid feeding behavior by RNA interference (Mutti et al. 2006).

Despite the fact that TPB is a serious and destructive pest on a wide host ranges, current understanding of biochemistry and physiology of salivary gland secretion in TPB is very limited. However, the detoxification and suppression of plant defense responses through secretion of certain enzymes from herbivores might be a common strategy for adaptation and evolution. Therefore, it is necessary to unravel what kinds of major toxic substances (enzymes) are injected from salivary gland to suppress plant defense mechanisms. Because TPBs have more than 300 host plant species (Young 1986), we hypothesized that the saliva in TPB may also contain a wide range of enzymes, enabling the bug to overcome a vast array of plant defense compounds.

To reveal salivary contents and enzymatic profiles in salivary glands of TPB, we constructed cDNA library from salivary gland mRNAs and sequenced ~7,000 clones by using ABI-Sanger sequencing techniques. Further analyses using bioinformatics tools helped identify hundreds of candidate genes and their transcript abundance in salivary glands. This study enhanced the understanding of TPB feeding biochemistry and mechanisms resulting in plant damages. Future research may benefit from this study to develop host plant resistance tools to neutralize damaging enzymes or toxic components secreted by TBP.

Materials and Methods

TPB Population (L. lineolaris) and Feeding Damage
More than 500 TPB adults (~1–2-weeks old) were collected from cotton field edges containing mixed vegetation of weeds (mainly pig weed) and cotton near Stoneville, Mississippi using a sweeping net (ratio of males to females was 1.13 according to Ridgway and Gyrisco [1960]). Bugs were immediately dissected for collecting salivary glands. When extra dissection time was needed, bugs were briefly maintained on excised pigweeds or green bean pods in laboratory.

Cotton bolls were picked in August 2015 from a control plot (no chemical control) ~4 miles south of Leland, MS. The cotton plot was infested by TPBs, and other piercing-sucking insects, such as stink bugs, were not seen. Both damaged and undamaged cotton bolls were cut transversely using a knife, and pictured using a camera.

Dissecting Salivary Glands and mRNA Extraction
The adults were immobilized in a −20°C freezer and transferred to a petri dish which was placed on ice. Salivary gland complex (SGC; Fig. 1) was dissected under a stereo light microscope (Nikon SMZ1000) in DEPC (diethylpyrocarbonate)-treated water. The SGC (including all lobes, accessory glands, and tubules) were exposed by holding and pressing the abdomen, and simultaneously removing the prothorax with forceps. The SGC were carefully picked out from the head-prothorax with fine-tipped forceps. A total of 500 salivary glands were dissected and pooled for RNA extraction. Total RNA was extracted from salivary glands using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. The quantity and quality of the total RNA were determined by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The purification of mRNA was performed using NucleoTrap mRNA purification kit (BD Bioscience Clontech, Palo Alto, CA).

cDNA Library Construction
The cDNA libraries were constructed with a ‘SMART’ library construction kit from Clontech (Palo Alto, CA). Plasmid libraries were made following the procedures provided by the manufacturer with modifications, e.g. the cDNA plasmids were transformed into TOPO Escherichia coli cells (Invitrogen, Carlsbad, CA) rather than using a phage vector. Approximately 1µg salivary gland mRNA was used as starting material for the first strand of cDNA synthesis with an oligo-dT primer. Although the cDNA library was not normalized, using 1 µg purified mRNA instead of total RNA may minimize selective amplifications of highly abundant gene transcripts over less expressed genes. We also optimized amplification cycles to 15. After cDNAs were digested with restriction enzyme Sfi1, we checked cDNA quality and found an expected faint smearing image of

![Salivary gland structure of L. lineolaris. AG, accessory salivary gland; SG, salivary gland; SD, salivary duct.](image-url)
Sequence Data Processing
The SeqMan module of DNAStar (Ver. 8, Madison, WI) was used to trim vector and assemble sequences. Assembling parameters were set at 80% for minimal match percentage, 100 bp for minimal sequence length, 0 for gap penalty, and 0.7 for gap length penalty. The assembled sequences (or contigs) were subjected to a similarity search for putative identity against the protein and nucleotide databases of the GenBank in the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using BlastX NR, BlastN, and tBlastX protocols. The Blast2GO software (https://www.blast2go.com/) with 10⁻² for cutoff e-value was further used to conduct BlastX, mapping, and annotation. Gene ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations were performed with Blast2GO to determine each putative protein’s function in biological process, molecular function, and cellular components at level 2.

Phylogenetic Analysis
To further characterize the highly abundant genes families, the predicted amino acids sequences of 45 PGs and 15 serine proteases from salivary gland were obtained by EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) nucleotide sequence translation software (http://www.ebi.ac.uk/tools/st). ClustalW (gap opening penalty = 10, gap extension penalty = 0.2) of the MEGA6 software (http://www.megasoftware.net/) was used to conduct multiple sequence alignments. The known PGs and serine proteases mRNA or predicted amino acid sequences of other hemipteran species were obtained from NCBI database and the accession number of each sequence is listed in Table 1. Phylogenetic analysis was performed using the maximum parsimony method (MEGA6) with bootstrapping (1,000 replications) to generate the phylogenetic tree which includes the predicted 45 PGs and 15 serine proteases from TBP salivary glands.

Results and Discussion
TPB Feeding Damage to Cotton
TPB is the most important pest of cotton from the time of first squaring to the initiation of bloom and boll. They are attracted to tender lush growth where they feed by inserting their mouthparts into the tender plant parts and sucking sap. TPB feeding damages were well documented by many researchers (Hanny et al. 1977; Greene et al. 1999; Musser et al. 2009). But graphic details of the symptoms and damages are hard to find in peer-reviewed publications, although certain pictures may be seen in internet sources. Here we provided more details of external and internal symptoms of TPB feeding damages to cotton bolls (Fig. 2). The feeding damage from TPBs leads to malformed bolls of cotton with dark color lesion (Fig. 2A). Under the external feeding spots, there are yellowish to tan to brown stains and the dark or brown pin prick spots on the inside of boll wall (Fig. 2C). The internal injury also has the small wart growths or even undeveloped locules. This feeding results in shedding of squares and small bolls, stunted plants, aborted terminals, boll deformation, lint staining, and yield loss. The feeding damage may be associated directly with piercing injury and extra-oral digestion (Cohen 1998) of plant tissues by salivary enzymes from TPB and indirect opportunistic microbial infection (Lee et al. 1993; Medrano et al. 2009). Therefore, the economic importance of the TPB feeding damages signify the revealing of responsible enzymes and genes in salivary glands for facilitating the further studies and development of novel control strategies to knock down the damaging genes in the pest.

Identification of Genes and Putative Enzymes from TPB Salivary Glands
Sequence Assembling and Relative Abundance of Gene Transcripts.
To identify candidate feeding/damaging genes and enzymes, we used
cDNA library and Sanger sequencing technique to obtain ~7,000 high-quality sequences. After assembling 666 unique clusters (cDNAs) were obtained from TPB salivary glands, including 261 contigs and 405 singletons. Each of the contigs in Table 2 was assembled from more than 50 sequences, especially the contig_19 and contig_13, assembled from 384 and 318 sequences, respectively, were the most abundantly clones in the cDNA library, potentially to be the most abundantly expressed genes in the salivary glands. We assumed that the numbers of sequences for assembling a contig may be closely and positively associated with the original abundance of corresponding gene transcripts in the mRNA used as starting templates at the beginning of cDNA synthesis and library construction. The selective gene expression in TPB salivary glands indicated that some more abundantly expressed genes might be associated with specific and important function in the insect-plant interaction. After sequence similarity search of GenBank (see details in Section 'Gene identities and annotations using Blast2GO analysis'), the transcripts, contig_19 and contig_13, were identified as PG and serine protease, respectively (Table 2).

**Gene Identities and Annotations Using Blast2GO Analysis.** Results from Blast2go analysis showed that 51.2% of cDNAs (347 out of 666 sequences) have been identified with significant Blast hits (E-value ≤ 10^-6). Other 329 sequences have not been matched to any sequence in GenBank database, suggesting that a unique set of genes exist in TPB salivary glands. Of the 347 identified genes, 228 cDNAs (34.2% of the 666 sequences) were successfully annotated with clear physiological and structural functions (Fig. 3A; Supp Table 1 [online only]). Salivary gland cDNAs of TPB were compared with the relevant cDNAs of other species using NCBI database, and
Table 2. The potential most abundantly expressed enzyme gene transcripts (more than 50 cDNAs from 7,000 clone sequences) in *L. lineolaris* salivary glands

| Sequence Name | Putative gene identity     | cDNA number |
|---------------|----------------------------|-------------|
| Contig_19     | PG 9                       | 384         |
| Contig_13     | venom serine protease-like  | 318         |
| Contig_77     | endopolygalacturonase       | 153         |
| Contig_143    | PG pg2-1                   | 111         |
| Contig_112    | PG pg1                     | 108         |
| Contig_86     | PG pg1                     | 105         |
| Contig_120    | PG 11                      | 104         |
| Contig_144    | PG pg3                     | 72          |
| Contig_100    | PG pg2-1                   | 58          |
| Contig_162    | venom serine protease-like  | 55          |
| Contig_6      | PG pg1                     | 50          |

Fig. 3. (A) Resulted distribution of identified, annotated, and non-identified salivary gland cDNAs of *L. lineolaris* based on Blast2GO analysis. (B) Top-hit insect species in Blast2GO analysis of salivary gland cDNAs of *L. lineolaris*, showing distributions of significant homologous sequences with an E-value of at least 1.0E-4 in different insect species.

Important Gene Functions in Primary Digestion and Interaction with Host Plant

**Genes for Detoxification Enzymes or Effectors.** Eight enzyme-coding cDNAs for 4 glutathione S-transferases (GSTs), 3 esterases, and one cytochrome P450 (CYP450) were identified (Table 4). These genes may play essential roles in detoxification of plant anti-herbivore toxic molecules and/or degradation of plant defense compounds. It is well-known that plants produce a variety of secondary metabolites after being attacked by herbivores (Schoonhoven 2005), including terpenoids, fatty acid derivatives, phenylpropnoids and benzenoids, etc. (Mumm and Hilker 2006). In the co-evolution of herbivores and plants, herbivores gradually adapt to the plant-based toxic molecules by their own detoxification mechanisms (Chapman and Boer 1995). GSTs, esterases and CYP450s are well-known detoxification enzymes against plant defense toxic compounds (Despres et al. 2007). In *M. persicae*, the over-production of GSTs is proposed to be associated with the adaptation to glucosinolates and isothiocyanates present in its *Brassicaceae* host plants (Francis et al. 2005). Recently, two esterases from armyworms (*Spodoptera* spp.) were reported to degrade plant volatiles (He et al. 2014). The well-documented CYP450 (CYP6B1 and CYP6B3) involvement in plant compound detoxification is associated with the adaptation of lepidopterans in *Papiliomidae* to cope with toxic furanocoumarin in their host plants (Petersen et al. 2001). Similarly, CYP6AE14 was identified from *Helicoverpa armigera*, which is essential for detoxifying cotton gossypol (Mao et al. 2007). Therefore, we hypothesized in this study that the detoxification enzymes in TPB salivary gland may also play essential roles in the detoxification of plant defense compounds.

**Genes Related to Extra-Oral Digestion of Cell Wall Components.** Plant cell wall offers protection against invading organisms and is mainly composed of the polysaccharides pectin, cellulose, and hemicellulose, which can be degraded by plant cell wall degrading enzymes, like cellulases and pectinases (Gilbert 2010). In caterpillars, gut endosymbionts are hypothesized to be responsible for the degradation of plant cell wall, where the insects depend heavily on the secretion of a range of digestion enzymes to efficiently digest plant cellulose networks. Alternately, piercing-sucking hemipterans mainly rely on enzyme secretions from salivary glands for extra-oral digestion prior to uptake of the pre-digested sap. Among the various digestive enzymes in the salivary glands of mirid bugs, PGs, one group of pectin hydrolases, are likely one of the most important enzymes in causing visible plant injury (Strong 1970). PGs, whose substrate are polygalacturonic acid-polysaccharides and monosaccharides of plant cells (α-1,4 polygalacturonic acid), function as associated with biological process, cell components, and molecular functions. Further analysis of the molecular functions showed that these genes are associated with binding, catalytic, and transporter activities (Fig. 4). The annotated transcripts of TPB were mapped to 18 KEGG pathways (Table 3) in which the majority of salivary gland genes were assigned to cell wall degradation and carbohydrate (starch, fiber, and sugar) metabolism. The presence of digestive enzyme genes and their specific functions consistently demonstrated potential extra-oral feeding of TPB for breaking down plant cell walls and pre-digestion of plant contents. Sequence data showed that PGs and serine proteases were abundantly present in the cDNA library, potentially to be the most abundantly expressed gene families in the TPB salivary glands, showing 45 and 15 enzyme-coding cDNAs, respectively, as multigene family members (Table 4).

the top Blast matching sequences were from *Aplysus lucorum*, *Halyomorpha halys*, *Cames lectularius*, *Lygus hesperus*, *L. lineolaris* and *Triatoma castaneum* (Fig. 3B).

GO terms were assigned to 228 contigs with copy number (Supp Table 1 [online only]). They were categorized into functional groups...
Two types of PGs were identified in *L. hesperus* (Celorio-Mancera et al. 2009), as endo-PG and exo-PGs with two hydrolytic modes (Cook et al. 1999). Endo-PGs degrade the polygalacturonic acid component of plant cell wall and generate galacturonic acid-containing oligosaccharides. Exo-PGs target the non-reducing end of polygalacturonic polymer to produce monosaccharide polygalacturonic acid. In *A. lucorum*, 14 PG genes were identified and their expression profiles were well-established in different body parts and different developmental stages (Zhang et al. 2015). The expression levels of 14 PGs were significantly higher in salivary gland cDNAs of *L. lineolaris*.
glands than other tissues. This indicated that *A. lucorum* depends on salivary gland enzyme secretion for extra-oral digestion of plant cell and making nutrient available. In *Lygus*, three PGs have also been identified and characterized (Allen and Mertens 2008; Celorio-Mancera et al. 2009). In this study, the gene transcripts of PGs were also identified as one of the most abundant transcripts in TPB salivary glands (Table 2). One PG gene (contig_19) also had the highest presence in the cDNA library (384 highly similar sequences out of nearly 7,000 sequenced clones, Table 2). The abundance of the sequences might be closely associated with abundant expression of the gene in salivary glands and the significance of PGs in extra-oral digestion in TPBs.

By using phylogenetic analysis, 45 PGs from TPB salivary glands were clearly separated and grouped into six clades (Fig. 5). Our results are similar to the finding that six groups of PGs were present in *L. lineolaris* (Showmaker et al. 2016) and in *A. lucorum* (Zhang et al. 2015), suggesting that mirids may have a common trait in evolving diverse PG genes for adaptation to wide host ranges. In addition, we found that 26 coded PGs (Table 5) from this study highly matched to the PGs of *L. lineolaris* in GenBank. The other 19 coded PGs were highly similar to the PGs of other species, indicating potential new PG cDNAs found in this study. Further analysis is needed to confirm the finding.

PG from salivary glands are important digestive enzymes. Expression levels of these genes may be altered when TPBs feed on different host plants (Habibi et al. 2001). Our microarray data (data not shown) indicated 26 PG genes were significantly down-regulated in the TPBs collected from the horseweed (*Conyza*...
in this study might be better for representing field populations of TPBs than the laboratory colony used by Showmaker et al. (2016).

In plants, starch is a common polysaccharide that is metabolized by a series of enzyme complexes, including α-amylose, glucosidase and glycan enzymes. All of these were identified from TPB salivary glands in this study (Table 4). Alpha-amylase breaks down the oligosaccharides and polysaccharides by catalyzing the hydrolysis of α-1,4-glucosidic linkage. The role of glucosidase is for breaking down complex carbohydrates (polymer carbohydrates), such as starch and glyco-
gen into monomers. Glucosidase, α-amylase, and maltase are very common enzyme found in salivary glands of leafhopper, E. fabae, another insect with piercing-sucking mouthparts (DeLay et al. 2012). From TPB salivary gland cDNAs, we also identified several gene transcripts for two α-amylases, one maltase, one glucosidase, and one glyc-

Table 5. Top-hit of 45 PGs from salivary glands of L. lineolaris to the PGs in Genbank

| Sequence Id | PGs in GenBank | e-value | GenBank accession no. | Full with ORF* or partial |
|-------------|----------------|---------|-----------------------|-------------------------|
| Contig_330  | PG 12 [A. lucorum] | 2e-93 | AFB33363 P |
| Contig_120  | PG 18c [L. lineolaris] | 0.0 | AHGS4226 F |
| Contig_78   | PG [L. hespera] | 2e-162 | ACC44844 F |
| Contig_97   | PG [L. hespera] | 2e-129 | ACC44844 P |
| Contig_153  | PG 4b [L. lineolaris] | 0.0 | AHGS4208 F |
| Contig_19   | PG 9 [A. lucorum] | 0.0 | AHGS4336 F |
| Contig_56   | PG 5-3 [A. lucorum] | 1e-139 | AIO4035 P |
| Contig_303  | PG 2-1 [A. lucorum] | 3e-110 | AFV15473. P |
| Contig_6    | PG 9 [L. lineolaris] | 0.0 | AHGS4214 F |
| Contig_32   | PG 18c [L. lineolaris] | 9e-177 | AHGS4226 F |
| Contig_86   | PG 22a [L. lineolaris] | 0.0 | AHGS4232 F |
| Contig_89   | PG 15a [L. lineolaris] | 0.0 | AHGS4220 F |
| Contig_98   | PG 14 [L. lineolaris] | 0.0 | AHGS4219 F |
| Contig_112  | PG PG1-2 [A. lucorum] | 0.0 | AIO4027 P |
| Contig_116  | PG 1b [L. lineolaris] | 3e-39 | AHGS4225 P |
| Contig_166  | PG 17 [L. lineolaris] | 2e-159 | AHGS4223 P |
| Contig_173  | PG 6 [L. argentum] | 2e-171 | AFP3336 F |
| Contig_203  | PG 16 [L. lineolaris] | 1e-139 | AHGS4222 P |
| Contig_304  | PG 18c [L. lineolaris] | 2e-64 | AHGS4226 P |
| Contig_331  | PG [L. hespera] | 2e-70 | ACC44844 P |
| Contig_341  | PG [L. hespera] | 4e-95 | ACC44845 P |
| Contig_355  | PG 10 [L. lineolaris] | 3e-151 | AHGS4215 P |
| Contig_356  | PG 13 [L. lineolaris] | 1e-124 | AHGS4218 P |
| Contig_328  | PG PG1-2 [A. lucorum] | 2e-41 | AIO4027 P |
| Contig_59   | PG 8 [L. lineolaris] | 0.0 | AHGS4213 F |
| Contig_100  | PG PG2 [L. lineolaris] | 0.0 | ABDS3921 F |
| Contig_136  | PG 1 [L. argentum] | 0.0 | AHGS4201 F |
| Contig_138  | PG 5b [L. lineolaris] | 0.0 | AHGS4210 F |
| Contig_140  | PG 23 [L. lineolaris] | 0.0 | AHGS4234 F |
| Contig_143  | PG 5a [L. lineolaris] | 0.0 | AHGS4209 F |
| Contig_169  | PG 6 [L. lineolaris] | 0.0 | AHGS4211 P |
| Contig_319  | PG PG2-1 [A. lucorum] | 1e-141 | AFV15474 P |
| Contig_359  | PG 5 [A. lucorum] | 8e-86 | AFPS3366 F |
| Contig_144  | PG 3b [L. lineolaris] | 0.0 | AHGS4205 F |
| Contig_148  | PG 19c [L. lineolaris] | 0.0 | AHGS4229 F |
| Contig_159  | PG 20 [L. lineolaris] | 0.0 | AHGS4230 F |
| Contig_238  | PG 21 [L. lineolaris] | 0.0 | AHGS4231 F |
| Contig_55   | PG PG3-3 [A. lucorum] | 6e-154 | AIO4035 P |
| Contig_314  | PG 20 [L. lineolaris] | 1e-123 | AHGS4230 P |
| Contig_167  | PG 25 [L. lineolaris] | 2e-138 | AHGS4236 F |
| Contig_46   | PG [L. hespera] | 4e-150 | ACC44844 F |
| Contig_77   | PG 14 [A. lucorum] | 3e-168 | AFB3336 F |
| Contig_152  | PG 12 [A. lucorum] | 2e-170 | AFPS3363 F |
| Contig_236  | PG [L. hespera] | 0.0 | ACC44798 F |

*Open reading frame

**Serine Proteases.** Insect digestive proteases play two essential roles in insect physiology. The main function is to break down proteins into free amino acids and to make them nutritionally available. Proteases may also inactive or degrade toxic compounds from food sources, e.g. plant-expressed toxins (such as Bacillus thuringiensis toxins) and protease inhibitors (PIs). Three major groups of proteases have already been well documented (trypsin, chymotrypsin, and elastase), that were highly expressed in caterpillar midguts (Jongsma and Bolter 1997). PIs are one of highly produced compounds in plants and work at the gut level by inhibiting digestion. This functions as a resistance trait against herbivores and pathogen infection (Lawrence and Koundal 2002). The major role of PIs in herbivores is to block the activity of endogenous proteases, like trypsin, chymotrypsin and elastase, which are responsible for initial digestion of dietary protein. However, phytophagous insects have evolved certain proteases capable of specifically degrading the host plant PIs, or produced large quantity (over-expression) of PIs-inhibition-insensitive proteases when sensitive proteases are inhibited (Jongsma and Beekwilder 2011). Serine proteases in TPB salivary glands might also contribute to both inactivation of host plant PIs and pre-digestion of plant tissues. Using specific proteinase inhibitors, we previously demonstrated that serine proteinases in TPBs are major proteinases in both salivary glands and gut tissues (Zeng et al. 2002; Zhu et al. 2003). Protease activities in salivary glands were more active than those in guts and four trypsin cDNAs were sequenced from salivary glands and only one was cloned from gut in previous attempt (Zhu et al. 2003). In this study, 15 unique serine proteases from TPB salivary glands (Table 4) were grouped into three different clades based on phylogenetic analyses (Fig. 6). Results of annotation indicated that most were trypsin and trypsin like serine proteases. In addition to multigene family of proteases, we also found from sequence assembling that serine proteases were quantitatively the second most abundant clones in cDNA library.
potentially being highly expressed gene family in TPB salivary glands. Besides the diversity of 15 different genes (≈2.3%, 15 of 666 cDNAs), the number of cDNA clones (potentially associated with serine protease gene transcripts) was high as well (Table 2), such as contig_13, encoding a serine protease-like enzyme, was assembled from 318 sequences out of 7,000 clones.

Lipase. Four lipase gene transcripts were identified from TPB salivary glands, including two lipases and two pancreatic lipase-like proteins. Lipases were well documented in blood feeding insects for inhibiting activation of host plasma and immunomodulatory properties (Anderson et al. 2003, 2006). In leaf-feeding lepidopterans, pancreatic lipases function as galactolipase and phospholipases, and gastric lipases function as triacylglycerol hydrolases (Christeller et al. 2010). For example, in *Epiphyas postvittana* and *H. armigera*, the high activities of galactolipase and phospholipase activities were associated with digestion of high galactolipid content in leaves (Christeller et al. 2011). In addition, lipases were also proposed to have an involvement in extra-oral digestion and host cell permeability in *Mayetiola destructor* (Shukle et al. 2009). However, the involvement of lipases in extra-oral digestion and inhibition of host plant defense activities in piercing-sucking phytophagous hemipteran species has not been well-documented. The expressions of pancreatic lipases in TPB saliva glands might be a consequence of adaptation to the high content of galactolipid in fresh plant tissues. Understanding the importance of lipase, especially why lipases are present in TPB saliva and how these lipases function...
enzymes interact with host plant tissues, deserves study to further clarify the detail functions of lipases in TPB feeding.

Duplex-Specific Nuclease. Blast search of GenBank showed that two contigs are similar to duplex-specific nuclease (DSN). Contig_107 matched to the DSN of Sequella paramamosain (GenBank Acc no. AFPI9103) with 35% sequence identity. Contig_131 matched to the DSN of Paralitobdes camtschaticus (GenBank Acc no. BAH02823) with 27% sequence identity. DSN shows a strong preference for cleaving double-stranded DNA and DNA in DNA- RNA hybrid duplexes, compared with single-stranded DNA (Shagin et al. 2002). The cleavage rate of short, perfectly matched DNA duplexes by this enzyme is essentially higher than that for non-perfectly matched duplexes of the same length. Thus, the capability of DSN in differentiating single nucleotide variations in DNA makes DSN a useful tool for single nucleotide polymorphism detection based on this unique property (Shagin et al. 2002). However, Allen and Walker III (2012) demonstrated that nuclease from TPB saliva was responsible for degrading dsRNA, resulting in repeatedly unsuccessful RNA interference via oral feeding. Therefore, the presence of nucleases in TPB saliva becomes a barrier for development of RNAi based bio-pesticide against this economically important pest. Nevertheless, sequencing DSN cDNAs may facilitate further studies to overcome this barrier.

The TPB has become the most destructive insect on southern row crops, especially on cotton. Currently, management of this serious pest relies almost exclusively on chemical insecticides. Considering the adverse impact of pesticides on environment and non-target organisms, alternative control strategies against TPB are urgently needed. Biotechnologies, such as RNAi, have already showed some successes in insect pest management (Price and Stehr. 1994). The bio-pesticide against this economically important pest. Nevertheless, sequencing DSN cDNAs may facilitate further studies to overcome this barrier.

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