FIRST 454 TRANSCRIPTOME TO THE PLANT BUG DIGESTIVE SYSTEM: NEW LEADS FOR NEXT GENERATION TRANSGENIC COTTON TO CONTROL SUCKING PESTS

R.M. Roe
K.V. Donohue
L.C. Magalhaes
Jaap Van Kretschmar
North Carolina State University, Department of Entomology
Raleigh, North Carolina

Abstract

Separate transcriptomes were constructed by pyrosequencing on the GS-FLX sequencer (Roche), for the whole body and digestive system of the adult Tarnished plant bug, *Lygus lineolaris*, collected from the field in North Carolina, USA. Each cDNA library from the two samples examined were sequence on one-half plates each. The total ouput from the two reads was 116, 635, 527 bps of data. The whole body library produced 292,714 reads which were assembled using the GS Assembler ver. 1.1.02.15 (Roche) with default parameters into 5,529 contiguous sequences (contigs). The digestive system library produced 229,919 reads which was assembled into 3,549 contigs. Contigs were putatively identified using the Tera-BLASTX algorithm with DeCypher (TimeLogic) against Genbank nr and est databases that were downloaded on June 2008. The contigs obtained from the tarnished plant bug gut transcriptome were analyzed using the blast2go program (www.blast2go.org). Blast2go also blasted the contigs using its blastx function and performed the gene ontologies. The analysis of the results from the whole bug and gut transcroptomes are in its infancy. However, the gut library has some obvious messages that should be of interest to plant, genetic pest management.

Introduction

The tarnished plant bug has been found on more than 300 plants and is the most common and abundant mirid species in North America (Coulson 1987). Consequently, it is a pest of more than 100 crops of economic importance, which includes fiber, fruit, vegetable and seed crops (Young 1986) such as strawberry (Sabbahi et al. 2008), peach, apple (Hardman et al. 2004), canola (Broadbent et al. 1999), soybean and cotton. *L. lineolaris* not only reduces plant vigor by removing nutrients, but also disrupts tissues by injecting salivary enzymes (Wheeler 2001). The green stink bug is also a polyphagous hemipteran species and a pest of fruits and seed crops, such as soybean and cotton. In soybean, the stink bug complex was responsible for a total loss of nearly $2.3 million in Georgia alone (Riley et al. 1997), and the pest status in general is expected to increase due to reductions or the elimination of broad-spectrum insecticides (Leskey and Hogmire 2005).

Since the mid 1990s, transgenic *Bt* cotton has been widely expanded (James 2006) and is highly efficient in controlling lepidopteran pests (Perlak et al. 1990). For this reason and the success of the boll weevil, *Anthonomus grandis* (Boheman), eradication program, chemical insecticide use is reduced considerably. As a result, a new group of pests has recently emerged in cotton. Pierce-sucking insects, such as plant bugs and stink bugs, which were indirectly being controlled (Hardee and Bryan, 1997; Greene et al. 1999) have now become important cotton pests in the US (Bauer et al. 2006). Among other injuries, *L. lineolaris* impact cotton by releasing salivary toxins that causes abortion and malformation of the attacked part, usually flowers (Layton 2000). Losses can exceed $40 million depending on the region and time of infestation (Williams, 1999). Stink bugs damage cotton mainly later in the season by feeding on developing bolls (Roberts et al. 2005), which reduces yield and fiber quality (Greene et al. 1996). Total cotton losses in 2004 related to stink bug damage were estimated around $10 million (Williams 2005). Insecticide applications and yield losses in cotton production due to plant and stink bugs have been increasing sharply (Greene et al. 2006), and tarnished plant bug resistance to both pyrethroids and organophosphates is now widespread (Snodgrass and Scott, 2003). *In toto*, the problem of secondary pest problems for sucking pests are exacerbated by (i) increased resistance to chemical insecticides, (ii) potential concerns about the bee toxicity of the neonicotinoids (important for the control of many sucking pests), (iii) on the paucity of information on the digestive physiology of sucking pests and especially stink and plant bugs but also limited information on gut transcriptomes and mechanisms for the regulation of digestion in insects in general; and (iv) the absence of a transgenic approach using gut toxins like Bt for sucking pests.

New opportunities have become available to study emerging pests in cotton and to elucidate possible new targets for insect control from advances in high throughput DNA sequencing, solid surface DNA synthesis, methods to monitor global gene expression, bioinformatics, and gene silencing. The goal of this project was to develop the first 454 whole body and digestive system transcriptome to a plant bug for the investigation of new plant transgenic approaches for the control of this pest.

Materials and Methods

Insect and tissue collection

Tarnished plant bug adults, *Lygus lineolaris* (Palisot de Beauvois), were collected with a sweep net from alfalfa at the Lake Wheeler Road Field Laboratory (North Carolina State University, Raleigh, NC) in June and July of 2008. Collected insects were kept in 1-gallon plastic tubs covered with one layer of cheesecloth, fed on artificial NI diet (Cohen, 2000), and held in a growth chamber (Percival Scientific Model I-66NL; Percival Scientific, Inc., Perry, IA) at 27 ± 1° C, 65% relative humidity, 14 hours light: 10 hours dark until needed for dissections. Dissections were conducted in 10 mM sodium phosphate buffer, pH 7.4. Once the digestive system was removed from the insect, it was immediately transferred to microcentrifuge tubes containing 200 ul of TRI Reagent. The tubes were kept on dry ice throughout the time of dissections to avoid RNA degradation. At the end of the day, tissue was homogenized for 5 s with a hand held mortar and pestle (Fisher, Pittsburg, PA) at room temperature, and the microcentrifuge tubes were stored at -80^{o}C until needed again. This procedure was repeated each day with new material until adequate amounts of tissues were obtained for cDNA library construction. Digestive systems from approximately 30 insects were used for the preparation of RNA. The whole insect homogenization was performed on different days by placing 8 adults per culture tube (17 X 100 mm)(Fisher), adding 2.5 ml of Tri Reagent per tube, and immediate homogenization for 1 min using a Brickmann (Westbury, NY) Polytron. RNA was extracted immediately after homogenization. Approximately 16 insects were used for RNA extraction and sequencing.

cDNA preparation and 454 sequencing

Whole plant bugs and pooled digestive system tissues were separately homogenized in TRI Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's recommendations. RNA pellets were rehydrated in 100 µM aurintricarboxylic acid to prevent degradation (Hallick et al., 1977). Approximately 3 µg of total RNA from each group were pooled, and mRNA was isolated using an Oligotex mRNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Purified mRNA was ethanol precipitated and rehydrated in 2 µl and combined with 10 picomoles of modified 3' reverse transcription primer (5'-ATTCTAGAGACCGAGGCGGCCGACATGT$_{(4)}$GT$_{(9)}$CT$_{(10)}$VN-3') (Beldade et al. 2006) and 10 picomoles SMART IV oligo (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3') (Zhu et al. 2001). The resulting 4 µl were incubated at 72°C for 2 min and then combined with the following reagents on ice: 1 µl RNase Out (40 U/µl), 2 µl 5X first strand buffer, 1 µl 20 mM DTT, 1 µl dNTP mix (10 mM each) and 1 µl Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction was incubated at 42°C for 90 min then diluted to 30 µl with TE buffer (10 mM Tris HCL pH 7.5, 1 mM EDTA) and stored at -20°C until further use. To synthesize second strand cDNA, 5 µl of first-strand cDNA was mixed with 10 picomoles of modified 3' PCR primer (5'-ATTCTAGAGGCCGAGGCGGCCGACATGT$_{(4)}$GTCT$_{(4)}$GTTCTGT$_{(3)}$CT$_{(4)}$VN-3') (Beldade et al. 2006), 10 picomoles of 5' PCR primer (5'-AAGCAGTGGTATCAACGCAGAGT-3') (Zhu et al. 2001), 5 µl 10X reaction buffer, 1 µl dNTP mix, 2 µl $MgSO_4$, 0.4 µl Platinum HiFi Taq Polymerase and 34.6 µl H_2O (Invitrogen). Thermal cycling conditions were 94°C for 2 min followed by 20 cycles of 94°C for 20 sec, 65°C for 20 sec and 68°C for 6 min. The first PCR reaction was conducted, and 5 µl aliquots from cycles 18, 22 and 25 were analyzed on a 1% agarose gel to optimize the number of cycles. Five reactions were then conducted at the optimized number of cycles to produce sufficient quantities of cDNA at a maximum length for 454 library preparation. The contents were combined, and the cDNA was purified by using a PCR purification kit (Qiagen) according to the manufacturer's recommendations.

The cDNA library was prepared with appropriate kits (Roche, Indianapolis, IN; Qiagen) for pyrosequencing on the GS-FLX sequencer (Roche) according to the manufacturer's recommendations which were described previously (Margulies et al. 2005). We conducted whole body on one-half plate and digestive system on one-half plate.

Bioinformatics

Removal of primer sequence contamination and assembly of GS-FLX sequencing reads were carried out with GS Assembler ver. 1.1.02.15 (Roche) using default parameters as follows: seed step, 12; seed length, 16; seed count, 1; minimum overlap length, 40; minimum overlap identity, 90%; alignment identity score, 2; and alignment difference score, -3. Assembled contiguous sequences, herein referred to as contigs, were initially identified using the Tera-BLASTX algorithm with DeCypher (TimeLogic) against Genbank nr and est databases (downloaded June 2008). The contigs obtained from the tarnished plant bug gut transcriptome were analyzed using the blast2go program (www.blast2go.org) in Dec. 2008. Blast2go blasted the contigs using its blastx function and also performed the gene ontology annotations reported in this study.

Results and Discussion

A cDNA library was sequenced separately for whole adult body and adult digestive system using half plates each by pyrosequencing on the GS-FLX sequencer (Roche) according to the manufacturer's recommendations. The total number of reads were 116, 635, 527 bases of data (Fig. 1) from the two, one-half runs. The individual reads were assembled separately. Removal of primer sequence contamination and assembly was conducted using the GS Assembler ver. 1.1.02.15 from Roche using their default parameters. For the whole body library, we obtained 292, 714 reads which were assembled into 5,529 contigs. For the digestive system library, we obtained 229, 919 reads which weres assembled into 3 549 contigs. Assembled contiguous sequences, referred to as contigs, were putatively identified using the Tera-BLASTX algorithm with DeCypher (TimeLogic) against Genbank nr and est databases that were downloaded on June 2008.

Output from 454 Sequencing

116, 635, 527 total bases of data from two, one-half runs

Whole Body Library

- 292, 714 reads
- 5,529 contigs

Gut Library

- 229, 919 reads
- 3,549 contigs

Figure 1. Results from 454 pyrosequening.

The contigs obtained from the tarnished plant bug gut transcriptome were analyzed using the blast2go program (www.blast2go.org). Blast2go also blasted the contigs using its blastx function and performed the gene ontologies. The summaries that follow were only conducted with the digestive system transcriptome. Fig. 2 shows the relative distribution of contig size in bps. The predominant size for the contigs that were assembled from the digestive system library was approximately 250 bps with the largest being as high as 2.3kb. As might be expected the greatest number of similarities were found for insects like *Drosophila melanogaster*, *Tribolium castaneum*, and *Aedes aegypti* (Fig. 3). These are insects where the genome is available along with a large amount of information on expressed genes. The functional analysis of the plant bug digestive system transcriptome is shown in Fig. 4. As would be expected, the largest hits were for proteolysis and carbohydrate metabolism. In addition, there were a number of expressed genes for cellular processing, for example, translation, transcription, electron transport, etc. Other less abundant expressed genes were involved in protein folding, glycolysis, lipid metabolism, etc.

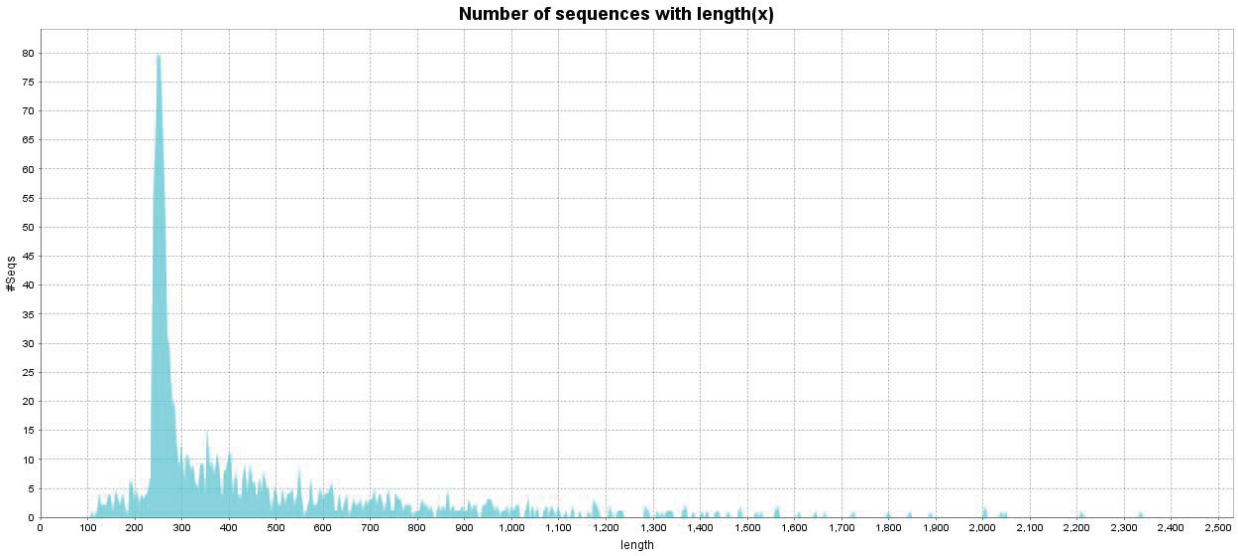

Figure 2. Size of contigs from the plant bug digestive system 454 library.

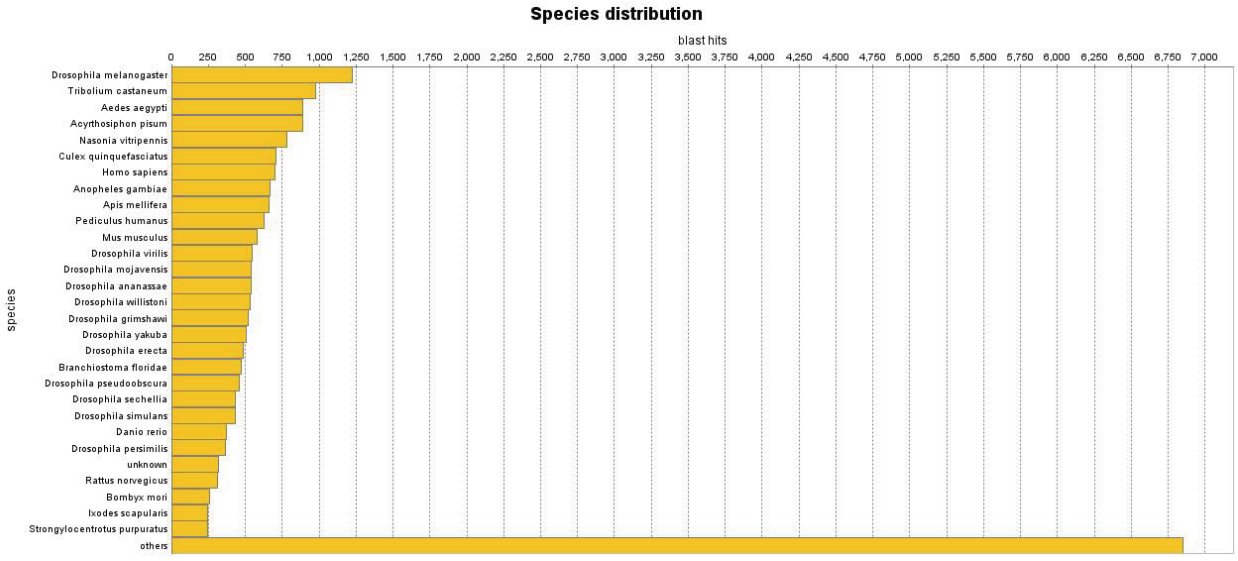

Figure 3. Species distribution of contigs from the plant bug 454 contigs.

Our transcriptome analysis was organized into an excel spreadsheet for further evaluation. An example of some of the columns of data in this spread sheet is shown in Fig. 5 for contig 3486. These data include the E-value, the number of deduced amino acids from the contig, the number of nucleotides in the contig, the number of 454 reads used to assemble the contig, and the putative identification from a blast analysis.

The analysis of the results from the gut transcroptome is in its infancy, especially since these data were only obtained a few weeks before this presentation. However, there are some obvious target messages of interest. For example, contig 3486 (Fig. 5) was identified as a putative JH epoxide hydrolase. We have developed a sensitive and specific assay for this enzyme (Share and Roe, 1988) which can be used with RNAi knockdown to optimize high throughput screening methods to evaluate the critical functions of the contigs in our database. A high throughput screening method is needed to identify the most critical genes for insecticide development using real-

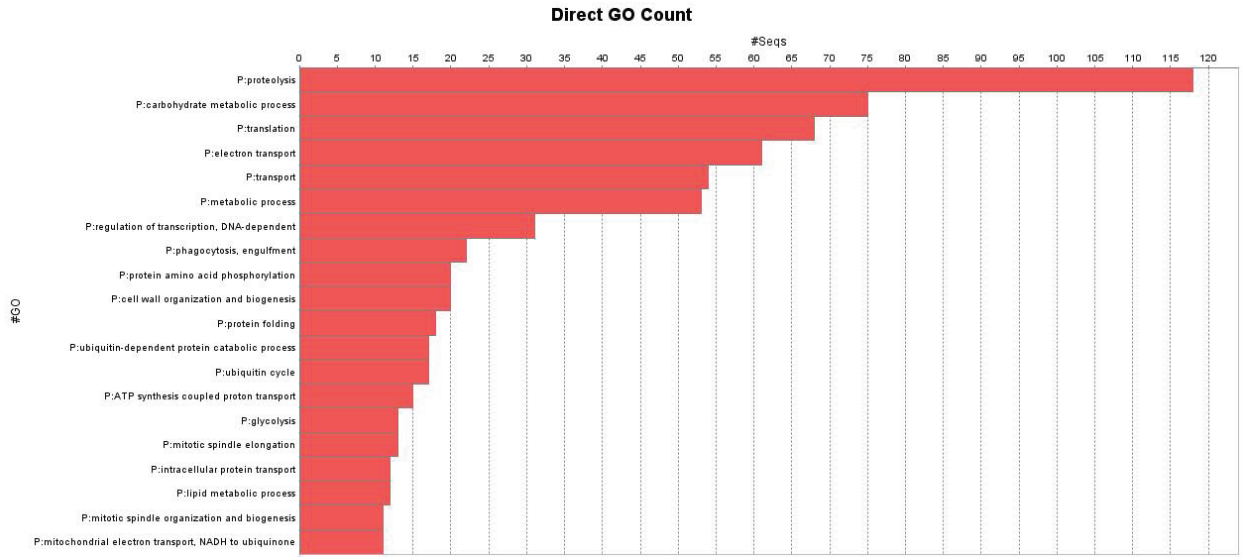

Figure 4. Functional analysis of the putative expressed proteins from the digestive system of the plant bug.

E-value	AA	Contig	nts	Read	Access#	Putative Function
4.20E-09	67	3486	204	107	AAB18243.1	microsom EH [Trichoplusia ni]
1.90E-09	67	3486	204	107	AAL13528.1	GH06241p [Drosophila mel]
2.30E-07	67	3486	204	107	AAM88326.1	JH EH [A. aegypti]
5.80E-11	67	3486	204	107	BAE48505.1	JH EH [Athalia rosae]
5.40E-09	67	3486	204	107	NP_001085033.1	hypothet protein [Xenopus]
1.40E-09	67	3486	204	107	NP_611385.1	JH EH [Drosophila mel]
1.10E-09	67	3486	204	107	Q8MZR5	JH EH 2
1.00E-07	67	3486	204	107	Q8MZR6	JH EH 1

Figure 5. Example of data in the excel file from the analsysis of the 454 transcriptome from the plant bug.

world approaches for delivery of dsRNA or plant antibodies in the context of plant, genetic pest management as well as traditional insecticide delivery methods. Another example from our data base is the identification of putative Bt toxin receptors. In addition, there are contigs that appear to be obvious targets for a plant, genetic pest management strategy for plant bugs as well as targets for the development of baits to control a variety of other insect pests outside of cotton. Further work will be needed to show proof of concept.

Summary

Separate 454 transcriptomes were constructed for the whole body and digestive system of the adult Tarnished plant bug, *Lygus lineolaris*, collected from alfalfa in North Carolina, USA. The individual reads for each library were assembled and a putative identification made where possible for each contig that was assembled using available nucleic acid and protein databases. Excel spread sheets were constructed with the relevant search data for future study of these contigs. Gene ontologies were also constructed. In our preliminary examination of the digestive system database, there are a few obvious messages of interest as a target for the development of a plant, genetic pest management strategy for the control of plant bugs. However, studies are needed to determine absolute proof of concept.

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