Transcriptome of the Plant Virus Vector *Graminella nigrifrons*, and the Molecular Interactions of *Maize fine streak rhadovirus* Transmission

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Abstract

**Background:** Leafhoppers (Hemiptera: Cicadellidae) are plant-phloem feeders that are known for their ability to vector plant pathogens. The black-faced leafhopper (*Graminella nigrifrons*) has been identified as the only known vector for the *Maize fine streak virus* (MFSV), an emerging plant pathogen in the *Rhabdoviridae*. Within *G. nigrifrons* populations, individuals can be experimentally separated into three classes based on their capacity for viral transmission: transmitters, acquirers and non-acquirers. Understanding the molecular interactions between vector and virus can reveal important insights in virus immune defense and vector transmission.

**Results:** RNA sequencing (RNA-Seq) was performed to characterize the transcriptome of *G. nigrifrons*. A total of 38,240 ESTs of a minimum 100 bp were generated from two separate cDNA libraries consisting of virus transmitters and acquirers. More than 60% of known *D. melanogaster, A. gambiae, T. castaneum* immune response genes mapped to our *G. nigrifrons* EST database. Real-time quantitative PCR (RT-qPCR) showed significant down-regulation of three genes for peptidoglycan recognition proteins (PGPR – SB1, SD, and LC) in *G. nigrifrons* transmitters versus control leafhoppers.

**Conclusions:** Our study is the first to characterize the transcriptome of a leafhopper vector species. Significant sequence similarity in immune defense genes existed between *G. nigrifrons* and other well characterized insects. The down-regulation of PGPRs in MFSV transmitters suggested a possible role in rhabdovirus transmission. The results provide a framework for future studies aimed at elucidating the molecular mechanisms of plant virus vector competence.

Introduction

Hemipteran insects such as aphids, whiteflies, planthoppers and leafhoppers are arguably the most important vectors of plant-infecting viruses. These insects have specialized mouthparts suitable for tissue specific feeding (often the phloem), and wide host ranges that provide ample opportunity for virus transmission [1,2]. Most insect vectors of plant viruses have significant associations with humans and agroecosystems. Recent rapid changes in these environments have increased crop exposure to viruses and vectors or altered evolutionary, ecological or genetic interactions leading to enhanced transmission [3]. A lack of understanding these factors, including the molecular mechanisms of virus transmission by vectors, reduces our ability to assess and manage risks posed by plant virus vectors, particularly for emerging diseases whose full impacts are not yet realized.

*Graminella nigrifrons* is one of the most common and abundant leafhoppers in the eastern half of the U.S., presently found in 35 states from southern Maine to Florida [4]. It has a wide plant host range, including oats (*Avena sativa* L.), maize (*Zea mays* L.), perennial rye grass (*Lolium perenne* L.) and johnsongrass (*Sorghum halepense* L.) [5]. *G. nigrifrons* is a natural and experimental vector of several pathogens (e.g. maize bushy stunt phytoplasma and corn stunt spiroplasma) as well as several types of viruses, including rhabdoviruses [6–10].

*G. nigrifrons* has recently been identified as a vector of the emerging Rhabdovirus *Maize fine streak virus* (MFSV) [7,11], which is a newly described member of *Nucleorhabdovirus*, first detected in Georgia, US in 1999 [7]. In maize, this emerging virus causes symptoms of dwarfing and fine chlorotic steaks along intermediate and small veins. Consistent with most rhabdoviruses, MFSV has high vector species specificity as *G. nigrifrons* is the only reported insect vector [7,11,12]. *G. nigrifrons* transmits MFSV in a persistent propagative manner, but, vector competence for MFSV is not consistent within *G. nigrifrons* populations. Individual insects can be
To expand leafhopper molecular resources, we constructed cDNA libraries using RNA from G. nigrifrons transmitters and acquirers, and sequenced the transcripts using RNA-seq. The resulting EST database provides the first transcriptome information on a leaffopper, and contains genes potentially involved in anti-pathogen defense. In addition, we used RT-qPCR to examine the expression of seven genes putatively involved in insect immune response for leaffoppers known to transmit MFSV and leaffoppers not previously exposed to MFSV.

**Methods**

**Insect rearing and virus maintenance**

*G. nigrifrons* was maintained on maize (*Zea mays* L.) hybrid Early Sunglow in growth chambers, and MFSV was maintained by serial transfer to maize at OARDC as previously described [11]. For virus maintenance, 300 *G. nigrifrons* individuals were reared on MFSV infected maize for three weeks then transferred to healthy maize seedlings. Inoculated plants with MFSV symptoms were subsequently used as source plants.

**Differentiating non-acquirers, acquirers, and transmitters**

Two hundred *G. nigrifrons* adults (100 males and 100 females) were collected, placed in a single cage, and allowed to mate and oviposit on MFSV infected maize seedlings for two days. After 14 days, the (F1) nymphs were observed, and this was denoted as day 0. The F1 nymphs were reared on MFSV infected maize seedlings for 21 days, then F1 adults were individually transferred to 4-day old healthy maize plants within a tube. After one week, F1 leafhoppers were collected, labeled according to the maize test plant, and stored at −80°C. Plants were moved to a growth chamber for three weeks for MFSV symptom development [11]. An insect was designated as a transmitter if MFSV symptoms developed on the test plant it fed on. For non-transmitter insects, RT-PCR was performed on individuals using the Access RT-PCR System (Promega, Madison, WI) following the manufacturer’s protocol. Primer pairs 361F (5'-GTGCGAATTGGCCCTATCC-3')/917R (5'-TCGAGCCATTCTGTATCC-3') and 5335F (5'-CTCCTCAATTGATAAGAAG-3')/6360R (5'-TATATGCCATTCTGTATCC-3') were used to amplify a 1120 bp and 1030 bp fragments of the viral N and G genes, respectively, based on MFSV genome sequence [12]. Reverse transcription was performed at 45°C for 45 min and followed by 2 min at 94°C. PCR included 40 cycles of 94°C for 30 s, annealing at 54°C and 94°C for 30 s, and extension at 68°C for 120 s. An insect was designated as an ‘acquirer’ if RT-PCR indicated the presence of MFSV, but no transmission of the virus to the test plant. ‘Non-acquirers’ were those insects for which MFSV was not detected by RT-PCR, and no symptoms developed on test plants.

**RNA isolation and cDNA library construction**

Total RNA from individual *G. nigrifrons* transmitters and acquirers was isolated with Trizol (Invitrogen, Grand Island, NY) following the manufacturer’s protocol. The concentration and the quality of RNA were analyzed using a NanoDrop 2000c spectrophotometer and Agilent 2100 Bioanalyzer. RNA (10 μg) was pooled from eight individuals from each class and used to construct two cDNA libraries following the mRNA sequencing sample preparation guide (Illumina, San Diego, CA). Paired-end DNA sequencing was done in two lanes (one per library) on an Illumina GA-II following manufacturer’s protocol.
Sequence assembly and annotation

The 76-bp paired-end Illumina reads from the acquirer (32,548,016 reads) and transmitter (30,541,892 reads) libraries were combined for de novo assembly. Low-quality ($\geq 80\%$ of the read with the Phred score of less than 20) and low complexity ($> 80\%$ of the read with single-nucleotide, di-nucleotide, or tri-nucleotide repeats) were removed. The processed reads were then assembled using a combination of the Velvet (ver. 1.2.01) [30] and Oases (ver. 0.2.06; http://www.ebi.ac.uk/zerbino/oases/) programs with the k-mer lengths of 41, 43, 45, 47, 49, 51, 53, and 55. The resulting assembled sequences and singletons were combined, processed to remove duplicates using a custom Perl Script [31], and further assembled after examining the overlapping regions identified by Vmatch (ver. z.z) [32]. Contigs were then further assembled using Phrap program (version: 0.20425.e) [33] to obtain the final transcriptome of sequences $> 100$ bp. Sequences have been submitted to the short read archive at NCBI GenBank under accession number SRP013390.3.

Functional annotation of the $G. nigrifrons$ transcriptome was performed by searching for analogous sequences in the Swiss-Prot database (http://www.ebi.ac.uk/uniprot) using an E-value cut-off of $10^{-4}$. Hierarchical functional categorization on gene ontologies (GO terms) was done using BLAST2GO (http://www.blast2go.de) [34]. BLAST2GO was also used to identify genes represented in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (http://www.kegg.com/kegg/kegg1a.html).

Comparison of $G. nigrifrons$ and other invertebrate transcriptomes and immune response genes

Each of the 32,480 $G. nigrifrons$ ESTs was subjected to pair-wise comparison to EST databases of eight invertebrate species using desktop downloaded tBLASTx software and a $10^{-10}$ E-value threshold. Seven invertebrate databases were constructed by retrieving cDNA sequences of characterized genomes from NCBI (ftp://ftp.ncbi.nih.gov) or Ensembl (ftp://ftp.ensembl.org/): Acyrthosiphon pisum (pea aphid, order Hemiptera, 37,994 sequences), Apis mellifera (honey bee, order Hymenoptera, 18,542 sequences), Nasonia vitripennis (parasitic wasp, order Hymenoptera, 27,287 sequences), Tribolium castaneum (red flour beetle, order Coleoptera, 882 ESTs), Drosophila melanogaster (fruit fly, order Diptera, 14,974 sequences), Crassostrea gigas (mollusk, order Bivalvia, 14,366 sequences), Anopheles gambiae (malaria mosquito, order Diptera, 14,974 sequences), and Caenorhabditis elegans (nematode, order Rhabditida, 32,201 sequences). A NCBI transcriptome database was also constructed for $P. maidis$ (maize plant hopper), order Hemiptera), which contained 10,636 sequences derived from the gut [28]. Immune gene homologs from $G. nigrifrons$ EST database were identified by similarity to annotated immunity genes from an insect immunity gene database constructed by Whitfield et al. [28] that contained over 300 immunity genes derived from $D. melanogaster$ (356 genes) [35], $A. gambiae$ (302 genes) [24], and $T. castaneum$ (243 genes) [36] using tBLASTx with an E-value cut-off of $10^{-10}$.

Validation of reference genes for gene expression studies

Six candidate housekeeping genes were selected: alpha tubulin (\textit{z-TUB}) (GniEST017131), elongation factor 1-alpha (\textit{EF-1a}) (GniEST068963), glyceraldehyde-3-phosphate dehydrogenase (\textit{GAPDH}) (GniEST000002), succinate dehydrogenase (\textit{SDH}) (GniEST008732), ribosomal protein L3 (\textit{RPL3}) (GniEST000330), ribosomal protein S13 (\textit{RPS13}) (GniEST004586). Six treatments were used including MFSV-transmitters, non-acquirers and leaffoppers raised on healthy plants, separated in groups of males and females. Total RNA was extracted from individual $G. nigrifrons$ adults using Trizol (Invitrogen). DNA was removed from the RNA samples with the TURBO DNA-free kit (Ambion, Grand Island, NY). The concentration and quality of the RNA were verified as outlined above. Total RNA (1\mu g) from individual insects was used for cDNA synthesis with the SuperScript III First-Strand Synthesis System (Invitrogen, Grand Island, NY). The cDNA was used as template for RT-qPCR using the iQ SYBR Green Mix (Bio-Rad, Hercules, CA), following the manufacturer’s recommendations and primers designed using Beacon Designer 7.0 (BioRad, Hercules, CA). PCR was performed with 5\mu l of SYBR Green Mix, 2\mu l of cDNA template (20ng/ml) and 5 pmole of each primer. Target genes were amplified at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Nuclease-free water was used for negative control reactions. PCR efficiency ($E$) was calculated by the equation $E = 10^{\text{1/C}_{\text{M}}}-1$ [37]. A standard curve was constructed using serial dilutions of pooled individual cDNAs and used to determine the relative expression values for these six reference genes (User Bulletin #2: ABI Prism 7700 Sequence Detection System vide supra) (http://www3.appliedbiosystems.com/). The software geNorm was used to determine the most stable reference gene among the six tested [38].

Expression of seven genes with known function in insect innate immunity was evaluated in transmitters and leaffoppers raised on healthy plants (i.e. never exposed to MFSV infected plants, used as control) using RT-qPCR: acetylcholine receptor subunit alpha-L1 (\textit{ACHR}) (GniEST002867), autophagy protein 5 (\textit{ATG5}) (GniEST012478), defensin (GniEST011432), peptidoglycan recognition protein SB1 (\textit{PGRP-SB1}) (GniEST015027), PGRP-SD (GniEST009213), PGRP-LC (GniEST006324), tripetidyl peptidase II (\textit{TPP II}) (GniEST021246). RNA isolation and RT-qPCR protocols were carried out as outlined above with primers listed in Table S1. The expression profiles of the seven genes were normalized to the internal control \textit{RPS13}. Nuclease-free water was used as the negative control reactions. For each treatment, three biological replicates composed of RNA isolated from 10 adult leaffoppers were analyzed. The relative accumulation of transcripts in MFSV transmitters and control leaffoppers were determined using the comparative $C_{\text{t}}$ method [39]. A T-test was used to compare mean $2^{-\Delta C_{\text{t}}}$ values.

Results and Discussion

Sequence assembly and annotation

A total of 38,240 good quality ESTs of a minimum 100 bp were generated for the $G. nigrifrons$ transcriptome. Approximately 34% of these transcripts (n = 13,036) mapped to the Swiss-Prot database (E<10^{-4}) based on deduced amino acid similarity. Of these, 177 homologs were of prokaryotic origin and therefore removed from further analysis. On a more conserved level (E-value <10^{-100}), 1,070 $G. nigrifrons$ ESTs have high similarity with genes in the Swiss-Prot database (listed in Table S2). Approximately one-third of these highly conserved genes (n = 355) had highest similarity to a $D. melanogaster$ gene. To guide biological interpretation, we used BLAST2GO to examine the associations between gene (GO) and enzyme (EC) ontologies assigned to the $G. nigrifrons$ ESTs. We identified 4,488 ESTs (12%) that mapped to GO or EC terms (Figure S1). Of these, 2,634 $G. nigrifrons$ ESTs were classified as having function in cellular pathways, 2,592 as having binding function, and 2,604 as cell parts. KEGG-based pathway analysis using BLAST2GO from ortholog cellular pathways indicated that 882 ESTs could be putatively assigned to one or more of 126 KEGG pathways (Table S3). Thus, our initial functional analysis of the $G. nigrifrons$ transcriptome indicates significant similarities.
with those of previously sequenced organisms, particularly *D. melanogaster*. However, fewer than half of the *G. nigrifrons* ESTs had significant similarity to genes in SWISS-PROT database, and less than 10% could be classified by GO term or KEGG pathway.

**Comparison of *G. nigrifrons* and other invertebrate ESTs**

Pair-wise comparison of the *G. nigrifrons* ESTs with cDNA databases for seven well-characterized invertebrate genomes and one insect dbEST collection indicated that ca. 37% (n = 14,259) of the *G. nigrifrons* ESTs had a significant match to at least one of the insect databases (E<10⁻¹⁵). When significant *G. nigrifrons* matches to the same predicted or known ortholog protein sequence in different species were collapsed into a single observation, there were 9,635 transcripts with at least one hit to an invertebrate database (~25% of the total ESTs). Partitioning of the significant matches among the eight invertebrate databases indicated *D. melanogaster* (n = 7,303) and *T. castaneum* (n = 7,028) had the greatest number of matches, whereas the most distantly related species, *C. elegans*, had the fewest (n = 4,777). The number of matches ranged between 6,309 and 6,907 for the remaining five invertebrate comparisons. Differences in the number of matches may be due to genomic evolution, or may more likely reflect different stages of transcriptome characterization and curation.

For the putative or known ortholog transcripts, the number of *G. nigrifrons* ESTs that match a sequence in only one of the eight comparison invertebrate species was determined. The number of significant ortholog matches as well as the number of transcripts exclusive to one invertebrate for all eight pair-wise comparisons are shown in figure 1. Roughly half of the unique matches to the most closely related species in the *vitripennis* or the hemipteran *N. vitripennis* of unique matches to the most closely related species in the comparison, the corn planthopper *P. maidis*, is likely due to the small number of *P. maidis* ESTs (10,636) compared to the transcriptomes of the other seven query species.

**Immune response ESTs in *G. nigrifrons***

The transcriptional responses of pathogen infection in insect human disease vectors are well described [40–46]. These studies suggest that innate immunity genes can have different functions in different genetic backgrounds, between closely related insect vector species, and even between different populations of the same species [47]. However, little is known about the transcriptome response in insect plant disease vectors due to pathogen invasion. To identify ESTs that play a putative role in the leafhopper immune response, we compared our dataset against >300 *A. gambiae*, *D. melanogaster*, and *T. castaneum* transcripts with known function in the insect immune response (including several transcript variants of some genes). The 25 *G. nigrifrons* ESTs with highest similarity to known immunity genes of *A. gambiae*, *D. melanogaster*, and *T. castaneum* are shown in Table 1.

A total of 194 *G. nigrifrons* ESTs were predicted to be functional in the immune response (Table S4) such as Toll and immune deficiency (IMD) pathways, Jun N-terminal kinase (JNK), and Janus Kinase/signal transducer of activator of transcription (JAK/STAT). In addition, 10 ESTs with the E<10⁻¹³, putatively function as pattern recognition proteins (PRPs) including different gram-negative bacteria binding proteins (GNBP), C-type lectin and scavenger receptors. Of particular interest in this group of PRPs are PGRP, which are the proteins responsible for sensing and binding of non-self molecules and which activate downstream immune responses. Since the Toll pathway was reported to play an important role in *Drosophila* X virus (DXV) defense [23], ESTs encoding putative key proteins involved in this pathway such as Spaetzle, protein Toll as well as its partners, Pelle and MyD88, Cactus, and Dorsal-related immunity factor (Dif) were identified. Other essential proteins function in IMD pathway such as DREDD and Relish, their corresponding ESTs were also present in *G. nigrifrons*. Although there are eight types of AMPs identified in *D. melanogaster*, there was only one putative AMP gene, defensin, identified in *G. nigrifrons* ESTs. The relatively large percentage of highly similar transcripts is expected, given the high degree of conservation of immune signaling pathways across diverse insect and mammalian taxa [48,49].

**G. nigrifrons** ESTs with similarity to RNAi pathway genes

RNAi is a highly conserved gene silencing process triggered by double-stranded RNAs and guided by small interfering RNA (siRNA) that involves post-transcriptional gene silencing (PTGS), which is essential for virus defense and development in insects [50,51]. Thirty-two *Drosophila* genes have been implicated in RNAi/PTGS. *G. nigrifrons* ESTs homologous to RNAi/PTGS

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Figure 1. Invertebrate comparative genomics. The number of significant ortholog matches (E-value <10⁻¹⁵) as well as the number of unique invertebrate transcripts for all eight pair-wise invertebrate comparisons with *Graminella nigrifrons*. These invertebrates are *Acrithosiphon pisum* (pea aphid, order Hemiptera), *Apis mellifera* (honey bee, order Hymenoptera), *Nasonia vitripennis* (parasitic wasp, order Hymenoptera), *Tribolium castaneum* (red flour beetle, order Coleoptera), *Anopheles gambiae* (malaria mosquito, order Diptera), *Drosophila melanogaster* (fruit fly, order Diptera), and *Caenorhabditis elegans* (soil nematode, order Rhabditida). doi:10.1371/journal.pone.0040613.g001

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**Number of Matches**

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transcripts were identified using tBLASTx with an (E-value <10^{-10}) against nucleotide sequences of D. melanogaster RNAi/PTGS genes. Forty-three G. nigrifrons ESTs matched to at least one D. melanogaster RNAi/PTGS transcript variant, and included 69% of D. melanogaster RNAi/PTGS genes (Table 2). Significant matches for some of the D. melanogaster RNAi/PTGS genes (e.g. FBgn0262447, FBgn0262432, FBgn0262391) were not identified, likely due to their small size (<100 bp).

**Validation and expression profiles of G. nigrifrons candidate genes**

Since reference genes for assaying G. nigrifrons gene expression have not been described, we examined expression of six genes previously selected for reference gene validation in other insects using RT-qPCR [52]. Primer pairs for all six genes had a $R^2$>0.99 and primer efficiency (E values) between 1.8 and 2.2 (Table S5). Stability analysis revealed RPS13 and 3′–TUB were the most stably expressed genes (Figure S2), and RPS13 was chosen based on comparisons with other insects [53].

Gene expression patterns for seven selected genes implicated in insect innate immunity were tested using RT-qPCR, including three PGRPs (two representing short class and one representing long class), as well as AChR, ATG 5, TPP-II and defensin (the only AMP EST in our database) [19,28,54]. RNAs were isolated from G. nigrifrons individuals that were either experimentally determined to be MFSV transmitters or control leafhoppers. RNA used for RT-qPCR had an A260/A280 between 1.8 and 2, and the PCR efficiencies for the candidate gene primers were between 1.8 and 2.2 (Table S1). The fold difference in expression for transmitters versus control leafhoppers ranged from 4.0 to 1.4 (Figure 2). No significant differences in ATG5, AChR, TPP-II or defensin expression were detected. However, expression of the PGRP genes was significantly lower in transmitters compared to control leafhoppers.

Previous studies suggested roles for ATG5, AChR, TPP-II or defensin in the innate immune response. A role for autophagy in controlling vesicular stomatitis virus (VSV) replication was indicated in D. melanogaster [55]. TPP-II, a multi-function peptidase, was reported to be involved in protein turnover and

### Table 1. G. nigrifrons ESTs with highest sequence similarity to known immunity genes of A. gambiae, and D. melanogaster.

| A. gambiae | D. melanogaster |
|-----------|----------------|
| **Genie** | **EST** | **E value** | **Ortholog** | **Immune Function** | **Genie** | **EST** | **E value** | **Ortholog** | **Immune Function** |
| 009993 0  | contactin-like putative cell adhesion molecule | 005095 0 | p38b |
| 019425 0  | thioester-containing protein | 002299 0 | Pelle associated protein Pellino (Pli) |
| 005039 0  | phenoloxidase | 000902 0 | Poly-(ADP-ribose) polymerase CG40411-RA, transcript variant A (Parp) |
| 012471 0  | signal transducer and activator of transcription | 004283 0 | Ect4 CG34373-RD, transcript variant D (Ect4), mRNA |
| 003430 0  | inhibitor of apoptosis protein | 019825 0 | MibI |
| 002299 0  | Pellino | 002293 0 | basket CG5680-RB (bsk) |
| 012767 0  | Emi domain; Emilin | 010860 0 | Helicase 89B CG4261-RA (Hel89B) |
| 008405 0  | Trypsin-like serine protease | 002299 0 | Pellino CG5212-RB, transcript variant B (Pli) |
| 019839 0  | Fibronectin type III/ Immunoglobulin | 003507 0 | scribbled CG5462-RD, transcript variant D (scrib) |
| 002849 0  | catalase | 010867 0 | Myosin 61F CG9155-RA, transcript variant A (Myo61F) |
| 018879 0  | Peroxidasin | 018879 0 | Peroxidasin CG12002-RE, transcript variant E (Pxn) |
| 003728 0  | scavenger receptor class A-like, C-type lectin | 009993 0 | Contactin CG1084-RB (Cont) |
| 011660 0  | thioester-containing protein | 011660 0 | Thioester containing protein II CG7052-RB, transcript variant B (Tepli) |
| 012471 0  | signal transducer and activator of transcription | 004310 0 | Hemocyanin |
| 005039 0  | phenoloxidase | 002293 0 | basket CG5680-RB (bsk) |
| 003430 0  | inhibitor of apoptosis protein | 004340 0 | Bruce CG6303-RB (Bruce) |
| 009993 9.0E-17 | contactin-like putative cell adhesion molecule | 006257 0 | epithelial membrane protein CG2727-RB, transcript variant B (emp), |
| 005039 2.0E-137 | phenoloxidase | 019839 0 | Neuroglian CG1634-RA, transcript variant C (Nrg) |
| 002271 2.0E-172 | scavenger receptor class B | 002849 0 | Catalase CG6871-RA (Cat) |
| 016562 2.0E-169 | START_STAR101-like; Ceramide-binding | 005821 0 | ion channel regulatory protein |
| 008405 2.0E-169 | Trypsin-like serine protease | 010898 0 | olf413; Copper type II ascorbate-dependent monooxygenase |
| 011660 8.0E-168 | thioester-containing protein | 019425 0 | Macroglobulin complement-related CG7586-RA (Mcr) |
| 012767 1.0E-161 | Emi domain; Emilin | 010860 0 | Helicase 89B CG4261-RA (Hel89B) |
| 002849 1.0E-161 | catalase | 008405 0 | TequilA CG4821-RD, transcript variant D (TequilA) |
| 002190 7.0E-161 | UAS family, FAS-associated factor 1, ubiquitin | 005095 0 | Mpk2 CG5475-RB, transcript variant B (Mpk2) |

**G. nigrifrons Transcriptome**

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[10] G. nigrifrons EST E value Ortholog Immune Function

| Genie-EST | E value | Ortholog Immune Function |
|-----------|---------|--------------------------|
| 009993    | 0       | contactin-like putative cell adhesion molecule |
| 019425    | 0       | thioester-containing protein |
| 005039    | 0       | phenoloxidase |
| 012471    | 0       | signal transducer and activator of transcription |
| 004340    | 0       | inhibitor of apoptosis protein |
| 009993    | 9.0E-177| contactin-like putative cell adhesion molecule |
| 005039    | 2.0E-137| phenoloxidase |
| 002271    | 2.0E-172| scavenger receptor class B |
| 016562    | 2.0E-169| START_STAR101-like; Ceramide-binding |
| 008405    | 2.0E-169| Trypsin-like serine protease |
| 011660    | 8.0E-168| thioester-containing protein |
| 012767    | 1.0E-161| Emi domain; Emilin |
| 002849    | 1.0E-161| catalase |
| 002190    | 7.0E-161| UAS family, FAS-associated factor 1, ubiquitin |
immune response through processing antigen epitopes [56,57].
AChR was suggested as a receptor for rabies virus (Rhabdoviridae)
[58]. Defensin is induced during bacterial infection after activation
of the Toll pathway by the recognition and binding of PAMPs to
PGRP-SA or SD [48,59,60]. However no significant expression
difference between transmitters and control leafhoppers was
detected for any of these four genes. A similar lack of an effect
of MMV infection was found in P. maidis for ATG3 and TPP-II
[28], and for defensin in Sigma virus (SIGMAV)-infected and non-
infected Drosophila [19]. Therefore, although these genes have roles
in innate insect immunity, their expression does not seem to

Table 2. Comparisons of RNAi related genes among D. melanogaster and G. nigrifrons.

| D. melanogaster Gene ID | D. melanogaster matching transcript ID | Gene Name | G. nigrifrons Ortholog Sequence(s) | E-value
|-------------------------|--------------------------------------|-----------|---------------------------------|--------|
| FBgn0262739 | FBtr0087613 | Argonaute 1 | GniEST000518 | 5.00E-89 |
| FBgn0262739 | FBtr0087613 | Argonaute 1 | GniEST002602 | 7.00E-89 |
| FBgn0087035 | FBtr0308124 | Argonaute 2 | GniEST001517 | 5.00E-129 |
| FBgn0250816 | FBtr0299881 | Argonaute 3 | GniEST010662 | 0 |
| FBgn0041164 | FBtr0073126 | armitage | GniEST001564 | 1.00E-61 |

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respond to long-term rhabdovirus infection in planthoppers or leafhoppers.

Recent studies have indicated a role of PGRPs in insect virus defense [19], and our data shows a potential interaction between putative G. nigrifrons PGRPs and rhabdovirus infection. All three putative-PGRPs tested (PGRP-SB1, PGRP-SD and PGRP-LC) showed significantly lower expression in MFSV transmitters compared to control leafhoppers, and the fold changes ranged from -2.9 to -4.0 (Figure 2). Pathogens, such as bacteria and fungi, are recognized by PGRPs, which subsequently trigger downstream AMPs production through Toll or IMD immune pathway [22]. In Drosophila, infection with the rhabdovirus SIGMAV also alters expression of PGRP-SB1 and PGRP-SD; however in this case, increased transcript levels were found with these genes in infected Drosophila compared to un-infected [19,23]. Several non-mutually exclusive factors may explain the different expression profiles of these three putative PGRPs in the G. nigrifrons/MFSV and D. melanogaster/SIGMAV systems. SIGMAV is known insect pathogens, whereas the pathogenicity of MFSV in G. nigrifrons has not been determined. For example, SIGMAV, which is also a member of Rhabdoviridae, is a natural viral insect pathogen widespread in D. melanogaster populations, and is vertically transmitted [61]. Alternatively, MFSV is transmitted only through feeding on infected plant hosts; vertical transmission has not been observed in G. nigrifrons [7,11]. The difference in virus acquisition and pathogenicity may elicit different immune responses.

Additionally, we hypothesize that MFSV might actively manipulate vector immune response in terms of reduced expression of these three PGRPs in order to successfully replicate within G. nigrifrons transmitters and acquirers. Virus manipulation has been observed with the Togavirus Semliki Forest Virus (SFV) within infected cell lines of the mosquito Aedes albopictus; in this case, activation of immune defense pathways was suppressed [62]. Manipulation may be through direct interactions with the insect vector, or by altering physiological processes of the plant hosts such that an elevated plant defense response impacts insect defenses which consequently favors virus replication. Although this hypothesis is untested with G. nigrifrons-MFSV, further work aimed at describing the expression profiles of other PGRPs among all three G. nigrifrons classes (transmitters, acquirers, non-acquirers) will help understand the interactions between PGRPs and MFSV proteins that lead to virus transmission.

Our study represents the first transcriptome characterization of a leafhopper species, which includes 38,240 transcripts. Significant sequence similarity existed in immune defense genes existed between G. nigrifrons and other well-characterized insects. Additionally, we have identified several putative components of the RNAi pathway, which will enable future development of functional evaluation of the role of this important pathway in transmission of MFSV. The down-regulation of PGRPs in MFSV transmitters suggests a possible interaction with rhabdovirus transmission by vectors, although additional research is required to define the mechanism. The results presented expand molecular characterization of plant virus vectors and will help further understand the mechanisms of plant virus vector competence.

Supporting Information

Figure S1 Gene ontology (GO) terms for G. nigrifrons EST (contigs and singletons). The pie charts were generated...
based on A. Biological process; B. Molecular function; C. Cellular component.

(PDF)

**Figure S2** Comparison of reference genes for *G. nigrifrons* using geNorm. Genes with lower average expression stability *M* are more stable among all treatments. *z-TUB*, alpha tubulin; *EF-1z*, elongation factor 1-alpha; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *SDH*, succinate dehydrogenase; *RPS3*, ribosomal protein L3; *RPS13*, ribosomal protein S13.

(PDF)

**Table S1** Primer sequences and efficiencies of candidate genes evaluated for differential expression among MFSV transmitters and control *G. nigrifrons* (RT-qPCR).

(DOCX)

**Table S2** *G. nigrifrons* ESTs that have the most significant alignment (E-value <10^-180) with genes in the Swiss-Prot database.

(XLSX)

**Table S3** Results from KEGG pathway analysis.

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(XLSX)

**Table S4** A total of 194 *G. nigrifrons* ESTs were predicted to be implicated in immune response.

(XLSX)

**Table S5** Primer sequences, efficiencies and correlation of potential RT-qPCR reference genes for *G. nigrifrons*.

(DOCX)

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**Author Contributions**

Conceived and designed the experiments: YC BJJC MGR APM. Performed the experiments: YC BJJC. Analyzed the data: YC BJJC XBM. Contributed reagents/materials/analysis tools: XBM MGR APM. Wrote the paper: YC BJJC XBM MGR APM.
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