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BRIEF REPORT

Larval stage *Lymantria dispar* microRNAs differentially expressed in response to parasitization by *Glyptapanteles flavicoxis* parasitoid

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Abstract MicroRNAs (miRNAs) are small RNA molecules that regulate gene expression by targeting messenger RNAs and causing cleavage or translation blockage. miRNAs induced after parasitization of the lepidopteran host *Lymantria dispar* by the parasitoid wasp *Glyptapanteles flavicoxis,* which introduces a polydnavirus and other parasitoid factors, were examined to identify induced miRNAs that might regulate host genes and contribute to host immunosuppression and other effects. miRNA profiling of parasitized larval hemocytes versus non-parasitized ones by microarray hybridization to mature insect and virus miRNAs identified 27 differentially expressed miRNAs after parasitization. This was confirmed by real-time relative qPCR for insect miRNAs (dme-mir-1, -8, -14, -184, -276, -277, -279, -289, -let-7) using miRNA-specific TaqMan™ assays. Certain cellular miRNAs were differentially expressed in larval tissues, such as the potentially developmentally linked mir-277, signifying a need for functional studies.

MicroRNAs (miRNAs) are novel small (~22 nt) noncoding RNAs that play key roles in eukaryotes, such as regulation of differentiation, development, metabolism, and gene expression [1–4]. They act by binding to partially complementary sites in mRNAs of targeted gene(s) and regulate gene expression by blocking translation and/or degrading the mRNA transcript, depending on the level of complementarity between the miRNA and the target. miRNAs are ubiquitous and may regulate gene expression profiles across various tissue types in plants, nematodes, insects, and vertebrates [5–8].

Approaches to identifying miRNAs include screening of miRNA expression profiles, cloning and sequencing of miRNAs associated with specific tissues, and high-throughput microarray analysis [9]. An miRNA that is differentially expressed in a particular tissue or cell type may play a role in gene regulation, while an miRNA expressed during a particular developmental stage could regulate development. Differential expression of insect miRNAs at different developmental stages with roles in developmentally regulated processes has been shown in *Drosophila* [10], the honeybee *Apis mellifera* [11] and most recently, in the silkmoth *Bombyx mori* [12, 13].

Certain parasitoid wasps possess dsDNA polydnaviruses (PDVs) that are important for successful parasitization. PDVs replicate only in ovarian calyx cells and are introduced into lepidopteran host larvae during oviposition, where they infect cells and cause suppression of the host immune system, developmental delay, or physiological effects (reviewed in Ref. [14]). Once in host cells, PDV genes are temporally and spatially expressed in a sustained or transient fashion [15–17], although the virus does not replicate. Several conventional viruses have been shown to encode miRNAs that repress host cellular gene expression, including transcriptional regulators, signal transducers, and immune-related cells [18–21]. miRNAs have not yet been predicted by computational methods to be encoded in sequenced PDV genomes. Host cellular miRNAs expressed after parasitization have not been

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examined in parasitoid-PDV-host systems, and therefore profiling of miRNAs that are differentially regulated in *L. dispar* in response to parasitization by *Glyptapanteles* parasitoids was undertaken to find induced cellular miRNAs that might potentially be involved in regulation in the host.

Glyptapanteles flavicoxis and *G. indiensis*, gregarious and solitary braconid parasitoids of *L. dispar*, respectively, were maintained at USDA, Beltsville, MD, USA. Both were reared under similar protocols, except *G. flavicoxis* parasitizes late third (Ld3) while *G. indiensis* parasitizes late first instar (Ld1) larvae. *L. dispar* larvae were reared on a high-wheat-germ diet according to Bell et al. [22]. Parasitization was conducted by exposing a synchronous individual larva to a single parasitoid female until oviposition was observed and then incubating on diet. Post-parasitization (pp) times were recorded from initiation of parasitization. Hemocytes were collected from surface-sterilized Ld3 by cutting a proleg and transferring hemolymph to PBS in chilled microfuge tubes. A crystal of *N*-phenylthiourea was added to inhibit melanization. Hemolymph was centrifuged at 14,000*g* for 10 min at 4°C to pellet hemocytes, followed by RNA preparation. Larvae were dissected for additional tissue types. Total RNAs from non-parasitized and parasitized *L. dispar* larvae were isolated using MirVana™ (Ambion Austin). Tissues were disrupted in lysis agent in a Fastprep® FP 120 (Q-Biogene, Solon, OH, USA) for 45 s, setting 4.5. Supernatants were transferred to sterile tubes, incubated 10 min on ice, and then extracted in acid-chloroform and processed. RNAs were used immediately for microarray or TaqMan assays, or stored at −80°C.

miRNA activity in response to *G. flavicoxis* parasitization was examined by microarray (LC Sciences, Houston, USA) profiling of larval Ld3 hemocytes (two biological replicates) collected 24 h pp for known miRNAs by hybridization on microfluidic chips (probe sets insect and virus; first replicate Sanger 9.2; second replicate Sanger 10.0) with appropriate dye swap. Small RNAs were 3′-extended using poly(A) polymerase, followed by ligation of an oligonucleotide tag to the poly(A) tail for staining with fluorescent dye; two different tags were used for RNA samples. Hybridization was done overnight on a ParaFlo™ microfluidic chip using a microcirculation pump (Atactic Technologies, Houston, USA) [9]. On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target miRNA (miRBase: http://microrna.sanger.ac.uk/) or internal controls. Signals were detected using fluorescence labeling (Invitrogen, Carlsbad, USA). Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Devices, Sunnyvale, USA) and quantified. Data were analyzed by subtracting background and normalized using a cyclic LOWESS filter [23]. Ratios of the two sets of detected signals ($\log_2$ transformed, balanced) and p values of t tests were calculated in which differentially detected signals were those with p values of <0.01. Data were visualized using Multiple Experimental Viewer Software (TIGR, Rockville, MD, USA). Microarray signals showed statistically significant differences in expression levels between non-parasitized and parasitized larval hemocytes for numerous defined insect miRNAs (Table 1). miRNA sequences exhibiting signal strengths >500 using probes designed to detect miRNAs from insect and virus species were considered detected. Of 50 miRNAs detected, 27 were unique. To date, there is limited *L. dispar* genome information in public databases, thus BLAST searches were not performed to identify precursor sequences or designate these as *L. dispar* miRNAs. Although they may have base differences in precursor miRNA species, *L. dispar* miRNAs hybridizing to mature miRNAs from other species on microarray should possess identical mature sequences, as mature miRNAs are conserved [25]. Several differentially regulated mature *Drosophila*-derived miRNAs (dme-mir) identified as expressed with significance in parasitized Ld3 hemocytes were validated using commercial mir-specific TaqMan™ assays (Fig. 1) (Applied Biosystems, Foster City, CA). A two-step protocol was conducted with reverse transcription with an miRNA-specific primer, followed by real-time qPCR with specific TaqMan™ major groove binder probe. Assays were conducted in triplicate on an ABI 7500 Real Time System. Relative quantification was performed using the comparative C_t method [24]. For each, eukaryotic 18S was employed as an endogenous internal control and $\log_2$-transformed miR/18S expression ratios were used for analysis. For each, control reactions were conducted without reverse transcription and/or template. *G. flavicoxis*-parasitized larval hemocytes showed highest levels of up-regulation of expressed miRNAs mir-1, mir-184, and mir-277, and up-regulation of mir-8 and mir-289, consistent with microarray assay predictions, though lower-level expression was obtained for mir-289. *G. flavicoxis*-parasitized larval hemocytes showed highest down-regulation of expressed miRNAs mir-279 and mir-let7, with down-regulation of mir-276a and mir-14 as compared with hemocytes from non-parasitized larvae. These results were consistent with microarray predictions, with the exception of mir-14.

RNAs isolated from parasitized (A) hemolymph, (B) fat body, (C) brain, and (D) midgut (Fig. 2a) were examined for expression of certain miRNAs that are differentially expressed in hemocytes. The up-regulated miRNAs mir-1 and mir-277 and the down-regulated mir-279 and mir-let- 7 were evaluated using miRNA-specific TaqMan assays. Of the *G. flavicoxis*-parasitized tissues examined, the least miRNA activity was observed in the larval midgut. Mir-1

Table 1 Differentially expressed miRNAs in *G. flavicoxis*-parasitized *Lymantria dispar* third-instar larval hemocytes versus non-parasitized microarray, differentially expressed with statistical significance

Reporter name	*p* value	Fold difference
dme-miR-281-2*	6.59E−07	2.45
dme-miR-289	0.00E+00	1.98
dme-miR-1	4.30E−03	1.76
hcmv-miR-UL70-3p	1.36E−05	1.63
C-ame-miR-1	2.74E−13	1.52
dme-miR-92b	5.14E−10	1.49
C-ame-miR-277	6.88E−15	1.45
dme-miR-277	0.00E+00	1.44
kshv-miR-K12-3	9.40E−04	1.37
C-ame-miR-92a	6.64E−04	1.34
dme-miR-92a	6.05E−05	1.19
C-ame-bantam	1.61E−12	0.72
ame-bantam	3.85E−12	0.68
aga-miR-2	1.05E−03	0.65
C-ame-miR-275	2.05E−10	0.56
dme-miR-14	3.28E−03	0.50
dme-miR-275	3.64E−13	0.49
C-ame-miR-7	3.97E−04	0.49
dme-miR-184	3.25E−09	0.47
aga-miR-184	4.42E−09	0.46
C-ame-miR-184	2.38E−09	0.45
dme-miR-7	1.24E−03	0.39
C-ame-miR-14	1.00E−07	0.30
dme-miR-8	1.31E−05	0.19
C-ame-miR-8	2.64E−04	0.18
bmo-miR-31	4.00E−14	−2.27
C-C6617	1.85E−05	−1.72
dps-miR-100	3.29E−07	−1.46
dme-miR-87	2.77E−05	−0.95
C-ame-miR-31a	2.17E−05	−0.92
dme-miR-276b	3.44E−10	−0.71
dme-miR-276*	8.25E−09	−0.71
dme-miR-279	6.21E−06	−0.66
C-ame-miR-279	9.00E−06	−0.64
bmo-miR-276	7.59E−11	−0.60
C-ame-miR-79	1.78E−09	−0.60
bmo-miR-279	4.01E−08	−0.57
dme-miR-79	2.10E−11	−0.55
C-ame-let-7	1.11E−08	−0.53
hsv1-miR-H1	2.02E−12	−0.51
aga-miR-317	4.04E−04	−0.51
dme-let-7	7.02E−05	−0.42
dme-miR-276a	1.69E−10	−0.41

The data are from two biological replicates

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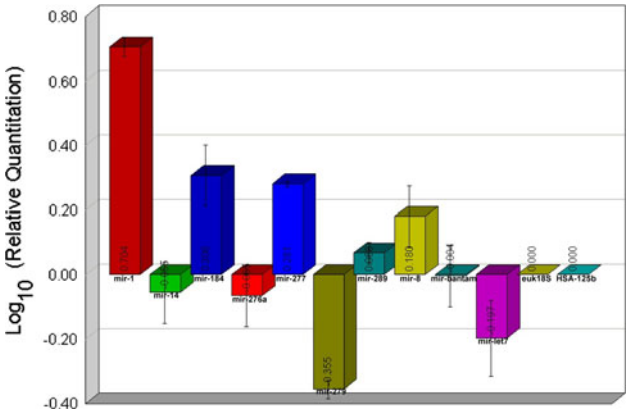

Fig. 1 *G. flavicoxis*-parasitized *L. dispar* miRNA expression profile 24 h post-parasitization. Real-time PCR using miRNA-specific TaqMan™ assays were conducted on hemocyte RNAs isolated from *G. flavicoxis*-parasitized *L. dispar* larvae (3rd instar) 24 h pp. Relative quantitation was employed to estimate differential expression of miRNAs in parasitized tissues by comparison to non-parasitized larvae in three biological replicates, using eukaryotic 18S as the endogenous standard to normalize cDNA quantities. Three technical replicates were conducted

was up-regulated in all parasitized tissues, most strongly in hemolymph and fat body. Mir-277 was up-regulated, most strongly in fat body. Mir-279 had tissue-variable expression patterns, with negligible expression in brain and midgut, down-regulation in hemolymph, and up-regulation in fat body. Similarly, mir-let-7 showed tissue-variable expression patterns, down-regulation in hemolymph and brain, slight up-regulation in midgut, and negligible expression in fat body.

Expression in *L. dispar* of cellular Ld-mir-277 in response to separate parasitization by related parasitoids was examined (Fig. 2b); *G. flavicoxis* and *G. indiensis* are parasitoids of Ld3 and Ld1, respectively. In both, Ld-mir-277 was strongly up-regulated at 24 h pp. Ld-mir-277 expression levels declined by 7 days pp but remained high compared with other miRNA levels.

The ability to distinguish miRNA expression patterns at the tissue, cellular and subcellular levels is important for understanding the biological roles of miRNAs and how they impact or regulate gene networks. Molecular studies of parasitism and PDV infection in lepidopteran cells have demonstrated high levels of viral gene transcription accompanied by spatial and temporal alteration of host genes with effects of immunosuppression, alteration of endocrine function, and developmental arrest. High transcriptional activity pp with a corresponding effect on host gene regulation suggests an involvement of cellular miRNAs as cellular transcriptional regulators. Microarray miRNA profiling of *G. flavicoxis*—parasitized *L. dispar* larval hemocytes yielded a wide array of differentially

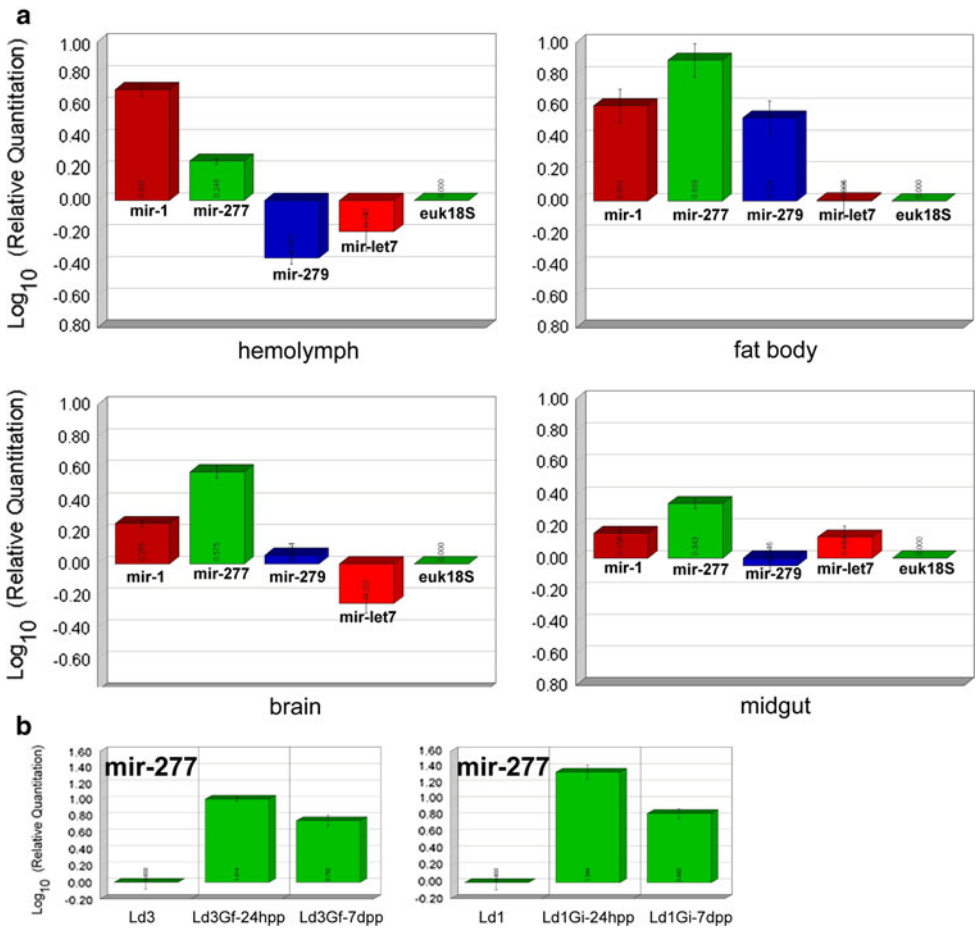

Fig. 2 miRNA expression in **a** *G. flavicoxis*-parasitized third instar and **b** *G. indiensis*-parasitized first instar *L. dispar* larvae. **a** Real-time PCR using miRNA-specific TaqMan™ assays were conducted with RNAs isolated from *G. flavicoxis*-parasitized *L. dispar* larvae (third instar) 24 h pp. miRNA-specific TaqMan™ dme-mir-1, dme-mir-277, dme-mir-279, and dme-mir-let7 assays were conducted for (*a*) hemocyte (*b*) fat body, (*c*) brain/nervous, and (*d*) midgut larval RNAs. **b** Real-time PCR specific mir-277 TaqMan™ assays were conducted to evaluate expression in two *L. dispar*—*Glyptapanteles* parasitoid systems at 24 h and 7 days pp. Relative quantitation was employed to estimate differential expression levels in the parasitized host by comparison to a non-parasitized host in three biological replicates, using eukaryotic 18S as the endogenous standard to normalize cDNA quantities. Three technical replicates were conducted

expressed known miRNAs associated with insects and viruses. Most, but not all, miRNAs identified in microarray profiling showed similar real-time qPCR profiles. The miRNAs mir-1, mir-184, mir-277, mir-289 were up-regulated, and mir-279 and mir-let-7 were down-regulated in validation assays. Of the more highly expressed miRNAs (mir-1, mir-277, mir-279 and mir-let-7) analyzed further for tissue-specific expression, all showed differential expression in diverse parasitized tissues. Mir-1 and -277 were up-regulated in all parasitized tissues examined. Lowest levels of miRNA activity were observed in parasitized midgut, consistent with limited cellular transcriptional activity associated with midgut pp.

Functional roles have been identified for few cellular miRNAs in insects, particularly lepidopterans. Recently, researchers have examined miRNAs in the silkmoth *B. mori* in several studies [12, 13, 26] that form the basis of our knowledge of miRNA activity in lepidopterans. Liu et al. [26] characterized the expression of *B. mori* miRNA let-7, the mature form of which is highly conserved, and found that *B. mori* development was triggered by a combination of ecdysone, *B. mori* (bmo)-let-7 miRNA and other genes, suggesting that bmo-let-7 might regulate metamorphosis from the late larval stage and function in earlier larval stages. In *L. dispar*, mir-let-7 was down-regulated in parasitized *L. dispar* larvae. In further analysis, He et al. [12] found that bmo-miR-1, bmo-let-7a, bmo-miR-8, bmo-miR-14, bmo-miR-276a, bmo-miR-279 miRNAs were highly and uniformly expressed in all developmental stages, suggesting that these miRNAs could play roles in regulation of constitutive processes in *B. mori.* They found that bmo-miR-277 was expressed only in adult moths and not in the larval or pupal stages, and similarly, bmo-miR-289 was expressed weakly in pupae and not in larvae or adults [26]. Our analysis suggests that parasitism and PDV of *G. flavicoxis* and *G. indiensis* induce expression of *L. dispar* cellular miRNA (mir-277) at the larval stage, earlier than the expected adult stage. Functional roles have been explored to a limited degree for differentially expressed miRNAs-277, -279, -289, -184, and –1 in *B. mori*.

miRNA profiling suggests future areas to be explored in analyzing parasitism and roles in host regulation by PDVs, including potential associations with conventional viruses. Microarray profiling has shown that two human herpes

virus-associated miRNAs, human cytomegalovirus miRNA hcmv-mir-UL70-3p and Kaposi's sarcoma-associated herpes virus kshv-miR-K12-3, are up-regulated in *G. flavicoxis*-parasitized *L. dispar* hemocytes. Interestingly, these are two of the many miRNAs that have been hypothesized to be involved in viral miRNA-mediated self-suppression of immediate-early genes as part of viral strategy to enter and maintain latency [21]. Functional assays are needed to decipher the roles of these and other miRNAs in parasitization, which appear to be involved in regulation of both cellular gene expression and the host anti-viral response.

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