Comparative Biochemistry and Physiology, Part D 20 (2016) 95–100

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part D

journal homepage: www.elsevier.com/locate/cbpd

Characterization of cold-associated microRNAs in the freeze-tolerant gall fly *Eurosta solidaginis* using high-throughput sequencing

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ARTICLE INFO

Article history:
Received 20 June 2016
Received in revised form 26 August 2016
Accepted 31 August 2016
Available online 04 September 2016

Keywords:
MicroRNAs
Cold hardiness
Freeze tolerance
Eurosta solidaginis
Next-generation sequencing

ABSTRACT

Significant physiological and biochemical changes are observed in freeze-tolerant insects when confronted with cold temperatures. These insects have adapted to winter by retreating into a hypometabolic state of diapause and implementing cryoprotective mechanisms that allow them to survive whole body freezing. MicroRNAs (miRNAs), a family of short ribonucleic acids, are emerging as likely molecular players underlying the process of cold adaptation. Unfortunately, the data is sparse concerning the signature of miRNAs that are modulated following cold exposure in the freeze-tolerant goldenrod gall fly *Eurosta solidaginis*. Leveraging for the first time a next-generation sequencing approach, differentially expressed miRNAs were evaluated in 5 °C and −15 °C-exposed *E. solidaginis* larvae. Next-generation sequencing expression data was subsequently validated by qRT-PCR for selected miRNA targets. Results demonstrate 24 differentially expressed freeze-responsive miRNAs. Notable, miR-1-3p, a miRNA modulated at low temperature in another cold-hardy insect, and miR-14-3p, a miRNA associated with stress response in the fruit fly, were shown to be significantly up-regulated in −15 °C-exposed larvae. Overall, this work identifies, for the first time in a high-throughput manner, differentially expressed miRNAs in cold-exposed *E. solidaginis* larvae and further clarifies an emerging signature of miRNAs modulated at low temperatures in cold-hardy insects.

1. Introduction

Several insects are able to tolerate freezing in order to cope with the frigid conditions associated with winter. Production of cryoprotectants such as glycerol and sorbitol, synthesis of antifreeze proteins, modulation of heat shock proteins and accumulation of acetylated triacylglycerols are examples of the molecular strategies used by freeze-tolerant insects to deal with prolonged exposure to cold temperatures (Morrissey and Baust, 1976; Lee and Denlinger, 1991; Duman, 2001; Zhang et al., 2011; Storey and Storey, 2012; Marshall et al., 2014). Larvae of the North American goldenrod gall fly *Eurosta solidaginis* have been amply studied as a model of insect freeze tolerance. Third instar *E. solidaginis* larvae overwinter in goldenrod stem galls that often protrude above the snowpack and are able to withstand temperatures as low as −40 °C in this environment (Williams and Lee, 2008).

MicroRNAs (miRNAs) are small non-coding transcripts that can regulate expression of several target mRNAs (Bartel, 2004). These molecules can repress transcript expression and subsequently influence a myriad of processes including glucose and lipid metabolism (Hartig et al., 2015) as well as cellular response to various stresses (Bhattacharyya et al., 2006). Interestingly, modulation of key molecular players involved in miRNA biogenesis has been reported in different animal models of cold adaptation including reduced Dicer protein levels in skeletal muscle of the freeze-tolerant wood frog *Rana sylvatica* during freezing and elevated Dicer protein levels in foot muscle and hepatopancreas of the intertidal gastropod *Littorina littorea* following exposure to sub-zero temperatures (Biggar et al., 2009, 2012). Accordingly, variations of miRNA transcripts have been observed in various species that can adapt to cold environments including cold-hardy insects (Lyons et al., 2013), mammalian hibernators (Liu et al., 2010a) and freeze-tolerant amphibians (Biggar et al., 2009) to name a few. On the other hand, data remains limited on differentially expressed miRNAs in the freeze-tolerant *E. solidaginis*.

The current study was undertaken to identify a signature of modulated miRNAs in cold-exposed *E. solidaginis* using next-generation sequencing. Recent work utilizing such an approach has revealed miRNA fingerprints in models of cold adaptation (Liu et al., 2010a; Lyons et al., 2015a). This study reports, for the first time using a high-throughput approach, differential expression of a series of miRNAs linked to cold adaptation including miR-1-3p, miR-14-3p, miR-31a-3p and miR-284-3p in cold-exposed *E. solidaginis* larvae. Predicted transcript targets and potential impact of these changes in insect freeze tolerance are also discussed.

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http://dx.doi.org/10.1016/j.cbd.2016.08.007
1744-117X/

2. Materials and methods

2.1. Insect collection

E. solidaginis larvae were sampled from goldenrod galls in fields around the Ottawa, Canada area as reported previously (Courteau et al., 2012). Galls harboring insects were initially acclimated for a period of ~3 weeks at a temperature of 5 °C in an incubator. Control insects were then sampled from the galls and flash frozen in liquid nitrogen. The remaining galls were rapidly placed at −5 °C for ~3 weeks and then moved to −15 °C for ~3 weeks before being sampled as above. Approximately 30 larvae were sampled at each temperature. Frozen larvae were shipped to Moncton on dry ice and immediately stored at −80 °C for long-term storage.

2.2. Small RNA isolation

Fractions containing small and large RNA were obtained from 5 °C and −15 °C-exposed *E. solidaginis* larvae using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific) following the protocol provided by the manufacturer and as previously reported (Lyons et al., 2015a). RNA isolates were prepared in duplicates for each group using three *E. solidaginis* larvae per replicate as starting material. Small RNA integrity was assessed with the High Sensitivity RNA ScreenTape on the Agilent TapeStation 2200 instrument.

2.3. Small RNA library construction and sequencing

A small RNA library was constructed and sequenced following Ion Torrent (Life Technologies) protocols. Samples were loaded on an Ion PI Chip v2 and subsequently sequenced using an Ion Proton Sequencer with the 260 flows parameter. Two *E. solidaginis* small RNA libraries were generated from control (5 °C) larvae and two from cold-exposed (−15 °C) larvae. FASTQ files were obtained for each sample and adaptor sequences were removed. Reads of <16 and of >60 nucleotides were eliminated from the sequencing data. Reads with bases having Q scores below 20 were also discarded. These steps were performed using the Python cutadapt script (version 1.2.1). Small RNA annotation was performed using the standalone sRNAbench tool from sRNAtoolbox populated with *Drosophila melanogaster* reference sequences (Rueda et al., 2015). The use of characterized genomes from other organisms as a reference to identify non-coding RNAs in non-model insect species has been performed elsewhere (Etebari and Asgari, 2014). The number of allowed mismatches for mapping to mature miRNA sequences was set to 3. Other settings were left to default values. Annotated sequences with fewer than 10 normalized read counts were filtered and log2 fold-changes were determined.

2.4. cDNA synthesis

cDNA was prepared from small RNA isolates as previously reported (Biggar et al., 2011). RNA isolates were prepared, as described above, in triplicates for each *E. solidaginis* group. Briefly, stem-loop primers to amplify the *E. solidaginis*–specific miRNAs of interest were synthesized using consensus alignment sequences of previously characterized miRNAs from other insects. Primers are presented in Table 1. Small RNA was mixed with 2 μl of 300 nM miRNA-specific stem-loop primer. The mixture was heated to 95 °C for 5 min, cooled to 60 °C for 5 min and rapidly placed on ice for 1 min. Following cold exposition, 4 μl of 5× first strand buffer, 2 μl of 0.1 M DTT, 1 μl of 10 mM dNTPs, 2 μl of DEPC-treated water and 1 μl of M-MLV reverse transcriptase (Thermo Fisher Scientific) were added. Samples were incubated at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min.

2.5. PCR and qRT-PCR amplification of miRNAs

Initial miRNA amplification reactions were conducted as before (Lyons et al., 2015a). PCR reactions were undertaken by combining 5 μl of cDNA template (10^{-1}) with 5.5 μl of DEPC-treated water, 1 μl of 25 μM miRNA forward primer, 1 μl of 25 μM universal reverse primer and 12.5 μl of 2× Taq FroggaBio mix (FroggaBio). PCR protocol included an initial step at 95 °C for 5 min, followed by 35 cycles at 95 °C for 15 s and 60 °C for 60 s. PCR was performed using a Mastercycler Pro S thermocyler (Eppendorf). Products were subsequently separated on 2% agarose gels and visualized on a UV transilluminator. Products were purified and sequenced by the Université Laval sequencing platform (Quebec City, Qc) and were confirmed using BLAST.

Amplification efficiencies for each primer couple were initially measured by amplifying target miRNAs in serial cDNA dilutions (10^{-1} to 10^{-4}) using similar conditions as above. Efficiencies were 94.7% at 56.6 °C, 94.9% at 57.8 °C and 85.9% at 64.0 °C for miR-31b-5p, miR-305-3p and miR-965-3p, respectively. The qRT-PCR protocol was undertaken by mixing 2.5 μl of 10^{-1} cDNA with 0.5 μl of DEPC-treated water, 1 μl of 5 μM forward primer, 1 μl of 5 μM reverse primer and 5 μl of iTaq Universal SYBR Green Supermix (Bio-Rad). Amplification was conducted with an initial step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, optimal annealing temperature of each primer pair for 30 s and 72 °C for 60 s. PCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad). U6 snRNA, a small nuclear RNA previously leveraged as a housekeeping RNA in cold-exposed *E. solidaginis* (Lyons et al., 2015b), was utilized as a reference gene and amplified in parallel using qRT-PCR at an annealing temperature of 56.7 °C where it displayed a 95.7% efficiency.

2.6. Quantification and statistics

The Bio-Rad CFX Manager software was leveraged to collect Cq values for qRT-PCR reactions. Levels of miRNA targets were normalized using U6 snRNA levels amplified in the same cDNA sample. MiRNA quantification was conducted following the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). Ratios of normalized transcript levels in −15 °C-exposed *E. solidaginis* larvae to average transcript expression in 5 °C-exposed *E. solidaginis* larvae were obtained. Statistical significant differences in miRNA relative expression between the two conditions were evaluated with the Student's *t*-test and were considered significant when $P < 0.05$. For next-generation sequencing, log2 ratios of

Table 1
Primer sequences, efficiencies and optimal annealing temperatures. Abbreviations: Forward (Fwd); Stem-loop (Stem); and Reverse (Rev) primers.

Primer		Sequence	Efficiency	Temperature
miR-31b-5p	Fwd	5′-ACACTCCAGCTGGGTGGCAAGATGTCGGAA-3′	94.7%	56.6 °C
	Stem	5′-CTCACAGTACGTTGGTATCCTTGTGATGTTCGATGCCATATTGTACTGTGAGTCAGCTAT-3’		
miR-305-3p	Fwd	5′-ACACTCCAGCTGGGCGGCACATGTTGAAGT-3′	94.9%	57.8 °C
	Stem	5′-CTCACAGTACGTTGGTATCCTTGTGATGTTCGATGCCATATTGTACTGTGAGTGAGTGTA-3′		
miR-965-3p	Fwd	5′-ACACTCCAGCTGGGTAAGCGTATAGCTTTT-3′	85.9%	64.0 °C
	Stem	5′-CTCACAGTACGTTGGTATCCTTGTGATGTTCGATGCCATATTGTACTGTGAGAAGGGGAA-3′		
U6	Fwd	5′-ACACTCCAGCTGGGCGGCAGCACATATACTA-3′	95.7%	56.7 °C
	Stem	5′-CTCACAGTACGTTGGTATCCTTGTGATGTTCGATGCCATATTGTACTGTGAGTGGAACGC-3′		
Universal	Rev	5′-CTCACAGTACGTTGGTATCCTTGTG-3′	–	–