Identification and Comparative Profiling of microRNAs at Different Diapause Stages of Galeruca Daurica Adults

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Research article

Keywords: MicroRNA, Diapause, Galeruca daurica, Small RNA sequencing, Juvenile hormone, Ribosomal protein, Signaling pathway

Posted Date: September 9th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-70765/v1

Abstract

Background: MicroRNAs (miRNAs) are a class of small noncoding RNAs of approximately 22 nt in length, which regulate gene expression at the post-transcriptional level. Although the regulatory roles of miRNAs in various physiological processes throughout insect development have been investigated, it is almost unknown about the roles of miRNAs involved in the regulation of diapause in insects.

Results: We constructed 12 small RNA libraries from *Galeruca daurica* adults at different diapause stages: pre-diapause (PD), diapause (D), post-diapause 1 (TD1), and post-diapause 2 (TD2). Using Illumina sequencing, a total of 95.06 million valid reads was obtained, and 230 miRNAs, including 143 conserved and 87 novel miRNAs, were identified from *G. daurica*. The expression profiles of these miRNAs were assessed across different diapause stages and miRNAs that were highly expressed at different diapause stages were identified. Comparative analysis of read counts indicated that both conserved and novel miRNAs were differently expressed among the four different diapause stages, and the differential expression was validated via qRT-PCR. The 25, 11, 15, 14, 26, and one miRNAs were differentially expressed in D/PD, D/TD1, D/TD2, TD1/PD, TD2/PD, and TD2/TD1, respectively. The KEGG and GO analysis of the predicted target genes suggested the essential roles of miRNAs in the regulation of summer diapause in *G. daurica*, especially via the juvenile hormone, ribosome, MAPK signaling, mTOR signaling, Ca^{2+} signaling, and G-protein coupled receptor signaling pathways.

Conclusion: Our research results indicate that miRNAs may be involved in the regulation of summer diapause in *G. daurica*, and these results also provide an important new small RNA genomics resource for further studies on insect diapause.

Background

Diapause is a programmed dormancy that allows organisms to tolerate predictable periods of unfavorable conditions by temporarily stopping development and reducing metabolism [1]. Understanding the mechanism of diapause is essential not only for predicting the geographic and seasonal distributions of insects but also for the development of effective pest management strategies [2]. Although physiological and biochemical aspects of diapause are well understood and a growing body of literature reports the changes in gene expression that drive the diapause program in insects, many details of the regulatory networks remain largely unknown. One particular gap in our understanding of diapause is how regulatory noncoding RNAs regulate diapause [3].

MicroRNAs (miRNAs) are a class of small noncoding RNAs of approximately 22 nt in length, which regulate gene expression at the post-transcriptional level by targeting sites in mRNA, and have been shown to regulate a variety of physiological processes throughout insect development [4]. Up to date, thousands of miRNAs have been identified from insect species, including *Drosophila melanogaster* [5], *Bombyx mori* [6,7], *Locusta migratoria* [8], *Manduca sexta* [9-11], *Plutella xylostella* [12,13], *Helicoverpa armigera* [14,15], *Mayetiola destructor* [16], *Nilaparvata lugens* [17], *Blattella germanica* [18], *Aphis gossypii* [19], *Lissorhoptrus oryzophilus* [20], *Leptinotarsa decemlineata* [21], *Grapholita molesta* [22], *Atrijuglans hetaohei* [23], *Apis cerana cerana* [24], *Laodelphax striatellus* [25], and *Plodia interpunctella* [26]. More recent evidence has shown that miRNAs may play an important role in regulation of insect diapause. Reynolds et al. [27] firstly found the diapause-related changes in the abundance of genes encoding core components of the miRNA silencing pathway in the pupal diapause of *Sarcophaga bullata*, and then identified ten conserved miRNAs which were differentially expressed in diapause pupae compared to non-diapause pupae by using high-throughput sequencing and qPCR [3]. Injection of three endocrine agents, including ecdysone, diapause hormone and a diapause hormone analog all capable of breaking pupal diapause in *Helicoverpa zea*, resulted in down-regulation of miR-277-3p, a miRNA related to the insulin/FOXO signaling pathway [28]. This pathway plays an important regulatory role in insect diapause [29].

Galeruca daurica (Coleoptera: Chrysomelidae) is a new pest on the grasslands of Inner Mongolia, China. Since its sudden outbreak in 2009, this pest has continually spread and caused great losses to pasture on the Inner Mongolia grasslands [30,31]. The leaf beetle occurs one generation each year, and adults survive summer as an obligatory diapause form. Summer diapause ends about three months later in autumn, and adults start to feed again, mate and lay eggs which overwinter under cow dung,

stone and thick grass [32]. However, the regulation mechanism of summer diapause in this pest has little known until now. Chen et al. investigated the biochemical change in *G. daurica* adults during oversummering [32]. Ma et al. compared the change of proteomic levels in adults at the pre-diapause, diapause and post-diapause stages using iTRAQ, and found a large number of differentially-expressed proteins involved in metabolic process, stress response, cytoskeletal reorganization, and phagosome pathway [30]. Moreover, juvenile hormone regulation and Ca^{2+} signaling may play an important role in the regulation of summer diapause in *G. daurica*. Our present research aims to discover potential contributions of miRNAs in regulating summer diapause in *G. daurica* adults.

Results

Analysis of sRNA data from libraries

In order to obtain the microRNAs regulating summer diapause in *G. daurica* adults, 12 sequencing libraries were constructed from the adult samples at different diapause stages (PD, D, TD_1, and TD_2). The raw data have been deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive under BioProject ID PRJNA660157 (Accessions SRR12545577-SRR12545588). A total of 170.14 million raw reads were generated, ranging from 27.42 to 10.01 million reads from each library (**Table S1**). After removing 5' and 3' adapter sequences, low-quality reads, and RNAs less than 18 nucleotides and longer than 25 nucleotides, the remaining reads were searched against the Rfam database (http://rfam.janelia.org) and the Repbase database (http://www.girinst.org/repbase). Thereafter, sRNAs were classified into different categories according to their annotations as 3' adapter (ADT) and length filter, junk reads, Rfam, repeats, rRNA, tRNA, snRNA, and snoRNA sequences, and other Rfam RNA sequences (**Table S1**). Finally, the total number of valid sequences was reduced to 95.06 million (**Table S1**). The number of unique sRNAs ranged from 2.16 to 0.82 million among the libraries (**Table S2**). The length distribution of unique sRNAs showed a bimodal pattern with peaks occurring at 21-22 and 25–26 nt (**Table S2**).

In this study, a total of 230 miRNAs, including 143 conserved and 87 novel miRNAs, were identified in these 12 combined libraries of *G. daurica*(**Table S3**), and the 143 conserved miRNAs can be divided into three groups: gp2a (1), gp2b (29), and gp3 (113). The number of members within conserved miRNA families (54) was different. Among the identified families, miR-10 family contained the most members (8), followed by miR-6497 (6), miR-216 (4), and miR-46 (4) (**Table S4**). The most number of miRNAs (130) was obtained during diapause (D), secondly in the PD (103) and TD1 stages (103), and lastly in the TD2 stage (99).

Expression profiles of miRNAs

Totally, the conserved miRNAs were much more abundant than novel miRNAs in all the libraries of *G. daurica* adults, and all of the top 25 most abundant miRNAs belonged to conserved miRNAs (**Table 2**). The top five most abundant miRNAs were miR-100_R-1, miR-275_R-1, miR-8-3p, miR-184-3p_R+1, and miR-276a-3p in the PD and TD1 libraries, and miR-100_R-1, miR-275_R-1, miR-14, miR-8-3p, and miR-184-3p_R+1 were the top five most abundant miRNAs in the D and TD2 libraries.

After the 83 miRNAs with very faint expression (reads <10) were excluded, the expression profiling of remaining 147 miRNAs was analyzed. The heatmap showed the various expression patterns of miRNAs of *G. daurica* adults in different developmental stages, which could be further divided into 8 clusters (**Fig. 1, Table S5**). The numbers of miRNAs varied greatly in different clusters. The most abundant cluster D contained 39 miRNAs whereas the smallest cluster C just included four miRNAs. Interestingly, the highly expressed miRNAs were much less during diapause (D) than those in the other stages (PD, TD1, and TD2), and only cluster A were highly expressed during diapause (D), including bmo−mir−6497−p3_1ss3AG, bmo−mir−6497−p3_1ss12AG, ame−miR−3759−3p_L−4R−2_1ss5CT, bmo−miR−2779_L−1_1ss2TA, bmo−miR−6497−5p_L−3_1ss11CT, bmo−miR−2779_L−2_1ss20AG, PC−5p−117266_63, PC−3p−361832_14, bmo−mir−6497−p5_1ss4AG, bmo−miR−2779_L−1R−1_1ss2TA, and bmo−mir−6497−p5_1ss18CT. The expression abundance of cluster B was highest in the TD2 stage, middle in the PD stage, and lowest in the D and TD1 stages. The clusters C were highly expressed in the TD2 stage, secondly in the TD1 stage, and lowest in the D and PD stages. The cluster D were highly expressed only in the TD1 stage while moderately or lowly in the other stages. The cluster E displayed high expression only in the PD stage

while low expression in the other stages. The cluster F had the highest expression level in the PD stage, second in the TD2 stage, third in the TD1 stage, and lowest in the D stage. The ranking of expression abundance for the cluster G was TD1>PD>TD2>D, and PD>TD1>TD2>D for the cluster H.

Identification of differentially expressed miRNAs

In total, five miRNAs were significantly up-regulated and 20 miRNAs were significantly down-regulated in the D libraries compared with the PD libraries (**Fig. 2**). Of these miRNAs, the top three most significantly up-regulated miRNAs were ame-miR-3759-3p_L-4R-2_1ss14TC, tca-miR-279e-3p_R-4_1ss10CG, and tca-miR-34-3p_R-2_1ss18CT, while the top three most significantly down-regulated miRNAs were tca-let-7-5p_R+1, PC-3p-260638_22, and PC-5p-126401_57 (**Table 1**). Five miRNAs were significantly up-regulated and six miRNAs were significantly down-regulated in the TD1 libraries compared with the D libraries (**Fig. 2**). Of these miRNAs, the top three most significantly up-regulated miRNAs were tca-miR-2944c-5p_L-1_1ss10TC, aga-miR-2944b-5p_L-1_1ss12CT, and dpu-miR-100_R-1, while the top three most significantly down-regulated miRNAs were ame-miR-3759-3p_L-4R-2_1ss14TC, tca-miR-279e-3p_R-4_1ss10CG, and dan-miR-279_R-2 (**Table 1**). Eight miRNAs were significantly up-regulated and seven miRNAs were significantly down-regulated in the TD2 libraries compared with the D libraries (**Fig. 2**). Of these miRNAs, the top three most significantly up-regulated miRNAs were ame-miR-316-5p_R-1_1ss21CG, PC-3p-545_10300, and tca-miR-2944c-5p_L-1_1ss10TC, while the top three most significantly down-regulated miRNAs were ame-miR-3759-3p_L-4R-2_1ss14TC, tca-miR-2788-5p, and tca-miR-193-3p_R-1 (**Table 1**). Four miRNAs were significantly up-regulated and 10 miRNAs were significantly down-regulated in the TD1 libraries compared with the PD libraries (**Fig. 2**). Of these miRNAs, the top three most significantly up-regulated miRNAs were tca-miR-34-5p_R+1, bmo-miR-317-3p_L-2R+1, and ame-miR-316-5p_R-1_1ss21CG, while the top three most significantly down-regulated miRNAs were ame-miR-3759-3p_L-4R-2_1ss14TC, pca-miR-1175-5p_1ss20AT, and tca-miR-11-3p (**Table 1**). Seven miRNAs were significantly up-regulated and 19 miRNAs were significantly down-regulated in the TD2 libraries compared with the PD libraries (**Fig. 2**). Of these miRNAs, the top three most significantly up-regulated miRNAs were PC-3p-545_10300, ame-miR-316-5p_R-1_1ss21CG, and bmo-miR-317-3p_L-2R+1, while the top three most significantly down-regulated miRNAs were hme-miR-193-3p_R+1_1ss11CT, bmo-miR-9a-5p_R-2, and PC-3p-260638_22 (**Table 1**). Only one miRNA, pca-miR-980-3p_R+3, was significantly down-regulated in the TD2 libraries compared with the TD1 libraries (**Fig. 2**). These differentially expressed miRNAs might play crucial roles in the summer diapause of *G. daurica* adults.

Prediction and functional analysis of targets for differentially expressed miRNAs

A total of 22561 putative target unigenes for 46 DEMs were predicted by TargetScan 50 and Miranda 3.3a. The GO and KEGG databases were used to further functionally annotate these predicted putative target unigenes. As comparing the D and PD (D/PD), the GO analysis showed that the predicted target genes for 25 DEMs (ame-miR-3759-3p_L-4R-2_1ss14TC, tca-miR-279e-3p_R-4_1ss10CG, tca-let-7-5p_R+1, PC-3p-260638_22, PC-5p-126401_57, tca-miR-2765-3p_L+1R-1_1ss9CT, tca-miR-2944c-5p_L-1_1ss10TC, aga-miR-2944b-5p_L-1_1ss12CT, aae-miR-970_1ss20AG, tca-miR-281-5p, bmo-miR-281-5p, bmo-miR-9a-5p_R-2, dpu-miR-100_R-1, tca-miR-34-3p_R-2_1ss18CT, tca-miR-2765-5p_R+1, tca-miR-2788-3p_R+3, tca-miR-87b-3p_R-2, pca-miR-1175-5p_1ss20AT, PC-3p-145150_48, bmo-miR-2779_L-2_1ss20AG, bmo-mir-6497-p5_1ss4AG, tca-miR-87b-5p, PC-3p-32095_277, PC-3p-117645_62, and tca-miR-193-3p_R-1) were significantly enriched in the 32 GO terms (**Fig. 3A, Table S6**). For the biological process, the three most significantly enriched terms included ‘G protein-coupled receptor signaling pathway’, ‘behavioral response to ethanol’, and ‘nucleosome assembly’. For the molecular function, the three most significantly enriched terms included ‘calmodulin binding’, ‘protein heterodimerization activity’, and ‘transferase activity’. For the cellular component, the three most significantly enriched terms included ‘nucleosome’, ‘mitochondrial proton-transporting ATP synthase complex’, and ‘coupling factor F(o) neuronal cell body’. For the KEGG analysis, the 11 pathways were significantly enriched (**Fig. 4A, Table S7**). The three most significantly enriched pathways included ‘nucleotide excision repair’, ‘mTOR signaling pathway’, and ‘MAPK signaling pathway – fly’.

As comparing the TD1 and D (TD1/D), the GO analysis showed that the predicted target genes for 14 DEMs (ame-miR-3759-3p_L-4R-2_1ss14TC, pca-miR-1175-5p_1ss20AT, tca-miR-11-3p, bmo-miR-2779_L-2, bmo-miR-9a-5p_R-2, PC-3p-32095_277, tca-miR-2788-3p_R+3, tca-miR-34-5p_R+1, tca-miR-279d-3p_R-2, tca-miR-193-3p_R-1, bmo-miR-317-3p_L-2R+1, ame-miR-316-5p_R-1_1ss21CG, tca-miR-277-3p_R-3, and PC-3p-145150_48) were significantly enriched in the 56 GO terms (**Fig. 3B, Table S6**). For

the biological process, the three most significantly enriched terms included 'ATP metabolic process', 'defense response to Gram-positive bacterium', and 'protein-chromophore linkage'. For the molecular function, the three most significantly enriched terms included 'dipeptidase activity', 'proton-transporting ATPase activity, rotational mechanism', and 'binding'. For the cellular component, the three most significantly enriched terms included 'plasma membrane proton-transporting V-type ATPase complex', 'proton-transporting V-type ATPase, V1 domain', and 'collagen trimer'. For the KEGG analysis, the 12 pathways were significantly enriched (**Fig. 4B, Table S7**). The three most significantly enriched pathways included 'MAPK signaling pathway – fly', 'glycosylphosphatidylinositol', and 'endocytosis'.

Verification of differentially expressed miRNAs by qRT-PCR

To confirm the results of small RNA sequencing, ten differentially expressed miRNAs were selected and the qRT-PCR analyses of ten miRNAs were performed (**Fig. 5**). Except for miR-970, nine out of ten miRNAs showed similar expression patterns as those revealed by small RNA sequencing, indicating the reliability of our small RNA sequencing data.

Discussion

In the present study, to explore characteristics and functions of miRNAs from *G. daurica*, 12 libraries from four developmental stages of adults were constructed. In total, 95.06 million valid reads were obtained, indicating *G. daurica* is rich in small RNAs. The length distribution of our small RNA libraries displayed a bimodal pattern with peaks occurring at 21-22 and 25–26 nt, similarly to previous studies in *N. lugens* [33], *B. mori* [34], *M. destructor* [16], and *Bactrocera dorsalis* [35], which have two peaks occurring at 22 and 25–27 nt. In contrast, many other species, such as *L. migratoria* [8], *A. albopictus* and *C.quinquefasciatus* [36], *L. striatellus* [25] and *P. interpunctella* [26] have unimodal patterns of length distributions with a peak occurring at 22 nt.

Many miRNAs are differentially expressed during insect diapause, suggesting that miRNAs may play an important role in regulation of insect diapause. Batz et al. found that seven miRNAs were differentially abundant in early diapause pharate larvae compared with non-diapause larvae of *Aedes albopictus*, including miR-183-5p, bantam-5p, miR-2942-3p, miR-286b-3p, miR-282-5p, miR-14-5p, and miR-1-3p [1]. Reynolds et al. identified 10 differentially abundant miRNAs during the pupal diapause of *S. bullata*, including miR-289-5p, miR-1-3p, miR-9c-5p, miR-13b-3p, miR-31a-5p, miR-92b-3p, miR-275-3p, miR-276a-3p, miR-277-3p, and miR-305-5p [3]. In this study, 25 miRNAs were differentially expressed in diapause adults (D) compared with adults before diapause (PD) in *G. daurica* (**Table 1**). However, there was no same differentially expressed miRNAs except for miR-1-3p which was more abundant in *S. bullata* during diapause relative to non-diapause whereas downregulated in diapause pharate larvae of *A. albopictus*, suggesting independent mechanisms of miRNA regulation of diapause in these three species.

In the present study, the down-regulated differentially expressed miRNAs (20) were much more than the up-regulated differentially expressed miRNAs (5) during diapause (D/PD), which was in accord with our iTRAQ proteome data that the differentially overexpressed proteins (82) were more than the differentially underexpressed proteins (57) during diapause [30], because miRNA abundance has most often been negatively correlated with expression of its target genes. Reynolds et al. also got similar results that the majority of differentially expressed miRNAs (8) were down-regulated during diapause and only two miRNAs were up-regulated in diapausing pupae than in their non-diapausing counterparts in *S. bullata* [3].

Developmental arrest is a defining feature of diapause, and many miRNAs have been implicated in regulation of developmental processes including developmental timing (let-7) and cell cycle progression (miR-100) [37,38]. Reynolds et al. indicated that let-7 abundance increased significantly 48 h post-diapause in *S. bullata*, and let-7 and miR-100 may be important regulators of developmental arrest in diapausing pupae [3]. In this study, let-7-5p_R+1 and miR-100_R-1 were also significantly up-regulated after diapause termination (TD2/D) while they were significantly down-regulated during diapause (D/PD) (**Table 1**). Therefore, these two miRNAs might play regulatory roles in insect diapause.

In many instances, miRNAs are essential to regulate the expression of their target genes to optimal levels [39]. Therefore, it is important to determine the target genes of miRNAs in order to understand their biological functions. In our study, a lot of miRNA target unigenes were predicted from the transcriptomes of *G. daurica* adults at different diapause stages. Although the accurate targets of these miRNAs are still unknown and much more work will be needed to confirm these exact target genes, these results

could also provide us valuable information about the roles of miRNAs in the diapause regulation of *G. daurica* adults. Juvenile hormone (JH) is produced and secreted by the corpora allata in insects, and regulates various aspects of their developmental and reproductive processes [40]. The absence of JH induces adult diapause [41,42]. Hao et al. found the four differentially regulated genes involved in JH biosynthesis or degradation during summer diapause in *D. antiqua* [43]. During diapause maintenance in *A. albopictus*, three genes related to JH binding and metabolism were also differentially expressed [44]. The transcript encoding juvenile hormone epoxide hydrolase (*JHEH*) was significantly increased during adult diapause in *Coccinella septempunctata* [45]. Our proteomic analysis found that the juvenile hormone binding protein (JHBP) levels increased significantly in *G. daurica* adults developing from the PD to TD stages, whereas the juvenile hormone esterase (JHE) levels decreased [30]. qRT-PCR indicated that *GdJHBP* was expressed in various developmental stages of *G. daurica* with the highest expression in larvae and the lowest in eggs and pupae. Expression of *GdJHBP* was relatively low during summer diapause of *G. daurica* adults and was higher both before and after diapause [46]. The 150 putative target unigenes for 39 DEMs are involved in the regulation of juvenile hormone synthesis and catabolic process, such as *juvenile hormone esterase*, *juvenile hormone acid methyltransferase*, *juvenile hormone binding protein5p2*, *juvenile hormone epoxide hydrolase-like protein 3*, *putative juvenile hormone-inducible protein*, *methoprene-tolerant isoform X1*, and *Kruppel-like protein 1* (**Table S8**). The main miRNAs targeting these genes included dpu-miR-100_R-1, tca-let-7-5p_R+1, aga-miR-13b, pca-miR-1175-5p_1ss20AT, tca-miR-11-3p, tca-miR-263a-5p_R-5_1ss10GA, tca-miR-277-3p_R-3, bmo-miR-9a-5p_R-2, tca-miR-2788-5p, tca-miR-2944c-3p_R+1, tca-miR-34-5p_R+1, and PC-3p-32095_277.

Ribosomal proteins are a kind of important proteins which are involved in the functions of protein synthesis, cellular metabolism, organism immunity, signal transduction and etc [47]. Robich et al. firstly found that *RpS3a* was significantly down-regulated during diapause in *Culex pipiens* [48]. Kim et al. further found that *RpS3a* was consistently expressed in nondiapausing females of *C. pipiens*, but in females programmed for diapause, expression of the *RpS3a* transcript was dramatically reduced for a brief period in early diapause [49]. RNA interference against *RpS3a* in nondiapausing females stopped follicle development, mimicking the diapause state. Another ribosomal protein gene, *RpS2*, was also expressed continuously in nondiapausing females reared under long-day conditions, while it was strongly down-regulated 5–18 days after adult eclosion in females reared under the short-day conditions that induce diapause, and an exogenous application of JHIII could rescue the arrest in follicle growth caused by *dsRpS2* [50]. These results suggest that ribosomal proteins may play a critical role in arresting the ovarian development associated with adult diapause. In our study, 184 putative target unigenes encoding ribosomal proteins for 49 DEMs were found (**Table S9**). The miRNAs targeting *RpS2* included ame-miR-316-5p_R-1_1ss21CG, bmo-miR-9a-5p_R-2, *tca-miR-87b-3p_R-2,* and PC-3p-32095_277 while the miRNAs targeting *RpS3a* contained bmo-mir-6497-p5_1ss4AG, tca-miR-277-3p_R-3, ame-miR-316-5p_R-1_1ss21CG, bmo-miR-9a-5p_R-2, PC-5p-69765_116, and PC-3p-545_10300. Our study showed that *GdRpS3a* was expressed in different developmental stages with the highest expression levels after termination of adult diapause and the lowest during the egg stage and adult diapause of *G. daurica* (unpublished). Next, we will furtherly study the roles of these miRNAs in the regulation of summer diapause in *G. daurica* adults.

Based on the results of the KEGG enrichment analysis, the MAPK signaling pathway was the common pathway significantly enriched by the target genes for the DEMs in both D/PD and TD1/D comparisons (**Table S7**). Transcriptomic and proteomic analysis indicated that this pathway was also significantly enriched in summer diapausing *Delia antiqua* [43] and winter diapausing *L. migratoria* [51]. These results suggested that the MAPK signaling pathway might be important for insect diapause regulation. The miRNAs involved in this pathway in *G. daurica* included tca-let-7-5p_R+1, dpu-miR-100_R-1, aae-miR-970_1ss20AG, bmo-miR-281-5p, tca-miR-87b-5p, bmo-miR-9a-5p_R-2, tca-miR-279e-3p_R-4_1ss10CG, ame-miR-3759-3p_L-4R-2_1ss14TC, bmo-mir-6497-p5_1ss4AG, tca-miR-2765-5p_R+1, bmo-miR-2779_L-2_1ss20AG, tca-miR-34-3p_R-2_1ss18CT, pca-miR-1175-5p_1ss20AT, tca-miR-193-3p_R-1, PC-3p-117645_62, PC-3p-145150_48, PC-3p-260638_22, and PC-3p-32095_277.

G-protein coupled receptor could play important roles in mediating developmental arrest [52]. In this study, G-protein coupled receptor signaling pathway was another common pathway significantly enriched by the target genes for the DEMs in both D/PD and TD1/D comparisons (**Table S7**), implying its potential importance in summer diapause of *G. daurica*. The miRNAs involved in this pathway in *G. daurica* included dpu-miR-100_R-1, tca-let-7-5p_R+1, aae-miR-970_1ss20AG, bmo-miR-281-5p, tca-miR-87b-5p, tca-miR-87b-3p_R-2, tca-miR-279e-3p_R-4_1ss10CG, ame-miR-3759-3p_L-4R-2_1ss14TC, bmo-mir-6497-p5_1ss4AG, tca-miR-2765-5p_R+1, tca-miR-2944c-5p_L-1_1ss10TC, tca-miR-2788-3p_R+3, bmo-miR-2779_L-2_1ss20AG, tca-miR-193-3p_R-1, tca-miR-

34-3p_R-2_1ss18CT, bmo-miR-9a-5p_R-2, pca-miR-1175-5p_1ss20AT, aga-miR-2944b-5p_L-1_1ss12CT, tca-miR-2765-3p_L+1R-1_1ss9CT, tca-miR-281-5p, PC-3p-260638_22, PC-3p-145150_48, and PC-3p-32095_277.

Insulin signaling and its target FOXO are implicated as a key regulator of diapause by effects on metabolic suppression, fat hypertrophy, and growth control [29]. The mTOR signaling pathway, which regulates cell growth and proliferation, overlaps and interacts with insulin signaling pathway, implying that this pathway might also play roles in the regulation of diapause. A transcriptome analysis showed that the differentially expressed genes after diapause termination in *Rhagoletis pomonella* were significantly enriched in the mTOR signaling pathway [53]. Liu et al. indicated that TOR signaling functions in the reproductive diapause of *N. lugens* by regulating the expression of *NlFoxA* and *NlVitellogenin* [54]. In the present study, the target genes for the DEMs during diapause (D/PD) were also significantly enriched in this pathway. The miRNAs involved in this pathway in *G. daurica* included dpu-miR-100_R-1, tca-let-7-5p_R+1, aae-miR-970_1ss20AG, bmo-miR-281-5p, tca-miR-87b-5p, tca-miR-279e-3p_R-4_1ss10CG, ame-miR-3759-3p_L-4R-2_1ss14TC, tca-miR-281-5p, bmo-mir-6497-p5_1ss4AG, tca-miR-87b-3p_R-2, tca-miR-2944c-5p_L-1_1ss10TC, tca-miR-2788-3p_R+3, bmo-miR-2779_L-2_1ss20AG, tca-miR-34-3p_R-2_1ss18CT, tca-miR-2765-5p_R+1, bmo-miR-9a-5p_R-2, pca-miR-1175-5p_1ss20AT, aga-miR-2944b-5p_L-1_1ss12CT, tca-miR-2765-3p_L+1R-1_1ss9CT, PC-3p-117645_62, PC-3p-145150_48, PC-5p-126401_57, PC-3p-32095_277, and PC-3p-260638_22.

Ca^{2+} is a universal and multifunctional carrier of signals that regulate diverse cell processes. Many signaling components are combined in different ways, amongst which calcium-binding proteins (CaBPs) are the important parts [55]. However, it is nearly unknown whether Ca^{2+} signaling is involved in regulating diapause in animals. More recent research indicated that lots of CaBPs were differentially expressed during diapause in insects and mites, such as *H. armigera* [56, 57], *Tetranychus urticae* [58], *Bombyx mori* [59], and *Culex pipiens pallens* [60], suggesting that Ca^{2+} signaling might play a critical role in the diapause regulation of insects and mites. In this study, the 51 putative target unigenes involved in Ca^{2+} signaling for 41 DEMs were obtained (**Table S10**). Our proteomic and qRT-PCR results showed that several CaPBs (calreticulin, calmodulin, troponin, and calcyphosine-like) were differentially expressed at the protein and mRNA levels during summer diapause of *G. daurica* [30, 61]. The main miRNAs targeting these CaBPs included tca-miR-71-3p, bmo-mir-6497-p5_1ss4AG, tca-miR-2788-5p, tca-miR-2944c-3p_R+1, tca-miR-87b-3p_R-2, aga-miR-2944b-5p_L-1_1ss12CT, ame-miR-3759-3p_L-4R-2_1ss14TC, bmo-miR-317-3p_L-2R+1, pca-miR-980-3p_R+3, tca-miR-2944c-5p_L-1_1ss10TC, aae-miR-970_1ss20AG, bmo-miR-9a-5p_R-2, tca-miR-2765-5p_R+1, and PC-3p-32095_277.

The most frequently mentioned miRNAs in above pathways, which might be involved in the regulation of summer diapause in *G. daurica*, included tca-let-7-5p_R+1, dpu-miR-100_R-1, bmo-miR-9a-5p_R-2, tca-miR-87b-3p_R-2, aae-miR-970_1ss20AG, ame-miR-3759-3p_L-4R-2_1ss14TC, tca-miR-2765-5p_R+1, and PC-3p-32095_277. It is especially worthy of further studying if these miRNAs play important roles in the regulation of summer diapause in *G. daurica*.

Conclusions

A total of 230 miRNAs was identified from *G. daurica*, and the expression analysis results demonstrated that the transcript levels of these miRNAs were changed in different developmental stages of *G. daurica* adults. The results of target prediction suggest that miRNAs may be involved in the regulation of summer diapause of *G. daurica*, especially via the juvenile hormone, ribosome, MAPK signaling, mTOR signaling, Ca^{2+} signaling and G-protein coupled receptor signaling pathways, and these results provide an important new small RNA genomics resource for further studies on insect diapause.

Methods

Insect rearing, sampling, and sample preparation

Eggs were collected from the Xilinguole grasslands of Inner Mongolia, China, on April 20, 2019. Egg collection was not necessary to be permitted. Eggs were incubated (RH: 70 ± 5%; temperature: 25 ± 1°C) until hatching. Larvae were transferred to natural conditions, and reared on *Allium mongolium*. As Ma et al. described [30], sampling was conducted at 3, 40, 90, and 100 days after adult emergence to obtain adult samples at the pre-diapause (PD, before diapause), diapause (D, during diapause),

post-diapause (TD1, diapause terminated), and post-diapause (TD2, diapause terminated and the female abdomen expanded, indicating that the ovary began to develop) stages. Each sample (10 females and 10 males) for each treatment (PD, D, TD1 and TD2) with three replicates was ground in liquid nitrogen, and stored at -80°C until sRNA-seq and quantitative real-time PCR (qRT-PCR) was performed.

Small RNA isolation, cDNA library construction and deep sequencing

Total RNA of each treatment was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. The quantity of the total RNA was accessed by NanoDrop™ 2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA), and the integrity of the RNA samples were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and only values of 28S/18S ≥ 0.7 and RIN ≥ 7.0 were considered qualified for the subsequent small RNA library construction. The total RNA samples were size-fractionated, and 10–30 nt long small RNAs were isolated through gel separation. These small RNAs were purified and then ligated with 3' and 5' adapters. The fragments with adaptors on both ends were enriched by PCR after reverse transcription. After purification, the PCR amplification products were sequenced by the Lc-Bio Technologies Company, Ltd. (Hangzhou, China) using the Illumina Hiseq 2500 platform.

Sequence analysis, miRNAs identification, and data processing

Raw reads were subjected to an in-house program, ACGT101-miR (LC Sciences, Houston, Texas, USA) to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, unique sequences with length in 18-26 nucleotide were compared with known insect miRNAs in miRBase 22.0 by BLAST search to identify known miRNAs and novel 3p- and 5p- derived miRNAs. Length variation at both 3' and 5' ends and one mismatch inside of the sequence were allowed in the alignment. The unmapped sequences were BLASTed against the *G. daurica* transcriptome, and the hairpin RNA structures containing sequences were predicated from the flank 80 nt sequences using RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The criteria for secondary structure prediction were: (1) number of nucleotides in one bulge in stem (≤12); (2) number of base pairs in the stem region of the predicted hairpin (≥16); (3) cutoff of free energy (kCal/mol ≤-15); (4) length of hairpin (up and down stems + terminal loop ≥50); (5) length of hairpin loop (≤20); (6) number of nucleotides in one bulge in mature region (≤8); (7) number of biased errors in one bulge in mature region (≤4); (8) number of biased bulges in mature region (≤2); (9) number of errors in mature region (≤7); (10) number of base pairs in the mature region of the predicted hairpin (≥12); (11) percent of mature in stem (≥80).

Data normalization followed the procedures with minor modification [62]. (1) Find a common set of sequences among all samples; (2) Construct a reference data set. Each data in the reference set is the copy number median value of a corresponding common sequence of all samples; (3) Perform 2-based logarithm transformation on copy numbers (Log2 (copy#)) of all samples and reference data set; (4) Calculate the Log2 (copy#) difference (ΔLog2 (copy#)) between individual sample and the reference data set; (5) Form a subset of sequences by selecting |ΔLog2 (copy#)| < 2, which means less than 4 (2^2) fold change from the reference set; (6) Perform linear regressions between individual samples and the reference set on the subset sequences to derive linear equations $y = a_i x + b_i$, where a_i and b_i are the slop and interception, respectively, of the derived line, x is Log2 (copy#) of the reference set, and y is the expected Log2 (copy#) of sample i on a corresponding sequence; (7) Calculate the mid value x_{mid} = (max(x)-min(x))/2 of the reference set. Calculate the corresponding expected Log2 (copy#) of sample i, $y_{i,mid} = a_i x_{mid} + b_i$. Let $y_{r,mid} = x_{mid}$, let $\Delta y_i = y_{r,mid} - y_{i,mid}$, which is the logarithmic correction factor of sample i. We then derive the arithmetic correction factor $f_i = 2^{\Delta y_i}$ of sample i; (8) Correct copy numbers of individual samples by multiplying corresponding arithmetic correction factor to original copy numbers.

Analysis of differentially expressed miRNAs

Differential expression of miRNAs based on normalized deep-sequencing counts at different diapause stages was analyzed by using Student t-test. The significance threshold was set to be 0.05 in each test. miRNAs with *P*-value<0.05 between different stages (D vs PD, TD_1 vs D, TD_2 vs D, TD_1 vs PD, TD_2 vs PD, and TD_2 vs TD1) were considered as differentially expressed miRNAs (DEMs).

Prediction of target genes of miRNAs

The miRNA sequences were aligned against the *G. daurica* transcriptome database assembled in our lab (Accession number: SRP147970) to identify possible target sites by using the TargetScan 50 and Miranda 3.3a software packages [63]. The default parameters of miRanda were set score and energy threshold of 140 and −10 kcal/mol, respectively, which demanded strict 5'seed region (nucleotide 2-8 from the 5'end of miRNA sequence) pairing as well. The default parameters of TargetScan were that the 2-8 nt sequences of 5' miRNA were selected as seed region to predict with 3'sequence of transcripts. Finally, the target genes simultaneously recommended by the two algorithms were defined as the final prediction.

Functional analysis of target genes

To better understand the function of the target genes and their corresponding metabolic network at different diapause stages of *G. daurica* adults, annotations of target gene functions were performed using the Gene Ontology (GO, http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) pathway databases. The most abundant DEM targets were annotated based on sequence similarity by performing a BLASTx search against the GO protein database. Furthermore, target genes were categorized according to their functions under biological processes, molecular functions, and cellular components using GO analysis. The enriched metabolic pathways or signal transduction pathways of potential miRNA target genes were validated using KEGG enrichment analysis to enrich the KEGG terms. Fisher's exact test was employed to filter the significant GO terms and KEGG pathways using R software 3.3.1., and $P < 0.05$ was considered statistically significant.

Validation of miRNA expression profiles

The RNA samples for small RNA library were also used for qRT-PCR to confirm the reliability of the sequencing results. For the analysis of miRNAs, cDNA was synthesized using the Mir-X miRNA First-Strand Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The qRT-PCRs were performed on FTC-3000 (Funglyn Biotech, Canada) using the GoTaq® qPCR Master Mix (2×) (Promega, USA). The reaction profile was as follow: starting with a 10 min incubation at 95℃ to activate the hot-start polymerase followed by 40 cycles at 95 ℃ for 15 s and 60℃ for 1 min, and then at 95℃ for 15 s, at 60℃ for 15 s and at 95℃ for 15 s. We performed three biological replicates (4 females and 4 males for each replicate) and four technical replications of each reaction, and U6 snRNA was used as an internal control gene for qRT-PCR of miRNAs. The relative expression values of the randomly chosen DEMs were calculated based on the $2^{-\Delta\Delta Ct}$ method [64]. The primers were designed by using the Primer Premier 5.0 (http://www.premierbiosoft.com/primerdesign/index.html) (**Table S11**).

Abbreviations

3ADT: 3'Adaptor; BLAST: Basic Local Alignment Search Tool; cDNA: complementary DNA; D: Diapause; DEM: Differentially expressed miRNA; DNA: Deoxyribonucleic acid; GO: Gene ontology; iTRAQ: Isobaric tags for relative and absolute quantification; FOXO: Forkhead box; JH: Juvenile hormone; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPK: mitogen-activated protein kinase; miRNA: MicroRNA; mTOR: Mammalian target of rapamycin; NCBI: National Center for Biotechnology Information; PD: Pre-diapause; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; RNA: Ribonucleic acid; Rfam: RNA family database; RP: Ribosomal protein; rRNA: Ribosomal RNA; snRNA: Small nuclear RNA; snoRNA: Small nucleolar RNA; sRNA: Small RNA; TD: Post-diapause; tRNA: Transfer RNA.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The sequence data generated during this study is deposited on the NCBI Sequence Read Archive, under BioProject ID PRJNA660157 (Accessions SRR12545577-SRR12545588). Other related data are available within the manuscript and its additional files.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was funded by the National Natural Science Foundation of China (No. 31760517). The funding body played no role in the design of the study and collection, analysis and interpretation of the data and in writing the manuscript.

Authors' contributions

TFD and BPP designed the research, analyzed the data and wrote the paper. TFD performed the experiments. LL, YT and YYL participated in the data analysis and discussion. All authors read and approved the final manuscript.

Acknowledgements

We thank the three anonymous reviewers for their valuable suggestions and comments. In addition, we thank the editor of BMC Genomics for improving the writing.

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References

1. Batz ZA, Goff AC, Armbruster PA. MicroRNAs are differentially abundant during *Aedes albopictus* diapause maintenance but not diapause induction. Insect Mol Biol. 2017; 26(6): 721–733.
2. Denlinger DL. Why study diapause?. Entomol Res. 2008; 38:1–9.
3. Reynolds JA, Peyton JT, Denlinger DL. Changes in microRNA abundance may regulate diapause in the flesh fly, *Sarcophaga bullata*. Insect Biochem Mol Biol. 2017; 84:1-14.
4. Lucas KJ**,** Zhao B, Liud S, Raikhe AS. Regulation of physiological processes by microRNAs in insects. Curr Opin Insect Sci. 2015; 11: 1–7.
5. Aravin AA, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B, Gaasterland T, Meyer J, Tuschl T. The small RNA profile during *Drosophila melanogaster* Dev Cell. 2003; 5: 337–50
6. Yu XM, Zhou Q, Li SC, Luo QB, Cai YM, Lin WC, Chen H, Yang Y, Hu SN, Yu J. The silkworm (*Bombyx mori*) microRNAs and their expressions in multiple developmental stages. PLoS ONE. 2008; 3: e2997.
7. Cao J, Tong C, Wu X, Lv J, Yang Z, Jin Y. Identification of conserved microRNAs in Bombyx mori (silkworm) and regulation of fibroin L chain production by microRNAs in heterologous system. Insect Biochem Mol Biol. 2008; 38: 1066–71.
8. Wei Y, Chen S, Yang P, Ma Z, Kang L. Characterization and comparative profiling of the small RNA transcriptomes in two phases of locust. Genome Biol. 2009; 10: R6
9. Zhang X, Zheng Y, Cao X, Ren R, Yu XQ, Jiang H. Identification and profiling of *Manduca sexta* microRNAs and their possible roles in regulating specific transcripts in fat body, hemocytes, and midgut. Insect Biochem Mol Biol. 2015; 62: 11–22.
10. Zhang X, Zheng Y, Jagadeeswaran G, Ren R, Sunkar R, Jiang H. Identification of conserved and novel microRNAs in *Manduca sexta* and their possible roles in the expression regulation of immunity-related genes. Insect Biochem Mol Biol.

2014; 47: 12–22.

11. Zhang X, Zheng Y, Jagadeeswaran G, Ren R, Sunkar R, Jiang H. Identification and developmental profiling of conserved and novel microRNAs in *Manduca sexta*. Insect Biochem Mol Biol. 2012; 42: 381–95.
12. Liang P, Feng B, Zhou XG, Gao XW. Identification and developmental profiling of microRNAs in diamondback moth, *Plutella xylostella* (L.). PLoS ONE. 2013; 8: e78787.
13. Etebari K, Hussain M, Asgari S. Identification of microRNAs from *Plutella xylostella* larvae associated with parasitization by *Diadegma semiclausum*. Insect Biochem Mol Biol. 2013; 43: 309–18.
14. Agrawal N, Sachdev B, Rodrigues J, Sree KS, Bhatnagar RK. Development associated profiling of chitinase and microRNA of *Helicoverpa armigera* identified chitinase repressive microRNA. Sci Rep. 2013; 3:2292.
15. Lomate PR, Mahajan NS, Kale SM, Gupta VS, Giri AP. Identification and expression profiling of *Helicoverpa armigera* microRNAs and their possible role in the regulation of digestive protease genes. Insect Biochem Mol Biol. 2014; 54:129–37.
16. Khajuria C, Williams CE, Bouhssini MEl, Whitworth RJ, Richards S, Stuart JJ, Chen MS. Deep sequencing and genome-wide analysis reveals the expansion of MicroRNA genes in the gall midge *Mayetiola destructor*. BMC Genomics. 2013; 14:187.
17. Zha W, Zhou L, Li S, Liu K, Yang G, Chen Z, Liu K, Xu H, Li P, Hussain S, You A. Characterization and comparative profiling of the small RNA transcriptomes in the Hemipteran insect *Nilaparvata lugens*. Gene. 2016; 595: 83–91.
18. Ylla G, Piulachs MD, Belles X. Comparative analysis of miRNA expression during the development of insects of different metamorphosis modes and germband types. BMC Genomics. 2017; 18:774.
19. Ma KS, Li F, Liu Y, Liang PZ, Chen XW, Gao XW. Identification of microRNAs and their response to the stress of plant allelochemicals in *Aphis gossypii* (Hemiptera: Aphididae). BMC Mol Biol. 2017; 18: 5.
20. Yang S, Zhang J, Wang S, Zhang X, Liu Y, Xi J. Identification and profiling of miRNAs in overwintering *Lissorhoptrus oryzophilus* via next-generation sequencing. Cryobiol. 2017; 74: 68-76.
21. Mathieu D. Morin, Pierre J. Lyons, Nicolas Crapoulet, Sébastien Boquel,and Pier Jr Morin. Identification of differentially expressed miRNAs in Colorado potato beetles (*Leptinotarsa decemlineata* (Say)) exposed to imidacloprid. Int J Mol Sci. 2017; 18: 2728.
22. Wang X, Li Y, Zhang J, Zhang Q, Liu X, Li Z. De novo characterization of microRNAs in oriental fruit moth *Grapholita molesta* and selection of reference genes for normalization of microRNA expression. PLoS ONE. 2017; 12(2): e0171120.
23. Zhang Z, Li T, Tang G. Identification and characterization of conserved and novel miRNAs in different development stages of *Atrijuglans hetaohei* Yang (Lepidoptera: Gelechioidea). J Asia-Pacific Entomol. 2018; 21: 9-18.
24. Chen D, Du Y, Chen H, Fan Y, Fan X, Zhu Z, Wang J, Xiong C, Zheng Y, Hou C, Diao Q, Guo R. Comparative identification of microRNAs in *Apis cerana cerana* workers' midguts in response to *Nosema ceranae* Insects. 2019; 10: 258.
25. Liu L, Zhang K-J, Rong X, Li Y-Y, Liu H. Identification of wolbachia-responsive miRNAs in the small brown planthopper, *Laodelphax striatellus*. Front. Physiol. 2019; 10: 928.
26. Chen EH, Tao YX, Song W, Shen F, Yuan ML, Tang PA. Transcriptome-wide identification of microRNAs and analysis of their potential roles in development of Indian meal moth (Lepidoptera: Pyralidae). J Econ Entomol. 2020; 113(3): 1535–1546.
27. Reynolds JA, Clark J, Diakoff SJ, Denlinger DL. Transcriptional evidence for small RNA regulation of pupal diapause in the flesh fly, *Sarcophaga bullata*. Insect Biochem Mol Biol. 2013; 43: 982-989.
28. Reynolds JA, Nachman RJ, Denlinger DL. Distinct microRNA and mRNA responses elicited by ecdysone, diapause hormone and a diapause hormone analog at diapause termination in pupae of the corn earworm, *Helicoverpa zea*. General Comp Endocrinol. 2019; 278: 68-78.
29. Sim C, Denlinger DL. Insulin signaling and the regulation of insect diapause. Front Physiol. 2013; 4: e00189.
30. Ma HY, Zhou XR, Tan Y, Pang BP. Proteomic analysis of adult *Galeruca daurica* (Coleoptera: Chrysomelidae) at different stages during summer diapause. Comp Biochem Physiol D. 2019; 29:351-357.
31. Zhou XR, Shan YM, TanY, Zhang ZR, Pang BP. Comparative analysis of transcriptome responses to cold stress in *Galeruca daurica* (Coleoptera: Chrysomelidae). J Insect Sci. 2019; 19(6): 8; 1–6.

32. Chen L, Zhou XR, Gao LJ, Tan Y, Pang BP. Change of carbohydrate, protein and lipid contents in *Galeruca daurica* (Coleoptera: Chrysomelidae) adults during oversummering. Acta Entomol Sin. 2018; 61(7): 808–814.
33. Chen Q, Lu L, Hua H, Zhou F, Lu L, Lin Y. Characterization and comparative analysis of small RNAs in three small RNA libraries of the brown planthopper (*Nilaparvata lugens*). PLoS One. 2012; 7: e32860.
34. Jagadeeswaran G, Zheng Y, Sumathipala N, Jiang H, Arrese EL, Soulages JL, Zhang W, Sunkar R. Deep sequencing of small RNA libraries reveals dynamic regulation of conserved and novel microRNAs and microRNA-stars during silkworm development. BMC Genomics. 2010; 11: 52.
35. Huang Y, Dou W, Liu B, Wei D, Liao CY, Smagghe G, Wang JJ. Deep sequencing of small RNA libraries reveals dynamic expression patterns of microRNAs in multiple developmental stages of *Bactrocera dorsalis.* Insect Mol Biol. 2014; 23: 656–667.
36. Skalsky RL, Vanlandingham DL, Scholle F, Higgs S, Cullen BR. Identification of microRNAs expressed in two mosquito vectors, *Aedes albopictus* and *Culex quinquefasciatus*. BMC Genomics. 2010; 11: 119.
37. Sempere LF, Sokol NS, Dubrovsky EB, Berger EM, Ambros V. Temporal regulation of miRNA expression in *Drosophila melanogaster* mediated by hormonal signals and broad-Complex gene activity. Dev Biol. 2003; 259: 9e18.
38. Caygill EE, Johnston LA. Temporal regulation of metamorphic processes in Drosophila by the let-7 and miR-125 heterochronic miRNAs. Curr Biol. 2008; 18: 943e950.
39. Bartel DP, Chen CZ. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat Rev Genet. 2004; 5: 396–400.
40. Qu Z, Bendena WG, Tobe SS, Hui JHL. Juvenile hormone and sesquiterpenoids in arthropods: Biosynthesis, signaling, and role of MicroRNA. J Steroid Biochem Mol Biol. 2018; 184: 69-76.
41. Denlinger DL. Regulation of diapause. Annu Rev Entomol. 2002; 47: 93–1.
42. Kang DS, Cotten MA, Denlinger DL, Sim C. Comparative transcriptomics reveals key gene expression differences between diapausing and non-diapausing adults of *Culex pipiens*. PLoS ONE. 2016; 11(4): e0154892.
43. Hao YJ, Zhang YJ, Si FL, Fu DY, He ZB, Chen B. Insight into the possible mechanism of the summer diapause of *Delia antiqua* (Diptera: Anthomyiidae) through digital gene expression analysis. Insect Sci. 2016; 23: 438–451.
44. Poelchau MF, Reynolds JA, Elsik CG, Denlinger DL, Armbruster PA. RNA-Seq reveals early distinctions and late convergence of gene expression between diapause and quiescence in the Asian tiger mosquito, *Aedes albopictus*. J Exp Biol. 2013; 216: 4082–4090.
45. Qi X, Zhang L, Han Y, Ren X, Huang J, Chen H. *De novo* transcriptome sequencing and analysis of *Coccinella septempunctata* in non-diapause, diapause and diapause-terminated states to identify diapause-associated genes. BMC Genomics. 2015; 16:1086.
46. Chen L, Zhou XR, Tan Y, Pang BP, Xinbayin. Cloning and expression profiling of the *Galeruca daurica* juvenile hormone binding protein gene, *GdJHBP*. Chin J Appl Entomol. 2020; 57(3): 623-631.
47. Warner JR, Mclntosh KB. How common are extraribosomal functions of ribosomal proteins?. Molecular Cell. 2009; 34(1): 3–11.
48. Robich RM, Rinehart JP, Kitchen LJ, Denlinger DL. Diapause-specific gene expression in the northern house mosquito, *Culex pipiens*, identified by suppressive subtractive hybridization. J Insect Physiol. 2007; 53(3): 235–245.
49. Kim M, Denlinger DL. A potential role for ribosomal protein S2 in the gene network regulating reproductive diapause in the mosquito *Culex pipiens*. J Comp Physiol. 2010; 180(2): 171–178.
50. Kim M, Sim C, Denlinger DL. RNA interference directed against ribosomal protein S3a suggests a link between this gene and arrested ovarian development during adult diapause in *Culex pipiens*. Insect Mol Biol. 2010; 19(1): 27–33.
51. Tu X, Wang J, Hao K, Whitman DW, Fan Y, Cao G, Zhang Z. Transcriptomic and proteomic analysis of pre-diapause and nondiapause eggs of migratory locust, *Locusta migratoria* (Orthoptera: Acridoidea). Sci Rep. 2015; 5:11402.
52. Poelchau MF, Reynolds JA, Denlinger DL, Elsik CG, Armbruster PA. A de novo transcriptome of the Asian tiger mosquito, *Aedes albopictus*, to identify candidate transcripts for diapause preparation. BMC Genomics. 2011; 12: 619.

53. Ragland GJ, Egan SP, Feder JL, Berlocher SH, Hahn DA. Developmental trajectories of gene expression reveal candidates for diapause termination: a key life-history transition in the apple maggot fly *Rhagoletis pomonella*. J Exp Biol. 2011; 214: 3948–3959.
54. Liu F, Li K, Cai W, Zhao J, Zou Y, Hua H. Knockdown of TOR causing ovarian diapause in a genetically stable brachypterous strain of *Nilaparvata lugens*. Arch. Insect Biochem Physiol. 2017; 95: e21400.
55. Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol. 2003; 4: 517–529.
56. Lu YX, Xu WH. Phosphoproteomic analysis at diapause initiation in the cotton bollworm, *Helicoverpa armigera*. J Proteome Res. 2010; 9(10): 5053-5064.
57. Zhang Q, Lu YX, Xu WH. Integrated proteomic and metabolomic analysis of larval brain associated with diapause induction and preparation in the cotton bollworm, *Helicoverpa armigera*. J Proteome Res. 2012; 11(2): 1042-1053.
58. Zhao JY, Zhao XT, Sun JT, Zou LF, Yang SX, Han X, Zhu WC, Yin Q, Hong XY. Transcriptome and proteome analyses reveal complex mechanisms of reproductive diapause in the two-spotted spider mite, *Tetranychus urticae*. Insect Mol Biol. 2017; 26(2): 215-232.
59. Hsieh HY, Gu SH. Expression of calcineurin in relation to the embryonic diapause process in the silkworm, *Bombyx mori*. Comp Biochem Physiol A. 2019; 228(2): 35-42.
60. Zhang CX, Wei DD, Shi GH, Huang XL, Cheng P, Liu GX, Guo XX, L LJ, Wang HW, Miao F. Understanding the regulation of overwintering diapause molecular mechanisms in *Culex pipiens pallens* through comparative proteomics. Sci Rep. 2019; 9(1): 6485.
61. Li S, Li L, Zhou XR, Pang BP, Shan YM. Identification and expression profiling of calcium-binding protein genes in *Galeruca daurica* (Coleoptera: Chrysomelidae). Acta Entomol Sin. 2020; .
62. Cer RZ, Herrera-Galeano JE, Anderson JJ, Bishop-Lilly KA, Mokashi VP. miRNA Temporal Analyzer (miRNATA): A bioinformatics tool for identifying differentially expressed microRNAs in temporal studies using normal quantile transformation. Gigascience. 2014; 3: 20.
63. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. Nucleic Acids Res. 2008; 36: D149–D153.
64. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Methods. 2001; 25: 402–408.

Tables

Table 1 Significantly differentially expressed miRNAs at different diapause stages of *G. daurica* adults

miRNA name	Log2(Fold change)	*P*-value	up/down	miRNA name	Log2(Fold change)	*P*-value	up/down
D vs PD				**TD1 vs PD**			
ame-miR-3759-3p_L-4R-2_1ss14TC	2.01	6.48E-05	up	tca-miR-34-5p_R+1	1.87	1.64E-02	up
tca-miR-279e-3p_R-4_1ss10CG	0.79	1.41E-03	up	bmo-miR-317-3p_L-2R+1	1.64	3.52E-02	up
tca-miR-34-3p_R-2_1ss18CT	1.65	2.06E-02	up	ame-miR-316-5p_R-1_1ss21CG	2.16	4.41E-02	up
bmo-miR-2779_L-2_1ss20AG	Inf.	2.61E-02	up	tca-miR-277-3p_R-3	3.53	4.45E-02	up
bmo-mir-6497-p5_1ss4AG	3.24	2.92E-02	up	ame-miR-3759-3p_L-4R-2_1ss14TC	-3.85	3.68E-03	down
tca-let-7-5p_R+1	-1.13	4.54E-03	down	pca-miR-1175-5p_1ss20AT	-3.84	7.45E-03	down
PC-3p-260638_22	-inf	5.97E-03	down	tca-miR-11-3p	-0.51	8.38E-03	down
PC-5p-126401_57	-inf	6.61E-03	down	bmo-miR-2779_L-2	-1.09	9.81E-03	down
tca-miR-2765-3p_L+1R-1_1ss9CT	-inf	6.67E-03	down	bmo-miR-9a-5p_R-2	-1.43	1.07E-02	down
tca-miR-2944c-5p_L-1_1ss10TC	-1.26	9.20E-03	down	PC-3p-32095_277	-3.11	1.31E-02	down
aga-miR-2944b-5p_L-1_1ss12CT	-1.26	9.20E-03	down	tca-miR-2788-3p_R+3	-2.17	1.61E-02	down
aae-miR-970_1ss20AG	-0.68	1.07E-02	down	tca-miR-279d-3p_R-2	-1.28	1.79E-02	down
tca-miR-281-5p	-1.88	1.17E-02	down	tca-miR-193-3p_R-1	-2.52	3.11E-02	down
bmo-miR-281-5p	-1.88	1.17E-02	down	PC-3p-145150_48	-2.23	4.59E-02	down
bmo-miR-9a-5p_R-2	-1.21	1.38E-02	down	**TD2 vs PD**			
dpu-miR-100_R-1	-0.69	1.42E-02	down	PC-3p-545_10300	1.89	2.95E-03	up
tca-miR-2765-5p_R+1	-1.87	2.08E-02	down	ame-miR-316-5p_R-1_1ss21CG	2.69	7.36E-03	up
tca-miR-2788-3p_R+3	-inf	2.12E-02	down	bmo-miR-317-3p_L-2R+1	1.50	1.13E-02	up
tca-miR-87b-3p_R-2	-0.84	2.21E-02	down	tca-miR-34-5p_R+1	1.85	3.21E-02	up
pca-miR-1175-5p_1ss20AT	-1.90	2.37E-02	down	tca-miR-2944c-3p_R+1	0.33	3.64E-02	up
PC-3p-145150_48	-inf	2.42E-02	down	tca-miR-34-3p_R-2_1ss18CT	1.83	4.51E-02	up
tca-miR-87b-5p	-inf	2.97E-02	down	tca-miR-263a-5p_R-5_1ss10GA	0.71	4.75E-02	up

PC-3p-32095_277	-1.97	3.04E-02	down	hme-miR-193-3p_R+1_1ss11CT	-inf	1.17E-03	down
PC-3p-117645_62	-inf	3.32E-02	down	bmo-miR-9a-5p_R-2	-1.86	5.49E-03	down
tca-miR-193-3p_R-1	-1.87	4.61E-02	down	PC-3p-260638_22	-inf	5.97E-03	down

Table 1 Continued

miR_name	Log2(Fold change)	*P*-value	up/down	miR_name	Log2(Fold change)	*P*-value	up/down
TD2 vs PD				bmo-miR-2779_L-2_1ss20AG	-3.32	1.50E-02	down
PC-5p-126401_57	-inf	6.61E-03	down	tca-miR-279d-3p_R-2	-1.21	3.31E-02	down
tca-let-7-5p_R+1	-0.74	8.95E-03	down	bmo-miR-6497-p5_1ss4AG	-2.29	3.78E-02	down
tca-miR-2765-3p_L+1R-1_1ss9CT	-2.90	9.02E-03	down	**TD2 vs D**			
tca-miR-71-3p	-0.54	9.48E-03	down	ame-miR-316-5p_R-1_1ss21CG	1.75	2.72E-03	up
tca-miR-2788-3p_R+3	-3.74	1.18E-02	down	PC-3p-545_10300	1.54	6.65E-03	up
pca-miR-1175-5p_1ss20AT	-2.20	1.20E-02	down	tca-miR-2944c-5p_L-1_1ss10TC	1.31	7.92E-03	up
tca-miR-11-3p	-0.35	1.25E-02	down	aga-miR-2944b-5p_L-1_1ss12CT	1.31	7.92E-03	up
ame-miR-2-3p_L-2R-1	-0.36	1.40E-02	down	tca-miR-87b-3p_R-2	1.06	1.53E-02	up
dme-miR-2a-3p_L-2R-1	-0.36	1.40E-02	down	tca-let-7-5p_R+1	0.38	2.00E-02	up
ame-miR-3759-3p_L-4R-2_1ss14TC	-2.01	1.50E-02	down	tca-miR-34-5p_R+1	1.18	3.81E-02	up
bmo-miR-2779_L-2	-0.96	1.55E-02	down	PC-3p-215_25171	1.30	4.91E-02	up
tca-miR-2765-5p_R+1	-1.78	2.06E-02	down	ame-miR-3759-3p_L-4R-2_1ss14TC	-4.02	3.98E-05	down
bmo-miR-2738_L-6_1ss24GA	-2.71	2.35E-02	down	tca-miR-2788-5p	-3.01	6.26E-03	down
tca-miR-193-3p_R-1	-3.65	3.18E-02	down	tca-miR-193-3p_R-1	-1.78	1.64E-02	down
PC-3p-145150_48	-2.23	4.55E-02	down	dan-miR-279_R-2	-1.04	1.90E-02	down
aga-miR-13b	-0.40	4.98E-02	down	bmo-miR-2779_L-2_1ss20AG	-3.04	2.27E-02	down
TD1 vs D				bmo-miR-6497-p5_1ss4AG	-2.62	3.64E-02	down
tca-miR-2944c-5p_L-1_1ss10TC	1.01	8.35E-04	up	PC-5p-69765_116	-1.97	4.56E-02	down
aga-miR-2944b-5p_L-1_1ss12CT	1.01	8.35E-04	up	**TD2 vs TD1**			
dpu-miR-100_R-1	0.66	1.41E-02	up	pca-miR-980-3p_R+3	-1.76	4.07E-02	down
tca-miR-34-5p_R+1	1.21	1.57E-02	up				
aae-miR-970_1ss20AG	1.03	1.84E-02	up				

ame-miR-3759-3p_L-4R-2_1ss14TC	-5.85	8.37E-05	down
tca-miR-279e-3p_R-4_1ss10CG	-0.77	1.80E-03	down
dan-miR-279_R-2	-0.99	1.49E-02	down

Table 2 The 25 most abundant miRNAs at different diapause stages of *G. daurica* adults

PD		D		TD1		TD2	
miRNA	Normalized data	miRNA	Normalized data	miRNA	Normalized data	miRNA	Normalized data
dpu-miR-100_R-1	178334.58	dpu-miR-100_R-1	110280.17	dpu-miR-100_R-1	174276.05	dpu-miR-100_R-1	182826.90
aga-miR-275_R-1	95763.85	aga-miR-275_R-1	100431.49	aga-miR-275_R-1	106634.46	aga-miR-275_R-1	116321.74
tca-miR-8-3p	81671.29	api-miR-14	93980.52	tca-miR-184-3p_R+1	77969.305	api-miR-14	82736.69
tca-miR-184-3p_R+1	63233.84	tca-miR-8-3p	63721.24	tca-miR-8-3p	64862.33	tca-miR-184-3p_R+1	58636.25
dgr-miR-276a-3p	56331.53	tca-miR-184-3p_R+1	61206.86	dgr-miR-276a-3p	59915.80	tca-miR-8-3p	52918.58
isc-miR-1	35725.48	dgr-miR-276a-3p	54648.54	isc-miR-1	43402.86	dgr-miR-276a-3p	51931.88
tca-miR-281-5p	32180.79	isc-miR-1	26166.29	api-miR-14	35051.53	isc-miR-1	34524.01
pxy-miR-281_L+1R-1	22748.19	bmo-miR-317-3p_L-2R+1	22061.56	tca-miR-34-5p_R+1	32141.02	tca-miR-34-5p_R+1	31519.45
api-miR-14	20495.37	bmo-mir-6497-p5_1ss18CT	16210.87	bmo-miR-317-3p_L-2R+1	24176.98	tca-miR-281-5p	27408.18
ame-miR-31a-5p_L+1R-2	14189.98	tca-miR-34-5p_R+1	13940.44	tca-miR-281-5p	19916.64	bmo-miR-317-3p_L-2R+1	21946.14
tca-let-7-5p_R+1	10954.05	pxy-miR-281_L+1R-1	12935.58	dpe-miR-133	14985.35	pxy-miR-281_L+1R-1	18552.71
aga-miR-13b	10350.55	aga-miR-13b	10548.92	pxy-miR-281_L+1R-1	12800.76	dpe-miR-133	10380.39
tca-miR-34-5p_R+1	8769.39	ame-miR-31a-5p_L+1R-2	9626.63	ame-miR-31a-5p_L+1R-2	9163.79	ame-miR-31a-5p_L+1R-2	9267.73
bmo-miR-317-3p_L-2R+1	7783.65	dpe-miR-133	8974.19	aga-miR-13b	9043.85	tca-miR-2944c-3p_R+1	8067.77
dpe-miR-133	7445.10	tca-miR-281-5p	8728.86	aae-miR-277-3p_R+1	8166.42	aga-miR-13b	7869.42
tca-miR-2944c-3p_R+1	6413.05	tca-miR-2944c-3p_R+1	7070.97	tca-miR-2944c-3p_R+1	7142.57	bmo-miR-263a-5p_1ss10GA	6959.88
ame-miR-2-3p_L-2R-1	6333.79	ame-miR-2-3p_L-2R-1	5226.84	tca-let-7-5p_R+1	6280.56	dqu-miR-263b-5p_R-1	6587.59
tca-miR-8-5p	6319.75	dme-miR-2a-3p_L-2R-1	5226.84	dqu-miR-263b-5p_R-1	5786.57	tca-let-7-5p_R+1	6545.55
bmo-miR-263a-5p_1ss10GA	5565.65	tca-let-7-5p_R+1	5017.57	bmo-miR-263a-5p_1ss10GA	5479.39	aae-miR-277-3p_R+1	5947.40
dan-miR-279_R-2	3403.14	dan-miR-279_R-2	4794.73	tca-miR-8-5p	4954.64	tca-miR-8-5p	5396.51

dqu-miR-263b-5p_R-1	3060.72	dqu-miR-263b-5p_R-1	4446.22	ame-miR-2-3p_L-2R-1	4909.74	ame-miR-2-3p_L-2R-1	4926.21
aae-miR-970_1ss20AG	3040.75	tca-miR-8-5p	4126.41	aae-miR-970_1ss20AG	3858.76	dme-miR-2a-3p_L-2R-1	4926.21
pca-miR-1175-3p_1ss24TG	2949.05	bmo-mir-6497-p5_1ss4AG	3996.89	ame-miR-283-5p_R+2	3368.70	aae-miR-970_1ss20AG	3947.68
aae-miR-277-3p_R+1	2736.32	bmo-miR-263a-5p_1ss10GA	3766.96	tca-miR-2796-3p	3297.99	ame-miR-283-5p_R+2	3892.11

Figures

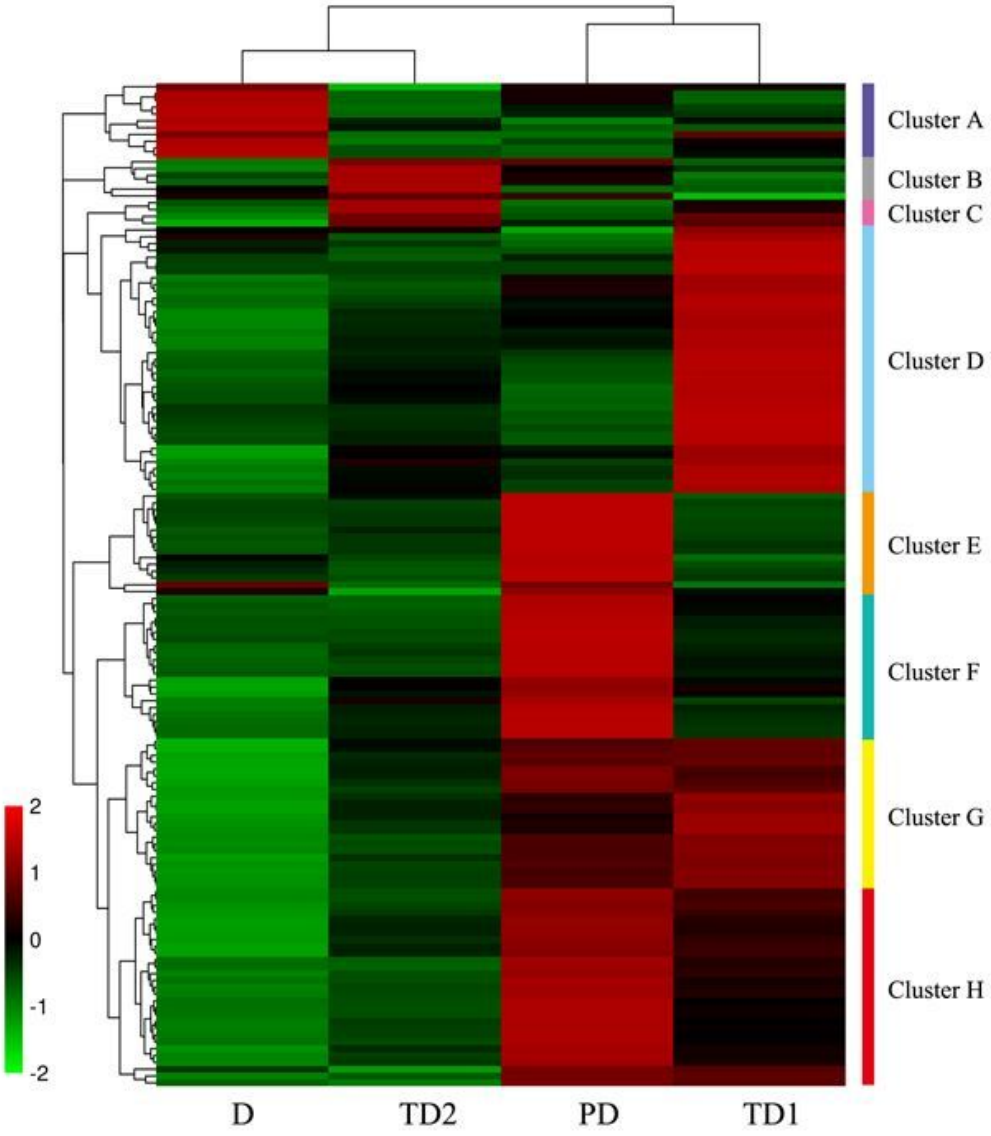

Figure 1

Expression profiles of miRNAs at different diapause stages. Hierarchical clustering revealed clusters of distinct expression profiles at different diapause stages of G. daurica adults. The expression levels of the miRNAs indicated by the different colors,

red indicates higher levels of miRNAs and green indicates lower levels of miRNAs.

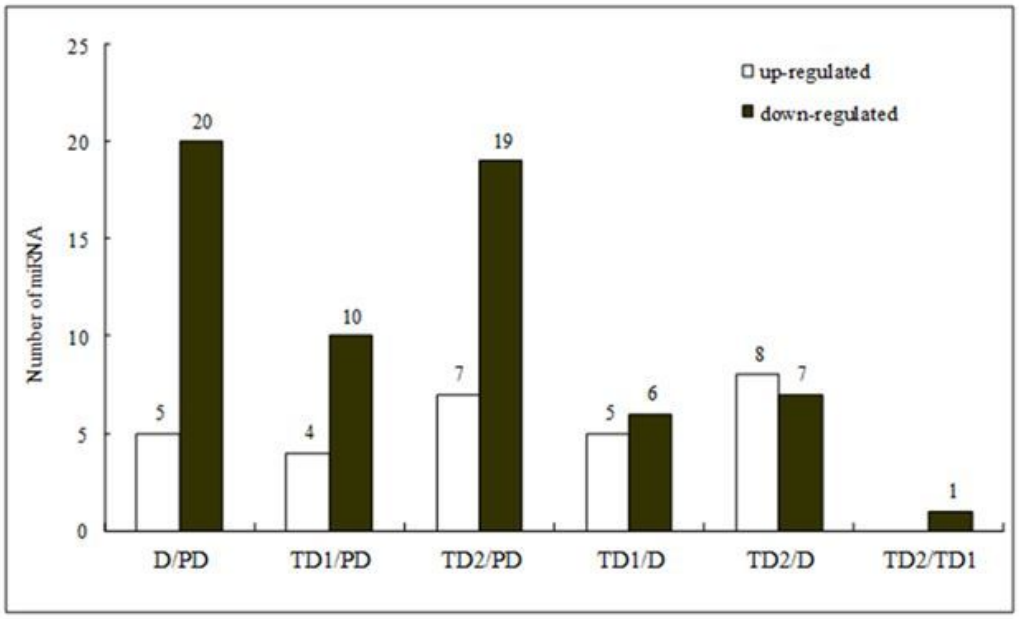

Figure 2

The number of differentially expressed miRNAs at different diapause stages of G. daurica adults. The differentially expressed genes were analyzed by comparing D to PD, TD1 to PD, TD2 to PD, TD1 to D, TD2 to D, and TD2 to TD1 based on a P-value of < 0.05 by t-test.

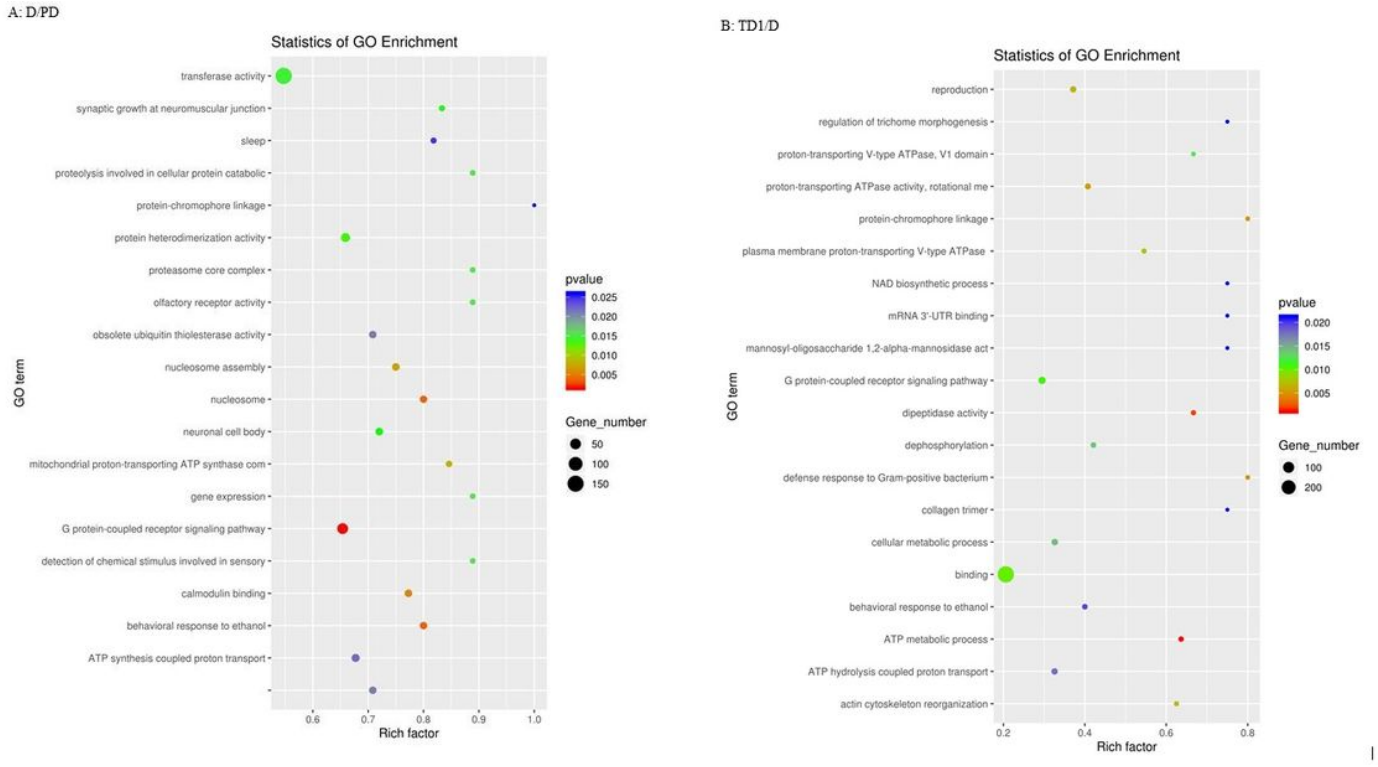

Figure 3

The GO enrichment for the target unigenes of differentially expressed miRNAs in G. daurica. A: D/PD; B: TD1/D

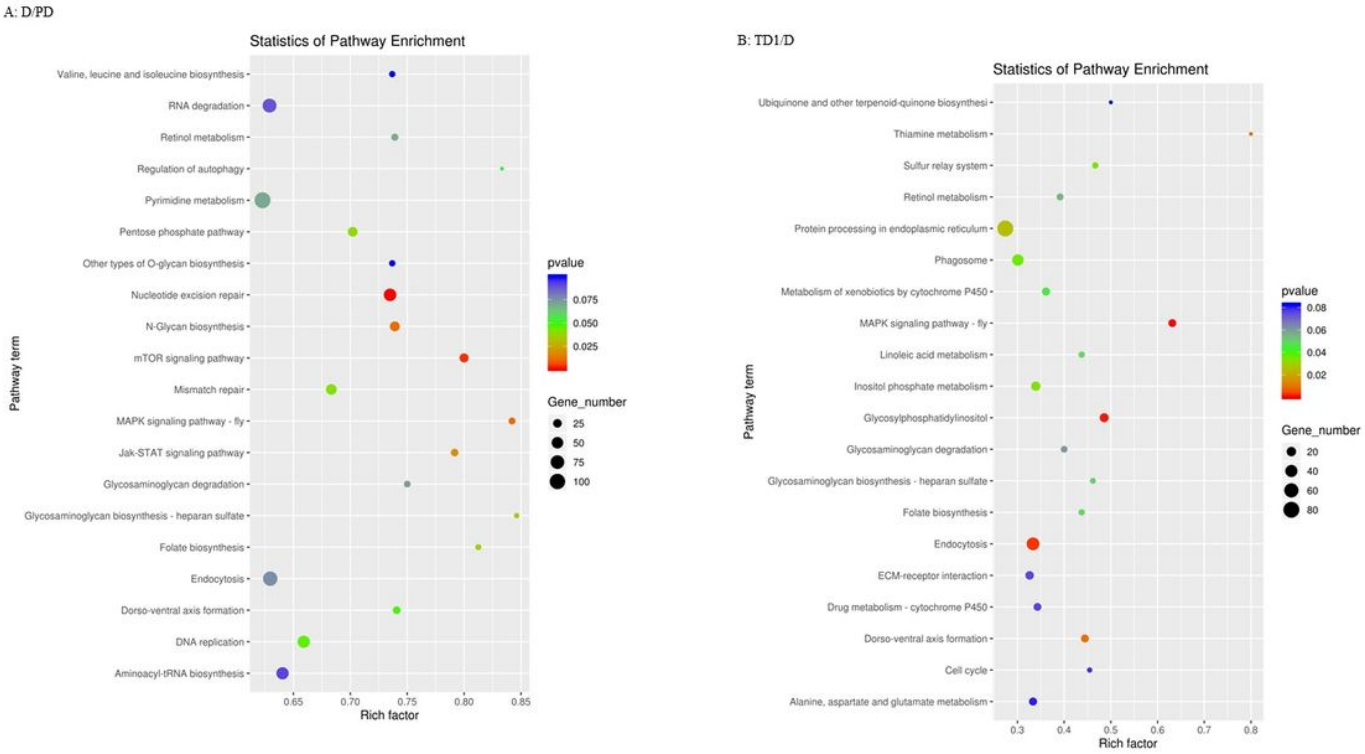

Figure 4

The KEGG enrichment for the target unigenes of differentially expressed miRNAs in G. daurica. A: D/PD; B: TD1/D

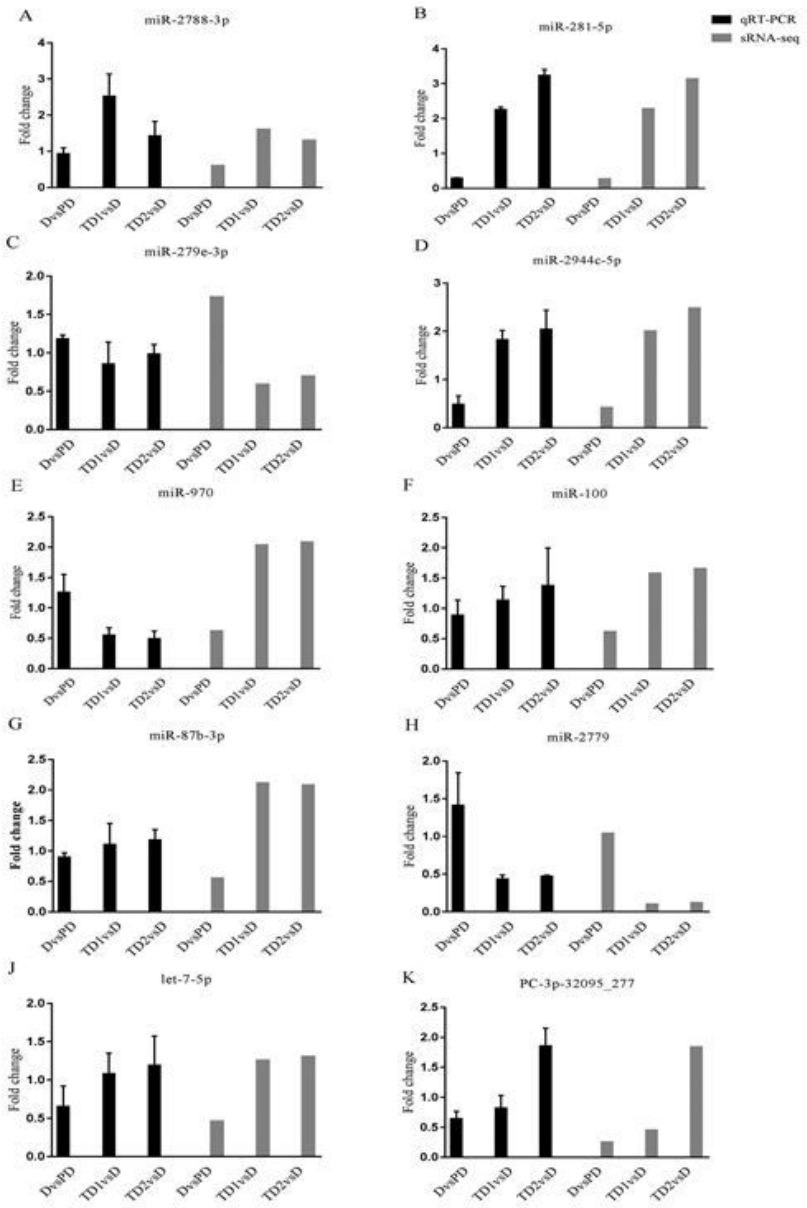

Figure 5

Validation of small RNA-seq derived changes in transcript abundance with qRT-PCR for 10 miRNAs. Fold change: normalization values in small RNA sequencing data and relative expression values obtained with qRT-PCR based on the 2-ΔΔCt method

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS11.PrimersusedforqRTPCRvalidation.docx
- TableS10Ca2signalinggenes.docx
- TableS9Ribosomalproteingenes.docx
- TableS8DEMsandtheirtargetgenesinvolvedinJH.docx
- TableS7SignificantlyenrichedKEGGpathways.docx
- TableS6SignificantlyenrichedGOterms.docx

- TableS5ClustersofmiRNAs.docx
- TableS4MiFambasedfamily.docx
- TableS3Summaryofconservedandnovelmi RNAs.docx
- TableS2Thelengthdistributionandabundance.docx
- TableS1SummaryofsmallRNAsequences.docx