Identification of cold stress responsive microRNAs in cold tolerant and susceptible *Hemerocallis fulva* by high throughput sequencing

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Abstract

Background: *Hemerocallis fulva* is a perennial herb belonging to Hemerocallis of Hemerocallis. Because of the large and bright colors, it is often used as a garden ornamental plant. But most varieties of *H. fulva* on the market will wither in winter, which will affect their beauty. It is very important to study the effect of low temperature stress on the physiological indexes of *H. fulva* and understand the cold tolerance of different *H. fulva*. MiRNA is a kind of endogenous non coding small molecular RNA with length of 21-24nt. It mainly inhibits protein translation by cutting target genes, and plays an important role in the development of organisms, gene expression and biological stress. Low temperature is the main abiotic stress affecting the production of *H. fulva* in China, which hinders the growth and development of plants. A comprehensive understanding of the expression pattern of microRNA in *H. fulva* under low temperature stress can improve our understanding of microRNA mediated stress response. Although there are many studies on miRNAs of various plants under cold stress at home and abroad, there are few studies on miRNAs related to cold stress of *H. fulva*. It is of great significance to explore the cold stress resistant gene resources of *H. fulva*, especially the identification and functional research of miRNA closely related to cold stress, for the breeding of excellent *H. fulva*.

Results A total of 5619 cold-responsive miRNAs, 315 putative novel and 5304 conserved miRNAs, were identified from the leaves and roots of two different varieties 'Jinyan' (cold-tolerant) and 'Lucretius' (cold-sensitive), which were stressed under -4 °C for 24 h. Twelve conserved and three novel miRNAs (novel-miR10, novel-miR19 and novel-miR48) were differentially expressed in leaves of 'Jinyan' under cold stress. Novel-miR19, novel-miR29 and novel-miR30 were up-regulated in roots of 'Jinyan' under cold stress. Thirteen and two conserved miRNAs were differentially expressed in leaves and roots of 'Lucretius' after cold stress. The differentially expressed miRNAs between two cultivars under cold stress include novel miRNAs and the members of the miR156, miR166 and miR319 families. A total of 6598 target genes for 6516 known miRNAs and 82 novel miRNAs were predicted by bioinformatic analysis, mainly involved in metabolic processes and stress responses. Ten differentially expressed miRNAs and predicted target genes were confirmed by quantitative reverse transcription PCR(q-PCR), and the expressional changes of target genes were negatively correlated to differentially expressed miRNAs. Our data indicated that some candidate miRNAs (e.g., miR156a-3-p, miR319a, and novel-miR19) may play important roles in plant response to cold stress.

Conclusions Our study indicates that some putative target genes and miRNA mediated metabolic processes and stress responses are significant to cold tolerance in *H. fulva*.

Background

H. fulva is a perennial flower of the family *Liliaceae*, *Hemerocallis* spp., with short rhizomes and thick spindle-shaped fleshy roots (Tuulos et al. 2015; An et al. 2014). Due to its rich varieties, flowers diversity, rich colors, and strong resistance to stress, it has been widely used in the construction of ecological cities, such as road greening, park green space, and roof gardens (An et al. 2014).

MicroRNAs (miRNAs), which are small (20-24nt), endogenous, non-coding, and single-stranded RNAs, are the most widely studied at present (Sunkar et al. 2004; Carvallo et al. 2011; Chen et al. 2018). As negative regulators of gene expression, miRNAs mainly play an important role in regulating gene expression at the post-transcriptional level (Bartel et al. 2009; Siomi . 2010). Recent studies have found that miRNAs are widely distributed in plants, animals, and microorganisms, regulating gene expression related to growth, development, and diseases of organisms (Sanz-Carbonell et al. 2019; Zhang , Stellwag . 2009; He , Hannon . 2004; Bartel . 2004). Besides, miRNAs have been recently confirmed as new regulator in plant development, environmental adaptation, and stress resistance. It plays a key role in the process of making adjustments and responses to abiotic stresses in plants (Jin , Zhu . 2010; Biggar , Storey . 2015; Yang et al. 2019; San et al. 2020).

Abiotic stresses, such as cold, salinity, drought, and nutrient, regulate the expression of genes in plants at both the transcriptional and post-transcriptional level. Cold stress, as one of the most common abiotic factors, adversely affects plant growth, development, geographical distribution and use as crops (Wang et al. 2019; Xu et al. 2019; Chen et al. 2012; Doherty et al. 2009). When tolerance is insufficient, either cold or freezing injuries result (Knight R, Knight H. 2012). Cold injury is associated with a complex array of cellular dysfunctions, and symptoms include loss of vigour, wilting, chlorosis, sterility and even death (Ou et al. 2015). Altered expression profiles of cold-regulated genes involved in lipid metabolism, chloroplast function, carbohydrate metabolism and free radical detoxification, observed in cold-sensitive *Arabidopsis* mutants, supports the suggestion that at least some cold-induced genes play a role in acclimation to cold temperatures (Abla et al. 2019; Provart et al. 2003). Zeng et al. (Zeng et al. 2018) studied cold stress responsive microRNAs in two winter turnip rape (*Brassica rapa* L.) by high throughput sequencing. The work indicated that miRNA and putative target genes mediated metabolic processes and stress responses are significant to cold tolerance in *H. fulva*. Chen et al. (Chen et al. 2015) studied the comparison of the low temperature transcriptomes of two tomato genotypes that differ in freezing tolerance. The results found that differences in gene expression, and miRNAs under cold stress may contribute to the observed differences in cold tolerance of these two tomato species. Low temperature also negatively affects the growth and development of *H. fulva*. The above ground parts of *H. fulva* are withered at low temperature, whereas the roots are capable of low temperature, indicating the root tolerance to cold is important for its survival in low temperature. Unlike *Arabidopsis* and other plants, cold-tolerance in *H. fulva* is rarely studied. 'Jinyan' is cultivated cold tolerant *H. fulva* variety that can survive at extremely low temperatures. And 'Lucretius' is a cold sensitive *H. fulva* variety. In presents, researches have been carried out in the fields of physiological and biochemical, classification and identification, tissue culture and breeding of *H. fulva* (Zhou. 1989), but there are relatively few studies on adversity stress of *H. fulva*. Whereas the miRNAs and target genes with putative functions in cold tolerance of *H. fulva* has not been rarely reported. An et al. (An et al. 2014) preliminarily constructed and analysed the MicroRNA expression profile of *H. fulva* at low temperature. Therefore we constructed and sequenced miRNA libraries from leaves and roots of two *H. fulva* varieties ('Jinyan' and 'Lucretius') treated with cold stress (4 °C), to research the expression differences of miRNAs related to cold stress in *H. fulva*. The miRNAs and target genes identified by high throughput Sequencing and bioinformatics analysis in this study could facilitate the understanding of molecular mechanisms of cold stress signaling in *H. fulva*. This work can provide theoretical guidance to the exploration of cold stress-responsive genes and molecular mechanism of stress-responsive proteins.

Results

Overview of sRNA sequencing in different cold resistance *H. fulva*

In order to understand the regulation of miRNAs in the process of cold resistance of *H. fulva* (leaf and root), 16s RNA libraries from eight samples (two biological replicates for each sample) were constructed. These libraries yielded a total of 203307248 raw reads by high through put sequencing (approximately 12.7 million raw reads per library). In a small RNA library of 4 leaf samples (4LCK, 4LTR, 7LCK, 7LTR) and 4 root samples (4RCK, 4RTR, 7RCK, 7RTR), 12868505, 12824175, 12877759, 11899075, 12855956, 12737633, 12720024 and 12870500 raw reads were obtained, respectively (Additional file: Table 1). After removing the low-quality reads, sequences with adapter and polyA, sequences less than 18 nucleotides and more than 30, we obtained 196320905 clean reads and an average of 12270057 reads (96.56%) per library. Of these reads, 77954289 clean reads were mapped to the database of miRBase (Kozomara et al. 2014; Quiroga et al. 2000) and Rfam (Rfam 12.2). The mapped sRNAs were annotated and classified into structural RNA (rRNA, tRNA, snRNA, snoRNA), known miRNAs (miRNAs in miRBase 21.0), precursor RNA, intergenic RNA, and unknown sRNAs (Additional file: Table 2). Notably, approximately 2% of the reads were mapped to mature miRNAs in each sample (Additional file: Table 2). The proportion of known miRNAs increased in 7LTR compared with 7LCK, implying that a number of miRNAs were induced in the leave of 'Jinyan' under cold stress. The distribution of various size of sRNAs showed that 24 nt sRNAs are the most abundant, accounting for 49.5% (4RTR) to 39.2% (7RTR), following by 22 nt sRNAs (Additional file: Fig.1). The distribution

is consistent with previous reports in various plants (Liu et al. 2017). To verify the results, we analyzed the Pearson correlation coefficients of the miRNA expression between the biological duplicates (Additional file: Figure S1), and the Pearson *r* value of miRNA expression were about 0.8.

Identification of conserved and novel miRNAs

Conserved miRNAs were identified by aligning against the miRBase 21.0 database. We obtained 5304 conserved miRNAs belonging to 315 miRNA families from 16 libraries (Additional file: Table S1). And 15 members of them belonged to the known miRNAs, and 5289 were similar to known miRNAs from other plant species. The result demonstrated that some conserved miRNAs in evolution also mediated the regulatory pathways of *H. fulva*. The expression profiles of the conserved miRNAs showed great variation in the amount of reads, which varied from 100,000 to less than 10 reads (Additional file: Table S1). Six miRNAs (miR159, miR166, miR396, miR319, miR167_1, miR167_2) presented high expression levels in all the samples. miR159 was identified with the highest expression level, covering about 100,000 reads in 7RTR, 4RTR and 4RCK. In contrast, over 2000 miRNAs were identified with less than 10 reads in all libraries. Besides, miR156 was identified as the largest miRNA family including 1131 members, following by miR172 and miR319. Moreover, 3560 and 3396 known miRNAs were common between control and treatment from leaves of 'Lucretius' and 'Jinyan', respectively (Additional file: Fig.2a). And 4056 and 3878 known miRNAs were common between control and treatment from roots of 'Lucretius' and 'Jinyan', respectively (Additional file: Fig.2b), implying that known miRNAs are stable in different cultivars even under cold stress. The lengths of most of these miRNAs was 21 nt long, similar to those observed in previous reports (Ye et al. 2014). Characteristic information of all the conserved miRNA sequences was summarized in Additional file : Table S1.

For the identification of novel miRNA candidates, the unannotated sRNAs matched to the Arabidopsis genome sequence were analyzed by miRDeep2. All the novel miRNA sequences were named in the form of *H. fulva* ovel-miR-number. Generally, we identified 1830 novel miRNAs (Additional file: Table S1), and 85 of them have star sequences (Additional file : Table S2). The lengths of novel miRNAs with star sequences varied from 20 nt to 24 nt, with the majority of 21 nt in length. The mean length of these miRNA precursors was 110 nt, ranging from 108 to 114 nt (Additional file: Table S2). Most of the novel miRNAs were identified with relatively low read counts compared with conserved miRNAs. Only seven novel miRNAs were identified with more than 100 reads, and most of the remaining novel miRNAs had less than 10 reads (Additional file: Table S2).

Differentially expressed miRNAs under cold stress

The heat map presenting the expressional pattern of conserved and novel miRNAs in stressed and non-stressed leaves and roots of two *H. fulva* cultivars were shown in Additional file: Figure S2 and Figure S3. 32 differentially expressed miRNAs with more than two fold change were identified in 'Jinyan' after cold stress, and 15 differentially expressed miRNAs were identified in 'Lucretius' under cold stress. In detail, seven miRNAs (six known and one novel miRNAs) were up-regulated and eight miRNAs (six known and two novel miRNAs) were down-regulated in leaves of 'Jinyan' under cold stress. And Bra-novel-miR3936-5p was up regulated in roots of 'Jinyan' under cold stress. Six known miRNAs were up regulated in leaves of 'Lucretius' under cold stress, while seven conserved miRNAs were down-regulated in leaves of 'Lucretius' after cold stress. miR166h-3p and miR396a were up regulated and down-regulated in stressed roots of 'Lucretius' compared with non-stressed roots of 'Lucretius', respectively (Additional file: Fig. 3).

Target prediction of differentially expressed miRNAs and gene enrichment analysis

To acknowledge the potential functions of differentially expressed miRNAs, we used psRobot and Target Finder to predict the target genes. All the target genes were subjected to GO enrichment analysis. A total of 6598 genes for 6516 known and 82 novel miRNAs were predicted as potential miRNA targets. GO annotation of these target genes showed that some stress-related GO terms were enriched, including response to stimulus and signaling. The most enriched GO terms were

metabolic process, cellular process, cell, cell part, and binding. The target genes of the differentially expressed miRNAs (novel_mir_19, novel_mir_28 and novel_mir_31) in 7RCK and 7RTR were involved in the regulation of metabolic processes and binding. The diverse functions of the predicted target genes suggested that the differentially expressed miRNAs may play important roles during plant response to cold stress.

It can be seen from the results that 781 predicted target genes of 'Lucretius' leaves were enriched to 108 pathway genes after cold treatment, and 8 of them were significantly enriched. It mainly includes selenocompound metabolism, monobactam biosynthesis, glycosphingolipid biosynthesis - ganglio series, basal transcription factors and other pathways. Compared with 0h, 85 predicted target genes of 'Lucretius' roots were enriched in 38 pathways after cold treatment, and only selenocompound metabolism was significantly enriched. Besides, for the 'Jinyan' leaves, 458 predicted target genes were distributed to 93 pathways which had 6 significant enriched including monobactam biosynthesis, selenocompound metabolism, lysine degradation, plant-pathogen interaction, mRNA surveillance pathway and sulfur metabolism. But when it came to 'Jinyan' roots, only plant-pathogen interaction and isoquinoline alkaloid biosynthesis were significant enriched. It can be found that miRNAs can improve the cold resistance of plants by regulating the expression of related genes in metabolic pathways under biological stress. At the same time, it is also proved that the cold resistance mechanism of *H. fulva* is a very complex process, involving the cooperation of multiple metabolic pathways.

Validation of expression patterns of miRNAs and predicted target genes by qRT-PCR

In order to verify the sequencing results, we selected ten miRNAs (mir398b, mir6478, mir395b, mir396a, newmir19, Stumir166d-5p, mdm-miR171i, bna-miR395d, ppe-miR395a-3p, pab-miR395) and corresponding target genes for qRT-PCR analysis. The results showed that the QRT PCR data of the ten miRNAs with different expression were consistent with the sequencing results, and the expression changes of the target genes were negatively correlated with the miRNAs with different expression (additional file: Figure 4). However, there were some differences in the expression amplitude multiples, which may be caused by the different detection sensitivity and data analysis methods of the two types of experiments. Under the cold stress of *H. fulva*, the miRNA expression characteristics of ten different genes showed the same trend with the sequencing results, which verified the reliability of the transcriptome sequencing results.

Discussion

Cold is a common stress facing by many plant species grown in southeast regions of China, which can induce cellular dehydration and membrane injury (Saddhe et al. 2018). *H. fulva* is a ornamental plant broadly grown in many regions, the selection and breeding of cold tolerance cultivars is of great importance to the vegetative supports in southeast regions. Plants enduring cold stress could adapt themselves with defense mechanisms, including physiological responses, adaptation in metabolic and signal transduction pathways, as well as gene expressional changes (Skyba et al. 2012). miRNAs as systemic regulators are involved in plant response to various abiotic stresses. Cold stress severely limits the geographic distribution of plant species by suppressing growth and development. Plants could adapt to cold stress by regulating gene expression. Moreover, 24 nt siRNAs can induce transcriptional gene silencing by DNA and histone modifications (Pontes et al. 2009; Ye et al. 2016). The relationship between miRNAs and cold response was reported in many plants (Yang et al. 2018; He et al. 2018; Shi et al. 2018). In *H. fulva*, the genome-wide expression profile of miRNAs in response to cold stress remains unreported. In the present study, millions of short reads were obtained from the sRNA libraries of two *H. fulva* cultivars under 20 °C and -4 °C treatments. Similar to other plants, *H. fulva* was enriched with 24 nt sRNAs (Arasu et al. 2017). The identified 5304 conserved miRNAs (Additional file : Table S1) and 1830 novel miRNAs (Additional file: Table S2) showed great variations in the amount of reads (from 1 to 100,000). The expression levels of novel miRNAs was relatively lower than that of conserved miRNAs, similar to the previous reports (Zhou et al. 2018). The novel miRNAs could enrich the B. rapa miRNA database, which may also provide a foundation for cold-tolerant regulation of miRNA.

miRNA is involved in almost all aspects of plant growth and development regulation, including morphogenesis of plant organs, hormone signal transduction and response to various external environmental stresses. In recent years, people have revealed the different expression patterns of miRNAs in Arabidopsis, poplar, rice, vegetable soybean, trifoliate orange and grape under cold stress, and discussed the regulatory mechanism of miRNAs in plant cold stress. The results showed that under cold stress, 376 miRNAs were differentially expressed in "Jinyan" leaves, while up to 90 miRNAs were up-regulated in "Jinyan" roots. The research shows that the cold tolerance of root is very important for the survival of Jinyan in winter (Zeng et al. 2018). In order to determine the difference of miRNAs of the same variety of *H. fulva* under control and cold stress. It is known that the above ground part of "Jinyan" is more sensitive to cold stress, showing poor growth and aging (Zeng et al. In 2018), the number of miRNAs differentially expressed in "Jinyan" leaves is relatively high, which may be related to the solution of "Jinyan" to alleviate cold induced damage such as ice induced cytoplasmic damage (Geyikoglu et al. 2017). Previous studies have shown that small changes in miRNA expression can lead to significant changes in mRNA levels and related biological processes (Maddaly et al. 2018). In this study, 5 619 cold responsive miRNAs were identified from leaves and roots of two different varieties of "Jinyan" (cold tolerance) and "lucrete" (cold sensitivity), of which 315 are speculated new miRNAs and 5 304 are conservative miRNAs.

miR164 plays an important role in the mechanism of wheat drought stress tolerance. In the study of rice senescence related genes, miR164 family mediates rice anti-aging by regulating target gene nap (NAC family member). The conserved miR164 targeted NAC gene is not only a growth and development factor, but also a negative regulator of rice drought resistance. It was also found that mir164g-3p was up-regulated in the leaves of "Jinyan" *Hemerocallis* under cold stress, which indicated that mir164g-3p might be the key regulatory factor of cold resistance of Jinyan. In addition, cold stress induced 658 differentially expressed miRNAs in 'Lucretius' leaves and 63 differentially expressed miRNAs in roots. Five miRNAs (MIR 398b, Mir 6478, Mir 395B, Mir 166h-3p, mir395a) were found in the leaves and roots of 'Lucretius', indicating that they were the most basic regulatory factors of 'Lucretius' cold stress. The differential expression of miRNAs suggested that the two varieties had different responses to cold stress, and the study showed that the induced differential expression of miRNAs in 'Jinyan' root might be more valuable than 'Lucretius'.

miRNAs are involved in many biological processes by regulating the expression of target genes. Therefore, it is important to recognize the function of target gene for understanding the mechanism of miRNA mediated. We predicted 6598 target genes with different functions of 6516 known miRNAs and 82 new miRNAs by Bioinformatics Method (additional file: table S3). Most of the predicted target genes encode transcription factors, such as athb, MYB, gamyb, TCP, bHLH transcription factors and scale promoter binding proteins. In addition, other target genes encoding glucosidase, glycylopeptidyltransferase, laccase, acyl lipid desaturase and galactitol synthetase are also involved in various physiological and metabolic processes. Many other biological processes, including development, signal transduction, metabolism and stress response, are also regulated by miRNA. In this study, mir156, miR164, mir166 and mir319 family members showed different expression patterns under low temperature stress. Scale promoter binding protein like (SPL) is the target gene of mir156, which has been reported to have the function of regulating leaf development (Wu et al. 2006; Bai et al. 2016). We found that mirna156a-3p was down regulated in the leaves of "Jinyan" under low temperature stress, but there was no difference in the expression in the roots (additional document: Figure 3). Similar results were found in barley under dehydration stress (Kantar et al. 2010). The expression of mir156 in *Medicago sativa* was down regulated under drought stress (Arshad et al. 2017). The changes of mir156a expression in "Jinyan" leaves indicate that it may respond to cold stress. Freezing injury can lead to dehydration and damage of cell membrane system (steponkus). 1984). Therefore, we speculated that the down-regulation of mirna156a-3p might be the result of dehydration and cell membrane damage in the leaves of auriferous rock under low temperature stress, but its mechanism is not clear. In addition, we found that the targets of new and preserved cold reactive miRNAs are usually related to development and other abiotic stresses such as heat, salt and drought (additional document: table S4). miR164 targets the NAC family of transcription factors (for Nam / ataf1, 2 / CUC2), which mediates stem and root development, flowering time, and response to cold, drought, salt, and flooding (nuruzzaman et al. 2012). The down-regulation of mir164g-3p expression may be one of the reasons for the early

senescence of gold rock roots under cold stress. *Tcp4* is the main target gene of *mir319*. A recent study found that *tcp4* mediated by *mir319* controls the formation of secondary cell walls by activating the expression of downstream *vnd7* transcriptional network in *Arabidopsis*. Overexpression of *tcp4* can significantly increase cell wall thickness, promote the formation of xylem vessels, and promote the deposition of lignin and cellulose (Sun et al., 2001). 2017). Increasing cell wall thickness and lignification can improve the cold tolerance of plants (Rajashekar, LAFTA). 1996). Under low temperature stress, we found that two *mir319* members (*mir319a* and *mir319b*) in the roots of the gold rock were up-regulated compared with *Lucretius*, and *TCP4* expression was enhanced in the roots of the gold rock. These changes may lead to the thicker cell wall of the root system of the gold rock than *Lucretius*, which is helpful to the cold resistance of the gold rock.

In conclusion, the cold reaction miRNAs in leaves and roots of two *Hemerocallis* cultivars were compared and identified. These miRNAs were selected for stem ring qRT-PCR validation. These data can be used to identify miRNAs related to cold tolerance. Through the prediction of target genes and functional annotation, we can understand the assumed function of miRNAs in cold stress response. On the other hand, whether these miRNAs expressed differently are the real regulators of the cold resistance of gold rocks, especially in the roots, needs more experimental verification. For example, the transformation of precursor genes of these candidate miRNAs into '*Lucretius*' to verify their molecular functions, and the analysis of cold tolerance phenotype in isolated populations with different miRNA expression levels will help to confirm the molecular functions of these miRNAs in regulating cold stress response.

Conclusion

In the present study, small RNA libraries of cold treated and non-treated leaves and roots of two *H. fulva* varieties were constructed separately, including two replicates for each sample. As a result, some conserved and novel miRNAs were identified by high throughput sequencing. The differentially expressed miRNAs were screened to find stress responsive miRNAs by comparing different organs and cultivars, as well as different treatments (cold treated and non-treated). The results indicated many candidate miRNAs and target genes with potential functions in regulating cold tolerance in '*Jinyan*', which are valuable for dissecting post-transcriptional and transcriptional regulations of cold responses, and *H. fulva* improvement in cold regions in the world.

Methods

Plant materials, cold stress treatment and physiological analysis

H. fulva '*Jinyan*' (cold-tolerant cultivar) and '*Lucretius*' (cold-sensitive cultivar) were provided by the College of Horticulture, Suzhou Polytechnic Institute of Agriculture (Jiangsu, China). Plants were grown in plastic pots until six true leaves in a ventilated greenhouse (20 °C, 16 h light/8 h dark cycle), and then moved into growth chamber (Safu, Ningbo, China) for treatment. Cold treatment was started by gradually decreasing the temperature from 20 °C to -4 °C. The plants were grown at 20 °C for 48 h and then induced at 10 °C and 4 °C for 48 h, respectively. Finally, they were stressed at -4 °C for 8 h. The stems and roots of plants grown at 20 °C (as control, CK) and -4 °C (as treatments, TR) were collected and frozen in liquid nitrogen for RNA extraction analysis. Generally, eight samples were collected and named 4 LCK (leaf of '*Lucretius*' at 20 °C), 4 LTR (leaf of '*Lucretius*' treated at -4 °C), 4 RCK (root of '*Lucretius*' at 20 °C), 4 RTR (root of '*Lucretius*' treated at -4 °C), 7 LCK (leaf of '*Jinyan*' at 20 °C), 7 LTR (leaf of '*Jinyan*' treated at -4 °C), 7 RCK (root of '*Jinyan*' at 20 °C), and 7 RTR (root of '*Jinyan*' treated at -4 °C), respectively. Each sample were polled from three plants and three biological replicates were included.

RNA isolation, library construction and sequencing

Total RNA was extracted using TRNzol Universal Reagent (Nanjing, China) following the manufacturer's instructions. The quality and concentration of RNA were tested with Agilent 2100 bio-analyzer. The sRNA libraries were constructed as previously described (Chen et al. 2005; Li et al. 2016). In brief, the 18-30 nt sRNAs of each sample were gel purified from the total RNA. Two adaptors (5' and 3' adaptors) were ligated to the purified sRNAs successively and then reversely transcribed and amplified for 15 cycles by PCR. Then sRNA libraries (including two biological replicates) of the eight samples were constructed. The quality and production of the libraries were further Tested by Agilent 2100 Bioanalyzer and ABI Step One Plus Real-Time PCR system, and then sequenced on an Illumina Genome Analyzer by the Beijing Genomics Institute in Shenzhen(<http://www.genomics.cn/en/index>).

MicroRNA data analysis

Analysis of small RNA sequencing data Raw data from high-throughput sequencing were filtered as previously described to obtain clean reads (Yi et al. 2013; Kozomara et al. 2011). The process included the removal of adapters and low quality sequences containing poly A and less than 18 nucleotides. Next, the clean sequences were mapped onto the Arabidopsis genome and Rfam12.2 (<http://rfam.xfam.org/>) to remove non coding RNAs, such as rRNAs, tRNAs, snRNAs, and snoRNA. The obtained sequences after screening were searched in the miRBase 21.0 database (<http://www.mirbase.org>) to identify known mature miRNAs. The sRNAs matched to known plant miRNAs with fewer than three mismatches, and could form typical stem-loop secondary structures, were identified as conserved miRNAs (Campos et al. 2003). Sequences that were unmapped to any miRNA in the miRBase and had less than two mismatches were taken as novel miRNAs by miRDeep2 (<https://www.mdc-berlin.de/8551903/en>). Differential expression analysis of miRNA expression level was calculated by Transcripts Per Kilobase Million (TPM), which can eliminate sequencing discrepancy (Love et al. 2014). Differential expression of miRNA between two samples was analyzed by DESeq2 (Wang et al. 2010). miRNAs were considered up-regulated when the fold change was greater than 2 or down-regulated when the Padj value (adjusted p-value) was less than 0.01. TPM and fold change were calculated as previously described (Yu et al. 2015).

Target gene prediction and analysis

Target gene predication and enrichment analysis Target gene prediction of miRNAs was analyzed by psRobot (<http://omicslab.genetics.ac.cn/psRobot>) and Target Finder (<https://github.com/carringtonlab/Target-Finder>) according to the related references (Allen et al. 2005). The targets of known and novel miRNAs with different expression between two samples were subjected to Gene Ontology (GO) annotations using the WEGO package (Schwab et al. 2005).

Fluorescent quantitative PCR verification

Expression analysis of miRNAs and predicted target genes using qRT-PCR Stem-loop quantitative reverse transcription PCR (qRT-PCR) was performed to analyze miRNA expression. According to the mature miRNA sequence, the specific stem-loop reverse transcription (RT) primers and forward primers of seven selected miRNAs were designed following the method described by Chen et al. (Chen et al. 2005). The universal reverse primer was used for all qRT-PCR reactions and U6 snRNA was used as an endogenous reference gene. For qRT-PCR validation of target gene, firststrand cDNA was generated using a SuperScript®III RT Reagent Kit (Invitrogen, China, Beijing). The Actin of *B. rapa* was used as an endogenous reference gene for normalization. qRT-PCR analysis was carried out on 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) using SYBR qPCR Mix (Invitrogen). The PCR conditions were 95 °C for 2 min, followed by 40- cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s. Triplicate technical replicates were run for qPCR, and amplification products were further confirmed by analyzing the melting curve and gel electrophoresis.

Abbreviations

4 LCK leaf of 'Lucretius' at 20 °C

4 LTR leaf of 'Lucretius' treated at −4 °C

4 RCK root of 'Lucretius' at 20 °C

4 RTR root of 'Lucretius' treated at −4 °C

7 LCK leaf of 'Jinyan' at 20 °C

7 LTR leaf of 'Jinyan' treated at −4 °C

7 RCK root of 'Jinyan' at 20 °C

7 RTR root of 'Jinyan' treated at −4 °C

H. fulva Hemerocallis fulva

q-PCR: quantitative reverse transcription PCR

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the conclusions of this article are available in the NCBI repository at website: <https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA642662> and accession No. [PRJNA642662](https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA642662).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HZ conceived and designed the experiments. Experiments were performed by CH. Data analysis was performed by CJ and LJ. All the authors have read and approved the final version of the manuscript..

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Tables

Table 1 The statistics of sRNA-seq reads and mapped reads

Sample		Raw tag count	Clean tag count	Mapped tag
4LCK	4LCK1A	12874209	12683702 (98.52%)	5270092 (41.55%)
	4LCK2A	12862800	12590794 (97.89%)	5402880 (42.91%)
4RCK	4RCK1A	12862796	12689995 (98.66%)	4693337 (36.98%)
	4RCK2A	12849115	12637155 (98.35%)	4809621 (38.06%)
7LCK	7LCK1A	12881718	12441106 (96.58%)	5892098 (47.36%)
	7LCK2A	12873799	12630390 (98.11%)	4973541 (39.38%)
7RCK	7RCK1A	12609519	12441204 (98.67%)	4551509 (36.58%)
	7RCK2A	12830529	12579606 (98.04%)	4770449 (37.92%)
4RTR	4RTR1A	12818670	12636170 (98.58%)	4567995 (36.15%)
	4RTR2A	12656595	12473415 (98.55%)	3897177 (31.24%)
7RTR	7RTR1A	12862908	12574664 (97.76%)	4877339 (38.79%)
	7RTR2A	12878091	12694404 (98.57%)	4056172 (31.95%)
7LTR	7LTR1A	10916132	10577907 (96.90%)	4446914 (42.04%)
	7LTR2A	12882017	10428232 (80.95%)	5133332 (49.23%)
4LTR	4LTR1B	12769411	11587180 (90.74%)	5449996 (47.03%)
	4LTR2B	12878939	12654981 (98.26%)	5161837 (40.79%)
Total		203307248	196320905	77954289
Average		12706703	12270057 (96.56%)	4872143 (38.34%)

Table 2 Distribution of smallRNAs among different categories in *H. fulva*

Category	Leaf				Root			
	4LCK	4LTR	7LCK	7LTR	4RCK	4RTR	7RCK	7RTR
Total	12637248 (100%)	12121081 (100%)	12535748 (100%)	10503070 (100%)	12663575 (100%)	12554793 (100%)	12510405 (100%)	12634534 (100%)
gene	35319 (0.28%)	35867 (0.30%)	45142 (0.36%)	31166 (0.30%)	34208 (0.27%)	28500 (0.23%)	33345 (0.27%)	37449 (0.30%)
miRNA	328085 (2.60%)	300101 (2.48%)	256698 (2.05%)	245534 (2.34%)	252107 (1.99%)	248332 (1.98%)	354363 (2.83%)	244109 (1.93%)
rRNA	1669829 (13.21%)	1449259 (11.96%)	1652353 (13.18%)	1386079 (13.20%)	1594315 (12.59%)	1290332 (10.28%)	1490248 (11.91%)	1547409 (12.25%)
repeat	1523032 (12.05%)	1341948 (11.07%)	1276196 (10.18%)	1090362 (10.38%)	1583497 (12.50%)	1698611 (13.53%)	1573545 (12.58%)	1544026 (12.22%)
snRNA	213040 (1.69%)	224167 (1.85%)	257773 (2.06%)	151123 (1.44%)	232253 (1.83%)	236786 (1.89%)	222272 (1.78%)	227744 (1.80%)
snoRNA	182266 (1.44%)	169577 (1.40%)	172700 (1.38%)	135484 (1.29%)	202353 (1.60%)	214598 (1.71%)	200111 (1.60%)	212498 (1.68%)
tRNA	257956 (2.04%)	957009 (7.90%)	274884 (2.19%)	665733 (6.34%)	235361 (1.86%)	174096 (1.39%)	162670 (1.30%)	286156 (2.26%)
intergenic	1126962 (8.92%)	827991 (6.83%)	1497075 (11.94%)	1084645 (10.33%)	617387 (4.88%)	341333 (2.72%)	624428 (4.99%)	367367 (2.91%)
unmap	7300762 (57.77%)	6815164 (56.23%)	7102929 (56.66%)	5712947 (54.39%)	7912096 (62.48%)	8322207 (66.29%)	7849426 (62.74%)	8167779 (64.65%)

Figures

Figure 1

Small RNA length distribution

Figure 2

Venn diagram of differentially expressed miRNAs (a: leaf b: root)

Figure 3

Significant different expression of miRNA in 7LCK/7LTR, 7RCK/7RTR, 4LCK/4LTR, 4RCK/4RTR

Figure 4

Validation of expression patterns of miRNAs and predicted target genes by qRT-PCR Note: the error line in QRT PCR results represents the standard deviation of the mean value (n = 3)