

Selection of suitable reference genes for miRNA expression normalization by quantitative RT-PCR under abiotic stress in *Larix olgensis*

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Abstract

Suitable selection of endogenous controls is necessary for obtaining reliable qRT-PCR results. However, insufficient attention has been paid to the selection of endogenous controls for normalization in different stresses in plant, especially *Larix olgensis*. In this study, we screened candidate internal reference genes for miRNA expression normalization after subjecting *Larix olgensis* PEG-induced drought, NaHCO₃, or NaCl stress. Needles samples were collected at 0, 12, 24, 48 and 96 h after treatment. Four common housekeeping genes and 11 mature miRNAs that were found to be stably expressed in small RNA sequencing data of *Larix olgensis* were selected as candidate internal reference genes. Their expression levels were assessed by qRT-PCR. GeNorm and NormFinder were used to analyse the stability of gene expression from the candidate internal reference genes. Finally, the expression of some miRNAs was analysed after stress. The results showed that miRNAs exhibited better expression stability compared to the analysed ncRNAs. Under PEG, NaHCO₃, or NaCl stress, miR83321, miR43169, and miR74940 were identified as the most suitable internal reference genes, respectively. Under PEG stress, the expression of most miRNAs reached its highest level at 12 h, showing a trend of first increasing expression and then decreasing. Similarly, under NaHCO₃ and NaCl stress, the expression of most miRNAs reached its maximum levels at 48 h and 12 h, respectively. This indicates that most miRNAs more quickly respond to PEG and NaCl stress than NaHCO₃ stress.

Keywords: internal reference genes; miRNA; *Larix olgensis*; qRT-PCR; stress

Introduction

Plants encounter various unfavourable environmental factors during their survival and growth. When plants are subjected to an abiotic stressor such as drought, salinity, alkali, and others, the stimulation of environmental stress causes certain plant genes to be expressed under specific conditions. Among these stressors, drought and salinity are considered the most severe and abundant abiotic stressors with detrimental impacts on plant development, growth and productivity (Du et al., 2023). MiRNAs, which are small non-coding RNAs, are abundant in plants and play a very important role in response to biotic stressors such as nutrient deficiency (Fujii et al., 2006; Kehr et al., 2013; Bai et al., 2018; Carrió-Seguí et al., 2019), abiotic stress (Zhang et al., 2006; Shriram et al., 2016; Li et al., 2020), drought (Yang et al., 2019b; Qiu et al., 2020; Islam et al., 2022; Mishra et al., 2022), cold (Zeng et al., 2018; Wang et al., 2019), and high salinity (Fu et al., 2017; Ma et al., 2019; Parmar et al., 2020). MiRNAs are key factors in the regulation of post-transcriptional gene expression, and stress-specific miRNAs have been identified in model plants under various biotic and abiotic stresses (Yasmin Begum, 2022). In recent years, high-throughput sequencing and bioinformatics approaches have been widely used in some plant species to identify large amounts of stress-responsive conserved and novel miRNAs, thereby expanding the field of miRNA research (Alzahrani et al., 2019; Parmar et al., 2020; Zhang et al., 2020b; Gao et al., 2022). A previous study found that miR169g was the only member of the miR169 family to be induced to be expressed after rice was treated with drought stress (Zhao et al., 2007). In addition, low temperature can induce the expression of miR172 in *Arabidopsis thaliana* (Zhou et al., 2008), while miR395 in *Arabidopsis thaliana* and rice under high salt stress was also induced (Covarrubias and JOSÉ, 2010). The miRNAs that respond to salt stress in different plants are not necessarily similar. In Arabidopsis, cotton,

wheat and tomato, the expression of miR398 after NaCl treatment was significantly downregulated (Wang et al., 2014; Jia et al., 2009; Wang et al., 2013; Luan et al., 2014). In other species, miR398 has been confirmed to undergo positive regulatory changes under salt stress: expression was induced during the first 3–4 h of salt stress, down-regulated at 48 h and then upregulated again at 72 h in poplar (Jia et al., 2009). In addition, miR156 was upregulated in *Arabidopsis thaliana* and downregulated in *Populus trichocarpa*. Similarly, miR396 was downregulated after salt stress in *Arabidopsis thaliana* and *Oryza sativa*, and upregulated in *Panicum virgatum* and *Suaeda maritima* (Kumar et al., 2018; Gao et al., 2022). These results suggest that miRNA expression was specifically regulated in various plants and dynamically regulated in specific individual plants (Yasmin Begum, 2022). Li et al. (2020) has reported on the reference gene screening of mRNA under abiotic stress. However, the expression patterns of miRNAs in *Larix olgensis* seedlings under drought, salinity and alkali stress have not been reported yet. Therefore, our study aims to investigate the expression of miRNAs in *Larix olgensis* under drought and saline-alkali stress. We expect to find specific miRNAs that are induced as a response to these stresses. These findings will provide new genetic resources and technical improvement in studying the stress adaptation mechanisms of *Larix olgensis*.

To ensure accurate detection of differences in the specific expression of target genes, it is crucial to utilize an internal reference gene for normalize, as highlighted in the study by Brunner et al. (2004). Most of the commonly used internal reference genes are housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin (ACT), elongation factor (EF-1a), 18S rRNA, among others. These housekeeping genes are expressed in all cells and are required for the maintenance of basic cellular life, so these genes are considered to be stably expressed (Nicot et al., 2005). However, previous studies have shown that there is a significant change in the expression of housekeeping genes under some conditions (Czechowski et al., 2005). An internal reference gene that exhibits stable expression in one test may be altered in another test (Jain et al., 2006; Tong et al., 2009; Vdal and Lillo, 2009; Bai et al., 2022; Li et al., 2023). Consequently, an internal reference gene that is stably expressed under all experimental conditions is almost non-existent (Rui et al., 2016; Wang et al., 2016). Therefore, it is important to screen out the internal reference genes that are stable under respective experimental conditions. This involves screening a large number of potential internal reference genes to identify new or more stable internal reference genes. Before the experiment, it is necessary to use the geNorm (<http://medgen.ugent.br/~jvdesomp/genorm/>) (Vandesompele et al., 2002) and NormFinder (<http://www.mdl.dk/publicationsnormfinder.html>) (Andersen et al., 2004) algorithms to comprehensively evaluate the internal reference genes based on the cyclic threshold (Ct) from real-time quantitative PCR. These two programs were developed by different laboratories based on different algorithms and have been widely used in the study of gene selection of animal and plant internal reference gene research (Chen et al., 2012). In this study, to normalize miRNA expression in *Larix olgensis*, 5.8S rRNA, U6, 5S rRNA, 18S rRNA,

and 11 miRNAs were used as candidate internal reference genes under different stress conditions (drought, salinity and alkali), and then use geNorm and NormFinder software to screen out the suitable genes under different stress conditions. Furthermore, quantitative expression analysis of differential miRNAs in different internal reference genes under drought and saline-alkali stress was carried out. This study will provide a theoretical basis for studying the stress resistance of *Larix olgensis*.

Materials and Methods

Plant materials and growth conditions

Larix olgensis seedlings from Heilongjiang province Jixi provenances were placed in a thermostatic seedling chamber and grown at 24 ± 1 °C with a light duration of 16 h/d⁻¹ and light intensity of 35 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. After the plants had grown for 6 months, some seedlings were treated with 25 % PEG (PEG₆₀₀₀), 50 mmol/L NaHCO₃, and 250 mmol/L NaCl solution, respectively (Zhang et al., 2021), while others were treated with deionized water as a control (0 h). Needles samples were collected at 0 h, 12 h, 24 h, 48 h and 96 h after treatment, and then total RNA was extracted.

Extraction of total RNA and cDNA synthesis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA of the miRNA was synthesized using TransScript[®] miRNA First-Strand cDNA Synthesis SuperMix (TRANS, Beijing, China) according to the manufacturer's instructions. cDNA was stored at -20 °C.

MiRNA qRT-PCR

The qRT-PCR reactions of miRNAs were performed using TransStart[®] Top Green q PCR SuperMix (TRANS, Beijing, China). The qRT-PCR instrument used for gene expression analysis was an Applied Biosystems ABI7500 (Applied Biosystems, USA). Three technical replicates were included for each reaction. The reaction procedure was as follows: 94 °C for 30 s; 40 cycles of 94 °C for 5 s, 60 °C for 15 s, and 72 °C for 35 s; followed by 95 °C for 15 s; 60 °C 1 min; 95 °C 30 s.

Candidate internal reference genes and miRNA qPCR primer design

Based on small RNA-Seq data from *Larix olgensis* (data not published), 11 mature microRNAs and 4 common housekeeping genes were selected as candidate reference genes. The forward primer of *Larix olgensis* miRNA was based on a mature miRNA sequence where U was replaced with T. The difference between the Tm value and GC content of the forward and reverse primers was achieved by adding or subtracting base pairs at both ends of the primers. The reverse primer was the universal primers provided by the kit, Universal miRNA qPCR Primer (Tm=58 °C). All designed primers were synthesized by Sangon Biotech (Shanghai, China). The amplification efficiency for each pair of primers was determined in the qRT-PCR assay

Table 1
Forward primer sequences of candidate internal reference genes and primers amplification efficiency

miRNA ID	Forward primer sequence (5'~3')	Length (nt)	Tm (°C)	GC Content (%)	R2	Amplification efficiency (%)
miR64366	GCGCGCTATTTGAGTTTCTGAGTTT	26	58.02	42.31	0.9912	98.9
miR79262	TCACATCTGGGCCACGATG	19	57.96	57.89	0.9925	99.6
miR43169	CTCCACAGCTTCTTGAGCTT	22	57.57	50	0.9996	106.3
miR84097	CGTGCTCTGCGATCTGATGTT	23	58.06	52.17	0.9995	100.9
miR74749	CGCGTTTCTGGATAAAGAACTGTGT	25	57.36	44	0.9978	101.8
5.8S rRNA	GAGCAGAGCAATGGCCTA CAGAG	23	60.45	56.52	0.9958	99.2
U6	AACGATGGCACGGATCAACGC	21	61.41	57.14	0.9984	108.4
5S rRNA	CACGACGTGCCAGCA AGAGAC	21	61.89	61.90	0.9972	100.5
18S rRNA	TAGCCCGCAGCCTTGCTAGT	20	58.76	55	0.9945	96.7
miR66438	AATTGCAGAAGGGCTGGTTAG C	22	58.2	50	0.9996	100.9
miR72447	CGAAGGGCAGGAGTTGTTTGG	21	59.36	57.14	0.9967	110.1
miR70959	AAAACCAACGGGGTCAAGAC	20	58.19	55	0.9992	109.6
miR83321	AGGTGTGAGCTGGAGGTTTG	20	57.67	55	0.9989	110.3
miR74940	CGCGCTTCTTCAATGTATCACTAATC	27	58	44.44	0.9991	108.9
miR60880	GTTACCATGAGGACGAGCTCATC	23	57.56	52.17	0.9967	99.8

as described above. To monitor the amplification efficiency, cDNA was serially diluted (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6}) for determination of the correlation coefficient (R^2), R^2 and slope values obtained from the standard curves, and the amplification efficiency (E) calculated according to the formula: $E \% = (10^{1/\text{slope}} - 1) \times 100 \%$ (Pfaffl et al., 2004). Optimizing qRT-PCR assay is essential for accurate and reproducible quantification of samples. The hallmarks of an optimized qRT-PCR assay are: correlation coefficient $R^2 > 0.99$, amplification efficiency ranges from 95 % to 105 % (Zhao et al., 2021). The primers sequences and amplification efficiency were listed in Table 1, with R^2 values ranging from 0.9912 to 0.9996 and amplification efficiency ranging from between 96.7 % to 110.1 %.

Data collation and statistical analysis

Two algorithms, GeNorm and NormFinder were used to evaluate suitable reference genes for normalization of miRNA expression in *Larix olgensis*. GeNorm software, developed by Vandesompele et al. (2002), is based on the Microsoft Excel platform and is used for analyzing candidate internal gene expression by real-time quantitative PCR. NormFinder software, written by Andersen et al. (2004), is another program used for screening the stability of candidate internal reference genes. The relative expression level, Q, of each gene was calculated using Excel 2003 to find the Ct value of each amplified sample according to the formula $Q = E^{\Delta Ct}$, where E is the efficiency of gene amplification (generally, gene amplification is set to the ideal amplification and the amplification efficiency, E, is set

at a default of 2), $\Delta Ct = Ct_{\text{min}} - Ct_{\text{sample}}$ (Ct_{min} is the lowest Ct value of all samples, Ct_{sample} is Ct value for each sample). For both GeNorm and NormFinder, the lowest M value suggests the most stable gene (Livak and Schmittgen, 2004).

The raw data from this study were processed using Graph-Pad Prism 5.0 (<https://q.115.com/101075/T1211440.html>, America). The relative expression of miRNAs in each treatment was quantitatively analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2004). Gene expression levels of miRNAs were normalized using suitable reference genes as an internal control, followed by calculation and analysis of miRNA differential expression.

Results

Analysis of the expression profiles of the candidate reference genes

Using qRT-PCR, the levels of gene expression are determined by Ct values. To investigate the suitability of 15 candidate internal reference genes, their Ct values were analyzed (Figure 1).

Under PEG stress, the overall variation ranged from 11.326 to 35.962, with miR74940 having the largest Ct value and 5S rRNA having the smallest Ct value. The average Ct value of all samples was 25.78. miR84097, 5S rRNA, miR66438, and miR70959 were highly expressed compared to other candidate

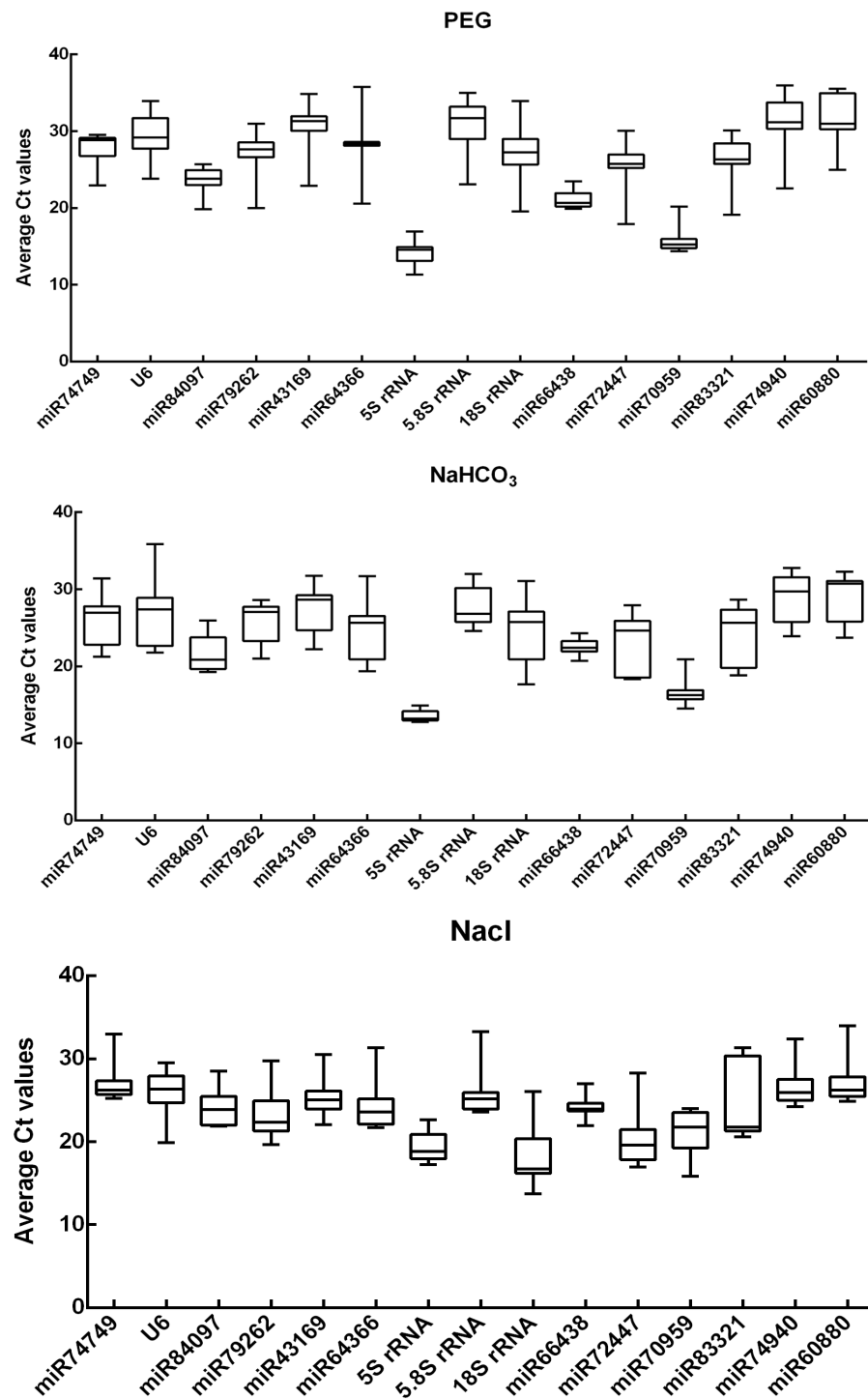


Figure 1

Box plots of the Ct values of different candidate reference genes among samples. Expression data for fifteen candidate reference genes in *Larix olgensis* by PEG, NaHCO₃, or NaCl treatment 0, 12, 24, 48, 96 h, respectively. The horizontal line in the box represents the median, the upper and lower sides of the box represent the upper quartile and the lower quartile, respectively and the whisker caps represent the maximum and minimum. All data are means means $\pm$ SD of three biological replicates.

genes. Some genes were expressed at very low levels, including U6, 5.8S rRNA, miR74940, and miR60880.

Following NaHCO_3 stress, the overall range of Ct value was 12.759-35.841, with U6 having the largest value, and 5S rRNA having the smallest value. The average Ct value of all samples was 24.12. miR84097, 5S rRNA, and miR70959 showed highly expressed levels compared to other candidate genes. On the other hand, some genes were expressed at relatively low levels, including U6, 5.8S rRNA, miR74940, and miR60880.

After NaCl stress, the overall range of Ct value was 13.702-33.934, with miR60880 having the largest value and 18S rRNA having the smallest value. The average Ct value of all the samples was 24.05. 5S rRNA, 18S rRNA, and miR70959 showed highly expressed level compared to the other candidate genes, while some genes were expressed at low levels, such as 5.8S rRNA, miR74749, miR74940, and miR60880.

The wide expression ranges of the 15 reference genes indicated that no one reference gene had a constant expression throughout the different conditions. Therefore, it is not sufficient to evaluate the stability of internal reference genes by the change of Ct value. Further analysis with relevant software is necessary to ensure the accuracy of the results.

Stability analysis of candidate reference genes

GeNorm analysis

GeNorm software, based on the Microsoft Excel platform, was used for candidate internal gene expression analysis from real-time quantitative PCR. The program screens out the internal reference genes with better stability by calculating the M value of the stability of each internal reference gene. $M=1.5$ is used as the threshold for the expression stability of the internal reference gene. $M<1.5$ indicates that the gene expression was stable, while $M>1.5$ indicates relatively unstable gene expression. During PEG stress treatment, there were 10 candidate internal reference genes with $M<1.5$, among which the most stable expressed genes were miR72447 and miR83321. During NaHCO_3 stress treatment, there were 8 candidate internal reference genes with $M<1.5$, among which the most stable expressed genes were miR74749 and miR43169. During NaCl stress treatment, there were 9 candidate internal reference genes with $M<1.5$, and the most stable expressed genes were miR74749 and miR74940 (Figure 2).

NormFinder analysis

To make the test results more accurate, the data was re-analyzed using NormFinder software. NormFinder is another program for screening the stability of candidate internal reference genes for stability, using the same data conversion methods as GeNorm. Here, a lower stability value indicates that the candidate internal reference gene is more stable. The program is used to analyze the gene expression stability of internal

reference genes under different stress conditions. The stability values are shown in Table 2.

Under different stress, it was found that the stability values of candidate internal reference genes were different, and the most stable internal reference genes were also different. When *Larix olgensis* seedlings were treated by PEG, NaHCO_3 , NaCl stress at different time, the most stable candidate internal reference genes were miR83321, miR79262, and miR74940, respectively. For common housekeeping genes, the stability value of 5S rRNA was 2.536 in PEG treatment, 2.199 in NaHCO_3 treatment and U6 was 4.1631 in NaCl treatment. The stability value of 18S rRNA was 4.1631, 1.636 in NaHCO_3 treatment and 1.618 in NaCl treatment. This indicated that miRNAs are more suitable as internal reference genes than the housekeeping genes used as miRNA expression by qPCR.

Combined analysis using two programs showed that the most stably expressed genes were miR72447 and miR83321 under PEG stress using GeNorm analysis, and the most stably expressed genes were miR83321 and miR79262 by NormFinder analysis. Therefore, miR83321 was selected as the internal reference gene under PEG stress. Under NaHCO_3 stress, the most stably expressed genes were miR74749 and miR43169 by GeNorm analysis, and the most stably expressed genes using NormFinder were miR43169 and miR79262, so miR43169 was selected as the internal reference gene under NaHCO_3 stress. Under NaCl stress, the most stably expressed genes were miR74749 and miR74940 by GeNorm analysis, and the most stably expressed genes were miR74940 and miR64366 by NormFinder analysis, so miR74940 was selected as the internal reference gene under NaCl stress.

Expression analysis of differential miRNAs after stress

Based on the findings of the internal reference gene study, miR83321, miR43169 and miR74940 were selected as internal reference genes for analyzing the expression levels of differentially expressed miRNAs under PEG, NaHCO_3 and NaCl stress (Figure 3-5). The primer design method for qPCR to detect these differentially expressed microRNAs was consistent with the description provided in the methods section. The primer sequences are presented in Table 3.

After treatment with 25 % PEG solution, miRNAs showed different expression trends when miR83321 was selected as internal reference genes. However, the expression level of most of the miRNAs was the highest after treated for 12 h, which suggested that these miRNAs faster respond to drought stress than other miRNAs. These miRNAs, miR84046, miR83720, miR56010, miR79508, miR40049, miR67876, miR73244, and miR97580 all showed a trend of first increasing and then decreasing. These results indicated that these miRNAs responded strongly to drought stress after 12 h. Meanwhile, the expression levels of miR51167, miR78971, and miR49391 were at the maximum levels without stress and were decreased after stress, indicating that they responded strongly during PEG stress. There were also some miRNAs that responded strongly to drought stress at different time points, such as miR79508, miR35203, and miR97580.

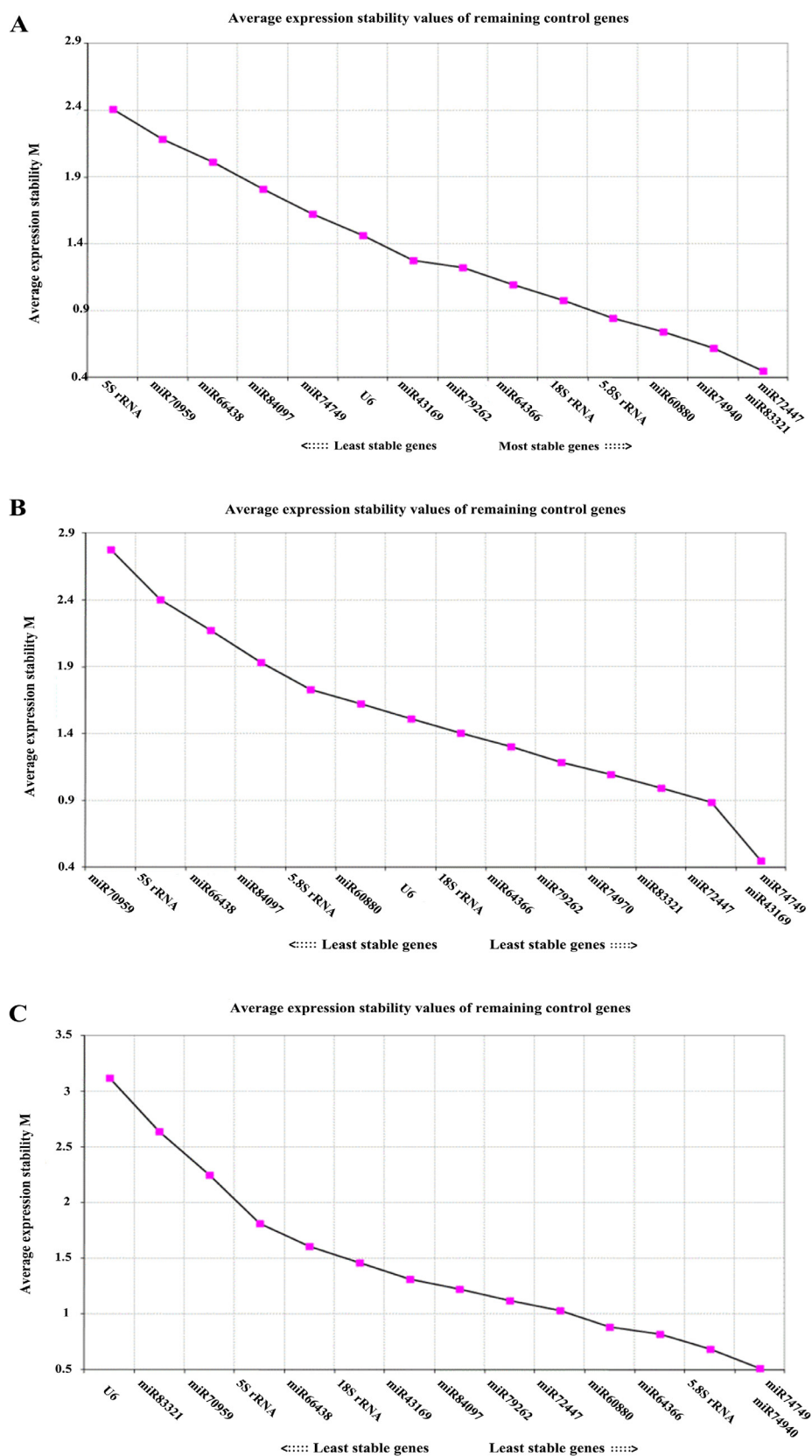


Figure 2

Stability of each candidate internal reference genes under PEG, NaHCO_3 , NaCl stress. Average expression stability values (M) of candidate reference genes after geNorm analysis: (A) PEG treatment 0, 12, 24, 48, 96 h; (B) NaHCO_3 treatment 0, 12, 24, 48, 96 h; (C) NaCl treatment 0, 12, 24, 48, 96 h. All data are means $\pm$ SD of three biological replicates.

Table 2
Stability of reference gene expression as calculated by NormFinder

Gene	PEG		NaHCO ₃		NaCl	
	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking
miR83321	0.657	1	1.163	7	3.301	14
miR79262	0.698	2	0.387	1	0.965	7
U6	0.907	3	1.727	12	4.134	15
miR60880	0.945	4	1.315	8	0.85	6
miR43169	0.963	5	0.587	2	1.287	11
miR72447	0.973	6	0.941	6	1.287	10
miR64366	1.01	7	1.325	9	0.607	2
miR74749	1.043	8	0.635	3	0.619	3
5.8S rRNA	1.067	9	0.903	5	0.735	5
18S rRNA	1.302	10	1.636	11	1.644	12
miR74940	1.352	11	0.86	4	0.497	1
miR84097	1.385	12	1.4	10	0.687	4
miR66438	1.644	13	1.325	9	0.607	2
miR70959	1.825	14	3.515	15	2.848	13
5S rRNA	2.536	15	2.199	14	1.27	9

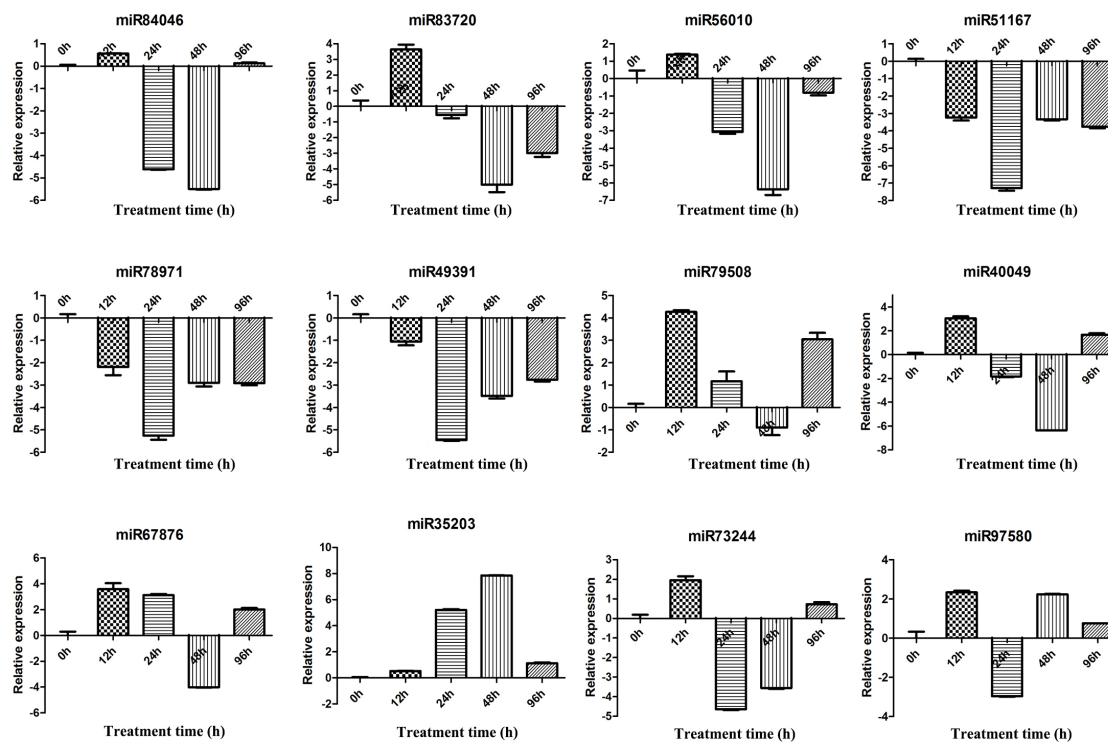


Figure 3

Differential miRNA expression under PEG stress. The expression levels of miR84046, miR83720, miR56010, miR51167, miR78971, miR49391, miR79508, miR40049, miR67876, miR35203, miR73244, miR97580 under PEG treatment 0, 12, 24, 48 and 96 h. miR83321 was used as the reference genes. All data are means $\pm$ SD of three biological replicates.

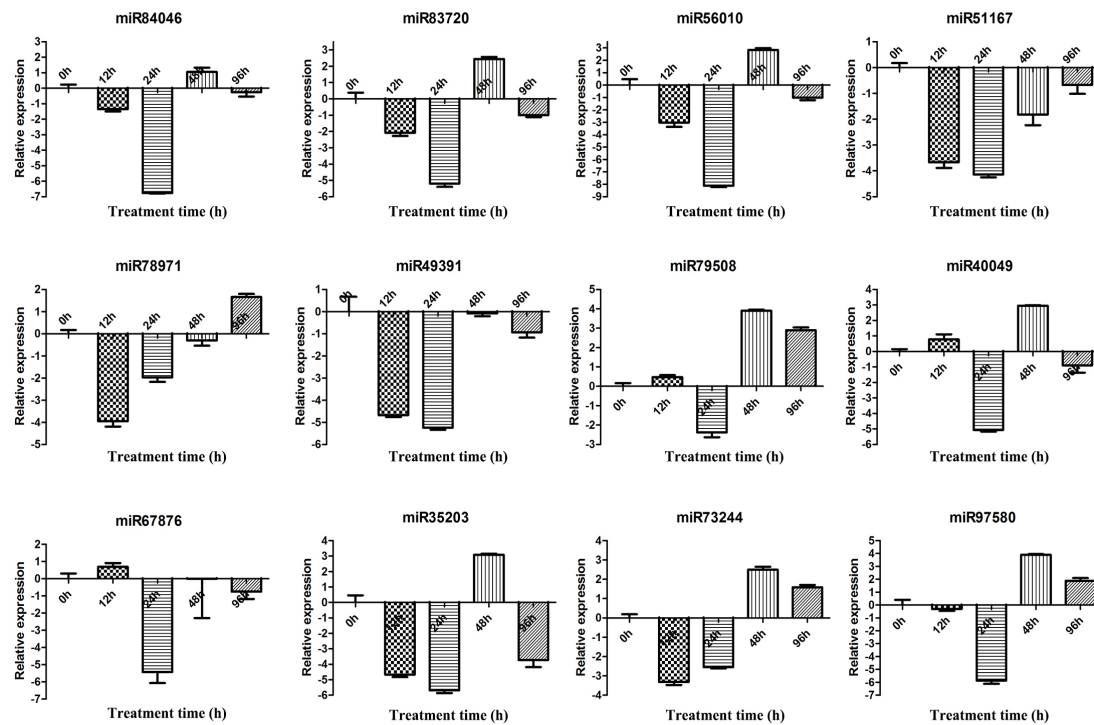


Figure 4

Differential miRNA expression under NaHCO₃ stress. The expression levels of miR84046, miR83720, miR56010, miR51167, miR78971, miR49391, miR79508, miR40049, miR67876, miR35203, miR73244, miR97580 under NaHCO₃ treatment 0, 12, 24, 48 and 96 h. miR43169 was used as the reference genes. All data are means $\pm$ SD of three biological replicates.

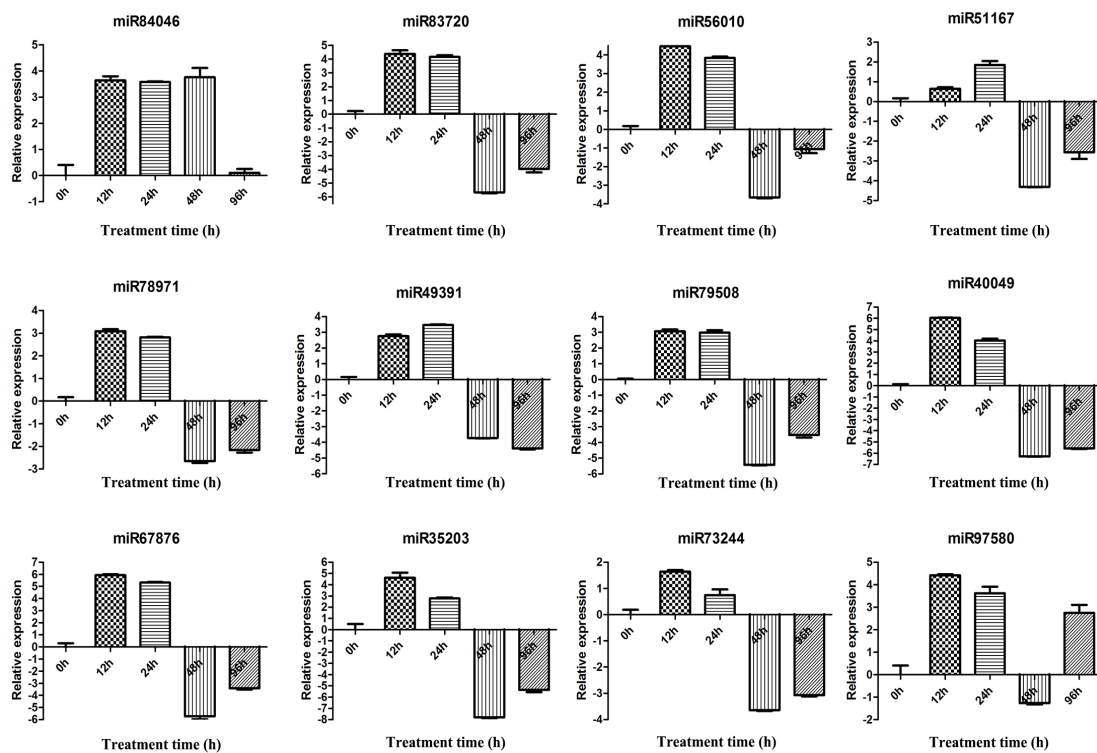


Figure 5

Differential miRNA expression under NaCl stress. The expression levels of miR84046, miR83720, miR56010, miR51167, miR78971, miR49391, miR79508, miR40049, miR67876, miR35203, miR73244, miR97580 under NaCl treatment 0, 12, 24, 48 and 96 h. miR74940 was used as the reference genes. All data are means $\pm$ SD of three biological replicates.

Table 3
Differentially expressed miRNA quantitative primers

miRNA ID	Forward primer sequence (5'~3')	Length (nt)	Tm (°C)	GC Content (%)
miR84046	GGGTCTGAGTTCTTTGGGGC	20	59	60
miR83720	CGCGAATTGATGCTCAGAAATCCTG	25	59	48
miR56010	GCGGTGGCTGGAAAACCTATTGAAG	25	59	48
miR51167	TCGATTGTAGCCAGATGGGC	20	58	55
miR78971	GCCGTGACCGTGGCGATC	18	63	72
miR49391	GCGGCTGAGGACCACTAG	18	59	67
miR79508	AACTGCGGAATGTACGCCTC	20	58	55
miR40049	TCTTCCCAACCCCTCCCAT	19	59	58
miR67876	TGCCTGGCTCCCTGTATG	18	58	61
miR35203	CGCGAATTGATGCTCAGAAATCCTG	25	59	48
miR73244	CGAGGCACATTGGTTCTCAGAG	23	58	52
miR97580	GGGTTTAGGAAATGTAGGGATGCC	24	58	50

After treatment with 50 mM NaHCO₃ solution, expression of different miRNAs showed variable trends, but the expression levels of most of the miRNAs peaked after 48 h of stress. These miRNAs, miR84046, miR83720, miR56010, miR79508, miR40049, miR35203, miR73244, and miR97580, indicating that these miRNAs responded strongly to alkali stress after 48 h. In addition, the expression levels of miR51167 and miR49391 were highest without stress, indicating that miR51167 and miR49391 responded strongly under NaHCO₃ stress conditions. The expression level of miR78971 reached the maximum at stress treatment 96 h, which indicated that these two miRNAs were not induced expressed under alkali stress. The expression level of miR67876 reached the maximum at stress treatment 12 h, indicating that this miRNA was faster responded to alkali stress at an earlier time than other miRNAs. In conclusion, most of the miRNAs responded strongly to NaHCO₃ stress when the seedlings were treated for 48 h when miR43169 was selected as internal reference genes.

After treatment with 250 mM NaCl solution, we found that most of the miRNAs reached the highest expression at 12 h. These miRNAs included miR83720, miR56010, miR78971, miR40049, miR67876, miR35203, miR73244, and miR97580, and their expression levels start decreased after 12 h, suggesting that they responded strongly to NaCl stress earlier than other miRNAs. There were also miRNAs which reached their maximum expression level after 24 h, such as miR51167, miR49391 and start decreased after 24h. In general, most of these miRNAs faster and stronger respond to NaCl stress at early time points when miR74940 was selected as internal reference genes.

Discussion and Conclusions

Screening of internal reference genes of miRNA after stress

Recent studies have found that miRNAs play an important role in the growth and development of many tissues. The detection of expression levels in different tissues play an important in their function. Therefore, it is especially important to choose the appropriate internal reference miRNA. There are some genes such as 5S rRNA (Tu et al., 2018; Song et al., 2016), 5.8 rRNA (Zhang et al., 2012), and U6 (Dini et al., 2017), which have previously been used as internal reference genes. In previous studies on miRNA expression, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sekalska et al., 2006), actin (Wu et al., 2011), tubulin (Jeong et al., 2006), and 18S rRNA (Miao et al., 2011) were often used as internal reference miRNAs, and many studies of miRNAs are in the context of cancer. There are few studies to screen miRNA internal reference genes in plants, and most of them have used common housekeeping genes to standardize miRNA expression (Dheda et al., 2004). However, recent studies have found that housekeeping genes such as GAPDH are not consistently expressed in different tissue types or under different experimental conditions. For example, GAPDH is one of the two most stable reference genes under drought and cold stress, but not under salt and heat stress, and GAPDH was often used for screening of mRNA reference genes (Li et al., 2020). Therefore, more and more studies are beginning to select U6 snRNA, rRNA or the miRNA of the species itself as the internal reference gene (Zhong et al., 2011). U6 is often chosen as the internal reference gene when studying the expression levels of Arabidopsis miRNAs (Ramachandran and Chen, 2008). U6 or 5S rRNA is usually selected as an internal reference miRNA in rice (Jiao et al., 2010). U6 and 5S rRNA, which act as housekeeping ncRNA, are widely used as reference genes in miRNA studies. U6 is stably expressed in citrus somatic embryogenic cells (Kou et al., 2012). However, many

recent studies have found that miRNAs in plants can be found not only in RNase-enriched cytosol, but also under harsh conditions such as high temperature, extreme pH environments, freeze-thaw cycles, and more. As miRNA research continues to expand, some small RNAs have been obtained from plant species, such as grapevine (Luo et al., 2018), wheat (Feng et al., 2012), peach (Luo et al., 2014), tea (Song et al., 2016), rice (Verstraeten et al., 2019), and sweet potato (Liu et al., 2020), demonstrating that miRNAs are more stable under certain conditions than reference genes commonly used today. Therefore, miRNA can be used as an internal reference gene and are sometimes more stable than the commonly used internal reference genes (Michael and Bowman, 2008). Recently, studies have been performed to screen for miRNAs in many species such as soybean (Kulcheski et al., 2010), wheat (Feng et al., 2012), watermelon (Kong et al., 2014), Chinese Cedar (Zhang et al., 2021), sugarcane (Nandakumar et al., 2021), *Medicago ruthenica* (Guo et al., 2022). miR167 and miR159 were used as the internal reference miRNAs under abiotic and biotic stress in wheat (Feng et al., 2012). However, miR167a is not expressed in the early stages of longan somatic embryonic development and therefore cannot as a reference gene. miR5059 and miR5072 were selected as internal reference miRNAs to study the function of miRNAs in different tissues of peach trees under drought and salt stress (Luo et al., 2014). These studies showed that different species and different internal tissues of the same tree species have different internal reference miRNAs. Therefore, screening of suitable internal reference miRNAs plays an important role in the accuracy of the final test results. Our study found that the selection of internal reference miRNAs at the same tissue differs under different stress conditions. This relatively stable miRNAs and commonly used housekeeping genes were screen out as candidate internal reference miRNAs through small RNA sequencing and additionally, miRNAs from sequencing data were also used as candidate internal reference miRNAs (Peltier and Latham, 2008; Kou et al., 2012). The stability of the candidate internal reference miRNAs was analysed by geNorm and NormFinder. The results showed that miR83321 is the most stable under PEG stress treatment and can be used as the internal reference miRNA. We also found that this same miRNA was the second least stable following NaCl stress treatment, and 5S rRNA was the least stable gene. The expression of miR43169 is the most stable under NaHCO₃ stress treatment, and it can be used as the internal reference miRNA of this stress treatment. However, it ranks 10th in stability following NaCl stress treatment, and the 5S rRNA under this stress treatment is the second least stable. On the other hand, the expression of miR74940 is the most stable under NaCl stress treatment, making it a suitable internal reference miRNA for this stress treatment. In contrast, U6 expression under this stress treatment was found to be the least stable, which is consistent with previous research conducted by Lin and Lai in 2013 on longan embryogenic tissues. In our study, we observed that the ncRNA genes were much less stable compared to the miRNAs. This suggests that the ncRNA genes may be not suitable as internal reference genes for stress treatment tissues in *Larix olgensis*. These findings highlight the importance of

screening for appropriate reference genes under different conditions and treatments to ensure the accuracy of gene expression patterns. It is crucial to note that the most stable internal reference miRNA can vary between different stress treatments. The miRNA that is the most stable in one stress treatment may be the most unstable in another stress treatment. Therefore, it is necessary to carefully select internal reference genes based on the specific test conditions and treatments employed. This will help ensure the reliability and accuracy of gene expression analysis.

Expression analysis of differential miRNAs after stress

Plants have complex regulatory mechanisms to cope with stress, and studies have demonstrated that miRNAs play an important role in a plant's response to multiple stresses. In this study, we employed qRT-PCR to analyze the expression changes of 12 differentially expressed miRNAs during the early growth stage and growth stage of *Larix olgensis* under drought and saline-alkali stress conditions. The results revealed that the expression profiles of different miRNAs varied even under the same stress. Moreover, the same miRNA did not consistently exhibit the same expression trend under the same stress treatments. For instance, the expression levels of miR51167 and miR49391 exhibited a continuous decrease over time following PEG and NaHCO₃ treatments, while under NaCl stress treatment, the expression level first increased and then decreased. In addition, some miRNAs showed similar expression trends after treatment 12 h. Under PEG and NaCl stress, some miRNA such as miR83720, miR56010, miR40049, miR73244, and miR97580, had the highest expression levels, indicating that these miRNAs responded earlier to drought and salt stress. After treatment 48 h, most miRNAs reached their maximum expression level under NaHCO₃ stress, whereas only one miRNA reached the maximum expression after treatment 48 h. Previous studies of the miR398 of *Arabidopsis thaliana* is regulated by almost all stress responses (saline, drought, high temperature, chilling, etc.) (Sunkar and Zhu, 2004). In this study, we found that miR67876 reached the maximum expression level after treatment 12 h under all three of the stress, indicating that miR67876 have an earlier response time to drought and saline-alkali stress. In addition, other miRNAs in this study showed a trend of "dynamic change" between the three different stress conditions, with different response time to each stress, suggesting that the same miRNA may act on multiple target genes simultaneously in response to stress, which is consistent with the previous finding that showed that miR398 of poplar exhibits dynamic changes under salt stress (Jia et al., 2009).

In previous studies, it has been found that the expression profiles of miRNAs varies in different tissues during stress. For example, in soybean, gma-miR169c was found to have the highest expression levels in roots and lowest in stems (Yu et al., 2019). In cotton, the expression profiles of different members of the miR166 family also varied in roots and leaves, and miR166g-5p was significantly upregulated in roots and stems and downregulated in leaves after PEG stress. These results

suggest that miRNAs in different tissues are important for regulating plant adaptation to a stress environment and may be specifically expressed in certain sites, which may play a role in plant growth and development. In conclusion, under certain abiotic stresses, plants can directly or indirectly induce the upregulation or downregulation of certain miRNAs in order to respond in time, which is also a manifestation of plant adaptation to stress.

Declaration of Interest Statement

The authors declare that they have no conflicts of interest associated with this work.

Author Contributions

L. Z and HG. Z planned and organized the study. SF. Z and SS.Y provided the plant material. SF. Z, PY. Y and L. Z performed the experiments. SF. Z and L. Z analysed the data, SF. Z drafted the manuscript.

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