Identification of novel and salt-stress-regulated miRNAs from root of Melilotus officinalis (L.) Pall. by qRT-PCR

Yuxin Zhao
Beijing Forestry University
Huixia Kao
Beijing Forestry University
Xuemei Chen
Beijing Xinghe Landscape Engineering Co., LTD
Tiemei Wang
Beijing Forestry University
Yu Zhang
Beijing Botanical Garden
Yongjiang Sun
Beijing Forestry University
Huijie Xiao
Beijing Forestry University
Shubin Dong
Beijing Forestry University
Jin Cheng (chengjin@bjfu.edu.cn)
Beijing Forestry University https://orcid.org/0000-0001-7041-8283

Research Article

Keywords: high-throughput sequencing, Melilotus officinalis, miRNAs, qRT-PCR, salt stress

Posted Date: January 5th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2411419/v1

Abstract

Salinity is regarded as the main environmental stress, which affects plant growth and physiological functions. miRNAs play crucial roles in plants salt stress response. *Melilotus officinalis* (L.) Pall. (MO) is an excellent forage and honey plant. In addition to its high medicinal values and outstanding ecological effects, MO also has the ability of salt tolerance. However, the miRNA expression mechanism of MO in response to salt stress is still unclear. To reveal the salt tolerance related miRNAs and predict their target genes in MO, we extracted a small RNA library from the roots of MO seedlings without salt treatment and used 300 mM NaCl treated roots of MO seedlings to construct another small RNA library. We identified 93 novel and 98 known miRNAs in control, 100 novel and 115 known miRNAs in case. 27 up-regulation and 20 down-regulated miRNAs were selected, 466 potential target miRNAs and 520 sites were predicted by differential expression analysis. 9 miRNAs were verified by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Our findings summary the miRNAs and their targets for regulating salt defense reactions in MO. The discussion will be helpful to study the molecular mechanisms of salt resistance of MO and other Leguminosae species.

Introduction

Environmental stresses are the most detrimental factors affecting plant growth and development, among them, salt is an important abiotic stresses (Eldem et al. 2012). Therefore, plants develop a variety of physio-molecular processes to adjust to unfavorable stress circumstances (Pennisi 2008). It is significant to reveal the salt response in plants for understanding the abiotic stresses (Deng et al. 2015). The studies of related gene expression and upstream and downstream regulatory networks have been popular topics in plant response to salt stress, making a solid basis for the molecular mechanism of plant salt tolerance. Many previous studies have demonstrated that plants under salt stress could improve their own salt stress resistance by controlling the expression of some miRNAs and their target genes (Zhou et al. 2011).

A class of small non-coding RNAs known as miRNAs are created from precursors and have distinctive stem-loop structures (Bartel 2004; Li et al. 2021). They are post transcriptional negative regulators and essential for long-term growth of plants (Chen 2005; Jones-Rhoades et al. 2006), such as regulation of flowering time (Aukerman and Sakai 2003), leaf and anther development (Palatnik et al. 2003; Mallory et al. 2004; Millar and Gubler 2005), cell differentiation (Garcia 2008), and organ polarity (Rhoades et al. 2002). Moreover, miRNAs also serves as the key players in plant responses to abiotic stresses (Li et al. 2008; Lu et al. 2008).

Melilotus officinalis (L.) Pall. (MO) is originated in Eurasia and is now widely cultivated in agricultural production areas around the world (Kim et al. 2019). MO is an excellent forage and honey plant. As a leguminous plant, it can fix nitrogen, so MO is widely cultivated for soil improvement and naturalized (Kita 1965; Di et al. 2015). A number of studies have explored miRNAs associated with salt stress in leguminous plants (Evans and Kearney 2003; Al Sherif 2009). However, up to now, the function of miRNAs in the molecular mechanism of salt tolerance in MO was still unclear. Our aim was to excavate and identify salt tolerance-related miRNAs and predict their target genes in MO. Herein, we utilized 300 mM NaCl treated roots of MO seedlings to construct a small RNA library compared with another library from the roots of MO seedlings without salt treatment. We also performed high-throughput sequencing and compared the two small RNA libraries in order to obtain differential expression miRNAs and predict their targets. The results will provide a molecular basis to study the regulation of salt stress resistance of MO. Moreover, it may provide a foundation for the selection and germplasm innovation of saline plants.

Materials And Methods

Plant treatment and RNA isolation

Sterilize wild and uniform seeds with 5% (v/v) sodium hypochlorite for 10 minutes, followed by multiple flushes with sterilize distilled water. The treated seeds were sown in a mixed matrix of vermiculite and perlite (3:1), and the seedlings with 2–3 true leaves were salt-stressed with 300 mM NaCl solution for 10 h, with the solution without sodium chloride as the control. For quantitative reverse transcription polymerase chain reaction (qRT-PCR), the roots in the two treatments were collected respectively at 2 h, 4 h, 8 h, 16 h, 32 h, and rapidly cryopreserved with liquid nitrogen. Total RNA was drawed from every sample using RNAQUEOUS KIT, Ambion-1912.

Identification of known and novel miRNAs

Generation of small RNA reads was performed using Illumina analysis (Shanghai OE Biotech, China). Converted basic reads to raw reads via base calling. Adapters or low-quality bases in raw reads may affect follow-up assembly and analysis. To get good-quality and clean reads, reads with low sequencing quality were filtered, and those containing connector primers and poly (A) at the 5′ end of the read sequence were removed. Sequences with fewer than 15 bases or more than 41 bases, and sequences missing adapters and insert tags were excluded.

The sequence length distribution of clean reads in the reference genome was analyzed. Through annotation analysis, these clean reads contained a variety of rRNAs, tRNAs, snRNAs, and snoRNAs. The non-coding RNAs were aligned to GeneBank databases and Rfam v.10.1 by BLAST (Altschul et al. 1990; Griffiths-Jones et al. 2003). The miRNAs with known functions were identified by aligning with the miRBase v.21 database (Griffiths-Jones et al. 2007). The remaining miRNAs were analyzed and predicted to be novel miRNAs using miRDeep2 (Friedländer et al. 2012). The secondary structure of the sequence that could be matched to the genome was examined by RNAfold. The sequence was regarded as a novel miRNA if it contained the hairpin structure in the miRNA precursor (Denman 1993). The miRBase database and the pre-miRNA hairpin structure were used to calculate the associated miRNA star sequence.

Differential expression analysis of miRNAs

The direct reflection of miRNA expression level is its abundance, and the higher its abundance, the higher the expression level. We estimated miRNA expression levels by locating the mature and novel sequences. The expression level of miRNA was assessed through TPM (Transcript per Million) (Sun et al.

2014). Differential expression analysis was designed to identify miRNA with differential expression among the two different samples (Sample-CMX 1 and Sample-CMX 2). The *p*-value for the samples lacking biological replicates was determined using the Audic_Claverie formula (Tino 2009). miRNAs with TPM differential multiple > 2 and *p*-value < 0.05 were screened out. After the differential expression, Heat-map between the two samples was constructed for the differential miRNAs. Based on the hypergeometric distribution, KEGG pathway enrichment analysis and GO enrichment of target genes of the miRNAs were performed using R, respectively.

Prediction of miRNA Target Genes

miRNAs could integrate to the target mRNAs (mainly coding regions) to indirectly regulate gene expression by blocking or inhibiting the translation of the target mRNAs. TargetFinder is used to predict target genes of differentially expressed miRNAs (Fahlgren and Carrington 2010). GO functional analysis is the functional annotation and classification. The GO enrichment analysis uses the total gene or transcription as a background, differences in gene or transcription as a filter from the list of background, hypergeometric test is used to calculate representative GO feature set differences in gene or transcription list whether significant enrichment of *p*-value, then the *p*-value by Benjamini & Hochberg multiple test corrected by FDR (Du et al. 2021).

Verification of miRNAs by qRT-PCR

To investigate differences in miRNAs expression under different treatment conditions, 9 miRNAs with log2foldChange (Sample_CMX2.TPM/Sample_CMX1.TPM) > 2 were selected for qRT-PCR analysis. In our study, total RNA and miRNAs were respectively extracted and separated from the collected roots of different treatments. The PrimerScript RT Reagent Kit (TaKaRa, Japan) was used to reverse transcribe RNA into cDNA. Using QuantiFast ® SYBR ® Green PCR Kit (Qiagen, Germany) in LightCycler ® 480 type fluorescent quantitative PCR reaction (Roche, the Swiss). The quantitative analysis process included reverse transcription (RT) and polymerase chain reaction (PCR). The RT reactions contained 0.5 μg RNA, 5 μL 2 × TS miRNA Reaction Mix, and 0.5 μL TransScrip miRNA RT Enzyme Mix for a total of 10 μL. The reactions were carried out in GeneAmp® PCR System 9700 (Applied Biosystems, USA) at 37 ℃ for 60 min, and then heat inactivated at 85 ℃ for 5 s. Then RT reaction products were after that diluted 10 times with nuclease-free water and kept in reserve at − 20 ℃.

A 10 μL PCR reaction set including 1 μL of cDNA, 5 μL of 2 × PerfectStartTM Green qPCR SuperMix, 0.2 μL universal primer, 0.2 μL microRNA-specific primer, 3.6 μL of nuclease-free water was used to perform qRT-PCR on LightCycler® 480 Real-time PCR Instrument (Roche, Swiss). Conditions and steps for the reactions were as follows: 94 ℃ for 30 s, then 35 cycles of 94 ℃ for 5 s, followed by 60 ℃ for 30 s. Additionally, every sample was run through three times at once. At the conclusion of the PCR cycles, the melting curve was analyzed to ascertain the anticipated specificity result. Using the comparative Ct ($2^{-\Delta\Delta Ct}$) approach, the relative expression levels of different miRNAs in various treated samples were determined (Livak and Schmittgen 2001). The specific primer sequences for miRNAs were devised and synthetized by Shanghai OE Biotech.

Results

Deep Sequencing of MO Small RNAs

Based on Illumina Hiseq sequencing, we obtained two small RNA libraries from the total RNAs of MO roots, sample-CMX1 and sample-CMX2. Raw reads of approximately 20.1 million and 20.7 million were obtained, respectively. After selecting the size and collapsing the reads, approximately 16, 780, 272 clean reads (4, 184, 619 uniq) from sample-CMX1 and 17, 168, 935 clean reads (5, 487, 327 uniq) from sample-CMX2 were obtained and used for further analysis. tRNA, rRNA, snRNA, Cis-reg, snoRNA, known miRNAs and unannotation small RNAs were annotated via clean reads mapped to Rfam database, species repeat sequence library, cDNA sequence and miRBase database. The data was submitted to the GenBank (accession number: PRJNA842188). Among them, 0.47% of known miRNAs and 87.35% of unannotated small RNAs in sample-CMX1, while 1.11% of known miRNAs and 88.22% of unannotated small RNAs in sample-CMX2. The results mean that many small RNAs have not been discovered in MO. Thus, unannotated small RNAs would be used for novel miRNA prediction.

Identification of known and novel miRNAs in MO

To identify novel miRNAs in MO, we compared the sequences of small RNAs with miRBase database (version 21.0) which contained a great quantity of known mature miRNAs. From sample-CMX1 and sample-CMX2, 98 and 115 known miRNAs were identified respectively with transcriptome sequences, which were used to look for inverted repeats and stem-loop structures. For novel miRNAs prediction, the unannotated sequences with length at least 18 bp were mapped to genome sequences by miRDeep2 software. Finally, in Sample-CMX1 and sample-CMX2, a total of 93 and 100 novel miRNAs were detected respectively.

Differential expression analysis of miRNAs

According to comparative analysis, 47 differentially expressed miRNAs were found between the sample-CMX1 and sample-CMX2. Among them, 27 miRNAs expression was up-regulation, while 20 miRNAs expression was down-regulated. 47 miRNAs were analyzed separately using a heat-map based clustering approach (Fig. 1). In general, the clustered miRNAs may have similar biological functions. We identified the specific clusters which were salt stress-responsive in MO roots. Expression profiles of 47 miRNAs included 37 known and 10 novel miRNAs. Among them, 8 known miRNAs (miR162, miR166, miR168, miR171, miR4414, miR319, miR390, miR5037) and 1 novel miRNA (NW_013657516.1_33208) with log2foldChange ratio (Sample_CMX2.TPM/Sample_CMX1.TPM) > 2 were selected for qRT-PCR (Table 1). GO analysis results demonstrated that the target genes were divided into three parts, 10 cellular components, 10 biological processes, and 10 molecular functions (Fig. 2).

Table 1
Differentially expressed miRNAs between Sample_CMX1.TPM and Sample_CMX2.TPM

miRNA_id	Sample_CMX1.TPM	Sample_CMX2.TPM	foldChange	log2foldChange	FDR	Up_
NW_013657516.1_33208	0.059593789659667	1.04840515733795	17.5925237063336	4.13689055196939	0.06290594	Up
miR162	21.1557953291818	93.0750800792245	4.39950749338357	2.13734202906301	9.779262E-28	Up
miR166	10.7268821387401	52.0125447501549	4.84880360177649	2.27762881931862	7.404381E-20	Up
miR168	20.5598574325851	87.6583200996451	4.26356653430468	2.09206077050861	4.967943E-24	Up
miR171	2.14537642774801	16.192035207775	7.54740986166781	2.91598162180828	0.0000000000009284121	Up
miR319	44.6357484550906	199.837671934806	4.47707675689294	2.16255705262598	1.873524E-61	Up
miR390	13.5277902527444	119.634677398452	8.84362302809815	3.14463752955435	3.071032E-108	Up
miR4414	0.357562737958002	2.85399181719775	7.98179316305876	2.99671289392113	0.009098624	Up
miR5037	0.655531686256337	5.64973890343227	8.61855959350684	3.10744677407691	0.00002118254	Up
sample_CMX1.TPM: nutrient solution without NaCl as control group						
sample_CMX2.TPM: 300 mM NaCl for 10 h salt stress treatment						
foldChange(Sample_CMX2.TPM/Sample_CMX1.TPM)						

Prediction of miRNA Target Genes

In total, 466 target miRNAs and 520 target gene binding sites of MO were predicted. Among them, 38 target genes were predicted for selected 9 miRNAs (Table 2). Some known salt-related responsive targets were also found. For example, *GRAS* family transcription factor, a class of plant-specific transcription factor with importance in plant growth, metabolism and stress, was targeted by miR5037. Various *GRAS* family genes have been reported to give play to resist different stresses. Pentatricopeptide repeat (PPR) proteins were targeted by miR5037. Leucine-Rich Repeat Receptor-Like Kinases was targeted by miR390 and played significant roles in effecting plant development, regulation of plant growth hormones, and signal transduction in response to stress (Zan et al. 2013). Studies have shown that the receptor-like kinase OsSIK1 could energetically activate the antioxidant system of plants and give plants the ability to resist drought and salt (Ouyang et al. 2010).

Table 2
Predicted target genes and their protein annotation

MiR id	Accession no. Of miRNA targets	Protein annotation
mtr-miR162	XM_013595222.1	Endoribonuclease dicer-like protein
	XM_003626572.2	Endoribonuclease dicer-like protein
	XM_013592774.1	Transmembrane protein%2C putative
	XM_013592576.1	Transmembrane protein%2C putative
	XM_003617281.2	Transducin/WD40 repeat protein
	XM_003596769.2	Myb-like transcription factor family protein
mtr-miR166e-5p	XM_003588858.2	Adenine nucleotide alpha hydrolase-like domain kinase
	XM_013592181.1	Hypothetical protein
mtr-miR168c-3p	No hit	-
mtr-miR171e-5p	XM_013609397.1	FAD-binding berberine family protein
	XM_013609401.1	FAD-binding berberine family protein
	XM_003598231.1	FAD-linked oxidoreductase
	XM_003598236.2	FAD-linked oxidoreductase
	XM_003598228.1	FAD-linked oxidoreductase
	XM_013589215.1	Pmr5/Cas1p GDSL/SGNH-like acyl-esterase family protein
	XM_013612451.1	Histidyl-tRNA synthetase
	XM_013612138.1	F-box/RNI/FBD-like domain protein
mtr-miR4414a-3p	No hit	-
mtr-miR319d-3p	XM_013609150.1	TCP family transcription factor
	XM_013609743.1	TCP family transcription factor
	XM_013590053.1	TCP family transcription factor
	XM_003592500.1	Hypothetical protein
	XM_013603427.1	Myb transcription factor
mtr-miR390	XM_003604503.2	Hypothetical protein
	XM_003599246.2	Hypothetical protein
	XM_003593327.2	Kinesin motor catalytic domain protein
	XM_003596197.2	LRR receptor-like kinase
NW_013657516.1_33208	No hit	-
mtr-miR5037a > mtr-miR5037b > mtr-miR5037c	XM_003604021.2	Replication factor C subunit%2C putative
	XM_003604021.2	Replication factor C subunit%2C putative
	XM_013613670.1	Haloacid dehalogenase-like hydrolase
	XM_013613673.1	Haloacid dehalogenase-like hydrolase
	XM_013613671.1	Haloacid dehalogenase-like hydrolase
	XM_013613672.1	Haloacid dehalogenase-like hydrolase
	XM_003608835.1	GRAS family transcription factor
	XM_003604689.1	Pyruvate decarboxylase
	XM_003614175.2	Pyruvate decarboxylase
	XM_013609033.1	PPR containing protein%2C putative
	XM_013609034.1	PPR containing protein%2C putative
	XM_013609035.1	PPR containing protein%2C putative
	XM_013609036.1	PPR containing protein%2C putative

Verification of miRNAs by qRT-PCR

We integrated the line diagram of 9 miRNAs expression levels changing with salt stress time (Fig. 3). The miRNAs expression significantly changed with the stress time. In 4 h salt stress treatment of 300 mM NaCl, the expression of miR5037, miR319, miR168 and miR4414 were all down-regulated, while the expression of miR171 was slightly up-regulation. After 8 h of salt stress, the expression levels of miR162, miR166, miR171, miR4414, miR5037 and the novel miRNA (NW_013657516.1_33208) showed a sharp up-regulation trend. However, after 16 h salt stress, the expression changes of 9 miRNAs were significantly divided into two groups. The expression reached the peak value at the 16-hour time node and then dropped sharply in group 1 (miR162, miR166, miR171, miR5037, NW_013657516.1_33208), and their expression even tended to 0 after 32 h of salt treatment. In contrast, the expression of other miRNAs (miR319, miR168, miR4414, miR390) showed a sharp up-regulation trend after 16 h salt treatment and increased even after 32 h.

Discussion

Salinity is a major abiotic factor affecting the growth and physiological functions of plants (Agarwal et al. 2013; Xu and Fujiyama 2013). Currently, the total area of saline soils worldwide is approximately 1.1×10^9 hm^2, and the soil salinization is still globally on the rise (Yang et al. 2021). miRNAs have been proved to play essential roles in plant reactions to salt stress. However, traditional sequencing methods of miRNAs identification are slow and complex. Not only that, the detection results of traditional technology are not comprehensive and accurate. The next-generation high-throughput sequencing technology is highly efficient, rapid and low cost, moreover, it has obvious advantages in miRNAs identification and expression profiling analysis, which has been used in many researches about miRNAs (Wu et al. 2016).

In our study, 20.1 and 20.7 million raw reads were respectively acquired from the untreated and salt-stressed groups by Illumina Hiseq sequencing technology. By TPM analysis (Transcript per Million), 47 differentially expressed miRNAs were predicted from two groups including 27 up-regulation and 20 down-regulated miRNAs. Heat-map of the 47 miRNAs was based to identify which were salt stress-responsive in MO roots. A total of 466 target miRNAs and 520 target gene binding sites of MO were predicted. The results of GO analysis provided useful information about their function in plant growth and development. Through KEGG analysis, some target genes of salt resistance miRNAs in MO roots were matched to pathways related to salt stress, such as cell apoptosis and peroxisomes.

qRT-PCR results manifested that the expression differences of the 9 miRNAs were divided into two groups significantly after 16 h salt stress. The first comprised miR162, miR166, miR171, miR5037 and NW_013657516.1_33208, the expression reached the peak value at the 16-hour time node and then sharply dropped, which may indicate that they were responsive to early salt stress. miR166 as a conserved miRNA family with the highest expression (TPM > 100, 000) was known to regulate the miRNA biosynthesis pathway through cleaving of AGO1. Previous studies have shown that AGO1 had a certain effect on the downstream expression of DCL1, HEN1, and HYL1 through the miRNA pathway, so that it could effectively cut off the target mRNAs (Vaucheret et al. 2004; Cheah et al. 2015). Boualem et al., (2008) that the involvement of miR166-mediated regulation in the morphology of root vascular bundles and root architecture in Leguminosae sp.; miR171 was down-regulated in *Spartina alterniflflora* leaf tissues under salt stress (Qin et al. 2015). miR171 regulated anthocyanin accumulation under phosphate stress conditions through the regulation of GRAS family transcription factors (Li et al. 2018). *GRAS* proteins, plant-specific transcription factors found in various plants, were also targeted by miR5037.

In contrast, the other group (miR319, miR168, miR4414, miR390) showed a sharp up-regulation trend, and increased even after 32 h salt stress treatment. It was indicated the miRNAs played a crucial part in the later stages of salt stress. Zhou et al., (2014) proposed that miR319 was closely related to the salt tolerance of creeping bentgrass. Mature miR319 rapidly accumulated under salt stress and resulted in the down-regulated of at least 4 miR319 target genes (*AsPCF5*, *AsPCF6*, *AsPCF8*, *AsTCP14*). Many researches further confirmed that less Na^+ accumulation in the cytoplasm and reduced toxicity may lead to enhance salt tolerance in plants with OsamiR319a. Interestingly, we found that the transcription factor MYB was involved in secondary metabolic regulation in response to hormones and environmental factors in plants, which emerged as the targets of miR319 and miR162. In addition, F-box proteins, the known target gene that played roles in salt stress response (Chen et al. 2015). Overexpression of F-box protein genes in rice reduced tolerance to abiotic stresses and promoted rice roots growth (Yan et al. 2011).

Herein, we firstly provide and recommend some salt-tolerant miRNAs in MO roots and their target genes are introduced. In recent years, the high-quality arable land area is gradually decreasing, and as one of the important land resources, the improvement and utilization of saline land is one of the key measures to achieve sustainable agricultural development (Abbasi et al. 2016). Planting some plants with salt tolerant and high nitrogen fixation capacity, such as MO, could effectively develop and utilize saline land.

Conclusions

In our study, two small RNA libraries were obtained from the control and salt stress groups of MO and and sequenced by Illumina Hiseq sequencing technology. 115 known and 100 novel miRNAs were identified in the salt stress treatment. 47 miRNAs were differentially expressed, 466 target miRNAs and 520 target gene binding sites were predicted. The differentially expressed miRNAs were analyzed by qRT-PCR, and the expression of 9 miRNAs was verified. The results will facilitate the study of the molecular mechanisms of salt resistance in MO and other species in Leguminosae.

Declarations

Funding This research was funded by the Project of Intergovernmental International Cooperation in Science and Technology Innovation (NO. 2019YFE0116500), National Natural Science Foundation of China (No. 31972948) and the Program of Introducing Talents of Discipline to Universities (111 Project, No. B13007).

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

Author Contributions Shubin Dong designed the experiments and developed the methodologies, Jin Cheng coordinated the project and supervised all methodological steps, Xuemei Chen, Tiemei Wang and Yu Zhang collected some experimental materials, Yuxin Zhao and Huixia Kao participated in part of the experiment and analyzed data, Yongjiang Sun and Huijie Xiao revised the manuscript. All authors have read and approved the manuscript.

Data Availablity The raw fastq reads files can be accessed on NCBI Sequence Read Archive (SRA), accession nos. BioProject: PRJNA842188; BioSample: SAMN28649170.

Consent for Publication All authors have consented for the publication.

References

1. Abbasi H, Jamil M, Haq A, Ali S, Ahmad R, Malik Z, Parveen Z (2016) Salt stress manifestation on plants, mechanism of salt tolerance and potassium role in alleviating it: a review. Zemdirbyste 103(2): 229–238
2. Agarwal PK, Shukla PS, Gupta K, Jha B (2013) Bioengineering for salinity tolerance in plants: state of the art. Mol Biotechnol 54(1): 102–123.
3. Altschul SF, Gish W, Miller W, Myers EW, Lipman D (1990) Basic local alignment search tool. J Mol Biol 215(3): 403–410
4. Al Sherif EA (2009) *Melilotus indicus* (L.) All., a salt-tolerant wild leguminous herb with high potential for use as a forage crop in salt-affected soils. Flora 204(10): 737–746
5. Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. Plant Cell 15(11): 2730–2741
6. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116(2): 281–297
7. Boualem A, Laporte P, Jovanovic M, Laffont C, Plet J, Combier JP, Niebel A, Crespi M, Frugier F (2008) MicroRNA166 controls root and nodule development in *Medicago truncatula*. Plant J 54(5): 876–887
8. Cheah BH, Nadaraja K, Divate MD, Wickneswari R (2015) Identification of four functionally important microRNA families with contrasting differential expression profiles between drought-tolerant and susceptible rice leaf at vegetative stage. BMC Genomics 16(1): 692
9. Chen XM (2005) MicroRNA biogenesis and function in plants. Febs Lett 579(26): 5923–5931
10. Chen Z, Hu L, Han N, Hu J, Yang Y, Xiang T, Zhang X, Wang L (2015) Overexpression of a miR393-resistant form of transport inhibitor response protein 1 (mTIR1) enhances salt tolerance by increased osmoregulation and Na^+ exclusion in *Arabidopsis thaliana*. Plant Cell Physiol 56(1): 73–83
11. Deng PC, Wang L, Cui LC, Feng KW, Liu FY, Du XH, Tong W, Nie XJ, Ji WQ, Song WN (2015) Global identifification of microRNAs and their targets in barley under salinity stress. PLoS One 10(9): e0137990
12. Denman RB (1993) Using RNAFOLD to predict the activity of small catalytic RNAs. Biotechniques 15(6): 1090–1095
13. Di HY, Duan Z, Luo K, Zhang DY, Wu F, Zhang JY, Liu WX, Wang YR (2015) Interspecific phylogenic relationships within genus *Melilotus* based on nuclear and chloroplast DNA. PLoS One 10(7): e0132596
14. Du QX, Guo P, Shi YS, Zhang J, Zheng DY, Li Y, Acheampong A, Wu P, Lin Q, Zhao WG (2021) Genome-wide identification of copper stress-regulated and novel microRNAs in mulberry leaf. Biochem Genet 1–15
15. Eldem V, Çelikkol Akçay U, Ozhuner E, Bakır Y, Uranbey S, Unver T (2012) Genome-wide identification of miRNAs responsive to drought in peach (*Prunus persica*) by high-throughput deep sequencing. PLoS One 7(12): e50298
16. Evans PM, Kearney GA (2003) *Melilotus albus* (Medik.) is productive and regenerates well on saline soils of neutral to alkaline reaction in the high rainfall zone of south-western Victoria. Aust J Exp Agr 43(4): 349–355
17. Fahlgren N, Carrington JC (2010) miRNA target prediction in plants. Methods Mol Biol 592: 51–57
18. Friedländer MR, Mackowiak SD, Li N, Chen W, Rajewsky N (2012) miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucleic Acids Res 40(1): 37–52
19. Garcia D (2008) A miRacle in plant development: role of microRNAs in cell differentiation and patterning. Semin Cell Dev Biol 19(6): 586–595
20. Griffiths-Jones S, Bateman A, Marshall M, Khanna A, Eddy SR (2003) Rfam: an RNA family database. Nucleic Acids Res 31(1): 439–441
21. Griffiths-Jones S, Saini HK, Van DS, Enright AJ (2007) miRBase: tools for microRNA genomics. Nucleic Acids Res 36: D154–158
22. Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol 57: 19–53
23. Kim JO, Ryu TB, Kim MJ, Kim DH, Lee NS (2019) Two unrecorded alien plants of genus *Melilotus* in Korea: *M. officinalis* and *M. indicus* (Leguminosae). Korean J Plant Res 32(1): 63–71
24. Kita F (1965) Studies on the genus *Melilotus* (sweetclover) with special reference to interrelationships among species from a cytological point of view. J Fac Agr Hokkaido U 54(2): 23–122
25. Li WX, Oono Y, Zhu J, He XJ, Wu JM, Iida K, Lu XY, Cui X, Jin H, Zhu JK (2008) The *Arabidopsis* NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. Plant Cell 20(8): 2238–2251
26. Li YY, Luo WR, Sun YD, Chang HC, Ma K, Zhao ZX, Lu L (2021) Identification and expression analysis of mir160 and their target genes in cucumber. Biochem Genet 1–26.
27. Li ZY, Xu HY, Li Y, Wan XF, Ma Z, Cao J, Li ZS, He F, Wang YF, Wan LQ, Tong ZY, Li XL (2018) Analysis of physiological and miRNA responses to Pi deficiency in alfalfa (*Medicago sativa* L.). Plant Mol Biol 96(4): 473–492

28. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4): 402–408
29. Lu SF, Sun YH, Chiang VL (2008) Stress-responsive microRNAs in *Populus*. Plant J 55(1): 131–151
30. Mallory AC, Reinhart BJ, Jones-Rhoades MW, Tang GL, Zamore PD, Barton MK, Bartel DP (2004) MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5' region. Embo J 23(16) 3356–3364
31. Millar AA, Gubler F (2005) The Arabidopsis *GAMYB-like* genes, *MYB33* and *MYB65*, are microRNA-regulated genes that redundantly facilitate anther development. Plant Cell 17(3): 705–721
32. Ouyang SQ, Liu YF, Liu P, Lei G, He SJ, Ma B, Zhang WK, Zhang JS, Chen SY (2010) Receptor-like kinase OsSIK1 improves drought and salt stress tolerance in rice (*Oryza sativa*) plants. Plant J 62(2): 316–329
33. Palatnik JF, Allen E, Wu XL, Schommer C, Schwab R, Carrington JC, Weigel D (2003) Control of leaf morphogenesis by microRNAs. Nature 425(6955): 257–263
34. Pennisi E (2008) The blue revolution, drop by drop, gene by gene. Science 320(5873): 171–173
35. Qin Z, Chen J, Jin L, Duns GJ, Ouyang P (2015) Differential expression of miRNAs under salt stress in *Spartina alterniflora* leaf tissues. J Nanosci Nanotechno 15(2): 1554–1561
36. Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP (2002) Prediction of plant microRNA targets. Cell 110(4): 513–520
37. Sun J, Wang S, Li C, Ren YJ, Wang JQ (2014) Novel expression profiles of microRNAs suggest that specific miRNAs regulate gene expression for the sexual maturation of female *Schistosoma japonicum* after pairing. Parasite Vector 7(1): 1–15
38. Tino P (2009) Basic properties and information theory of Audic-Claverie statistic for analyzing cDNA arrays. BMC Bioinformatics 10(1): 1–9
39. Vaucheret H, Vazquez F, Crété P, Bartel DP (2004) The action of *ARGONAUTE1* in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Gene Dev 18(10): 1187–1197
40. Wu Y, Guo J, Cai YM, Gong XL, Xiong XM, Qi WW, Pang QY, Wang XM, Wang Y (2016) Genome-wide identifification and characterization of *Eutrema salsugineum* microRNAs for salt tolerance. Physiol Plantarum 157(4): 453–468
41. Xu R, Fujiyama H (2013) Comparison of ionic concentration, organic solute accumulation and osmotic adaptation in Kentucky bluegrass and Tall fescue under NaCl stress. Soil Sci Plant Nutr 59(2): 168–179
42. Yan YS, Chen XY, Yang K, Sun ZX, Fu YP, Zhang YM, Fang RX (2011) Overexpression of an F-box protein gene reduces abiotic stress tolerance and promotes root growth in rice. Mol plant 4(1): 190–197
43. Yang JS, Yao RJ, Wang XP, Xie WP, Zhang X, Zhu W, Zhang L, Sun RJ (2021) Research on salt-affected soils in China: History, Status Quo and Prospect. Acta Pedologica Sinica 59(1): 10–27
44. Zan YJ, Ji Y, Zhang Y, Yang SH, Song YJ, Wang JH (2013) Genome-wide identification, characterization and expression analysis of *populus* leucine-rich repeat receptor-like protein kinase genes. BMC Genomics 14(1): 318
45. Zhou M, Li DY, Li ZG, Hu Q, Luo H (2011) Overexpression of a rice microRNA319 gene enhances drought and salt tolerance in transgenic creeping bentgrass (*Agrostis stolonifera* L.). In Vitro Cell Dev-An 47: S37–S37
46. Zhou M, Luo H (2014) Role of microRNA319 in creeping bentgrass salinity and drought stress response. Plant Signal Behav 9(4): 1375–1391

Figures

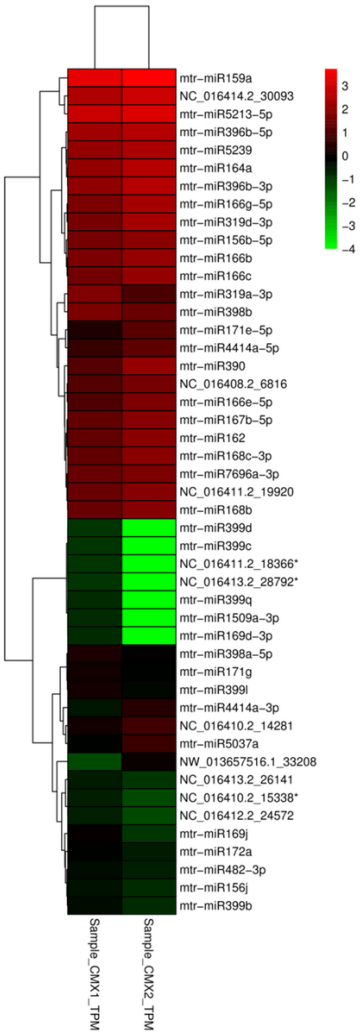

Figure 1

Heatmap based on 47 different expressed miRNAs in MO roots. sample-CMX1-TPM (nutrient solution without NaCl as control group); sample-CMX2-TPM (300 mM NaCl for 10 h salt stress treatment)

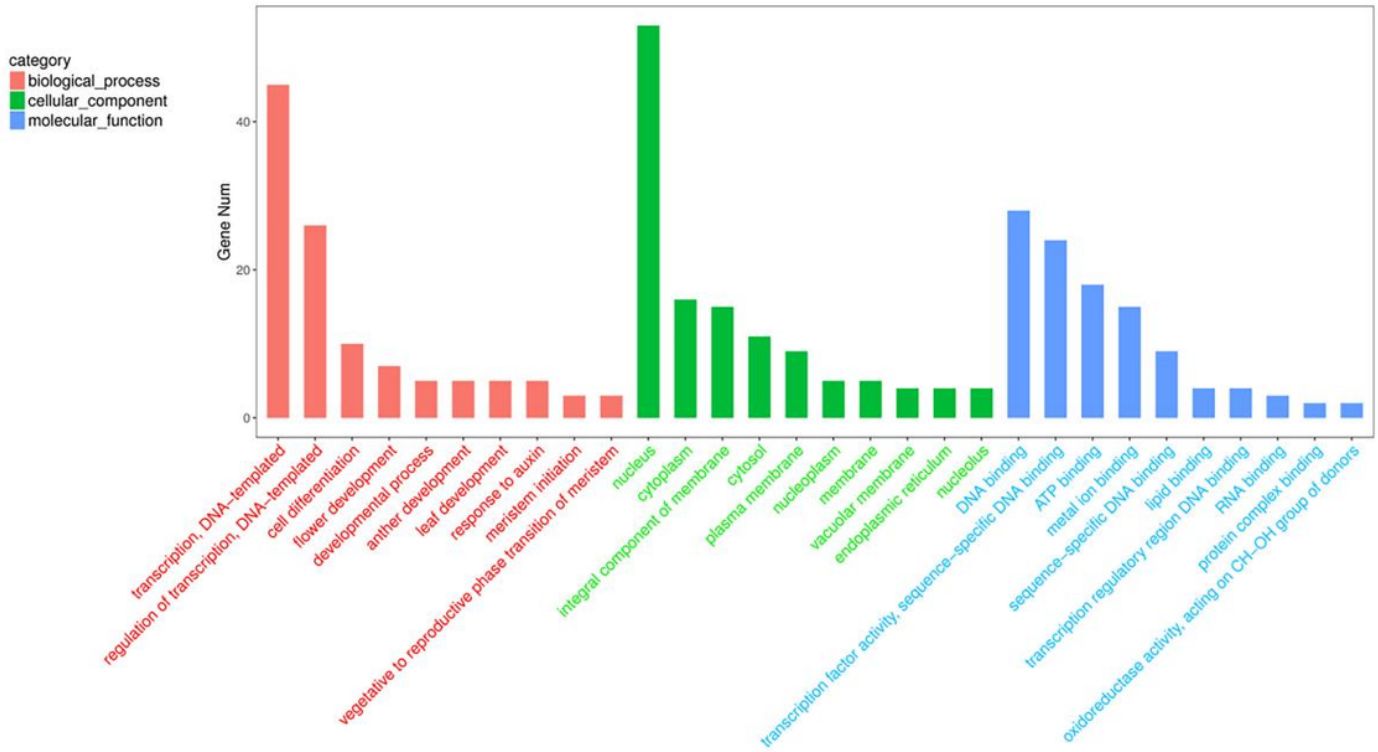

Figure 2

Gene Ontology (GO) enrichment analysis of target genes of the differentially expressed miRNAs

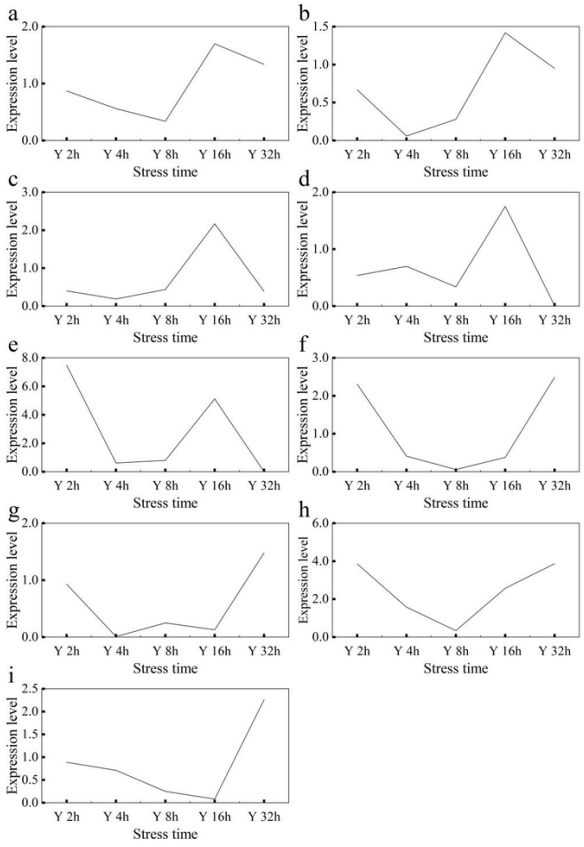

Figure 3

Relative expression of 9 conserved miRNAs changed with salt stress time in MO roots. The 9 miRNAs are NW_013657516.1_33208, miR162, miR166, miR171, miR5037, miR319, miR168, miR4414, miR390 (**a**–**i**)