Global identification and analysis of microRNAs involved in salt stress responses in two alfalfa (Medicago sativa ‘Millennium’) lines
Jin Ma, Yichun Wang, and Jiayun Li

Abstract: Alfalfa is an important economic crop; a mutant (M) strain was identified during planting and production. M plants consistently had better relative water content and relative electrical conductivity under higher salt conditions compared with the wild type (WT) plants, suggesting that M plants have higher tolerance for salt. To understand the microRNAs (miRNAs) involved in salt stress response in alfalfa, 128 miRNAs were identified from the WT and M alfalfa plants under normal and saline conditions. Of the 128 miRNAs, 29 and 23 differentially expressed miRNAs were identified in the M vs. WT control (M-CK vs. WT-CK) and salt-stressed M vs. WT (M-salt vs. WT-salt) comparison, respectively. These miRNAs responded to salt stress and showed different expression patterns after salt treatment. Their potential target genes were predicted and further analysed by GO classification and KEGG pathway analysis, where the majority of target genes were associated with plant growth and development, and exhibited significant changes in WT and M plants. In addition, compared with the WT plants, miR172-CNGC, miR319-CAX2, miR408-NHX and miR2590-CHX14/15 showed significant upregulation in M alfalfa plants, suggesting that M plants have higher ion transport levels. The differential expression profiles of miRNAs and putative target genes were further validated by quantitative real-time polymerase chain reaction. It is speculated that these miRNAs are involved in the increased salt tolerance of the M alfalfa plants.

Key words: microRNAs, salt stress, growth and development, alfalfa.

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Résumé : La luzerne est une culture importante pour l’économie et un mutant (M) a été identifié lors de la plantation et la de production. Sur les sols salinisés, les plants M se caractérisent constamment par une plus grande concentration relative d’eau et une meilleure conductivité de l’électricité que les plants de type sauvage (WT — « wild type »), ce qui laisse supposer qu’ils tolèrent mieux le sel. Pour comprendre comment l’ARNmic (l’ARNmi) concourt à la réaction de la luzerne au stress salin, les auteurs ont identifié 128 ARNmi chez des plants M et WT dans des conditions de culture normales et en présence de sel. Sur les 128 ARNmi, 29 et 23 s’exprimaient différemment lors des comparaisons entre les groupes M-CK c. WT-CK et M-sel c. WT-sel, respectivement. Ces ARNmi ont été associés à la réaction au stress de la salinité et leur expression diffère après l’application de sel. Les auteurs ont prévu les gènes qui pourraient en être responsables et les ont examinés après les avoir classifiés par ontologie génétique et analyse avec la base de données KEGG, qui associe la plupart des gènes à la croissance et au développement de la plante. Les gènes en question présentent d’importantes modifications chez les plants WT et M. En outre, contrairement à ce qui se passe chez les plants WT, les gènes miR172-CNGC, miR319-CAX2, miR408-NHX et miR2590-CHX14/15 semblent passablement régulés en amont chez les plants M, signe que le transport des ions pourrait être plus important chez ces derniers. La différence entre les profils d’expression des ARNmi et les gènes supposément responsables ont été validés par une réaction en chaine par polymérase quantitative en temps réel. Les auteurs spéculent que ces ARNmi concourent à la meilleure tolérance au sel des plants M de luzerne. [Traduit par la Rédaction]

Mots-clés : ARNmicro, stress de la salinité, croissance et développement, luzerne.

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Introduction

High soil salinity is a widespread and serious problem facing irrigated lands throughout the world; soil salinity can lead to lower yields and limited capacity to grow crops. Salinity is one of the most significant abiotic factors that plants are exposed to. Salt tolerance of plants is a very complex phenomenon involving many biological processes and pathways which operate at the whole-plant level (Deinlein et al. 2014). When plants are subjected to salt stress, an early response involving osmotic stress and the accumulation of Na+ and Cl− occurs (Zhu 2001), which inhibits plant growth by affecting metabolic processes and photosynthetic efficiency. Plants experiencing salt exhibit phenotypes of drought, wilting, and even death (Hasegawa 2013). Molecular mechanisms of salt tolerance in plants have been studied in many plant species including model plants, crops, and halophytes (Bartels and Sunkar 2005). Various genes involved in the response to salt stress that regulate ion balance to facilitate the intra- and inter-cellular homeostasis of plants. The plasma membrane Na+/H+ antiporter SOS1 is responsible for mediating Na+ efflux and controls long-distance Na+ transport in plants, and SOS2 and SOS3 regulate SOS1 transport activity in Arabidopsis (Qiu et al. 2002). In addition, one particularly important class of exchangers is the NHX-type, which regulates vacuolar Na+/H+ exchange, thereby maintaining ion balance (Jiang et al. 2010).

Small RNAs (sRNAs) are important regulators of growth and development, and of biotic and abiotic stress responses of plants (Khraiwesh et al. 2012; Dong et al. 2016). Several classes of sRNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs), regulate stress response by altering the expression of target genes at the post-transcriptional level (Leung and Sharp 2010). miRNAs are 20–24 nt short non-coding RNAs that are produced from a precursor hairpin structure, and they downregulate genes by targeting mRNAs for cleavage or translational repression (Bartel 2004). Many miRNAs are associated with the salt response. In Arabidopsis, 12 miRNAs (miR156, miR158, miR159, miR165, miR167, miR168, miR169, miR171, miR319, miR393, miR394 and miR396) are upregulated in response to high salt stress, while no significantly downregulated miRNAs are known to date (Liu et al. 2008). In Populus euphratica Olivier, a total of 211 known miRNAs and 162 new miRNAs were identified under salt treatment (Li et al. 2013). In Vigna unguiculata (L.) Walp. (cowpea), 18 conserved miRNAs belonging to 16 miRNA families are differentially expressed under salt stress using a comparative genomic approach (Paul et al. 2011). Furthermore, Jia et al. (2009) identified miR398 in response to abscisic acid (ABA) and salt stress, and showed its differential and dynamic regulation in Populus tremula L. and Arabidopsis thaliana (L.) Heynh. (Jia et al. 2009). miR169 members (miR169g, miR169n and miR169o) are induced by high-salinity treatment, and transiently inhibit the expression of NF-YA transcription factor (Zhao et al. 2009). Taken together, miRNAs are widely involved in the salt stress response in plants.

Alfalfa is an important economic crop that is used as animal forage. However, salinity significantly limits alfalfa production and quality. Although miRNAs regulate tolerance to salt stress, this has not been widely studied in alfalfa. Here, a mutant of alfalfa with high salt tolerance was identified during planting and production. The aim of this study was to identify miRNAs involved in salt stress response in alfalfa, and their downstream targets, using high-throughput sequencing technology.

Materials and Methods

Plant materials and growth conditions

Wild type (WT) and mutant (M) alfalfa (Medicago sativa L.’Millennium’) were kept in plastic pots and cultured in a greenhouse under 16 h light/8 h dark (25 °C/20 °C) cycles with a relative humidity of 70%–75%. All materials were obtained from Zhejiang Agriculture and Forest University (Zhejiang, People’s Republic of China). After 3 mo of culturing, at least six uniformly sized individual plants were treated with a 250 mmol L−1 NaCl solution, and at least six individuals were treated with water as control. Leaves were harvested at 0 and 72 h of the treatment. All the samples were collected from three randomly selected plants, immediately frozen in liquid nitrogen, and stored at −80 °C.

Determination of physiological parameters

A total of 1 g of fresh leaves (fresh weight, FW) were collected from different salt treatment stages (0, 24, 36, 48, 72, and 96 h after treatment). Relative water content (RWC) was measured according to Yamasaki and Dillenburg (1999). All samples were floated in distilled water for about 24 h to measure the turgid weight (TW). Then the leaves were dried for 30 min at 105 °C, and the dried leaves were used to measure dry weight (DW). The RWC was calculated as RWC (%) = [(FW − DW)/(TW − DW)] × 100. For the relative electrical conductivity (REC), fresh leaves (0.5 g) were obtained from different salt treatment stages (0, 24, 36, 48, 72, and 96 h after treatment). The REC was measured using a conductivity meter (DDS-11A) following methods described by Li et al. (2000). Three biological replicates were measured for each parameter.

RNA extraction and sRNA sequencing

Total RNA was isolated from 0.5 g of leaves using the RNAiso Plus reagent (TaKaRa, Dalian, People’s Republic of China) according to the manufacturer’s protocol. RNase-free DNase I (TaKaRa) was used to remove residual DNA. Equal amounts of leaf RNA from the three independent biological replicates were pooled for sRNA library construction. The 0 and 72 h samples from the WT and M
plants were sequenced using the Illumina HiSeq™ 2000 platform. The datasets are available in the NCBI repository (http://trace.ncbi.nlm.nih.gov/Traces/sra_sub/sub.cgi) under the accession number SRP150034. Adapter sequences were removed from the raw sequencing reads, and 10% of the nitrogen sequences and low-quality (Q < 5) reads were removed to obtain high-quality sequences. In addition, the length distributions of clean reads, total reads, and unique sequences were summarized.

**miRNA annotation and target prediction**

The set of sRNAs (18–30 nt) from the libraries were filtered to remove ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs) and small nuclear RNAs (snRNAs). The remaining sequences were compared with equivalent plant noncoding sRNA sequences deposited in either GenBank (http://www.ncbi.nlm.nih.gov/genbank/) or the Rfam (http://www.sanger.ac.uk/science/tools/rfam) database. The filtered sRNA sequences were aligned in the miRNA database (http://www.mirbase.org) to identify known miRNAs using BLASTN with a maximum of two mismatches. The secondary structures of all known miRNA precursors were obtained using the Mfold version 3.5 software. In addition, the novel miRNAs were predicted using the MIREAP software (http://sourceforge.net/projects/mireap/). miRNAs that had a \(|\log_2(\text{fold change})| \geq +1\) and a \(P\) value < 0.05 were considered significantly differentially expressed. In addition, a reads per minute (RPM) value of zero was assigned a nominal RPM of 0.01. Targets for differentially expressed miRNAs were predicted using the alfalfa genome (ftp://ftp.ncbi.nlm.nih.gov/ genomes/all/GCF/000/219/495/GCF_000219495.3_MedtrA17_4.0/GCF_000219495.3_MedtrA17_4.0_genomic.fna.gz). Target prediction was conducted following methods from Allen et al. (2005). The predicted target genes were functionally classified using the Gene Ontology (GO) database (http://www.geneontology.org) and assigned to a pathway through the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/kegg1.html).

**Real-time quantitative polymerase chain reaction (RT-qPCR) assay**

Total RNA samples were reverse transcribed using a PrimeScript miRNA qPCR starter kit version 2.0 (Takara) following the manufacturer’s protocol. Each RT-qPCR reaction was 20 μL RT-qPCR and contained 10 μL SYBR R Premix Ex Taq™ II (Takara), 5 μL cDNA (100 ng μL⁻¹), 1.0 μL 10 μM forward primer, and 1.0 μL UnimiR qPCR Primer. The primers of target genes were designed using Primer version 5.0, and the sequences are shown in Supplementary Tables S1 and S2. The PCR programme was as follows: 95 °C for 2 min; 40 cycles of 95 °C for 15 s and 55 °C for 15 s; and 72 °C for 20 s. The gene Elongation Factor 1-alpha (EF-1α) (GenBank accession number: JZ818475) was used as the reference gene (Castonguay et al. 2015). Three biological replicates were tested for each sample, and relative expression levels were calculated using the \(2^{-\Delta \Delta CT}\) method (Livak and Schmittgen 2001).

**Results**

**M plants have higher salt resistance than WT plants**

After salt treatment WT plants began to wilt and turn yellow, and some plants died (Fig. 1). In contrast, M plants exhibited higher salt tolerance (Fig. 1). Next, the physiological parameters of the plants, including RWC and REC, were measured after salt treatment. RWC rapidly decreased in WT plants relative to M plants after salt treatment (Fig. 2A). REC values were lower in M plants when compared with WT plants at any length of salt treatment time (Fig. 2B). These results suggested that M plants have higher salt tolerance than WT plants.

**Overview of sRNA profiles in response to salt stress**

The libraries were constructed from leaves of WT (control, WT-CK) and mutant (control, M-CK) alfalfa, and a total of 16 734 794 and 15 093 918 raw reads were generated, respectively. In addition, a total of 17 132 714 and 14 726 403 raw reads were generated from the salt-stressed M and WT (M-salt vs. WT-salt) libraries, respectively (Table 1). Clean reads were obtained from raw reads of the eight libraries after removing adapters, low-quality reads, <18 nt sequences, and poly-A sequences. A total of 16 081 690 (96.2%) and 13 853 206 (91.9%) clean reads resulted in 617 558 and 1 181 185 unique reads in the WT-CK and M-CK libraries, respectively (Table 1). Clean reads were obtained from raw reads of the eight libraries after removing adapters, low-quality reads, <18 nt sequences, and poly-A sequences. A total of 16 089 790 (94.1%) and 13 701 060 (93.2%) clean reads included 981 663 and 964 568 unique reads in the WT-salt and M-salt libraries, respectively (Table 1). In addition, 2 437 924...
(58.70%) and 1 797 183 (59.95%) total reads, and 35 546 (5.76%) and 59 527 (5.04%) unique reads mapped to the alfalfa genome in the WT-CK and M-CK libraries, respectively. Similarly, a total of 1 885 857 (45.68%) and 1 532 793 (52.49%) total reads and 61 261 (6.24%) and 60 775 (6.30%) unique reads mapped to the alfalfa genome in the WT-salt and M-salt libraries, respectively (Table 1).

The length distribution of the sRNAs is shown in Fig. 3. Reads with a length of 21 or 24 nt were abundant in the four libraries, followed by those with a length of 22 nt or 23 nt (Fig. 3). When these libraries were compared with the NCBI GenBank and miRbase databases, many of the sRNAs were matched to different types of sRNAs, including miRNA, siRNA, rRNA, tRNA, snRNA, snoRNA, and repeats. Noncoding sRNAs were removed except for miRNAs for subsequent analysis (Table 2).

### Analysis of differentially expressed miRNAs from WT and M plants

A total of 128 miRNAs were obtained from four libraries (Supplementary Table S3). To explore the expression patterns of miRNAs, differentially expressed miRNAs were identified using the following criteria: $|\log_2(\text{fold change})| \geq 1$ and $P$ value $< 0.05$. The number of differentially expressed miRNAs is shown in Fig. 4. Twenty-nine miRNAs were differentially expressed between the WT-salt vs. WT-CK comparison, and 16 downregulated and 13 upregulated miRNAs were identified under salt stress (Supplementary Table S4).

Similarly, 28 differentially expressed miRNAs were found between the M-salt vs. M-CK. Of these miRNAs, 13 were downregulated, and 15 were upregulated in the M-salt library (Supplementary Table S5). Furthermore, a total of 29 differentially expressed miRNAs were obtained from the M-CK vs. WT-CK comparison, including 17 downregulated and 12 upregulated miRNAs (Table 3). Meanwhile, 23 differentially expressed miRNAs were identified between the M-salt vs. WT-salt comparison, including 10 miRNAs that were upregulated.

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**Table 1.** Summary of reads mapping small RNA from wild type (WT) and mutant (M) alfalfa.

| Reads                  | WT-CK | M-CK | WT-salt | M-salt |
|------------------------|-------|------|---------|--------|
| Raw reads              | 16 734 794 | 15 093 918 | 17 132 714 | 14 726 403 |
| High quality           | 16 717 529 | 15 070 186 | 17 105 831 | 14 707 118 |
| Clean reads            | 16 081 690 (96.2%) | 13 853 206 (91.9%) | 16 089 790 (94.1%) | 13 701 060 (93.2%) |
| Unique reads           | 617 558 | 1 181 185 | 981 663 | 964 568 |
| Total reads mapped to genome | 2 437 924 (58.70%) | 1 797 183 (59.95%) | 1 885 857 (45.68%) | 1 532 793 (52.49%) |
| Unique reads mapped to genome | 35 546 (5.76%) | 59 527 (5.04%) | 61 261 (6.24%) | 60 775 (6.30%) |

**Note:** WT-CK, control wild type; M-CK, control mutant; WT-salt, salt-stressed wild type; M-salt, salt-stressed mutant.
Table 2. The reads count of small RNA among different categories in the four libraries.

| Libraries | miRNA   | rRNA  | tRNA   | snRNA   | snoRNA | repeat | other   |
|-----------|---------|-------|--------|---------|--------|--------|---------|
| WT-CK     | 3 724 650 (23.16%) | 2 533 037 (15.75%) | 107 556 (0.67%) | 41 064 (0.26%) | 26 341 (0.16%) | 873 085 (5.43%) | 8 775 957 (54.57%) |
| M-CK      | 2 055 672 (14.84%) | 4 948 398 (35.72%) | 93 024 (0.67%) | 46 337 (0.33%) | 29 707 (0.21%) | 553 434 (3.99%) | 6 126 634 (44.23%) |
| WT-salt   | 3 400 373 (21.13%) | 5 137 101 (31.93%) | 100 160 (0.62%) | 35 809 (0.22%) | 32 790 (0.20%) | 606 920 (3.77%) | 6 776 637 (42.12%) |
| M-salt    | 2 491 306 (18.18%) | 5 387 906 (39.32%) | 9 304 2 (0.68%) | 34 350 (0.25%) | 32 146 (0.23%) | 463 177 (3.38%) | 5 199 133 (37.95%) |

Note: miRNA, microRNA; rRNA, ribosomal RNA; tRNA, transfer RNA; snRNA, small nuclear RNA; snoRNA, small nuclear RNA; WT-CK, control wild type; M-CK, control mutant; WT-salt, salt-stressed wild type; M-salt, salt-stressed mutant.

Table 3. Differential microRNAs identified from WT-CK and M-CK libraries.

| microRNA | WT-CK Log2FC | M-CK Log2FC | mRNAs | mRNAs |
|----------|---------------|--------------|-------|-------|
| miR156   | 3.96          | 1.74         | -1.19 | 8.22E-03 |
| miR159   | 5.48          | 2.08         | -1.28 | 7.44E-04 |
| miR160   | 2.45          | 0.08         | -1.76 | 3.30E-02 |
| miR166   | 9.15          | 3.63         | -1.49 | 3.30E-06 |
| miR167   | 2.23          | 2.23         | -1.76 | 3.30E-02 |
| miR172   | 0.84          | 1.71         | 1.03  | 3.30E-02 |
| miR319   | 144.58        | 4.04         | -5.16 | 5.26E-16 |
| miR396   | 3299.63       | 14.93        | -7.84 | 3.30E-07 |
| miR398   | 321.25        | 14.16        | -4.50 | 1.43E-06 |
| miR408   | 224.84        | 35.44        | -2.67 | 2.39E-05 |
| miR482   | 4.47          | 44.02        | 3.30  | 8.22E-03 |
| miR1507  | 317.63        | 54.32        | -2.55 | 7.82E-04 |
| miR1509  | 3.51          | 1.27         | -1.47 | 1.17E-03 |
| miR2590  | 118.11        | 2.90         | -5.35 | 1.17E-03 |
| miR2610  | 1.25          | 0.37         | -1.76 | 4.99E-04 |
| miR2616  | 0.25          | 1.33         | 2.41  | 1.16E-03 |
| miR2661  | 10.15         | 1.25         | -3.02 | 7.75E-09 |
| miR3630  | 0.29          | 1.21         | 2.06  | 7.75E-03 |
| miR3711  | 3298.63       | 14.43        | -3.83 | 3.14E-03 |
| miR3696  | 5481.39       | 12.95        | -4.94 | 5.00E-02 |
| miR4414  | 1.82          | 22.93        | 3.66  | 2.05E-08 |
| miR5072  | 4.26          | 16.36        | 1.94  | 6.68E-03 |
| miR5213  | 147.52        | 16.76        | 1.30  | 1.88E-02 |
| miR5214  | 8.94          | 9.94         | 0.25  | 1.06E-05 |
| miR5256  | 2.01          | 0.08         | 1.47  | 9.99E-06 |
| miR5272  | 5.38          | 5.38         | 0.29  | 9.99E-06 |
| miR7696  | 2.45          | 2.45         | 0.08  | 9.99E-06 |
| miR7701  | 0.43          | 0.43         | 0.29  | 9.99E-06 |
| miR7767  | 35.86         | 16.96        | 1.30  | 8.27E-08 |
| miR9749  | 3.91          | 2.34         | -1.86 | 5.43E-04 |

Note: log2FC, log 2-fold change; FDR, false discovery rate; WT-CK, control wild type; M-CK, control mutant.

Fig. 4. The number of miRNAs differentially expressed in the wild type (WT) and mutant (M) alfalfa. WT-CK control wild type; M-CK, control mutant; WT-salt, salt-stressed wild type; M-salt, salt-stressed mutant.
Table 4. Differential microRNAs identified from WT-salt and M-salt libraries.

| microRNA | WT-salt | M-salt | log2FC | P     |
|----------|---------|--------|--------|-------|
| miR156   | 38.22   | 15.19  | −1.33  | 2.29E-06|
| miR160   | 0.02    | 0.22   | 3.46   | 2.60E-03|
| miR166   | 199.04  | 58.42  | −1.77  | 1.22E-02|
| miR168   | 1.30    | 16.93  | 3.70   | 4.99E-09|
| miR169   | 12.47   | 1.05   | −3.57  | 2.37E-02|
| miR319   | 2.59    | 0.50   | −2.37  | 1.35E-03|
| miR390   | 64.62   | 178.35 | 1.46   | 3.50E-03|
| miR395   | 7.64    | 23.11  | 1.60   | 5.21E-05|
| miR396   | 357.25  | 80.67  | −2.15  | 4.07E-05|
| miR397   | 13.01   | 85.73  | 2.72   | 5.12E-05|
| miR398   | 1270.45 | 160.28 | −2.99  | 4.29E-02|
| miR408   | 34.06   | 10.23  | −1.74  | 3.07E-03|
| miR894   | 23.06   | 7.34   | −1.65  | 4.59E-07|
| miR1507  | 23.23   | 3.17   | −2.87  | 2.07E-02|
| miR1510  | 377.23  | 149.68 | −1.33  | 8.60E-06|
| miR2590  | 50.37   | 3.17   | −3.99  | 4.10E-04|
| miR2610  | 0.24    | 0.12   | −1.00  | 1.85E-02|
| miR2612  | 0.01    | 0.17   | 4.09   | 5.02E-03|
| miR2661  | 1.59    | 0.11   | −3.85  | 5.39E-15|
| miR5049  | 0.28    | 1.02   | 1.87   | 3.25E-02|
| miR5211  | 5.93    | 20.22  | 1.77   | 3.63E-02|
| miR7767  | 19.07   | 76.47  | 2.00   | 9.54E-03|

Note: log2FC, log 2-fold change; WT-salt, salt-stressed wild type; M-salt, salt-stressed mutant.

and 13 miRNAs that were downregulated in the M plants (Table 4).

Identification of target genes associated with differentially expressed miRNAs

To further analyse the response to salt stress in the M and WT plants, target genes of the miRNAs that were identified in the previous analysis were characterized using the four libraries through NR (non-redundant), Swiss-Prot, GO, KEGG, Clusters of Orthologous Groups (COG), EuKaryotic Orthologous Groups (KOG) and Pfam databases. A total of 1833, 874, 1698 and 491 potential target genes were predicted for the WT-salt vs. WT-CK, M-salt vs. M-CK, WT-CK vs. M-CK and M-salt vs. WT-salt comparisons, respectively (Supplementary Table S61). The GO functional categories of the target genes were analysed, including cellular components, molecular functions, and biological processes for WT and M alfalfa under salt treatment (Fig. 5). In the cellular components, GO terms associated with cell, cell part, and organelle were the most abundant in the four comparisons. For molecular functions, the most abundant GO terms were binding, catalytic activity, and transport activity. Metabolic process and single-organism process were the two most dominant GO categories in the biological processes. In the KEGG database, these potential genes were assigned to 84 pathways (Supplementary Fig. S13). The results of COG and KOG were further analysed and shown in Supplementary Figs. S2 and S31.

Salt stress impacts plant growth, development, and ion transport. Therefore, the targets of differentially expressed miRNAs involved in growth, development, and ion transport were identified in the WT and M alfalfa (Table 5).

RT-qPCR validation of miRNAs and target genes

Validation of the differential expression of miRNAs and their target genes that were identified using RNA-Seq analysis was performed using RT-qPCR. Ten differentially expressed miRNAs were identified in the WT and M plants (Fig. 6). These miRNAs included miR156, miR166, miR168, miR169, miR319, miR390, miR395, miR396, miR398 and miR408. The other differentially expressed miRNAs are shown in Supplementary Fig. S41. Their target genes that were associated with ion transport, growth, and development were analysed including SPL2 (MTR_8g080680), ARFI (MTR_5g061220), GAI (MTR_3g065980), AGO (MTR_4g094858), GRF5 (MTR_4g125490), CAX2 (MTR_2g105640), CHX14 (MTR_5g074360), NHX (MTR_7g099800), PYL8 (MTR_3g090980), and bZIP60 (MTR_1g050502), and their expression patterns are shown in Fig. 7.

Discussion

miRNAs are an important class of endogenous sRNAs that are widely distributed in plants, where they regulate growth, development, and environmental stress responses by silencing the mRNAs of target genes. Here, the leaf materials from two alfalfa lines with or without salt stress were used to identify the miRNAs involved in plant response to salt stress.

Bioinformatic analysis of miRNAs under salt stress in WT and M alfalfa

Based on the length distribution of sRNAs, 18–30 nt sRNAs were dominant, and 21 and 24 nt sRNAs were abundant in both WT and M libraries, followed by 22 and 23 nt sRNAs (Fig. 3). This is consistent with previous studies in other species, such as *Triticum compactum* Host (Li and Sun 2017), rice (Sunkar et al. 2008), and potato (Zhang et al. 2013). In addition, four libraries of alfalfa exposed to salt stress were sequenced, and a total of 128 miRNAs were identified in the WT and M libraries (Supplementary Table S31). Among these libraries, most miRNAs were differentially expressed in WT and M plants after salt stress (Fig. 4). These results indicated that salt stress might regulate miRNA expression, and that these miRNAs directly or indirectly affect plant growth and development to reduce salt damage (Frazier et al. 2011). Additionally, the differentially expressed miRNAs in M plants are likely involved in the M plant’s ability to adapt to saline conditions.

Analysis of miRNA responses to salt stress in M alfalfa

The M alfalfa plants were more resistant to salinity than the WT plants (Figs. 1 and 2). The expressions of miRNAs associated with salt stress were compared between the M-CK vs. WT-CK and M-salt vs. WT-salt libraries. In total,
29 and 22 differently expressed miRNAs were identified in the M-CK vs. WT-CK and M-salt vs. WT-salt comparisons, respectively (Tables 3 and 4). Compared with WT-CK, miR156, miR159, miR160, miR166, miR167, miR319, miR396, miR398 and miR408 were downregulated in the M plants. A similar result was observed in the WT-salt vs. M-salt comparison, where miR156, miR166, miR169, miR319, miR396, miR398, miR408 and miR894 were also downregulated. These homologous miRNAs are involved in environmental stress responses (Kantar et al. 2011), and they contribute to salinity stress response in many plant species (Sunkar et al. 2012; Xie et al. 2014). They are necessary and sufficient for development and response to environmental stress in Arabidopsis (Stief et al. 2014), and are significantly downregulated during the early stages of salt stress (Jatan et al. 2018). The expression of miR1507, miR1509, miR1510 and miR2590 becomes strongly downregulated after stress treatment in plants (Jiang et al. 2014; Khandal et al. 2017). miR2661 regulates the plant–pathogen interaction and may regulate resistance against Phytophthora infestans (Mont.) de Bary in tomatoes (Luan et al. 2015). In addition, miR172 and miR482 were more upregulated in M-CK than in WT-CK plants (Table 3), but their expression was similar in M-salt vs. WT-salt. After salt treatment, miR160, miR168, miR390, miR395, and miR397 were upregulated in the M plants (Table 4). Overall, these miRNAs were differentially expressed in WT and M plants under normal and salt conditions, and they are likely involved in salt stress response, as well as growth and development of alfalfa, through translational repression or mRNA decay.

**Targets genes associated with salt response in M alfalfa**

Usually miRNAs downregulate or silence their target genes, thereby responding to environmental stresses and modulating plant growth and development (Chen et al. 2010). A series of target genes, such as SPL, GRAS, ARF, GRE, TCP, WRKY, MYB and bHLH (Table 5), were associated with the regulation of salt stress response and the growth and development in the alfalfa plants that were included in this study. Under salt stress these genes were upregulated in M plants compared with WT plants, except for SPL genes. miR156 post-translationally regulate SPL genes, and 35S:MIM156 can increase the sensitivity of stress tolerance in Arabidopsis (Cui et al. 2014). The expression of the SPL2 gene (MTR_8g080680) was significantly decreased in WT and M plants after salt stress (Fig. 7), implying that salt stress negatively affected the growth and development of M and WT plants. The putative target of miR166, GRAS family transcription factor GAI (MTR_3g065980), was upregulated in M-salt compared with WT-salt (Fig. 7). This transcription factor is involved in salt and drought stress response (Golldack et al. 2014). In addition, other potential targets such as miR168-AGO (MTR_4g094858), miR396-GRF5 (MTR_4g125490) and...
miR397-PYL8 (MTR_3g090980), also showed differential expression patterns in the WT and M groups after salt stress (Fig. 7).AGO protein allows RNA silencing to carry out its biological functions (Vaucheret 2008). GRF protein binds to the promoter of dehydration-responsive element binding protein2a (DREB2A) and represses its expression to increase osmotic and heat tolerance (Kim et al. 2012). The 35S:PYL8/RCAR3 transgenic plants show ABA-resistant drought response and strongly inhibit early root growth (Saavedra et al. 2010). In alfalfa, PYL8 (MTR_3g090980) expression was strongly induced after salt stress, demonstrating that PYL8 may upregulate ABA signalling during salt stress in WT and M plants. Furthermore, we found that the target genes miR172-CNGC (MTR_7g074360), miR319-CAX2 (MTR_2g105640), miR408-NHX (MTR_7g099800) and miR2590-CHX14/CHX15 (MTR_5g074360, MTR_5g074380) showed significant differential expression in the M plants after salt treatment, and these target genes were involved in ion transport. Cyclic nucleotide-gated ion channel (CNGC) is an important component of Ca^{2+} signal transduction which regulates various biological processes, such as development and response to salt stress (Saand et al. 2015). CAX2 is an exchanger that maintains intracellular Ca^{2+} ion.

### Table 5. Identified targets for differentially expressed microRNAs responding to salt stress in wild type and mutant alfalfa.

| microRNA ID | Gene name | Annotation |
|-------------|-----------|------------|
| miR156      | SPL2      | Squamosa promoter-binding-like protein 2 |
| miR160      | ARF       | Auxin response factor |
| miR166      | GAI       | DELLA domain GRAS family transcription factor GAI |
| miR167      | ARF6      | Auxin response factor 6 |
| miR168      | AGO       | Argonaute protein |
| miR169      | MATE efflux family protein |
| miR171      | CDPK4     | Calcium-dependent protein kinase 4 |
| miR172      | AP2-like  | AP2 domain transcription factor |
| miR319      | TCP4      | Transcription factor TCP4 |
| miR390      | LOB domain protein |
| miR393      | AUXIN-induced protein 5NG4 |
| miR396      | GRF5      | Growth-regulating factor GRF5 |
| miR397      | E3 ubiquitin-protein ligase |
| miR408      | NIHX      | K(+)H(+) antiporter |
| miR894      | PSY       | Phytoene synthase |
| miR1507     | bZIP60    | bZIP transcription factor 60 |
| miR1510     | WRKY16    | Probable WRKY transcription factor 16 |
| miR2590     | CHX14     | Cation/H(+) antiporter 14 |
| miR2592     | CHX15     | Cation/H(+) antiporter 15 |
| miR5049     | TOM20     | plant-specific import receptor subunit TOM20 |
| miR6214     | ATP-citrate synthase alpha chain protein 2-like |
| miR8736     | Ubiquitin-like-specific protease 1D |

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homeostasis (Kumar et al. 2013). NHX and CHX14/15 are involved in Na\(^+\) transport, and NHX is a salt tolerance determinant that affects cellular ion homeostasis (Deinlein et al. 2014). CHX14/15 were strongly induced by salt stress, and they are involved in K\(^+\) accumulation and homeostasis (Cellier et al. 2004). Taken together, these differently expressed miRNAs play important roles in regulating salt stress in M plants by downregulating target genes, and they likely contribute towards the higher salt tolerance observed in M plants.

**Conclusion**

The M alfalfa plants are more resistant to high salinity than WT plants. Using sRNA-seq results, changes in miRNA expression patterns in M and WT plants under normal and high salt conditions were studied.
Differentially expressed miRNAs and their target genes were identified, and these genes not only responded to salt stress but also contributed to plant growth and development. Furthermore, CNGC (miR172), CAX2 (miR319), NHX (miR408) and CHX14/CHX15 (miR2590) were identified as genes involved in ion exchange and transport that were differentially expressed in M compared with WT. Therefore, miRNAs may regulate these genes to provide salt resistance displayed by M alfalfa plants.

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References

Allen, E., Xie, Z., Gustafson, A.M., and Carrington, J. C. 2005. microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell, 121(2): 207–221. doi:10.1016/j.cell.2005.04.004. PMID:15851028.

Bartel, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116(2): 281–297. doi:10.1016/S0092-8674(04)00045-5. PMID:14744438.

Bartels, D., and Sunkar, R. 2005. Drought and salt tolerance in plants. Crit. Rev. Plant Sci. 24(1): 23–58. doi:10.1080/073526805909010410.

Castonguay, Y., Michaud, J., and Dubé, M.-P. 2015. Reference genes for qRT-PCR analysis of environmentally and developmentally regulated gene expression in alfalfa. Am. J. Plant Sci. 6(1): 132–143. doi:10.4236/ajps.2015.61015.

Cellier, F., Conéjéro, G., Ricaud, L., Luu, D.T., Lepetit, M., Gosti, F., and Casse, F. 2004. Characterization of AtCHX17, a member of the cation/H+ exchangers, CHX family, from Arabidopsis thaliana suggests a role in K+ homeostasis. Plant J. 39(6): 834–846. doi:10.1111/j.1365-3104.2004.02177.x. PMID:15341627.

Chen, X., Zhang, Z., Liu, D., Zhang, K., Li, A., and Mao, L. 2010. SQUAMOSA promoter-binding protein-like transcription factors: star players for plant growth and development. J. Integr. Plant Biol. 52(11): 946–951. doi:10.1111/j.1744-7909.2010.00987.x.

Cui, L.G., Shan, J.X., Shi, M., Gao, J.P., and Lin, H.X. 2014. The miR156-SPL-9-DFR pathway coordinates the relationship between development and abiotic stress tolerance in plants. Plant J. 80(6): 1108–1117. doi:10.1111/tpj.12712. PMID:25345491.

Deinlein, U., Stephan, A.B., Horie, T., Luo, W., Xu, G., and Schroeder, J.J. 2014. Plant salt-tolerance mechanisms. Trends Plant Sci. 19(6): 371–379. doi:10.1016/j.tplants.2014.02.001.

Dong, B., Wang, H., Song, A., Liu, T., Chen, Y., Fang, W., et al. 2016. miRNAs are involved in determining the improved vigor of autotetraploid Chrysanthemum nankingense. Front. Plant Sci. 7: 1412. doi:10.3389/fpls.2016.01412.

Frazer, T.P., Sun, G., Burkiew, C.E., and Zhang, B. 2011. Salt and drought stresses induce the aberrant expression of microRNA genes in tobacco. Mol. Biotechnol. 49(2): 159–165. doi:10.1007/s12033-011-9397-5.

Goldack, D., Li, C., Mohan, H., and Probst, N. 2014. Tolerance to drought and salt stress in plants: unraveling the signaling networks. Front. Plant Sci. 5: 151. doi:10.3389/fpls.2014.00151.

Hasegawa, P. M. 2013. Sodium (Na+) homeostasis and salt tolerance of plants. Environ. Exp. Bot. 92: 19–31. doi:10.1016/j.envexpbot.2013.03.001.

Jatan, R., Chauhan, P.S., and Lata, C. 2018. Pseudomonas putida modulates the expression of miRNAs and their target genes in response to drought and salt stresses in chickpea (Cicer arietinum L.). Genomics, 111: 509–519. doi:10.1016/j.ygeno.2018.01.007. PMID:29336160.

Jia, X., Wang, W.-X., Ren, L., Chen, Q.-J., Mendu, V., Willcut, B., et al. 2009. Differential and dynamic regulation of miR398 in response to ABA and salt stress in Populus tremula and Arabidopsis thaliana. Plant Mol. Biol. 71(1–2): 51–59. doi:10.1007/s11103-009-9508-8.

Jiang, Q., Wang, F., Li, M.Y., Tan, H.-W., Ma, J., and Xiong, A.S. 2014. High-throughput analysis of small RNAs and characterization of novel microRNAs affected by abiotic stress in a local celery cultivar. Sci. Hortic. 169: 36–43. doi:10.1016/j.scienta.2014.02.007.

Jiang, X., Leidi, E.O., and Pardo, J.M. 2010. How do vacuolar NHX exchangers function in plant salt tolerance? Plant Signaling Behav. 5(7): 792–795. doi:10.4161/psb.5.7.11767.

Kantar, M., Lucas, S.J., and Budak, H. 2011. miRNA expression patterns of Triticum dicoccoides in response to shock drought stress. Planta, 233(3): 471–484. doi:10.1007/s00425-010-1309-4. PMID:21069383.

Khandal, H., Parween, S., Roy, R., Meena, M.K., and Chattopadhyay, D. 2017. MicroRNA profiling provides insights into post-transcriptional regulation of gene expression in chickpea root apex under salinity and water deficiency. Sci Rep. 7(1): 4632. doi:10.1038/s41598-017-04906-2.

Khrawiresh, B., Zhu, J.K., and Zhu, J. 2012. Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. Biochim. Biophys. Acta Gene Regulat. Mech. 1819(2): 137–148. doi:10.1016/j.bbagen.2011.05.001.

Kim, J.S., Mizol, S., Kidokoro, S., Maruyama, K., Nakajima, J., Nakashima, K., et al. 2012. Arabidopsis GROWTH-REGULATING FACTOR7 functions as a transcriptional repressor of abscisic acid– and osmotic stress–responsive genes, including DREB2A. Plant Cell. 24: 3393–3405. doi:10.1105/tpc.112.100933. PMID:22942381.

Kumar, K., Kumar, M., Kim, S.R., Ryu, H., and Cho, Y.G. 2013. Insights into genomics of salt stress response in rice. Rice, 6(1): 27. doi:10.1186/1939-8433-6-27. PMID:24280112.

Leung, A.K., and Sharp, P.A. 2010. MicroRNA functions in stress responses. Mol. Cell. 40(2): 205–215. doi:10.1016/j.molcel.2010.09.027.

Li, B., Duan, H., Li, J., Deng, X.W., Yin, W., and Xia, X. 2013. Global identification of miRNAs and targets in Populus euphratica under salt stress. Plant Mol. Biol. 81(6): 525–539. doi:10.1007/s11103-013-0010-y.

Li, H., Sun, Q., Zhao, S., and Zhang, W. 2000. Principles and techniques of plant physiological biochemical experiment. Higher Education, Beijing. pp. 195–197.

Li, B., and Sun, G. 2017. microRNA s contribute to enhanced salt adaptation of the autopolyploid Hordeum bulbosum compared with its diploid ancestor. Plant J. 91(1): 57–69. doi:10.1111/tpj.13546. PMID:28370696.

Liu, H.H., Tian, X., Li, Y.-J., Wu, C.A., and Zheng, C.C. 2008. Principles and techniques of plant physiological biochemical experiment. Higher Education, Beijing. pp. 195–197.

Liu, H.H., Tian, X., Li, Y.-J., Wu, C.A., and Zheng, C.C. 2008. Principles and techniques of plant physiological biochemical experiment. Higher Education, Beijing. pp. 195–197.

Liu, H.H., Tian, X., Li, Y.-J., Wu, C.A., and Zheng, C.C. 2008. Principles and techniques of plant physiological biochemical experiment. Higher Education, Beijing. pp. 195–197.

Luan, Y., Cui, J., Zhao, J., Li, J., Han, L., and Meng, J. 2015. High-throughput sequencing reveals differential expression of miRNAs in tomato inoculated with Phytophthora infestans. Planta, 241(6): 1405–1416. doi:10.1007/s00425-015-2267-7. PMID:25697288.
Paul, S., Kundu, A., and Pal, A. 2011. Identification and validation of conserved microRNAs along with their differential expression in roots of *Vigna unguiculata* grown under salt stress. Plant Cell, Tissue Organ Cult. 105(2): 233–242. doi:10.1007/s11240-010-9857-7.

Qiu, Q.S., Guo, Y., Dietrich, M.A., Schumaker, K.S., and Zhu, J.-K. 2002. Regulation of SOS1, a plasma membrane Na⁺/H⁺ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. Proc. Natl Acad. Sci. 99(12): 8436–8441. doi:10.1073/pnas.122224699.

Saand, M.A., Xu, Y.P., Munyampundu, J.P., Li, W., Zhang, X.R., and Cai, X.Z. 2015. Phylogeny and evolution of plant cyclic nucleotide-gated ion channel (CNGC) gene family and functional analyses of tomato CNGCs. DNA Res. 22(6): 471–483. doi:10.1093/dnares/dsv029. PMID:26546226.

Saavedra, X., Modrego, A., Rodríguez, D., González-García, M.P., Sanz, I., Nicolás, G., and Lorenzo, O. 2010. The nuclear interactor PYL8/RCAR3 of *Fagus sylvatica* FsPP2C1 is a positive regulator of abscisic acid signaling in seeds and stress. Plant Physiol. 152(1): 133–150. doi:10.1104/pp.109.146381. PMID:19889877.

Stief, A., Altmann, S., Hoffmann, K., Pant, B.D., Scheible, W.R., and Bäurle, I. 2014. Arabidopsis miR156 regulates tolerance to recurring environmental stress through SPL transcription factors. Plant Cell, 26(4): 1792–1807. doi:10.1105/tpc.114.123851. PMID:24769482.

Sunkar, R., Zhou, X., Zheng, Y., Zhang, W., and Zhu, J.-K. 2008. Identification of novel and candidate miRNAs in rice by high throughput sequencing. BMC Plant Biol. 8(1): 25. doi:10.1186/1471-2229-8-25.

Sunkar, R., Li, Y.F., and Jagadeeswaran, G. 2012. Functions of microRNAs in plant stress responses. Trends Plant Sci. 17(4): 196–203. doi:10.1016/j.tplants.2012.01.010.

Vacheret, H. 2008. Plant argonautes. Trends Plant Sci. 13(7): 350–358. doi: 10.1016/j.tplants.2008.04.007.

Xie, F., Stewart, C.N., Jr., Taki, F.A., He, Q., Liu, H., and Zhang, B. 2014. High-throughput deep sequencing shows that micro RNA s play important roles in switchgrass responses to drought and salinity stress. Plant Biotechnol J. 12(3): 354–366. doi:10.1111/pbi.12142.

Yamasaki, S., and Dillenburg, L.R. 1999. Measurements of leaf relative water content in *Araucaria angustifolia*. Revista Brasileira de Fisiologia Vegetal, 11(2): 69–75.

Zhang, R., Marshall, D., Bryan, G.J., and Hornyik, C. 2013. Identification and characterization of miRNA transcriptome in potato by high-throughput sequencing. PLoS ONE, 8(2): e57233. doi:10.1371/journal.pone.0057233.

Zhao, B., Ge, L., Liang, R., Li, W., Ruan, K., Lin, H., and Jin, Y. 2009. Members of miR-169 family are induced by high salinity and transiently inhibit the NF-YA transcription factor. BMC Mol. Biol. 10(1): 29. doi:10.1186/1471-2199-10-29.

Zhu, J.K. 2001. Plant salt tolerance. Trends Plant Sci. 6(2): 66–71. doi:10.1016/S1360-1385(00)01838-0.