Integrative analysis of microRNA and mRNA expression in response to salt stress by UMI

Jing jing Zhan

Cotton Research Institute

Yang yang Diao

Cotton Research Institute

Yan yan Zhao

Cotton Research Institute

Chao Li

Cotton Research Institute

Xiao Han

Cotton Research Institute

Long yu Huang

Cotton Research Institute

muhammad sajjad

Cotton Research Institute

Peng Wang

Cotton Research Institute

Li sen Liu

Cotton Research Institute

Qing di Yan

Cotton Research Institute

Xi Wei

Cotton Research Institute

Yan peng Ding

Cotton Research Institute

Yu Chu

Cotton Research Institute

xiao yang Ge (✉ gexiaoyang@caas.cn)

institution of cotton research of CAAS

Research article

Keywords: Cotton, Salinity, miRNA, Transcriptome, Stress responses

Posted Date: July 16th, 2019

DOI: <https://doi.org/10.21203/rs.2.10362/v2>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background Small RNAs play an important role in regulating plant responses to abiotic stress through modulation of the processing, stability, and translation of larger RNAs. Plants employ complex mechanisms of gene regulation to respond to salinity stress. Results In this study, we constructed 12 small RNA libraries, 12 mRNA libraries, and a degradome library to systematically investigate the miRNAs response to salt stress. A total of 312 cotton miRNAs were identified and of these, 80 were known ghr-miRNAs. 144 genes showed significant differential expression under salt stress combined with the targeting relationship between the sRNA data and mRNA data. We found 56 miRNA-mRNA pairs which were positively correlated, and 91 pairs which were negatively correlated. Using degradome sequencing, 72 target genes were identified associated with 25 miRNA families. Gene ontology and KEGG analysis indicated some targets were involved in vital biological pathways of salinity stress tolerance: 14 were involved in responses to abiotic stress, two were associated with an environmental adaptation pathway, and a total of 16 NAC and MYB family transcription factors were related to salinity stress. Conclusions The present study identified a large number of cotton miRNAs and identified relationships between miRNA and mRNA, with function annotation revealing their possible biological roles in response to salt stress. Our findings will further functional studies of cotton miRNAs and the salt tolerance mechanism.

Background

Cotton (*Gossypium hirsutum* L.) is a paramount crop in the world for being a preponderant source of lint and oil. The productivity and quality of cotton are adversely affected by abiotic conditions including soil salinization and land desertification. Increased study of cotton can improve environmental adaptation to salinity [1, 2]. Although upland cotton is a major cultivated cotton with good resistance to a range of stresses, salt stress is still the most severe and wide-ranging factor that limits upland cotton growth and influences its biological and metabolic pathways, which causes huge losses in cotton fiber and seed output worldwide, especially at important periods of growth and development [3, 4]. Many studies has already attempted to elaborate the differential expression of genes in reaction to salt stress through several approaches , such as gene engineering, transcriptome and high-throughput sequencing .in cotton crop and other plant species. A large number of genes play key functions and their related regulatory mechanisms were elucidated. For instance, ABP9 gene, encodes a bZIP transcription factor, transgenic *Arabidopsis* plants showed remarkably improved tolerance to drought, salt and other abiotic stresses [5]. Cotton plants over-expressing ABP9 have enhanced tolerance to salt and osmotic stress [6]. B3 DNA-binding domain proteins and auxin response factor (ARF) are involved in the response to environmental changes [7, 8].

Soil salinity is one of the most damaging abiotic stresses to plants which affects plant growth and even lead to plant death. Plants have evolved a range of self-regulatory contrivances to avoid or resist the salt stress at various phases . Except for those protein-coding genes, other most important in the regulation of gene expression are miRNAs, which are endogenous non-coding RNAs (18nt to 25nt in length) which regulate target genes at the post transcriptional or translation level [9, 10]. Found mostly in eukaryotes,

they include miR156, miR168, miR159, miR167, miR171, miR319 and miR396, and exhibit altered expression levels upon salt stress [11, 12]. miRNAs bind to reverse complementary sequences to form RNA-induced silencing complexes (RISC) which leads to cleavage or translational inhibition of the target RNAs [13]. miRNAs are in this way included in different biological, physiological and molecular processes and elucidate the links with stress response, suchlike plant growth, development, signal transmittal, and tolerance to many biotic and abiotic stresses[14, 15].

RNAseq and PCR are important tools for biological research and detecting the expression level of miRNAs. These techniques have two challenges: one is to limit detection for the small copy numbers and the other is the amplification bias, both reducing quantitative accuracy [16, 17]. Unique molecular identifiers (UMIs) are highly sensitive, accurate, and reproducible and can directly count molecules to correct amplification bias. The UMI method begins with a reverse-transcription reaction using a primer designed with an anchored polyT and a unique barcode. The number of samples have unique barcodes and are essentially unlimited. The quantitative precision of this method is thus improved, especially at low molecule counts [18].

In the study, to get a better comprehension of molecular basis of the salinity stress reaction in cotton , we combined small RNA (sRNA) sequencing with transcriptome and degradome sequencing to identify conserved and novel miRNAs. We analyzed the targets and expression levels of miRNA in twelve libraries. We established relationships between miRNA and mRNA to further investigate the regulatory mechanisms. We found that ghr-miR156a/b, ghr-miR7502, ghr-miR7459a/b, ghr-miR172 and ghr-miR399c have high correlation with Ghir_A02G007740, Ghir_A02G015000, Ghir_A03G019050, Ghir_A12G010210 and Ghir_D11G003180, respectively. These targets are closely linked to stress and ghr-miR156a/b, ghr-miR172, ghr-miR399c are reported to play a critical role in response to stress This study illuminates the siRNAs and putative targets involved in salt tolerance and facilitates the molecular breeding of cotton.

Results

Phenotypic response of E911 to salt treatment

Cotton seedlings grown under a 300mM NaCl solution treatment provided a phenotype of salt stress: the leaves turned yellow and wilted from 0h to 3h, then were partially restored at 6h (Fig. 2A). Furthermore, the conductivity was significantly increased at three time points in leaves under the 300 mM NaCl treatment as compared to the control and became stable after 3h (Fig. 2B). Fresh weight decreased gradually, and finally stabilized, while plant water contents increased gradually (Fig. 2C). Therefore, miRNA-seq and RNA-seq was used to profile the gene expression at each time point and in different tissues to understand the salt stress response in cotton.

Deep sequencing of small RNA libraries

A total of 1,107,343,118 reads were generated from the twelve cotton small RNA libraries generated from the salinity treatment as well as the control, generating 80.37% of the clean readings included small RNA, rRNA, snRNA, snoRNA and tRNA. and degraded fragments of mRNA introns or exons and several other unannotated reads, length ranging from 18nt to 44nt. The sequences of 18–28nt in length, which accounted for 70.31% of all the clean reads, were extracted to analyze small RNA length distribution (Fig. 3). The length of the small RNAs was not evenly distributed in each library, and a high abundance of 21–24nt sequences was found, accounting for 53.97% of the total reads, therefore, it represents the typical length of mature miRNAs in plants. The number of 24nt sequences was significantly more than that of other sequences, followed by 21nt.

Identification of conserved and novel miRNAs

In order to avoid the deviation of sequence composition caused by species differences, we only collected known cotton miRNA for siRNA-seq analysis. Statistical results of every library illustrated that distributions of siRNA families is similar in the CK, 1h, 3h, and 6h salt-treatment samples, including rRNA (~1.83%, ~2.52%, ~3.33% and ~3.10% for the unique reads), snRNA (~0.03%, ~0.07%, ~0.07% and ~0.04% for the unique reads), snoRNA (~0.03%, ~0.05%, ~0.06%, and ~0.05% for the unique reads), and tRNA (~0.31%, ~0.40%, ~0.52% and ~0.42% for the unique reads) (Table 1). sRNAs that differed barely by two mismatches from known miRNAs were characterized as conserved miRNAs within the central miRNA Registry Database, miRBase. The miRNA genes primarily originate from independent transcriptional units, so RNAseq was performed to identify primary transcripts of novel miRNAs. Total reads were derived from 12 sRNA-seq libraries produced under CK, 1h, 3h, and 6h salt-treatment (three biological replicates per treatment). After read cleaning, 2490.2M clean reads were retained and 2090.3M were mapped genome, and a total of 422.2M unigenes were identified. The results of RNAseq were used for mapping reads and prediction of the hairpin structure of precursors of miRNAs. As a result, a total of 232 miRNA sequences were predicted in all libraries (Additional File 1: Table S1). We detected 80 ghr-miRNAs belonging to 53 miRNA families, including 31 miRNA families evolutionarily conserved with more than two members, and 22 non-conserved miRNAs with only one member. Most conserved small RNA families had two or more members, while most non-conserved small RNA families have only one member.

Identification of the targets via online software and degradome analysis

Plant miRNAs usually have perfect or near-perfect complementarity with their targets allowing the identification of targets using TargetFinder and psRobot14 [19, 20] with position-dependent scoring systems to predict miRNA targets. These analyses identified 1662 putative targets (Additional File 2: Figure S1; Additional File 3: Table S2). To further verify the predicted miRNA-target interactions, a degradome library was constructed, generating 28.31M sequence reads. After consecutive steps of filtering, 27.55M reads were obtained which were processed for identification of cleavage sites. Through degradome analysis, a total of 72 targets were identified for miRNAs (Additional File 4: Table S3). The

maximum number of targets was obtained for members of the miR164 family, followed by miR172 and miR393.

Differential transcript levels of miRNA in response to salt stress

The amplex of the conserved miRNAs varied considerably that ranged from 0 to 470,902 in the twelve libraries. In order to distinguish miRNAs responsive to salt stress, we studied the identified miRNAs expression patterns across all libraries. The majority of the identified miRNAs were expressed in more than one sample (Additional File 5: Table S4). Cluster analysis revealed four clusters constructed based on the expression patterns of 56 miRNAs in response to different stress regimes across root, leaf, and stem tissues (Fig. 4). Cluster I contains miRNAs with a high expression pattern; Cluster II shows a low expression pattern. Cluster III contains primarily miRNAs with lower expression in roots; Cluster IV contains many miRNAs with higher expression in roots. 144 miRNAs showed significant differential expression patterns across different combinations. The majority of miRNAs (down-regulated 284 miRNAs VS up-regulated 160 miRNAs) showed a trend of down-regulation under stress conditions, especially in root. These results supported that miRNA expression pattern varied in different tissues, and the same miRNAs may play contrary roles in response to salt stress in different tissues (Fig. 5). Hierarchical clustering analysis of differentially expressed miRNAs was conducted using the heatmap function. First, the intersection analysis found 155 miRNAs differentially expressed between the different samples and union DEGs for nine groups. Hierarchical clustering of the intersection showed two groups, and union DEGs clustered into three groups (Fig. 6). Relative expression level of miRNAs in response to salt stress was analyzed at different time points in different tissues. Among these, miR68 and miR119 exhibited the highest down- and up-regulated changes after salt stress, respectively (Additional File 5: Table S4). There are unique regulation modes for abiotic stresses, and roots, stems and leaves respond to salt stress differently, suggesting that there were special response pathways in different tissues after salt stress

Association analysis of mRNA and small RNA expression data

The expression of stress responsive miRNAs (from small RNA-seq) and their target genes (from RNA-seq) were integrated to infer the mediatory role of miRNAs during stress conditions. Correlation analysis of miRNA and their target mRNA expression profiles using Pearson's correlation coefficient ($r > 0.6$) identified a total of 147 miRNA-mRNA interaction pairs across all combinations under different stress conditions. These correlation pairs comprised 126 genes and 38 miRNAs (Additional File 6: Table S5). 56 pairs were positive correlations and 91 were negative correlation. Multiple potential target genes of the conserved miRNA ghr_miR7504a/b and novel miRNAs novel_mir83 were listed (Additional File 7: Figure S2). These results indicated that a single miRNA has the capability to cleave multiple targets, and a regulatory network consist of different targets responded to salt stress.

GO and KEGG pathway analysis

GO-based analysis lets you to determine which GO terms (biological process, molecular function, and cellular component) a gene belongs to [21]. Therefore, GO-based analysis can deliver better perception to understand miRNA function. We carried out GO enrichment analysis on the substantially up- and down-regulated genes detected by pairwise comparisons in various tissues.

A total of 735 DEGs targets were classified into 464 molecular functions, 754 biological processes, and 567 cellular components (Additional File 8: Figure S3). Twenty-six genes belonged to the biological process of response to stimulus which was most likely associated with resistance, and further analysis showed that Ghir_A02G007740, Ghir_A03G019050, Ghir_A08G008470, Ghir_A09G017710, Ghir_A09G023410, Ghir_A10G002160, Ghir_A11G003190, Ghir_A12G010210, Ghir_D06G002780, Ghir_D08G008560, Ghir_D10G002930, Ghir_D11G003180, Ghir_D11G034240 and Ghir_D12G011240 responded to abiotic stress. Fifty-six genes were associated with the biological process of biological regulation, including Ghir_A02G013350, Ghir_A02G015000, Ghir_A07G005320, Ghir_A08G004810, Ghir_A08G007740, Ghir_A09G000800, Ghir_D10G002930, Ghir_D12G011240, Ghir_D06G020660, Ghir_D08G004960, Ghir_A12G014750 and Ghir_A10G002160, which responded to stimulation. KEGG analysis of a set of DEGs identified 735 targets from 20 pathways (Additional File 9: Figure S4). The genes of the signal transduction and environmental adaptation pathways may be related to salt stress. Of these, Ghir_A04G000780, Ghir_D03G001070, Ghir_D05G038710, Ghir_D05G039730, Ghir_D11G027430, Ghir_A04G000770, Ghir_A11G027330, Ghir_D05G038660, Ghir_D05G038670, Ghir_D05G038680, Ghir_D05G038730, Ghir_D05G038740 and Ghir_D11G030710 (ko04626//Plant-pathogen interaction, ko04016 //MAPK signaling pathway - plant) can respond to environmental stress. In addition, the considerably enriched pathways encompass that of plant hormone signal transduction, and those genes may be involved in regulation of stress by plant hormones.

Discussion

As an important drought-tolerant crop, cotton provides an ideal system to study drought tolerance. Under environmental stress, some miRNAs are induced or inhibited to regulate the downstream targets in plants [22, 23]. For example, high osmotic stress decreased the miR167a expression, whereas IAA-Ala Resistant 3 (IAR3), a target of miR167a, showed increased expression level responding to drought tolerance [24]. The expression level of osa-MIR393 depicts obvious downregulated expression under salinity stress, and target genes of osa-MIR393 were activated to responding to abiotic stress. The osa-MIR393 over-expressed transgenic rice and Arabidopsis showed more susceptibility to salt [25]. Constitutively over-expressing osaMIR396c showed reduced salt and stress tolerance in plants [26]. miR399f participates in plant responses to abiotic stresses, especially salt and drought [27]. miR417 plays a role as a negative regulator of seed germination in *Arabidopsis* under salt stress conditions [28]. Increasing evidence indicates that miRNAs play important roles in plant response to drought. Many stress related mRNAs including the NAC, MYB and MAPK families are regulated by known miRNAs, and play important roles in drought and salinity environments [29, 30]. Target prediction as well as degradome sequencing data revealed that a set of cotton miRNAs are allied with these top-poster genes including miR167-IAR3, miR172-AP2 and miR164-NAC [31, 32]. As expectedly, degradome sequencing data in our research

confirmed those stress-related miRNA targets in cotton (Additional File 4: Table S3.), supporting the data obtained using the UMI method was available.

RNAseq is a powerful tool for transcriptome analysis in tissues. Even so, shortcomings in cDNA synthesis, and bias in cDNA amplification cause extreme quantitative errors. To correct for amplification bias, UMIs is used for direct molecular counting. This method corrects for PCR errors and provides an absolute scale of measurement with a defined zero level. Standard RNA-seq on the other hand obscure dissimilarities in total mRNA content, by using relative measures such as reads per kilobase per million reads (RPKM). In this study, we counted molecules by the total number of distinct UMIs to quantitatively assess miRNA-seq data, and provide the accurate expression patterns of the miRNAs response to salt stress in cotton .

In this study, conserved miRNAs miR164, miR167, miR399, miR7052 and novel miRNAs mir45, mir46, mir223, mir227, mir1, mir68 and mir86 had significant differential expression. Compared with the 0h samples, mir1, mir68 and mir86 all showed down-regulation at 1h, 3h and 6h, and mir45, mir46, mir223 and mir227 showed up-regulation at 1h, 3h and 6h. Similar to the results of previous studies, we found that miR164, miR167 and miR399 in cotton were responded to salt stress [23, 27, 28]. Therefore, we hypothesized that mir45, mir46, mir223, mir227, mir1, mir68 and mir86 may also be closely related to salt stress. In future studies, we will verify these presumed results. In addition, there are a number of unannotated small RNA sequences in the small RNA data which are likely to include functional small RNAs such as siRNA with functions yet to be revealed. Therefore, continued efforts are needed to identify the complete set of miRNAs and other small RNAs from cotton.

Conclusion

In this study, miRNA and mRNA expression pattern in response to salt stress was firstly detected by UMIs ,and provided the more accurate expression profile and inhibited the bias produced by PCR amplification . Our results indicate that miRNA-mRNA pairs participate in gene expression regulation and response to salt stress. The discovery of these miRNAs and their function allows better understanding of salt stress mechanisms in cotton and other drought-resistant crops.

Methods

Plant materials and stress treatment

The salt-tolerant cotton cultivar 'E911' (E911 was provided by the Institute of Cotton Research of Chinese Academy of Agricultural Sciences) was used in this study to test miRNA-target response to salt stress. Seeds were sterilized for 2h at 28 °C and then germinated on 1/2 MS medium under a 16h light/8h dark cycle. After 3-5 d, better germinated seedlings were transferred to pots enclosing aerated Hoagland nutrient solution [33]. Growth conditions of seedlings were 28/20°C day/night temperature, 55-70% relative humidity, and a 14/10h light/dark cycle. At the three-true leaf stage, seedlings showing normal

growth were randomly divided into two groups: one was treated with 300mM salt stress, and experienced salt treatment for 1 h, 3 h and 6 h. The remaining seedlings served as the control. In all experiments, 50 seedlings were used. After salt-treatment the seedlings, we measured relative electric conductivity and plant water content [34]. Then, roots, stems and leaves were harvested, immediately plunged in liquid nitrogen and stored at -80°C for lateral RNA isolation.

Construction of small RNA libraries

Before small RNA and mRNA library construction, total RNA was isolated and purified using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions and was then tested using the Agilent 2100 bioanalyzer system to guarantee RNA quality. The samples of mock, 1h, 3h, and 6 hours' salt-treatment with three biological replicates were executed, in total 36 small-RNA and RNA-seq libraries for sequencing. The general process used for constructing the small RNA library is as follows: small RNA 18 to 30nt in length were isolated on a 15% polyacrylamide gel and the 5-adenylated and 3-blocked adaptor were ligated to the 3' end of the small RNA fragment. Unique molecular identifiers (UMI) [35] labeled Primer (8-10nt sequences) were added and 5' end adaptor was ligated, and the unligated adaptor was digested. Purified RNAs were reverse-transcribed to cDNA with UMI labeled primer and the library was validated (Fig. 1). Ultimately, all prepared RNA samples were sequenced at the Beijing Genomics Institute (BGI) in Wuhan using an Illumina Genome Analyzer.

Identification of conserved and novel miRNAs

To evacuate the adulterations of raw data and the low quality reads, raw reads from small-RNA libraries were screened.(5' primer contaminants, no-insert tags, oversized insertion tags, low quality tags, poly A tags and small tags, and/or tags without 3' primer) to obtain clean reads. The clean sequences were used to search GenBank and the Rfam database [36] to annotate rRNA, tRNA, snRNA and snoRNA. After eliminating sequences of snRNAs, snoRNAs, rRNAs and tRNAs sequences, the left over sequences were used in a blast hunt against miRBase18 (<http://www.mirbase.org/>) to identify conserved miRNA[37]. Only the sequences containing less than two mismatches that known miRNAs in miRBase were considered as conserved miRNAs.

Degradome sequencing of 5' RACE libraries and data analysis

Degradome sequencing provides a method for prediction the miRNA-target interactions. The libraries were constructed according to the previous description [38]. Twenty micrograms of control and salt-treated RNA samples were used for degradome sequencing. Briefly, a 5'RNA adapter was ligated to the cleavage products, which possessed a free phosphate at the 5'end. The purified ligated products were reverse-transcribed to cDNA. After amplification, The Mmel enzyme was used to digest the PCR products and ligated to the 3'adapter... The Illumina Genome Analyzer was used to amplify and sequence the final cDNA library, resulted in 49nt raw reads. After filtering, the clean data were classified by alignment to the

database, and ncRNAs were removed. Finally, the miRNA–mRNA pairs were identified and mapped to the reference genes and the true miRNA cleavage site.

GO and KEGG analysis

Bioinformatics analysis uncovers the miRNA-gene regulatory network on the basis of biological process and molecular function. The functional enrichment analysis was applied to assign gene ontology (GO) annotations (<http://www.geneontology.org/>), exploring the candidate genes associated with miRNAs which may play important biological functions in cotton. Similarly, The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used, to assign the pathway annotations (<http://www.genome.jp/kegg/>). Gene numbers for every GO term and pathway and hyper geometric distribution were used to detect significantly enriched GO terms and pathways with a P-value threshold of ≤ 0.05 .

Differential expression analysis

UMI was connected to cDNA molecules by marking each molecule in the original sample at the early stage of library construction. It was used to reduce the quantitative bias introduced by PCR amplification and is conducive to obtaining sufficient readings for testing. We calculated the species numbers for accurate quantitative comparison of sRNAs. The expression levels of known miRNAs in each library were calculated and the miRNA expression levels between different samples were compared.

DEGseq [39] is novel method based on the MA-plot, which is a statistical analysis tool widely used to detect and visualize intensity-dependent ratio of microarray data [40]. To improve accuracy of the DEG results, we defined a gene as a DEG (differentially expressed gene) when reads number fold change was ≥ 2 and Q-value was ≤ 0.001 .

Association analysis of miRNAs and mRNAs

Pearson correlation coefficients (r values) between expression rates of small RNA and mRNA were constructed and calculated by Graph Pad Prism version 6 with two-tailed *t*-tests. A P-value < 0.05 was regarded statistically significant. We screened the differentially expressed microRNAs and their predictive target genes. Based on the difference between processed and control samples, the interaction between the genomic data and the gene targeting relationship was shown with VisNetwork.

Statistical Analysis

The statistical significance of expression profiles of miRNAs and genes was compared with one-way analysis of variance (ANOVA), followed by a Duncan's multiple range test. All data were analyzed by SPSS10.0 software. P-values < 0.05 were regarded as statistically significant.

Declarations

Acknowledgements

The authors wish to thank the National Natural Science Foundation of China (grants 31621005 and 31701476) for the financial support provided to this project.

Availability of data and materials

The supporting data are included within the article and additional files and authors are pleased to share analyzed/raw data and plant materials upon reasonable request.

Funding

This work was supported by funding from the National Natural Science Foundation of China (grants 31621005 and 31701476).

Authors' contributions

JZ, YD and XYG designed the experiments and drafted the manuscript. LSL, QDY and XW prepared samples for small RNA sequencing. JZ, CL, XH, LYH, MS, YPD, YC and PW performed the high-throughput sequencing data analysis. YD and YZ contributed in the design and discussion of the work, and assisted in drafting the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Reference

1. Yan R, Liang C, Meng Z, Malik W, Zhu T, Zong X, Guo S, Zhang R: Progress in genome sequencing will accelerate molecular breeding in cotton (*Gossypium* spp.). *Biotech* 2016, 6(2):217.
2. Wei Y, Xu Y, Lu P, Wang X, Li Z, Cai X, Zhou Z, Wang Y, Zhang Z, Lin Z: Salt stress responsiveness of a wild cotton species (*Gossypium klotzschianum*) based on transcriptomic analysis. *Plos One* 2017, 12(5):e0178313.

3. Munns R, Tester M: Mechanisms of salinity tolerance. *Annual Review of Plant Biology* 2008, 59(1):651-681.
4. Ashraf M: Salt Tolerance of Cotton: Some New Advances. *Critical Reviews in Plant Sciences* 2002, 21(1):1-30.
5. Zhang X, Wang L, Meng H, Wen H, Fan Y, Zhao J: Maize ABP9 enhances tolerance to multiple stresses in transgenic Arabidopsis by modulating ABA signaling and cellular levels of reactive oxygen species. *Plant Molecular Biology* 2011, 75(4-5):365-378.
6. Wang C, Lu G, Hao Y, Guo H, Guo Y, Zhao J, Cheng H: ABP9, a maize bZIP transcription factor, enhances tolerance to salt and drought in transgenic cotton. *Planta* 2017, 246(6):1-17.
7. Fedoroff NV: Cross-Talk in Absciscic Acid Signaling. *Sci Stke* 2002, 2002(140):re10.
8. Himmelbach A, Yang Y, Grill E, Grill E: Relay and control of abscisic acid signaling. *Current Opinion in Plant Biology* 2003, 6(5):470-479.
9. Rae Eden Y, Ju KY, Xigang L, Ruozhong W, Junhui D, Langtao X, Xuemei C: POWERDRESS and diversified expression of the MIR172 gene family bolster the floral stem cell network. *Plos Genetics* 2013, 9(1):e1003218.
10. Dong C, Song Y, Tian M, Zhang D: Methylation of miRNA genes in the response to temperature stress in *Populus simonii*. *Frontiers in Plant Science* 2015, 6(173).
11. Cui J, Sun Z, Li J, Cheng D, Luo C, Dai C: Characterization of miRNA160/164 and Their Targets Expression of Beet (*Beta vulgaris*) Seedlings Under the Salt Tolerance. *Plant Molecular Biology Reporter* 2018, 36(5-6):790-799.
12. He F, Xu C, Fu X, Shen Y, Guo L, Leng M, Luo K: The microRNA390/TRANS ACTING SHORT INTERFERING RNA3 module mediates lateral root growth under salt stress via the auxin pathway. *Plant Physiology* 2018, 177(2):pp.01559.02017.
13. Axtell MJ: Classification and comparison of small RNAs from plants. *Annual Review of Plant Biology* 2013, 64(1):137-159.
14. Babar IA, Slack FJ, Weidhaas JB: miRNA modulation of the cellular stress response. *Future Oncology* 2008, 4(2):289-298.
15. Lu XY, Huang XL: Plant miRNAs and abiotic stress responses. *Biochem Biophys Res Commun* 2008, 368(3):458-462.
16. Hashimshony T, Wagner F, Sher N, Yanai I: CEL-Seq: single-cell RNA-Seq by multiplexed linear amplification. *Cell Reports* 2012, 2(3):666-673.

17. Ramsköld D, Luo S, Wang YC, Li R, Deng Q, Faridani OR, Daniels GA, Khrebtukova I, Loring JF, Laurent LC: Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells. *Nature Biotechnology* 2012, 30(8):777-782.
18. Islam S, Kjällquist U, Moliner A, Zajac P, Fan JB, Lönnerberg P, Linnarsson S: Characterization of the single-cell transcriptional landscape by highly multiplex RNA-seq. *Genome Research* 2011, 21(7):1160.
19. Fahlgren N, Carrington JC: miRNA Target Prediction in Plants. *Methods Mol Biol* 2010, 592:51-57.
20. Wu HJ, Ma YK, Chen T, Wang M, Wang XJ: PsRobot: a web-based plant small RNA meta-analysis toolbox. *Nucleic Acids Research* 2012, 40(Web Server issue):22-28.
21. Eilbeck K, Lewis SE, Mungall CJ, Yandell M, Stein L, Durbin R, Ashburner M: The Sequence Ontology: a tool for the unification of genome annotations. *Genome Biology* 2005, 6(5):R44.
22. Jones-Rhoades MW, Bartel DP: Computational Identification of Plant MicroRNAs and Their Targets, Including a Stress-Induced miRNA. *Molecular Cell* 2004, 14(6):787-799.
23. Khraiweh B, Zhu JK, Zhu J: Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochimica Et Biophysica Acta* 2012, 1819(2):137-148.
24. Natsuko K, Huan W, Hiroyuki K, Jun L, Cameron M, Yasunori M, Yuji K, Hannah MA, Nam-Hai C: IAA-Ala Resistant3, an evolutionarily conserved target of miR167, mediates Arabidopsis root architecture changes during high osmotic stress. *Plant Cell* 2012, 24(9):3590-3602.
25. Peng G, Xi B, Liang Y, Dekang L, Xin P, Yong L, Hua C, Wei J, Qin C, Yanming Z: osa-MIR393: a salinity- and alkaline stress-related microRNA gene. *Molecular Biology Reports* 2011, 38(1):237.
26. Peng G, Xi B, Liang Y, Dekang L, Yong L, Hua C, Wei J, Dianjing G, Yanming Z: Over-expression of osa-MIR396c decreases salt and alkali stress tolerance. *Planta* 2010, 231(5):991-1001.
27. Baek D, Chun HJ, Kang S, Shin G, Su JP, Hong H, Kim C, Kim DH, Sang YL, Min CK: A Role for Arabidopsis miR399 in Salt, Drought, and ABA Signaling. *Molecules & Cells* 2016, 39(2):111-118.
28. Jung HJ, Kang H: Expression and functional analyses of microRNA417 in Arabidopsis thaliana under stress conditions. *Plant Physiology & Biochemistry* 2007, 45(10):805-811.
29. Kim JH, Nguyen NH, Chan YJ, Nguyen NT, Hong SW, Lee H: Loss of the R2R3 MYB, AtMyb73, causes hyper-induction of the SOS1 and SOS3 genes in response to high salinity in Arabidopsis. *Journal of Plant Physiology* 2013, 170(16):1461-1465.
30. Liu G, Li X, Jin S, Liu X, Zhu L, Nie Y, Zhang X: Overexpression of rice NAC gene SNAC1 improves drought and salt tolerance by enhancing root development and reducing transpiration rate in transgenic cotton. *Plos One* 2014, 9(1):e86895.

31. Man Z, Dayong L, Zhigang L, Qian H, Chunhua Y, Lihuang Z, Hong L: Constitutive expression of a miR319 gene alters plant development and enhances salt and drought tolerance in transgenic creeping bentgrass. *Plant Physiology* 2014, 161(4):1375-1391.
32. Millar AA, Frank G: The Arabidopsis GAMYB-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development. *Plant Cell* 2005, 17(3):705-721.
33. Yao D, Zhang X, Zhao X, Liu C, Wang C, Zhang Z, Zhang C, Wei Q, Wang Q, Yan H: Transcriptome analysis reveals salt-stress-regulated biological processes and key pathways in roots of cotton (*Gossypium hirsutum* L.). *Genomics* 2011, 98(1):47-55.
34. Dai HF, Hui WU, AmanguliMaimaitiali, Wang LH, MaimaitiApizi, Zhang JS: Analysis of Salt-Tolerance and Determination of Salt-Tolerant Evaluation Indicators in Cotton Seedlings of Different Genotypes. *Scientia Agricultura Sinica* 2014, 47(7):1290-1300.
35. Saiful I, Amit Z, Simon J, Gioele LM, Pawel Z, Maria K, Peter LN, Sten L: Quantitative single-cell RNA-seq with unique molecular identifiers. *Nature Methods* 2014, 11(2):163-166.
36. Gardner PP, Jennifer D, Tate JG, Nawrocki EP, Kolbe DL, Stinus L, Wilkinson AC, Finn RD, Sam GJ, Eddy SR: Rfam: updates to the RNA families database. *Nucleic Acids Research* 2009, 37(Database issue):136-140.
37. Romanel E, Silva TF, Corrêa RL, Farinelli L, Hawkins JS, Schrago CEG, Vaslin MFS: Global alteration of microRNAs and transposon-derived small RNAs in cotton (*Gossypium hirsutum*) during Cotton leafroll dwarf polerovirus (CLRDV) infection. *Plant Molecular Biology* 2012, 80(4-5):443-460.
38. Hafner M, Landgraf P, Ludwig J, Rice A, Ojo T, Lin C, Holoch D, Lim C, Tuschl T: Identification of microRNAs and other small regulatory RNAs using cDNA library sequencing. *Methods* 2008, 44(1):3-12.
39. Likun W, Zhixing F, Xi W, Xiaowo W, Xuegong Z: DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics* 2010, 26(1):136-138.
40. Yee Hwa Y, Sandrine D, Percy L, Lin DM, Vivian P, John N, Speed TP: Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Research* 2002.

Table

Table1.Dataset summary of small RNAs from different salt-treatment.

category	WT	1h	3h	6h
total reads	258,594,758	273,103,499	297,933,959	277,710,902
high quality	255,979,918	270,838,290	295,260,105	275,601,191
ployA	7024	8461	6277	8227
invalid	6,820,607	7,961,725	10,141,136	6,377,366
clean reads	222,965,736	224,541,353	219,152,920	223,053,752
Unique sRNAs	213,062,991	211,618,778	209,845,637	212,424,881
Mapped to genome	179,572,503(84.28%)	192,924,234(90.55%)	183,927,175(86.33%)	196,629,412(92.29%)
Exon	3,981,290(1.87%)	4,539,444(2.13%)	5,370,304(2.52%)	5,422,106(2.54%)
miRNA	1,341,383(0.63%)	1,140,948(0.54%)	997,800(0.47%)	1,067,406(0.50%)
snoRNA	66,721(0.03%)	101,831(0.05%)	131,388(0.06%)	105,628(0.05%)
tRNA	655,924(0.31%)	844,016(0.40%)	1,099,397(0.52%)	887,019(0.42%)
snRNA	74,014(0.03%)	140,876(0.07%)	155,570(0.07%)	86,163(0.04%)
rRNA	3,896,863(1.83%)	5,376,499(2.52%)	7,091,959(3.33%)	6,605,936(3.10%)

Additional File Legend

Additional File 1: Table S1. Sequence of novel-miRNAs

Additional File 2: Figure S1. Summary of miRNA target prediction. Venn diagram of miRNA targets predicted by different softwares. Red and blue represent the result of TagetFinder and TAPIR, respectively.

Additional File 3: Table S2. Prediction miRNA targets using different software

Additional File 4: Table S3. Targets of new miRNA verified by degradome sequencing

Additional File 5: Table S4. Differentially expressed miRNAs in different tissues

Additional File 6: Table S5. Correlation coefficients between miRNAs and targets

Additional File 7: Figure S2. Vis Network of the interaction. Positive and negative differences in genes are expressed in red and blue, respectively.

Additional File 8: Figure S3. Pathway classification Analysis and Pathway Enrichment Analysis of Target DEGs. “Rich Ratio”: the ratio of the differential expressed gene (DEG) numbers and the number of genes have been annotated in this pathway.

Additional File 9: Figure S4. Gene Ontology classification Analysis and Gene Ontology Enrichment Analysis of Target DEGs. Red, green, and blue represent the three GO ontologies cellular component, molecular function and biological progress, respectively.

Figures

Figure 1

Experimental Pipeline Steps for Small RNA Sequencing. (1) Small RNA enrichment and purification. (2) 3' end adaptor ligation: Ligate the 5-adenylated and 3-blocked adaptor to the 3' end of the small RNA fragment. (3) Add unique molecular identifiers (UMI) labeled Primer. (4) Unligated adaptors digestion. (5) 5' end adaptor ligation. (6) First strand synthesis with unique molecular identifiers (UMI) labeled Primer. (7) Second strand synthesis. (8) Fragment selection. (9) Library quantitative and pooling cyclization. (10) Library QC. (11) Sequencing on BGISEQ0000

Figure 2

Response of three-leaf-stage cotton seedlings to salt stress. A. Performance of cotton seedlings under different salt stresses for 6h. B., Relative Electric Conductivity (REC). C. Effects of salt stress on plant water content of E911. FW: Fresh weight, DW: Dry weight, PWC: Plant water content.

Figure 3

Length distribution of the small RNAs in the twelve libraries. Y-axis represents percentages of miRNAs identified in this study; X-axis represents the length of miRNAs. A: leaf; B: root; C: stem. Blue, yellow, red, and green lines represent 0h, 1h, 3h and 6h, respectively.

Figure 4

Clustering of miRNAs expression profiles. Heat map diagram of miRNA expression prepared with hierarchical clustering of miRNA expression in different tissues at different times. miRNAs are given in the rows and each column represents a sample. The miRNA clustering tree is shown on the right (clusters I, II, III, and IV). Abbreviations: L, Leaf; R, Root; S, Stem.

Figure 5

Differentially expressed miRNAs. X-axis represents the DESs pair, Y-axis represents the number of screened DESs, with green indicating down-regulation and red indicating up-regulation.

Figure 6

Hierarchical clustering of differentially expressed genes (DEGs) across all comparisons. X-axis represents each pair of differences, Y-axis represents Differently Expressed Genes (DEGs). The colors indicate the fold change, with red showing up-regulation, and green showing down-regulation.

Supplementary Files

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

- [supplement1.png](#)
- [supplement2.png](#)
- [supplement3.xlsx](#)
- [supplement4.xlsx](#)
- [supplement5.xlsx](#)
- [supplement6.xlsx](#)
- [supplement6.png](#)
- [supplement8.png](#)
- [supplement9.xlsx](#)