Comparative transcriptome analysis of sweet sorghum provides insights into new lncRNAs acting as ceRNAs during salt responses

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Abstract

LncRNAs can act as competitive endogenous RNAs (ceRNAs) to competitively bind miRNAs, thereby indirectly regulating the transcription levels of other RNA transcripts to confer resistance to plants. But how specific ceRNAs respond to salt stress in sweet sorghum is still unclear. In this study, 126 and 133 differentially expressed lncRNAs were identified in salt-tolerant sweet sorghum (M-81E) and a salt-sensitive line (Roma) by high-throughput RNA-seq, respectively. And five new lncRNAs were identified in M-81E and Roma after salt stress treatment, lncRNA13472, lncRNA11310, lncRNA2846, lncRNA26929 and lncRNA14798 acted as ceRNAs to regulate the expression of target genes related to salt resistance by binding the five miRNAs sbi-MIR169b-p3, sbi-MIR5567-p3-2ss16CT17TC, sbi-MIR5567-p5-2, sbi-MIR5567-p5-2ss17CT18TC and PC-3p-270284-34, respectively. The target genes mainly included proton pump proteins, transport proteinsantioxidants, signal transduction proteins and transcription factors. However M-81E had more complex ceRNAs network than in Roma, which might be related with its different salt tolerance. In summary, this study identified a new ceRNA network within the transcriptome and revealed the effect of lncRNAs in the salt stress response.

Background

Salt stress has now become one of the main abiotic stress factors constraining agricultural development worldwide. A considerable amount of arable soil in the world is affected by salinity. When the local topsoil contains a lot of soluble salts, it forms a saline-alkali soil, which causes different degrees of damage to plants and even plant death. Salt stress mainly reduces the quality and quantity of crops by affecting plant growth and metabolism, through ionic stress, osmotic stress, oxidative stress and nutrient stress[1–4].

In recent years, numerous studies have shown that non-coding RNA can enhance the resistance of plants to stress by regulating the expression of plant functional genes [5–9]. Based on their length, non-coding RNAs can be divided into small non-coding RNAs (sncRNA) and long non-coding RNAs (lncRNA). MicroRNA is an important type of small non-coding RNA, and has only 21 nucleotides of single-stranded small RNA in plants [10]. Mature miRNAs can form miRNA-mediated silencing complexes (MIRISCs) with AGO complexes, and then cleave and degrade their target genes, resulting in gene silencing [11]. By contrast, lncRNA refers to non-coding RNAs over 200 nt in length, and there is increasing evidence that lncRNAs are potential regulatory molecules [12]. lncRNAs can act as competing endogenous RNAs (ceRNAs) to competitively bind to the same miRNAs as mRNAs through miRNA response elements (MREs), resulting in changes in target gene expression and thereby conferring resistance to various stresses [13]. In plants, the standard for target mimics is much stricter than in animals and the positive position of the sequence must not conform to the principle of base pairing (resulting in protrusions). Recent studies have shown that lncRNAs can regulate the expression levels of target genes through *cis*- or *trans*-regulation and can be cleaved by miRNA to generate siRNA to silence target genes. In addition, some natural antisense lncRNAs are involved in the regulation of target gene expression [14–20]. ceRNA

has been well studied in animals. However, in plants, current research on the regulatory mechanism of ceRNA still represents the tip of the iceberg.

Sweet sorghum [*Sorghum bicolor* (L.) Moench], also known as sugar sorghum, is an annual C4 crop of the Gramineae family that has high biomass accumulation and high tolerance to adverse conditions such as salt, drought and flooding [21]. In our previous study, after treatment with 150 mM NaCl for 24 h, the concentration of Na^+ in the roots and shoots of M–81E was lower than that in Roma, and the Na^+ content in both shoots was much lower than that in the roots. It shows that the root plays a very good role in limiting the transport of Na^+ to reduce the ionic toxicity caused by salt stress. Similarly, the growth of Roma root was significantly inhibited under salt stress, but M–81E was not. M–81E showed more tolerance to salt stress than Roma. The transcriptional profiles of leaves and roots revealed some of the salt tolerance mechanisms of sweet sorghum [22, 23]. However, the regulatory mechanism of salt tolerance by ceRNA in sweet sorghum is still unclear. To better understand the dynamic process of salt tolerance and the regulatory mechanism of non-coding RNA, we analyzed the root transcriptome of a salt-tolerant sweet sorghum line (M–81E) and a salt-sensitive line (Roma) through high-throughput Illumina RNA sequencing (RNA-seq). By comparing the transcriptomes of the two sweet sorghum strains under salt stress, we identified differentially expressed lncRNAs, miRNAs and mRNAs, and constructed ceRNA networks related to differentially expressed mRNAs for network analysis. The results of this study provide a deeper understanding of the complex regulatory networks underlying the tolerance of sweet sorghum to salt stress.

Methods

Plant material and salt treatment

According to our previous research [22], we selected two sweet sorghum inbred lines, the salt-tolerant strain M–81E and salt-sensitive strain Roma, as experimental materials for subsequent experiments such as RNA extraction and RNA-seq. Following Yang et al.[23], seeds of the two sweet sorghum strains were cultured at a light intensity of 600 μmol m^{-2} s^{-1} (15 h photoperiod) and 70% relative humidity at 28 ± 3℃ (day/night). Previous studies have shown that 150 mM NaCl is an appropriate concentration that results in a significant difference in physiological parameters between M–81E and Roma [22]. Therefore, at the three-leaf phase, we started to water the plants with nutrient solutions containing 0 and 150 mM NaCl. In the experimental group, the NaCl concentration was increased by 50 mM every 12 h up to the final concentration. The plants were used in subsequent experiments after treatment with 150 mM NaCl for 24 h.

RNA sequencing and whole transcriptome library construction

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. The total RNA amount and purity were analyzed using a Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with a RIN value > 7.0. Libraries of small RNAs < 50 nt was prepared using approximately 1 μg of total RNA according to the protocol of the TruSeq Small RNA Sample Preparation Kit (Illumina, San Diego, USA). According to the manuscript of the Epicentre Ribo-Zero Gold Kit (Illumina, San Diego, USA), approximately 10 μg of total RNA was used to consume ribosomal RNA, and then purified and fragmented, using the mRNA-Seq sample preparation kit (Illumina, San Diego, USA) Reverse transcribed cleavage of RNA fragments to generate de-RNA strand-specific de-RNA > 200 nt. Then, single-end sequencing was performed on the Illumina Hiseq 2500 and Illumina Hiseq 4000 at LC-BIO (Hangzhou, China). The SE50 strategy was used for the small RNA library and the 150PE on-board strategy was used for the de-RNA chain-specific library.

Assembly and identification of lncRNAs

First, we used Cutadapt to clean the reads, then use FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to verify the sequence quality. The reads were mapped to the assembled transcripts of the sweet sorghum genomic sequence using Bowtie2 and Tophat2, and assembled with StringTie [24], which excluding known transcripts encoding proteins and <200 bp transcripts [25, 26]. We then used CPC, CNCI and Pfam to predict transcripts with coding potential. We discarded all transcripts with a CPC score < −1 and a CNCI score < 0, and considered the remaining transcripts with the class codes (i, j, o, u, x) to be lncRNAs [27, 28].

Analysis of DEGs and DELs

StringTie was used to calculate Fragments Per Kilobase of exon model per Million mapped fragments (FPKM) values to represent the expression levels of genes and lncRNAs [24]. Then, DEGs and DELs were selected using the R package Ballgown with $\log_2$(fc) > 1 or < −1 and *p*-value < 0.05 [29].

Predicting and analyzing lncRNA-miRNA-mRNA relationship pairs

lncRNA-miRNA-mRNA relationships were predicted using StarBase (http://starbase.sysu.edu.cn/). Predictions are usually made according to the following rules. (1) The middle of the miRNA is complementary to the base of the lncRNA, but this position produces a 3 to 5-nt base bulge on the lncRNA. (2) No more than four total mismatches are allowed in the non-intermediate region of each miRNA, and consecutive mismatches should not exceed two nucleotides. (3) No protrusions are allowed in the non-intermediate region of the miRNA. Then, Cytoscape (http://www.cytoscape.org/) was used to construct a putative interaction network.

Quantitative real-time PCR analysis

The relative expression levels of lncRNAs, miRNAs and mRNAs were analyzed by qRT-PCR. A total plant RNA extraction kit (Hua Yueyang, Beijing, China) was used on samples of M−81E and Roma treated with 0 and 150 mM NaCl for 24 h according to the manufacturer's protocol [22]. Total RNA was isolated from the primary root. The RNA was quantified using a Nanodrop-ND−1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The sweet sorghum actin gene was used as an endogenous reference gene for lncRNAs and mRNAs, and U6 was used as an endogenous reference gene for miRNAs. We used the stem-loop method to reverse-transcribe miRNAs. Primer sequences are listed in Supplemental Table 2.

Results

High-throughput sequencing and DEG analysis

The whole-transcriptome sequencing included three biological replicates of each sample (M−81E-CK1, M−81E-CK2, M−81E-CK3, M−81E-salt1, M−81E-salt2, M−81E-salt3, Roma-CK1, Roma-CK2, Roma-CK3, Roma-salt1, Roma-salt2, Roma-salt3). Single-end sequencing on the Illumina Hiseq 2500 and paired-end sequencing on the Illumina Hiseq 4000 were used to generate more than 363,000,000 raw reads from the four samples. A total of approximately 54.5 G of raw reads and 53.7 G of valid reads were detected (Table S1). As shown in Table S1, more than 98% of the raw reads were valid reads. To assess the quality of the RNA-seq data, fast QC was used to assign a quality score (Q) to each base in the reads with a phred-like algorithm [30], and the analysis showed that the data was highly reliable.

After mapping to the sweet sorghum genome, about 86.14%, 84.93%, 80.62% and 87.53% of reads were mapped in the M−81E-CK, M−81E-salt, Roma-CK, and Roma-salt samples, respectively, and over 74.59%, 72.44%, 69.04% and 76.17% were unique mapped reads (Table. S1). The set of unique mapped reads was mapped to exons (94.14% of M−81E-CK, 94.04% of M−81E-salt, 93.64% of Roma-CK and 94.10% of Roma-salt), introns (4.72% of M−81E-CK, 4.78% of M−81E-salt, 5.14% of Roma-CK, 4.65% of Roma-salt) and intergenic regions (1.14% of M−81E-CK, 1.18% of M−81E-salt, 1.22% of Roma-CK, 1.25% of Roma-salt) (Fig. 1a). The above results indicate that lncRNA in sweet sorghum is mainly derived from exon transcripts.

After calculating the expression level of each gene (FPKM), 714 genes were found to be significantly differentially expressed ($\log_2(fc) \leq -1$ or ≥ 1, p-value < 0.05) between M−81E-CK and M−81E-salt. Among them, 293 differentially expressed genes (DEGs) were up-regulated and 421 DEGs were down-regulated. Similarly, 1331 DEGs were detected between Roma-CK and Roma-salt, of which 623 were up-regulated and 708 were down-regulated (Fig. 1b).

A Venn diagram of the DEGs showed that 228 genes in M−81E and 588 genes in Roma were uniquely up-regulated and that 47 up-regulated genes were co-expressed in the M−81E and Roma samples. Among the down-regulated genes, 331 were M−81E-specific, 611 were Roma-specific, and 97 were co-expressed

in both M–81E and Roma. In addition, 31 genes were down-regulated in Roma and up-regulated in M–81E and 13 genes were down-regulated in M–81E and up-regulated in Roma (Fig. 1c).

Gene ontology (GO) analysis was performed to assign functional information to the DEGs between the control and NaCl-treated plants. In M–81E, 21,754 genes were assigned 900 Level–2 GO terms, of which 11 significant differences genes with specific GO were related to salt stress responses. By comparison, in Roma, 21,754 genes were assigned to 1,324 Level–2 GO terms, of which 31 significant differences genes with specific GO were associated with salt stress responses. In the Biological Process domain, the “biological process” and “regulation of transcription, DNA-templated” categories were the most prominent in M–81E and Roma. The most highly enriched cell groups in the Cellular Component domain in M–81E and Roma were the nuclear and plasma membrane fractions. In the Molecular Function domain, “molecular function” and “ATP binding” were the most represented GO terms in M–81E, but “molecular function” and “protein binding” were the most represented GO terms in Roma (Fig. S1). The above data indicate that salt stress may affect plants mainly by changing the structure and function of the plasma membrane.

Identification and characterization of lncRNA

After analysis of all transcripts from the RNA-seq results, annotation and filtration, 2,176 unique lncRNAs were obtained from the M–81E and Roma samples. Moreover, our results showed that these lncRNAs were evenly distributed on the 10 chromosomes in sweet sorghum (Fig. 2a).

Based on the locations of the lncRNAs in the genome of M–81E, 7% of lncRNAs showed exonic overlap with reference to the opposite strand; 7% of lncRNAs were transfrags falling entirely within a reference intron; 3% of lncRNAs were potentially novel isoforms (fragments) with at least one splice junction shared with a reference transcript; 2% of lncRNAs showed generic exonic overlap with a reference transcript; and 81% of lncRNAs were unknown and intergenic transcripts. In Roma, 8% of lncRNAs had exonic overlap with reference to the opposite strand; 8% of lncRNAs were transfrags falling entirely within a reference intron; 3% of lncRNAs were potentially novel isoforms (fragments) with at least one splice junction shared with a reference transcript; 2% of lncRNAs had generic exonic overlap with a reference transcript; and 79% of lncRNA were unknown and intergenic transcripts (Fig. 2b).

Of these 2,176 lncRNAs, 126 and 133 lncRNAs were specifically expressed in the M–81E and Roma samples, respectively. A total of 126 differentially expressed lncRNAs (DELs) ($\log_2$(fc) ≤ −1 or ≥ 1, *p*-value < 0.05) were identified in the salt-treated M–81E samples compared with the CK samples, including 71 up-regulated and 55 down-regulated lncRNAs (Fig. 2c). A total of 133 differentially expressed lncRNAs (DELs) ($\log_2$(fc) ≤−1 or ≥ 1, *p*-value < 0.05) were identified in the Roma samples, including 68 up-regulated and 65 down-regulated lncRNAs (Fig. 2c).

Analysis of lncRNA-related ceRNA networks in sweet sorghum roots and functional enrichment analysis of related DEGs

Previous studies have shown that lncRNAs can act as ceRNAs and regulate gene expression after transcription [31, 32]. lncRNAs act as target mimics to absorb miRNA like a sponge and prevent miRNA from completely degrading the target [14, 33, 34]. To assess the regulatory patterns of non-coding RNA-related ceRNA interactions in sweet sorghum roots, we predicted lncRNA-miRNA-mRNA pairs in the experimental and control groups of the two strains using StarBase (http://starbase.sysu.edu.cn/). Then, we used Cytoscape (http://www.cytoscape.org/) to construct a putative interactive network. Consequently, 672 lncRNA-miRNA-mRNA interactions were predicted in the global ceRNA network, of which 477 lncRNA-miRNA-mRNAs were predicted in M−81E (Fig. S2a) and 195 lncRNA-miRNA-mRNA relationship sets were predicted in Roma (Fig. S2b). Thirteen lncRNAs were involved in the 672 lncRNA-miRNA-mRNA regulatory networks, of which seven were in M−81E and six were in Roma.

To further elucidate how the DELs confer salt tolerance to sweet sorghum, we performed GO annotation analysis on the genes regulated by the 13 lncRNAs (Fig. 3). In the Biological Process domain, besides the term “transcriptional regulation” in M−81E, the protein-encoding genes were enriched in terms such as “redox process” and “defense reaction”, while in Roma, the terms “defense reaction” and “protein phosphorylation” were enriched. In the Cellular Component domain, besides term the “nucleus”, “cytoplasm” was most enriched in M−81E. However, the term “plasma membrane” was most enriched in Roma. In the Molecular Function domain, M−81E was mainly enriched in “protein binding”, but Roma was mainly enriched in “ATP binding”.

After colocalization and expression analysis using Cytoscape and network construction, we detected five DELs (3 in M−81E and 2 in Roma) from the 13 lncRNAs that might be related with salt responses. The five DELs (lncRNA13472, lncRNA 11310, lncRNA2846, lncRNA26929 and lncRNA14798) were identified as modulators of miRNA that regulated five miRNAs and 14 target genes (Fig. 4a, b). lncRNA13472 might compete with SORBI−3010G218400 for binding to sbi-MIR169b-p3. lncRNA11310 might compete with SORBI−3001G158100, SORBI−3001G223100, SORBI−3002G237000, SORBI−3002G302000, SORBI−3003G327000 and SORBI−3009G182800 for binding to sbi-MIR5567-p3−2ss16CT17TC. lncRNA2846 might compete with SORBI−3001G158100, SORBI−3001G223100, SORBI−3002G237000, SORBI−3002G302000, SORBI−3003G327000, SORBI−3004G116300, SORBI−3004G302400, SORBI−3006G123500, SORBI−3007G046900, SORBI−3009G182800, SORBI−3009G208000 and SORBI−3010G081800 for binding to sbi-MIR5567-p5−2. lncRNA26929 might compete with SORBI−3003G327000, SORBI−3001G158100, SORBI−3001G223100 for binding to sbi-MIR5567-p5−2ss17CT18TC. Finally, lncRNA14798 might compete with SORBI−3009G042700 for binding to PC−3p−270284- 34. The expression levels of the five DELs were different in M−81E and Roma. lncRNA13472 was up-regulated in both M−81E and Roma, but its expression level was higher in M−81E than in Roma. However, the expression of lncRNA11310, lncRNA2846 and lncRNA26929 was increased in M−81E and

decreased in Roma. The expression level of lncRNA14798 was not changed in M–81E and decreased in Roma (Fig. 4c).

To demonstrate that the ceRNA network is involved in the potential regulation of salt tolerance in sweet sorghum roots, we performed a functional enrichment analysis of the 14 target proteins based on GO annotations. It is worth noting that these protein-coding genes were mainly enriched in stress-related terms such as “regulation of transcription”, “response to salt stress”, “nucleus”, “protein binding” and “transport activity” (Fig. 5). These results indicate that under salt stress, lncRNAs can act as ceRNAs and compete with miRNAs for mRNA binding, and may regulate the metabolism of several protein-coding genes involved in important biological processes.

qRT-PCR validation of differentially expressed transcripts from RNA-seq

To verify the putative relationships between the five DELs, five miRNAs and 14 DEGs, their expression levels were examined by qRT-PCR. The results were consistent with the RNA-seq data, indicating that our RNA-seq results were authentic (Fig. 6).

Discussion

Salt stress induces ion toxicity, osmotic stress and oxidative stress, which can affect plant physiological metabolism and growth [5, 35]. To survive under salt stress, plants can regulate their salt tolerance by responding to and adapting to high salt habitats at the transcriptional and post-transcriptional levels [36–39]. Regulation at the transcriptional level is mainly achieved through transcription factors, DNA methylation, histone modification and other processes to activate or inhibit gene-specific expression[40]. At the post-transcriptional level, regulation is mainly achieved by RNA alternative splicing, RNA methylation and multiple RNA–RNA interactions [41–43]. Nonetheless, at present, there is limited detailed information on the functions and regulatory mechanisms of lncRNA, and how it plays a role in the regulation of salt stress in sweet sorghum is still little known.

In recent years, many studies have shown that lncRNA can be used to characterize the regulation of physiological metabolism and growth and development because it regulates the modification of histones, nucleic acid structure modification, nucleic acid methylation and RNA–RNA interactions [44, 45]. lncRNAs are actively involved in the salt stress response in cotton, which was confirmed by Deng et al. [14]. However, their study was focused on *cis*-acting regulation in which lncRNAs act as ubiquitous regulators. Moreover, Wang et al. [46] observed that there were differences in the number of lncRNAs in the roots and leaves of *Medicago truncatula* under salt stress. The number of lncRNAs in the roots was much larger than in leaves, which seems to indicate lncRNAs are especially active in plant roots. In this study, we selected the salt-tolerant genotype M–81E and salt-sensitive genotype Roma as experimental materials to explore how lncRNA acts as ceRNA to actively respond to salt stress in sweet sorghum roots.

To investigate this problem, we performed RNA-seq on M–81E and Roma roots after NaCl treatment. After annotating, characterizing and analyzing the RNA-seq data, our transcriptome profiling results were similar to previous results [47]. The DEGs of the different inbred lines were mainly enriched in transcription factors, transport proteins, antioxidants and proton pumps. We predicted the ceRNA relationships of the DELs using StarBase, and identified 477 and 195 lncRNA-miRNA-mRNA relationships in M–81E and Roma, respectively (Fig. S2). Many more interactions were found in M–81E than in Roma, which suggests that M–81E has a more accurate and complex ceRNA regulation mechanism for the response to salt stress, which may be one of the reasons that M–81E is more tolerant to salt stress.

Furthermore, we selected five lncRNAs that were related to salt stress for in-depth study. Their expression levels were different in the two lines under salt stress. Among them, lncRNA13472 was up-regulated in both M–81E and Roma, but had higher expression in M–81E than in Roma. lncRNA11310, lncRNA2846 and lncRNA26929 were up-regulated in M–81E and down-regulated in Roma. The expression level of lncRNA14798 decreased in Roma and did not change in M–81E (Fig. 4c). These lncRNAs were predicted to compete with 14 DEGs for binding to five miRNAs (Fig. 4a, b).

To clarify how ceRNAs participate in the response to salt stress, we further analyzed the lncRNA-miRNA-mRNA relationships and the roles of the 14 DEGs. The DEGs mainly encoded proton pumps, transport proteins, certain important enzymes and transcription factors (Table S2). Similarly, miRNAs target many transcription factors, signaling factors, and transporter-encoding genes, which have been shown to be involved in response to salt stress [48]. Interestingly, miRNAs also play an important role in the ceRNA network. Among them, sbi-MIR169b can be down-regulated under drought stress [49]. However, its expression level was up-regulated in our transcriptome profile. In this study, *SORBI–3010G218400* was predicted to bind sbi-MIR169b in competition with lncRNA13472. *SORBI–3010G218400* encodes V-type proton ATPase catalytic subunit A (VHA-A). V-ATPase (VHA) is an ATP-dependent proton pump located on the eukaryotic plasma membrane that is involved in the transmembrane transport of protons and in responses to salt stress [50–54]. The A subunit is an important component of VHA. Here, the expression level of *SORBI–3010G218400* increased, which might be helpful for establishing a transmembrane proton gradient. Coincidentally, sbi-MIR5567 was detected as a sorghum-specific miRNA [55], but how sbi-MIR5567-p3–2ss16CT17TC, sbi-MIR5567-p5–2 and sbi-MIR5567-p5–2ss17CT18TC participate in the complex ceRNA regulatory networks and the response to salt stress is unknown. The target genes of sbi-MIR5567-p3–2ss16CT17TC are *SORBI–3001G158100, SORBI–3001G223100, SORBI–3002G237000, SORBI–3002G302000, SORBI–3003G327000,* and *SORBI–3009G182800.*They can compete with lncRNA11310 for binding to sbi-MIR5567-p3–2ss16CT17TC. *SORBI–3001G158100* encodes BTB/POZ and MATH domain-containing protein 1 (BPM1). Members of the BPM family act as regulators of ABA responses and play important roles in plant development and stress responses. In the cytoplasm, BPM1 binds to CUL3 E3 ubiquitin ligase to degrade the 26S proteasome. However, in the nucleus, it can bind to transcription factors to regulate transcription. Moreover, studies have shown that reducing the function of BPM affects the opening and closing of stomata and ABA responsiveness, which in turn affects plant development and reproduction [56]. BPM1 was found to be induced by hormones and to enhance the resistance of host plants to pathogenic microorganisms [57] in *A. thaliana [*58, 59] and soybeans [60].

SORBI–3001G223100 encodes the protein NRT1/PTR FAMILY 5.2 (NPF5.2), a transporter of a variety of substances including nitrates, chlorides and phytohormones [9, 61, 62], which have been shown to respond to drought stress [63]. *SORBI–3002G237000* encodes fasciclin-like arabinogalactan protein 1 (FLA1). FLA1 has been shown to be involved in the salt stress response [64], and plays a crucial role in plant development [65–67]. *SORBI–3002G302000* encodes xyloglucan endotransglucosylase/hydrolase protein 31 (XTH31), a cell wall modifying enzyme that is involved in plant cell wall formation in plants such as wheat [68, 69], regulates root hair development in *A. thaliana* [70, 71], and responds to drought stress in maize [72]. *SORBI–3003G327000* and *SORBI–3009G182800* both encode basic leucine zipper 23 (bZIP23). bZIP proteins in *Arabidopsis* are involved in drought and high salinity responses and the ABA-dependent signal transduction pathway [73, 74]. Except for FLA1, the expression levels of the above five genes regulated by sbi-MIR5567-p3–2ss16CT17TC were increased under salt stress. These six genes encode ion transporters, cell wall modifying enzymes, and transcription factors. Thus, we speculate that lncRNA1310 confers high salt tolerance to plants by binding with sbi-MIR5567-p3–2ss16CT17TC and reducing the degradation of these target genes. *SORBI–3001G158100, SORBI–3001G223100, SORBI–3002G237000, SORBI–3002G302000, SORBI–3003G327000, SORBI–3004G116300, SORBI–3004G302400, SORBI–3006G123500, SORBI–3007G046900, SORBI–3009G182800, SORBI–3009G208000* and *SORBI–3010G081800* may compete with lncRNA2846 for binding to sbi-MIR5567-p5–2. *SORBI–3001G158100* and *SORBI–3007G046900* encode BPM1, *SORBI–3001G223100* encodes NPF5.2, *SORBI–3002G237000* encodes FLA1, *SORBI–3002G302000* encodes XTH31, and *SORBI–3003G327000* and *SORBI–3009G182800* both encode bZIP23. *SORBI–3004G116300* encodes *Bcl–2*-associated athanogene (BAG6).BAG6 selectively promotes mislocalized proteasomal degradation by ubiquitination [75]. Moreover, Kang et al. [76] confirmed that *AtBAG6* is a stress-regulated calmodulin-binding protein associated with programmed cell death in plants. Studies have shown that loss of BAG6 leads to reduced resistance to the fungal phytopathogen *Botrytis cinerea* [77]. *SORBI–3004G302400* and *SORBI–3010G081800* encode NAC domain-containing protein 7 (NAC007) and bZIP transcription factor 23 (bZIP TF 23), respectively. These proteins serve as two important transcription factors that regulate the expression of target genes at the transcriptional level to improve plant disease resistance [78] and can improve the salt tolerance and drought resistance of plants such as *Arabidopsis,* rice, corn and tomato [79–83]. *SORBI–3006G123500* is an important antioxidant gene [84, 85] that encodes the peptide methionine sulfoxide reductase A2–1 (MSRA2–1). MSRA2–1 participates in oxidative stress, is induced by salt stress in plants, and increases the salt tolerance of plants. As molecular chaperones, heat shock proteins (HSPs) help to maintain the structural integrity of cells and normal processes of cellular metabolism by assisting in the correct folding of proteins [86]. Fragkostefanakis et al. believe that heat stress transcription factors (Hsfs) interact with HSPs to confer plants the ability to resist stress [87]. The Hsf A–4d (HSFA4D) is an important transcription factor with transcriptional activator function that is encoded by *SORBI–3009G208000.* Hsfs in plants are not only related to heat stress, but also play an important role in responses to drought, oxidation, and biological stress [88–91]. In this study, the expression of most genes was up-regulated. We predict that *SORBI–3003G327000, SORBI–3001G158100* and *SORBI–3001G223100* may compete with lnCRNA26929 to bind sbi-MIR5567-p5–2ss17CT18TC. As mentioned above, *SORBI–3003G327000* encodes bZIP23, *SORBI–3001G158100*

encodes BPM1 and *SORBI–3001G223100* encodes NPF5.2. Interestingly, in this set of predicted target relationships, the expression levels of *SORBI–3003G327000* and *SORBI–3001G158100* increased, but the expression of *SORBI–3001G223100* was down-regulated. PC–3p has been shown to regulate plant development [92], but it is not known how PC–3p–270284–34 regulates plant responses to salt stress. *SORBI–3009G042700,* which can bind to PC–3p–270284–34 in competition with lncRNA14798, encodes sodium/hydrogen exchanger 2-like (NHX2), a Na^+, K^+/H^+ antiporter mainly located on the tonoplast membrane. As an ion homeostasis transporter, it regulates intracellular ion homeostasis and reduces ionic toxicity in plants due to salt stress [93]. Barragán et al. showed that the NHX2 protein confers stress tolerance by participating in the regulation of K^+ homeostasis, intracellular pH, and stomatal opening and closing [94].

Conclusion

In conclusion, our results revealed five unknown lncRNAs in M–81E and Roma under salt stress. These DELs act as ceRNAs that affect the salt tolerance of plants by regulating the transcription levels of certain proton pumps, transporters, important enzymes and transcription factors. These ceRNAs are highly correlated with the salt tolerance of sweet sorghum, and can therefore help to explain the mechanism of salt stress tolerance in plants and discover new genes that regulate plant salt tolerance.

Abbreviations

BAG6: Bcl–2-associated athanogene

BPM1: BTB/POZ and MATH domain-containing protein 1

bZIP TF 23: bZIP transcription factor 23

bZIP23: basic leucine zipper 23

ceRNAs: competitive endogenous RNAs

DEGs: differentially expressed genes

DELs: differentially expressed lncRNAs

FLA1: fasciclin-like arabinogalactan protein 1

FPKM: Fragments Per Kilobase of exon model per Million mapped fragments

GO: Gene ontology

HSFA4D: Hsf A–4d

Hsfs: heat stress transcription factors

HSPs: heat shock proteins

lncRNA: long non-coding RNAs

MicroRNA: miRNA

MIRISCs: miRNA-mediated silencing complexes

MREs: miRNA response elements

MSRA2–1: methionine sulfoxide reductase A2–1

NAC007: NAC domain-containing protein 7

NHX2: sodium/hydrogen exchanger 2-like

NPF5.2: NRT1/PTR FAMILY 5.2

RNA-seq: high-throughput Illumina RNA sequencing

sncRNA: small non-coding RNAs

VHA-A: V-type proton ATPase catalytic subunit A

XTH31: xyloglucan endotransglucosylase/hydrolase protein 31

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Competing interests

No competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Authors' Contributions

XS, HZ and JL imitated the manuscript. XS and HZ performed experiments; XS and NS collected data and carried out all analyses; NS conceptualized the idea and revised the manuscript. All authors read and approved the final manuscript.

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Figures

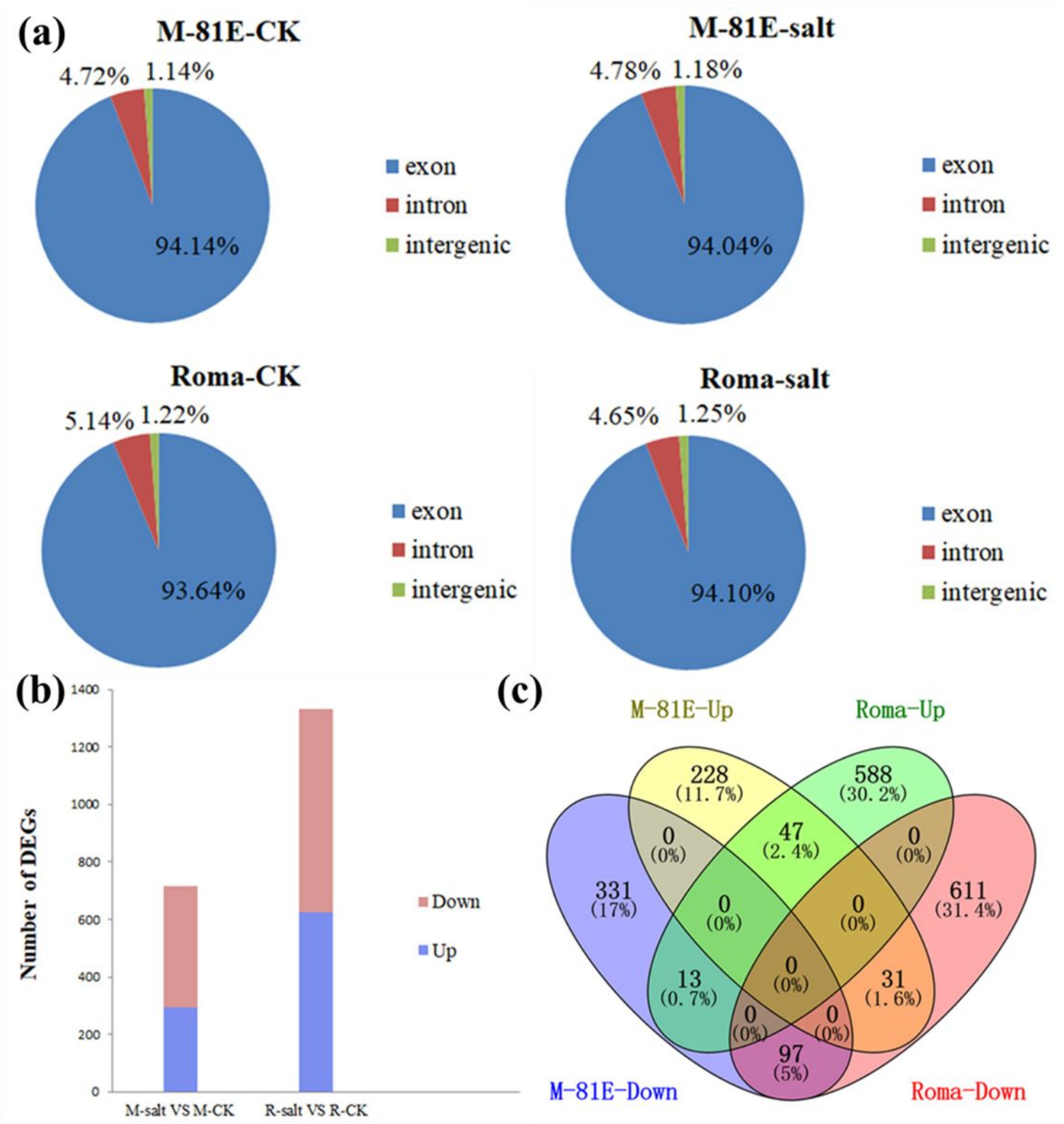

Figure 1

Unique reads mapped to various genomic regions, and identification and characterization of DEGs in M-81E and Roma between salt stress and control samples. (a) Unique reads mapped to various genomic regions. (b) Numbers of DEGs. (c) Venn diagram showing DEGs typically expressed in M-81E and Roma samples.

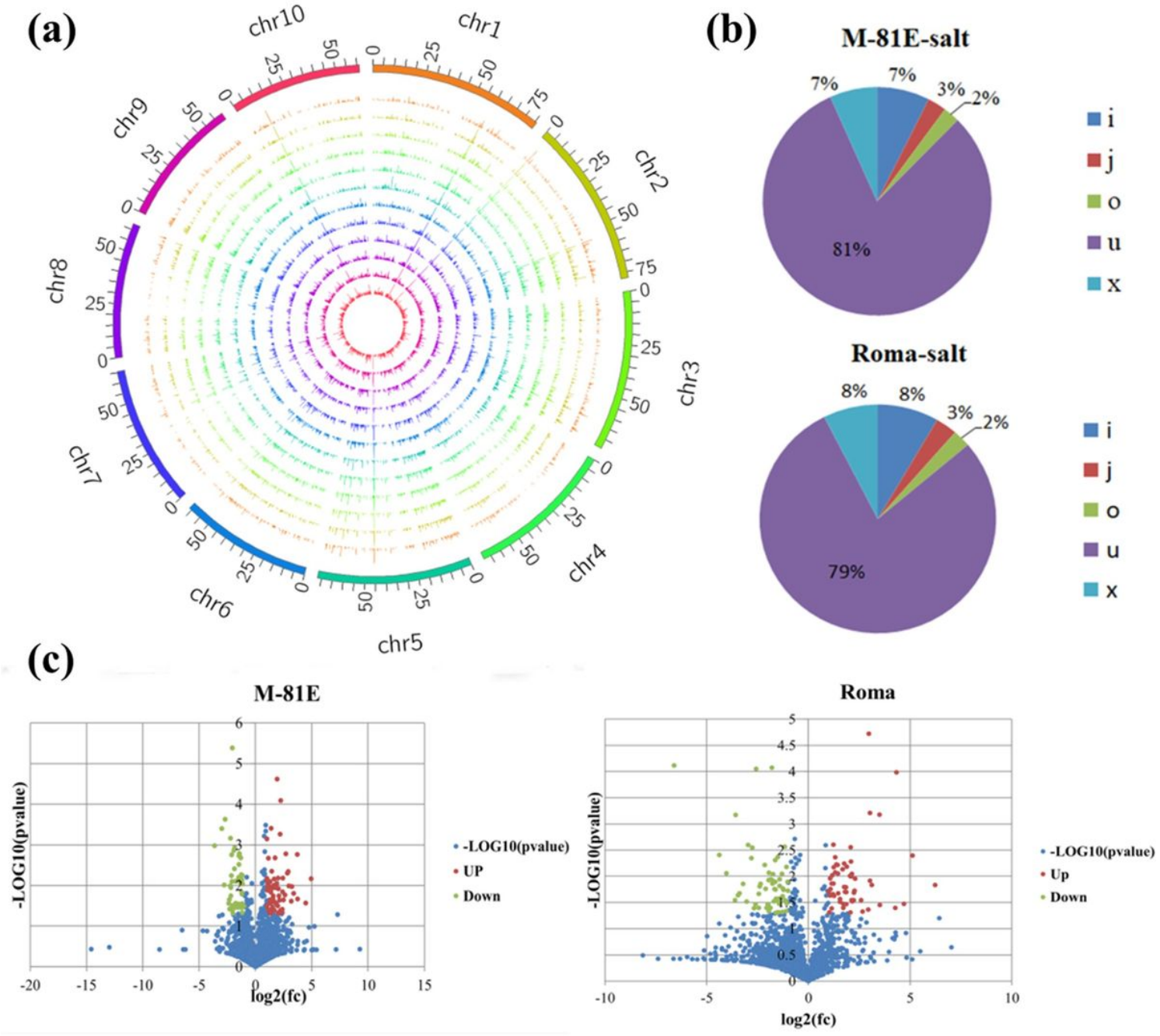

Figure 2

Identification and characterization of DELs. (a) Distribution of lncRNA on the chromosomes. (b) Unique reads mapped to various genomic regions. i: A transfrag falling entirely within a reference intron; j: potentially novel isoform (fragment) with at least one splice junction shared with a reference transcript; o: generic exonic overlap with a reference transcript; u: unknown, intergenic transcript; x: exonic overlap with reference on the opposite strand. (c) Number of DELs identified in M-81E-CK vs M-81E-salt and Roma-CK vs Roma-salt. Among them, the blue points indicate the DELs (-1>log2(fc)>1), the green points indicate

the downward adjustment of DELs (log2(fc)<-1, P≤0.05), and the red points indicate the Upward adjustment of DELs(log2(fc)>1, P≤0.05).

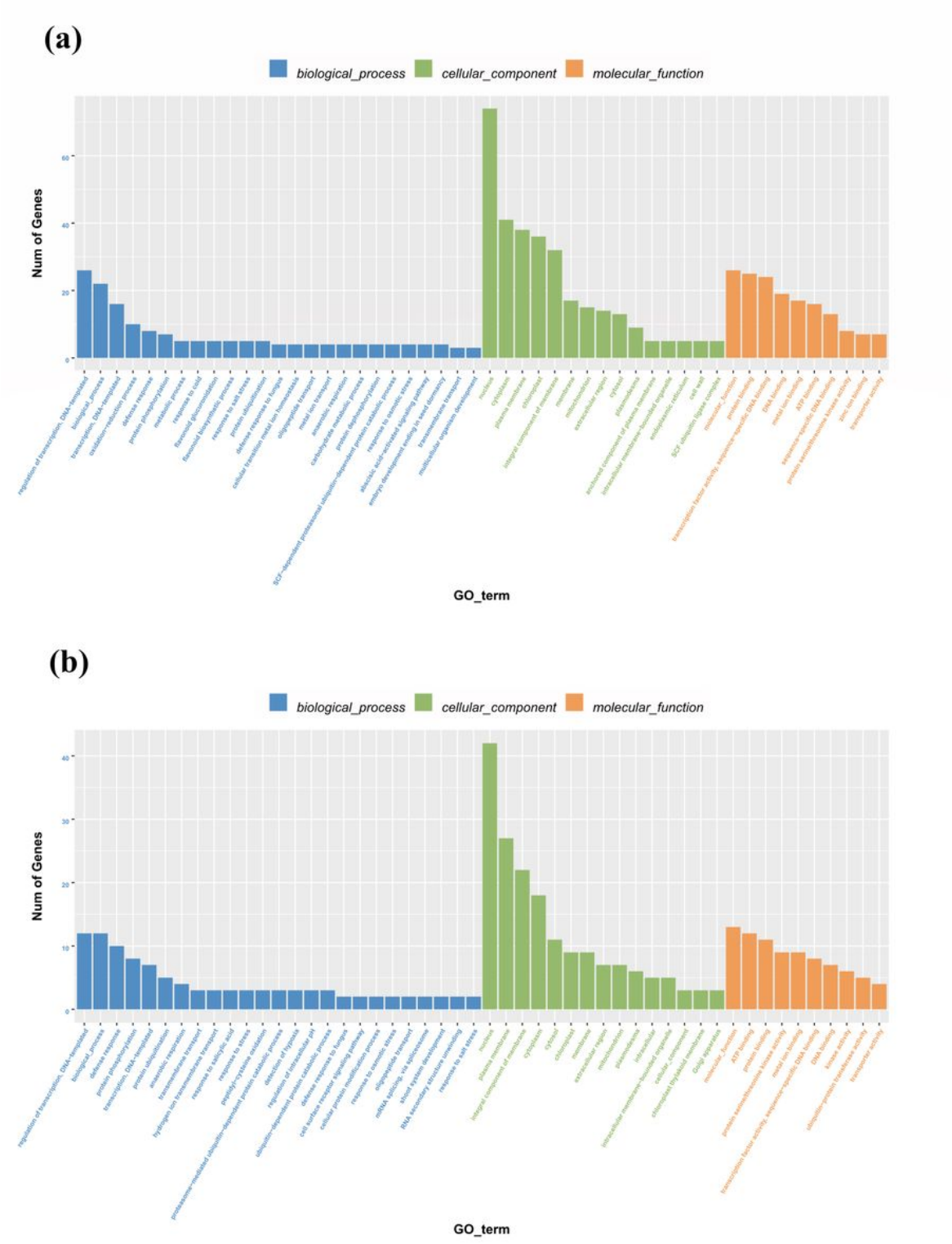

Figure 3

Gene ontology enrichment of ceRNA-regulated protein-coding genes.

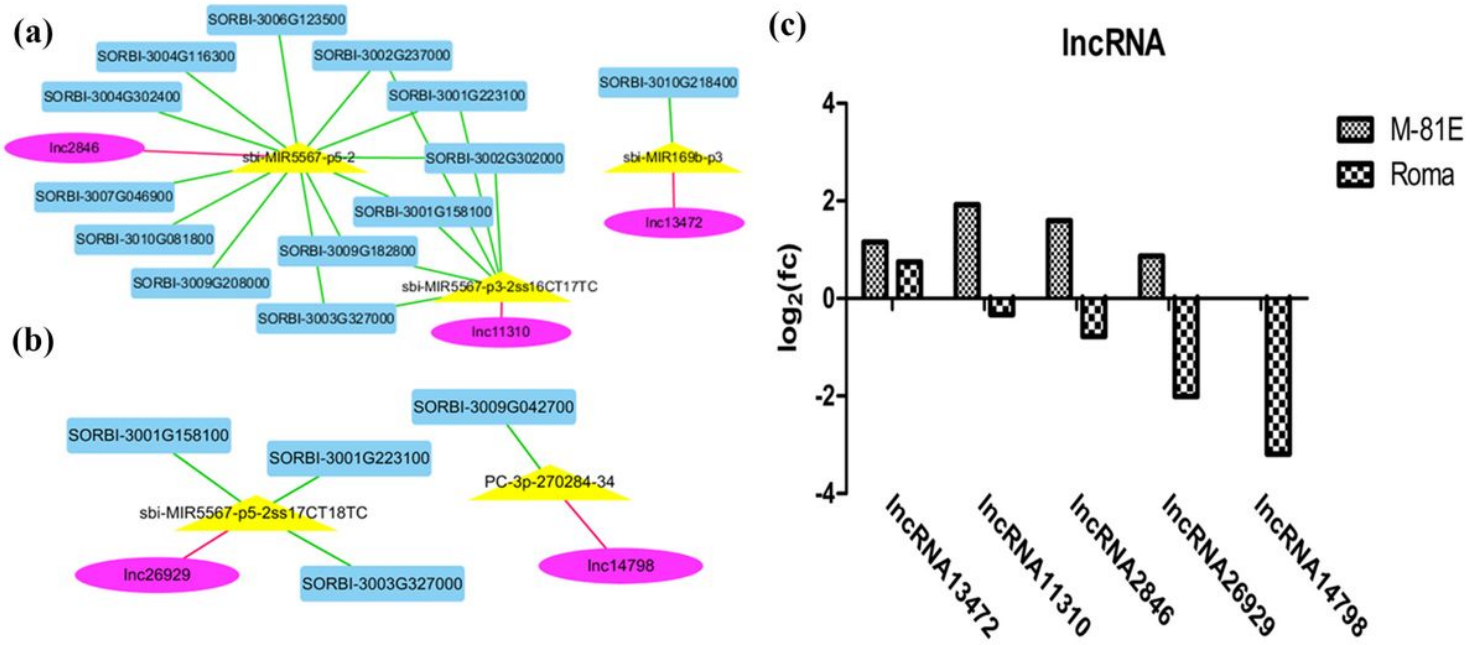

Figure 4

A ceRNA network enriched in salt-tolerant pathways in M-81E (a) and Roma (b). Red, yellow, and blue nodes represent lncRNAs, miRNAs, and mRNAs, respectively. Green edges represent miRNA-target interactions, while blue edges represent a competitive relationship. (c) Expression of five unknown lncRNAs in M-81E and Roma.

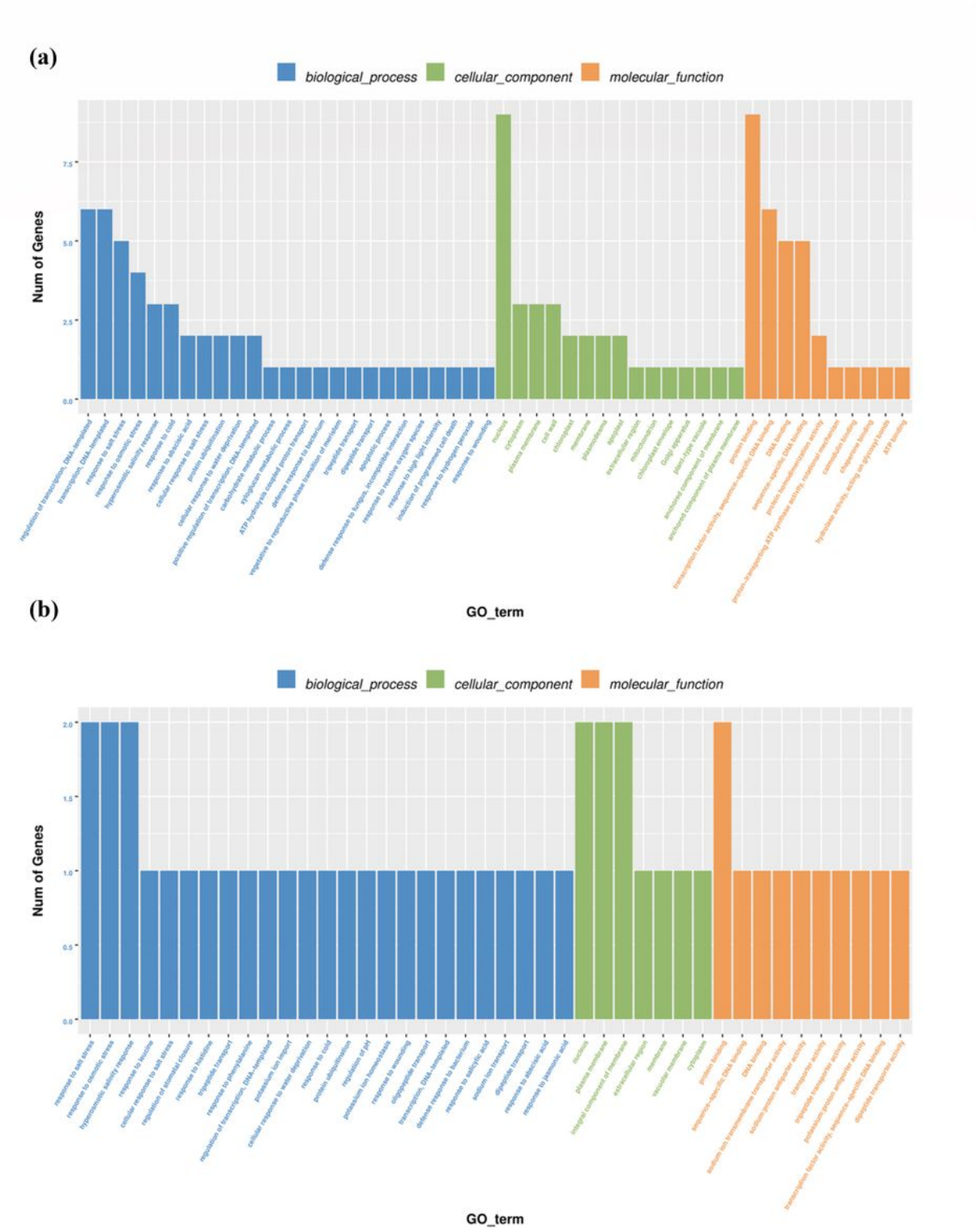

Figure 5

Gene ontology enrichment of ceRNA-regulated protein-coding genes. The results summarize the three main categories: Biological Processes, Molecular Functions and Cellular Components.

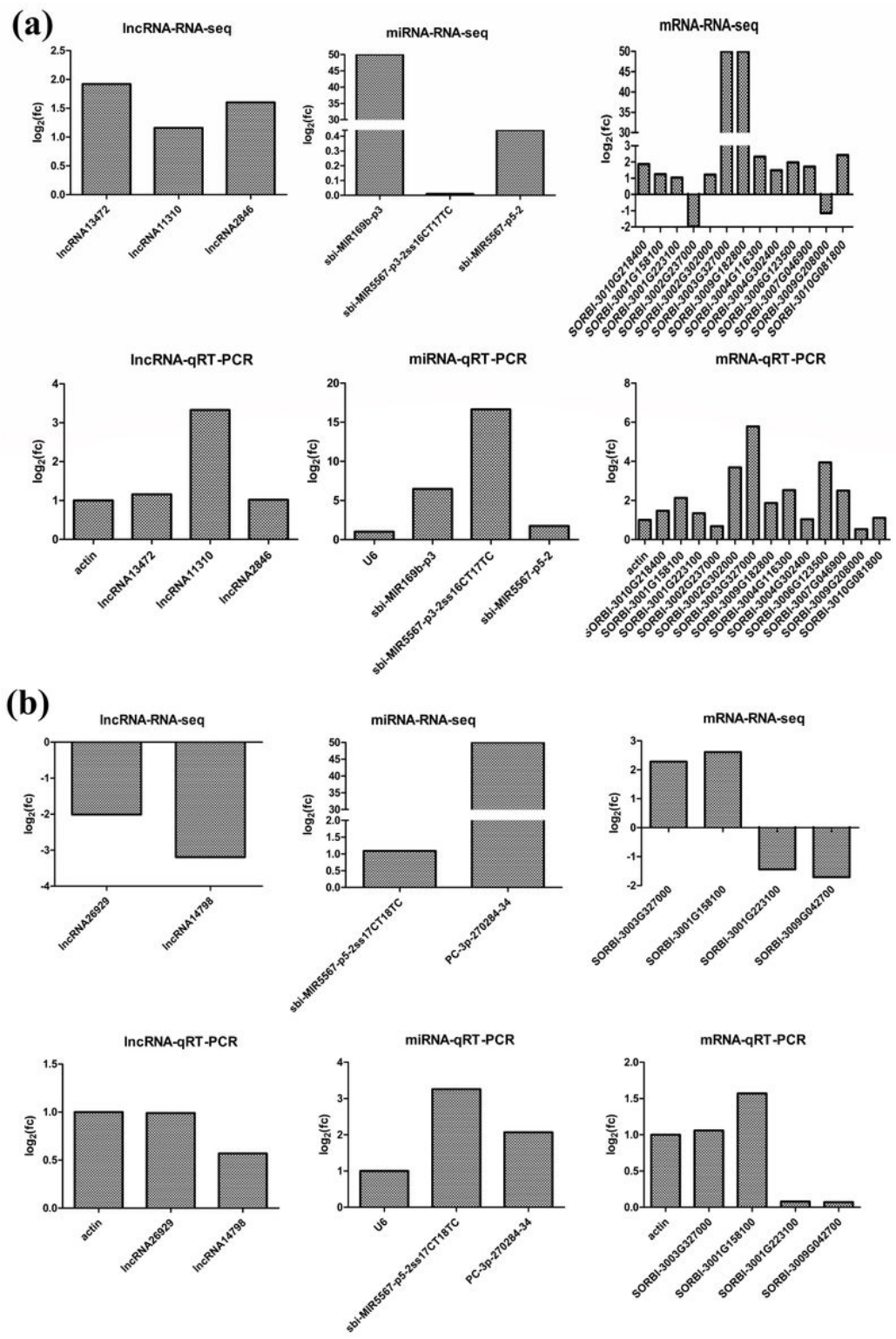

Figure 6

Quantitative RT-PCR analysis and RNA-seq results of ceRNA regulatory networks in M-81E (a) and Roma (b). Three biological replicates were prepared for each of these samples. Actin is the endogenous reference gene for lncRNAs and mRNAs, and U6 is the endogenous reference gene for miRNAs.

Supplementary Files

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