

Transcriptome analysis of sweet Sorghum inbred lines differing in salt tolerance provides novel insights into salt exclusion by roots

Zhen Yang · Hongxiang Zheng · Xiaocen Wei ·
Jie Song · Baoshan Wang · Na Sui

Received: 30 November 2017 / Accepted: 25 June 2018 / Published online: 6 July 2018
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Abstract

Backgrounds and aims Sweet sorghum is an annual C4 crop with a high salt tolerance. However, little is known about the molecular mechanisms of salt exclusion in roots of sweet sorghum. In this study, the physiological

Zhen Yang and Hongxiang Zheng contributed equally to this work.

Responsible Editor: Ian Dodd

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11104-018-3736-0>) contains supplementary material, which is available to authorized users.

Z. Yang · H. Zheng · X. Wei · J. Song · B. Wang (✉) ·
N. Sui (✉)
Shandong Provincial Key Laboratory of Plant Stress, College of
Life Sciences, Shandong Normal University, Jinan, Shandong
250014, People's Republic of China
e-mail: bswang.t@163.com; suina800101@163.com

Z. Yang
e-mail: gina35@126.com

H. Zheng
e-mail: 2992477319@qq.com

X. Wei
e-mail: Xiaocenwei@163.com

J. Song
e-mail: songjieever@163.com

Z. Yang
Shandong Provincial Key Laboratory of Microbial Engineering,
School of Biologic Engineering, Qilu University of Technology
(Shandong Academy of Sciences), Jinan 250300 Shandong, China

parameters and transcript profiles of two inbred lines of sweet sorghum roots (salt-tolerant M-81E and salt-sensitive Roma) were analyzed in the presence of 0 or 150 mM NaCl in order to elucidate the molecular mechanisms of salt exclusion.

Results We found that the Na⁺ concentrations in both shoots and roots of M-81E were lower than that of Roma. Moreover, we identified 2085 and 3172 differentially expressed genes between control plants and those subjected to salt stress in M-81E and Roma strains, respectively. The differentially expressed genes involved in pathways related to salt exclusion such as formation of root casparian bands and suberin lamellae, membrane-bound ion translocating proteins. Many of these genes underwent greater change in M-81E compared to Roma. These results revealed that the better ability of salt exclusion in M-81E may be caused by the combination of physical barrier effect of root apoplastic barriers and the transportation of Na⁺ out of the xylem by HKT1;5. Moreover, some genes encoding transcription factors were also differentially expressed, which may be involved in the regulation of genes related to salt exclusion.

Conclusions This RNA-seq dataset provide comprehensive insights into the transcriptomic landscape to reveal molecular mechanisms of salt exclusion in roots of sweet sorghum.

Keywords Genes · Roots · Salt exclusion · Sweet sorghum · Transcriptomic profile

Introduction

Salt stress is one of the major environmental stresses affecting plant growth and crop production worldwide. Salt stress involves a combination of two types of stress to plants, which include osmotic shock and ionic toxicity (Zhu 2001). Maintaining a low cytoplasmic concentration of Na^+ in shoots is quite important for plant survival under salt stress.

Plants have several strategies to maintain low Na^+ concentration in shoots: **A)** Root apoplastic barriers can block Na^+ transport to shoots. Apoplastic transpiration bypass flow of water and solutes has been shown to play a significant role in entry of Na^+ to shoots (Ochiai and Matoh 2002). Na^+ leakage into the transpiration stream via the apoplast account for a major portion of Na^+ entry into plants in some monocotyledons such as sorghum, rice and wheat (Zhu 2003). However, the bypass flow of water and solutes can be blocked by the apoplastic barriers which consists of casparian bands (CB) and suberin lamellae (SL) in the endo- and exodermis (Krishnamurthy et al. 2011; Steudle and Peterson 1998). **B)** Na^+ compartmentalization such as transport into vacuole can lower Na^+ concentration in the cytoplasm particularly in dicotyledons (Blumwald 2000). NHX proteins, which are Na^+/H^+ antiporters located in the vacuole, are regarded as key players in the sequestration of Na^+ into the vacuole (Jiang et al. 2010). **C)** The plasma membrane Na^+/H^+ antiporter encoded by *SOS1* gene can catalyze the efflux of Na^+ (Zhu 2003). The expression of *SOS1* can be up-regulated by salt stress which is partly regulated by *SOS2* and *SOS3* (Qiu et al. 2002). Plasma membrane H^+ -ATPases generate the driving force for Na^+ transport by *SOS1* (Zhu 2003). **D)** The high-affinity potassium transporter (HKT) *HKT1;5* has been proven taking part in retrieving Na^+ from the xylem vessels thus restricting the transport of Na^+ from the roots to the leaves (Byrt et al. 2007; Munns et al. 2012; Byrt et al. 2014; Zhu et al. 2016). As we know, salt stress can regulate the expression levels of many genes. The salt-response reactions are regulated by many different genes involved in different pathways, such as ion compartmentalization, ion extrusion, ion selectivity, compatible solute synthesis and reactive oxygen species scavenging (Munns and Tester 2008; Shi et al. 2003; Zhu 2001). In the past few decades,

researchers have come to regard the use of these genes as a way to improve the salt tolerance of plants.

Sweet sorghum [*Sorghum bicolor* (L.) Moench] is an annual C4 crop which is characterized by high sugar content, high efficiency biomass accumulation and high tolerance to saline and drought conditions (Guo et al. 2017). Sweet sorghum has been suggested as a good source for ethanol production because of its rapid growth rate and high sugar content (Almodares and Hadi 2009). Furthermore, sweet sorghum can complete its life cycle on saline land (E118°53'41", N37°38'39", salt concentration was about 0.34%) (Ding et al. 2013) as it is highly tolerant to saline conditions. Several mechanisms were proposed to contribute to salt tolerance of sweet sorghum, such as salt exclusion (Dai et al. 2014), antioxidative mechanisms (Chai et al. 2010) and osmoregulation (Oliveira et al. 2011). Salt exclusion is considered to be the pivotal process in salt resistance of monocotyledon crops such as sweet sorghum.

Although there are numerous studies on the response mechanism of sweet sorghum to salt stress, most are restricted to the physiological level or to the study of a single pathway. The molecular mechanisms of salt exclusion in roots of sweet sorghum are still unclear. In recent years, expression profiling has been widely used to identify genes involved in adaptive responses to abiotic stresses. The comparative study of different genotypes or cultivars in the tolerance to the abiotic stress is a common strategy to identify genes related to salt stress (Bazakos et al. 2012; Du et al. 2017; Yuan et al. 2015). In our previous study, transcript profiles of two sweet sorghum inbred lines' leaves were performed to reveal the mechanism of higher sugar content under salt stress in sweet sorghum (Sui et al. 2015). However, how sweet sorghum survive and maintains low Na^+ content and high biomass of aboveground parts under salt stress is still not clear. In this study, transcriptomes of inbred lines' roots of both salt-tolerant and salt-sensitive sweet sorghum roots were analyzed by high-throughput Illumina RNA-sequencing (RNA-seq). By comparing the transcriptomes of the two sweet sorghum inbred lines under salt stress, we identified 2085 and 3172 differentially expressed genes (DEGs) between control plants and those subjected to salt in M-81E and Roma, respectively. Results of this study provide further insight into the complex regulatory networks underlying the mechanism of high tolerance to salt stress of sweet sorghum.

Materials and methods

Plant materials and growth conditions

Seeds of two sweet sorghum inbred lines (M-81E and Roma) were selected as the experimental materials. M-81E was regarded as salt tolerant inbred line and Roma was regarded as salt-sensitive inbred line according to our previous study (Sui et al. 2015).

Sand culture was performed for physiological parameters measurement and RNA extraction. Plants were grown in pots and cultured at $28 \pm 3/23 \pm 3$ °C {day/night} at a light intensity of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ {15 h photoperiod) and 70% relative humidity. When they grew to the four leaf stage, salt treatment was performed. In our previous study, 150 mM was regarded as a proper concentration of NaCl which caused a significant difference in physiological parameters between M-81E and Roma (Sui et al. 2015). Therefore, the treated plants were irrigated with nutrient solution supplemented with 0 and 150 mM NaCl. The NaCl concentrations were increased stepwise towards the final concentrations by 50 mM each day. Physiological parameters were measured every 12 h until 48 h.

Measurement of parameters

Shoot and root fresh weight (FW) of 5 plants per treatment were determined every 12 h. After oven drying of tissues, Na^+ and K^+ concentration were measured by flame emission spectrometry (Flame Photometer 410, UK) as previously described (Sui et al. 2015). The *p* value between different data of content of Na^+ , K^+ of shoots and roots of M-81E and Roma under 150 mM salt treatments for different hours were showed in Table S1.

Total RNA extraction

Total RNA was isolated from the primary roots of each genotype treated with 0 and 150 mM NaCl for 48 h using a Total Plant RNA Extraction Kit (Karrotan, Beijing, China) following the manufacturer's protocols as described previously (Sui et al. 2015). The RNA was quantified using a Nanodrop-ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). A 1% agarose gel buffered by Tris–acetate–EDTA was also run to determine the integrity of the RNA.

Library construction and Illumina sequencing

Libraries were constructed following a High Throughput Illumina Strand-Specific RNA Sequencing Library protocol (Zhong et al. 2011). Briefly, mRNA was purified from 5 μg of total RNA using oligo (dT) magnetic beads. The purified mRNA was fragmented into small pieces using fragmentation buffer. Taking these short fragments as templates, first-strand cDNA was synthesized using reverse transcriptase and random hexamer primers. Second-strand cDNA synthesis was followed using DNA polymerase I and RNase H. Sequencing adapters were ligated to short fragments after purification with the QiaQuick PCR extraction kit, and which were used to distinguish different sequencing samples. Fragments with different lengths were then separated by agarose gel electrophoresis and selected for PCR amplification as sequencing templates. The final cDNA library was sequenced using Illumina HiSeq™ 2500 at BioMarker Technologies Co Ltd., Beijing. RNA-seq data of the untreated control and salt-treated samples were obtained from two to three biological replicates, respectively.

Mapping and detection of differentially expressed genes (DEGs)

Clean reads were mapped to the sorghum genome (Paterson et al. 2009; Zheng et al. 2011) using TopHat version 2.0.10 (Trapnell et al. 2009). Mapping results generated by TopHat were filtered to retain only unique mapped reads before being piped into Cuffdiff (<http://cufflinks.cbc.umd.edu/index.html>) to estimate read counts for each gene. Reads per KB per million (RPKM) values were calculated by an in-house script based on the count table of Cuffdiffs output. Mapping and detection of DEGs were performed as described previously (Sui et al. 2015). A RPKM threshold value of 0.1 was set to detect the presence of a transcript for a particular gene. DEGs were defined using DESeq (Anders and Huber 2010) as fold changes ≥ 2 with a false discovery rate (FDR) adjusted *p* value ≤ 0.01 .

Gene annotation and classifications

The optimal assembly results were chosen according to the assembly evaluation. The assembled sequences were compared against the NCBI non-redundant database (Pruitt et al. 2005), Swiss-Prot (Apweiler et al. 2004), GO (Ashburner et al. 2000), COG (Tatusov et al. 2000)

and KEGG (Kanehisa et al. 2004) database using BLAST with E-value $\leq 1e-10$ as the cutoff. To annotate the assembled sequences with GO, COG and KEGG terms, Blast2GO, COG database and KEGG web server (<http://www.genome.jp/kegg/>) were used, respectively.

Quantitative real-time PCR analysis

Eleven DEGs annotated to pathways related to salt exclusion were selected for quantitative real-time PCR (qRT-PCR) to verify the RNA-Seq results. Primers for these 11 genes were designed using the Beacon Designer software (version 7.0) and showed in Table S2. *S. bicolor*'s house-keeping gene β -actin (GenBank ID: X79378) was used as an internal standard. The measurement and calculation were performed as described previously (Sui et al. 2015).

Statistical analysis

Comparisons among samples were performed using two-way analysis of variance (ANOVA) followed by Student's t-test. Variables were presented as means \pm standard error of the means (SEM) and a $p < 0.05$ was considered significant. All tests were performed with SPSS Version 16.0 for Windows (SPSS, Chicago, IL, USA).

Availability of data and material The RNA-seq data in the current study are available in the National Center for Biotechnology Information (NCBI) SRA database with accession number of SRX1090369. Access to the data is available upon publication at <http://www.ncbi.nlm.nih.gov/sra/SRP060580/>. Other relevant data are within the paper and its additional files.

Results

Effects of salt stress on FW, Na^+ , K^+ contents and Na^+/K^+ ratio

Under salt stress, the growth rate of both shoots and roots in Roma was lower than that in M-81E. The growth of roots in Roma was inhibited after 24 h salt treatment. In M-81E, roots growth inhibition by salt stress was not apparent until 36 h. The growth of shoots was inhibited after 36 h salt treatment and the inhibition was stronger in Roma (Fig. 1).

After treated with 150 mM NaCl, Na^+ content in both shoots and roots of M-81E and Roma increased, and

Na^+ content in shoots was much lower than that in roots. Na^+ content in both shoots and roots of M-81E was significantly lower than that in Roma after treated with NaCl for 48 h (Fig. 1a and c).

However, the K^+ content in both roots and shoots of M-81E and Roma decreased under NaCl stress. The decrease was much more significant in Roma than that in M-81E (Fig. 1b and d).

As the increase of content of Na^+ and the decrease of content of K^+ , the Na^+/K^+ ratio of both shoots and roots increased in both genotypes. The increase was much more significant in Roma than that in M-81E. Additionally, the Na^+/K^+ ratio in shoots was much lower than that in roots in both genotypes (Fig. S1).

Sequencing output, assembly and exploration of DEGs

After stringent quality assessment and data filtering, 78.31 million clean reads were generated. More than 87% of them had Phred-like quality scores at the Q30 level (an error probability of 0.1%) (Table S3).

Numbers of DEGs between different genotypes and treatments were shown in Fig. 2. All of these DEGs were selected for further analysis.

Validation of RNA-seq data

We performed quantitative real-time PCR on 11 DEGs to validate the RNA-seq gene expression analysis. As shown in Fig. 3, a high correlation ($R^2 = 0.86$) between RNA-seq and qRT-PCR was observed, confirming the reliability of the RNA-seq data.

Functional categorization of stress-regulated genes in roots

Functional classification by GO

In order to assign functional information to the DEGs between control plants and those treated with NaCl, Gene Ontology (GO) analysis was performed. In M-81E, there were 1960 unique transcripts assigned to 48 level-2 GO terms, which were summarized under three main GO categories, including 14 for cellular component, 14 for molecular function and 20 for biological process. In Roma, there were 2963 unique transcripts assigned to 49 level-2 GO terms including 16 for cellular component, 13 for molecular function and 20

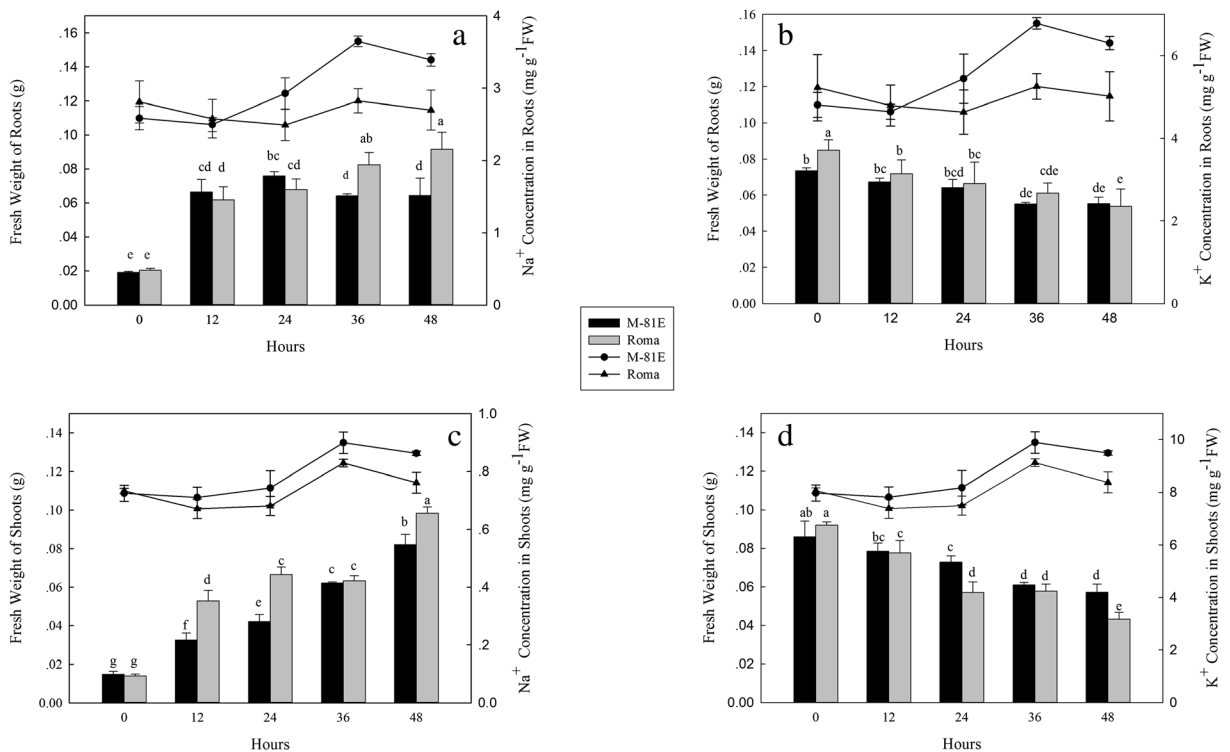


Fig. 1 Content of Na⁺ (a, c), K⁺ (b, d) and the fresh weight of shoots and roots of M-81E and Roma under 150 mM salt treatments for different hours (0, 12, 24, 36 and 48). Timing and indicators measuring started when the salt concentration reached 150 mM. The line shows the change in fresh weight, and the histogram shows the change in ion content. Values are means \pm SD of five replicates. Bars with the different letters are significantly different at $p = 0.05$. Bars with same letters are not significantly different. ANOVA for Na⁺ concentration in roots showed a significant effect for the factor treated time ($F(4, 22) = 86.782$; $p = 0.000$), a significant effect for the genotype ($F(1, 22) = 8.886$; $p = 0.007$), and a significant effect for the interaction between treated time and genotype ($F(4, 22) = 9.033$; $p = 0.000$). ANOVA for K⁺ concentration in roots showed a significant effect for the factor

treated time ($F(4, 21) = 20.155$; $p = 0.000$), a significant effect for the genotype ($F(1, 21) = 5.627$; $p = 0.027$), and no significant effect for the interaction between treated time and genotype ($F(4, 21) = 1.230$; $p = 0.328$). ANOVA for Na⁺ concentration in shoots showed a significant effect for the factor treated time ($F(4, 23) = 409.588$; $p = 0.000$), a significant effect for the genotype ($F(1, 23) = 91.968$; $p = 0.000$), and a significant effect for the interaction between treated time and genotype ($F(4, 23) = 17.149$; $p = 0.000$). ANOVA for K⁺ concentration in shoots showed a significant effect for the factor treated time ($F(4, 20) = 53.795$; $p = 0.000$), a significant effect for the genotype ($F(1, 20) = 8.593$; $p = 0.008$), and no significant effect for the interaction between treated time and genotype ($F(4, 20) = 5.262$; $p = 0.005$). The p value between different sets are listed in supplementary Table S1

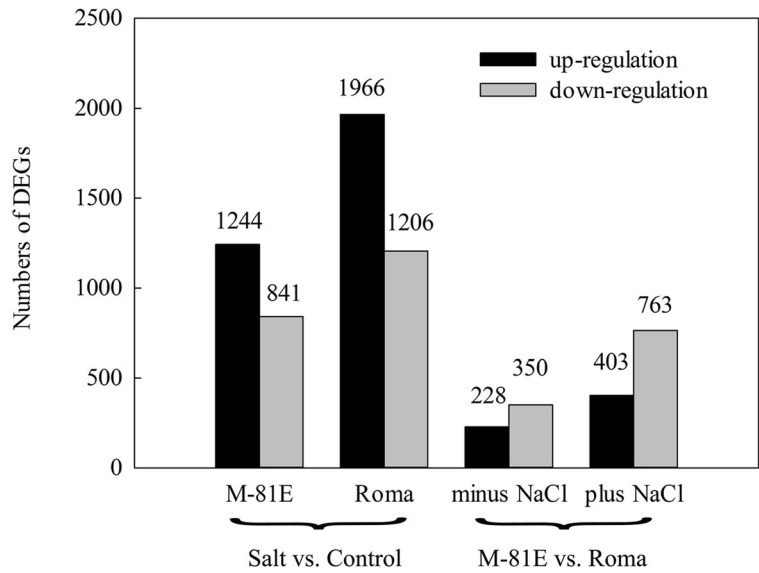
for biological process. The categories with the highest enrichment for cellular group in both M-81E and Roma were cell part, cell and organelle. For molecular function, the binding category was the most highly represented GO term, which was followed by catalytic activity. Regarding biological process, NCBI UniGene for cellular process, metabolic process and single-organism process were highly represented (Fig. 4).

Functional classification by COG

In addition, all the DEGs were subjected to a search against the Clusters of Orthologous Groups (COG) classification. Among the 2085 DEGs, 1274 sequences

showed a COG classification in M-81E (Fig. S2A). Among the mapped 23 COG categories, the cluster for “general function prediction only” was the largest group, followed by “carbohydrate transport and metabolism”, “amino acid transport and metabolism”, “replication, recombination and repair” and “posttranslational modification, protein turnover, chaperones”. The categories “nuclear structure” and “extracellular structures” had no corresponding genes. The 1939 sequences of the 3172 DEGs could be assigned to COG classifications in Roma (Fig. S2B). The cluster for “general function prediction only” represented the largest group, followed by “replication, recombination and repair”, “signal transduction

Fig. 2 Numbers of DEGs of different genotypes affected by salt stress



mechanisms”, “transcription” and “carbohydrate transport and metabolism”. The categories “nuclear structure” and “extracellular structures” had no corresponding genes.

Functional classification by KEGG

Kyoto Encyclopedia of Genes and Genomes database (KEGG) was used to identify potential biological

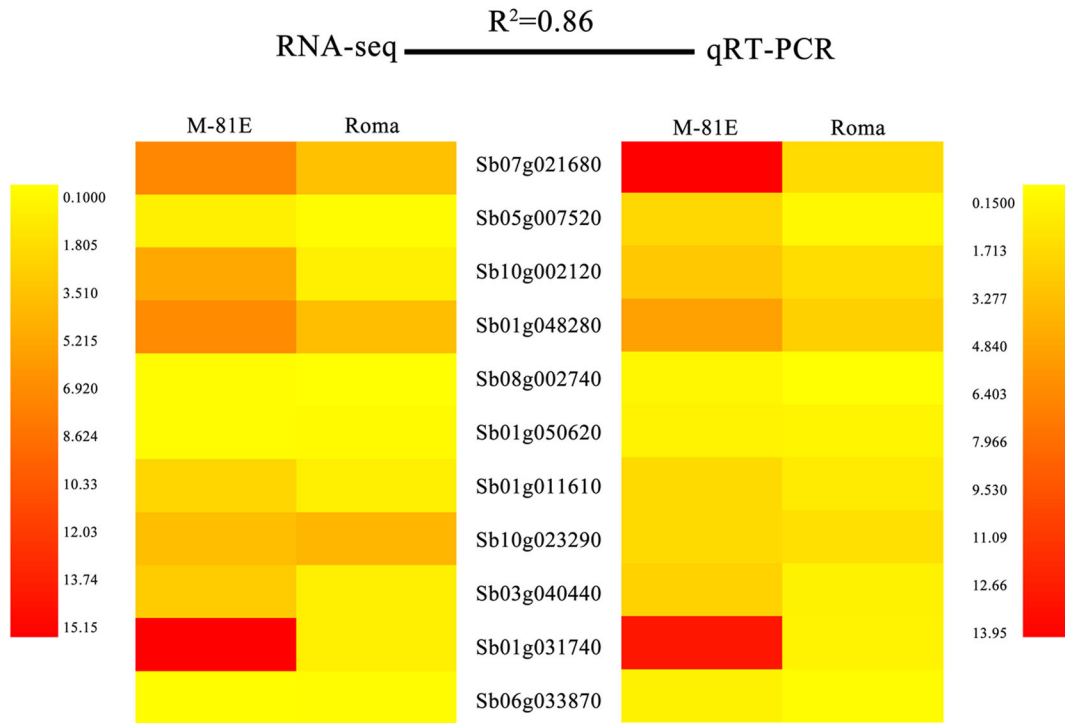


Fig. 3 Validation of RNA-seq results by RT-qPCR. Expression levels of 11 DEGs in the four samples used in this study were detected by RT-qPCR. R^2 represents the correlation coefficient value between the two platforms. The numbers in the scale bar

stand for RPKM values in RNA-seq and $\Delta\Delta Ct$ in qRT-PCR, which were used to evaluate the correlation (R^2). Primers and annotation of the 11 DEGs are listed in Supplementary Table S2

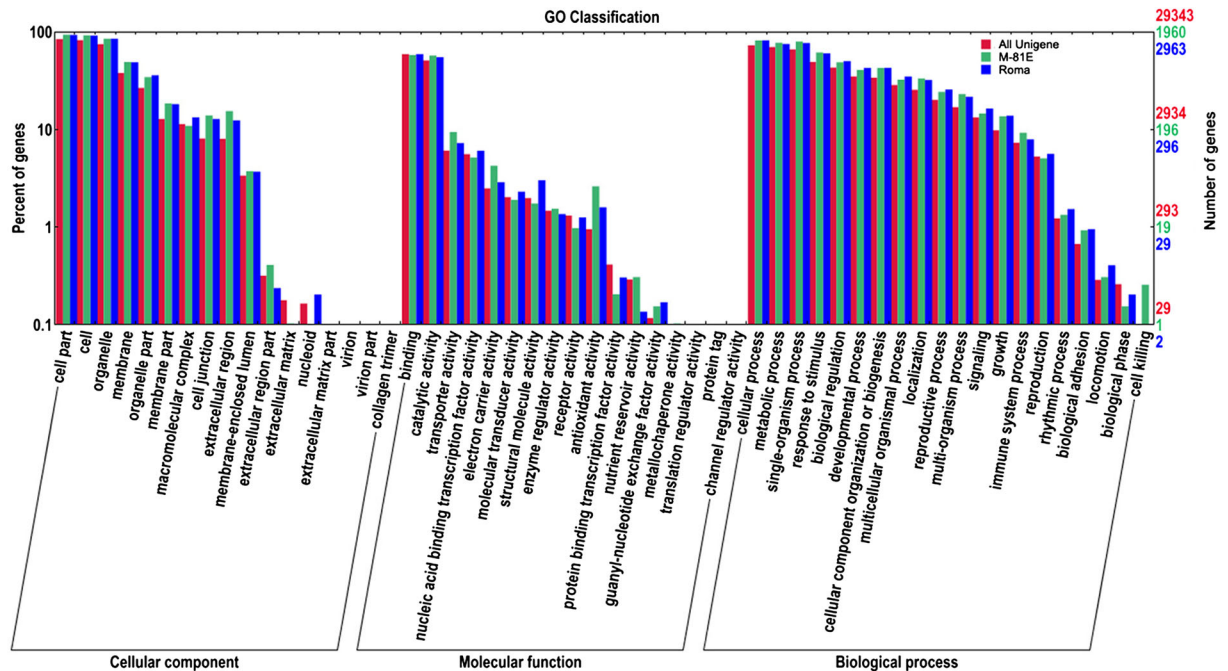


Fig. 4 Functional annotation of assembled sequences based on gene ontology (GO) categorization. Results are summarized for three main Go categories: Biological Process, Molecular Function, and Cellular Component

pathways represented in the sweet sorghum transcriptome. There were 557 DEGs of M-81E and 825 DEGs of Roma assigned to 100 and 103 KEGG pathways, respectively. A large number of these DEGs mapped to categories such as “phenylpropanoid biosynthesis”, “fatty acid biosynthesis” and “biosynthesis of unsaturated fatty acids”. This may be related to the formation of apoplastic barriers such as SL. Moreover, there were also many DEGs mapped to categories related to synthesis and metabolism of different amino acids and “protein processing in endoplasmic reticulum” which may contribute to changes in membrane transport proteins and some other defense proteins. “Plant hormone signal transduction” which plays important roles in abiotic stress response is also a category which contain many DEGs (Fig. 5).

Identification of genes related to salt resistance

Genes related to apoplastic barriers

The apoplastic barriers which consist of CB and SL in the endo- and exodermis can block the bypass flow of water and solutes. Lignin is one of the main components of apoplastic barriers. It has been proven that the monomers

of lignin are synthesized through the phenylpropanoid pathway (Whetten and Sederoff 1995). In the present study, 14 and 10 DEGs were mapped to the phenylpropanoid pathway related to lignin biosynthesis in M-81E and Roma, respectively (Fig. S3, Table 1). Most of these DEGs were up-regulated, suggesting that lignin biosynthesis was enhanced by salt stress in both genotypes. Cinnamoyl CoA reductase (CCR, EC:1.2.1.44) and cinnamyl alcohol dehydrogenase (CAD, EC:1.1.1.195) are considered as key enzymes for lignin biosynthesis. After 2 days treatment with 150 mM NaCl, the expression of DEG (Sb07g021680) encoding CCR was enhanced in both genotypes, though the expression level of Sb07g021680 in M-81E was higher than that in Roma. The expression of genes encoding CAD were unchanged during salt stress. Additionally, the expression of other genes encoding enzymes involved in lignin biosynthesis such as 4-coumarate-CoA ligase (4CL), trans-cinnamate 4-monooxygenase and p-coumarate 3-hydroxylase (Sb04g031010, Sb03g038160 and Sb09g024210 respectively) were also induced in both genotypes, particularly in M-81E. Moreover, five (Sb02g000470, Sb03g024460, Sb03g030630, Sb07g001280 and Sb10g027490) and three (Sb03g024460, Sb03g030630 and Sb02g027330) genes encoding peroxidases which play roles in lignin

Fig. 5 The heat map display of DEGs assigned to different KEGG pathways. The numbers in the scale bar show the percentage of the number of DEGs assigned to a certain KEGG pathway in which assigned to all KEGG pathways. Red indicates that more genes are enriched in this pathway



Table 1 DEGs mapped to phenylpropanoid pathway

Gene ID	annotation	M-81E		Roma	
		FDR	log ₂ FC	FDR	log ₂ FC
Sb01g010830	Beta-glucosidase 7 (Precursor)	0.000157	-1.523	–	–
Sb01g010840	Beta-glucosidase 7 (Precursor)	0.006449	1.280427	4.47E-05	1.459969
Sb01g041760	Peroxidase N (Precursor)	8.31E-14	-3.18323	3.06E-10	-2.62365
Sb02g000470	Peroxidase 1 (Precursor)	0.00584	1.229081	–	–
Sb03g024460	Peroxidase 72 (Precursor)	0.006355	1.189848	1.57E-08	2.003783
Sb03g030630	Peroxidase 19 (Precursor)	0.000386	2.136814	2.93E-06	2.125997
Sb03g038160	Trans-cinnamate 4-monooxygenase	4.50E-08	2.637436	0.001809	2.413054
Sb04g031010	Probable 4-coumarate-CoA ligase	0.002771	2.221379	0.002767	2.174296
Sb04g038610	Peroxidase 64 (Precursor)	4.42E-07	-2.90919	–	–
Sb07g001280	Peroxidase 47 (Precursor)	1.33E-05	1.812576	–	–
Sb07g021680	Cinnamoyl-CoA reductase	1.08E-12	2.842039	8.35E-07	1.920045
Sb09g024210	p-coumarate 3-hydroxylase P450 98A1	0.000158	1.536418	–	–
Sb10g010040	Peroxidase 11 (Precursor)	2.29E-05	-1.74935	–	–
Sb10g027490	Peroxidase 3 (Precursor)	6.93E-07	2.061833	–	–
Sb02g027330	Peroxidase 17 (Precursor)	–	–	0.005278	2.749369
Sb10g023000	Aldehyde dehydrogenase family 2 member C4	–	–	0.003219	2.644443
Sb10g026130	Probable 4-coumarate-CoA ligase 4	–	–	0.004708	1.471672

Legend: DEGs mapped to phenylpropanoid pathway. “FDR” means false discovery rate, “FC” means fold change, “-” means the expression of the gene was not changed under salt stress

biosynthesis were up-regulated under salt stress in M-81E and Roma, respectively.

Suberin is the other main component of apoplastic barriers. Fatty acid elongation and ω -carbon oxidation represent two characteristic processes of suberin biosynthesis. According to our RNA-seq data, lots of genes related to suberin biosynthesis showed differential expression. Sb10g023290, which encodes the β -ketoacyl-CoA synthase (KCS), was up-regulated in both genotypes. Among the genes encoding NADPH-dependent cytochrome P450 monooxygenases (P450) catalyzing the hydroxylation of fatty acids in the ω -position, 1 gene (Sb03g040440) and 2 genes (Sb09g021890 and Sb08g003110) were enhanced by salt stress in M-81E and Roma, respectively. Furthermore, Sb09g006170 encoding FAR1 involving suberin synthesis was significantly up-regulated in both genotypes. Meanwhile, another 2 genes (Sb10g024700 and Sb04g022400) related to FAR1 were down-regulated only in Roma (Table 2). In addition, DEGs encoding ABCG transporters participating in the transport of precursors of suberin in both genotypes were up-regulated after treated with NaCl.

Genes related to the ion transport

Among all DEGs, we identified 21 and 27 genes related to K⁺ and Na⁺ transport in M-81E and Roma, respectively (Table 3). The expression of Sb03g012590, which encodes HKT8 (HKT1;5) was enhanced in both genotypes after treated with NaCl. However, the increase in the expression level was more significant in M-81E. According to our RNA-seq data, 6 and 4 genes related to K⁺ transport were differentially expressed under salt stress in M-81E and Roma, respectively. Most of these genes were up-regulated under salt stress. CBL (calcineurin B-like protein)-interacting protein kinase (CIPK) have been implicated in regulating cellular sodium and potassium homeostasis. In M-81E, there were only 4 DEGs encoding CIPK, and only one gene of which was up-regulated. However, 10 CIPK encoding DEGs were found in Roma, four of which were up-regulated (Table 3). Two and one genes encoding plasma membrane ATPase, which generates the proton gradient to provide energy for various transporters on the plasma membrane, were differentially expressed in M-81E and Roma, respectively. Sb01g050620 encoding plasma

Table 2 DEGs related to suberin synthesis

Gene ID	annotation	M-81E		Roma	
		FDR	log ₂ FC	FDR	log ₂ FC
Sb10g023290	3-ketoacyl-CoA synthase 11 KCS11	7.49E-06	1.954551	3.24E-09	2.138424
Sb03g040440	Cytochrome P450 94A1	0.0001658	1.655476	–	–
Sb08g001320	Cytochrome P450 94A1	0.0013684	–2.52925	–	–
Sb09g021890	Cytochrome P450 94A2	–	–	0.0003816	1.493168
Sb08g003110	Cytochrome P450 94A1	–	–	0.0000584	2.638363
Sb09g006170	FAR1-RELATED SEQUENCE 5	0.0004716	2.85013	0.0002032	2.985953
Sb10g024700	FAR1-RELATED SEQUENCE 4	–	–	0.0019157	–1.37522
Sb04g022400	FAR1-RELATED SEQUENCE 5	–	–	0.0016168	–1.42401
Sb07g023550	ABC transporter G family member 4	0.003223	1.67846	–	–
Sb05g004940	ABC transporter G family member 25	–	–	5.25E-05	1.466189

Legend: DEGs mapped to suberin synthesis. “FDR” means false discovery rate, “FC” means fold change, “–” means the expression of the gene was not changed under salt stress

membrane ATPase was down-regulated in both genotypes. Whereas, only M-81E showed a 2.51-fold induction of the gene (Sb01g011610). In plants, the Na⁺/H⁺ antiporter in vacuolar membranes transports Na⁺ from the cytoplasm into vacuoles using the electrochemical H⁺ gradient generated by vacuolar H⁺-inorganic pyrophosphatase and vacuolar H⁺-ATPase. In M-81E and Roma, expression of vacuolar H⁺-inorganic pyrophosphatase related DEGs (Sb04g005710 and Sb10g025280, respectively) were down-regulated after NaCl treatment (Table 3).

Genes related to the ROS scavenging system

In M-81E, there were 31 DEGs categorized into peroxidase, of which 21 were up-regulated by salt stress (Table S4). Furthermore, the expression of Sb01g004160 and Sb10g028480, which encode the precursor of cationic peroxidase 2 and peroxidase 45, were significantly enhanced by salt stress. Thirty-five DEGs categorized into peroxidase were found in Roma, 25 DEGs of which were up-regulated by salt stress. While, the increases in expression level of these DEGs were not as significant as those of M-81E. Three genes encoding CATs were differentially expressed in both M-81E and Roma. The expression of Sb01g048280 which encodes catalase isozyme 2, was up-regulated by salt stress in both M-81E and Roma. However, this increase in the expression level was more significant in M-81E. Two genes encoding ascorbate peroxidase (APX)

differentially expressed in both M-81E and Roma according to our RNA-seq data. These 2 genes showed similar expression levels in M-81E and Roma under salt stress. There were 10 and 7 DEGs categorized into glutathione-S-transferases (GST) in M-81E and Roma, respectively. Most of these DEGs were up-regulated by salt stress. In M-81E, the expression levels of 3 genes encoding germin-like protein were significantly enhanced after 48 h treatment with 150 mM NaCl. However, Roma had only one germin-like protein encoding gene differentially expressed under salt stress.

Up-regulated genes related to transcription factors

As expected, there were 50 and 21 up-regulated transcription factors (TFs) under salt stress in M-81E and Roma, respectively (Table S5).

In M-81E, up-regulated TFs were categorized into many different families, and the greatest enrichment was for the *bHLHs* and *MYBs*. In addition, members of the *WRKY*, *ERF*, *RF* and *NAC* which have been previously implicated to play critical roles in abiotic stress were also differentially expressed following NaCl treatment. Three heat stress transcription factor encoding genes (Sb01g008380, Sb04g008300, and Sb06g025710) were highly induced (48.17-, 43.41- and 6.32-fold, respectively) by salt stress. In Roma, however, DEGs related to TFs only categorized into a few families, and the majority of which categorized into *bHLHs*. Members of *RF*

Table 3 DEGs related to the ion transport

Gene ID	annotation	M-81E		Roma	
		FDR	log ₂ FC	FDR	log ₂ FC
Sb03g000700	Salt stress root protein RS1	–	–	4.44E-10	–2.2919745
Sb08g021840	K ⁺ efflux antiporter 3, chloroplastic	0.003025	–1.4466245	7.12E-05	–1.6404701
Sb01g048670	K ⁺ efflux antiporter 5	0.00300451	1.4315531	–	–
Sb03g012590	Cation transporter HKT8	7.21E-18	5.2589382	2.22E-08	3.0232756
Sb07g022685	Probable potassium transporter 4	7.86E-07	3.9500162	0.0066322	3.2573801
Sb03g000700	Salt stress root protein RS1	0.00023617	–2.0413971	–	–
Sb09g021160	Potassium channel AKT2	–	–	6.09E-05	1.7252641
Sb03g029520	Potassium channel AKT1	–	–	0.00109296	2.7976411
Sb01g011630	Calcium-dependent protein kinase 10	–	–	0.00445807	1.1769026
Sb10g008960	Potassium channel KOR1	0.00341438	1.5000486	–	–
Sb09g018980	Annexin D3	0.00874144	–1.239857	–	–
Sb02g000340	Potassium transporter 27	0.0001048	1.8329655	–	–
Sb05g002070	Protein ZINC INDUCED FACILITATOR 1	–	–	0.0019163	–1.5849044
Sb03g034980	Potassium channel KAT3	5.58E-08	–2.587228	–	–
Sb04g011100	Plasma membrane-associated cation-binding protein 1	3.76E-08	2.3620005	7.71E-11	2.9976177
Sb09g018910	Cation/H ⁺ antiporter 20	–	–	0.00726751	2.191702
Sb08g021720	Cation/H ⁺ antiporter 19	1.33E-07	–2.1333373	0.00140537	–2.3404881
Sb09g002900	CBL-interacting protein kinase 17	–	–	7.07E-05	–1.4658384
Sb08g020690	CBL-interacting protein kinase 4	–	–	0.00027912	–2.3430821
Sb07g021660	CBL-interacting protein kinase 6	0.00038423	2.5458847	0.00289129	3.6021476
Sb09g025420	CBL-interacting protein kinase 19	–	–	0.00055258	1.6967653
Sb01g048750	CBL-interacting protein kinase 9	–	–	0.00226559	1.1212687
Sb01g036020	CBL-interacting protein kinase 10	1.18E-34	–5.6438371	4.96E-10	–2.415617
Sb03g035170	CBL-interacting protein kinase 19	–	–	0.00425493	1.5789395
Sb05g002000	CBL-interacting protein kinase 32	–	–	0.00341144	–1.0931847
Sb08g002740	CBL-interacting protein kinase 14	0.00146763	–1.4514006	1.91E-06	–3.1510508
Sb02g024770	CBL-interacting protein kinase 16	–	–	0.00292796	–1.0967604
Sb02g024780	CBL-interacting protein kinase 6	4.00E-08	–3.4671062	–	–
Sb01g050620	Plasma membrane ATPase 1 LHA	0.00030139	–1.5009787	0.00049896	–1.2579605
Sb01g011610	ATPase 8, plasma membrane-type AHA8	0.00143895	1.331467	–	–
Sb04g005710	Pyrophosphate-energized vacuolar membrane proton pump	0.00046977	–1.449252	–	–
Sb10g025280	Pyrophosphate-energized vacuolar membrane proton pump	–	–	0.00576836	–1.9336548

Legend: DEGs related to the ion transport. “FDR” means false discovery rate, “FC” means fold change, “–” means the expression of the gene was not changed under salt stress

and *MYB* were also differentially expressed under salt stress.

Other identified genes

Plants have to adjust their water balance in response to salt stress. Aquaporin proteins have been shown to transport water and other small molecules through

biological membranes, which is crucial for plants to combat salt stress. After treated with 150 mM NaCl for 48 h, 9 genes encoding aquaporins were differentially expressed in M-81E, of which 6 categorized into plasma membrane intrinsic proteins (PIPs) and 3 categorized into tonoplast intrinsic proteins (TIPs) (Table S6). All the DEGs encoding PIPs were up-regulated more than 4-fold. The genes Sb02g010780,

Sb02g010800 and Sb10g007610, which respectively encode PIP 2–6, PIP 2–6 and PIP 1–6, were highly induced by salt stress. Two of the DEGs encoding TIPs in M-81E were up-regulated. In Roma, 6 genes which encode aquaporins were differentially expressed in response to salt stress. Three of these DEGs were related to PIPs and were up-regulated. Sb09g007940, which encodes TIP4–2 was down-regulated by 5.54-fold. To our surprise, the expression of Sb07g003270 which encodes NIP3–2 was significantly enhanced under salt stress in Roma. Gene which encodes SIP1–1 was down-regulated after treated with NaCl. In M-81E, 9 DEGs categorized into heat shock proteins (HSPs) were found after 48 h NaCl treatment. All of these genes were significantly enhanced by salt stress. In Roma, 10 genes encoding HSPs were differentially expressed, with 9 of which were up-regulated (Table S7).

Discussion

Plants have evolved various mechanisms to overcome salt stress in the long-term natural selection. Salt tolerance in monocotyledonous species is strongly correlated with salt exclusion which characterized by low Na⁺ content in leaves and relative high Na⁺ content in roots (Läuchli et al. 2008; Munns et al. 2006; Munns et al. 2003; Yeo and Flowers 1986). In the present study, Na⁺ content in shoots is much lower than that in roots in both genotypes, suggesting that salt exclusion plays important roles in response to salt stress in sweet sorghum. Moreover, Na⁺ content in shoots of the salt-sensitive Roma increased more significantly than that in salt-tolerant M-81E (Fig. 1a and c), indicating that M-81E had better ability to limit the transport of Na⁺ into shoots. Na⁺ content in roots of M-81E was also significantly lower than that in Roma, suggesting roots of M-81E may have better abilities to limit the entry of Na⁺ or/and to promote the efflux of Na⁺. High Na⁺ concentration impairs the ability of plants to accumulate K⁺ (Munns and Tester 2008). K⁺ content decreased with raised NaCl concentration and the decrease was more severe in Roma (Fig. 1b and d). These results indicated that M-81E has a higher salt exclusion and the net capacity of selection for transport of K⁺ over Na⁺ than Roma being consistent with previous studies (Sui et al. 2015).

Root apoplastic barriers consist of CB and SL play a pivotal role in blocking apoplastic bypass flow of water

and ions into the stele and Na⁺ transport to shoots (Ma and Peterson 2003). It has been reported that salt stress strengthened the root apoplastic barriers (Krishnamurthy et al. 2014; Krishnamurthy et al. 2009). However, little is known about the genes involved in root apoplastic barriers and their possible roles in salt exclusion under salinity.

Suberin and lignin are the main components of apoplastic barriers. Fatty acid elongation and ω -carbon oxidation represent two characteristic processes of suberin biosynthesis. KCS is rate-limiting step in the fatty acid elongation products (Millar and Kunst 1997). The ω -carbon oxidation is typically catalyzed by P450, particularly by the CYP86 and CYP94 families (Höfer et al. 2008; Le Bouquin et al. 1999; Le Bouquin et al. 2001; Serra et al. 2009). According to our RNA-seq data, DEGs encoding KCS and CYP94A1 which are key enzymes of suberin biosynthesis were up-regulated in both genotypes, indicating that the biosynthesis of suberin precursors was enhanced by salt stress. This result was consistent with Benveniste's study (Benveniste et al. 2005). Subfamily G ABC transporters (ABCG transporters) have been reported involved in the transport of precursors of suberin (Landgraf et al. 2014; Vishwanath et al. 2015; Yadav and Reed 2014). DEGs encoding ABCG transporters were induced by salt stress in both genotypes (Sb07g023550 in M-81E and Sb05g004940 in Roma) (Table 2). Salt stress also strengthened apoplastic barriers in rice (Krishnamurthy et al. 2009). In addition, genes encoding key enzymes for lignin biosynthesis such as Sb07g021680 encoding CCR, Sb04g031010 encoding 4CL and Sb03g038160 encoding trans-cinnamate 4-monooxygenase were also induced in both genotypes (Table 1). Moreover, the increases were more significant in M-81E than that in Roma, suggesting that lignin biosynthesis was induced by salt stress in both genotypes, particularly in M-81E. In particular, Sb09g024210 encoding p-coumarate 3-hydroxylase (C3H, 1.14.13.-) was up-regulated only in M-81E, which may cause an increase of G-type lignin. The biosynthesis of lignin was also affected by various biotic and abiotic stresses (Vanholme et al. 2010). These results suggested that salt exclusion in sweet sorghum can be regulated by changing expression level of genes encoding not only the biosynthesis of suberin and lignin but also the transport process of its monomer transport process.

Through prior exposure to mild stress, plants can accumulate many TFs to defend against lethal levels of

stress and may be related to signal transduction pathways under stress (Yang et al. 2017). There are many evidences that TFs are involved in improving stress tolerance in plants. MYB family TFs play important roles in the regulation of lignin biosynthesis (Zhong and Ye 2009), suberin biosynthesis (Kosma et al. 2014) and secondary cell wall deposition (McCarthy et al. 2009). In the present study, many DEGs encoding MYBs were up-regulated in M-81E, which may be involved in the regulation of lignin and suberin biosynthesis in M-81E. While, there were only 2 genes encoding MYB86 up-regulated by salt stress in Roma. In addition, *OsMYB59* and *ScMYBAS1* are thought to play positive roles in stress response (Prabu and Prasad 2012; Quan et al. 2010). DEGs encoding these may also contribute to improving the salt tolerance of M-81E. In addition, other TFs such as bHLHs, NACs, HSFs and WRKYs were also up-regulated in both genotypes, particularly in M-81E. These TFs are involved in plant stress responses (Liu et al. 2014; NIU et al. 2012; Yokotani et al. 2008; Yokotani et al. 2009). These results revealed that the genes encoding TFs such as MYBs and bHLHs contribute to improving the salt tolerance of sweet sorghum.

Although root apoplastic barriers can block or reduce the Na^+ transport to shoots, they also limit the absorption and transport of water. Aquaporin proteins play important roles in transportation of water through biological membranes (Xin et al. 2014). According to our RNA-seq data, 8 and 4 DEGs encoding aquaporin proteins were up-regulated in M-81E and Roma, respectively. Most of these DEGs categorized into PIPs, and the up-regulation was more significant in M-81E. In addition to PIPs, TIPs have also been proven play roles in enhancing the tolerance to salts stress (Xin et al. 2014). In the current study, there were 2 DEGs encoding TIPs up-regulated in M-81E, whereas there were no up-regulated DEGs related to TIPs in Roma (Table S6). As salt stress presumably induced the synthesis of apoplastic barriers, water transport may be inhibited. The up-regulation of aquaporins may promote water transport across apoplastic barriers.

According to our RNA-seq data, most of the DEGs encoding plasma membrane ATPase and vacuolar membrane proton pumps were down-regulated in both genotypes, suggesting that the generation of electrochemical H^+ gradient was inhibited by salt stress. This may further inhibit the transportation of Na^+ into vacuoles or out of cells via Na^+/H^+ antiporter. However, it was contrast to

most previous published reports (Chen et al. 2010; Zhang et al. 2017). We speculated that changes in proton pumps are the early reactions which driving salt stress responses, therefore it may be induced by salt stress at an earlier stage. In addition, the response of proton pump to salt stress is mainly through the post-translational regulation rather than the transcriptional regulation (Chen et al. 2007). Therefore, changes in expression level accounts for a relatively small proportion. Further genetic and molecular analysis will be critical to verify these speculation. Additionally, according to our RNA-seq data, a DEG encoding plasma membrane ATPase was increased only in M-81E, which may contribute to Na^+ exclusion. Moreover, more K^+ transport related genes were differentially expressed under salt stress in M-81E, which may serve important functions for K^+ homeostasis in M-81E. HKTs have been demonstrated to be involved in the regulation of Na^+ and K^+ transportation (Corratgé-Faillie et al. 2010) and maintenance of Na^+/K^+ balance (Wang et al. 2014) in higher plants. Group 1 HKT genes, such as *AtHKT1;1* and *OsHKT1;5*, are Na^+ specific and may be involved in inhibiting Na^+ transportation from the roots to the leaves (Byrt et al. 2007; Munns et al. 2012; Byrt et al. 2014; Zhu et al. 2016). Expression of the HKT 1;5 encoding Sb03g012590 was enhanced by salt stress in both genotypes, particularly for M-81E with a 38.32-fold increase (Table 3). As a result, Na^+ accumulation in shoots can be limited. CBL and CIPK mediate plant responses to a variety of stresses. While, only a few CIPKs' functions are clear. The CBL1 (or CBL9)-CIPK23 complex is required for the activation of the Arabidopsis K^+ transporter 1 (AKT1) channel (Li et al. 2006; Xu et al. 2006) and function in maintaining ion homeostasis under salt stress (Luan 2009). As well, CIPK6 and CIPK16 interacted with AKT1 and enhanced the activity of AKT1 (Lee et al. 2007). Sb07g021660 encoding the CIPK6 was up-regulated in both genotypes, whereas there were also some CIPK6 or CIPK16 encoding DEGs down-regulated. These may be related with the regulation of K^+ balance under salt stress.

HSPs belong to a class of proteins that are conserved in prokaryotes and eukaryotes and are especially abundant in plants. Most HSPs functions molecular chaperones in maintaining homeostasis of protein folding and are thought to be responsible for the acquisition of thermal tolerance. Certain proteins such as HSPs and antioxidant enzymes can be induced by several stresses (Sun and MacRae 2005; Yan et al. 2005). Overexpression of these genes

enhanced the tolerance of transgenic plant to various stresses (Sun et al. 2001). In the present study, most DEGs related to HSPs and ROS scavenging system were up-regulated in both genotypes (Table S4 and

S7). These indicated that salt stress strongly induced the ability to scavenge ROS and to accumulate HSPs, which may be a general salt resistance strategy of sweet sorghum.

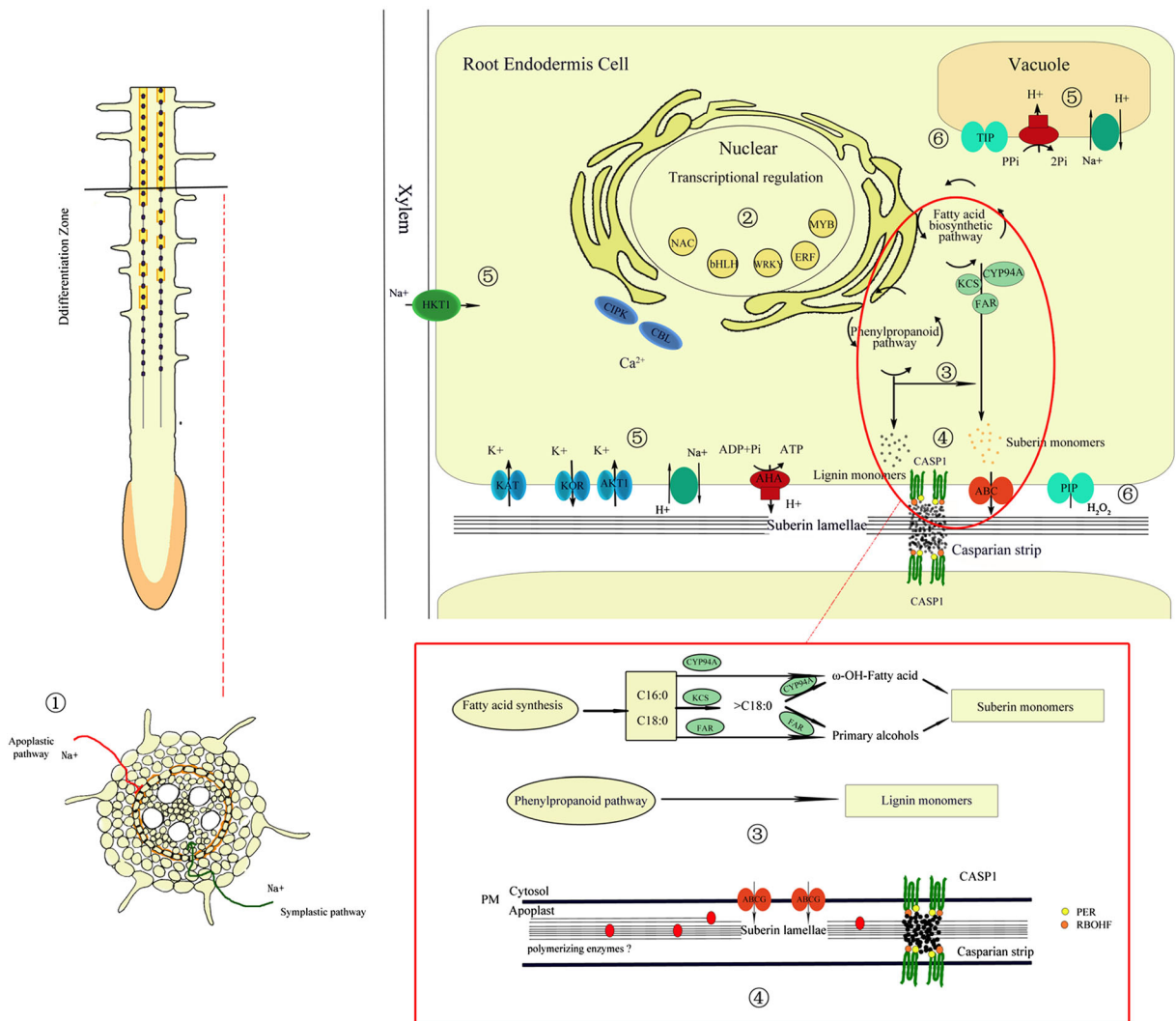


Fig. 6 Possible roles of salt-regulated genes in salt exclusion of sweet sorghum. A. Schematic longitudinal section of a root showing the formation zone of casparian strip (black point) and suberin lamellae (yellow plates). B. A cross section of maturation zone of the root showing Na^+ uptake pathway. Na^+ is transported into roots through apoplastic pathway and symplastic pathway. Apoplastic barriers can block or limit Na^+ transportation across endodermis and Na^+ will accumulate outside the endodermis (1). C. A schematic pathway of salt exclusion process in sweet sorghum root showing the possibly critical position such as endodermis cell and physical barriers (casparian strip and suberin lamellae). Endodermis cells firstly sense Na^+ by an unknown receptor and initiate the expression of some transcription factors such as NAC, bHLH, MYB (2). These transcription factors then activate the expression of genes encoding enzymes of biosynthesis of suberin and lignin

monomers (3). The genes encoding Subfamily G of ATP-binding-cassette transporters (ABCG) involved in transport of lignin and suberin monomers across the plasma membrane are up-regulated to enhance the formation of casparian strip and suberin lamellae (4). Then, root apoplastic barriers consisting of casparian strip and suberin lamellae could block Na^+ transport to shoots. Coordinate regulation of the genes encoding ion transporters and H^+ -pumps ensure cytosolic ion homeostasis via Na^+ sequestration into vacuole and out of cells by Na^+/H^+ antiporter or Na^+ unloading from xylem sap by HKT1 to limit Na^+ to the shoot (5). And the Na^+ finally out of the root cells via SOS1. The up-regulation of aquaporins genes may promote water transport across apoplastic barriers of membrane (6). D. A detail process of biosynthesis of suberin and lignin monomers and ABCG-mediated monomer transport across plasma membrane

Conclusions

In conclusion, sweet sorghum achieves relatively high salt tolerance mainly by improving the ability to exclude Na^+ and to maintain ion homeostasis. Compared with Roma, M-81E has a better ability to limit the transport of Na^+ from roots to shoots. Apoplastic barriers may play a critical role in this process by blocking or reducing Na^+ across endodermis. The H^+ -pumps and ion transporters also contribute to Na^+ exclusion, particularly unloading of Na^+ out of the xylem by HKT1;5 and finally out of the root cells via SOS1. In addition, aquaporins and antioxidants of scavenging ROS also participate salt exclusion under salt stress. Briefly, root cells sense Na^+ rise by an unknown sensor and start transcription of TFs such as bHLH, MYB and WRKY leading to enhancement of all the above processes involved in salt exclusion (Fig. 6). This RNA-seq dataset is an important resource for future studies aimed at improving salt tolerance of monocotyledon crops such as sweet sorghum. Further genetic and molecular analysis will be critical to understanding the detail roles of these genes in salt exclusion of sweet sorghum.

Acknowledgements We are grateful for financial support from Natural Science Research Foundation of Shandong Province (ZR2016JL028, ZR2014CZ002), Major Program of Shandong Provincial Natural Science Foundation (2017C03), the NSFC (National Natural Science Research Foundation of China, project No. 31770288), Independent innovation and achievement transformation of special major key technical plans of Shandong Province (2015ZDJS03002).

Authors' contributions ZY wrote this manuscript; ZY, HZ and XW performed experiments; ZY and JS collected data and carried out all analyses; NS and BW conceptualized the idea and revised the manuscript.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Abbreviation *CB*, casparian bands; *SL*, suberin lamellae; *FW*, fresh weight; *CCR*, cinnamoyl CoA reductase; *CAD*, cinnamyl alcohol dehydrogenase; *4CL*, 4-coumarate-CoA ligase; *KCS*, β -ketoacyl-CoA synthase; *CBL*, calcineurin B-like protein; *CIPK*, CBL-interacting protein kinase; *APX*, ascorbate peroxidase; *GST*, glutathione-S-transferases; *HSP*, Heat shock proteins; *RNA-seq*, RNA-sequencing; *DEGs*, differentially expressed genes; *RPKM*, Reads per KB per million; *FDR*, false discovery rate; *GO*, Gene Ontology; *COG*, Clusters of Orthologous Groups; *KEGG*, Kyoto

Encyclopedia of Genes and Genomes; *qRT-PCR*, quantitative real-time PCR; *TF*, transcription factors; *PIP*, plasma membrane intrinsic proteins; *TIPs*, tonoplast intrinsic proteins; *C3H*, p-coumarate 3-hydroxylase

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