Environmental and Experimental Botany 155 (2018) 619–627

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Comparative *de novo* transcriptomic profiling of the salinity stress responsiveness in contrasting pearl millet lines

Harshraj Shinde[a], Keisuke Tanaka[b], Ambika Dudhate[a], Daisuke Tsugama[c], Yoko Mine[d], Takehiro Kamiya[e], Shashi K. Gupta[f], Shenkui Liu[g], Tetsuo Takano[a,*]

[a] Asian Natural Environmental Science Center (ANESC), The University of Tokyo, Nishitokyo-shi, Tokyo, 188-0002, Japan
[b] NODAI Genome research center, Tokyo University of Agriculture, Setagaya-ku, 156-8502, Tokyo, Japan
[c] Laboratory of Crop Physiology, Research Faculty of Agriculture, Hokkaido University, Sapporo-shi, Hokkaido, Japan
[d] Tokyo University of Agriculture, Funako, Atsugi, 243-0034, Kanagawa, Japan
[e] Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Japan
[f] International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, 502 324, India
[g] State Key Laboratory of Subtropical Silviculture, Zhejiang A and F University, Hangzhou, 311300, China

ARTICLE INFO

Keywords:
Pearl millet
Salinity
Differentially expressed genes
Metabolic pathways
Transcription factors

ABSTRACT

Pearl millet (*Pennisetum glaucum* (L) R. Br.) is a staple crop for more than 90 million poor farmers. It is known for its tolerance against drought, salinity, and high temperature. To understand the molecular mechanisms underlying its salinity tolerance, physiological analyses and a comparative transcriptome analysis between salinity tolerant (ICMB 01222) and salinity susceptible (ICMB 081) lines were conducted under control and salinity conditions. The physiological studies revealed that the tolerant line ICMB 01222 had a higher growth rate and accumulated higher amount of sugar in leaves under salinity stress. Sequencing using the Illumina HiSeq 2500 system generated a total of 977 million reads, and these reads were assembled *de novo* into contigs corresponding to gene products. A total of 11,627 differentially expressed genes (DEGs) were identified in both lines. These DEGs are involved in various metabolic pathways such as plant hormone signal transduction, mitogen-activated protein kinase signaling pathways, and so on. Genes involved in ubiquitin-mediated proteolysis and phenylpropanoid biosynthesis pathways were upregulated in the tolerant line. In contrast, unigenes involved in glycolysis/gluconeogenesis and genes for ribosomes were downregulated in the susceptible line. Genes encoding SBPs (SQUAMOSA promoter binding proteins), which are plant-specific transcription factors, were differentially expressed only in the tolerant line. Functional unigenes and pathways that are identified can provide useful clues for improving salinity stress tolerance in pearl millet.

1. Introduction

Salinity stress severely limits crop production. Low precipitation, irrigation with saline water, a rising water table, and poor irrigation practices generally cause salinity stress. More than 6% of the world's total land area is affected by soil salinity (Munns and Tester, 2008). The adverse effects of salinity on plants includes ion toxicity, nutrient constraints, oxidative stress, and osmotic stress (Shrivastava and Kumar, 2015).

Salinity tolerance involves complex responses at the molecular, cellular, metabolic, and physiological levels. At the molecular level, genes encoding ion transporters, transcription factors, protein kinases, and osmolytes are able to confer salinity tolerance (Tuteja, 2007; Kasuga et al., 1999). Pathways such as plant hormone signaling pathway, SOS (salt overly sensitive) pathway, calcium-signaling pathway, MAPK (mitogen-activated protein kinase) signaling pathway, and proline metabolism also have key roles in the salinity stress tolerance (Zhu, 2002; Danquah et al., 2014; Ji et al., 2013; Knight, 1999; Kavi Kishor et al., 2005).

Pearl millet is an important grain crop grown in adverse agro-climatic conditions where other crops fail to produce sufficient yields. It is grown mostly in arid and semi-arid tropical regions of Asia and Africa (Vadez et al., 2012). It is a glycophyte but has the inbuilt capacity to withstand soil salinity. The pearl millet variety named "HASHAKI I" has

* Corresponding author.
E-mail addresses: hsshinde@anesc.u-tokyo.ac.jp (H. Shinde), kt205453@nodai.ac.jp (K. Tanaka), ambika_dudhate@anesc.u-tokyo.ac.jp (A. Dudhate), tsugama@res.agr.hokudai.ac.jp (D. Tsugama), y3mine@nodai.ac.jp (Y. Mine), akamiyat@mail.ecc.u-tokyo.ac.jp (T. Kamiya), S.Gupta@cgiar.org (S. K. Gupta), shenkuiliu@nefu.edu.cn (S. Liu), takano@anesc.u-tokyo.ac.jp (T. Takano).

https://doi.org/10.1016/j.envexpbot.2018.07.008
Received 1 February 2018; Received in revised form 11 July 2018; Accepted 11 July 2018
Available online 15 August 2018

been released to be grown in salinity affected areas of Uzbekistan (Shivhare and Lata, 2017). Limited information is available on the responses of pearl millet to salinity stress. According to previous studies, the reduced shoot nitrogen content and increased K+ and Na+ contents are associated with the salinity tolerance in pearl millet (Krishnamurthy et al., 2007; Dwivedi et al., 2011). A transcriptome study of pearl millet using the suppression subtractive hybridization approach discovered salinity stress-related genes (Mishra et al., 2007). Functions of only a small number of salinity stress-responsive genes such as *PgDREB2A* (dehydration responsive element binding), *PgNHX1* (Na+/H + antiporter), *PgDHN* (dehydrin), *PgVDAC* (voltage-dependent anion channel), and *PgLEA* (late embryogenesis abundant) have been studied (Agarwal et al., 2010; Verma et al., 2007; Reddy et al., 2012; Desai et al., 2006; Singh et al., 2015). Recently, comprehensive transcriptome analysis for drought stress response has been performed in pearl millet (Dudhate et al., 2018; Jaiswal et al., 2018). However, a comprehensive understanding of salinity stress tolerance in pearl millet still remains to be obtained.

Among the different transcriptome analysis methods, RNA sequencing (RNA-Seq) has become a widely used method to study gene expression and identify novel genes and pathways. RNA-Seq can efficiently detect unknown genes and novel transcripts (Hrdlickova et al., 2017).

In this study, we conducted a comparative transcriptome analysis of the pearl millet salinity tolerant line and the salinity susceptible line using the high-throughput Illumina HiSeq platform. Genome sequences of pearl millet have been published (Varshney et al., 2017) but the genome has only been partially annotated. Thus, we performed *de novo* assembly of our transcriptome data. We identified many genes and metabolic pathways involved in the salinity stress tolerance of pearl millet. Comparative physiological studies of the two lines were also conducted. To our knowledge, this is the first study conducted to understand the molecular basis of salinity tolerance of pearl millet using the RNA-Seq approach.

2. Materials and methods

2.1. Plant material and stress treatment

Seeds of two pearl millet lines, ICMB 01222 and ICMB 081 were provided by the International Crop Research Institute of Semi-Arid Tropics (ICRISAT), India. ICMB 01222 had been evaluated as a salinity-tolerant line in ICRISAT and hardly withered under a salinity stressed condition in our study, whereas ICMB 081 has been evaluated as a salinity-susceptible line and did wither under the stressed condition (see Fig. 1). Seeds were sown in composite soil in a greenhouse at 28 °C during the day and at 25 °C during the night with a relative humidity between 55%–75%. After 18 days, 250 mM salinity (NaCl) stress was imposed for 6 days.

2.2. Physiological responses of contrasting pearl millet lines against salinity stress

Chlorophyll content were measured using SPAD 502 plus chlorophyll meter, relative water content (RWC) was calculated as previously described (Smart and Bingham, 1974). Total soluble sugar was determined using the anthrone reagent method using the glucose as the standard (Yemm and Willis, 1954), Na+ contents in leaves were determined using inductively coupled plasma-mass spectrometry [ICP-MS (Agilent 7800, Agilent Technologies, U.S.)].

2.3. RNA isolation, library construction, and sequencing

The total RNA was isolated from leaves of ICMB 01222 and ICMB 081 under control and salinity stress condition (250 mM NaCl for 18 h) with three biological replications. The RNA was extracted with a Trizol reagent (Invitrogen). RNase free DNase (Qiagen, Germany) was used to eliminate genomic DNA contamination. To check the purity of the RNA, gel electrophoresis, nanodrop, and the Agilent 2100 bioanalyzer were used. Highly pure Messenger RNA (mRNA) was isolated from the total RNA using oligo (dT) beads. The Illumina TruSeq RNA Library Prep Kit v2 was used to synthesize the second strand cDNAs library. The Illumina HiSeq 2500 platform was used to sequence the constructed cDNA libraries. Sequencing results were obtained as paired-end reads (2 × 100 bp each) in the FASTQ format.

2.4. De novo assembly, ORF detection, and clustering

Raw reads were subjected to quality control by fastaQC (an online tool). Any poor-quality reads and adaptor sequences were filtered by the Trimmomatic and the FASTX-toolkit (Bolger et al., 2014; Gordon et al., 2014). The clean reads were deposited in the sequence read archive (SRA) of the National Center for Biotechnology Information (NCBI) under the accession number SRP128956. The obtained clean reads were assembled into transcriptome, *de novo*, by Bridger (Chang et al., 2015). After the transcriptome was assembled, a TransDecoder was used for the identification of long open reading frames (ORFs) within the transcripts and to score them according to their sequence similarity (Haas et al., 2013). In order to filter redundancies and to reduce noise in the generated contigs, clustering was performed by the CD-HIT program (Li and Godzik, 2006).

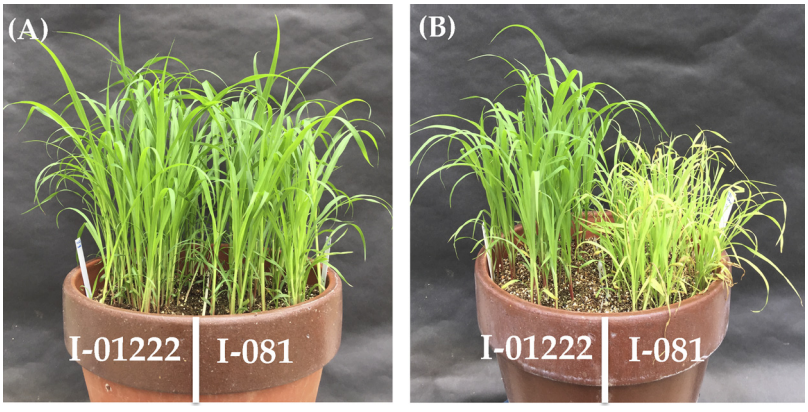

Fig. 1. Differential responses of two pearl millet lines to salinity stress. The left and right sides of the pots contained the ICMB 0122 (tolerant) and ICMB 081 (susceptible) lines, respectively. (A) Control condition; (B) Salinity stressed condition (250 mM NaCl for 6 days).

2.5. Gene annotations

Contigs generated by the de novo assembly were regarded as the products of pearl millet genes and used as queries for the BLASTX search (Altschul et al., 1990) to examine which protein they encode. For the BLASTX search, non-redundant protein sequences were used as the database. On the basis of the results of the BLASTX search, functional annotations were assigned to the contigs.

2.6. Differential gene expression and pathway enrichment analysis

To obtain the lists of differentially expressed genes (DEGs) in the control and salinity stress conditions, the CLC genomic workbench version 9.5 (QIAGEN) was used. The Empirical Analysis of Differential Gene Expression (EDGE) test was implemented to calculate the p-values and the false discovery rate (FDR). The DEGs were filtered as upregulated and downregulated based on the FC (fold change) ≥ 2 or FC ≤ -2 respectively with FDR-corrected p values < 0.01. KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis was performed on the upregulated and downregulated differentially expressed genes in order to map them with various biological pathways (Ogata et al., 1999; Kanehisa, 2002). The PlantTFcat online tool (http://plantgrn.noble.org/PlantTFcat/) was used to identify genes encoding transcription factors (Dai et al., 2013).

2.7. Validation of the RNA-Seq results by the real-time PCR

Ten randomly selected DEGs were chosen for validation by qRT-PCR. The reaction was performed using TB Green™ Premix Ex Taq™ II Kit (Tli RNaseH Plus) (TaKaRa, Tokyo, Japan) in a volume of 20 µl containing 10 µl of TB Green premix Ex Taq II (2X), 0.4 µl of ROX Reference Dye II, 200 ng of cDNA template, and 0.4 µM of each of the primers. Amplification was performed as follows: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. All the experiments were performed in biological triplicate. The pearl millet actin gene *PgActin* was used as a reference gene as previously described (Shivhare and Lata (2016)). Relative gene expression was calculated using the ΔΔCT method (Livak and Schmittgen, 2001). The primers used for real-time PCR study are listed in the Supplementary Table S7.

3. Results

3.1. Physiological changes in contrasting lines under salinity stress

The two pearl millet lines ICMB 01222 and ICMB 081 exhibited considerable variation in their Na + content, chlorophyll content, relative water content and total soluble sugar content during the salinity stress. The tolerant line ICMB 01222 was found to have less Na + content in its leaf under salinity stress than the susceptible line ICMB 081. ICMB 01222 maintained higher chlorophyll content and relative water content in leaves under salinity stress. Total soluble sugar was 1.13 fold higher under salinity stressed condition than in a control conditions, whereas, total soluble sugar content in ICMB 01222 was 1.64 fold higher under salinity stressed condition. (Table 1)

Fresh weight and plant height of ICMB 01222 were not affected by salinity as compared to susceptible line. ICMB 081 showed leaf tip drying and leaf yellowing under the salinity stressed condition but ICMB 01222 did not (Fig. 1) These results confirm that ICMB 01222 is more tolerant to salinity stress than ICMB 081 at the seedling stage.

Table 2
Raw and clean reads obtained from salinity tolerant and susceptible pearl millet lines (ICMB 01222 and ICMB 081, respectively) grown under control and salinity-stressed condition.

Sample	Raw reads	Clean reads	Contigs	Min. Length	N50	Max. Length
ICMB 01222 control	241,037,282	221,619,342	55,745	201	1,897	32,776
ICMB 01222 Salinity	221,832,510	206,656,132	53,634	201	1,768	32,978
ICMB 081 control	264,234,540	245,012,716	54,021	201	1,816	31,640
ICMB 081 Salinity	250,850,634	180,110,152	53,517	201	1,724	34,155
Total	97,79,54,966	85,33,98,342				

3.2. Sequencing and de novo transcriptome assembly

The total number of raw reads obtained by RNA-Seq was 977,954,966, and 853,398,342 (87.26%) of them passed a quality check. The clean reads were subjected to *de novo* assembly and generated 54,229 contigs on average in each sample. The length of the contigs varied between 201 and 34,155 nucleotides (Table 2).

3.3. Open reading frame detection, cluster analysis, and annotation

The TransDecoder software detected 35,357 (control) and 34,711 (salinity-stressed) open reading frames (ORFs) on average in each set of the contigs. These ORFs were clustered to reduce redundancies or noise and 100,260 ORFs remained in total. These ORFs were defined as unigenes in this report. BLASTX results were obtained for 94,592 (94.3%) of these ORFs. The species with the highest numbers of BLASTX hits were foxtail millet, sorghum, dichanthelium, maize, and rice (Supplementary table S1).

3.4. Differential gene expression in response to salinity stress

Differential gene expression analysis of the two lines grown under salinity stress and control conditions discovered a total of 11,627 DEGs. In ICMB 01222, 2965 unigenes were upregulated by salinity stress and 2946 unigenes were downregulated. In ICMB 081, 2243 unigenes were upregulated by salinity stress, and 3473 unigenes were downregulated (Fig. 2a). Of these DEGs, 1287 upregulated unigenes and 1451 downregulated unigenes were common across both lines (Fig. 2b and c).

Table 1
Physiological responses of two contrasting lines, ICMB 01,222 (tolerant) and ICMB 081 (susceptible) to salinity stress. All results are mean values of three biological replications and each replicate is mean of ten readings.

Physiological characteristics	Tolerant line ICMB 01222		Susceptible line ICMB 081	
	Control	Stress	Control	Stress
Relative water content (%) in Leaves	87.88 ± 2.3	72.12 ± 4.0	86.74 ± 1.95	48.10 ± 2.8
Chlorophyll content (SPAD)	38.5 ± 2.1	30.6 ± 3.7	37.6 ± 2.3	16.18 ± 2.2
Total soluble sugar (TSS), mg/gm	20.7 ± 0.84	34.07 ± 0.56	21.64 ± 0.79	24.6 ± 0.64
Leaf Na + content (ppm)	216.16 ± 58	1793.87 ± 169	287.13 ± 48	3084 ± 192
Na + injury symptoms	None	None	None	Leaf tip drying, leaf yellowing and stunting

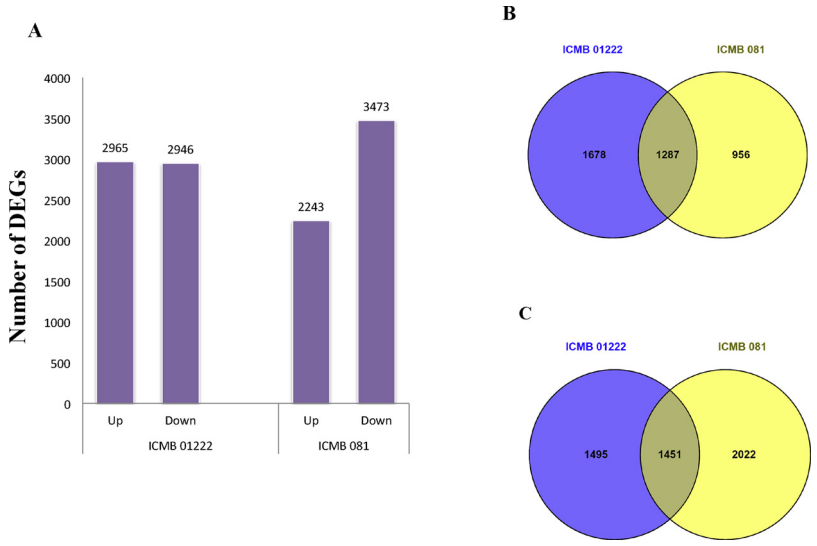

Fig. 2. (**a**) Differentially expressed genes (DEGs) of ICMB 01222 and ICMB 081; (**b**) Venn diagram showing the common and unique upregulated unigenes; (**c**) Venn diagram showing the common and unique downregulated unigenes.

3.5. Differentially expressed genes encoding transcription factors

Transcription factors are important regulators of salinity stress responses. Our analysis identified 935 and 906 unigenes that possibly encode transcription factors (TFs) in ICMB 01,222 and ICMB 081, respectively. These transcription factors belong to 56 different families and 60% of them belong to the Zinc finger (C2H2), MYB, AP2-EREBP, NAC, bHLH, WD 40, bZIP, WRKY, homeobox-wox, and HSF-DB families. The zinc finger (C2H2) TF family contained the largest numbers of TFs encoded by the DEGs (144 in ICMB 01222 and 125 in ICMB 081; around 15% in both lines). Unigene encoding members of the SBP (SQUAMOSA promoter binding protein) family, a major plant-specific TF family, were differentially expressed (two upregulated and three downregulated) only in the tolerant line ICMB 01222. (Fig. 3; Supplementary Tables S2–S5)

3.6. Differentially expressed ion transporter families

Ion transporters play important roles in adaptation to salinity stress by regulating cellular ion concentrations. In our transcriptome data, we found six different ion transporter families rich in proteins encoded by DEGs (Fig. 4; Supplementary Tables S6 and S7). Among them, ABC (ATP-binding cassettes) transporters were the most abundant. In ICMB 01222 and ICMB 081, 40 and 32 DEG encoding ABC transporters were found, respectively. Other salinity stress-related ion transporters such as HKTs (high-affinity potassium transporters), NHXs (sodium hydrogen exchangers), Ca^{2+}ATPases (calcium ATPases), CAXs (cation hydrogen exchangers), and sugar transporters were also discovered.

3.7. Metabolic pathways involved in salinity stress responses

Twenty pathways that are most enriched in DEGs were compared (Fig. 5). Among these pathways, the MAPK signaling pathway, cysteine and methionine metabolism, and plant hormone signal transduction were all pathways associated with many upregulated unigenes. Among the pathways associated with many downregulated unigenes, ribosomes, ribosome biogenesis in eukaryotes, and the purine metabolism pathways were the most notable. These downregulated pathways were significantly enriched in the susceptible line ICMB 081.

Hormone signaling pathways, especially the abscisic acid (ABA) signaling pathway and auxin signaling pathway were associated with many DEGs in both lines. The PYL-ABA-PP2C (pyrabactin resistant-like regulatory components of ABA (abscisic acid) receptors-protein phosphatase 2C) complex is the ABA receptor. In this pathway, NCED (nine-cis-epoxycarotenoid dioxygenase) is involved in the ABA biosynthesis during abiotic stress. Once ABA is synthesized during salinity stress

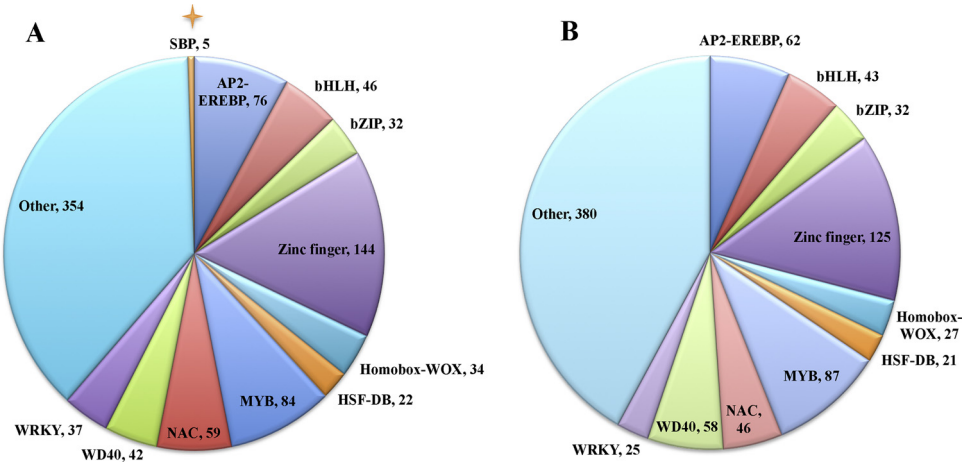

Fig. 3. The number of transcription factors encoded by differentially expressed genes (DEGs). (**a**) TFs of ICMB 01222. The asterisk shows ICMB 01222-specific SBPs (SQUAMOSA promoter binding protein) transcription factors; (**b**) TFs of ICMB 081.

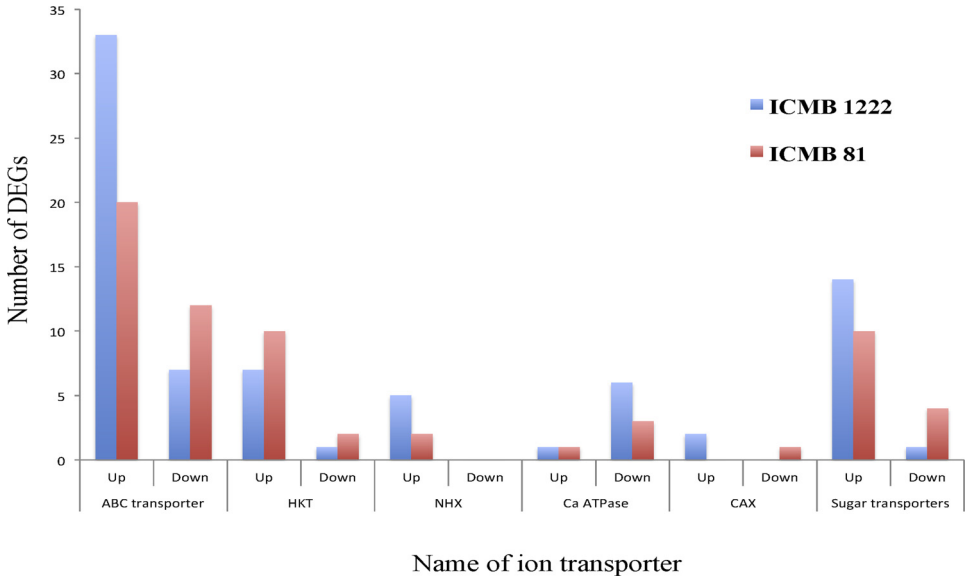

Fig. 4. The number of differentially expressed genes encoding ion transporters in both lines.

conditions, it binds to PYL (pyrabactin resistant like). Then, the ABA-bound PYL receptors interact with PP2C (protein phosphatase 2C) and inhibit its phosphatase activity. SnRK2 (SNF1-related protein kinase 2) is then released from negative regulation by the PP2C, turning on the ABA signals by phosphorylation of downstream factors such as ABF (ABA-responsive element binding factor), bZIP transcription factors proteins, S anion channels, MAPK signaling, and stomata closure through ABF. All the components of the PYL-ABA-PP2C pathway were differentially expressed in both lines. We further verified their expression with real-time PCR. MAPK signaling mediated by MAPKKK17, MKK3, and MPK1 is known to regulate ABA signaling, and the unigenes encoding MAPKKK17 and MPK1 were differentially expressed in both lines (Fig. 6).

Plant hormone signaling pathways are also involved in salinity stress adaptation through ubiquitin-mediated proteolysis. The ubiquitin-mediated proteolysis pathway was associated with 11 upregulated unigenes in the tolerant line but with only 5 upregulated unigenes in the susceptible line. Ubiquitin-mediated proteolysis (Fig. 7) can be divided into three steps: in step 1, the UBE1 subunit of the activating enzyme E1 works and the unigene encoding UBE1 was upregulated not in the susceptible line ICMB 081 but in the tolerant line ICMB 01222; in step 2, the ubiquitin-conjugating enzyme complex E2 works, and

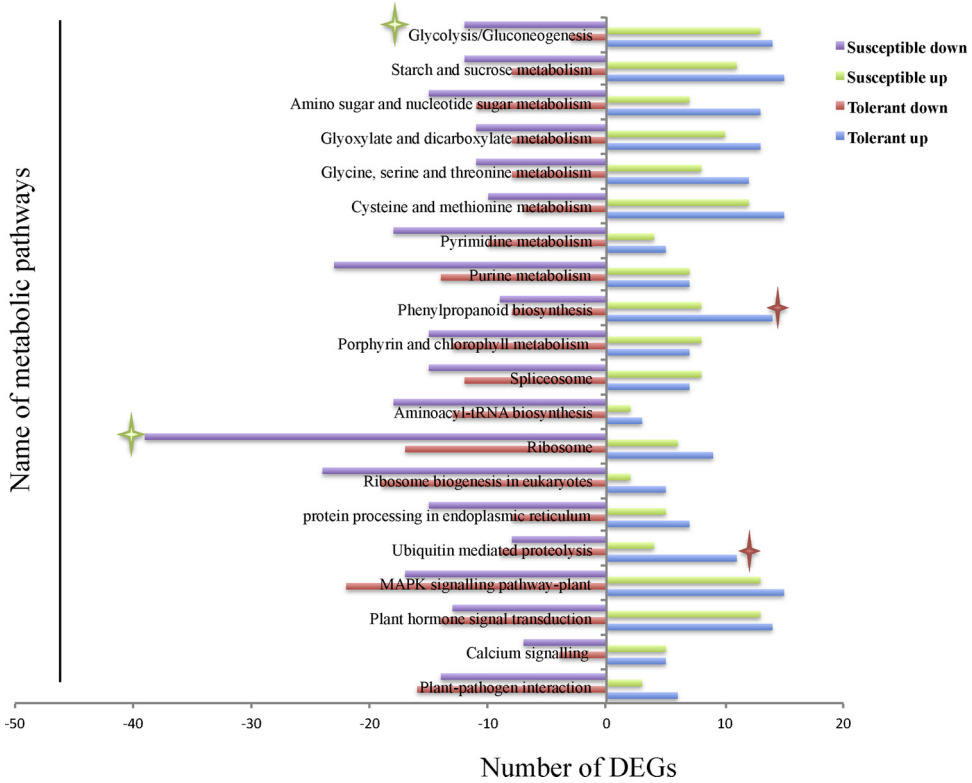

Fig. 5. Metabolic pathways enriched under salinity stress in the tolerant (ICMB 01222) and susceptible (ICMB 081) lines. Red asterisks show pathways extensively upregulated in the tolerant line by salinity stress. Green asterisks show pathways extensively downregulated in the susceptible line by salinity stress.

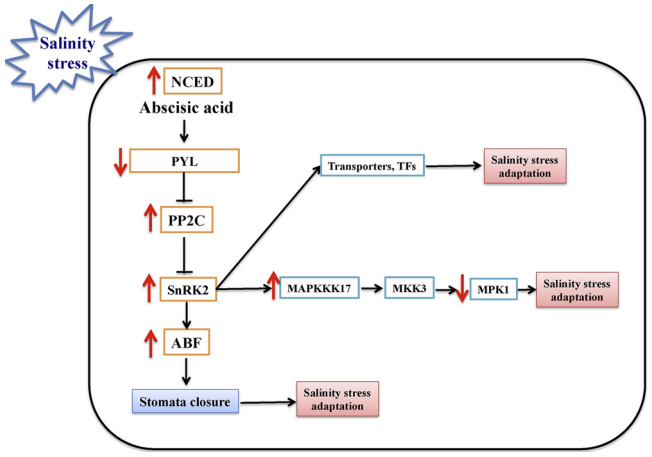

Fig. 6. The PYL-ABA-PP2C pathway. NCED: Nine-cis-epoxycarotenoid dioxygenase; PYL: pyrabactin resistant like; PP2C: protein phosphatase 2C; SnRK2: SNF1-related protein kinase 2; ABF: ABA-responsive element binding factor; MAPKKK17: mitogen-activated protein kinase kinase kinase 17; MKK3: mitogen-activated protein kinase kinase 3; MPK1: mitogen-activated protein kinase 1.

unigenes encoding components of the E2 complex (UBE2D, UBE2J, UBE2O, and UBE2W) were upregulated in the tolerant line, while only one of them (UBE2D) was upregulated in susceptible line; and in step 3, the ubiquitin ligase complex E3 works, and six unigenes encoding components of the E3 complex (TRIP2, COP1, PIRH2, F-box, Cul3, and DCAF) were upregulated in the tolerant line, while only five of them (TRIP2, COP1, PIRH2, F-box, and DCAF) were upregulated in the susceptible line (Fig. 7).

The phenylpropanoid metabolism pathway serves as a source of metabolites, and these metabolites contribute to stress tolerance. In our KEGG analysis, the phenylpropanoid pathway was associated with 14 upregulated unigenes in the tolerant line, while it was associated with only 8 upregulated unigenes in the susceptible line.

3.8. Validation of DEGs by Quantitative Real-Time PCR

To validate the sequencing results, 10 DEGs were selected based on their different roles in salinity stress (5 DEGs were part of the PYL-ABA-PP2C pathway), and subjected to reverse transcription-PCR. For seven of the 10 DEGs, the PCR expressions were consistent with the RNA-Seq data. However, the PCR-detected fold changes of three of the genes (PYR/PYL, PP2C and HSP) were less than those in RNA-Seq data. (Fig. 8)

4. Discussion

Our *de novo* transcriptomic data from the first report could offer an insight into the genes and metabolic pathways involved in the salinity stress tolerance of pearl millet.

Physiological analysis showed that total soluble sugar content was higher in the salinity stressed tolerant pearl millet line than in the susceptible line under salinity stress (Table 1). Sugar plays roles in the detoxification of Na + ions under salinity stress (Kanai et al., 2007), and also acts as an osmoprotectant (Pattanagul and Thitisaksakul, 2008). In finger millet, tolerant line showed 2.63 higher accumulation of sugar than susceptible line under salinity stress (Rahman et al., 2014). In our KEGG pathway analysis, sugar metabolism related pathways such as glycolysis/gluconeogenesis and starch and sucrose pathways were downregulated in the susceptible line and were upregulated in the tolerant line. In many cases, activation of starch metabolism under abiotic stress is a common plant response, as it contributes to sugar accumulation and stress tolerance (Thalmann and Santelia, 2017). Together the sugar metabolism related pathways and total soluble sugar are likely to contribute to salinity stress tolerance of pearl millet. The sodium content (Na+) of ICMB 01222 was lower than that of ICMB 081 during salinity stress (Fig. 1). This result is consistent with the finding from the RNA-Seq that the genes encoding salt transporters such as NHXs (sodium hydrogen exchangers), Ca^{2+}ATPases (calcium ATPases), CAXs (cation hydrogen exchangers) and ABC (ATP-binding cassettes) transporters are strongly expressed in ICMB 01222 (Fig. 4).

Differential gene expression analysis revealed that the salinity tolerant line has more upregulated DEGs and fewer downregulated DEGs than the susceptible line in pearl millet. These results were consistent with a previous comparative transcriptome analysis (Kulkarni et al., 2016). Among these DEGs, around 15.8% of DEGs were found to be transcription factors (TFs) (15.81% in the tolerant line and 15.85% in the susceptible line), similar to a previous finding of the comparative transcriptome analysis study on banana (Muthusamy et al., 2016). TFs are master regulators of salinity stress as well as regulators of the downstream stress-responsive genes (Wang et al., 2016). Most of these reported TFs belong to zinc finger, MYB, AP2-EREBP, NAC, and bZLH

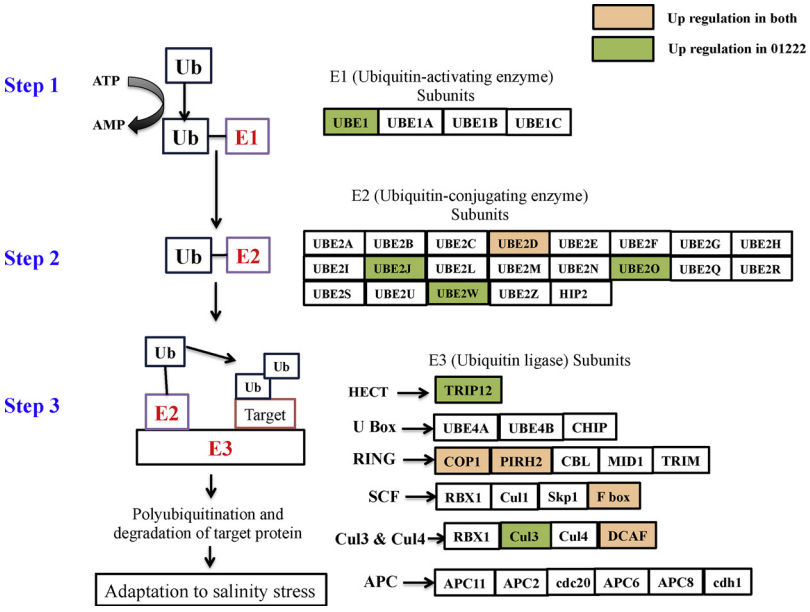

Fig. 7. Ubiquitin-mediated proteolysis pathway. Red boxes show upregulated unigenes in both lines (the tolerant line ICMB 01222 and the susceptible line ICMB 081). Green boxes show only the upregulated unigenes in the ICMB 01222. E1 is a ubiquitin-activating enzyme complex consisting of 4 subunits, E2 is a ubiquitin-conjugating enzyme consisting of 21 subunits and E3 is a ubiquitin ligase complex consisting of many subunits; only the important subunits are shown in this figure.

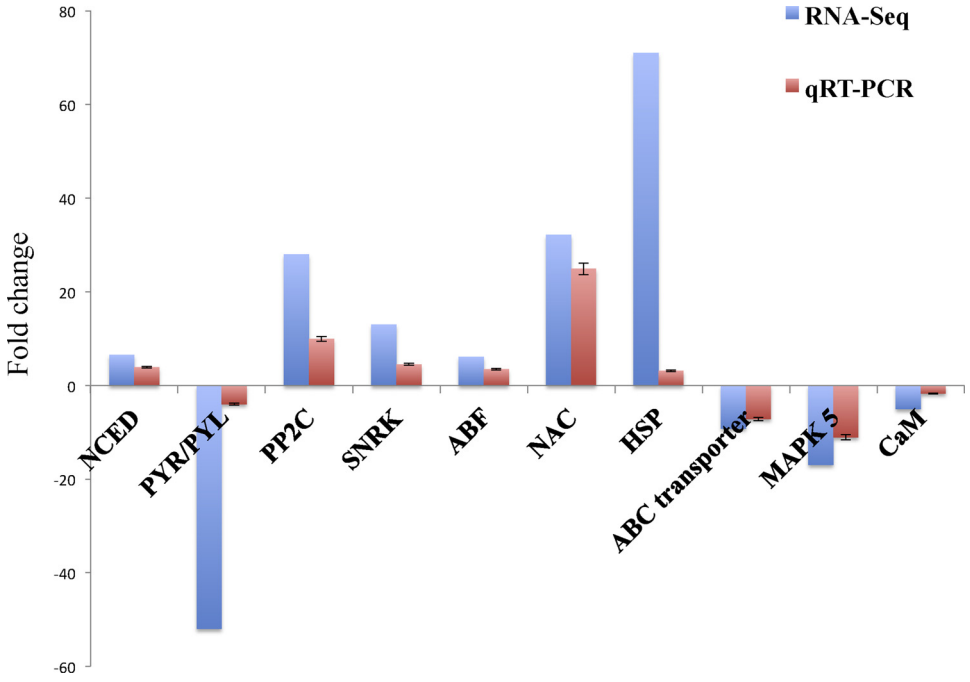

Fig. 8. qRT-PCR validation of the RNA-Seq data of 10 DEGs. Blue bars represent fold changes in the RNA-Seq data and red bars represent fold changes in the real-time PCR analysis.

families as was the case in a study of the jute transcriptome (Yang et al., 2017). In arabidopsis, zinc finger TF, *ZFP3* changes proline and chlorophyll accumulation during salinity stress to give salinity stress tolerance (Zhang et al., 2016). The wheat salinity responsive R2R3 MYB TF, *TaSIM* confers salinity stress tolerance by activating transcription of desiccation-responsive genes (Yu et al., 2017). Plant-specific SBP family TFs function in a variety of developmental processes including abiotic stress response (Song et al., 2016). Overexpression of a grapevine SBP TF, *VpSBP16* in arabidopsis improved salinity stress tolerance (Hou et al., 2018). Differential expression of the SBP TF family was specific to the tolerant line in this study. The differential expression of such TF genes likely to contribute to the salinity stress tolerance of ICMB 01222.

Differential expression of the ion transporter family was seen in both pearl millet lines. Among them, the ABC transporter family was the most enriched. In a previous study, the arabidopsis ABC transporter, *AtABCG36*, was found to promote salinity stress adaptation by reducing the shoot sodium content (Kim et al., 2010). Another ABC transporter MRP5 affected Na+/K+ homeostasis and elicited a salt stress response in arabidopsis (Lee et al., 2004). A ABC transporter G family member, AtABCG25, was reported to be involved in abscisic acid transport and responses (Kuromori et al., 2010). Interestingly, in our data upregulation of a larger number of ABC transporter genes were observed in tolerant line than in the susceptible line. Roles of other ion transporters are well documented in previous studies (Brini and Masmoudi, 2012). For example, Ca^{2+}ATPase promote salt stress adaptation through the generation of salt-induced Ca^{2+} signatures (Qudeimat et al., 2008). The genes encoding these transporters are also likely to regulate the salinity stress tolerance in pearl millet.

ABA is a plant hormone that plays a critical role in the adaptive responses to stressors such as drought and high salinity (Vishwakarma et al., 2017). This ABA signaling is mediated by the PYL-ABA-PP2C protein complex, and this is considered one of the key model to study abiotic stress adaptation in plants (Umezawa et al., 2010; Park et al., 2010). The salinity stress adaptation mechanism of pearl millets is also found to be through the PYL-ABA-PP2C pathway.

In the presence of abiotic stress, abnormal proteins are degraded by ubiquitin-mediated proteolysis to control the protein load in the cell (Hershko and Ciechanover, 1998). In plants, the process of ubiquitin-mediated proteolysis is complex, which requires three enzymes E1, E2, and E3 ubiquitin ligase, among them E3 ubiquitin ligase is a multi-protein complex (Zeng et al., 2006). Previous studies showed that E3 ubiquitin ligase is largely involved in ABA signaling and abiotic stress responses. The role of the subunits of the E3 ubiquitin ligase such as the CUL4 (cullin based ligase 4) and the COP1 (constitutively photomorphogenic 1) in abiotic stress has been studied. In tomato DWD motif-containing protein DDI1 interacts with the CUL4–DDB1-based ubiquitin ligase to promote the salinity stress tolerance (Sharma et al., 2016; Miao et al., 2014). Our study shows that unigenes encoding components of the E3 ubiquitin ligase, unigenes encoding subunits of the E1 activating enzymes and unigenes encoding subunits of the E2 conjugating enzymes are upregulated by salinity stress. Elucidation of the functions of these stress-responsive E1 and E2 enzymes will help to understand ubiquitin-mediated proteolysis and its role in abiotic stress response.

Protein synthesis is one of the fundamental biological processes. Ribosomal proteins are well known for their role in mediating protein synthesis. The rice ribosomal protein large subunit gene, *OsRPL23A*, is involved in salinity stress tolerance, as RPL23A-overexpressing transgenic rice lines showed a significant increase in fresh weight, root length, proline and chlorophyll contents under salinity stress (Moin et al., 2016). The majority of rice ribosomal small protein subunit genes, manifested significant expression under all abiotic stress treatments with ABA, PEG, NaCl, and H_2O_2 (Saha et al., 2017). In our study, the downregulation of 39 ribosome-associated genes was observed in the susceptible line ICMB 081. In the tolerant line ICMB 01222, 17 ribosome-associated genes were downregulated. We speculate that the downregulation of a large number of ribosomal protein genes in the susceptible line is one of the reasons for its salinity susceptibility.

Based on all our findings, we have proposed a scheme describing the events happening in pearl millet during salinity stress (Fig. 9). According to this scheme, different metabolic pathways, transcription factors, and ion transporters act synergistically to mediate salinity stress tolerance in pearl millet.

Our study provides several new insights into the transcriptome responses of the salinity-tolerant and susceptible pearl millet lines. The salinity tolerance mechanism of the tolerant line might be attributable

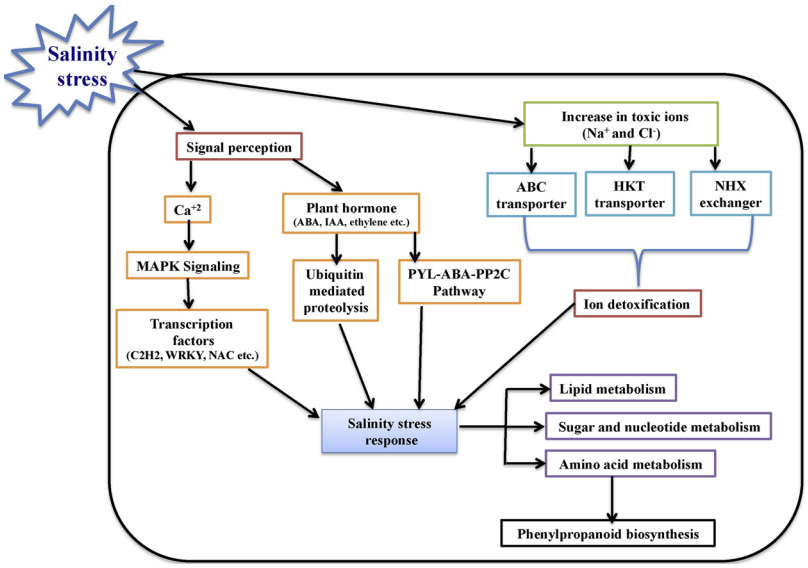

Fig. 9. A Scheme for the response of pearl millet to salinity stress. Upon salinity stress, the pearl millet cells sense the stress and initiate calcium and plant hormone signaling. Calcium acts as a secondary messenger and activates the MAPK signaling pathway, which in turn activates the transcription factors that further activates stress responses. Plant hormones also activate the PYL-ABA-PP2C pathway and the ubiquitin-mediated proteolysis pathway to initiate salinity stress responses. Ion transporters and different metabolic pathways also get involved in the salinity stress responses.

to the upregulation of ion transporters, SBP family TFs and pathways such as the ubiquitin-mediated proteolysis and sugar metabolism. On the other hand, the downregulation of key metabolic pathways such as the glycolysis/gluconeogenesis pathway and the ribosome pathway might be responsible for the salinity susceptibility of the ICMB 081 line. These insights will be useful for future pearl millet improvement programs.

5. Conclusions

Our comparative transcriptomic analysis between the salinity tolerant and salinity susceptible pearl millet lines provides useful clues for understanding the salinity stress tolerance mechanism of this crop. We found that DEGs encoding transcription factors, ion transporters, and regulators of metabolic pathways are extremely useful in improving the pearl millet productivity under salinity stress condition. The most conspicuous differences between the two lines are pathways related to sugar metabolism and total sugar accumulation pattern during the salinity stress. Identified DEGs and metabolic pathways with contrasting expression patterns between two contrasting lines are excellent targets for future functional studies to understand mechanism of salinity stress tolerance. It is also possible to use the tolerant line, ICMB 01222 as a donor of useful genes to further improve the salinity-tolerance in pearl millet using either cross breeding or transgenic approach.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This study was supported by the Co-operative Research Grant of the Genome Research for Bio Resource, NODAI Genome Research Center, Tokyo University of Agriculture Setagaya-ku Tokyo, Japan.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2018.07.008.

References

Agarwal, P., Agarwal, P.K., Joshi, A.J., Sopory, S.K., Reddy, M.K., 2010. Overexpression of PgDREB2A transcription factor enhances abiotic stress tolerance and activates downstream stress-responsive genes. Mol. Biol. Rep. 37 (2), 1125–1135. https://doi.org/10.1007/s11033-009-9885-8.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Brini, F., Masmoudi, K., 2012. Ion transporters and abiotic stress tolerance in plants. ISRN Mol. Biol. https://doi.org/10.5402/2012/927436.

Chang, Z., Li, G., Liu, J., Zhang, Y., Ashby, C., Liu, D., et al., 2015. Bridger: a new framework for de novo transcriptome assembly using RNA-seq data. Genome Biol. 16 (1), 30. https://doi.org/10.1186/s13059-015-0596-2.

Dai, X., Sinharoy, S., Udvardi, M., Zhao, P.X., 2013. PlantTFcat: an online plant transcription factor and transcriptional regulator categorization and analysis tool. BMC Bioinformatics 14 (1), 321. https://doi.org/10.1186/1471-2105-14-321.

Danquah, A., de Zelicourt, A., Colcombet, J., Hirt, H., 2014. The role of ABA and MAPK signaling pathways in plant abiotic stress responses. Biotechnol. Adv. 40–52. https://doi.org/10.1016/j.biotechadv.2013.09.006.

Desai, M.K., Mishra, R.N., Verma, D., Nair, S., Sopory, S.K., Reddy, M.K., 2006. Structural and functional analysis of a salt stress inducible gene encoding voltage dependent anion channel (VDAC) from pearl millet (Pennisetum glaucum). Plant Physiol. Biochem. 44 (7–9), 483–493. https://doi.org/10.1016/j.plaphy.2006.08.008.

Dudhate, A., Shinde, H., Tsugama, D., Liu, S., Takano, T., 2018. Transcriptomic analysis reveals the differentially expressed genes and pathways involved in drought tolerance in pearl millet [Pennisetum glaucum (L.) R. Br]. PLoS ONE 13 (4). https://doi.org/10.1371/journal.pone.0195908.

Dwivedi, S., Upadhyaya, H., Senthilvel, S., Hash, C., Fukunaga, K., Diao, X., et al., 2011. Millets: genetic and genomic resources. Plant Breed. Rev. 247–375. https://doi.org/10.1002/9781118100509.ch5.

Gordon, A., Hannon, G.J., Gordon, 2014. FASTX-Toolkit. [Online] Http://Hannonlab.Cshl. Edu/Fastx_ Toolkit Http://Hannonlab. Cshl. Edu/Fastx_ Toolkit. .

Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., et al., 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat. Protoc. 8 (8), 1494. https://doi.org/10.1038/nprot.2013.084.

Hershko, A., Ciechanover, A., 1998. The ubiquitin system. Annu. Rev. Biochem. 425–489. https://doi.org/10.1146/annurev.biochem.67.1.425.

Hou, H., Jia, H., Yan, Q., Wang, X., 2018. Overexpression of a SBP-box gene (VpSBP16) from chinese wild vitis species in arabidopsis improves salinity and drought stress tolerance. Int. J. Mol. Sci. 19 (4), 940. https://doi.org/10.3390/ijms19040940.

Hrdlickova, R., Toloue, M., Tian, B., 2017. RNA-Seq methods for transcriptome analysis. Wiley Interdiscip. Rev. RNA 1364. https://doi.org/10.1002/wrna.

Jaiswal, S., Antala, T.J., Mandavia, M.K., Chopra, M., Jasrotia, R.S., Tomar, R.S., et al., 2018. Transcriptomic signature of drought response in pearl millet (Pennisetum glaucum (L.) and development of web-genomic resources. Sci. Rep. 8 (1), 3382. https://doi.org/10.1038/s41598-018-21560-1.

Ji, H., Pardo, J.M., Batelli, G., Van Oosten, M.J., Bressan, R.A., Li, X., 2013. The salt overly sensitive (SOS) pathway: Established and emerging roles. Mol. Plant 6 (2), 275–286. https://doi.org/10.1093/mp/sst017.

Kanai, M., Higuchi, K., Hagihara, T., Konishi, T., Ishii, T., Fujita, N., et al., 2007. Common reed produces starch granules at the shoot base in response to salt stress. New Phytol. 176 (3), 572–580. https://doi.org/10.1111/j.1469-8137.2007.02188.x.

Kanehisa, M., 2002. The KEGG databases at GenomeNet. Nucleic Acids Res. 30 (1), 42–46. https://doi.org/10.1093/nar/30.1.42.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., Shinozaki, K., 1999. Improving

plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat. Biotechnol. 17 (3), 287. https://doi.org/10.1038/7036.
Kavi Kishor, P.B., Sangam, S., Amrutha, R.N., Sri Laxmi, P., Naidu, K.R., Rao, K.R.S.S., et al., 2005. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. Curr. Sci. 424–438. https://doi.org/http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.471.372&rep=rep1&type=pdf.
Kim, D.-Y., Jin, J.-Y., Alejandro, S., Martinoia, E., Lee, Y., 2010. Overexpression of AtABCG36 improves drought and salt stress resistance in Arabidopsis. Physiol. Plant. 139 (2), 170–180. https://doi.org/10.1111/j.1399-3054.2010.01353.x.
Knight, H., 1999. Calcium signaling during abiotic stress in plants. Int. Rev. Cytol. 195, 269–324. https://doi.org/10.1016/S0074-7696(08)62707-2.
Krishnamurthy, L., Serraj, R., Rai, K.N., Hash, C.T., Dakheel, A.J., 2007. Identification of pearl millet [Pennisetum glaucum (L.) R. Br.] lines tolerant to soil salinity. Euphytica 158 (1–2), 179–188. https://doi.org/10.1007/s10681-007-9441-3.
Kulkarni, K.S., Zala, H.N., Bosamia, T.C., Shukla, Y.M., Kumar, S., Fougat, R.S., et al., 2016. De novo transcriptome sequencing to dissect candidate genes associated with Pearl Millet-Downy Mildew (Sclerospora graminicola Sacc.) interaction. Front. Plant Sci. 7, 847. https://doi.org/10.3389/fpls.2016.00847.
Kuromori, T., Miyaji, T., Yabuuchi, H., Shimizu, H., Sugimoto, E., Kamiya, A., et al., 2010. ABC transporter AtABCG25 is involved in abscisic acid transport and responses. Proc. Natl. Acad. Sci. 107 (5), 2361–2366. https://doi.org/10.1073/pnas.0912516107.
Lee, E.K., Kwon, M., Ko, J.-H., Yi, H., Hwang, M.G., Chang, S., et al., 2004. Binding of sulfonylurea by AtMRP5, an Arabidopsis multidrug resistance-related protein that functions in salt tolerance. Plant Physiol. 134 (1), 528–538. https://doi.org/10.1104/pp.103.027045.
Li, W., Godzik, A., 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22 (13), 1658–1659. https://doi.org/10.1093/bioinformatics/btl158.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25 (4), 402–408. https://doi.org/10.1006/meth.2001.1262.
Miao, M., Zhu, Y., Qiao, M., Tang, X., Zhao, W., Xiao, F., et al., 2014. The tomato DWD motif-containing protein DDI1 interacts with the CUL4-DDB1-based ubiquitin ligase and plays a pivotal role in abiotic stress responses. Biochem. Biophys. Res. Commun. 450 (4), 1439–1445. https://doi.org/10.1016/j.bbrc.2014.07.011.
Mishra, R.N., Reddy, P.S., Nair, S., Markandeya, G., Reddy, A.R., Sopory, S.K., et al., 2007. Isolation and characterization of expressed sequence tags (ESTs) from subtracted cDNA libraries of Pennisetum glaucum seedlings. Plant Mol. Biol. 64 (6), 713–732. https://doi.org/10.1007/s11103-007-9193-4.
Moin, M., Bakshi, A., Saha, A., Dutta, M., Madhav, S.M., Kirti, P.B., 2016. Rice ribosomal protein large subunit genes and their spatio-temporal and stress regulation. Front. Plant Sci. 7, 1284. https://doi.org/10.3389/fpls.2016.01284.
Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59 (1), 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911.
Muthusamy, M., Uma, S., Backiyarani, S., Saraswathi, M.S., Chandrasekar, A., 2016. Transcriptomic changes of drought-tolerant and sensitive banana cultivars exposed to drought stress. Front. Plant Sci. 7, 1609. https://doi.org/10.3389/fpls.2016.01609.
Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H., Kanehisa, M., 1999. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 27 (1), 29–34. https://doi.org/10.1093/nar/27.1.29.
Park, S., Fung, P., Nishimura, N., Jensen, D.R., Zhao, Y., Lumba, S., et al., 2010. Abscisic acid inhibits PP2Cs via the PYR/PYL family of ABA- binding START proteins. Science 324 (5930), 1068–1071. https://doi.org/10.1126/science.1173041.Abscisic.
Pattanagul, W., Thitisaksakul, M., 2008. Effect of salinity stress on growth and carbohydrate metabolism in three Rice (Oryza sativa L.) cultivars differing in salinity tolerance. Indian J. Exp. Biol. 46 (10), 736–742.
Qudeimat, E., Faltusz, A.M.C., Wheeler, G., Lang, D., Brownlee, C., Reski, R., et al., 2008. A PIIB-type Ca2+-ATPase is essential for stress adaptation in Physcomitrella patens. Proc. Natl. Acad. Sci. U. S. A. 105 (49), 19555–19560. https://doi.org/10.1073/pnas.0800864105.
Rahman, H., Jagadeeshselvam, N., Valarmathi, R., Sachin, B., Sasikala, R., Senthil, N., et al., 2014. Transcriptome analysis of salinity responsiveness in contrasting genotypes of finger millet (Eleusine coracana L.) through RNA-sequencing. Plant Mol. Biol. 85 (4–5), 485–503. https://doi.org/10.1007/s11103-014-0199-4.
Reddy, P.S., Reddy, G.M., Pandey, P., Chandrasekhar, K., Reddy, M.K., 2012. Cloning and molecular characterization of a gene encoding late embryogenesis abundant protein from Pennisetum glaucum: protection against abiotic stresses. Mol. Biol. Rep. 39 (6), 7163–7174. https://doi.org/10.1007/s11033-012-1548-5.
Saha, A., Das, S., Moin, M., Dutta, M., Bakshi, A., Madhav, M.S., et al., 2017. Genome-wide identification and comprehensive expression profiling of ribosomal protein small subunit (RPS) genes and their comparative analysis with the large subunit (RPL) genes in rice. Front. Plant Sci. 8, 1553. https://doi.org/10.3389/fpls.2017.01553.
Sharma, B., Joshi, D., Yadav, P.K., Gupta, A.K., Bhatt, T.K., 2016. Role of ubiquitin-mediated degradation system in plant biology. Front. Plant Sci. 7, 806. https://doi.org/10.3389/fpls.2016.00806.
Shivhare, R., Lata, C., 2016. Selection of suitable reference genes for assessing gene expression in pearl millet under different abiotic stresses and their combinations. Sci. Rep. 6, 23036. https://doi.org/10.1038/srep23036.
Shivhare, R., Lata, C., 2017. Exploration of genetic and genomic resources for abiotic and biotic stress tolerance in Pearl Millet. Front. Plant Sci. 7, 2069. https://doi.org/10.3389/fpls.2016.02069.
Shrivastava, P., Kumar, R., 2015. Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi J. Biol. Sci. 22 (2), 123–131. https://doi.org/10.1016/j.sjbs.2014.12.001.
Singh, J., Reddy, P.S., Reddy, C.S., Reddy, M.K., 2015. Molecular cloning and characterization of salt inducible dehydrin gene from the C4 plant Pennisetum glaucum. Plant Gene 4, 55–63. https://doi.org/10.1016/j.plgene.2015.08.002.
Smart, R.E., Bingham, G.E., 1974. Rapid estimates of relative water content. Plant Physiol. 53 (2), 258–260. https://doi.org/10.1104/pp.53.2.258.
Song, A., Gao, T., Wu, D., Xin, J., Chen, S., Guan, Z., et al., 2016. Transcriptome-wide identification and expression analysis of chrysanthemum SBP-like transcription factors. Plant Physiol. Biochem. 102, 10–16. https://doi.org/10.1016/j.plaphy.2016.02.009.
Thalmann, M., Santelia, D., 2017. Starch as a determinant of plant fitness under abiotic stress. New Phytol. 214 (3), 943–951. https://doi.org/10.1111/nph.14491.
Tuteja, N., 2007. Mechanisms of High Salinity Tolerance in Plants. Meth. Enzymol. 428, 419–438. https://doi.org/10.1016/S0076-6879(07)28024-3.
Umezawa, T., Nakashima, K., Miyakawa, T., Kuromori, T., Tanokura, M., Shinozaki, K., et al., 2010. Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. Plant Cell Physiol. 51 (11), 1821–1839. https://doi.org/10.1093/pcp/pcq156.
Vadez, V., Hash, T., Bidinger, F.R., Kholova, J., 2012. II.1.5 Phenotyping pearl millet for adaptation to drought. Front. Physiol. 3, 386. https://doi.org/10.3389/fphys.2012.00386.
Varshney, R.K., Shi, C., Thudi, M., Mariac, C., Wallace, J., Qi, P., et al., 2017. Pearl millet genome sequence provides a resource to improve agronomic traits in arid environments. Nat. Biotechnol. 35 (10), 969. https://doi.org/10.1038/nbt.3943.
Verma, D., Singla-Pareek, S.L., Rajagopal, D., Reddy, M.K., Sopory, S.K., 2007. Functional validation of a novel isoform of Na+/H+ antiporter from Pennisetum glaucum for enhancing salinity tolerance in rice. J. Biosci. 32 (3), 621–628. https://doi.org/10.1007/s12038-007-0061-9.
Vishwakarma, K., Upadhyay, N., Kumar, N., Yadav, G., Singh, J., Mishra, R.K., et al., 2017. Abscisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. Front. Plant Sci. 8, 161. https://doi.org/10.3389/fpls.2017.00161.
Wang, H., Wang, H., Shao, H., Tang, X., 2016. Recent advances in utilizing transcription factors to improve plant abiotic stress tolerance by transgenic technology. Front. Plant Sci. 7, 67. https://doi.org/10.3389/fpls.2016.00067.
Yang, Z., Lu, R., Dai, Z., Yan, A., Tang, Q., Cheng, C., et al., 2017. Salt-stress response mechanisms using de novo transcriptome sequencing of salt-tolerant and sensitive corchorus spp. Genotypes Genes 8 (9), 226. https://doi.org/10.3390/genes8090226.
Yemm, E.W., Willis, A.J., 1954. The estimation of carbohydrates in plant extracts by anthrone. Biochem. J. 57 (3), 508. https://doi.org/10.1042/bj0570508.
Yu, Y., Ni, Z., Chen, Q., Qu, Y., 2017. The wheat salinity-induced R2R3-MYB transcription factor TaSIM confers salt stress tolerance in Arabidopsis thaliana. Biochem. Biophys. Res. Commun. 491 (3), 642–648. https://doi.org/10.1016/j.bbrc.2017.07.150.
Zeng, L.R., Vega-Sánchez, M.E., Zhu, T., Wang, G.L., 2006. Ubiquitination-mediated protein degradation and modification: an emerging theme in plant-microbe interactions. Cell Res. https://doi.org/10.1038/sj.cr.7310053. (p. 16(5), 413).
Zhang, A., Liu, D., Hua, C., Yan, A., Liu, B., Wu, M., et al., 2016. The arabidopsis gene zinc finger protein 3(ZFP3) is involved in salt stress and osmotic stress response. PLoS ONE 11 (12), 1–16. https://doi.org/10.1371/journal.pone.0168367.
Zhu, J.-K., 2002. Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53 (1), 247–273. https://doi.org/10.1146/annurev.arplant.53.091401.143329.