博士論文（要約）

Doctorate thesis (Abridged)

Studies on the salinity stress responses of pearl millet (*Pennisetum glaucum* (L.)

トウジンビエの塩ストレス応答に関する研究

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LIST OF CHAPTERS

Chapter 1

General introduction

1.1. Pearl millet, An abiotic stress tolerant, staple food crop.

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] or [*Cenchrus americanus* (L.) Morrone] is a highly cross-pollinating, monocot crop of the family Poaceae. It has diploid genome (2n = 2x = 14) with estimated 38, 579 genes (Varshney et al., 2017). It originated in the northern–central Sahel in West Africa (Mariac and Vigouroux, 2008). Pearl millet is C_4 species with high photosynthetic efficiency. Pearl millet is the short duration crop, usually needs 65-85 days. It grows to a height of three meters, and its stems are 10-20 mm thick. Leaves are flat, green and up to 8 cm wide. Its seeds are small, wedge-shaped to spherical and of different colours. Pearl millet is known by several common names including Pearl millet, Cattail, Spiked millet, Bulrush millet, Ddukn, Bajra and so on. In Asia, India is the major pearl millet growing country. In Africa, Sudan and Niger are the major pearl millet growing countries. Pearl millet is very nutritious crop, contains high level of micro-nutrients (Fe and Zn content), high fibre and balanced amino-acid composition. It is the main source of energy, protein, mineral and vitamins for millions of poor people in India and Africa (Shivhare and Lata, 2017).

Pearl millet is grown mostly in arid and semi-arid tropical regions of Asia and Africa. (Debieu et al., 2017). It has high drought tolerance capacity (Yadav et al., 2012). It is also high temperature stress tolerant crop (Djanaguiraman et al., 2018). It is a glycophyte but has the inbuilt capacity to withstand soil salinity. Its cultivar named “HASHAKI I” has been released to be grown in salinity affected areas of Uzbekistan (Shivhare and Lata, 2017). Very limited information is available on the responses of pearl millet to salinity stress at physiological and

molecular level. 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).

1.2. Salinity stress, one of the abiotic stress which limits world agriculture production

Salinity stress is one of the abiotic stresses that severely affect 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 effect of salinity on plants includes ion toxicity, nutrient constraints, oxidative stress, and osmotic stress(Shrivastava and Kumar, 2015). Soil salinity which is characterized by a high concentration of soluble salts has become a major limitation to agricultural production. Saline soil defined as ECe $\geq$ 4 dS m^{-1}, which is equivalent to about 40mM NaCl with an osmotic pressure of nearly 0.2 MPa, would reduce yield of most of the agriculturally important crops.

In plants, 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. Pathways such as the plant hormone signaling pathway, the SOS (salt overly sensitive) pathway, the calcium-signaling pathway, the MAPK (mitogen-activated protein kinase) signaling pathway, and proline metabolism also have key roles in the salinity stress tolerance. (Danquah et al., 2014; Ji et al., 2013; Kavi Kishor et al., 2005; Knight, 1999; Zhu, 2002).

1.3. Scope and Applications of the research

Pearl millet is important crop of food security. It is considered as future crop, as it stands well to different abiotic stresses including salinity stress. Pearl millet has already proved its salinity stress tolerance capacity, as pearl millet is growing well in salt affected areas of Uzbekistan. (Yadav et al., 2012)

To study and research the salinity stress tolerance potential of pearl millet we used different biotechnological approaches. The aim of study was to understand the molecular and biochemical responses of pearl millet to salinity stress.

We were able to find out salinity stress responsive genes and pathways using the transcriptome analysis. We found that genes encoding transcription factors, ion transporters, and regulators of metabolic pathways are extremely useful in improving the pearl millet productivity under salinity stress condition. Functional salinity stress responsive genes and pathways that are identified in our study can provide useful clues for improving salinity stress tolerance in crop plants (Shinde et al., 2018b)

By small RNA sequencing, we identified salinity stress responsive micro RNAs (miRNAs). In total 81 salinity stress responsive miRNAs were found, of which 30 miRNAs were identified as an upregulated miRNA and 51 miRNAs were identified as a downregulated miRNA. In total, 448 target genes of 81 miRNAs were identified using the online server. Pathway analysis found that Auxin signaling pathway is the key pathway targeted by salinity stress responsive miRNAs. Salinity stress responsive miRNA identified in our studies could facilitate future research to understand the mechanism of salinity stress tolerance in pearl millet. Identified key miRNAs and target genes can be successfully used to breed new stress tolerant crop plants.

In our functional analysis studies, function of pearl millet stress-responsive NAC gene *PgNAC21* was characterized. Gene expression analysis revealed that *PgNAC21* expression is induced by salinity stress and abscisic acid (ABA) treatment. *In silico* promoter analysis showed the presence of *ABA response elements* (ABREs) and MYB TF binding sites. Relative to control plants, *Arabidopsis* plants overexpressing *PgNAC21* exhibited better seed germination, heavier fresh weight and greater root length under salinity stress. Overexpression of *PgNAC21* in *Arabidopsis* plants also enhanced the expression of stress-responsive genes such as *GSTF6* (*GLUTATHIONE S-TRANSFERASE 6*), *COR47* (*COLD-REGULATED 47*) and *RD20* (*RESPONSIVE TO DEHYDRATION 20*). Our data demonstrate that *PgNAC21* functions as a stress-responsive NAC TF and can be utilized in transgenic approaches for developing salinity stress tolerance in crop plants (Shinde et al., 2018a).

Our all studies generate useful information to understand the molecular mechanism of salinity stress tolerance in pearl millet. This information can be used by breeders to breed new salinity stress tolerant crop plants by transgenic approaches.

Chapter 2

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

2.1. Introduction

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 (Kasuga et al., 1999; Tuteja, 2007). 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. (Danquah et al., 2014; Ji et al., 2013; Kavi Kishor et al., 2005; Knight, 1999; Zhu, 2002)

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 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 (Dwivedi et al., 2011; Krishnamurthy et al., 2007). 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; Desai et al., 2006; Reddy et al., 2012; Singh et al., 2015; Verma et al., 2007). 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.

2.2. Materials and Methods

2.2.1. 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 figure 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.

2.2.2. 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.)].

2.2.3. 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 (250mM NaCl for 18 hours) 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 x 100 bp each) in the FASTQ format.

2.2.4. *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).

2.2.5. 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.

2.2.6. 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 (Kanehisa, 2002; Ogata et al., 1999). The PlantTFcat online tool

(http://plantgrn.noble.org/PlantTFcat/) was used to identify genes encoding transcription factors (Dai et al., 2013).

2.2.7. 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 seconds followed by 40 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds. 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 (Kenneth J Livak and Schmittgen, 2001).

2.3. Results

2.3.1 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 (Figure 1) These results confirm that ICMB 01222 is more tolerant to salinity stress than ICMB 081 at the seedling stage.

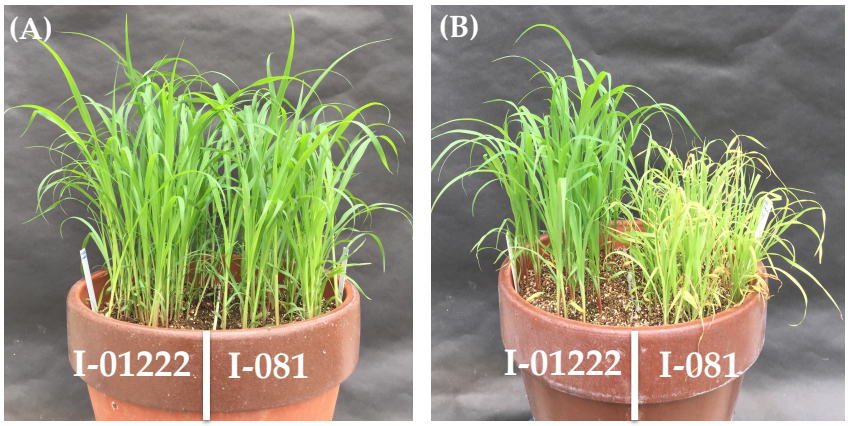


Figure 1- Differential responses of two pearl millet lines to salinity stress. The left and right sides of the pots contained the ICMB 0122 (tolerant) and ICMB 081 (susceptible) lines, respectively. (A) Control condition; (B) Salinity stressed condition (250mM NaCl for 6 days).

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

Table 1. Physiological responses of two contrasting lines, ICMB 01222 (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.

2.3.2. 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).

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.

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				

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

2.3.4. 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 (Figure 2a). Of these DEGs, 1287 upregulated unigenes and 1451 downregulated unigenes were common across both lines (Figure 2b and 2c).

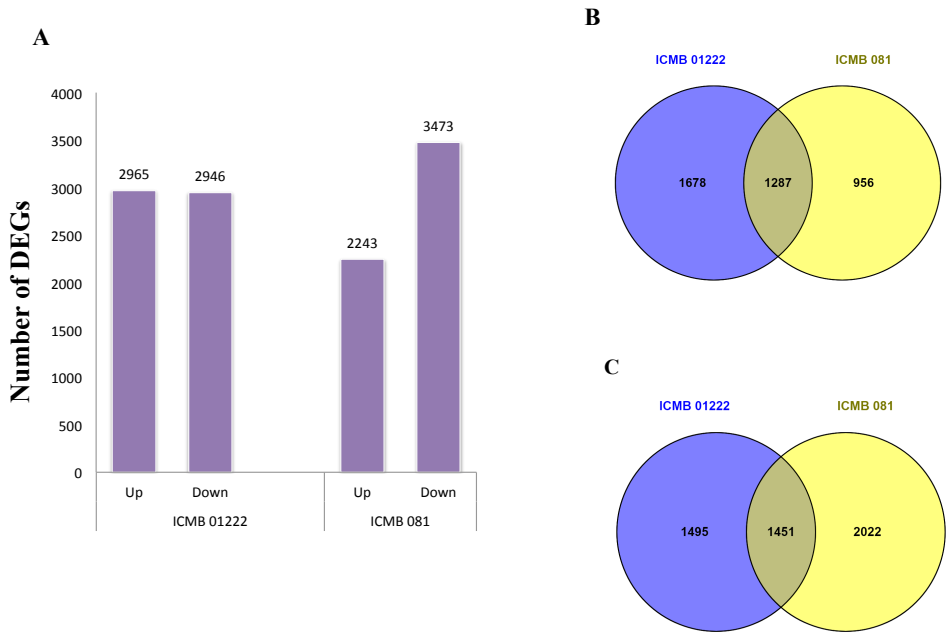


Figure 2. (**a**) Differentially expressed genes (DEGs) of ICMB 01222 and ICMB 081; (**b**) Venn diagram showing the common and unique upregulated unigenes; (**c**) Venn diagram showing the common and unique downregulated unigenes.

2.3.5. 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 01222 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. (Figure 3)

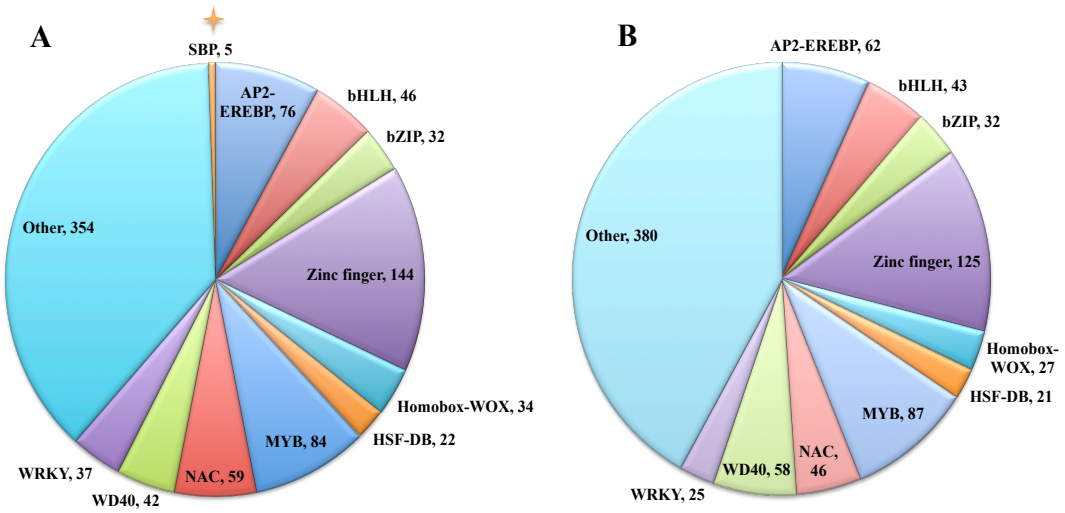


Figure 3. The number of transcription factors encoded by differentially expressed genes (DEGs). (**a**) TFs of ICMB 01222. The asterisk shows ICMB 01222-specific SBPs (SQUAMOSA promoter binding protein) transcription factors; (**b**) TFs of ICMB 081.

2.3.6. 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 (Figure 4). 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.

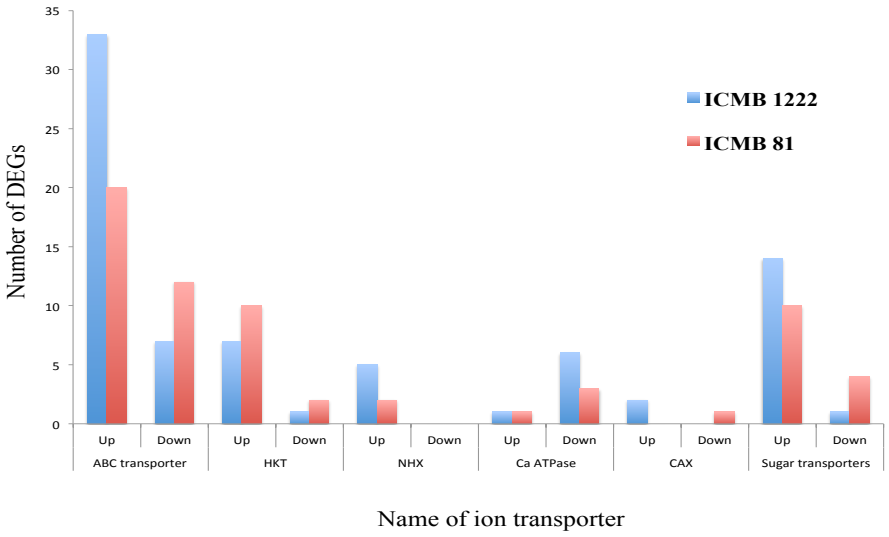


Figure 4. The number of differentially expressed genes encoding ion transporters in both lines.

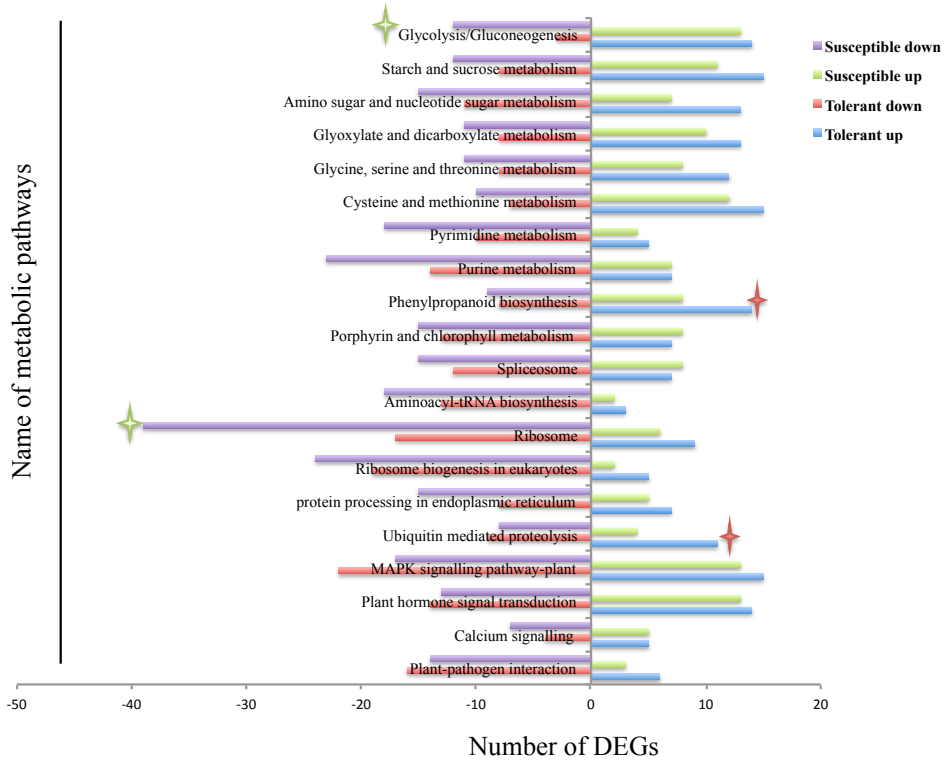


Figure 5. Metabolic pathways enriched under salinity stress in the tolerant (ICMB 01222) and susceptible (ICMB 081) lines. Red asterisks show pathways extensively upregulated in the tolerant line by salinity stress. Green asterisks show pathways extensively downregulated in the susceptible line by salinity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

2.3.7. Metabolic pathways involved in salinity stress responses

Twenty pathways that are most enriched in DEGs were compared (Figure 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.

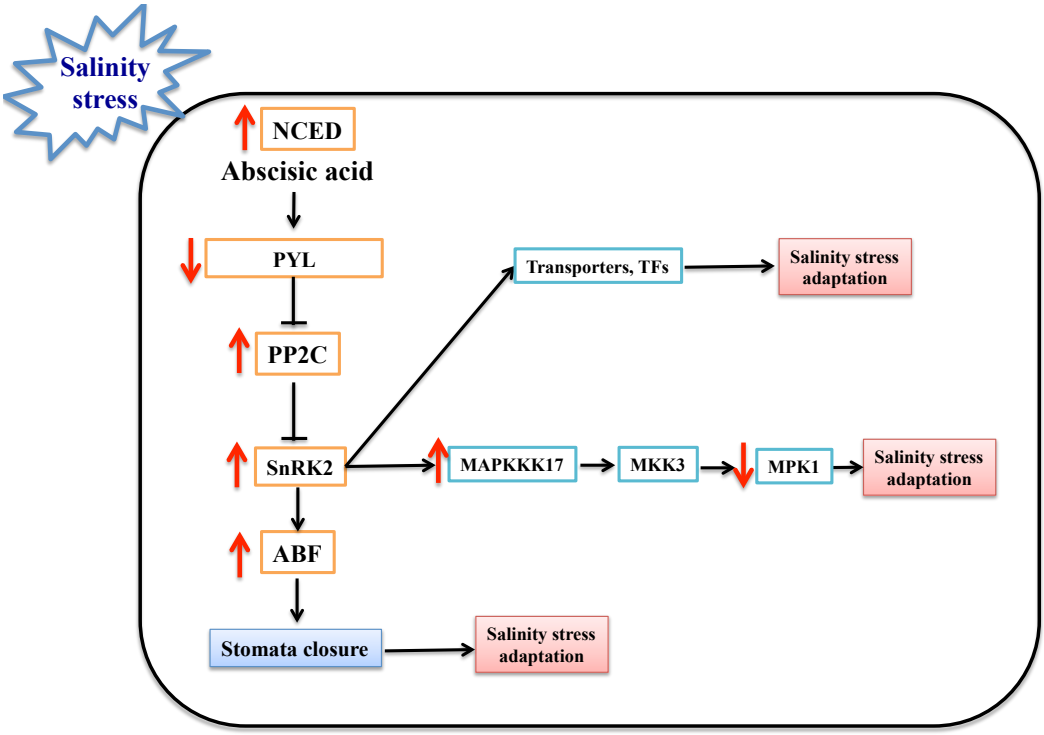


Figure 6. The PYL-ABA-PP2C pathway. NCED: Nine-cis-epoxycarotenoid dioxygenase; PYL: pyrabactin resistant like; PP2C: protein phosphatase 2C; SnRK2: SNF1-related protein kinase 2; ABF: ABA-responsive element binding factor; MAPKKK17: mitogen-activated protein kinase kinase kinase 17; MKK3: mitogen-activated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

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 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 (Figure 6).

Plant hormone signaling pathways are also involved in salinity stress adaptation through ubiquitin-mediated proteolysis. The ubiquitin-mediated proteolysis pathway was

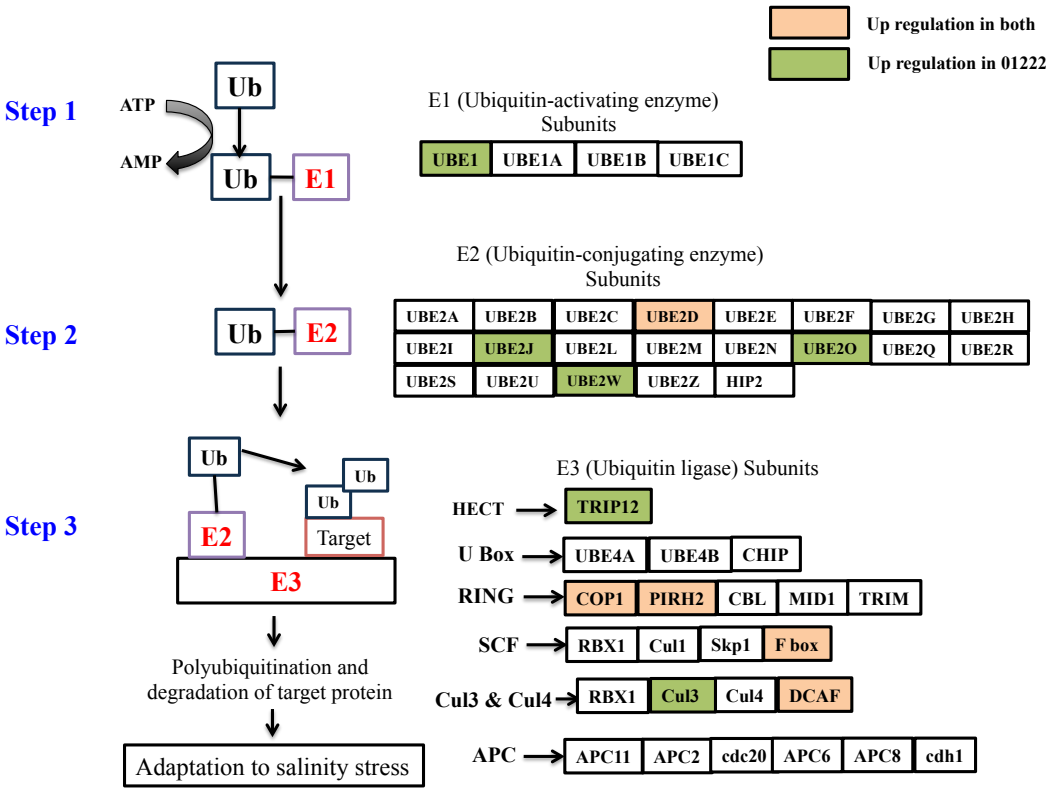


Figure 7. Ubiquitin-mediated proteolysis pathway. Red boxes show upregulated unigenes in both lines (the tolerant line ICMB 01222 and the susceptible line ICMB 081). Green boxes show only the upregulated unigenes in the ICMB 01222. E1 is a ubiquitin-activating enzyme complex consisting of 4 subunits, 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.

associated with 11 upregulated unigenes in the tolerant line but with only 5 upregulated unigenes in the susceptible line. Ubiquitin-mediated proteolysis (Figure 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 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 (Figure 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.

2.3.8. 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. (Figure 8). Primer pairs used this study are given in Table. 3 (at the end of chapter).

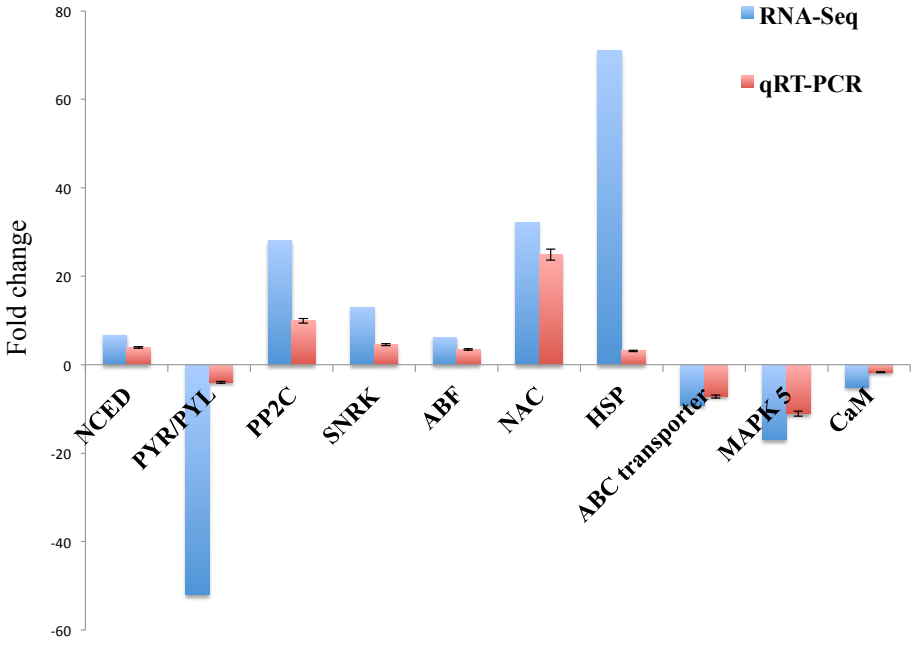


Figure 8. qRT-PCR validation of the RNA-Seq data of 10 DEGs. Blue bars represent fold changes in the RNA-Seq data and red bars represent fold changes in the real-time PCR analysis.

2.4. 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.

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 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, 2012a). 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 (Park et al., 2010; Umezawa 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 (Miao et al., 2014; Sharma et al., 2016). 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 (Figure 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 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.

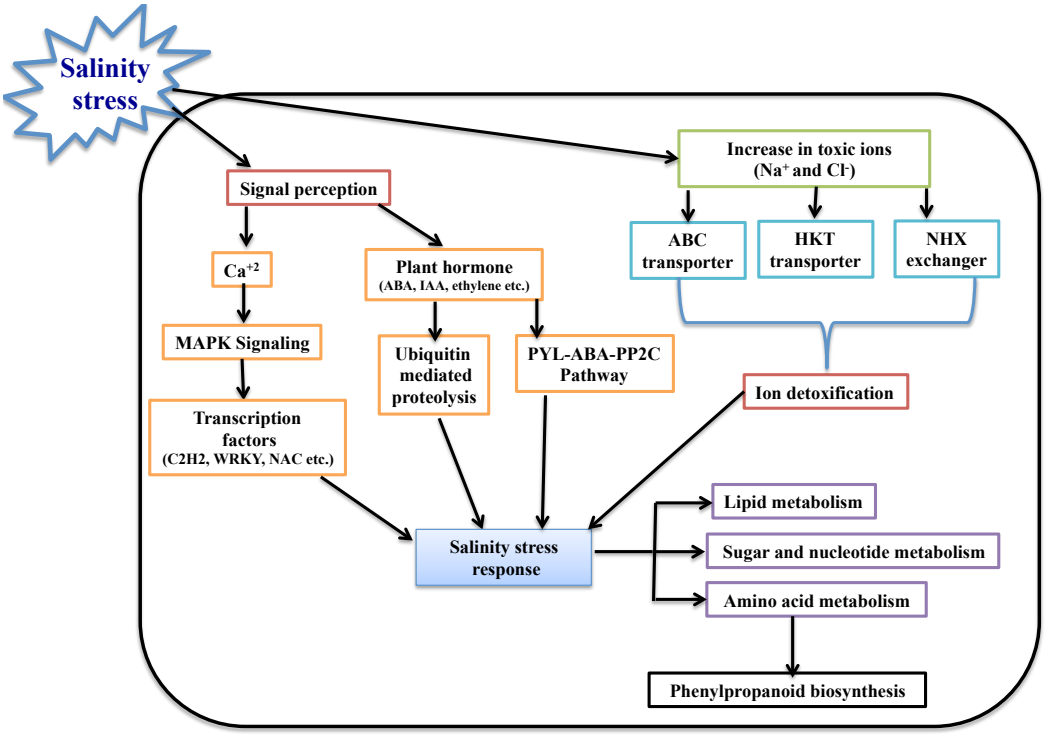


Figure 9. A Scheme for the response of pearl millet to salinity stress. Upon salinity stress, the pearl millet cells sense the stress and initiate calcium and plant hormone signaling. Calcium acts as a secondary messenger and activates the MAPK signaling pathway, which in turn activates the transcription 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.

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.

Unigene Name	Annotation	Primer sequence
NCED	PREDICTED: 9-cis-epoxycarotenoid dioxygenase 1, chloroplastic [*Setaria italica*]	Fw. GGCTCCCTCTGGTGACTTCTC Rv. CGTGCCCTTTGGCTTCC
PYL	PREDICTED: abscisic acid receptor PYL4-like [*Setaria italica*]	Fw. AGGCAACAGCCACAGAAATG Rv. CGGGACGTTGTTCCTTGA
PP2C	PREDICTED: probable protein phosphatase 2C [*Setaria italica*]	Fw. CCCGCCATTTATAGTCGGTA Rv. GCACTTTCATCCAACCACCT
SNRK	predicted protein [*Hordeum vulgare subsp. vulgare]*	Fw. CGACCCAACCAAATCCC Rv. ACCAACAAGCACCAACAGC
ABF	hypothetical protein SETIT_013904mg [*Setaria italica*]	Fw. CTGTGCCGTACCCGTTTG Rv. TGCGGACTCCCTGTTCTT
NAC	PREDICTED: NAC domain-containing protein 67-like [*Setaria italica*]	Fw. TCGTCTTCTACGCCGGCAAG Rv. GTCATCCAACCTGAGCGACC
HSP	Small heat shock protein Hsp17.0B (*Cenchrus americanus*]	Fw: CCCTTCTCCCTCGACCTCTG Rv: TCGGGCGTCTCCTTCCAAT
ABC transporter	PREDICTED: ABC transporter C family member 4-like isoform [S*etaria italica*]	Fw. GCACCAGAAATACAAGC Rv. ATACGATAGCCAATAGTCA
MAPK 5	PREDICTED: mitogen-activated protein kinase 5 isoform X2 [*Setaria italica*]	Fw. CGTCAGCGATGTCGTGTA Rv. TTCGTTTGGGTCGTGTC
CaM	PREDICTED: calmodulin-binding protein 25 [*Setaria italica*]	Fw. CGGTGATGTAGGTGGTCTGC Rv. TGCTGCTGCTGGTTGCC

Table 3. Primers used for real-time PCR in this study.

Chapter 3

As the content of this chapter are anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published within 2 years from the date of submission.

Chapter 4

Pearl millet stress-responsive NAC transcription factor *PgNAC21* enhances salinity stress tolerance in *Arabidopsis*

4.1. Introduction

Soil salinity severely limits plant growth and yields worldwide (Shrivastava and Kumar, 2015). Plants have developed numerous mechanisms to cope with salinity stress at physiological, biochemical and molecular levels. At the molecular level, plants activate stress-responsive genes and pathways. Genes encoding ion transporters, transcription factors (TFs), protein kinases, and osmolytes can confer salinity tolerance (Kasuga et al., 1999). TFs activate or suppress the expression of such stress-responsive genes. Major stress-responsive TF families include the NAC (NAM, ATAF1/2 and CUC), DREB, bZIP, ERF, zinc finger, WRKY and MYB families (Joshi et al., 2016); these families can be individually or synergistically involved in regulating the expression of other stress-response genes in plants. Among these different TF families, the NAC TF family is a plant-specific family with many members (Nuruzzaman et al., 2013). NAC TFs in *Arabidopsis*, rice and wheat are relatively well characterized and are known to have versatile functions (Nuruzzaman et al., 2013; Saidi et al., 2017). NAC TF proteins have highly conserved N-terminal domains with DNA binding function and highly variable C-terminal domains (Olsen et al., 2005). NAC TFs with specific combinations of N- and C-terminal domains have been functionally characterized with respect to their roles in responding to salinity stress (Puranik et al., 2012).

The stress-responsive TF ONAC022 enhances drought and salinity stress tolerance in rice by modulating a pathway mediated by the stress-related phytohormone abscisic acid (ABA) (Hong et al., 2016). When overexpressed, the *SlNAC8* gene of the halophyte plant *Suaeda liaotungensis* improves the salt stress tolerance of *Arabidopsis* plants by regulating the expression of stress-responsive genes (Wu et al., 2018). The *Miscanthus* NAC TF gene *NAC9*

enhances the salt stress tolerance of *Arabidopsis* plants by enhancing scavenging capacity for reactive oxygen species (Zhao et al., 2016). *AtNAC2*, a salt stress-responsive NAC TF gene in *Arabidopsis*, promotes salt stress tolerance via the ethylene and auxin signaling pathways; *AtNAC2* is also involved in lateral root development during salt stress (He et al., 2005).

Pearl millet is an abiotic stress tolerant crop that has been attracting attention since its genome was sequenced (Varshney et al., 2017). QTLs for salinity tolerance were identified earlier on Linkage group 2 in pearl millet (Sharma et al., 2011) . Recently, many drought and salinity stress-related genes in pearl millet were found using transcriptome (RNA-sequencing) analyses (Dudhate et al., 2018; Shinde et al., 2018b). *PgNAC21* is among the genes found to be upregulated by both drought and salinity stress in such analyses. Here, we cloned and characterized *PgNAC21* and found that *PgNAC21*-overexpressing *Arabidopsis* plants show enhanced tolerance of salinity stress. To our knowledge, this article is the first report describing the functional characterization of a pearl millet NAC TF.

4.2. Materials and methods

4.2.1 Plant material and stress treatments

The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in India provided pearl millet seeds. The salinity-tolerant pearl millet genotype ICMB 01222 (Shinde et al., 2018b; Yadav et al., 2012) was used to isolate *PgNAC21* and to examine its expression pattern. ICMB 01222 seeds were sown in composite soil in a greenhouse maintained at 28°C during the day and at 25°C at night with a relative humidity of 55-75%. To induce salinity stress, 18-day-old pearl millet seedlings were transferred to a Hoagland liquid medium supplemented with 250 mM NaCl, and sampled for RNA isolation at the time points indicated in Fig. 1.

4.2.2 Quantitative real-time PCR assay

Total RNA was isolated from pearl millet leaves and Arabidopsis seedlings as previously described [14]. cDNA was synthesized using PrimeScript reverse transcriptase (Takara Bio, Japan), and quantitative real-time PCR (qRT-PCR) was performed using this cDNA and TB Green Premix Ex Taq II (Takara). Relative gene expression was calculated using the ΔΔCT method (Kenneth J. Livak and Schmittgen, 2001). The pearl millet actin gene *PgActin* was used as a reference gene, as described previously (Shivhare and Lata, 2016)..

4.2.3 Analysis of cis-acting elements in the promoter region

The presence of cis-acting regulatory elements in the promoter region (2000 bp from the start codon) of *PgNAC21* was predicted using the online search tool at the PlantCARE website (Lescot, 2002; Rombauts et al., 1999).

4.2.4 Yeast one-hybrid analysis

For the yeast one-hybrid analysis, the 100-bp *PgNAC21* promoter sequence with two MYBHv1-binding sites (CAACGG) was amplified by genomic PCR and cloned into the yeast expression vector pHIS2 to generate the pHIS2-PgNAC21 promoter. The coding region of pearl millet MYB1 (PgMYB1) was amplified by PCR and cloned into the pGADT7-Rec2 vector to generate pGADT7-Rec2-PgMYB1. The yeast strain Y187 was co-transformed with pGADT7-Rec2-PgMYB1 and the pHIS2-PgNAC21 promoter. pGAD-Rec2-53, which has the murine p53 transcription factor gene, and p53HIS2, which has three tandem copies of p53-binding elements upstream of the *HIS3* reporter gene, were co-transformed as a positive control according to Yeast Protocols Handbook (TaKaRa Bio USA). The pGADT7-rec2 and pHIS2 empty vectors were co-transformed as a negative control. DNA-protein interactions were evaluated by growing transformed yeast cells on SD/-His/-Leu/-Trp selective medium

(synthetic dextrose medium lacking histidine, leucine and tryptophan) with 100 mM 3-amino-1,2,4-triazole (3-AT) for 3 days at 28°C.

4.2.5 Transactivation assay

The full-length coding sequences of *PgNAC21* and the coding sequences of the N-terminal and C-terminal domains of PgNAC21 were separately cloned into the pGBKT7 vector. The resulting vectors were then transformed into the yeast strain AH109 with the pGBKT7 vector. The transformed yeast cells were streaked on SD (-Trp) and SD (-His) media (synthetic dextrose medium lacking tryptophan and histidine, respectively). LacZ activity was assessed via a β-galactosidase assay (Yeast Protocols Handbook; Clontech, USA).

4.2.6 Generation of *Arabidopsis* overexpression lines

The coding sequence of *PgNAC21* was amplified and cloned into the pCAMBIA1300 binary vector. This vector was then transformed into the *Agrobacterium* strain EHA 105. Transgenic plants were generated using the floral dip method as previously described (Clough and Bent, 1998). Transformed plants were selected using 30 μg/ml hygromycin media plates. T3 homozygous transgenic lines harbouring a single copy of transgene were selected on the basis of the segregation ratio of antibiotic-resistant plants and susceptible plants in their progeny, and used for further analysis.

4.2.7 Analysis of salinity stress tolerance

Wild-type (Col-0) seeds and seeds from three T3 homozygous transgenic lines were sown on half-strength MS medium containing 125 mM NaCl to measure germination rates and perform a survival assay. In a primary root length assay, plants were grown on half-strength MS medium for 6 days and transferred to half-strength MS medium containing 100 mM NaCl.

They were grown for an additional 6 days, and their root length was then measured using ImageJ software (Rasband, 2015; Schindelin et al., 2015; Schneider et al., 2012).

4.2.8 qRT-PCR analyses of stress-responsive genes in *Arabidopsis*

Total RNA was isolated from transgenic *Arabidopsis* plants under salinity-stressed and control conditions. qRT-PCR was performed using TB Green Premix Ex Taq II (Takara). The primers used in this study were used in prior research (Wu et al., 2018).

4.3. Results

3.1 Gene expression analysis of *PgNAC21* under salinity stress

PgNAC21 expression in pearl millet leaves under salinity stress (250 mM NaCl) was characterized using qRT-PCR. Under salinity stress, there was higher *PgNAC21* expression at 6 h, 12 h, 18 h and 28 h than at 0 h. The *PgNAC21* expression level was highest at 18 h (23.8 times higher than the corresponding expression level at 0 h). *PgNAC21* expression was also upregulated (2.8-fold) by exogenous ABA application (10 μM) (Figure 1). These results suggest that *PgNAC21* is a salinity stress- and ABA-responsive gene.

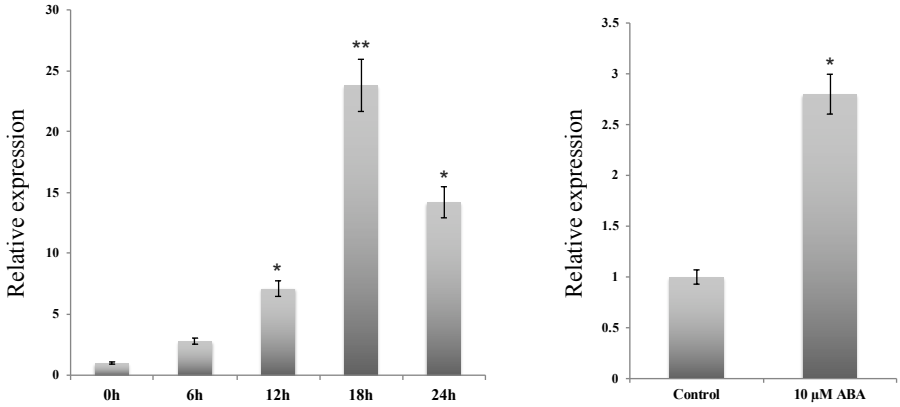


Figure 1. Induction of P*gNAC21* by salinity stress and ABA treatment in pearl millet. For the left panel, plants were incubated for the indicated periods in 250 mM NaCl, and used for the analysis. For the right panel, plants were treated with 0 (Control) or 10 µM ABA for 24 hours, and used for the analysis. The presented data represent means ± SD from three independent experiments. *: $P < 0.01$ vs. non-stressed samples in Student's t-test

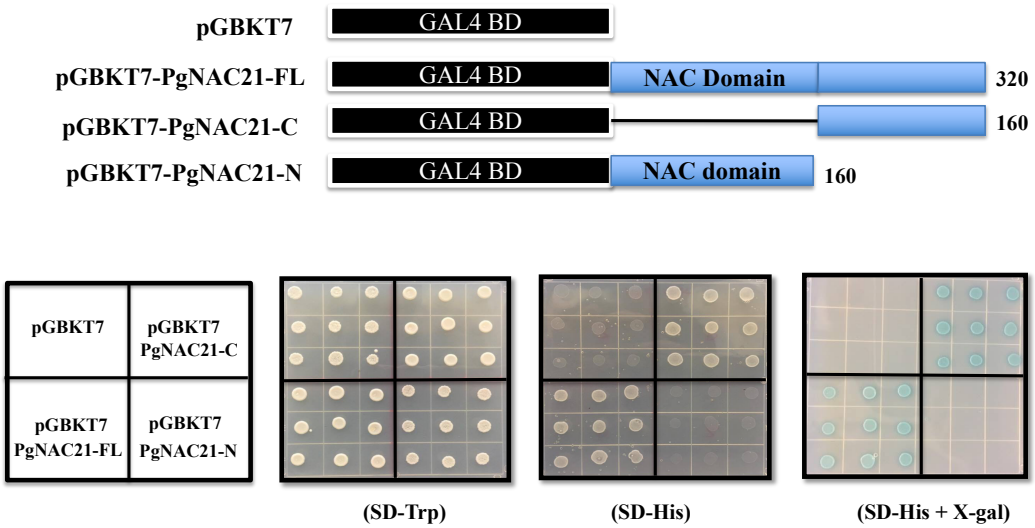


Figure 2. PgNAC21 exhibits transactivation activity. Yeast cells containing the pGBKT7 empty vector ("pBD"), pGBKT7-PgNAC21-FL ("pBD-PgNAC21-FL", which is to express full-length PgNAC21 as GAL4 DNA-binding domain-fused protein), pGBKT7-PgNAC21-N ("pBD-PgNAC21-N", to express an N-terminal region of PgNAC21) or pGBKT7-PgNAC21-C ("pBD-PgNAC21-C", to express a C-terminal region of PgNAC21) were streaked on either the SD-His medium or the SD-His + X-gal medium to examine reporter gene activation. The SD-Trp medium was used for a control. The media with yeast cells were incubated for 3 days at 28°C.

4.3.2 *PgNAC21* is a transcriptional activator gene

The transcriptional activation activity of the PgNAC21 was assessed in yeast. Yeast cells containing the pGBKT7 empty vector or the pGBKT7-PgNAC21-N vector (which included the coding sequences of the N-terminal region of PgNAC21) could not grow on SD (-His) medium. However, cells containing the pGBKT7-PgNAC21-C vector (which included the coding sequences of the C-terminal region of PgNAC21) or pGBKT7-PgNAC21 (which included full-length PgNAC21) could grow on SD (-His) medium and turned blue in that context, indicating enhanced β-galactosidase activity (Figure 2). These results suggest that PgNAC21 functions as a transcription activator and that its activation domain is located in its C-terminus.

4.3.3 *In silico* promoter analysis

In silico analysis of the *PgNAC21* promoter showed the presence plant hormone-responsive element, such as an ABA-responsive element (ABRE). In addition, four MYB1 binding sites (*Hordeum vulgare* MYB protein (MYBHv1)-binding elements) were detected in the vicinity of the putative transcription start site of *PgNAC21*. Drought responsive elements and MYC transcription factor binding sites were also detected.

4.3.4 Putative PgMYB1 protein binds to the promoter of *PgNAC21*

To confirm that the *PgNAC21* promoter is bound by PgMYB1, which is highly similar to MYBHv1, we performed yeast one-hybrid analysis. In this analysis, yeast cells grew on the selection medium when cotransformed with pGADT7-Rec2-PgMYB1 and the pHIS2-PgNAC21 promoter (Figure 3), suggesting that PgMYB1 binds to the *PgNAC21* promoter.

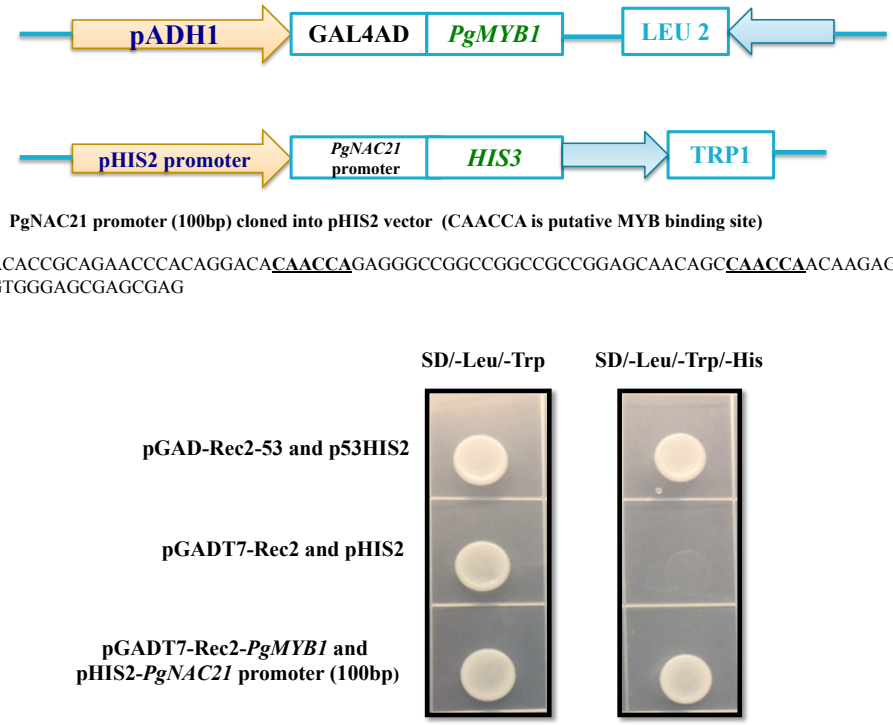


Figure 3. Yeast one-hybrid analyses using the PgMYB1 and PgNAC21 promoters. The upper panel shows the schematic structure of the effector construct pGADT7-Rec2-PgMYB1 and the reporter construct pHIS2-PgNAC21 promoter (100 bp region) utilized for the yeast one-hybrid analysis. These constructs were transformed into the yeast strain Y187, and the growth of transformed cells on SD/-Leu/-Trp and SD/-Leu/-Trp/-His medium was observed to examine reporter gene activation in yeast. pGAD-Rec2-53, which can express the murine p53 transcription factor, and p53HIS2, which has p53-binding elements upstream of the reporter gene, were used as a positive control. pGADT7-Rec2 and pHIS2 were used as a negative control (lower panel).

4.3.5 Analysis of PgNAC21-overexpressing *Arabidopsis* plants under salinity stress

Germination rates of Col-0 and the *PgNAC21*-overexpressing Arabidopsis plants were similar under control conditions (72-85%). Under salinity stress (125 mM NaCl), the germination rate of Col-0 was 21%, whereas the germination rates of the transgenic lines were 42-80% (Figure 4A and B). The average fresh weight of 6-day-old Col-0 plants under salinity stress was 1.1 mg, whereas significantly heavier fresh weights (3.11 to 4.8 mg) were observed for the transgenic plants (Figure 4C). When salinity stress was imposed at a post-germination stage, the *PgNAC21*-overexpressing plants showed less severe reduction in primary root length than the wild type (Figure 5). The *PgNAC21*-overexpressing plants did not show any abnormal phenotypes under normal growth conditions at later developmental stages (Shinde et al., unpublished data).

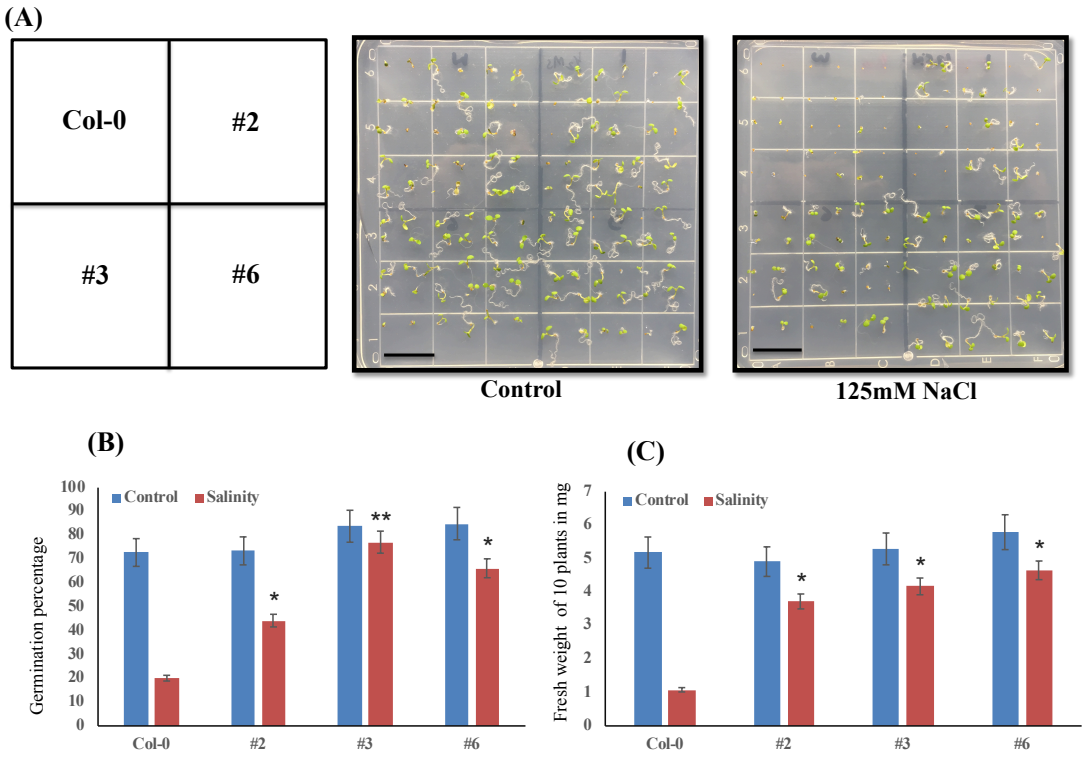


Figure 4. Characterization of PgNAC21-overexpressing *Arabidopsis* T3 lines (#2, #3 and #6) with respect to salinity stress tolerance. (A) Germination on MS media with 0 ("Control") and 125 mM NaCl. Scale bars = 1 cm. (B) Germination rates of seeds sown on MS media with 0 ("Control") and 125 mM NaCl ("Salinity"). Data are means ± SD of three replicates. Thirty plants were assessed in each replicate. *: $P < 0.05$ vs. "Control" in the Tukey-Kramer test. (C) Fresh weights of plants grown on MS media with 0 ("Control") and 125 mM NaCl ("Salinity").Data are means ± SD of three biological replicates. Ten plants were weighed for each replicate

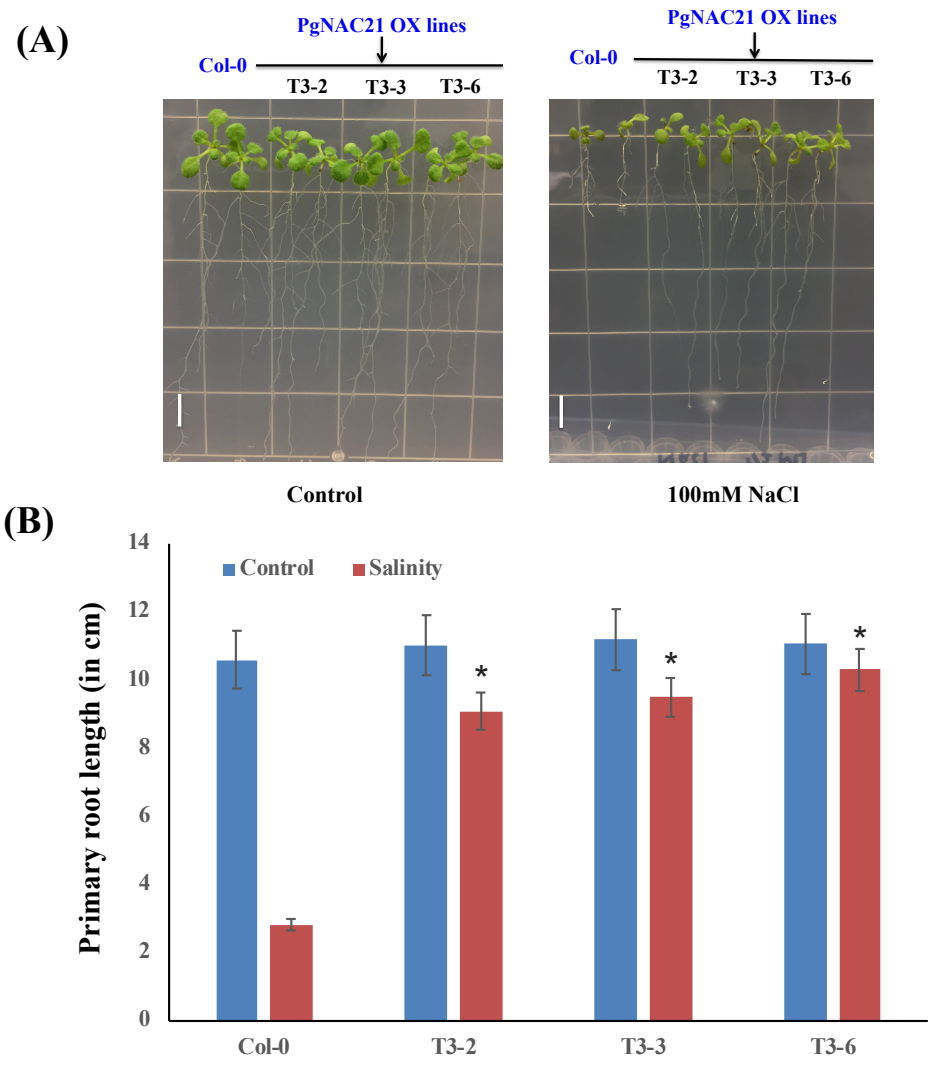


Figure 5. Analysis of root length for *PgNAC21*-overexpressing lines (T3-2, T3-3 and T3-6). Plants were grown for six days on an MS medium with 0 mM NaCl, transferred to an MS medium with either 0 ("Control") or 100 mM NaCl ("Salinity"), and further grown for six days. (A) Plant images. Scale bars = 1 cm. (B) Primary root lengths. Data are means ± SD of three representative plants for each genotype for each treatment. To choose the representative plants, at least 20 plants were observed. *: $P < 0.05$ vs. "Control" in the Tukey-Kramer test.

4.3.6 Analysis of the expression of stress-responsive genes in transgenic *Arabidopsis*

We analyzed the expression of stress-responsive genes in PgNAC21-overexpressing *Arabidopsis* plants and Col-0 plants. The expression levels of the three stress-responsive genes *GSTF6* (*GLUTATHIONE S-TRANSFERASE 6*), *COR47* (*COLD-REGULATED 47*) and *RD20* (*RESPONSIVE TO DEHYDRATION 20*) were higher in *PgNAC21*-overexpressing plants than in Col-0 plants (Figure 6).

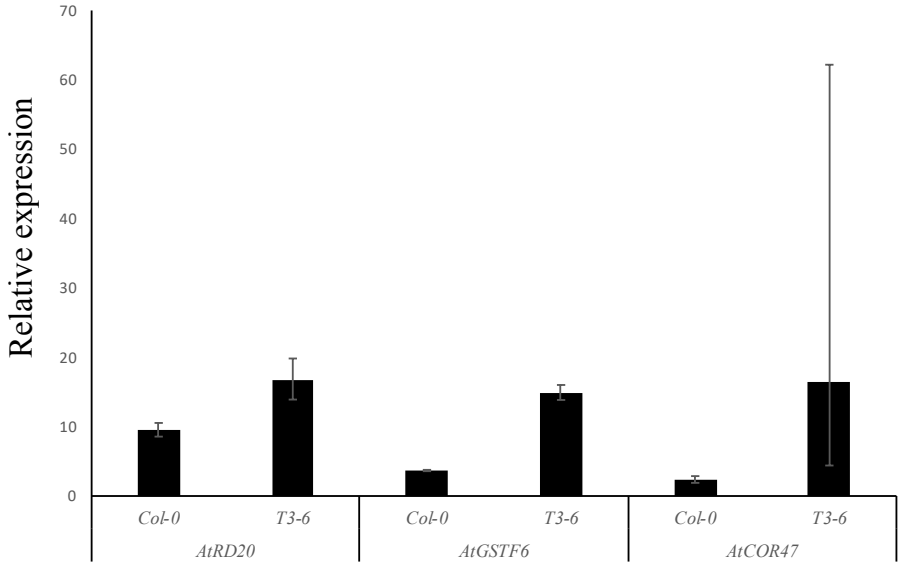


Figure 6. Analysis of the expression of stress-responsive genes (*RD20, GSTF6* and *COR47*) in *PgNAC21*-overexpressing *Arabidopsis* plants ("OX plants", the line T3-6) and Col-0 plants. Seven-day-old seedlings were exposed to salinity stress (100 mM NaCl) for 7 days. Levels of gene transcripts were assessed using qRT-PCR. Each value represents the mean ± SE of three biological replicates. *: $P < 0.01$ vs. Col-0 in Student's t-test

4.4. Discussion and conclusion

Soil salinity is one of the major abiotic stresses limiting agricultural productivity worldwide. Climate change is expected to increase soil salinity, posing serious threat to food security (Su and Hock, 2016). Developing the salinity tolerant crop varieties will help to improve the agricultural production in such a situation. Conventional plant breeding approaches take some time in achieving this goal (Ashkani et al., 2015). Use of biotechnologies such as genetic engineering can reduce the time required for this process. Salinity stress causes both dehydration stress and ion toxicity-derived stress in plants. Salinity stress tolerance of plants should therefore be a complex trait, acquired by functions of multiple genes. Utilization of stress-responsive TFs that regulate wide array of downstream genes is one of the most powerful approaches to enhance plant stress tolerance (Wang et al., 2016). Among the different TF families, the NAC TF family has gained lots of attention because of their functional diversity and importance (Nakashima et al., 2007; Tran et al., 2010). Our results also show that one of the NAC TFs, PgNAC21, is important in regulating salinity stress tolerance in plants.

Pearl millet is the sixth most important staple crop. It is grown in arid and semi-arid tropical regions of Asia and Africa (Vadez et al., 2012). This is because pearl millet has strong tolerance to abiotic stresses, and several genes such as *PgDREB2A* (dehydration responsive element-binding

protein), *PgNHX1* (Na+/H + antiporter), *PgDHN* (dehydrin), *PgVDAC* (voltage-dependent

anion channel), and *PgLEA* (late embryogenesis abundant protein) are linked to the strong tolerance of pearl millet (Agarwal et al., 2010; Desai et al., 2006; Reddy et al., 2012; Singh et al., 2015; Verma et al., 2007). It should be interesting to determine whether *PgNAC21* contributes to the expression of these genes.

NAC TFs represent a major plant-specific TF family, and pearl millet has 155 members of the NAC TF family. This article is the first report of functional characterization of a pearl millet NAC TF. In this study, *PgNAC21* was induced by salinity stress, exogenous ABA treatment (Fig. 1), PEG-induced dehydration stress and cold (4°C) stress. Because salinity stress, dehydration stress and cold stress all can activate ABA biosynthesis (Tuteja, 2007b), and because the promoter of *PgNAC21* contains an ABRE, the *PgNAC21* induction by these stresses may partially be dependent on ABA. However, because the salinity stress induced *PgNAC21* more greatly than the ABA treatment (Fig. 1), an ABA-independent yet salinity stress-dependent inducer of *PgNAC21* may be present. This idea is consistent with a previous finding that some genes such as *NCED3*, *AAO3* and *ABA1* in *Arabidopsis* are induced by both ABA-dependent and ABA-independent pathways under a salinity-stressed condition (Barrero et al., 2006) . The promoter of *PgNAC21* contains not only the ABRE but also four MYB TF-binding elements, and the *PgNAC21* promoter fragment containing two of these elements were bound by PgMYB1 (Fig. 3). These results raise the possibility that PgMYB1 regulates the expression of *PgNAC21*. This idea is consistent with a recent finding that *Arabidopsis* MYB108 binds the promoter of ANAC003 (*Arabidopsis* NAC 003), although neither MYB108 nor ANAC003 has been shown to be involved in regulating salinity stress tolerance. The promoter of *PgNAC21* also contains abiotic stress responsive element (such as DRE) and MYC transcription factor binding site. Thus, it should also be interesting to examine whether these elements mediate the salinity-dependent induction of *PgNAC21*. (Chou et al., 2018).

In this study, *PgNAC21*-overexpressing *Arabidopsis* plants showed enhanced salinity stress tolerance. *PgNAC21* should therefore be a positive regulator of salinity stress tolerance. This idea is consistent with previous findings that certain NAC TFs act as positive regulators of salinity stress tolerance (Chou et al., 2018; Hong et al., 2016; Huang et al., 2015). *GSTF6, COR47* and *RD20* showed significant upregulation in *PgNAC21*-overexpressing plants.

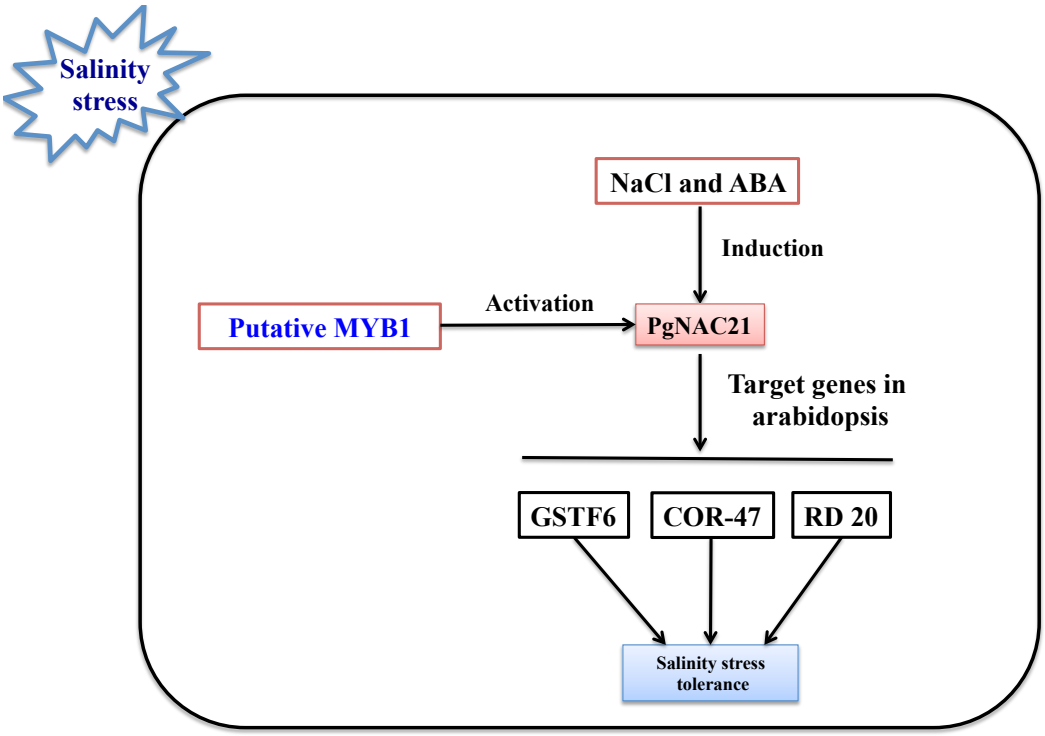
Promoters of these genes have multiple copies of the NAC TF-binding site CACG. *PgNAC21* may therefore directly bind to these promoters to regulate the expression of these genes. It is known that NAC TFs bind to promoters of their target genes, preferentially to the NAC core binding site CACG (Tran et al., 2004). Levels of GSTF6 protein are significantly increased in roots by NaCl-induced salinity stress, and this protein plays a role in scavenging reactive oxygen species generated by such stress (Jiang et al., 2007). COR47 belongs to the dehydrin protein family and responds to osmotic stress, ABA and cold stress (Puhakainen et al., 2004). RD20 is a salinity and drought stress-inducible caleosin protein that participates in regulating stomatal apertures, transpiration and abiotic stress tolerance in *Arabidopsis* (Aubert et al., 2010). The salinity stress tolerance of PgNAC21-overexpressing Arabidopsis plants may be attributed to strong expression of these genes (Figure 7).

N-terminal regions (i.e., NAC domains) of NAC TF members are highly conserved between each other, and cause these proteins to localize in the nucleus, to homo- and heterodimerize, and to bind DNA (Nuruzzaman et al., 2013). Their C-terminal regions are less conserved, and can fine-tune their DNA-binding specificity. Interestingly, the C-terminal region of *PgNAC21* is highly homologous to that of rice SNAC1, overexpression of which enhances tolerance to drought and salinity stresses in rice (Liu et al., 2014). Functions of *PgNAC21* examined in our study are shared with *SNAC1*: The C-terminal region of SNAC1 exhibits transcriptional activation potential in a yeast one-hybrid system (Hu et al., 2006); *SNAC1* is induced by drought stress, salinity stress, cold stress and ABA treatment. *SNAC1* overexpression increases ABA sensitivity in rice, not consistent with our finding that *PgNAC21* overexpression does not affect ABA sensitivity in Arabidopsis. Nevertheless, these results together support the idea that *SNAC1* and *PgNAC21* are both useful to enhance salinity stress tolerance of cereal crops.

We also found that under a salinity stress condition *PgNAC21*-overexpressing *Arabidopsis* plants had longer primary roots and more lateral roots than wild type plants (Figure 5). This indicates a possible role of *PgNAC21* in regulating the plant root system under salinity stress and thereby improving the salinity stress tolerance. This result is consistent with several previous findings that root system has a role in abiotic stress tolerance (Jeong et al., 2010; Redillas et al., 2012). Our analysis also showed that under salinity stress *PgNAC21*-overexpressing Arabidopsis plants had better seed germination than wild type plants. This result is consistent with previous finding that *ThNAC13* from *Tamarix hispida* confers salinity stress tolerance in Arabidopsis by improving its germination rate under salinity stress (Wang et al., 2017).

In summary, our study demonstrates that *PgNAC21* is a stress-responsive gene and encodes a transcription activator that regulates salinity stress tolerance. *PgNAC21* can improve salinity stress tolerance in not only *Arabidopsis* but also pearl millet and other crops. Further investigation of *PgNAC21* target genes in pearl millet will be helpful for elucidating mechanisms underlying salinity stress tolerance in this plant species.

Figure 7. A schematic model for *PgNAC21*-mediated salinity stress tolerance. NaCl and ABA



induce PgNAC21 expression; PgMYB1 also activates PgNAC21 expression by binding to the activator region of the promoter. PgNAC21 then activates stress-responsive genes, such as GSTF6, COR47 and RD20, to increase salinity stress tolerance.

Chapter 5

As the content of this chapter are anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published within 2 years from the date of submission.

Chapter 6

6.1. General discussion

Pearl millet is the staple food crop grown on 27 million hectare area in Asia and Africa. A better understanding of the effects of salinity on pearl millet and mechanisms of salinity stress tolerance are vital for breeding new salinity stress tolerant cultivars.

6.1.1. Physiological responses of pearl millet to salinity stress

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. Sugar plays roles in the detoxification of Na+ ions under salinity stress (Kanai et al., 2007) and also acts as an osmo-protectant (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. 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.

6.1.2. Molecular responses of pearl millet to salinity stress

At molecular level, we were able to find out the metabolic pathways and genes involved in salinity stress tolerance of pearl millet. Our comparative transcriptome analysis between salinity tolerant line, ICMB 01222 and salinity susceptible line, ICMB 081 were able to find out many important pathways and genes. Metabolic pathways like, Ubiquitin mediated proteolysis and phenylpropanoid biosynthesis were found to be upregulated only in tolerant genotype, ICMB 01222. So we think that these pathways are important regulators of salinity stress tolerance in pearl millet. In previous studies, it has proved that ubiquitin mediated proteolysis pathway plays critical role in abiotic stress responses (Sharma et al., 2016). Among the transcription factors, we found different transcription factor families in both lines. But interestingly, SQAMOSA promoter binding transcription factors were found to be up-regulated only in tolerance line ICMB 01222. In previous studies it has proved that SQAMOSA promoter binding transcription factors are important regulators of abiotic stress tolerance in plants (Hou et al., 2018; Song et al., 2016). Other than that, ABA responsive metabolic pathways were found to expressed in both tolerant and susceptible genotypes. ABA responsive pathway play important role in activation of many stress tolerance related pathways and genes. (Barrero et al., 2006; Park et al., 2010)

Our RNA sequencing analysis able to find out many salinity stress responsive genes in pearl millet. We performed functional studies of one of the gene called, PgNAC21. PgNAC21 belong to NAM, CUC, ATAF family NAC transcription factor family. NAC transcription factors are key regulators of salinity stress responses in plants (Lu et al., 2018; Nuruzzaman et al., 2013). In our research, gene expression analysis revealed that *PgNAC21* expression is induced by salinity stress and abscisic acid (ABA) treatment. *In silico* promoter analysis showed the presence of *ABA response elements* (ABREs) and MYB TF binding sites.

A yeast one-hybrid assay indicated that a putative MYB TF in pearl millet, PgMYB1, binds to the promoter of *PgNAC21*. A transactivation assay in yeast cells revealed that *PgNAC21* functions as a transcription activator and that its activation domain is located in its C-terminus. Relative to control plants, *Arabidopsis* plants overexpressing *PgNAC21* exhibited better seed germination, heavier fresh weight and greater root length under salinity stress. Overexpression of *PgNAC21* in *Arabidopsis* plants also enhanced the expression of stress-responsive genes such as *GSTF6* (*GLUTATHIONE S-TRANSFERASE 6*), *COR47* (*COLD-REGULATED 47*) and *RD20* (*RESPONSIVE TO DEHYDRATION 20*). Our data demonstrate that *PgNAC21* functions as a stress-responsive NAC TF and can be utilized in transgenic approaches for developing salinity stress tolerance in crop plants.

We perform the small RNA sequencing to find out the salinity stress responsive small RNAs, especially MicroRNAs (miRNAs). miRNAs are small regulatory RNAs of 20-22 base pairs, play versatile roles in plant growth, development and stress responses. However, the regulatory mechanism is unclear on miRNA-mediated response to salinity stress in pearl millet. In this study, we performed small RNA sequencing to identify miRNAs from salinity tolerant pearl millet genotype ICMB 01222. In total, 126 million sequence reads were generated. In total, 81 miRNAs were identified as a salinity stress responsive miRNA (30 miRNAs as an up-regulated miRNAs and 51 miRNAs as a down-regulated miRNAs). Interestingly, significantly upregulated pearl millet miRNAs belong to miR159 family, which was previously reported to be involved in various abiotic stress responses. We performed the target prediction for these miRNAs using the psRNATarget analysis server. In total, 448 mRNAs genes of pearl millet were identified as target genes. Among these target genes 122 mRNAs were identified as a transcription factor (25 % of total target gene). Pathway analyses showed that differentially expressed miRNAs and their target genes might regulates Auxin responsive pathway. *In silico*

mapping of these 81 differentially expressed miRNAs on chromosomes, indicated uneven distribution on chromosomes. Chromosomal localization studies also indicated that miR394 family and miR408 family on chromosome 2 and miR162 family on chromosome 5 undergoes duplication and may have expanded in pearl millet during the salinity stress. Quantitative real-time PCR analysis of miRNAs and target genes showed consistent expression pattern with RNA-Sequencing. This suggests that miRNAs play an important role in salinity stress tolerance in pearl millet. This result could be used to improve salinity stress tolerance in upland crops.

6.1.2. Future study plan.

Our physiological analysis found that ICMB 01222 has relatively good salinity stress tolerance capacity. And transcriptome analysis has found that tolerant line ICMB 01222 is reservoir of salinity stress tolerance related genes. So in future it is possible to use the salinity tolerant line, ICMB 01222 as a donor of useful genes to further improve the salinity stress tolerance in pearl millet either by cross breeding or transgenic approach. Our studies has identified many salinity stress responsive genes and miRNAs, functional studies of these genes in future will help to understand the in detail role of these genes in salinity stress tolerance. CRISPER-Cas9 mediated genome editing can be successfully used for functional studies of these genes. Currently we are performing the CRISPER-Cas9 mediated related experiments in pearl millet but unfortunately we are not successful. But we strongly believe that, in future, we will be able to created mutant pearl millet lines using the CRISPER-Cas9 method. We have overexpressed salinity stress responsive gene in Arabidopsis and overexpression lines showed enhanced salinity stress tolerance. Along with that, we have also overexpressed PgNAC21 gene in pearl millet. In future we will analyse PgNAC21-overexpressing pearl millet lines of salinity stress responses.

Knowledge generated in our studies, can be successfully used in agriculture to produce salinity stress tolerant plants.

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