Unravelling the distinctive growth mechanism of proso millet (Panicum miliaceum L.) under salt stress: From root-to-leaf adaptations to molecular response

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Abstract

Proso millet (Panicum miliaceum L.) has potential applications as a new source of bioenergy owing to its high-yielding C₄ attribute. Besides, it has a short life cycle, high harvest index, low planting costs, and strong adaptability and resistance. This study comprehensively reveals the sodium ion (Na⁺) toxicity resistance strategies from germination to the seedling stage, root to leaf, outside to inside, macro to micro, and the phenotype to the mechanism. Comparative phenotypic and physiological analysis suggested that salt-tolerant proso millet (ST 47) had better salt tolerance, biomass accumulation, and osmo-protection than salt-sensitive proso millet (SS 212). Microstructural analysis indicated that ST 47 could maintain better internal surfaces and intact structures to resist Na⁺ toxicity and maintain optimal growth. Further, digital RNA sequence analysis indicated that ST 47 could maintain better Na⁺ and K⁺ homeostasis by coordinated regulation of transporter genes; the abundance of transcripts involved in chlorophyll metabolism and photosynthesis pathways was higher in ST 47 than SS 212, which contributed to the biomass accumulation. These results suggest that ST 47 resists Na⁺ toxicity via coordinated physiological, morphological, and molecular mechanisms. Therefore, this crop can serve as an emerging salt-tolerant bioenergy crop for sustainable saline agriculture and simultaneous phytoremediation.

KEYWORDS

biomass accumulation, Na⁺ toxicity, Na⁺/K⁺ homeostasis, photosynthesis, physiological and structural adaptation, proso millet

1 | INTRODUCTION

Long-term climate change and periodic environmental extremes threaten the global crop productivity and yield of energy crops and impede the sustainable development of modern agriculture (Cappelli et al., 2015; Gupta et al., 2020). These challenges are exacerbated by soil salinization caused by natural or human activities, leading to a more bioenergy-scarce world (Litalien & Zeeb, 2020; Sahab et al., 2021). Therefore, the development of salt-tolerant energy crops is becoming increasingly important. High salinity typically results from the excessive accumulation...
of salts (mainly sodium chloride [NaCl]) in soil, leading to osmotic, ionic, and secondary stresses in crops, which impedes water and nutrients absorption from the soil (Yang & Guo, 2018). Unlike animals, crops are sessile organisms and should develop strategies to tolerate high salinity conditions through phenotypic plasticity and evolutionary adaptation (Zhu, 2016). This adjustment involves changes in plant physiology (redistribution of ions, soluble compounds, and antioxidants), organ (leaf surface) or cell structure, and gene expression (Huang et al., 2018). An evaluation of the morphological, physiological, microstructural, and molecular mechanisms by which salt-tolerant plants respond to the saline soils is needed to utilize salinized soil better.

Although research on crop responses to salt stress has produced extensive literature, only few cultivars resistant to saline soils have been developed (Loescher, 2011). A comprehensive understanding of the associated physiological and molecular mechanisms is lacking because of ineffective criteria for identifying and evaluating salt tolerance (Jafar et al., 2012; Lenis et al., 2011). However, crop salt tolerance is a comprehensive and complex reflection of multiple developmental stages and traits whose comprehension needs an integrated approach (Liu et al., 2015). The seed germination and early seedling growth stages of most crop species are more sensitive to salt stress than the later stages, which are the most crucial periods for the crop life cycle under salt stress (Khan et al., 2003). Therefore, it is necessary to strengthen the identification and evaluation of the salt tolerance capacity of crops during the germination and seedling stages.

Crop osmotic adjustment involves accumulating soluble compounds in the cells, including glycine betaine, free amino acids, soluble sugars, soluble proteins, and proline (Hasegawa et al., 2000). Other essential mechanisms for avoiding osmotic stress include the reduction of transpiration water loss (Hepworth et al., 2015) and transpiration flux by controlling stomatal opening (short-term response) and modifications of stomatal density (long-term response; Franks et al., 2015; Hepworth et al., 2015). Plant cells resist sodium ion (Na⁺) toxicity by restricting Na⁺ uptake, sequestering Na⁺ in the vacuoles, and promoting Na⁺ exclusion from the cytoplasm (Albaladejo et al., 2016; Zhao et al., 2020). First, root cells down-regulate Na⁺ uptake in the root epidermis via non-selective cation channels (NSCCs) or high-affinity K⁺ transporters (HKTs; Mian et al., 2011). Secondly, the sodium/hydrogen antiporter (NHX) family of transporters facilitates the vacuolar sequestration of excess cytosolic Na⁺, which effectively avoids the cytoplasmic accumulation of Na⁺ (Li et al., 2020). Finally, the salt-overly-sensitive (SOS) pathway facilitates Na⁺ extrusion from root cells into the xylem via the SOS1 Na⁺/H⁺ exchanger (Wang et al., 2020). The entry of Na⁺ into the cell causes membrane depolarization, and the subsequent change in membrane voltage influences ion transport (K⁺ and Ca²⁺). However, the high K⁺ retention facilitates the cytosolic over-accumulation of Na⁺ without compromising the K⁺/Na⁺ ratio, while depletion of the cytosolic K⁺ pool triggers programmed cell death (Demidchik, 2014). Thus, the ability of plant tissues to retain K⁺ under salt stress is essential for salinity tolerance. Salinity-induced K⁺ loss from plant cells occurs via depolarization-activated outward-rectifying K⁺ channels (GORK) or K⁺-permeable ROS-activated NSCCs (Riedelsberger et al., 2015; Wu et al., 2018). However, most salt stress studies have been done using Arabidopsis, the model plant, and glycophytic crops (van Zelm et al., 2020). Most plants cannot tolerate high salt concentrations in growth media and thus fail to complete their life cycle under high salt conditions (Flowers et al., 2015; Munns & Tester, 2008; van Zelm et al., 2020).

Proso millet ( Panicum miliaceum L.) is an allotetraploid with two sub-genomes. It is among the earliest cereal crops to be domesticated worldwide, dating back to 8000–10,000 years before the present (Barton et al., 2009; Lu et al., 2009; Miller et al., 2016; Xu et al., 2019). The crop originated from Northern China and is now widely grown in the semiarid regions of Asia, Europe, and other continents (Lu et al., 2009). Owing to various attributes of proso millet, it is considered a potential feedstock for ethanol production. For instance, it has efficient C₄ photosynthesis, it is a close relative of the bioenergy crop switchgrass (Panicum virgatum L.), and its ethanol yield is similar to highly fermentable corn (Zea mays L.) after 72 h of fermentation (Lu et al., 2013; Rose & Santra, 2013; Zou et al., 2019). Further, the proso millet exhibits exceptional resistance to abiotic stress due to its higher salt and temperature tolerance relative to most cereal crops (Zou et al., 2019). Proso millet is, therefore, the crop of choice for sustainable saline agriculture. Since the release of the proso millet genome (Shi et al., 2019; Zou et al., 2019), this species has evidenced an increase in research interest. In the present study, the germination indices of 100 proso millet cultivars from 21 sources were evaluated under salt conditions to develop and optimize a system for evaluating and identifying the salt-tolerant proso millet cultivars. Based on the germination stage identification results, two proso millet cultivars with contrasting salt tolerance attributes, SS 212 (salt-sensitive) and ST 47 (salt-tolerant), were selected for subsequent experimentation. The cultivars were subjected to salt stress and subsequent re-watering during seed germination and seedling growth stages. The shoot length, root length, survival percentages, salinity tolerance, and biomass of both cultivars were measured at the seed germination and seedling growth stages to determine salt stress responses. In addition, the
physiological, microstructural, digital RNA-sequencing, and gene expression patterns of both cultivars were also determined to identify the crucial traits and genes determining salinity adaptation in ST 47. This study aimed to identify the primary traits and genes involved in Na\(^+\) toxicity resistance to enhance understanding of the molecular mechanisms of Na\(^+\) toxicity and the application of these genes in breeding programs.

2 | MATERIALS AND METHODS

2.1 | Plant materials

A total of 100 accessions were selected from the Multigrain Center for Northwest A&F University, Shaanxi, China, including 88 landraces and 12 breeding lines (Table S1). The accessions were initially acquired from different countries/organizations, including India (1), Russia (1), Poland (2), USA (1), and China (95, Heilongjiang, Xinjiang, Xizang, Shaanxi, Shanxi, Shandong, Qinghai, Ningxia, Inner Mongolia, Liaoning, Jiangsu, Jilin, Hebei, Hainan, Gansu, Beijing, and Anhui).

2.2 | Evaluation and identification of salt tolerance in 100 accessions at germination

Thirty seeds of each accession were sterilized with 0.1% mercuric chloride (HgCl\(_2\)) for 5 min and rinsed with sterile water five times. Then, sterile seeds were placed on a double-layered filter paper in Petri dishes containing either 8-ml sterile water (control) or 8 ml of NaCl solution (170 mM; −1.0%). Each treatment was done in three replications in a greenhouse under controlled conditions (30°C day/18°C night temperature, 14 h light/10 h dark cycle, and 60% relative humidity). Seeds were considered to have germinated when the plumule length was equal to half of the seed length, and the radicle length was equal to the seed length. The number of germinated seeds in each replicate was evaluated daily from the fourth day. Germination energy (GE) is used to evaluate germination speed capacity (Domin et al., 2019). It was determined using the formula GE (%) = \(M_1/M \times 100\), where \(M_1\) is the number of germinating seeds on the fourth day, while \(M\) is the total number of seeds tested (Domin et al., 2019; Huang et al., 2017). The germination percentage (GP) was determined using the number of germinated seeds at 10 days post-seed treatment (Sharma et al., 2013). The sprout length, root length (RL), sprout fresh weight (SFW), and root fresh weight (RFW) were also determined on the 10th day. The germination index (GI) was determined using the formula \(GI = \sum(Gt/Dt)\), where \(Gt\) represents the number of germinated seeds on the day \(t\) of salt stress and \(Dt\) is the corresponding day number (Karaguzel et al., 2004). Vigor index (VI) was determined using the formula \(VI = GI \times RFW\), where GI represents the germination index, and RFW is the root fresh weight. Salt damage index (SDI) was determined following the formula \(SDI(\%) = (GP_{CK} - GP_T)/GP_{CK} \times 100\), where \(GP_{CK}\) represents germination percentage under the control conditions, while \(GP_T\) is the germination percentage under salt stress (Liu et al., 2015). The relative GE, GP, GI, SL, RL, SFW, RFW, VI, and the relative salt damage rate (RSDR) were determined as the ratios of the respective parameter values under saline conditions to those under control conditions.

2.3 | Salt tolerance of the selected cultivars at germination under salt stress and after re-watering

Based on the preliminary evaluation at germination, two proso millet cultivars with contrasting salt tolerance attributes, SS 212 (salt-sensitive) and ST 47 (salt-tolerant), were selected for further study. The seeds were first cultured for 10 days on a double-layered filter paper in Petri dishes containing 8-ml sterile water (control) or 8 ml of NaCl solution (170 mM; −1.0%). Each treatment was done in three replications in a greenhouse under controlled conditions (30°C day/18°C night temperature, 14 h light/10 h dark cycle, and 60% relative humidity). Seven days after germination, the seeds in the treatment regime were subjected to post-NaCl recovery by washing three times with sterile water and allowed to germinate for an additional 7 days in sterile water. The RL, shoot length (SL), seedling survival percentages, and salinity tolerance were determined after 7 days of salt stress and subsequent re-watering for 7 days. Seedlings were considered to have survived when demonstrating vibrant growth, or the heart leaves remained green. The roots and shoots of 15 seedlings for the treatment and control (in three biological replicates) were separated and dried in a forced-air oven at 80°C for 3 days to determine the biomass. Salinity tolerance was determined based on increases in dry shoot biomass after growth in 170-mM salt-nutrient solution for 7 days, relative to growth in the control.

2.4 | Salt tolerance of selected cultivars at seedling stage

The seeds of cultivars SS 212 and ST 47 were surface disinfected (0.1% HgCl\(_2\), 5 min), rinsed (sterile water, three
times), sprouted (3–4 days), and cultured in a hydroponics system until the three-leaf stage (about 10 days). The hydroponic growth was done in a seedling pot (12.4 cm x 17.5 cm) under 14h light/10h dark cycles, day/night temperatures of 28/18°C, and relative humidity of 60%. Seedlings were stressed by ½ Hoagland’s nutrient solution containing 0 mM (control) and 170 mM NaCl for 7 days and then transplanted into ½ Hoagland’s nutrient solution for another 7 days. The RL and SL were measured under the 7-day salt stress and after the 7 days of subsequent re-watering. Survival percentages, biomass, and salinity tolerance were evaluated, as described in Section 2.3.

2.4.1 | Ion flux measurement

The net fluxes of Na$^+$ and K$^+$ were measured noninvasively using ion-selective vibrating microelectrodes (the MIFE technique) as described by Cuin et al. (2010) and Wu et al. (2015). The third fully expanded leaves were collected and cut off laterally to expose the mesophyll cells, and then immersed in a 5-ml Perspex glass measuring chamber containing 5-ml measuring solution (0.1 mM KCl, 0.5 mM NaCl, and pH 5.8) and preincubated for 4 h. The measuring solution was then discarded and 5-ml fresh measuring solution was added for sample testing. The Na$^+$ and K$^+$ flux microsensor was placed 10 μm away from the root and mesophyll cell surface detection site and 5 min ion flux data for each sample were collected. Net ion fluxes were determined from the electrochemical potential differences of the ions between the two positions (Newman, 2001).

2.4.2 | Evaluation of root vigor and relative electrolyte leakage

Root viability (RV) was measured by the 2,3,5-triphenyl tetrazolium chloride method, as described previously (Del Egido et al., 2017; Lin et al., 2019). Meanwhile, the relative electrolyte leakage (EL) was evaluated as described by Zhang et al. (2013).

2.4.3 | Evaluation of soluble substances

Roots (0.1 g) and leaves (0.1 g) for evaluating soluble substances were harvested after the 7-day salt stress and 7-day subsequent re-watering. The glycine betaine and free amino acid contents were measured using the Solarbio Assay Kit (Beijing Solarbio Science & Technology).

2.4.4 | Microstructural analysis of the root and leaf surfaces

The tips of fresh roots and leaves were cut, prepared as described previously (Pan et al., 2012), then dried in a critical-point drying machine (Emitech K850; Quorum) to observe the root and leaf surface morphology. After metal spraying, the tissues were observed under an S-4800 field-emission scanning electron microscope (Hitachi, Ltd.). For further evaluation of the toxicity of Na$^+$ on proso millet, root tips were prepared for transmission electron microscopy (TEM) using the method described by Meng et al. (2014). Ultrathin sections were stained with uranyl acetate and lead citrate then examined under a TEM (HT7700; Hitachi, Ltd.).

2.4.5 | Determination of the chlorophyll content

Fresh leaves were punched into 1-cm$^2$ discs and soaked in 10-ml acetone with 20% (v/v) water. The chloropropyl was then extracted in the dark at room temperature until the leaves are completely white. Chlorophyll $a$ (Chl $a$) and Chlorophyll $b$ (Chl $b$) was then quantitatively measured at wavelengths of 663.2, 646.8, and 470 nm (UV-1800; Shimadzu). Measurements were done using 80% acetone as a blank. The concentrations of Chl $a$ and Chl $b$ were determined using equations described by Lichtenthaler and Buschmann (2001):

$$\text{Chl}_a(\mu g/ml) = 12.25A_{663.2} - 2.79A_{646.8},$$

$$\text{Chl}_b(\mu g/ml) = 21.50A_{646.8} - 5.10A_{663.2}.$$  

2.4.6 | Chlorophyll fluorescence and photosynthetic characteristics

Chlorophyll fluorescence was determined in vivo using a MINI-PAM-II system (Heinz Walz GmbH). Whole plants were placed in the dark for 30 min before in vivo chlorophyll fluorescence determination. All measurements were taken at the same time during the day to minimize the effect of circadian rhythms on photosynthesis efficiency. The $F_v/F_m$ ratio, effective photochemical efficiency of photosystem II (PSII), actual light quantum yield (YII)), quantum yields of non-photochemical quenching, constitutive heat dissipation (YNO), electron transport rate (ETR), photochemical quenching (qp), and non-photochemical quenching (qN) properties of the chlorophyll were also analyzed. The photosynthetic characteristics of fully expanded third leaves (the third knob) were measured using a portable photosynthesis system (Li-6400; Li-Cor Biosciences) equipped with a leaf
chamber fluorometer (Li-Cor part no. 6400-40, area² cm⁻²). The flow rate through the chamber was 500 μmol s⁻¹, and the leaf temperature was 30°C during measurements. The ambient CO₂ concentration was approximately 380 μmol CO₂ mol⁻¹ air while the vapor pressure deficit was approximately 2.0 kPa. Gas exchange parameters were also measured in leaves of control and salt-treated plants, including the photosynthetic rate (Pn, μmol CO₂ m⁻² s⁻¹), conductance to H₂O (Gs, mol H₂O m⁻² s⁻¹), transpiration rate (Tr, mmol H₂O m⁻² s⁻¹), and intercellular CO₂ concentration (Ci, μmol CO₂ mol⁻¹).

2.4.7 | RNA extraction, cDNA library construction, and digital RNA sequencing

Total RNA was extracted from the root and leaf tissues of three replicate samples for each treatment time point using an RNA Plant Plus Reagent (Tiangen) following the manufacturer's instructions. RNA purity was checked by a NanoDrop 2000 Spectrophotometer (Thermo Scientific), and RNA was resolved on a 1% agarose gel to check for contamination and degradation. The total RNA integrity was assessed on an Agilent Bioanalyzer 2100 system (Agilent Technologies).

The mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads and then interrupted into short fragments by a fragmentation buffer. Complementary DNA (cDNA) was synthesized using random hexamer primer, M-MuLV reverse transcriptase (RNase H), and DNA Polymerase I. The remaining overhangs were then converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3’ ends, NEBNext Adaptors with a hairpin loop structure were ligated to the cDNA to prepare for hybridization and barcoded with a large set of barcode sequences (Smith et al., 2017). Then, cDNA fragments (200–250 bp in length) were purified by an AMPure XP system (Beckman Coulter), and PCR was performed using a Phusion High-Fidelity DNA polymerase, universal PCR primers, and Index (X) Primer. Finally, PCR products were purified by the AMPure XP system, and library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies).

All the 18 libraries were sequenced on an Illumina Hiseq™ 4000 platform (Illumina) using the PE-150 module, and paired-end 150 bp reads were generated. The adaptor sequences and low-quality reads were filtered by the Trimmomatic tool (v0.33) to clean raw reads (Bolger et al., 2014). The clean reads obtained after data processing were mapped to the P. miliaceum L. genome sequence using TopHat (v2.1.0). Gene expression levels were estimated by fragments per kilobase of transcript per million fragments mapped values.

2.4.8 | Analysis of differentially expressed genes

Differentially expressed genes (DEGs) after 7 days of salt treatment and RW 7 d were identified using the DESeq R package (v1.10.1) by comparing the expression levels at these time points with those at 0 days. Genes with a minimal twofold change (|log2 (FC)| ≥ 1) and a false discovery rate of p ≤ 0.05 were identified as significantly differentially expressed between the two time points (Storey & Tibshirani, 2003).

2.4.10 | Gene expression analyses

The GOseq R package (v1.12) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology Based Annotation System software (v2.0) were used for the determination of DEGs in the Gene Ontology (GO) and KEGG pathways, respectively. A corrected P-value <0.05 was chosen as the significance cutoff (Mao et al., 2005; Young et al., 2010).

2.4.11 | Data statistics and analysis

All results were reported as means ± SE, and experiments were performed in three biological replicates. The data were analyzed by SPSS software version 23.0 (IBM SPSS Inc., Chicago, IL, USA). Duncan’s multiple comparison
test was used at the $p < 0.05$ level to evaluate significant differences among treatment means. Pearson correlation coefficients and significance levels among traits were computed in SPSS v23.0 (IBM SPSS Inc.). Meanwhile, correlations were generated using the R project for statistical analysis and computing. The principal component analysis (PCA) was performed using smartpca in the EIGENSOFT package (v 3.0), following the method described by Tang et al. (2013) to rank the salt tolerance levels of different genotypes. The PCA ranking value for each genotype was calculated using the formula: $\text{PCA rank value} = \sum_{j=1}^{n} (PC_j \times \text{contribution of PC}_j\%)$, $j = 1, 2, 3$, etc. “PC$_j$” represents the value of principal component $j$ and “contribution of PC$_j$ (%)” represents the variance in response to stress treatment that principal component j could explain. The cluster analysis was also conducted using SPSS v23.0.

3 | RESULTS

3.1 | Evaluation of salt tolerance in 100 proso millet accessions at the germination stage

The relative values of all measurement indices in the 100 proso millet cultivars were generally less than 1 when subjected to salt stress (170 mM NaCl solution) in the mass fraction. The relative germination energy (RGE), relative germination percentage (RGP), relative germination index (RGI), relative sprout length (RSL), relative root length (RRL), relative sprout fresh weight (RSFW), relative root fresh weight (RRFW), relative vigor index (RVI), and relative salt damage rate (RSDR) ranged from 7.4% to 98.7%, 40.7% to 102.3%, 26.8% to 109.9%, 8.6% to 95.6%, 2.4% to 77.1%, 14.5% to 99.6%, 0% to 63.6%, 0% to 58.3%, and 9.9% to 73.2%, respectively (Table S1). These results suggest that the salt tolerance of the different proso millet cultivars is different. Correlation analysis showed a significant correlation between the RGE, RGP, RGI, RSL, RSFW, RRFW, and RVI ($p < 0.05$), which were significantly negatively correlated with RSDR ($p < 0.01$), except for RRL (Figure 1a).

Principal component analysis and cluster analysis divided the 100 cultivars into four groups (Figure 1b). The first group consisted of two strongly sensitive accessions (SS 212 and Linheshuanglishu), while the second group had 17 sensitive accessions. The third group had 71 tolerant accessions, while the fourth group consisted of 10 strongly tolerant accessions (including ST 47, Wujushu, and Baishu).

3.2 | Growth response of proso millet cultivars to salt stress and re-watering at germination and seedling stages

Compared with control conditions, salt stress severely inhibited the growth of the two proso millet cultivars at seed germination and seedling stages (Figure 2). However, at germination, the decrease in RL and SL was higher in ST 47 than SS 212 (Figure 2b,c,g,h). The survival percentages and salinity tolerance of ST 47 (91.3% and 60.7%, respectively) were significantly higher than that in SS 212 (14.0% and 21.5%, respectively; Figure 2d,e,i,j). Moreover, the RL,
SL, and survival percentages of both proso millet cultivars were similar under control conditions, and no significant difference was detected at the seedling stage (Figure 2g–i). The RL, SL, and survival percentage of SS 212 were significantly lower than that under normal conditions ($p < 0.001, p < 0.05, p < 0.001$, respectively). However, the RL and survival percentage of ST 47 was almost unaffected by salt treatment (Figure 2g,i), and SL was only reduced by 1.38-fold but was still significantly higher than that in SS 212 ($p < 0.001$). Following RW 7 days, the RL of SS 212 was not restored, and the SL was even lower, while the SL of ST 47 showed a dramatic increase (Figure 2g,h). Thus, the salinity tolerance of ST 47 was higher than that of SS 212 (64.7%, 85.8%, respectively) at the germination and early seedling stages (Figure 2e,j).

3.3 Cultivar differences in biomass accumulation at germination and seedling stages

The relative root biomass, relative shoot biomass, and relative total biomass were significantly higher in ST 47 than SS 212 under salt stress during germination (Figure 3a–d). After re-watering, the root, aboveground, and total biomass of both cultivars increased significantly relative to control conditions. Besides, the root-shoot biomass ratio was substantially higher in ST 47 than SS 212 under salt stress, but no significant difference was observed between the cultivars after re-watering (Figure 3d). In particular, the root-shoot ratio of ST 47 increased significantly after re-watering (Figure 3d).

At the seedling stage, the root biomass, shoot biomass, and total biomass of ST 47 were significantly higher than those in SS 212 (Figure 3e–h). Re-watering significantly increased the root, aboveground, and total biomass of both cultivars relative to the control conditions. Also, the root-shoot ratio of ST 47 was considerably higher than that of SS 212 before re-watering, but no significant difference was observed between the two treatment groups after re-watering (Figure 3h). Therefore, salt stress induced the formation of small root structures in proso millet while re-watering increased the root size. These results indicate that ST 47 exhibits greater salt tolerance and recovery than SS 212 at germination and seedling stages.

3.4 Cultivar differences in root and leaf ion flux responses to salt stress and re-watering

In the control solution, Na$^+$ and K$^+$ from the root and leaf mesophyll epidermis showed influx (Figure 4). However, the net Na$^+$ efflux of roots and leaves in both cultivars was high under salt stress. It reached 6461.20 and 652.06 pmol m$^{-2}$ s$^{-1}$ in root and leaves, respectively, in SS 212 within 300 s. In ST 47, the net Na$^+$ efflux levels in root and leaves increased to 1561.83 and 442.54 pmol m$^{-2}$ s$^{-1}$, respectively, which was approximately 4.14-fold and 1.47-fold lower than that in SS 212 (Figure 4a,c,e,g). In contrast, the net K$^+$ flux of roots and leaves (−45.11 and 34.69 pmol m$^{-2}$ s$^{-1}$, respectively) in ST 47 was higher than that of SS 212 (−73.01 and −145.98 pmol m$^{-2}$ s$^{-1}$, respectively; Figure 4b,d,f,h). Upon re-watering, the net Na$^+$ flux of both cultivars showed a significant decline but did not return to normal levels. The Na$^+$ flux declined to 77.85 and 146.78 pmol m$^{-2}$ s$^{-1}$ in SS 212 and ST 47 roots, respectively. The net Na$^+$ efflux of SS 212 and ST 47 leaves declined to significantly different levels (393.59 and 210.61 pmol m$^{-2}$ s$^{-1}$, respectively). Moreover, the net K$^+$ efflux of ST 47 roots and leaves was higher than that of SS 212 under salt stress (Figure 4a,e,g). Compared with SS 212, ST 47 showed lower Na$^+$ influx and K$^+$ efflux. As such, ST 47 could maintain a higher K$^+$/Na$^+$ ratio, which was ideal for tolerating salt stress.

3.5 The synthesis of soluble substances synthesis is activated to varying degrees between SS 212 and ST 47 under salt stress and re-watering

In both cultivars, glycine betaine and free amino acid contents under salt stress were higher than under the control conditions but quickly recovered upon re-watering (Figure 5). Salt treatment increased the glycine betaine content of SS 212 and ST 47 roots and leaves by 2.78- and 3.77-folds, and 3.55- and 4.73-folds, respectively (Figure 5a,b). However, SS 212 roots showed slower recovery upon re-watering, and the glycine betaine content decreased by 1.34- and 2.94-fold in SS 212 and ST 47 roots, respectively (Figure 5a,b). The free amino acid contents of ST 47 roots and leaves (1.72- and 1.77-folds increase, respectively) also increased faster than SS 212 in response to salt stress (1.51- and 1.43-folds increase, respectively). However, the free amino acid contents of ST 47 roots and leaves recovered to the basal level upon re-watering, while the free amino acid contents of SS 212 were significantly higher than the control (Figure 5c,d).

3.6 Differences between SS 212 and ST 47 in root and leaf damages in response to salt stress and re-watering

To examine the deterioration of proso millet under salt stress, root viability and EL were characterized (Figure S1). The RV of both SS 212 and ST 47 under salt stress were...
significantly lower than under control conditions, but the RV of ST 47 (3.1) was substantially higher than that of SS 212 (2.8). With re-watering, the RV of both proso millet was restored but not to normal levels. Upon re-watering, the RV of ST 47 (3.6) exhibited faster recovery than that of SS 212 (3.1). Moreover, the leaf EL of salt-stressed SS 212 (44.2%) was significantly higher than that of ST 47 (26.7%). Of note, re-watering decreased the EL of both cultivars. However, the EL of ST 47 was still significantly lower than that of SS 212.

The root morphology under untreated conditions (CK) was smooth, and the root tip had a full and integrated cellular structure, with many intact parenchyma cells (Figure 6a,d,g,j). However, the surface of SS 212 roots became coarse after 7-day salt stress, whereas that of ST 47 remained smooth (Figure 6b,e). Moreover, the root epidermal cells of both cultivars were severely damaged; the parenchyma cells of SS 212 showed severe shrivel and had started to slough off, whereas ST 47 cells were only slightly shriveled and still intact (Figure 6b,e,h,k). Re-watering restored the morphology of SS 212 parenchyma cells but still shriveled, whereas ST 47 cells were normal (Figure 6c,f,i,l).

The SEM of leaf surfaces showed that salt stress caused the stomata of SS 212 to close and decompose the leaves (Figure 6n) and did not recover after re-watering (Figure 6o). Meanwhile, the entire stomata in ST 47 remained full under salt treatment (Figure 6w). Compared with seedlings under control conditions, the stomatal density of ST 47 was significantly higher under salt stress, whereas that of SS 212 was significantly lower (Figure S1c).

### 3.7 Cultivar differences in root and leaf cell damages in response to salt stress and re-watering

Transmission electron microscopy revealed an intact root cell structure in untreated conditions, with mitochondria well distributed around the cell wall in an ellipsoidal shape with clear cristae. The endoplasmic reticulum was evenly distributed on the plasma membrane close to the cell wall (Figure...
FIGURE 3  Biomass and root-shoot ratio. (a–d) Biomass and root-shoot ratio at germination stage; (a) Root biomass; (b) Shoot biomass; (c) Total biomass; (d) Root-shoot ratio. (e–h) Biomass and root-shoot ratio at seedling stage; (e) Root biomass; (f) Shoot biomass; (g) Total biomass; (h) Root-shoot ratio. Different letters (a, b, c, d, and e) indicate significant differences ($p < 0.05$) between the control and 170 mM NaCl.
FIGURE 4 Net Na\(^+\) and K\(^+\) fluxes measured from the epidermal root and leaf cells in response to salt stress and re-watering. (a) Transient Na\(^+\) fluxes measured from the root of both cultivars. (b) Transient K\(^+\) fluxes measured from the root of both cultivars. (c) Transient Na\(^+\) fluxes measured from the leaf of both cultivars. (d) Transient K\(^+\) fluxes measured from the leaf of both cultivars. (e, f) Mean Na\(^+\), K\(^+\) fluxes in the root of both cultivars. (g, h) The mean rate of Na\(^+\), K\(^+\) fluxes in the leaf of both cultivars. The sign convention for all MIFE measurements is “influx positive.” Different letters (a, b, c, d, and e) indicate significant differences ($p < 0.05$) between the control and 170 mM NaCl.
7a,d). However, salt treatment thinned the root cell wall due to decomposition, caused severe plasmolysis, blurred the mitochondrial structure, and disordered the cristae of SS 212. Meanwhile, the cell wall was thickened in ST 47 root cells. Subsequent re-watering did not restore the internal root structure in SS 212, but ST 47 returned to its normal state (Figure 7b,c,e,f). In untreated conditions, the chloroplast of SS 212 and ST 47 exhibited the typical ellipsoidal shape with well-arranged and smooth thylakoid membranes in distinct grana. The numerous plastoglobuli showed clear cristae (Figure 7g,j). However, salt stress significantly changed the internal chloroplast structure in SS 212; damaged membrane systems, deformed and vacuolized the chloroplasts, dissolved most stroma lamellae, and dilated, loosened, distorted, and even disintegrated the thylakoids (Figure 7h). Meanwhile, chloroplast autolysia (self-digestion) was not found in the leaves of salt-stressed ST 47; the edges of most chloroplasts were clear and complete, and the thylakoid membranes and internal lamellar systems were also well preserved (Figure 7k). With subsequent re-watering, the external shape and internal structure of SS 212 chloroplasts did not recover. However, ST 47 returned to its normal state (Figure 7i,l).

### 3.8 Differences in photosynthetic parameters between SS 212 and ST 47 in response to salt stress and re-watering

This study analyzed the Chl contents to further understand the phenotype of the two proso millet cultivars. Compared with seedlings grown under control conditions, the Chl a and Chl b contents of both cultivars exhibited a decrease. However, the decrease in the contents of Chl a and Chl b was greater in SS 212 (3.80- and 17.95-fold, respectively) than in ST 47 (1.37- and 1.86-fold, respectively; Figure 8a,b). The Chl a/b ratio of SS 212 increased by 3.46-folds that of ST 47 under salt stress (Figure 8c). Re-watering restored the Chl a and Chl b contents of the two proso millet cultivars, though not to normal levels. Notably, more Chl content recovery was observed in ST 47 (1.47 and 1.29 mg/ml), relative to SS 212 (1.35 and 0.81 mg/ml). Similar to the change in chlorophyll (Chl a and Chl b) content, Fv/Fm, NPQ, and Y(II) significantly decreased in both proso millet cultivars under salt stress, while ST 47 levels remained higher than that of SS 212 (Figure 8d,f; Figure S2a). However, the opposite response was observed in the third phase NO and qP (Figure 8g; Figure S2b). Moreover, photosynthetic parameters also significantly decreased in the two varieties, including Pn, Gs, Tr, and Ci, while ST 47 still maintained higher photosynthesis than SS 212 (Figure 8i–l). With re-watering, the photosynthetic parameters of ST 47 were restored to normal levels, while SS 212 was also significantly lower than the control (Figure 8i–l).

### 3.9 Analysis of DEGs under salt stress

This study conducted RNA-seq analysis of roots and leaves of SS 212 and ST 47 grown in salt stress, re-watering, and
control conditions to determine how salt stress affects Na⁺ balance and photosynthesis. Under salt stress, 3031 and 5559 DEGs were detected in SS 212 root and leaf tissues, respectively, whereas ST 47 had 2355 and 4686 DEGs, respectively (Figure S3). However, 1745 and 5375 DEGs were detected in SS 212 root and leaf tissues with re-watering, respectively, while 1498 and 1940 genes were differentially expressed in ST 47 (Figure S3). GO and KEGG analysis suggested that the DEGs were mainly involved in chlorophyll metabolism, ion transport, and photosynthesis (Figure S4).

3.10 | The transcription program differences for chlorophyll metabolism, K⁺/Na⁺ homeostasis, and photosynthesis in SS 212 and ST 47 under salt stress and re-watering

The gene expression levels under salt stress, re-watering, and control conditions were analyzed using digital RNA-Seq to determine the molecular mechanisms of differential Chl accumulation in the two cultivars. There were 15
DEGs mainly involved in Chl biosynthesis (Figure 8m). Three genes in the Chl biosynthesis pathway were upregulated in both cultivars compared with the controls, including 1 oxygen-dependent coproporphyrinogen III oxidase (HemF), 1 uroporphyrinogen-III synthase (HemD), and 1 protoporphyrinogen oxidase (HemY) upregulated more than 1.21-fold, 1.57-fold, and 4.05-fold in ST 47 compared with SS 212. However, the expression levels of genes encoding for the essential enzymes involved in the Chl synthesis pathway, including magnesium-chelatase subunit

**FIGURE 7** The root and leaf subcellular structure under salt stress and re-watering conditions. SS 212 root (a–c); ST 47 root (d–f). SS 212 leaf (g–i); ST 47 leaf (j–l). Control, untreated root; salt stress 7 days, 170 mM NaCl treatment for 7 days. Re-watering, re-watering treatment for 7 days. Ch, chloroplast; CW, cell wall; ER, endoplasmic reticulum; Gr, granum; La, lamellae; Mt, mitochondria; Nu, cell nucleus; PM, plasmalemma; Va, vacuole. (a–l) ×4000 magnification, scale bars = 2 μm

**FIGURE 8** Effects of salt stress and re-watering on plant chlorophyll content, photosynthetic trait, and chlorophyll metabolism. Chlorophyll content (a–c). Chlorophyll fluorescence characteristics (d–h). Photosynthetic characteristics (i–l). Chlorophyll metabolism (m). Different letters (a, b, c, d, e, and f) indicate significant differences ($p < 0.05$) between the control and 170 mM NaCl.
(CHLD) and magnesium protoporphyrin IX methyltransferase (CHLM), was repressed in both cultivars. This phenomenon catalyzed the first step in the Chl biosynthesis pathway. However, these genes’ expression level was higher in ST 47 than in SS 212 under salt stress. Notably, the two primary genes encoding for protochlorophyllide oxidoreductase and catalyzing the photoreduction of protochlorophyllide to chlorophyllide were up-regulated in SS 212 under salt stress, but not in ST 47. Similarly, genes encoding for chlorophyllide an oxygenase and chlorophyll b reductase were highly up-regulated in SS 212 than in ST 47 under salt stress. Chl synthase was downregulated in SS 212 but lacked significant changes in ST 47 under salt stress. Chlorophyllase-2, which catalyzes the photoreduction of protochlorophyllide to chlorophyllide (Chl a and Chl b into chlorophyllide a and chlorophyllide b), was up-regulated in SS 212 under salt stress, but not in ST 47. These findings suggested that ST 47 had a greater Chl a and Chl b synthesis capacity than SS 212 under salt stress.

Among the DEGs, the gene encoding for HKT_{1,2} high-affinity K\(^+\) transporters, was highly expressed in the roots of both cultivars but repressed in the leaves (Figure 9). In contrast, the expression of genes encoding HKT_{2} transporter and CNGCs were up-regulated in the leaves of both proso millet cultivars and repressed in roots (Figure 9). Notably, the expression of the CNGCs encoding gene was up-regulated in SS 212 leaves than in ST 47 under salt stress. Although the HKT_{2} transporter was repressed in both proso millet cultivars, its expression in SS 212 was 10.01-fold higher than ST 47. These results support that ST 47 had a higher ability to resist the uptake of Na\(^+\). Additionally, the genes encoding NHX transporters were repressed after salt stress in SS 212 but up-regulated in ST 47. These results suggest that ST 47 had a higher capacity for vacuolar transfer of Na\(^+\). The expressions of two genes encoding SOS_{1} transporters were up-regulated in ST 47 leaves than in SS 212 under salt stress and repressed in roots. However, the expressions of the two genes in ST 47 were still 10.01-fold higher than in SS 212. These results suggest that ST 47 had a higher ability to exclude Na\(^+\) from the cells. The genes regulating the GORK channel were significantly downregulated in ST 47 leaves than in SS 212 under salt stress, while the genes encoding TPK_{1} and AKT_{1} transporters were up-regulated. These results support that ST 47 had a higher ability to maintain K\(^+\) homeostasis via vacuolar K\(^+\) buffering, activating high-affinity K\(^+\)-uptake systems, and limiting the K\(^+\) lost from the cytosol.

Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis indicated that proso millet’s response to salt stress and subsequent re-watering potentially involves a rearrangement of the photosynthetic apparatus. Interestingly, substantial repression of genes encoding proteins related to light-harvesting complex and subunits, including oxygen-evolving enhancer proteins 2 and 3, and a 22 kDa protein of PSII was observed in both proso millet cultivars under salt stress. Meanwhile, the expression of these genes in ST 47 was higher than in SS 212. Similarly, various genes that encode proteins related to the photosystem and electron transport system, PC, Fd, and FNR, were substantially repressed. The expression levels of these genes in ST 47 were also higher than in SS 212. The genes encoding cytochrome c6 were activated to higher levels in ST 47. Substantial repression was observed in genes encoding proteins related to F-type ATPase (gamma, delta, b) in both proso millet cultivars. In contrast, the expression of these genes in ST 47 was higher than in SS 212 (Figure 9). These data suggest that ST 47 exhibits a more significant reduction in light-harvesting capacity under salt stress than SS 212. Moreover, the number of genes, enzymes, and metabolite transporters involved in C\(_{4}\) carbon fixation in the photosynthetic tissues were analyzed. The expressions of most genes encoding key enzymes, including phosphoenolpyruvate carboxylase (PEPC), aspartate aminotransferase, and alanine aminotransferase. For example, the transcript levels of two candidate PEPC (longmi042938, longmi028031, and longmi053924) were over 1.75-fold, 1.88-fold, and 43-fold in ST 47 than in SS 212 under salt stress (Figure 4e). Proteins specific for the NADP-dependent malic enzyme (NADP-ME) subtype, such as NADP-dependent malate dehydrogenase (NADP-MDH), were highly repressed in SS 212 than in ST 47 under salt stress. Notably, the expression of most genes encoding the key enzymes in C fixation in SS 212 was still repressed after re-watering. These findings suggested that ST 47 maintained higher photosynthetic rates than SS 212 under salt stress.

3.10.1 | Expression patterns of key genes involved in Na\(^+\) homeostasis in SS 212 and ST 47 under salt stress and re-watering

The basal levels (CK) of HKT_{2} were similar in SS 212 and ST 47 at germination and seedling stages (Figure 10). No significant changes were detected in the root expression of HKT_{2} for both proso millet cultivars. Changes in NHX expression were similar in the roots of both proso millet cultivars at germination, while that of ST 47 was higher than SS 212. Contrary to the expression changes at germination, significantly higher expression was found in ST 47 at the seedling stage. Under salt stress, the changes observed in SOS_{1} expression were similar to those observed in NHX in the root of both cultivars at the germination and seedling stages (Figure 10). Meanwhile, the basal levels of HKT_{2}, NHX, and SOS_{1} were similar in SS 212 and ST 47 leaves at both germination and seedling stages. The expression of HKT_{2} was down-regulated in both cultivars, while that of ST
was significantly higher than in SS 212. The leaf expression levels of NHX and SOS1 differed substantially between the cultivars under salt stress; the expressions were strongly increased in ST 47 but down-regulated in SS 212 (Figure 10).

4 | DISCUSSION

4.1 | Cultivar differences in growth under salt stress

The ability of seeds to germinate at high salt concentrations is crucial for crop survival (Kan et al., 2015). Most studies involved in identifying salt-tolerant resources during seed germination focus on glycophytic crops and Arabidopsis (Foti et al., 2019; Huaran et al., 2018; Oyiga et al., 2016). Although older domesticated crops such as millets possess unique resilience features to adverse environments (Nadeem et al., 2020), salt tolerance capability has been rarely studied (Ardie et al., 2015; Liu et al., 2015). Similar to these findings, previous studies have also reported that the ability of plants to tolerate high salinity varies within species (Liu et al., 2015; Munns & Tester, 2008). The 100 proso millet cultivars examined in this study were divided into four groups (Figure 1b). The first group consisted of 10 entries, accounting for 10% of all the tested entries, that were strongly salt-tolerant. These entries provide valuable salt-tolerant cultivar resources for rehabilitating saline lands. Plant salt tolerance is a comprehensive reflection of multiple developmental stages and multiple salt tolerance traits (Jafar et al., 2012; Lenis et al., 2011). The seed germination
and early seedling growth stages are more sensitive to salt stress than the later growth stages of most crop species, which are the two most critical stages of the crop life cycle (Almansouri et al., 2001). Herein, the proso millet cultivars exhibited retarded growth under salt stress at seed germination and early seedling stages. Under salt stress, the decrease in RL and SL in both cultivars was more significant at the seed germination stage than during early seedling growth. Similarly, the survival percentages and salinity tolerance of both cultivars at seed germination were significantly lower than those at early seedling growth. These results suggest the seed germination stage was more sensitive to salt stress than the seedling stage, which is a significant stage limiting plant establishment (Zapata et al., 2004).

**4.2 The salt-tolerant cultivar ST 47 exhibits stronger resistance to Na\(^+\) toxicity**

Salinized soil is of poor quality and hinders plant growth by inducing osmotic stress and ion toxicity. Besides, most plants cannot tolerate high salt concentrations in growth media (Litalien & Zeeb, 2020). Therefore, breeding plant varieties that are tolerant to salt can help in the reclamation of salt impacted sites. Herein, SS 212 (salt-sensitive) and ST 47 (salt-tolerant) cultivars were selected for further evaluations based on the response to salt stress (170 mM NaCl) during the germination stage. Consistent with the survival percentages and salinity tolerance results, the adverse effects of salt stress on the RL, SL, and biomass at seed germination and seedling stages were less in ST 47 than those in SS 212 (Figure 2). These observations indicate that ST 47 could better adapt to saline conditions than SS 212 at the germination and seedling growth stages. Moreover, the ability of plants to recover after stress (re-watering) can be used to measure the plant resistance capability (Chaves et al., 2003; Xu et al., 2010). Re-watering is an effective measure to recover the growth of stressed plants (Azeem et al., 2017; Javed et al., 2017). Most studies have focused on the compensation effect and drought stress mechanism after re-watering (Miao et al., 2020; Zhang et al., 2015). However, the compensation or
overcompensation effect of salt-stressed plants after re-watering has received little attention (Javed et al., 2017; Khorsandi & Anagholi, 2010). Herein, the two cultivars showed positive responses upon re-watering. Some compensation effects in macro, micro, and phenotypic properties were also observed, especially in ST 47. Further, soluble substances (glycine betaine and amino acid) contents, chlorophyll content, chlorophyll fluorescence parameters (Fv/Fm, Y[NPQ], and Y[NO]), and photosynthetic ability of ST 47 recovered to almost that of the control plants. Therefore, ST 47 exhibits stronger resistance to Na⁺ toxicity than SS 212 at the germination and seedling stages.

4.3 | ST 47 had a better osmo-protection than SS 212 under salt stress

Salt stress can induce osmotic, ionic, and secondary stresses in plants. Therefore, plants must develop strategies to overcome highly saline conditions through phenotypic plasticity and evolutionary adaptation (Zhu, 2016). This adaptation involves changes in the physiology (redistribution of ions, soluble compounds), of plant organs (root and leaf surface), cell structure, and gene expression levels (Huang et al., 2018). The current study revealed that the cultivar ST 47 resists the osmotic stress caused by salt damage via synthesizing more soluble substances. Other mechanisms for avoiding osmotic stress are those for reducing transpirational water loss. Plants can regulate transpiration flux through better control of the stomatal opening (short-term response) and modifications of stomatal density (long-term response; Abbruzzese et al., 2009; Barbieri et al., 2009). In the present study, the cultivar ST 47 was shown to close the stomata under salt stress to avoid water loss. Also observed was a stomata density difference among leaves of SS 212 and ST 47. While ST 47 showed a significant decrease in stomata density under salt stress and significantly smaller epidermal cells, the stomata density of SS 212 did not change significantly. A similar increase in stomata density has been observed in salt-tolerant Populus alba L. (14P11; Abbruzzese et al., 2009).

4.4 | ST 47 had a stronger structure adaptation than SS 212 under salt stress

The maintenance of intact cell surface and internal structures is conducive to plant growth and salt stress tolerance (Feng et al., 2018; Zhao et al., 2018). Since cell wall polymers are negatively charged, they can reversibly bind to cations (O’Neill et al., 2004; Shomer et al., 2003), such as Na⁺. During salt stress, the excessive accumulation of Na⁺ replaces Ca²⁺ and directly binds to cell wall components, affecting its chemical properties and reducing cell expansion (Byrt et al., 2018). In this study, Na⁺ toxicity destroyed the morphology of SS 212 roots, resulting in irregular surfaces and severely broken epidermal cells, while the surface of ST 47 remained smooth. Besides, the surface and stomatal structure of ST 47 leaves remained intact under salt stress, with closed stomata. However, the stomata of SS 212 decomposed and lost the capacity to regulate water loss from the plant. These results indicate that ST 47 could maintain the integrity of the root and leaf surface structures to reduce Na⁺ toxicity. The results of the TEM examination of root and leaf surface tips revealed that the root cell wall of ST 47 was significantly thickened under salt stress. Plants respond to abiotic stresses such as salt stress by promoting secondary cell wall thickening through increased deposition of hemicellulose, cellulose, and lignin in the cell wall (Shafi et al., 2017). Under salt stress, the chloroplast of ST 47 could maintain the typical internal structure. The chloroplast edges remained clear and complete, and the thylakoid membranes and internal lamellar systems were also well preserved. Since chloroplasts are the primary photosynthetic sites in plants (Allen et al., 2011), ST 47 ensured continuous photosynthesis by maintaining a standard chloroplast structure under salt stress, thereby achieving more biomass accumulation.

4.5 | ST 47 showed more efficient Na⁺, K⁺ homeostasis, and photosynthesis than SS 212

Ion homeostasis is associated with an optimal K⁺/Na⁺ ratio (Flowers et al., 2015; Orsini et al., 2010). Salt-tolerant plants in high-salt environments respond by reducing Na⁺ uptake, vacuolar sequestration of Na⁺, and promoting Na⁺ exclusion to prevent the cytoplasmic accumulation of excess Na⁺ (Munns & Tester, 2008; van Zelm et al., 2020). Herein, ST 47 maintained a better K⁺/Na⁺ ratio than SS 212 through higher Na⁺ efflux and K⁺ influx under highly saline conditions (Figure 4). Subsequently, the two cultivars should also have differences in Na⁺, K⁺ homeostasis, and the expression levels of Na⁺ transporters and channels (Maathuis, 2014). It has been reported that the cyclic nucleotide-gated (CNGCs) non-selective cation channels and HKT₂ high-affinity K⁺ transporters mediate Na⁺ uptake in plants (Mian et al., 2011). In this study, soil salinity did not cause significant changes in the expression levels of CNGCs in the roots of SS 212 and ST 47. However, the transcript levels were higher in the leaves of both cultivars. A significant increase in HKT₂ expression was observed in the roots of SS 212 and ST 47 but not in the leaves. This
finding supported the phenomenon of Na\(^+\) uptake in roots by HKT2\(_{1}\) high-affinity K\(^+\) transporters and Na\(^+\) uptake in leaves by CNGCs transporters (Figure 9). The expression of OsHKT2;2/1 is regulated by Na\(^+\) concentrations in roots (Jabnoune et al., 2009). The transport activity of OsHKT2;1 is also regulated by external Na\(^+\) in *Xenopus* oocytes (Oomen et al., 2012). The expression pattern of gene transcription is generally synchronized with the activity of transporters under salt stress (Hamada et al., 2001; Horie et al., 2011; Huang et al., 2019). Therefore, the down-regulation of the HKT2 gene is generally synchronized with repressed HKT2 high-affinity K\(^+\) transporters activity in ST 47, thus limiting Na\(^+\) uptake. Excessive Na\(^+\) in the cytosol is deposited in the vacuole by NHX-type cation/H\(^+\) antiport activity (Conde et al., 2011). The remarkable increase in the expression of NHX in the roots and leaves suggests that ST 47 sequestered Na\(^+\) into the vacuoles. Moreover, SOS1 was upregulated in ST 47, thus maintaining low intracellular levels of toxic Na\(^+\) under salt stress (Shi et al., 2002). Jarvis et al. (2014) postulate that the expression of SOS1 in the halophytic *E. salsugineum* (EsSOS1) confers stronger salt tolerance than *Arabidopsis homologs* (AtSOS1). Herein, Na\(^+\) fluxes in the roots and leaves interface were directly measured using the MIFE technique. ST 47 had a higher Na\(^+\) efflux than SS 212, which led to the synchronization of gene transcription with Na\(^+\) flux and SOS1 Na\(^+\)/H\(^+\) exchange activity under salt stress.

In general, visual symptoms of plant damage due to K\(^+\) and Na\(^+\) imbalance first appear in the leaves (Vijayan et al., 2008). In this study, ST 47 exhibited a remarkable tolerance to salt stress than SS 212. ST 47 seedlings displayed no salt-stress-induced symptoms such as chlorosis or necrosis in the leaves after 7 days of treatment, indicating a strong adaptive mechanism to combat salinity-induced ionic imbalance (Figure 2f). Maintaining chloroplast's structural integrity is a prerequisite for photosynthesis under salt stress (Zhao et al., 2020). Excessive accumulation of Na\(^+\) and Cl\(^-\) reduces K\(^+\) influx. K\(^+\) regulates pH, volume, and electron transport in the chloroplast (Finazzi et al., 2015; Shabala et al., 2016). The Chl content also affects the assembly of photosynthetic reactions and chloroplast development (Zhang et al., 2020). Under salt stress conditions, thylakoid membranes were almost completely degraded in SS 212 plants. However, ST 47 remained almost unaffected by salt stress (Figure 7h,k). The Chl content was significantly higher in ST 47 than in SS 212 under salt stress, especially for Chl \(a\), which participates in the reaction centers of photosystems I and II and in the pigment antenna (Lichtenthaler & Buschmann, 2001). However, the ratio of Chl \(a\) and Chl \(b\) significantly increased by 4.79-fold in SS 212 compared with the control. Notably, there was no significant change in the Chl \(a/b\) ratio between ST 47 control and salt-treated plants, indicating that salt stress caused insignificant damage to the chloroplasts' mesophyll cells (Barhoumi et al., 2017). The Chl content and Chl \(a/b\) ratio of ST 47 seedling recovered almost to that of the control upon re-watering (Figure 8c). Digital RNA-seq data demonstrated that ST 47 exhibited a higher Chl \(a\) and Chl \(b\) synthesis capacity than SS 212 under salt stress. This finding supported the high light absorption capacity of SS 212 (Figure 8m). The maximum quantum yield \((F_\text{v}/F_\text{m})\) levels of ST 47 were almost similar to those of control plants (Figure 8d), suggesting that photoinhibition was less under salt stress. In addition, decreased \(F_\text{v}/F_\text{m}\) levels led to a gradual decrease in the qP efficiency and relative ETR. Similar results were also reported by Marriboina and Reddy (2020) in *Pongamia pinnata* L. pierre subjected to salt stress. The ETR and qP levels in ST 47 decreased upon salt exposure. However, the observed increase in the quantum yields of non-photochemical energy loss of PSII (NPQ and qN) can protect the photosynthetic apparatus against photodamage. The non-regulated energy dissipation (YNO) levels were not changed significantly in ST 47 under salt stress (Figure 8f), suggesting that salt-induced irreversible damage to the photosynthetic machinery was not triggered (Maxwell & Johnson, 2000). Thus, the ST 47 leaves and chlorophyll pigments remained almost unaffected by salt treatment (Figure 7k). These results suggest photosynthetic machinery was not damaged (Marriboina et al., 2017; Santos et al., 2015).

The detrimental effects of salinity on plant morphology (Figure 2a,f) are attributed to the direct effects on photosynthesis (Parida & Das, 2005). Salinity causes a reduction in photosynthesis through its adverse impact on gas exchange parameters such as stomatal conductance and photosynthetic rate. Studies postulate that exposing plants to salinity results in a significant decrease in \(Pn\), \(Gs\), \(Tr\), and \(Ci\) (Marriboina & Reddy, 2020). This pattern of gas exchange rates was similar to this finding (Figure 8i-l). Given that stomatal density and stomatal aperture are related to photosynthesis (Tanaka et al., 2013), the higher stomatal density and stomatal aperture observed in ST 47 explain its stronger photosynthesis ability under stress conditions (Figure 6w; Figure S1c). Increased stomatal density is associated with \(CO_2\) diffusion, which positively impacts the photosynthetic performance under salinity (Abbrouzse et al., 2009; Boughaleb et al., 2009). There were phenotypic variations between the two cultivars in control plants, with the ST 47 having significantly smaller epidermal cells and higher stomatal density (Figure S1c). Both PSII and PSI are inactivated by increasing NaCl levels in the cytosol. Na\(^+\) influx affects photosynthesis by
disrupting the proton-motive force and chloroplast function and by interfering with the CO2-fixing enzymes (van Zelm et al., 2020; Zhang et al., 2010). Some chloroplast-encoded genes, including oxygen-evolving enhancer proteins 2 and 3 (OEE 2 and 3), PSI1I 22 kDa protein, and F-type ATPase, are associated with the maintenance of the photosystem and electron transport activity under salt stress (Saleethong et al., 2016). Herein, these genes were suppressed in both cultivars under salt stress. However, the expression levels in ST 47 were higher than in SS 212 but were restored with re-watering. This phenomenon was severe in SS 212 than in ST 47. ST 47 had a higher biomass accumulation than SS 212 under salt stress, which further supported the higher photosynthetic capacity of ST 47 in resisting Na+ toxicity.

5 CONCLUSIONS

The integrated approach employed herein has revealed the strategies adopted by the salt-tolerant proso millet to resist Na+ toxicity: (1) The accumulation of soluble substances is enhanced to resist the osmotic stress caused by Na+ toxicity. (2) The maintenance of a better hydric status via adaptation of cell structures to enhance osmoprotection, including stomatal closure and increased stomatal density. (3) Maintaining intact cell surface and internal structures to block Na+ entry into the cells. (4) Coordinated regulation of CNGCs, HKT2, HKT1, NHX, and SOS1 transporter genes to reduce Na+ accumulation in cytoplasm, contributing to resisting Na+ toxicity. Overall, these results highlight the significance of coordinated regulation of physiological, anatomical, and molecular mechanisms in the salinity adaptation of the salt-tolerant proso millet cultivar ST 47. The results reveal the molecular mechanisms by which salt-tolerant proso millet resists salt stress and provides experimental and sequencing data for adaptability studies and breeding salt-resistant energy crops.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

AUTHOR CONTRIBUTIONS

Yuhao Yuan: Conceptualization, data curation, formal analysis. Writing—original draft. Caoyang Wu: Methodology, data curation. Long Liu: Methodology, software. Qian Ma: Formal analysis. Qianghua Yang: Editing, project administration, resources, formal analysis. Baili Feng: Project administration, resources, formal analysis.

ACCESSION CODES

The RNA-seq data obtained herein were deposited in the NCBI Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra/) under the accession number PRJNA592319 (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA592319).

DATA AVAILABILITY STATEMENT

RNA-seq data are available on NCBI under the accession number PRJNA592319, and the URL is https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA592319.

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