

Lipid metabolism and antioxidant system contributed to salt salinity tolerance induced by Na⁺ accumulation in halophytic grass seashore paspalum

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Abstract

Background and aims

Soil salinization is a growing problem for agriculture worldwide. To elucidate the mechanism underlying the salt tolerance of halophytes can offer a new angle for developing salt-tolerant crops. Seashore paspalum (*Paspalum vaginatum* Sw.) is a halophytic turfgrass and genomic model system for salt tolerance research in cereals and other grasses. However, knowledge regarding the tolerance mechanism of this halophyte remains largely unknown.

Methods

The two *P.vaginatum* accessions with contrasting salinity tolerance were employed to investigate the relationship between Na⁺ accumulation, lipid metabolism, antioxidant response and tissue-dependent salt tolerance using ICP-MS-based ion analysis, lipidomic profiling analysis, enzyme assay and integrated transcriptomic and metabolomic analysis, respectively. Gene-metabolite network analysis was carried to identify the significant TF genes and metabolites associated with salt tolerance in *P.vaginatum* plants.

Results

We found that salt-tolerant *P.vaginatum* built up tissue-specific strategies accompanied with Na⁺ accumulation in response to salt stress. Antioxidant system and amino acid metabolism were curial to maintain high salinity tolerance in leaves of *P. vaginatum* plants. On the contrary, lipid upregulation is the important components of the salt-tolerant mechanism in roots of *P. vaginatum*. Furthermore, 109 TF genes were identified to be linked to salt tolerance, conferring to salinity tolerance in this halophytic grass.

Conclusions

Our results expand our understanding of the underlying salt tolerance of seashore halophyte grass for the breeding and genetic engineering of salt tolerance in crop plants.

Introduction

The increased salt accumulation in arable soils is a growing problem for agriculture worldwide (Flowers and Colmer 2015). Such high salinity within soils adversely limits plant growth and yield formation, as most crop plants are hypersensitive to saline conditions (Mishra and Tanna 2017). To address this global issue, halophytes play increasingly important roles as models for understanding the molecular basis of salt tolerance, thus conferring on developing plant germplasm with salinity capability for the utilization of salinized global agricultural land (Flowers and Colmer 2015; Ventura et al. 2015).

To achieve salt tolerance, halophytes have developed physiological and biochemical mechanisms in response to saline conditions (Ismail et al. 2014). When entering high Na^+ from saline soil into plant tissues, excess Na^+ induces osmotic stress and oxidative stress in cells, thus causing salt – induced changes in metabolic status, membrane remodelling, and abundance of genes and proteins involved in biological processes such as photosynthesis and translation (Flowers and Colmer 2015; Rozema and Schat 2013). Researchers have concluded a clear correlation of plant superior salinity tolerance with ion homeostasis, lipid adaptation, and an effective antioxidant system (Muchate et al. 2016).

The antioxidant response, also referred to as the oxidative stress tolerance, is becoming an increasingly focused trait for the genetic engineering of salt – tolerant crops on account of the energy cost of adaptation (Marco et al. 2015; Vinocur and Altman 2005; Zulfiqar and Ashraf 2021). Halophytes tend to employ a complex antioxidant system to alleviate the toxic effect of salt stress – induced reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2) is the most stable form of ROS. Many reports have demonstrated that ROS – scavenging enzymes such as superoxide dismutase, ascorbate peroxidase (APX), and catalase (CAT) (Adachi et al. 2019; Wu et al. 2020), and non – enzyme antioxidants consisting of ascorbate, proline, glutathione, flavonoids, polyamines, and polyols are necessary to avoid the harmful effects of ROS and offer exceptional salinity tolerance (Liu et al. 2021; Bose et al. 2013). However, the class and quality of ROS players differ in a range of halophyte species and even in various tissues within an individual (Choudhary et al. 2019). For example, glutathione is a non – enzymatic antioxidant and its activity was significantly decreased in the halophytic grass *Desmostachya bipinnata* and *Cakile maritima* at condition of high salinity (Asrar et al. 2020; Amor et al. 2006), but extremely increased in the halophyte *salicornia brachiata* (Parida and Jha 2010). Therefore, the mechanistic details of the salt – induced antioxidant response in halophyte species remain largely unclear.

Salt stress is known to result in oxidative damage in the biological membrane and changes in membrane properties in plants (de Azevedo Neto et al. 2006). Plant membrane lipids are classified into three groups: sphingolipids, sterols, and glycerolipids. In plants, membrane lipids are recognized as important players that regulate membrane fluidity and permeability for water, ions, and metabolites in response to abiotic stress (Guo et al. 2019; Guo et al. 2022). The few studies that have offered evidence for salt – induced alterations to the membrane lipid composition to maintain membrane homeostasis in halophytes under salt stress (Guo et al. 2022; Lin and Wu 1996; Salama et al. 2007). While, the correlation between salt – induced specific lipid species and salt tolerance mechanism has not yet been fully exploited.

Seashore paspalum (*Paspalum vaginatum* Sw.) is recognized as an important halophytic grass for agriculture and the improvement of saline soils in tropical and subtropical regions worldwide (Duncan and Carrow 2000). A wide range of genetic variation presents in tolerance to plant salinity across *P. vaginatum* species (Gao et al. 2020). Discovering the molecular basis of salt tolerance in this halophyte has gained much attention in recent years (Gao et al. 2020; Wu et al. 2020). However, knowledge of the deciphering the halophyte tolerance to this halophytic grass remains largely unknown. In this study, we employed salt – tolerant and salt – sensitive *P. vaginatum* accessions to compare and reveal the tissue – specific salt tolerance mechanism accompanied with Na^+ uptake in salt – tolerant *P. vaginatum* plants.

We also performed gene – metabolite interaction networks to attain significant TFs encoded by salt – responsive genes linked to plant salinity tolerance in *P. vaginatum*.

Materials And Methods

Plant growth conditions and salt treatment

Two contrasting *P. vaginatum* accessions, the salt – tolerant accession (PI UPG145) and salt – sensitive accession (PI 647910), were used for the present study. Stolons from two – month – old *P. vaginatum* plants were hydroponically cultivated in plastic containers (90 – mm top diameter × 57 – mm diameter width × 135 – mm depth) filled with full – strength Hoagland solution (pH = 6.0) in a growth chamber with a 16 h:8 h light: dark at 30/25 °C, 50%–60% relative humidity and 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ standard light intensity.

The salt treatments and non – stress treatments were performed on two – week – old plants in the hydroponic plastic containers by supplementing with or without 400 mM NaCl. To avoid the undesirable effects of salt shock on plants, NaCl concentrations gradually increased 100 mM each day until reached 400 mM NaCl. On the fifth days of salt stress, leaves and roots were sampled separately from salt – stressed and non – stressed plants. The treatments were each replicated in three hydroponic plastic contains, and six individual plants from each treatment were considered as a biological replicate. Three to six biological replicates were used for the following experiments.

ICP – MS based ion measurement

The concentrations of Na^+ and K^+ in the leaves and roots were quantitated through ICP–MS analysis using iCAP – Qc ICP – MS spectrometer (Thermo Fisher Scientific, USA). Approximately 50 mg of oven – dried root samples were ground and dissolved in 2% 5 ml HNO_3 and diluted for preparing five – point calibration standards for Na^+ and K^+ . The iCAP Qc ICP – MS was operated in a single KED mode using the following parameters: 1550 W forward power, 14 L/min coolant gas flow, 1.0749 L/min nebuliser gas flow, 0.8 L/min auxiliary gas flow and 0.02 s dwell time. The Selective Transport (ST) capacities was calculated as $\text{ST} = (\text{K}^+/\text{Na}^+ \text{ in leaves})/(\text{K}^+/\text{Na}^+ \text{ in roots})$ (Wang et al. 2002).

H_2O_2 measurement and antioxidant enzymatic assay

The concentration of H_2O_2 were measured using commercial kit (Product ID: BC3590, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The Mn^{2+} –SOD activity and Cu/Zn – SOD were measured using SOD antioxidant capacity assay kit (Product ID: A001 – 2–1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Catalase (CAT) was measured using commercial kits (Solarbio Beijing, China).

Lipid Peroxidation Assay

The levels of lipid peroxidation in plant cells were assayed with the TBA test and determined with a spectrophotometer using a malondialdehyde (MDA) assay kit (Product ID: BC0020, Solarbio Beijing, China).

Lipidomic Profiling Analysis

Approximately 100 mg of fresh leaves and roots from three biological repeats were frozen in liquid nitrogen, ground to powder and extracted with 300 μ L Methanol/1 mL MTBE/300 μ L water. LC – MS analysis of lipids was carried out using the Nexera UHPLC LC – 30A system (Shimadzu, Kyoto, Japan) coupled to the TripleTOF5600⁺ (AB SCIEX™) mass spectrometer. The column temperature was set at 40°C. The mobile phase was 0.1% HCOOH – H₂O (A) – acetonitrile (B) at a flow rate of 0.3 mL/min in gradient elution. The ESI source conditions were set as follows: Ion Source Gas1 (Gas 1): 50, Ion Source Gas2 (Gas 2): 50, Curtain Gas (CUR): 25, Source Temperature: 500 °C/450 °C (positive ion/negative ion), Ion Spray Voltage Floating (ISVF) 5500 V/4400 V(positive ion/negative ion), TOF MS scan range: 100–1200 Da, TOF MS scan accumulation time 0.2 s, Declustering potential (DP): $\pm$ 60V. The lipid identification was performed using LipidSearch software 4.0 (Thermo Fisher Scientific, CA, USA).

Metabolite Profiling and Identification

Approximately 100 mg of freeze – dried leaves and roots from three biological repeats were used for metabolite extraction. Sample extracts were analyzed using an UPLC – ESI – MS/MS system (UPLC, Shimpack UPLC SHIMADZU CBM30A system, www.shimadzu.com.cn/; MS, Applied Biosystems 4500 Q TRAP, www.appliedbiosystems.com.cn/). The analytical conditions were as follows, UPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μ m, 2.1 mm*100 mm); The mobile phase consisted of solvent A, pure water with 0.04% acetic acid and solvent B, acetonitrile with 0.04% acetic acid. Sample measurements were made with a gradient program (the starting conditions of 95% A, 5% B. Within 10min, a linear gradient to 5% A, 95% B was programmed, and a composition of 5% A, 95% B was kept for 1min, finally a composition of 95% A, 5.0% B was adjusted within 0.10 min and kept for 2.9 min). The column oven was set to 40°C; The injection volume was 4 μ L.

LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole linear ion trap mass spectrometer (Q TRAP), API 4500 Q TRAP UPLC / MS / MS system, equipped with an ESI Turbo Ion – Spray interface, operating in positive and negative ion mode, and controlled by Analyst 1.6.3 software (AB Sciex, USA). The operation parameters of the ESI source were as follows: ion source, turbo spray; source temperature 550 °C; ion spray voltage (IS) 5500 V (positive ion mode) / 4500 V (negative ion mode); ion source gas I (GSI), gas II (GSII), curtain gas (CUR) were set at 50, 60, and 30.0 psi, respectively; the collision gas (CAD) was high. Instrument tuning and mass calibration were performed with 10 μ mol/L and 100 μ mol/L polypropylene glycol solutions in the QQQ and LIT modes, respectively. The QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set at 5 psi. DP and CE for individual MRM transitions was done with further DP and CE optimization.

The SIMCA 14.1 software (Umetrics, Umea, Sweden) was used to process multivariate modelling in metabolic profiling. Orthogonal partial least squares discriminant analysis (OPLS – DA) was performed to identify significantly abundant metabolites. Only identified metabolites with fold change 2 or ≤ 0.5 and $VIP \geq 1$ were defined as significant metabolites. Pathway enrichment analysis of metabolomics data was performed using MetaboAnalyst 5.0. KEGG pathway visualization of metabolomic data was achieved using KEGG Mapper (https://www.genome.jp/kegg/tool/map_pathway2.html).

Transcriptomic profiling analysis and gene expression analysis

Total RNA was extracted using a Plant RNA Kit (Omega Bio – Tek, Norcross, GA, United States) according to the manufacturer’s protocol. Transcriptome sequencing and de novo assembly Paired – end sequencing of cDNA libraries was performed using the HiSeq 2000 (Illumina Technologies) platform. The raw reads were evaluated for quality, and then the adapter sequences and low – quality bases were filtered using Trimmomatic (v0.32). Clean reads were aligned to the reference genome using HISAT2 (v2.1.0). Gene expression was normalized by calculating Reads per Kilobase per Million Mapped Reads (RPKM) as described by Mortazavi and colleagues. DEGs were determined using the DESeq2 package of R software, and the false discovery rate ($FDR \leq 0.05$ and $|\log_2 FC| \geq 1$) were used as the threshold to determine the statistically significant differences in gene expression. GO Gene Ontology (GO) and KEGG pathway analyses were performed to identify biological roles and functions of the DEGs. The RNA – seq reads with quality scores were deposited in the NCBI Sequence Read Archive (SRA) with Bioproject accession number: PRJNA874860.

Transcription factors analysis and network construction

Transcription factors (TFs) from the whole – genome protein sequences and transcriptomic – based DNA sequences of *P. vaginatum* were identified and categorized based on conserved domains using PlantTFcat (<http://plantgrn.noble.org/PlantTFcat/>) (Dai et al. 2013). Pearson correlation coefficient between TFs were calculated via R (v4.0.1). The biomarkers were selected based on $|R^2| > 0.8$ to construct gene co – expression network. To build the network between TF genes and metabolites, we first selected significant TF genes and significant metabolites in salt – treated leaves and roots, respectively. Then the Pearson correlation coefficients were calculated between the TF genes and metabolites. Only the gene – metabolite pair with $|R^2| > 0.8$ were kept for constructing the network. Gene – metabolite interaction networks were visualized using Cytoscape (v3.9.1) (<https://cytoscape.org/>).

Results

Genotypic variation in salinity tolerance was associated with Na^+ accumulation in *P. vaginatum*

After 5 days of salt stress, salt – sensitive plants showed clear symptoms of leaf chlorosis and wilting, while salt – tolerant plants displayed a phenotype much closer to control plants (Fig. 1D). To test whether salt – tolerant accession of *P. vaginatum* tended to absorb more Na^+ compared to salt – sensitive

accession under salinity stress. ICP – MS technology was used to quantify concentrations of Na^+ and K^+ in non – salted and salted roots from plants of the two accessions. Roots and leaves of tolerant plants were inclined to uptake higher amounts of Na^+ in response to high salinity compared to sensitive ones (Fig. 1A). Endogenous K^+ concentration in salt – treated roots failed to alter between plants of the two accessions in response to high salinity (Fig. 1B), which leads to ST values showing no difference between the two accessions treated with salt stress (Fig. 1C). These results hint that salt – tolerant plants preferentially uptake more Na^+ in *P. vaginatum* under saline conditions.

Na^+ accumulation induced enzymatic antioxidant system in roots of salt – tolerant plants under high salinity

To investigate whether Na^+ accumulation leads to H_2O_2 accumulation in excess after salt stress, we measured the H_2O_2 levels and the activities of H_2O_2 -scavenging enzymes in salt – treated roots of tolerant and sensitive accessions. A significant increase in root – sourced H_2O_2 was detected in tolerant plants compared to sensitive plants (Fig. 2A). Correspondingly, activities of CAT and Mn^{2+} -SOD increased in salt – treated roots of tolerant plants rather than sensitive plants (Fig. 2B and 2D). We found that the activity of Cu/Zn – SOD in the roots and leaves of salt – tolerant plants did not change after application of 400 mM NaCl in comparison with neither salt – sensitive plants nor their corresponding controls (Fig. 2C). The levels of MDA showed an increase in the roots of salt – sensitive plants compared to salt – tolerant plants under salt stress, while the MDA did not alter in the leaves when treating with or without high salinity (Fig. 2E). These results indicate that increased uptake of Na^+ -induced H_2O_2 production to partly stimulate activities of enzymatic ROS scavengers.

To detect whether increased H_2O_2 induced non – enzymatic antioxidative system in salt – tolerant *P. vaginatum*, we profiled the metabolic status of non – enzymatic antioxidants consisting of amino acids, flavonoids, alkaloids, phenolic acids and polyols in salt – treated plants from tolerant and sensitive accessions. We found that high salinity indeed induced increased abundance of amino acids (i.e., proline and pipecolic acid), phenolic acids (i.e., caffeic acid, vanillylmandelic acid), and polyols (i.e., mannitol and inositol) in leaves and roots of tolerant plants (Fig. 3C). These results indicated that both enzymatic and non – enzymatic antioxidants attributed to Na^+ -induced H_2O_2 confers to plant salinity tolerance.

Na^+ accumulation induced upregulation of lipid species in the roots of salt – tolerant plants under high salinity

To test whether the elevated H_2O_2 induced by Na^+ accumulation disrupted the membrane lipid bilayer, we quantified alterations in the MDA concentration, recognized as the end product of lipid peroxidation (Yalcinkaya et al. 2019). We found that MDA levels in the roots of salt – tolerant plants did not elevate after application of 400 mM NaCl in comparison with corresponding controls and salt – sensitive plants (Fig. 3A). Lipidomic data showed an observably upregulation of different lipid species in salt – treated

roots of salt – tolerant plants compared to salt – sensitive plants, which included glycolipid (i.e., MDGDs, DGDGs and SGDGS), phospholipids (i.e., PCs and PEs), fatty acids (FAs), and triacylglycerols (TGs) (Fig. 3B – Fig. 3D). Conversely, leaf lipid profiling of salt – tolerant plants seems not to be affected by salt stress (Fig. 3B – Fig. 3D).

Metabolomic profiling revealed non – enzymatic antioxidants and amino acid metabolism conferred salt tolerance to salt – treated *P. vaginatum* plants

To detect the metabolic alteration associated with salt tolerance in *P. vaginatum* plants, we profiled and compared the significant metabolites consisting of amino acids, flavonoids, alkaloids, phenolic acids and polyols in salt – treated plants from tolerant and sensitive accessions (Fig. 4A – 4C). High salinity induced an increased abundance of amino acids (i.e., proline and pipecolic acid), phenolic acids (i.e., caffeic acid, vanillylmandelic acid) and polyols (i.e., mannitol and inositol) in both leaves and roots of tolerant plants (Fig. 4B). Noticeably, salt – tolerant plants tended to accumulate higher amounts of amino acids (i.e., cysteine, glutamine, lysine, histidine, phenylalanine, N – Acetyl – tryptophan), alkaloids (such as putrescine, putrescine, agmatine, N – Feruloyl – tyramine) and flavonoids (quercitrin, orientin, luteolin, cynaroside, apigenin – 8-C – glucoside and genistein 8 – C-glucoside) in salt – stressed leaves, instead of treated roots, compared to salt – sensitive plants (Fig. 4B). KEGG pathway analysis showed that leaves of salt – tolerant plants produced significant metabolites associated with amino acids in response to salt stress (Fig. 4C). However, a few of amino acids (pipecolic acid, acetyltryptophan, proline, homocysteine, and pyroglutamic acid), a flavonoid (isorhamnetin) and an alkaloid (glucopyranoside) were found to be highly accumulated in salted roots in salt – tolerant plants relative to those of salt – sensitive plants (Fig. 4B). In contrast, the abundance of phenolic acids (including caffeic aldehyde, vanillylmandelic acid, rosmarinic acid, protocatechuic aldehyde, p – coumaraldehyde and coniferaldehyde) showed an increase in stressed roots of salt – tolerant plants compared to salt – sensitive plants (Fig. 4B). Furthermore, four polyols (ribitol, mannitol, inositol, and dulcitol) had a significant and unique abundance in roots of salt – tolerant plants in roots compared to salt – susceptible plants.

Comparative transcriptomics showed that higher amounts of DEGs were induced to involve in biological processes in roots of *P. vaginatum* plants

To investigate the significant genes related to salt tolerance in *P. vaginatum* plants. we separately identified differentially expressed genes (DEGs) in leaves and roots between the two *P. vaginatum* accessions under salt – stressed or non – stressed conditions (Fig. 5). Salt – treated roots were able to increase number of regulated DEGs over that of treated leaves in *P. vaginatum* plants (Fig. 5A and 5B). The gene enrichment analyses the DEGs revealed DEGs from salt – treated roots were involved in more pathways of biological processes between salt – tolerant and salt – susceptible cultivars (Fig. 5C). Under non – stressed conditions, DEGs enriched in metabolic process of aromatic compound, and organic compound were upregulated in both leaves and roots (Fig.S1). When plants exposed to high salinity stress, DEGs identified from leaves were positively related to the process of organic substance metabolic processes in leaves while negatively enriched in processes related to amino acid import and transport

(Fig. 5C). In salt – treated roots, DEGs significantly and positively enriched in organic acids metabolic process and aromatic and organic cyclic biosynthetic process, while showed a downregulation in amino acid transport in salt – treated roots (Fig. 5C).

Integration of transcriptomic and metabolomic analysis highlighted amino acid metabolism is curial to plant salt tolerance in *P. vaginatum*

To combine the important metabolites with their corresponding DEGs on the gene – metabolite regulatory network, we initially assessed the correlation between salt – tolerant metabolites and differential genes using the Partial Least Squares (PLS) regression method (Fig. 6A). Individual metabolomes and transcriptomes of roots and leaves were well separated based on the sPLS – DA model (Fig. 6A). By performing an integrated pathway – level analysis, we combined significant metabolites with their corresponding DEGs in the metabolic networks. Apparently, most pathways related to amino acid metabolism were activated in salt – tolerant plants under salt stress, which play important roles in switch – on biosynthesis of phenolic acids and flavonoids (Fig. 6B and 6C).

The involvement of transcription factors in regulating salinity tolerance in *P. vaginatum*

To investigate the important transcription factors (TFs) regarding sanity tolerance in *P. vaginatum*, we identified TFs genes from DEGs between the tolerant and sensitive accessions treated with or without different salt conditions. We totally achieved 109 TF genes ($|\log_2\text{Fold Change}| \geq 1$ and $\text{FDR} < 0.05$) in the leaves and roots of *P. vaginatum* under salt stress (Fig. 7A). The TF genes primarily belonged to C2H2 (14 genes), MYB – HB – like (12 genes), bHLH (9 genes), WD40 – like (7 genes) and WRKY (7 genes) family. The TF genes encoding the AP2 – EREBP family seem to be up – regulated in salt – tolerant *P. vaginatum* plants. Gene co – expression networks showed that the TF genes from roots (1390 gene pairs) were more correlated than the TF genes from leaves (878 gene pairs) (Fig. 7B and 7C). The thirty shared TFs from C2H2, GAGA, NAM, WD40 – like were observed in both leaves and roots (Fig. 7A – 7C). Additionally, to attempt to understand the functional relationship between significant metabolites and identified TF genes, we connected significant TFs and different metabolites using their correlation coefficients. The networks showed that 151 gene – metabolite pairs in leaves were positively correlated, while only 2 gene – metabolite pairs showed a negative correlation. While only 18 gene – metabolite pairs showed an either positive and negative correlation in roots (Fig.S2).

Discussion

The halophytic grass *P. vaginatum* implements mechanisms to mitigate Na^+ induced osmotic stress and oxidative stress by limiting Na^+ delivery from roots to leaf tissues and by compartmentalization of Na^+ into vacuoles and leaf papillae (Wu et al. 2020). Therefore, we assumed that Na^+ uptake did not cause ROS accumulation in salt – tolerant plants. However, surprisingly, the increase in the accumulation of Na^+ resulted in H_2O_2 in roots, accompanied by a higher accumulation of antioxidants and upregulated lipids, conferring the ability to improve the tolerance of plant salinity (Fig. 2 and Fig. 3). Chloroplasts and

mitochondria are the main source of ROS in plants in response to stress conditions (Bartoli et al. 2004). SOD and CAT are recognized as the first and important lines of defense against oxidative stresses induced by salt stress. Much reports have defined the two enzymes as key regulators for achieving the optimal H₂O₂ signature for contributing to plant salinity tolerance in glycophytes (Hu et al. 2016), but whether stress – induced ROS production is attributed to superior salinity tolerance is arguable (Foyer and Noctor 2003). Until recent studies have demonstrated the relationship between root – sourced H₂O₂ and salt tolerance in both halophytes and glycophytes (Ben Rejeb et al. 2015; Ma et al. 2011; Qiu et al. 2002). Corresponding to previous reports, we observed that Na⁺ accumulation did not trigger H₂O₂ generation in leaf tissues, but high salinity produced hyperosmotic stress on the roots of this halophytic grass (Fig. 2C and 2D). we concluded that root – sourced H₂O₂ was necessary for the regulation of salt – tolerant mechanism in *P. vaginatum* plants.

Na⁺ accumulation is usually believed to decrease lipid contents in terms of lipid peroxidation (Gao et al. 2020), due to the presence of Na⁺–induced ROS in many plants under salt stress (Biswas and Mano 2015; Yalcinkaya et al. 2019). Many studies have offered evidence that salt – induced membrane lipid alterations are curial to maintain membrane homeostasis in halophytes under salt stress (Guo et al. 2022; Lin and Wu 1996). In fact, halophytic plants prone to increase their lipidic contents for enhanced salt tolerance (Castillo et al. 2022). The tolerance accession of *P. vaginatum* showed greatly increased accumulation of lipids after salt treatment compared to the sensitive ones (Fig. 3B). The increase was particularly noticeable in the galactolipids DGDGs, resulting in the tolerant accession to decrease their MGDG/DGDG ratio compared to the sensitive accession. Both the overall increase in lipids and the particular decrease in MGDG/DGDG ratio have been linked to the maintenance of membrane fluidity and permeability during osmotic stress (Castillo et al. 2022).

The contribution of amino acids to the efficient antioxidant mechanism and oxidative tolerance mechanism in salt – tolerant or halophytic plants has been extensively reported (Tzin and Galili 2010; Xu et al. 2017). Given the antioxidative properties of amino acids – tryptophan, phenylalanine, and phenylpropanoids seem to be widely required for plants to minimize ROS caused by saline conditions (Batista – Silva et al. 2019). The amino acids were indeed involved in conferring salt tolerance to *P. vaginatum* plants (Fig. 4B). In addition to act as antioxidants, a few of amino acids such as arginine is an essential precursor molecule for the synthesis of secondary metabolites associated with salt tolerance (Winter et al. 2015). We thereby assumed that the strongly decreased arginine probably contributed to the synthesis of polyamines during salt stress in the leaves of *P. vaginatum* plants (Fig. 4B). Moreover, other amino acids can serve as precursors for the synthesis of non – enzymatic scavengers – phenolic compounds and alkaloids to minimize salt stress – induced oxidative stress (Oliva et al. 2021). For instance, plant phenolics are mainly synthesized from phenylalanine and tyrosine (Tyagi et al. 2020). In agreement to our results, amounts of phenolic compounds were produced in the roots of salt – tolerant *P. vaginatum* plants for dealing with salt – induced stress (Fig. 4B). Proline, as the key player, confers tolerance to salt – treated plants via involvement of regulation of antioxidant system and ion homeostasis in plants (Abdelrahman et al. 2021). We found that roots tended to accumulate proline in

response to salt stress, but no alteration of proline was found in treated leaves (Fig. 4B), indicating that proline is essential to root salt tolerance of *P. vaginatum* plants.

To explore the regulatory network connected to non – enzymatic antioxidants and amino acid metabolism, a correlation test was performed on significant TF genes and metabolites (Fig. 7). Gene – metabolite interaction networks showed that five TF genes (emOS117.89_AP2 – EREBP, emOS124.282_GRAS, emOS174.433_C2H2, emOS79.423_C2H2 and emOS43.2_MYB – HB – like) were positively correlated with proline in roots, which were predicted as the key TF genes for proline metabolism in *P. vaginatum* roots (Fig. 7B). The importance of root system for salt tolerance in *P. vaginatum* has also been highlighted by previous studies (Hu et al. 2022). However, salt – tolerant *P. vaginatum* tended to accumulate higher amounts of amino acids, alkaloids, and flavonoids in salt – stressed leaves compared to salt – sensitive *P. vaginatum* plants, and we also identified 151 pairs of positive correlation gene – metabolite pairs in leaves (Fig. 7B). Noticeably, four TF genes (emFS18.314_MYB – HB – like, emFS27.666_MYB, emOS79.423_C2H2 and emOS99.692_bZIP) showed strong correlation with amino acids, alkaloids and flavonoids in leaves (Fig.S2). Since MYB (Wang et al. 2021), C2H2 (Han et al. 2020) and bZIP (Gai et al. 2020) have been shown to be related to salt tolerance in other plants, we speculate that these genes may perform similar functions in *P. vaginatum*. The transcriptomic and metabolomic dataset could be harnessed by researchers to utilize genetic approaches to clarify the mechanism of non – enzymatic antioxidants and amino acid metabolism.

Declarations

Author contributions

Conceptualization, L.P. and Z.W.; funding acquisition, Z.W. and L.L; investigation, L.P., M.T., Q.S. and Z.C.; writing—original draft, L.P. and X.H.; writing—review and editing, L.P. and X.H. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors have no conflict to declare.

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References

1. Abdelrahman M, Nishiyama R, Tran CD, Kusano M, Nakabayashi R, Okazaki Y, Matsuda F, Chávez Montes RA, Mostofa MG, Li W, Watanabe Y, Fukushima A, Tanaka M, Seki M, Saito K, Herrera –

- Estrella L (2021) Defective cytokinin signaling reprograms lipid and flavonoid gene – to – metabolite networks to mitigate high salinity in *Arabidopsis*. *Proc Natl Acad Sci* 118:e2105021118 Tran L – SP. <https://doi.org/10.1073/pnas.2105021118>
2. Adachi S, Yamamoto T, Nakae T, Yamashita M, Uchida M, Karimata R, Ichihara N, Soda K, Ochiai T, Ao R, Otsuka C, Nakano R, Takai T, Ikka T, Kondo K, Ueda T, Ookawa T, Hirasawa T (2019) Genetic architecture of leaf photosynthesis in rice revealed by different types of reciprocal mapping populations. *J Exp Bot* 70:5131–5144. <https://doi.org/10.1093/jxb/erz303>
 3. Amor NB, Jiménez A, Megdiche W, Lundqvist M, Sevilla F, Abdelly C (2006) Response of antioxidant systems to NaCl stress in the halophyte *Cakile maritima*. *Physiol Plant* 126:446–457. <https://doi.org/10.1111/j.1399-3054.2006.00620.x>
 4. Asrar H, Hussain T, Qasim M, Nielsen BL, Gul B, Khan MA (2020) Salt induced modulations in antioxidative defense system of *Desmostachya bipinnata*. *Plant Physiol Biochem* 147:113–124. <https://doi.org/10.1016/j.plaphy.2019.12.012>
 5. Bartoli CG, Gómez F, Martínez DE, Guiamet JJ (2004) Mitochondria are the main target for oxidative damage in leaves of wheat (*Triticum aestivum* L.). *J Exp Bot* 55:1663–1669. <https://doi.org/10.1093/jxb/erh199>
 6. Batista – Silva W, Heinemann B, Rugen N, Nunes – Nesi A, Araújo WL, Braun HP, Hildebrandt TM (2019) The role of amino acid metabolism during abiotic stress release. *Plant Cell Environ* 42:1630–1644. <https://doi.org/10.1111/pce.13518>
 7. Rejeb KB, Benzarti M, Debez A, Bailly C, Saviouré A, Abdelly C (2015) NADPH oxidase – dependent H₂O₂ production is required for salt – induced antioxidant defense in *Arabidopsis thaliana*. *J Plant Physiol* 174:5–15. <https://doi.org/10.1016/j.jplph.2014.08.022>
 8. Biswas MS, Mano J (2015) Lipid Peroxide – Derived Short – Chain Carbonyls Mediate Hydrogen Peroxide – Induced and Salt – Induced Programmed Cell Death in Plants. *Plant Physiol* 168:885–898. <https://doi.org/10.1104/pp.115.256834>
 9. Bose J, Rodrigo – Moreno A, Shabala S (2013) ROS homeostasis in halophytes in the context of salinity stress tolerance. *J Exp Bot* 65:1241–1257. <https://doi.org/10.1093/jxb/ert430>
 10. Castillo JM, Mancilla – Leytón JM, Martins – Noguerol R, Moreira X, Moreno – Pérez AJ, Muñoz – Vallés S, Pedroche JJ, Figueroa ME, García – González A, Salas JJ, Millán – Linares MC, Francisco M, Cambrollé J (2022) Interactive effects between salinity and nutrient deficiency on biomass production and bio – active compounds accumulation in the halophyte *Crithmum maritimum*. *Sci Hortic* 301:111136. <https://doi.org/10.1016/j.scienta.2022.111136>
 11. Choudhary A, Kumar A, Kaur N (2019) ROS and oxidative burst: Roots in plant development. *Plant Divers* 42:33–. <https://doi.org/10.1016/j.pld.2019.10.002>. 43
 12. Dai X, Sinharoy S, Udvardi M, Zhao PX (2013) PlantTFcat: an online plant transcription factor and transcriptional regulator categorization and analysis tool. *BMC Bioinf* 14:1–6. <http://www.biomedcentral.com/1471-2105/14/321>

13. de Azevedo Neto AD, Prisco JT, Enéas – Filho J, de Abreu CE, Gomes – Filho E (2006) Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt – tolerant and salt – sensitive maize genotypes. *Environ Exp Bot* 56:87–94.
<https://doi.org/10.1016/j.envexpbot.2005.01.008>
14. Duncan RR, Carrow RN (2000) *Seashore paspalum: The environmental turfgrass*. John Wiley & Sons
15. Flowers TJ, Colmer TD (2015) Plant salt tolerance: adaptations in halophytes. *Ann Bot* 115:327–331.
<https://doi.org/10.1093/aob/mcu267>
16. Foyer CH, Noctor G (2003) Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. 119. <https://doi.org/10.1034/j.1399-3054.2003.00223.x>. 355 – 364
17. Gai WX, Ma X, Qiao YM, Shi BH, Ul Haq S, Li QH, Wei AM, Liu KK, Gong ZH (2020) Characterization of the bZIP Transcription Factor Family in Pepper (*Capsicum annuum* L.): CabZIP25 Positively Modulates the Salt Tolerance. *Front Plant Sci* 11:139. <https://doi.org/10.3389/fpls.2020.00139>
18. Gao Y, Li M, Zhang X, Yang Q, Huang B (2020) Up – regulation of lipid metabolism and glycine betaine synthesis are associated with choline – induced salt tolerance in halophytic seashore paspalum. *Plant Cell Environ* 43:159–173. <https://doi.org/10.1111/pce.13657>
19. Guo Q, Liu L, Barkla BJ (2019) Membrane Lipid Remodeling in Response to Salinity. *Int J Mol Sci* 20:4264. <https://doi.org/10.3390/ijms20174264>
20. Guo Q, Liu L, Rupasinghe TW, Roessner U, Barkla BJ (2022) Salt stress alters membrane lipid content and lipid biosynthesis pathways in the plasma membrane and tonoplast. *Plant Physiol* 189:805–826. <https://doi.org/10.1093/plphys/kiac123>
21. Han G, Lu C, Guo J, Qiao Z, Sui N, Qiu N, Wang B (2020) C2H2 Zinc Finger Proteins: Master Regulators of Abiotic Stress Responses in Plants. *Front Plant Sci* 11:115.
<https://doi.org/10.3389/fpls.2020.00115>
22. Hu T, Chen K, Hu L, Amombo E, Fu J (2016) H₂O₂ and Ca²⁺ – based signaling and associated ion accumulation, antioxidant systems and secondary metabolism orchestrate the response to NaCl stress in perennial ryegrass. *Sci Rep* 6:1–13. <https://doi.org/10.1038/srep36396>
23. Hu X, Hao J, Pan L, Xu T, Ren L, Chen Y, Tang M, Liao L, Wang Z (2022) Genome – wide analysis of tandem duplicated genes and their expression under salt stress in seashore paspalum. *Front Plant Sci* 13:971999. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9562133/>
24. Lin H, Wu L (1996) Effects of salt stress on root plasma membrane characteristics of salt – tolerant and salt – sensitive buffalograss clones. *Environ Exp Bot* 36:239–254.
[https://doi.org/10.1016/0098-8472\(96\)01025-8](https://doi.org/10.1016/0098-8472(96)01025-8)
25. Liu J, Fu C, Li G, Khan MN, Wu H (2021) ROS Homeostasis and Plant Salt Tolerance: Plant Nanobiotechnology Updates. *Sustainability* 13:3552. <https://doi.org/10.3390/su13063552>
26. Ma L, Zhang H, Sun L, Jiao Y, Zhang G, Miao C, Hao F (2011) NADPH oxidase AtrbohD and AtrbohF function in ROS – dependent regulation of Na⁺/K⁺ homeostasis in *Arabidopsis* under salt stress. *J Exp Bot* 63:305–317. <https://doi.org/10.1093/jxb/err280>

27. Marco F, Bitrián M, Carrasco P, Rajam MV, Alcázar R, Tiburcio AF (2015) Genetic Engineering Strategies for Abiotic Stress Tolerance in Plants. In *Plant Biology and Biotechnology* 579 – 609. https://doi.org/10.1007/978-81-322-2283-5_29
28. Mishra A, Tanna B (2017) Halophytes: Potential Resources for Salt Stress Tolerance Genes and Promoters. *Front Plant Sci* 8:829. <https://doi.org/10.3389/fpls.2017.00829>
29. Muchate NS, Nikalje GC, Rajurkar NS, Suprasanna P, Nikam TD (2016) Plant Salt Stress: Adaptive Responses, Tolerance Mechanism and Bioengineering for Salt Tolerance. *Bot Rev* 82:371–406. <https://doi.org/10.1007/s12229-016-9173-y>
30. Oliva M, Guy A, Galili G, Dor E, Schweitzer R, Amir R, Hacham Y (2021) Enhanced Production of Aromatic Amino Acids in Tobacco Plants Leads to Increased Phenylpropanoid Metabolites and Tolerance to Stresses. *Front Plant Sci* 11:604349. <https://doi.org/10.3389/fpls.2020.604349>
31. Parida AK, Jha B (2010) Antioxidative Defense Potential to Salinity in the Euhalophyte *Salicornia brachiata*. *J Plant Growth Regul* 29:137–148. <https://doi.org/10.1007/s00344-009-9129-0>
32. Qiu QS, Guo Y, Dietrich MA, Schumaker KS, Zhu JK (2002) Regulation of SOS1, a plasma membrane Na⁺/H⁺ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. *Proc Natl Acad Sci U S A* 99:8436–8441. <https://doi.org/10.1073/pnas.122224699>
33. Rozema J, Schat H (2013) Salt tolerance of halophytes, research questions reviewed in the perspective of saline agriculture. *Environ Exp Bot* 92:83–95. <https://doi.org/10.1016/j.envexpbot.2012.08.004>
34. Salama KH, Mansour MM, Ali FZ, Abou – hadid AF (2007) NaCl – induced changes in plasma membrane lipids and proteins of *Zea mays* L. cultivars differing in their response to salinity. *Acta Physiol Plant* 29:351–359. <https://doi.org/10.1007/s11738-007-0044-3>
35. Tyagi K, Shukla P, Rohela GK, Shabnam AA, Gautam R (2020) Plant Phenolics: Their Biosynthesis, Regulation, Evolutionary Significance, and Role in Senescence. In: R Lone, R Shuab, AN Kamili (eds) *Plant Phenolics in Sustainable Agriculture*: 1: 431 – 449. https://doi.org/10.1007/978-981-15-4890-1_18
36. Tzin V, Galili GJM (2010) New insights into the shikimate and aromatic amino acids biosynthesis pathways in plants. *Mol Plant* 3:956–972. <https://doi.org/10.1093/mp/ssq048>
37. Ventura Y, Eshel A, Pasternak D, Sagi M (2015) The development of halophyte – based agriculture: past and present. *Ann Bot* 115:529–540. <https://doi.org/10.1093/aob/mcu173>
38. Vinocur B, Altman A (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Curr Opin Biotechnol* 16:123–132. <https://doi.org/10.1016/j.copbio.2005.02.001>
39. Wang S, Zheng W, Ren J, Zhang C (2002) Selectivity of various types of salt – resistant plants for K⁺ over Na⁺. *J Arid Environ* 52:457–472. <https://doi.org/10.1006/jare.2002.1015>
40. Wang X, Niu Y, Zheng Y (2021) Multiple Functions of MYB Transcription Factors in Abiotic Stress Responses. *Int J Mol Sci* 22:6125. <https://doi.org/10.3390/ijms22116125>

Figures

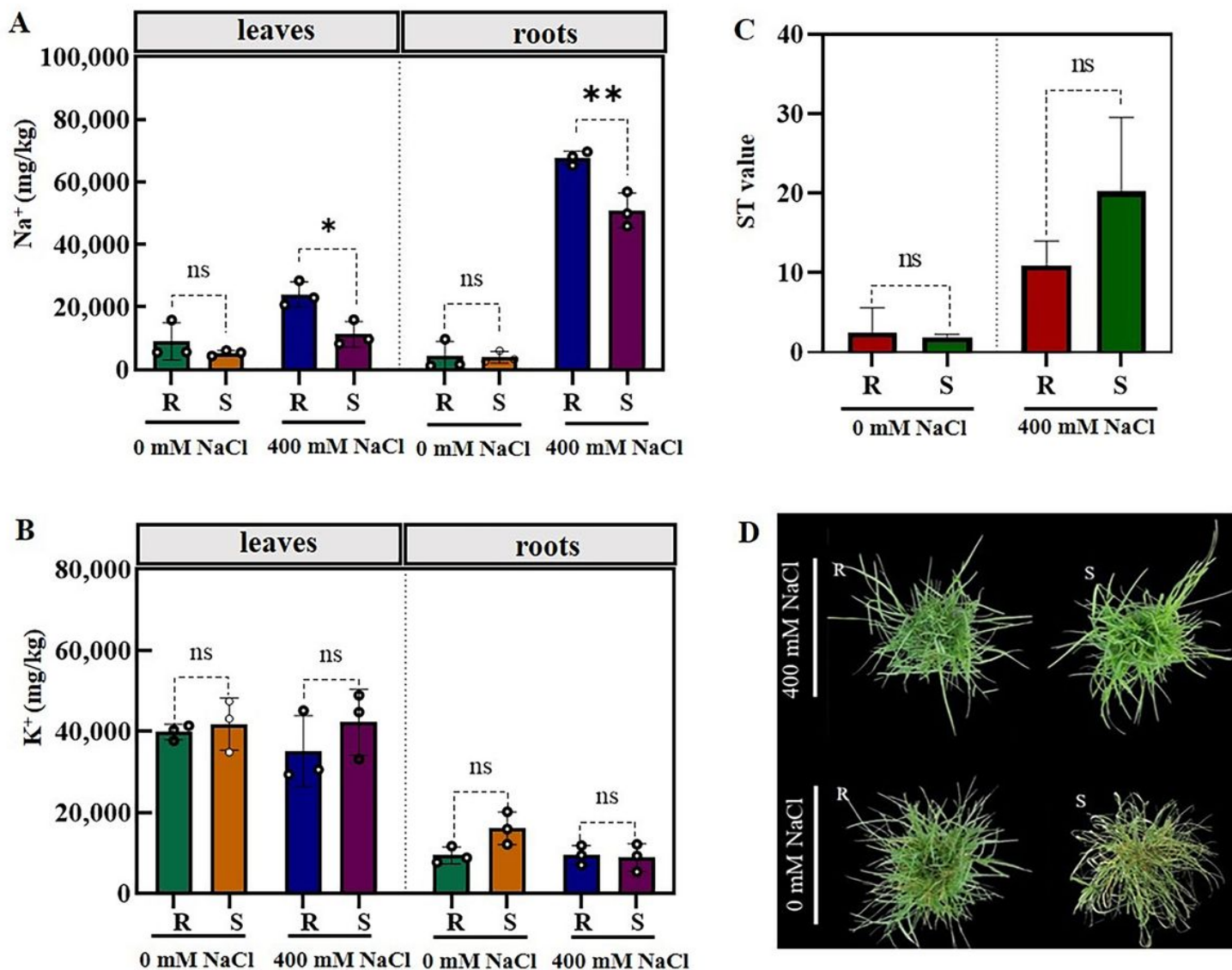


Figure 1

Distinct Na^+ (A) and K^+ (B) concentrations in the roots and leaves of salt-sensitive and salt-tolerant plants treated with or without high salinity for 5 days (** significant at $p < 0.001$; ns, not significant; two-sided Student's t -test). (C) ST values of the two *P. vaginatum* were calculated after 5 days of salt stress. (D) Visual comparison of the two *P. vaginatum* accessions with different salt tolerance.

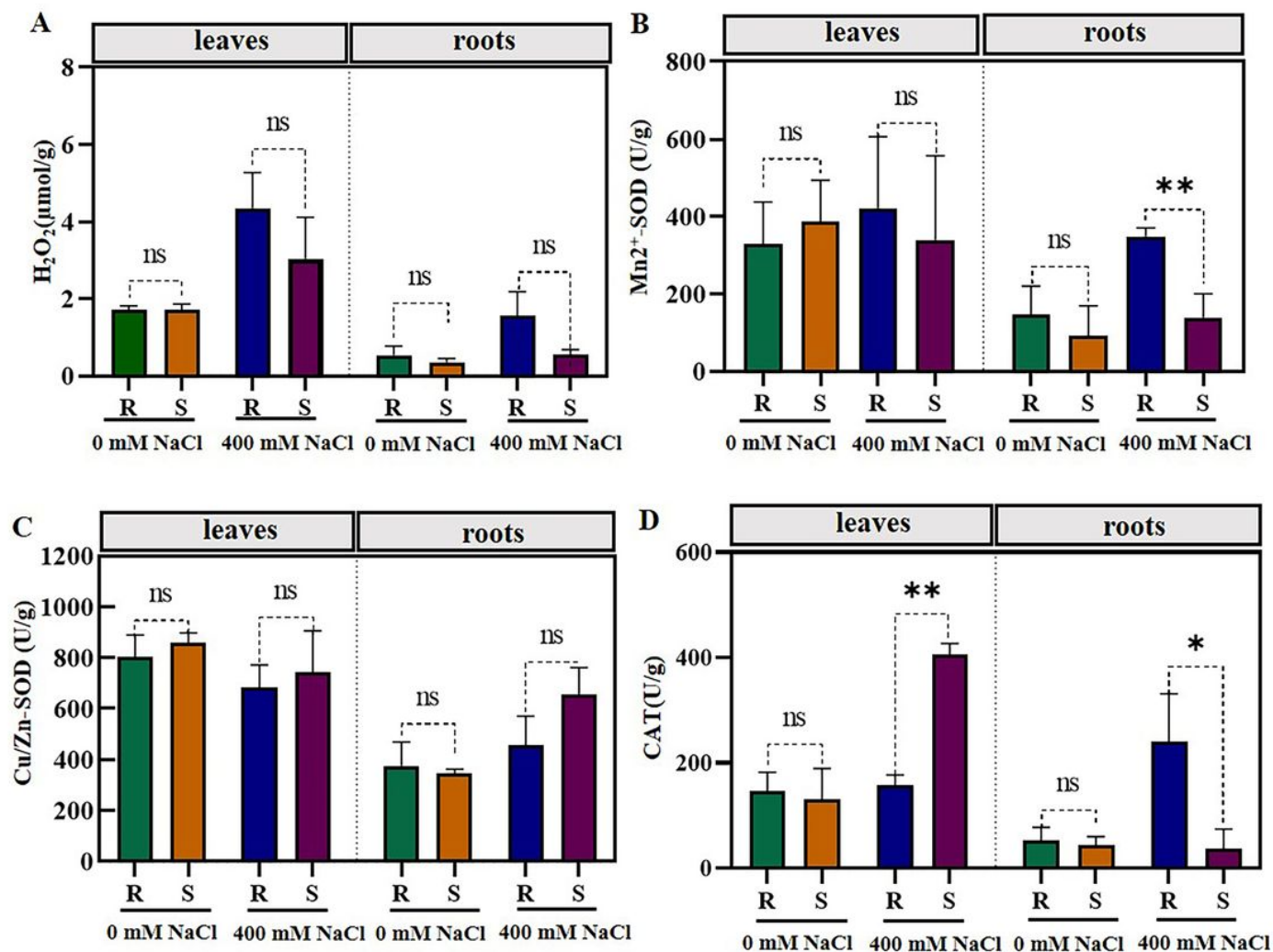


Figure 2

(A) Effect of salinity (400 mM NaCl) on H_2O_2 concentration in roots and leaves of tolerant and sensitive plants. **(B–D)** Distinctive alterations in the activity of enzymatic players (Mn^{2+} -SOD, Cu/Zn-SOD and CAT) in the two *P. vaginatum* plants under salinity (* significant at $p < 0.05$; ** significant at $p < 0.001$; ns, not significant; two-sided Student's t -test).

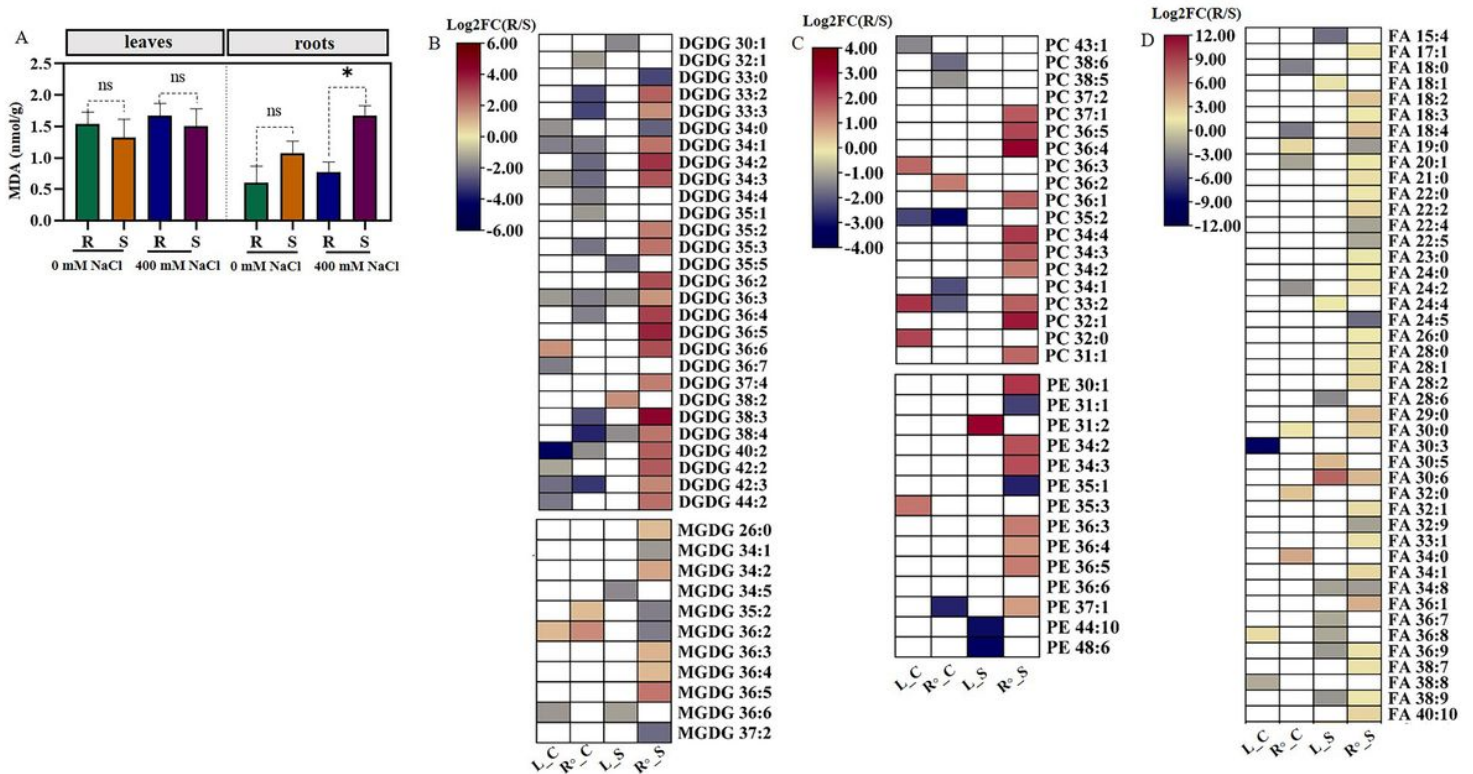


Figure 3

(A) Alterations in the concentration of MAD between the two *P. vaginatum* plants under non-salted and salted conditions. **(B-D)** Effect of salinity (400 mM NaCl) on lipid metabolism in roots and leaves of salt-sensitive and salt-tolerant plants. (* significant at $p < 0.05$; ** significant at $p < 0.001$; ns, not significant; two-sided Student's *t*-test).

Abbrev: 'R': salt-tolerant accession; 'S': salt-sensitive accession; 'T': salt treatment; 'C': unsalted treatment; 'L': leaf; 'R': root).

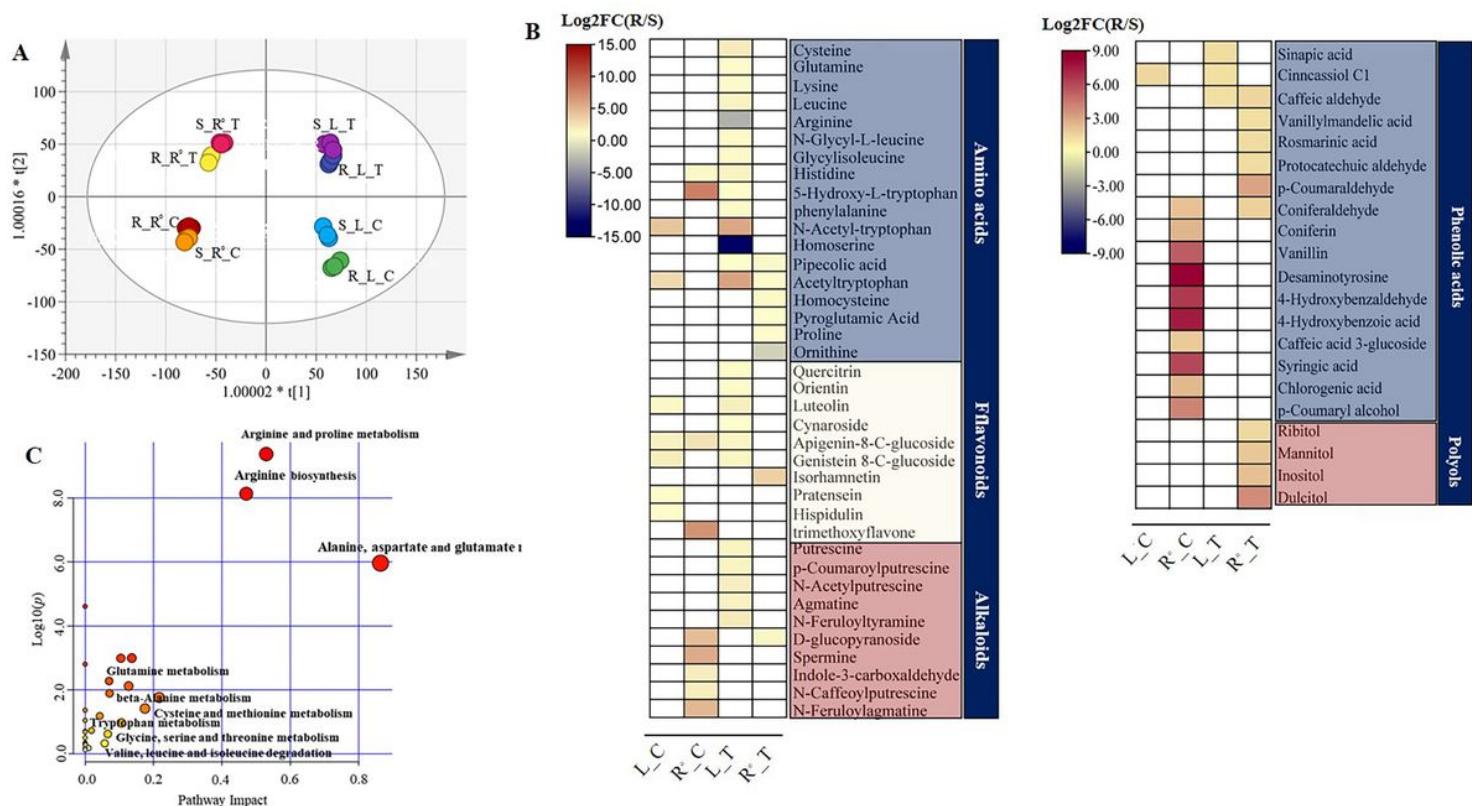


Figure 4

(A) Scatter plots of O2PLS-DA model of different samples for profiling metabolomics. Each symbol represents an independent sample in the score scatter plot and an independent annotated peak in the loading plot; **(B)** Effects of salinity (400 mM NaCl) on metabolic changes in salt-tolerant plants compared to salt-sensitive plants. Heat maps indicate the log_2 transferred value of the fold change between salt-tolerant plants and salt-sensitive plants treated with or without 400 mM NaCl. **(C)** The top enriched KEGG terms of DEGs from leaves in the salt-treated tolerant plants of *P. vaginatum* compared to the salt-sensitive plants.

Abbrev: 'R': salt-tolerant accession; 'S': salt-sensitive accession; 'T': salt treatment; 'C': unsalted treatment; 'L': leaf; 'R': root).

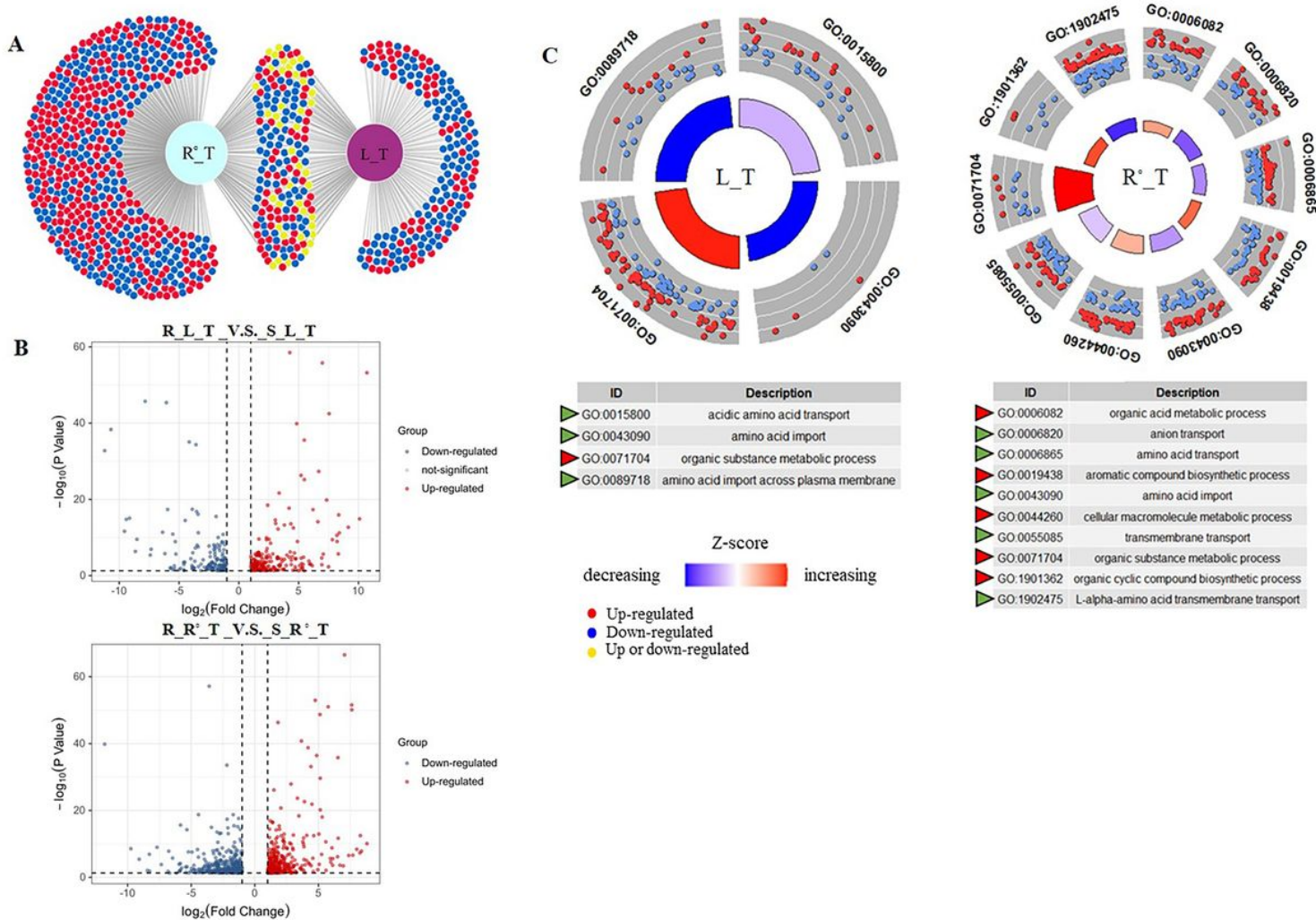


Figure 5

(A) Venn diagram and **(B)** Volcano plot presenting the number and regulation of differentially expressed genes (DEGs) identified from comparative transcriptome analyses of the two *P. vaginatum* accessions under salt stress and non-stress conditions. **(C)** GO graphs showing the GO terms most enriched involved by DEGs of compound metabolic process in salt-treated leaves and roots.

Abbrev: 'R': salt-tolerant accession; 'S': salt-sensitive accession; 'T': salt treatment; 'C': unsalted treatment; 'L': leaf; 'R': root).

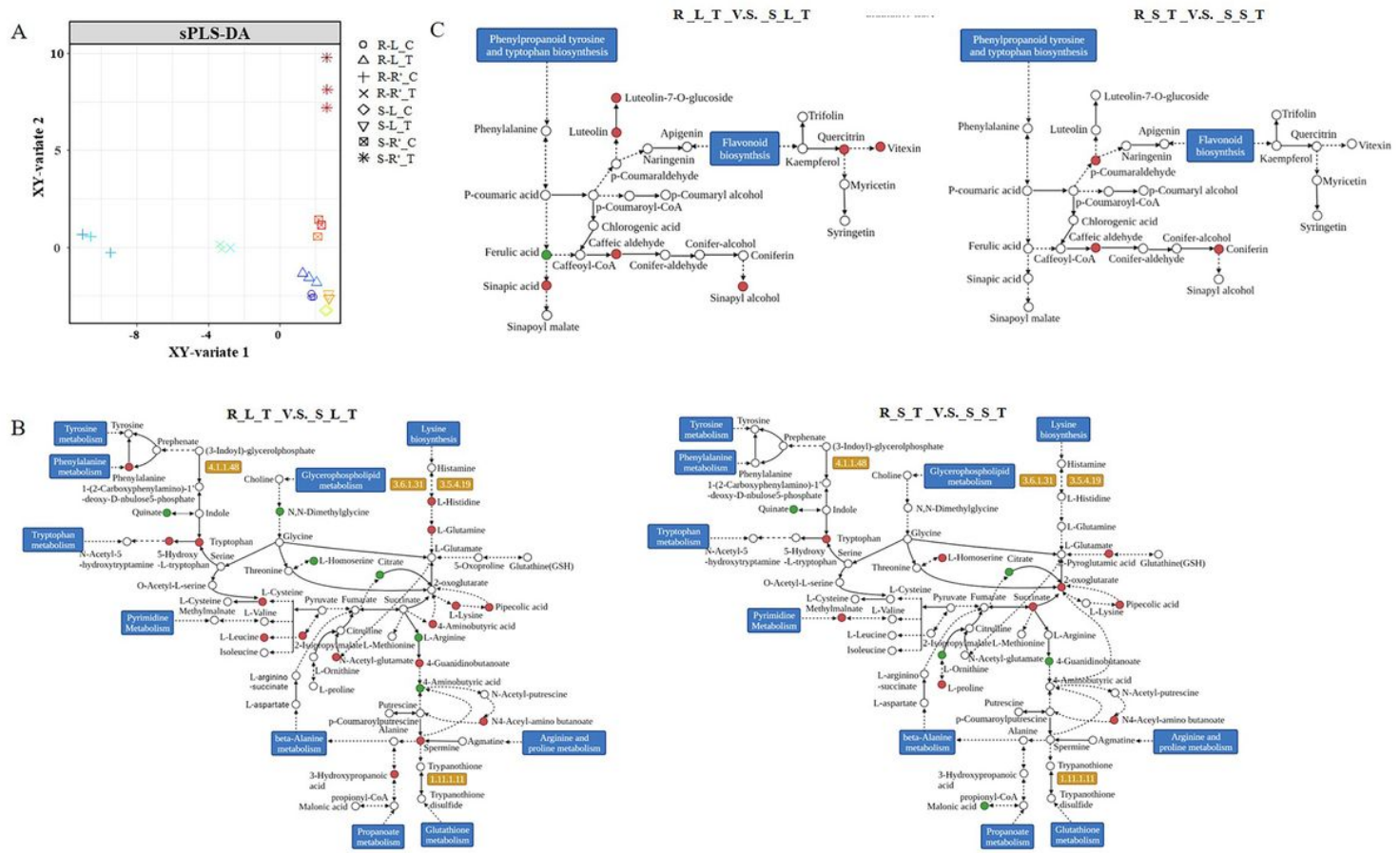


Figure 6

(A) Scatter plots of the sparse PLS-DA model on the first two components using significant metabolites and DEGs based on comparison analysis. **(B-C)** Complex dynamic regulatory networks of important metabolites involved in amino acid metabolism and their corresponding genes in roots of salt-tolerant *P. vaginatum* accession. Solid red or green circles represent either increased or decreased changes in metabolite abundance and gene expression.

Abbrev: 'R': salt-tolerant accession; 'S': salt-sensitive accession; 'T': salt treatment; 'C': unsalted treatment; 'L': leaf; 'R': root).

