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Early-responsive molecular signatures associated with halophytic adaptation in *Sesuvium portulacastrum* (L.)

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ABSTRACT

Sesuvium portulacastrum (L.) is a halophyte, adapted to grow naturally in saline environments. Under low and high salt conditions, an inverse correlation is seen between K and Na accumulation, suggesting its facultative halophytic nature and ability to use K or Na interchangeably. No significant growth reduction occurs in seedlings up to 250 mM NaCl, except for curling of the youngest leaf. Within 8 h of salt treatment seedlings accumulate proline, glycine betaine and other amino acids in both root and shoot. Despite a continued increase of tissue Na content, the number of differentially expressed genes (DEGs) decreases between 8 h and 24 h of salt exposure, indicating transcriptional restoration after the initial osmotic challenge. At 8 h, up-regulated genes mainly encode transporters and transcription factors, while genes in growth-related pathways such as photosynthesis and ribosome-associated biogenesis are suppressed. Overexpression of *SpRAB18* (an ABA-responsive dehydrin), one of the most strongly induced DEGs, in soybean was found to increase biomass in control conditions and the growth benefit was maintained when plants were grown in 100 mM NaCl, indicating conservation of function in halophyte and glycophyte. An open-access transcriptome database “SesuviumKB” (<https://cb.imsc.res.in/sesuviumkb/>) was developed to involve the scientific community in wide-scale functional studies of *S. portulacastrum* genes, that could pave the way to engineer salt tolerance in crops.

Keywords: Halophyte, transporters, transcription factors, salt tolerance, osmotic adjustment, abscisic acid.

1. INTRODUCTION

Salinity stress limits crop productivity across the globe (Raza et al., 2022). About 1125 million hectares of land throughout the world are saline and one fifth of the total irrigated land are affected by salinity (Hossain, 2019). Most crop plants are glycophytes, susceptible to soil salinity with the electrical conductivity of more than 4 dS m⁻¹ (~40 mM NaCl) (Bello et al., 2021). In contrast, a unique category of plants representing less than 2% of the global flora, can survive in high salinity, and are referred to as halophytes (Flower and Colmer, 2017; Rahman et al., 2021). Over the last couple of decades, research has been carried out on a range of halophytes to characterize the responses of halophytes to salt at multiple organizational levels including physiological, molecular, biochemical, metabolic, proteomic and ionomic (Panda et al., 2020; Duan et al., 2023; Chen et al., 2023). Understanding the molecular mechanisms underpinning salinity tolerance in halophytes offers opportunities for improving salinity tolerance in crop plants. Several attempts have been made to identify salt responsive gene(s) in halophytes and use them as transgene(s) for improving the salt tolerance in glycophytic plants (see Table 2 in Rahman et al., 2021). For instance, the transcriptomic studies in roots of *Avicennia officinalis* revealed upregulation of cytochrome P450 gene (CYP94B subfamily) (Krishnamurthy et al., 2017). Heterogeneous expression of *AoCYP94B1* increased salt stress tolerance in *atcyp94b1* mutant of *Arabidopsis* (Krishnamurthy et al., 2020). Similarly, other genes including C2H2 zinc finger gene (*ZjZFN1*) from *Zoysia japonica* (Xie et al., 2015; Teng et al., 2018) and phosphatidylserine synthase-encoding gene from *Salicornia europaea* (Fan et al., 2013; Lv et al., 2021) have been identified from transcriptomics studies in halophytes and later on used for improving stress tolerance in glycophytes. While these studies are encouraging, it often remains unclear whether the halophytic genes have specific functional or regulatory properties compared to the glycophytic homologues that are critical for salt tolerance in the endogenous system. The endogenous functions of the differentially expressed genes and the related physiological traits of the halophytic donor often remain uncharacterised, due to lack of amenability to genetic manipulation. Novel tissue regeneration and gene editing techniques might provide opportunities to close this knowledge gap in the future (Zafar et al., 2020; Cardi et al., 2023). However, most of these studies lack detailed physiological evidence about the plant's capacity to use Na-K interchangeably without negative impacts on growth, which is one of the signatures of halophytic traits.

Sesuvium portulacastrum (L.) is a fast-growing, dichotomous, perennial, facultative halophyte, commonly termed as sea purslane (Lokhande et al. 2009). *S. portulacastrum* shows high photosynthetic efficiency when grown under salinity (Muchate et al., 2016). It principally assimilates CO₂ via C₃, however, a recent study showed that it also operates a CAM at low level (Winter et al., 2019). Earlier studies suggest that several processes contribute to the halophytic potential of *S. portulacastrum* including increased osmolyte accumulation (Yi et al., 2014), up-regulation of sodium (Na⁺) transporters, increased antioxidant capacitance (Nikalje et al. 2018), altered energetic and redox status (Lokhande et al., 2011), regulation of the stability of PS II complex and improved carbon fixation (Peng et al., 2019), induced activity of V-ATPase/P-ATPase (Yi et al., 2014, Ding et al., 2022) and resilience of primary metabolism (Kulkarni et al., 2022). Fan et al. (2009) reported that fructose-1,6-bisphosphate aldolase (*SpFBA*) from *S. portulacastrum* improves salt tolerance of *E. coli*. Subsequent studies on salt-responsive genes from *S. portulacastrum* have reported increased salt tolerance of tobacco and *Arabidopsis* expressing the aquaporin *SpAQP1* (Chang et al., 2016) and the Na-transporter *SpSOS1* (Fan et al., 2019), respectively. Besides, increased osmotic/drought tolerance has been reported in transgenic *Arabidopsis* expressing *SpBADH*, a key enzyme in glycine betaine biosynthesis (Yang et al., 2015). These studies have demonstrated the potential usefulness of salt-responsive genes

from *S. portulacastrum*, but until now the lack of a comprehensive analysis of its transcriptome has prevented wider exploration of this halophytic gene pool.

With this background, the main aim of the present study was to perform *de-novo* assembly of the root and shoot transcriptomes of *S. portulacastrum*, gene annotation and identification of DEGs after salt treatment. To put the transcriptional responses into a physiological context, we also performed metabolomics analysis and Na/K measurements. One of the most strongly salt-induced genes (Response to ABA 18, *SpRAB18*) was tested for its ability to increase salt tolerance in soybean. Finally, we developed an open-access transcriptome database “SesuviumKB” based on results from this study to facilitate data access by the scientific community and enable future exploration of this halophytic gene pool.

2. MATERIALS AND METHODS

2.1. Plant material and treatments

Stem cuttings of *S. portulacastrum* were collected from Thane creek, near Mumbai (GPS location - 19° 3' 35.28" N, 72° 57' 0" E). Collected stem cuttings (~5-6 cm long) were washed with Tween-20 for five minutes followed by five washes with water. Washed stem cuttings were incubated at 25 ± 2 °C under 12/12 h photoperiod (white fluorescent tube lights) in hydroponics with half strength Murashige & Skoog (MS) medium to allow rooting and seedling establishment. Different tissues of shoot (upper leaf, middle leaf, lower leaf and stem) were harvested immediately after transfer to hydroponics (T0) and at several time points thereafter (8 h to 15 d), and tissue Na and K levels were quantified. Change in the level of Na and K at each time point were compared with those of T0. To study the impact of NaCl on the rooting potential of *S. portulacastrum*, another set of stem cuttings were hydroponically established either in control (half-strength MS with 0 mM NaCl added) or in variable NaCl concentrations (half-strength MS with 50 - 250 mM NaCl added), and root length variation and average fresh weight/seedling were measured after 20 d (Figure S1a). Further sets of experiments were started once the seedlings were fully established (15 d growth after transfer of stem cuttings to hydroponics, T0). The 15-d old seedlings were treated with various NaCl concentrations (0 - 250 mM in half strength MS) and differential phenotypes were recorded at 20 d after treatment (Figure S1b). At 250 and 0 (control) mM NaCl concentrations, different plant parts (upper leaf, middle leaf, lower leaf, stem and root) were harvested over a time course of 8 h to 15 d after treatment for determination of K and Na contents (Figure S1c).

For hormonal profiling, osmolyte quantification, LC-MS-based metabolomics and RNAseq studies, the 15-d old hydroponically grown seedlings were divided into three sets. In the first set, fresh half strength MS medium was added and root and upper leaf (shoot) tissues were then harvested immediately as control (CR-root; CS-shoot). Second and third sets were treated with 250 mM NaCl (in half strength MS medium). The second set was harvested after 8 h (NR8, root; NS8, shoot) and the third was harvested after 24 h (NR24, root; NS24, shoot) (Figure S1d).

2.2. Analysis of ion content

For elemental analysis, all the harvested tissues were washed thrice with chilled Milli-Q water and then dried in an oven at 65°C for 10 d, when they had reached constant weight. The dried tissues were acid digested in concentrated nitric acid and thereafter dissolved in Milli-Q water. Na and K concentrations were determined using flame atomic absorption spectroscopy and represented as mg g⁻¹ dry weight (DW).

2.3. Hormone and osmolyte profiling

Abscisic acid (ABA) and zeatin concentrations were measured using LC-MS (LC- Agilent 1290 Infinity UHPLC; MS- Thermo Fisher-TSQ Vantage). Briefly, 500 mg harvested samples were ground in liquid nitrogen and 500 mg samples were homogenized in 90% methanol (twice the volume of sample). Samples were sonicated for 2 minutes, followed by centrifugation at 14500 rpm for 5 min at 4°C. Supernatant was transferred to a new tube and spiked with Tryptamine-D4 (internal standard) (10 ml of 0.25 mg/ml stock). Spiked samples were vacuum dried and re-dissolved in 50 µl of 20% of methanol. 10 µl of these samples were injected in LC-MS. The standard curve for each hormone was prepared by serially diluting standards and processed similarly as samples. Peaks of ABA (10.58) and zeatin (4.67) were identified based on retention times. Peak area of each sample was compared with that of respective standards for quantification.

The levels of proline and glycine betaine were quantified through spectrophotometric methods, according to Bates et al. (1973) and Grieve and Grattan (1983), respectively.

2.4. LC-MS- based metabolite profiling

Briefly, 100 mg liquid-nitrogen grounded samples were homogenized in methanol by vortexing for 10 min. The mixture was sonicated for 20 min, followed by centrifugation at 13,000 rpm for 10 min. The supernatant was filtered by 0.2 µm syringe filter and used for LC-MS-based metabolite profiling. The protocol described by Ghate et al. (2022) was used for LC-MS-based metabolite profiling. All the data was uploaded on XCMS online (<https://xcmsonline.scripps.edu/>) for deconvolution, deisotoping and peak detection, and further retention time alignment and peak integration were performed on the same server (Smith et al., 2006). Moreover, the identities of four major amino acids, tyrosine, phenylalanine, glutamine and tryptophan, were confirmed by acquiring MS-MS fragmentation data at 10, 20 and 40 eV collision energies and comparing daughter ion spectra with theoretical fragmentation generated using CFM-ID webserver (<https://cfmid.wishartlab.com/>; Figure S2a-d). Minimum matches of 3 daughter ions were used as criteria for identity confirmation.

2.5. Transcriptomics and quantitative real-time PCR

Total RNA was isolated using PureLink RNA Mini Kit (Invitrogen) and RNA quality check was performed by running on agarose gel and by checking 260/280 and 260/230 ratio. RNA sequencing libraries were prepared with Illumina-compatible NEBNext® Ultra™ Directional RNA Library Prep Kit (New England BioLabs, MA, USA). For mRNA isolation, fragmentation and priming, 200 ng of total RNA were taken. Fragmented and primed mRNA was further subjected to first strand synthesis in the presence of Actinomycin D (Gibco, life technologies, CA, USA) followed by second strand synthesis. The HighPrep magnetic beads (Magbio Genomics Inc, USA) were used for the purification of the double-stranded cDNA. Purified cDNA was end-repaired, adenylated and ligated to Illumina multiplex barcode adapters as per NEBNext® Ultra™ Directional RNA Library Prep Kit protocol. The adapter-ligated cDNA was purified using HighPrep magnetic beads and was subjected to 13 cycles of Indexing-PCR (37 °C for 15 min followed by denaturation at 98° C for 30 sec, cycling (98 °C for 10 sec, 65 °C for 75 sec) and 65 °C for 5 min) to enrich the adapter-ligated fragments. The final PCR product (sequencing library) was purified with HighPrep magnetic beads, followed by a library quality control check. The Illumina-compatible sequencing library was quantified by a Qubit fluorometer (Thermo Fisher Scientific, MA, USA) and its fragment size distribution was analyzed on Agilent 2100 Bioanalyzer. The libraries were pooled in equimolar quantities and sequenced on Illumina HiSeq XTen sequencer (Illumina, San Diego, USA) using 150 bp paired-end chemistry and HiSeq XTen SBS reagents. De-novo

assembly of the reads, gene-annotation and differential gene expression was performed using the customized pipeline (Supplementary method-1). The script code used for generating de-novo assembly is given as supplementary method-2. The detailed R notebook rendered to HTML containing codes for DESeq2-based identification of DEGs for root and shoot-specific are given as supplementary method-3 and 4, respectively. Detailed methodology for downstream analysis of RNAseq data is provided in supplementary method-5. The quantitative real-time PCR was performed, as per the method described previously (Sahoo et al., 2023).

2.6. Development of web-based *SesuviumKb* database

An online knowledge base (*SesuviumKb*) was generated, which comprised of the entire transcriptome data from shoot and root of *Sesuvium portulacastrum* (L.). To create the web interface of *SesuviumKb*, we used PHP (<http://php.net>), HTML, CSS, Bootstrap 5, jQuery (<https://jquery.com>) and Google Charts (<https://developers.google.com/chart/>) to enable interactive visualization. *SesuviumKb* utilizes MariaDB (<https://mariadb.org/>) for data storage, Structured Query Language (SQL) for information retrieval, and Apache (<https://httpd.apache.org>) web server running on Debian 9.4 Linux Operating System for hosting. The detailed methodology for developing *SesuviumKB* is given as supplementary method-6.

2.7. Heterologous expression of *SpRAB18*

The ORF of the *SpRAB18* gene sequence was amplified from cDNA using gene-specific primers (with Kpn I and Sal I at the end). The amplified product was cloned in the plant expression vector (pCambia1305) and further gene sequence of the cloned vector was confirmed using gene-specific primers. Construct of *SpRAB18* and empty vector (pCambia1305) were transformed into *Agrobacterium rhizogenes* strain ARqua1. Further, the *A. rhizogenes* separately carrying *SpRAB18* construct and empty vector (vector control), along with water (for water control) were injected into soybean seedlings, according to the protocol reported by Kuma et al. (2015). Transformed soybean seedlings were tested by PCR amplification of genomic DNA, with both gene-specific (*SpRAB18*) and hygromycin (selection marker on the vector) primers. Soybean lines with at least 15-fold transient expression of *SpRAB18* were selected for phenotyping. 20-d old seedlings of the transgenic lines were treated independently with control and 100 mM NaCl, and the phenotypes were recorded after 9 days.

2.8. Statistical analysis

All experiments were performed in a completely randomized design and were carried out with at least three biological replicates, that represent independent set of experiments. Student's t-test was executed using MS-Excel for testing the significance of differences between two means. One-way ANOVA followed by Duncan's multiple range test (DMRT) based *post-hoc* ranking ($P < 0.05$) was carried out to determine statistical significance using SPSS 17.0 software. LC-MS-based metabolite analysis was carried out using MetaboAnalyst 5.0 software (Chong et al. 2019). Principle component analysis (PCA) and sparse partial least squares-discriminant analysis (sPLS-DA) were performed to explain the pattern of variation between different treatment groups. Cluster analysis was carried out to evaluate the relative abundance and relation among detected metabolites by constructing a heatmap.

3. RESULTS AND DISCUSSION

3.1. Rooting response and spatio-temporal dynamics of Na and K in *S. portulacastrum* under NaCl stress

Spatio-temporal measurement of Na and K during rooting of *S. portulacastrum* in control medium (half-strength MS with no NaCl added) revealed that a considerable amount of Na was already present in the tissues due to previous growth in the naturally saline environment. Na-levels initially increased slightly in upper leaf by 1.4-fold at 1 d and then decreased to reach 0.1-fold at 15 d, compared to T0 time-point (start of the rooting period). Similar dynamics were observed in middle leaf and stem, where Na-levels at 15 d were decreased to 0.6 and 0.7-fold of the initial values, respectively. No significant change in Na-level was observed in the lower leaf at any of the time-points, except for a 1.9-fold increase at 1 d, compared with T0 time-point (Figure 1a). Given that the control medium contains only trace levels of Na, it can be assumed that the observed dynamic changes reflect the re-distribution of pre-existing Na, with an initial influx of Na from the surface of the cuttings into tissues followed by leakage out of the tissues. The initial transient increase of tissue Na levels was followed by a strong accumulation of K over the following days in all the tissues, with maximum increases of 4.0 and 3.9-fold at 15 d (compared to T0) in upper and middle leaf, respectively (Figure 1b). Further, a clear inverse correlation was observed between Na and K accumulation for 15 d, especially in the upper-leaf ($r = -0.84$), middle leaf ($r = -0.96$) and stem ($r = -0.73$), suggesting that K tend to replace Na when grown under the non-saline conditions (Figure 1a-b). This highlighted the possibility that *S. portulacastrum* is evolved in such a manner that both Na and K can be used interchangeably to perform osmotic function, as suggested previously (Wakeel et al., 2011; Pilon-Smits et al., 2009).

Successful rooting from stem cuttings was still observed upto 250 mM NaCl concentrations. Compared to control (0 mM NaCl) the root length was maximally increased (1.7-fold. $p = 0.0001$) in 100 mM NaCl (Figure 1c; Figure S3). After 20 d of growth period, no significant difference in fresh weight of the seedlings was observed between the tested NaCl concentrations (Figure 1d). When NaCl was applied after a 15-d period of seedling pre-establishment in control conditions, leaf curling response was noticed, especially at higher NaCl concentrations (Figure 2a). The curling or rolling of the young leaf is considered as a signature phenotype that can help plants to reduce light interception, water transpiration and hence, avoids dehydration under NaCl-induced osmotic stress (Ali et al., 2022). Shoot length (Figure 2b), shoot biomass (Figure 2c), root length (Figure 2d) and root biomass (Figure 2e) varied slightly between seedlings exposed to different salt concentrations, but shoot and root lengths were significantly higher in 250 mM NaCl compared with those of 0 mM NaCl control (1.3-fold), while biomass of both tissues was lower in 250 mM compared to control (0.6 fold). The opposite changes in biomass and length, reflect either altered thickness (both root and shoot) and/or succulence (leaf), that needs further investigations. Beyond 250 mM NaCl, the significant biomass reduction was seen. At 500 mM NaCl, shoot biomass was reduced by 0.13-fold, indicating compromised halophytic potential of *S. portulacastrum* (Figure S4), which has also been substantiated by previous studies (Nikalje et al., 2018; Wang et al., 2022). Considering this, the subsequent experimentations were performed at a fixed dose of 250 mM NaCl, to ensure that the halophytic potential should be maintained. The maximum level of Na-accumulation in seedlings after 15 d treatment duration (70 mg/g) was found to be comparable with the initial Na-level in shoot-cuttings harvested from natural habitat (68 mg/g). This further suggested that the selected 250 mM NaCl concentration mimicked the salt level at which *S. portulacastrum* grows naturally.

Application of 250 mM NaCl to the pre-established, rooted seedlings, led to a strong increase of Na-concentration in the upper leaf reaching 11.8-fold of higher concentrations Na at 15 d than the

control seedlings grown without NaCl (Figure 3a). Considerable Na accumulation was also recorded in the root (6.2-fold), while lower leaf (1.3-fold) and stem (1.2-fold) showed only a small increase, while middle leaf showed no consistent change compared to control (Figure 3a). It is possible that this spatial difference is due to the fact that the older tissues were already fully established at cutting and contained considerable amounts of Na at the start of the experiment, whereas the upper leaf and the roots mostly developed/grew in the NaCl-free MS medium after cutting, and contained very little Na at the start of the experiment. K-concentrations in root and stem did not differ between control and salt treated seedlings and were only slightly decreased in lower and middle leaves of salt-treated seedlings (Figure 3b). By contrast, K-concentrations in the upper leaf were significantly lower in salt-treated seedlings than in control seedlings (0.6 of control at 15 d). This was due to the fact that the upper leaves in the NaCl-treated seedling did not show the strong K accumulation that occurred in the upper leaves of the control seedlings. Till 15 d of NaCl treatment, K-level remained unchanged in root and stem, except for 0.8- and 0.7-fold reduction at 3 and 12 d post-NaCl stress, respectively in stem, compared with those of respective control. In contrast, decreased K-level was noticed in other tested tissues. (Figure 3b).

In summary, *S. portulacastrum* showed NaCl stress tolerance up to 250 mM with respect to seedling establishment, rooting and growth but exhibits some leaf curling. Furthermore, at 250 mM NaCl, K accumulation in the upper leaf is increasingly replaced with Na accumulation. Using Na as a 'cheap' osmoticum and tolerating high Na levels in the leaves substantiates that *S. portulacastrum* showed the characteristic traits of halophytes, even if adapted to grow under non-saline conditions.

3.2. Differential responses of phytohormones, osmolytes and amino acids

Phytohormones and organic osmolytes play an important role in plant salt tolerance, particularly for adaptive responses to osmotic challenge. We therefore measured levels of the 'stress' hormone ABA as well as zeatin, a growth hormone of the cytokinin family (Schäfer et al., 2015), and of the well-known compatible osmolytes proline and glycine betaine in *S. portulacastrum* seedlings before (CR/CS; Control root/control shoot) and after treatment with 250 mM NaCl (8 h; NR8/NS8 and 24 h; NR24/NS24). In roots, ABA was only detected under NaCl treatment. At 8 h after adding NaCl, the ABA level in NR8 treatment was 2.3-fold higher than at NR24 (Figure 4a). In shoots, ABA was already detected before addition of NaCl. No change was seen at NS8 and a small decrease (to 0.7-fold of control) was observed at NS24 treatment (Figure 4a). The zeatin level remained unaltered in roots, while in shoots, it was slightly decreased at 8 h and 24 h (~0.8-fold of control, Figure 4b). The results demonstrate a strong ABA response in roots, particularly during the early phase of the salt challenge, which is dominated by hyperosmotic stress (Munns and Tester, 2008). Thus, *S. portulacastrum* shows an osmotic stress-induced ABA response in roots, similar to other plants (Li et al., 2019). The absence of an ABA increase in the shoots could be due to the potential of *S. portulacastrum* to utilize Na and K interchangeably (see Figures 1a-b and 3) and therefore might not perceive an additional challenge within the given time period. More localized studies, specifically in guard cells, might reveal changes of leaf ABA that were not resolved here.

The proline level increased by 4.3- and 9.9-fold at NR8 and NR24, respectively, compared with CR. In shoot, proline was increased ~1.8-fold at NS8 and NS24, respectively, compared with CS (Figure 4c). Glycine betaine was increased by 1.4- and 1.7- after NR8 and NR24, respectively, and by 1.4-fold at NS8 (Figure 4d) treatments. Under NaCl stress, the early hours are known to be dominated by osmotic-stress component; while, ionic stress becomes prominent only at the later of prolonged stress (Munns and Tester, 2008). Therefore, accumulation of ABA and osmolytes clearly indicated that *S.*

portulacastrum responded to NaCl-induced osmotic disturbances within 8-24 h stress duration. This was also supported by our earlier observations, wherein the activation of antioxidant enzymes and modulation of primary metabolites was observed within 8-24 h after NaCl stress imposition (Kulkarni et al., 2022). Besides, SEM imaging analysis also revealed changes in surface morphology of young leaves, within 24 h of NaCl treatment (Figure S5; Supplementary method-7).

In an untargeted approach, we used LC-MS based metabolite profiling to explore alteration in the metabolome 8 and 24 h after addition of 250 mM NaCl (Figure S6). PCA analysis revealed no visible separation of different treatment groups (Figure S6a-b), while the supervised sPLS-DA analysis showed well separated clusters of different treatment groups in both root (Figure S6c) and shoot (Figure S6d). Heatmap analysis indicated that NaCl treated groups (NR8-NR24 and NS8-NS24) clustered together, but separately from control groups (CR and CS) in both root (Figure S6e) and shoot (Figure S6f). The recent meta-analysis of 30 independent metabolomics datasets including those of *S. portulacastrum* highlighted amino-acid metabolism among the top-ranked salt-stress responsive pathways in halophytes (Kulkarni et al., 2022). Inspired from this, LC-MS based quantification of amino acid was attempted. Four amino acids (tyrosine, glutamine, phenylalanine and tryptophan) were identified and their peak-annotation was confirmed on the basis of MS-MS fragmentation spectra (Figure S2; Figure 4e-h). In both root and shoot, phenylalanine and tryptophan levels were increased at least 1.4-fold at 8 h and 24 h after NaCl addition compared with their respective controls (Figure 4f,h), and tyrosine level was increased by 1.4 and 1.6-fold at 8 h and 24 h (Figure 4e). By contrast, the glutamine level showed a ~0.5-fold decrease at NR8 and NR24 treatments, compared with those of CR. In the shoot, glutamine level was increased by 1.5 and 1.4-fold at NS8 and NS24, respectively, compared with CS (Figure 4g). The increased accumulation of amino acids indicated suppression of active protein biosynthesis, that might contribute to stress adaptation, as demonstrated earlier (Good and Zaplachinski, 1994).

In summary, the metabolite analysis revealed a strong increase of proline and a lesser increase of glycine betaine. Both amino acids are well known to being produced in other plants, including glycophytes, in response to osmotic challenge, albeit their precise function remains a matter of discussion (Dikilitas et al., 2020). We also include here metabolomics data for *S. portulacastrum* seedlings which exhibited differences in the metabolome within the first 24 hours of salt treatment, that can now be explored for further identification of the metabolites underlying these differences. In a first approach, we identified four amino acids that showed small changes upon salt indicating reprogramming of primary metabolism.

3.3. RNA sequencing-based identification of DEGs and database development

After establishing the physiological basis of halophytic-trait, the next objective was to identify the early-responsive molecular signatures associated with halophytic adaptation in *S. portulacastrum*. Towards this endeavour, we selected 8 and 24 h time points, as treatment durations, and de novo assembly and differential transcriptomics was carried out, independently in root and shoot. Previously, a transcriptome analysis has been carried out in *S. portulacastrum* at higher doses of NaCl including 400 mM (Wang et al., 2022), 600 mM (Chen et al., 2022) and 800 mM (Li et al., 2023). However, the significant reduction in shoot biomass was noticed at these concentrations, both in the present (Figure S4) as well as previous studies (Wang et al., 2022), indicating at least the partial loss of halophytic potential at these NaCl concentrations. To avoid this, we intentionally selected 250 mM NaCl for the present study, at which no significant shoot biomass reduction was observed even after 20 d stress duration (Figure 2c). PCA, based on normalised transcript levels, grouped replicate together

while separating different treatment-groups (Figure 5a). Based on FDR value less than 0.05, 675 (up/down: 344/331) and 191 (up/down: 44/147) differentially expressed genes (DEGs) were identified at NR8 and NR24, respectively (Figure 5b); while, 2028 (up/down: 481/1547) and 93 (up/down: 19/74) DEGs were identified at NS8 and NS24, respectively (Figure 5b). Of these, 123 and 42 DEGs were shared between NR8-NR24 and NS8-NS24, respectively (Figure 5b; Table S1 and S2). The number of DEGs was significantly higher at 8 h than at 24 h, in both shoot and root, which was clearly seen in the volcano plots (Figure 5c) and MapMan analysis (Figure S7). Such type of transcriptional restoration has also been shown in other halophytes such as *Atriplex canescens* (Guo et al., 2019) and *Achnatherum splendens* (Liu et al., 2016), indicating the existence of a well-conserved mechanism for salt stress adaptation. Incidentally, these transcriptome studies were also performed between 100–300 mM NaCl dose-range, which is close to the concentration used in the present study. Additionally, the expression changes of the 25 top-ranked DEGs from the NR8/CR comparison were re-analysed using qPCR at variable time points. The obtained results not only correlated well with those from RNAseq ($R^2=0.88$ and 0.61 at 8 and 24 h, respectively), indicating accuracy of our experimental approach and data-analysis pipeline (Figure 5d). In contrast, at 800 mM NaCl, the number of DEGs were found to be increased with stress duration in *S. portulacastrum* (Li et al., 2023), and resembled with those seen in glycophytes like *Arabidopsis* (Gong et al., 2005) and soybean (Liu et al., 2019) (Figure S8). These longer changes in the transcriptome are likely to reflect symptomatic damage rather than early adaptive responses. Therefore, the DEGs identified in the present study are likely to be more relevant for representing the halophytic trait of *S. portulacastrum*.

In root, 635 DEGs were annotated and 108 DEGs were unannotated, while in shoot, 1807 and 272 DEGs were annotated and unannotated, respectively (Figure S9). Additional expression data was also obtained using qPCR from the samples harvested 48 and 72 h after salt addition (Figure 5d). This substantiated that transcriptional restoration goes beyond 24 h stress duration as most of the tested genes were not differentially expressed within the cut-off of ± 1.5 fold, except that *Myb27* and *2OGFe* were upregulated 1.64 (NR72) and 2.39 (NR72) fold compared to CR, respectively, and *HKT1* which was down-regulated 1.84-fold in NR48 compared to CR (Figure 5d). KEGG-based enrichment analysis revealed that the most significantly altered pathways were associated with down-regulated DEGs, such as ribosome (NR8, FDR: $9.49E-11$ and NR24, FDR: $7.87E-13$), biosynthesis of secondary metabolites (NS8, FDR: $1.01E-20$) and photosynthesis (NS8, FDR: $2.6E-7$) (Figure 5e,f). The down-regulation of photosynthesis related GO terms were also observed specifically under NS8 treatment (Figure S10). The phenyl-propanoid biosynthesis pathway was down-regulated in all the treatments (NS8, FDR: 0.0013; NS24, FDR: 0.0063; NR8, FDR: $4.80E-07$ and NR24, FDR: 0.0042) (Figure 5f). Additionally, at NR8, up-regulated DEGs associated with phenyl-propanoid biosynthesis pathway were also significantly enriched (FDR: 0.0297) (Figure 5e). The raw RNAseq data has been deposited at NCBI (GSE235196) and the analysed datasets were used to develop an open-access resource named “SesuviumKB” (<https://cb.imsc.res.in/sesuviumkb/>) (Supplementary method-6).

3.4. Functional classification of DEGs across different treatments

To provide opportunities to link the transcriptomics and the metabolomics results, DEGs were functionally classified using MapMan and the normalized score was calculated for each functional process separately for up- and down-regulated DEGs, based on their $\log_2$ FC and number of DEGs (Supplementary method-5). Positive and negative value of normalized score for each functional process represented their extent of up- and down-regulation, respectively. The maximum MapMan score was obtained for transcription factors (NS8: 795 and -5743.9; NR8: 1367.2 and -343.1) and

transporters (NS8: 206 and -8744.3; NR8: 313 and -494.5) (Figure 5g). Further, transcription factor and transporters were analyzed using heat map-based clustering approach that identified five independent clusters in both transcription factors (Figure 6a) and transporters (Figure 6b). Cluster-1 of transcription factors represents DEGs specifically down-regulated at NR8. Among transcription factors of cluster-1, two *bHLH* family of transcription factors (*ABS5* and *bHLH2*) and *RAP2.11* were down-regulated by more than 2.5-Log₂ fold. Cluster-2 represented up-regulated transcription factors specifically at NS8 and this cluster was dominated by Constans-like zinc finger family. Among 17 transcription factors of cluster-2, three Constans-like zinc finger family (*COL12*, *BBX13* and *BBX12*) were up-regulated more than 4-Log₂ fold. Cluster-3 consisted of down-regulated DEGs at NS8, with lower expression at NS24 at least 1-Log₂ fold. This cluster included two of each AP2/EREBP (*RAV1* and *RAV2*) and WRKY (*WRKY25* and *WRKY26*) family of transcription factors. The expression level of cluster-4 was specifically up-regulated at NR8. Out of 19 cluster-4 transcription factors, *COL7*, *ABR1*, *RAP2.6* and *MYB27* were up-regulated by more than 4-Log₂ fold. Among all five clusters, cluster-5 was the largest cluster and consist of 33 transcription factors which were down-regulated at NS8. Among them, two MYB (*CCA1* and *LHY*), two pseudo ARR (*APRR3* and *APRR5*) and one constans-like zinc finger (*BBX28*) family of transcription factors were down-regulated by more than 5-Log₂ fold (Figure 6a). For transporter category, cluster-1 included NS8 specific up-regulated DEGs and among them, 2 transporters (1 sugar and 1 phosphate) were down-regulated by more than 2- Log₂ fold. Cluster-2 contained down-regulated transporters specifically in the root at both time points. Of these, *HAK5*, *TMT2* and *PHT3* were down-regulated at both time points by more than 2-Log₂ fold. Cluster-3 comprised of NR8-specific up-regulated transporters and the expression level of *ABCA2*, *AAP7*, *AVT6C* and *ABCG21* were up-regulated by more than 2-Log₂ fold. Cluster-4 was the largest cluster among all the identified clusters of transporters and composed of specifically down-regulated DEGs at NS8. Among them, the expression of *OCT-1*, *BOU*, *PHT4;5*, *ABCG5*, *ABCG11* and *DIT1* were up-regulated by more than 4-Log₂ fold. Cluster-5 represented transporters with 8 h specific down-regulated expression in both root (NR8) and shoot (NS8). Of these, the expression of *PPT2* and *PIP2;5* was reduced by more than 2.5-Log₂ fold in both root (NR8) and shoot (NS8) (Figure 6b).

3.5. Differential phenotyping of *SpRAB18*-overexpression lines of soybean

In plants, the ABA signalling pathway is comprised of ABA-PYR/PYL-PP2C ternary complex, which functions to activate the expression of stress-responsive genes in ABA-dependent manner (Cutler et al., 2010). In the present study, an increase of ABA was seen within 8 h of salt treatment (Figure 4a), which coincides with the up-regulation of ABA receptor RCAR1/PYL9 and RCAR3/PYL8 in NR8 and NS8. Besides, PP2C was significantly up-regulated at all treatment groups (Figure S11). The expression of 'Responsive to ABA' RAB18 genes which encode dehydrin family proteins is known to be induced under various diverse ABA-signalled abiotic stresses including cold, salt and drought (Sun et al., 2021). Therefore, *SpRAB18*, which was significantly up-regulated *S. portulacastrum* at NR8 (Log₂FC NR8/C = -5.8), was selected for heterologous expression in soybean to test its potential to increase salt-tolerance in a glycophytic crop. The overexpression lines were confirmed using genomic-DNA PCR with primers for *SpRAB18* (Figure 7a) and for hygromycin resistance gene in the vector (Figure 7b). Three independent lines having the highest *SpRAB18* expression were selected for phenotypic analyses. Compared to WT, the *SpRAB18*-overexpressed lines had increased shoot biomass (control 1.5-fold; NaCl 1.6-fold; Figure 7c-d) and length (control 1.3-fold; NaCl 1.3-fold; Figure 7e) and root biomass (control 1.7-fold; NaCl 1.2-fold; Figure 7f) in both control conditions and 100 mM

NaCl stress conditions. No significant change in root length was noticed (Figure 7g) and no differences were seen in the empty-vector expressing lines.

In summary, ectopic over-expression of *SpRAB18* was found to increase growth of soybean compared with wild-type (Figure 7), and this growth benefit was maintained in saline conditions. Earlier, the *AtRAB18* co-overexpression along with *AtCOR47* has been shown to provide freezing stress tolerance (Puhakainen et al., 2004). However, whether and how *SpRAB18* is integrated with the canonical ABA pathway remains to be studied. Additionally, considering the significant difference in amino acid sequence (~53%; Figure S12), it will be interesting to compare *SpRAB18* and *GmRAB18*, in terms of their ability to impart salt-tolerance in soybean.

4. Conclusion

Halophytes offer opportunities to characterise physiological and metabolic adaptations to salinity and to explore their genomes and transcriptomes for novel components of the regulatory pathways. In the present study, we showed that *S. portulacastrum* is a facultative halophyte which successfully develops and grows in NaCl concentrations up to 250 mM. We further show that similar to other halophytes *S. portulacastrum* can replace K with Na accumulation, in the growing leaf without negative impact. To enable future exploration of this halophytic model by the scientific community, we have provided here extensive data on metabolome and transcriptome over a 24-h time course of NaCl treatment. The raw dataset has been deposited in public repositories. Easy access to the obtained results and possibilities to enhance these datasets in the future with input from the scientific community is offered through a dedicated new database website.

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Conflict of interest

All authors declare no conflict of interest.

Data availability

Raw RNA sequencing data has been submitted to GEO repository of NCBI with accession number GSE235196.

List of the supplementary information

Supplementary method-1: Methodology for the De-novo assembly of the reads, gene-annotation and differential gene expression.

Supplementary method-2: Script containing code used for generating de-novo assembly.

Supplementary method-3: R Notebook rendered to HTML containing code for the DESeq2-based root-specific DEG analysis.

Supplementary methods-4: R Notebook rendered to HTML containing code for the DESeq2-based shoot-specific DEG analysis.

Supplementary method- 5: Downstream analysis of RNAseq data.

Supplementary method-6: SesuviumKb: A transcriptome database.

Supplementary method-7: Field Emission-Scanning Electron Microscopy (SEM) imaging of *S. portulacastrum* leaves.

Figure S1: Experimental layout for figure 1c-d (a), 2 (b), 3 (c) and 4-5 (d).

Figure S2: MSMS fragmentation data of metabolites.

Figure S3: Comparison of root length variation under different NaCl concentration with that of the control.

Figure S4: Phenotyping of seedlings of *S. portulacastrum* subjected to 500 mM NaCl stress under hydroponic condition.

Figure S5: SEM imaging of leaf surface at 8 and 24 h after NaCl treatment.

Figure S6: LC-MS-based metabolite profiling in *S. portulacastrum* under NaCl stress.

Figure S7: MapMan analysis of DEGs in *S. portulacastrum*.

Figure S8: The fold change variations in DEGs of *S. portulacastrum* and glycophytes (*Arabidopsis* and *Soybean*) are given as box-plots.

Figure S9: Percentages of annotated and unannotated transcripts in both root and shoot.

Figure S10: Gene ontology-based functional enrichment analysis.

Figure S11: Schematic representation and expression of ABA signaling under salt stress in *S. portulacastrum*.

Figure S12: Multiple sequence alignment of *SpRAB18* and its orthologous gene from *Glycine max* (soybean) origin.

Table S1: Venn analysis of distribution of DEGs in root.

Table S2: Venn analysis of distribution of DEGs in shoot.

Table S3: List of genes and primers used. a: for gene expression analysis of selected 25 genes by qPCR. b: for the molecular confirmation of transformed soybean seedlings.

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Figures

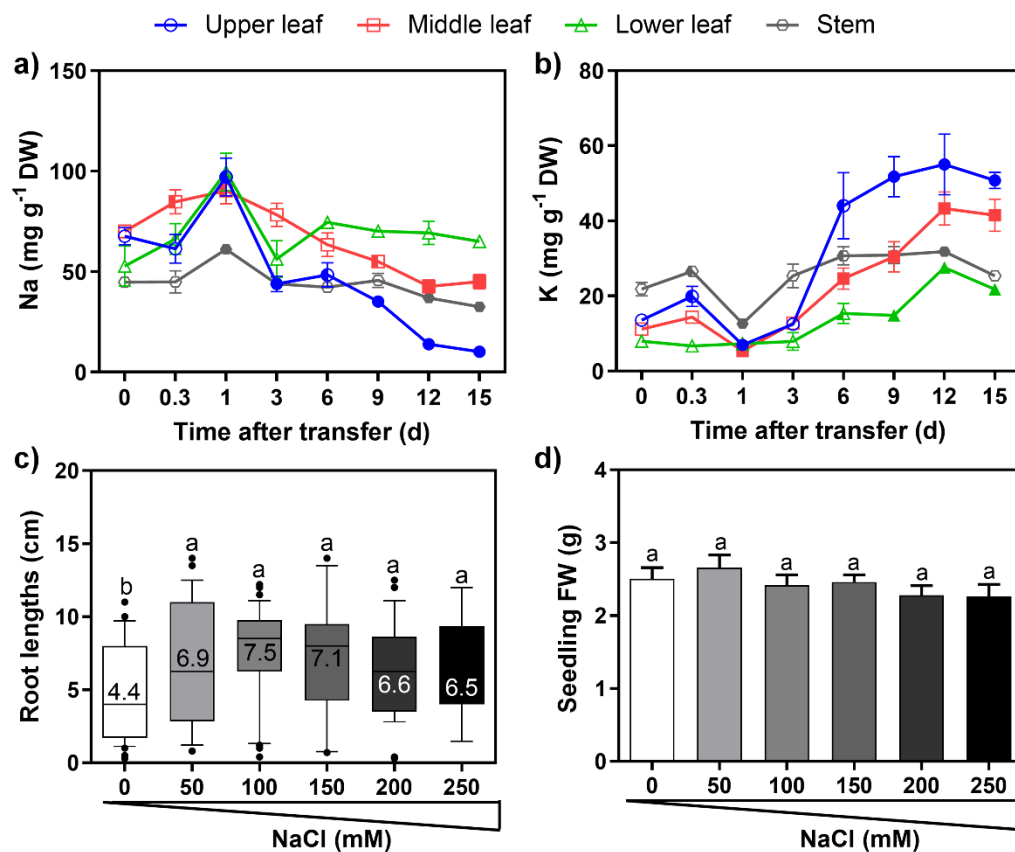


Figure 1

Figure 1: Ion contents, rooting and growth during the establishment of *S. portulacastrum* seedlings. *S. portulacastrum* stem cuttings were transferred to hydroponics for rooting at T0 time point. a, b: Na (a) and K (b) contents in upper leaf, middle leaf, lower leaf and stem during 15 days growth in half-strength MS media (with no salt added). Values are mean $\pm$ standard error of three independent set of experiments (five seedlings/set). Significant differences compared to T0 time point are represented at $P < 0.05$ (half closed symbols), $P < 0.01$ (closed symbols) or non-significant (open symbols), as determined by Student's t-test (for experimental layout refer Figure S1a). c, d: Root length (c) and seedling fresh weight (d) after 20 days of rooting in half-strength MS media with increasing concentrations of NaCl added (0-250 mM; refer Figure S1a for experimental layout). Number in the box-plot represent average root length (c). Duncan's multiple range test (DMRT) using SPSS software was performed and different letters on the bar graph indicate significant difference at $p \leq 0.05$.

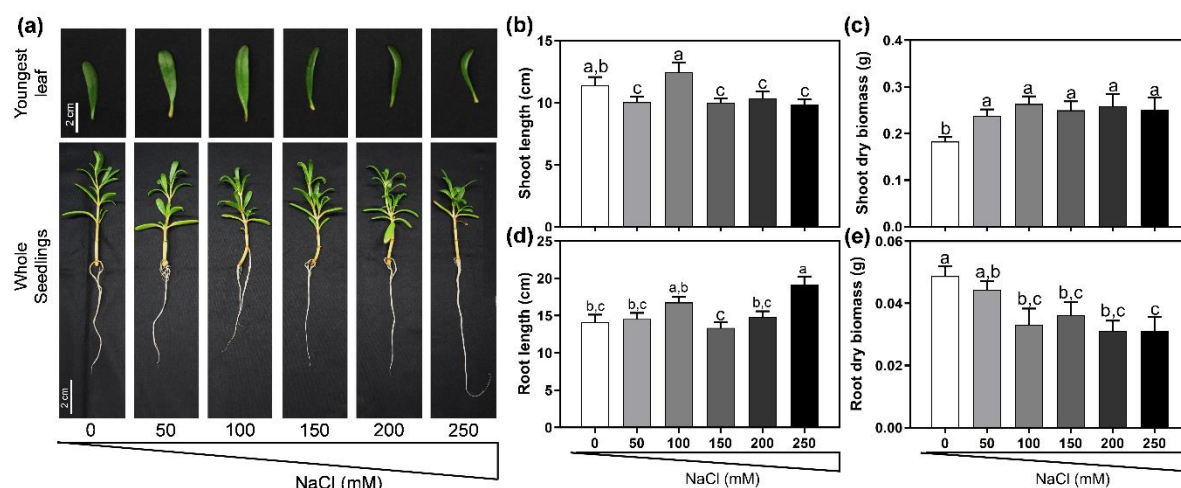


Figure 2: Growth phenotypes of *S. portulacastrum* under different NaCl concentrations. *S. portulacastrum* seedlings were allowed to establish for 15 d in half-strength MS media before being subjected to different NaCl concentrations (half-strength MS media with 0 - 250 mM NaCl added). At 20 d after treatment, phenotypes were recorded. Refer Figure S1b for experimental layout. a: photos of whole seedlings and youngest leaves. b-e: Quantitative data for shoot length (b), shoot dry biomass (c), root length (d) and root dry biomass (e). Values are mean±standard error of three independent set of experiments (five seedlings/set). Duncan's multiple range test (DMRT) using SPSS software was performed and different letters on the bar graph indicate significant difference at $p \leq 0.05$.

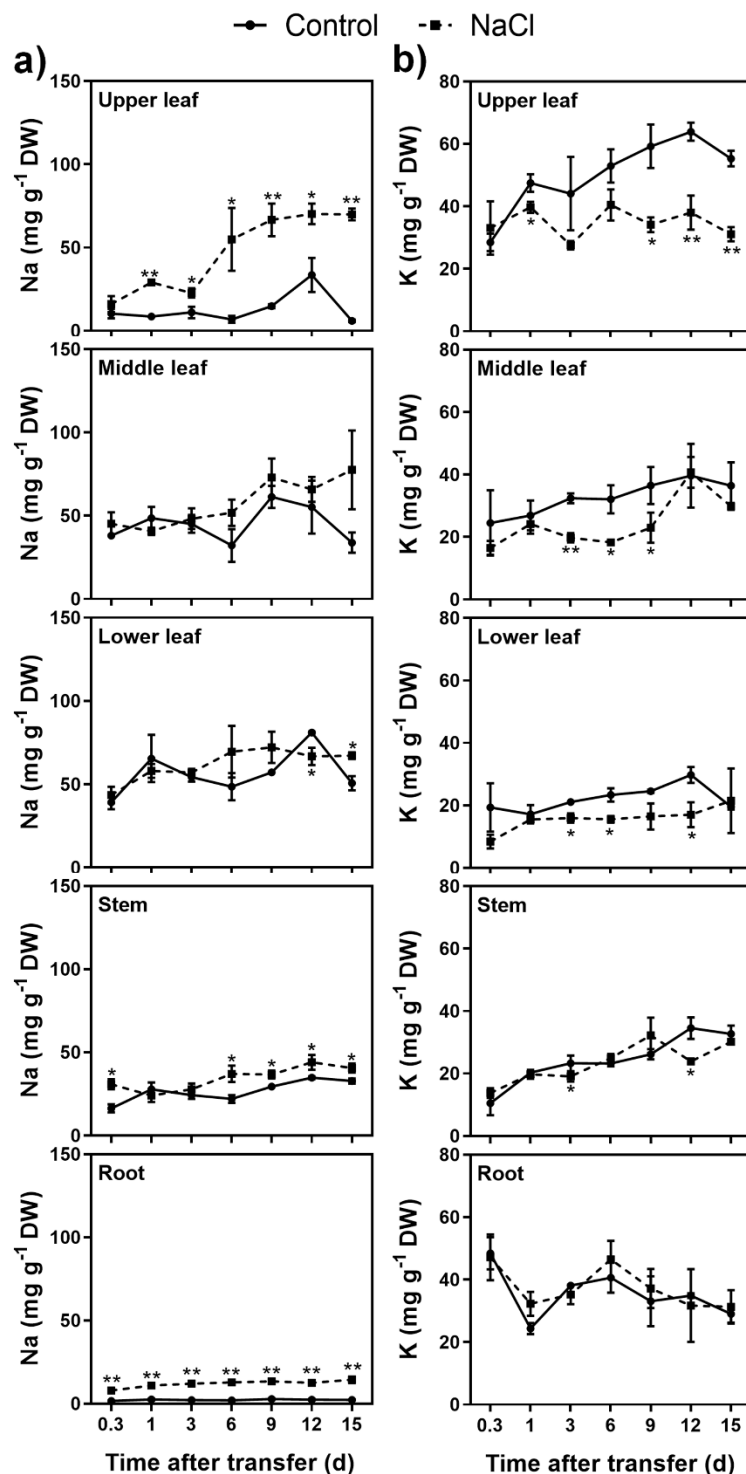


Figure 3: Ion accumulation in *S. portulacastrum* seedlings under NaCl stress. *S. portulacastrum* seedlings were allowed to establish for 15 d in half-strength MS media before being transferred to fresh half-strength MS media with 0 (control, solid lines) or 250 mM NaCl (dashed lines) added. Na (a) and K (b) contents in upper leaf, middle leaf, lower leaf, stem and root were measured for 15 days after transfer. Refer Figure S1c for experimental layout. Values are mean±standard error of three independent set of experiments (five seedlings/set). Error bars indicate standard error. Asterisks indicate significant differences between treatments at $p < 0.05$ (*) or $p < 0.01$ (**) as determined by Student's t-test.

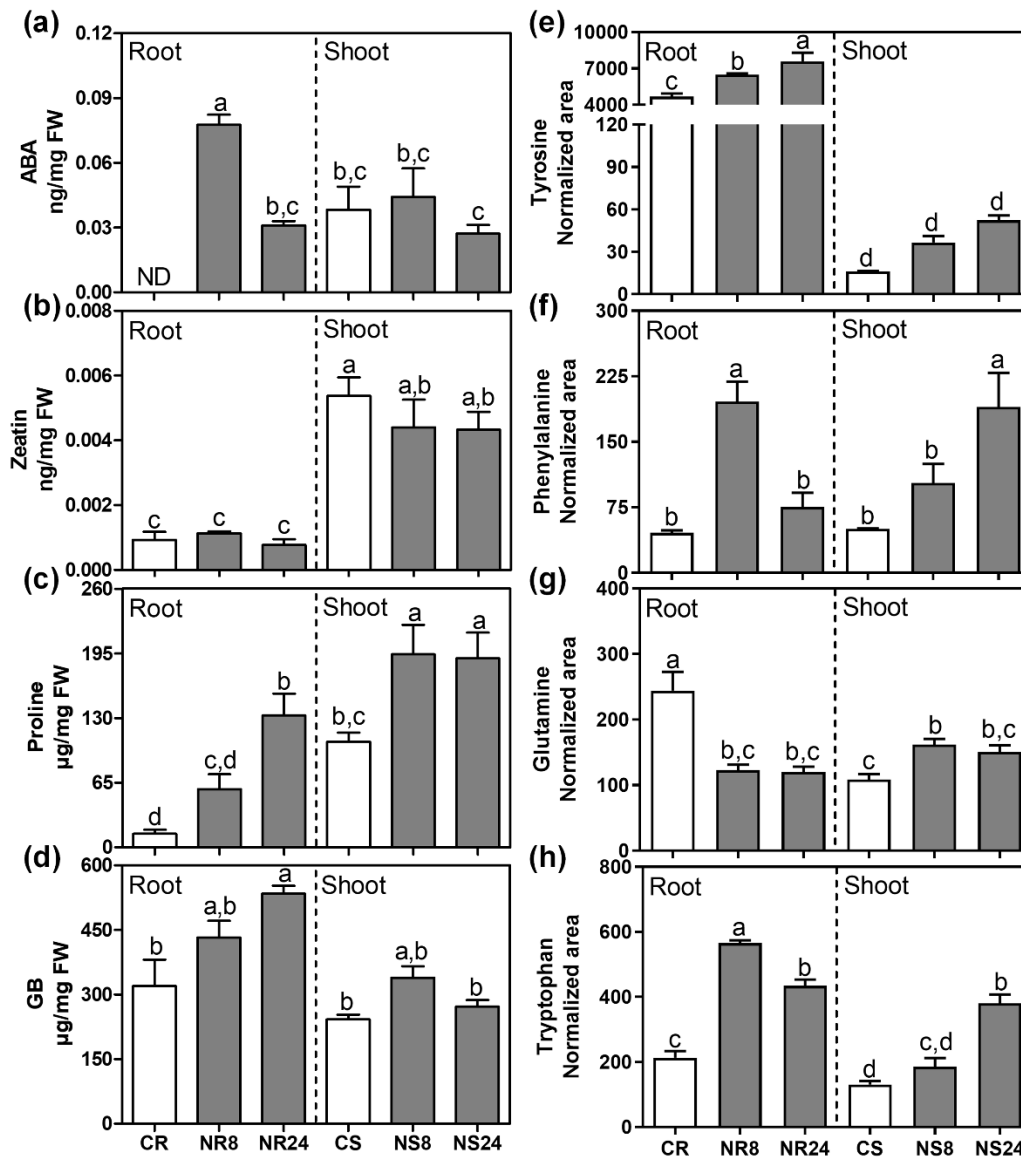


Figure 4: Concentration of phytohormones, osmolytes and amino acids and surface morphology of *S. portulacastrum* seedlings in response to NaCl treatment. The 15-d old hydroponically grown seedlings of *S. portulacastrum* were divided into three sets. In the first set, fresh half strength MS medium was added and root and shoot tissues were then harvested immediately as control (CR-root; CS-shoot). Second and third sets were treated with 250 mM NaCl (in half strength MS medium) and harvested after 8 (NR8-root; NS8-shoot) and 24 h (NR24-root; NS24-shoot) time-point, respectively. Refer Figure S1d for experimental layout. The levels of ABA (a), zeatin (b), proline (c), GB (d), tyrosine (e), phenylalanine (f), glutamine (g) and tryptophan (h) were measured in shoot (upper leaf, S) and root (R) tissues and at 8 (N8). Values are mean±standard error of three independent set of experiments (five seedlings/set). Duncan's multiple range test (DMRT) using SPSS software was performed and different letters on the bar graph indicate significant difference at $p \leq 0.05$.

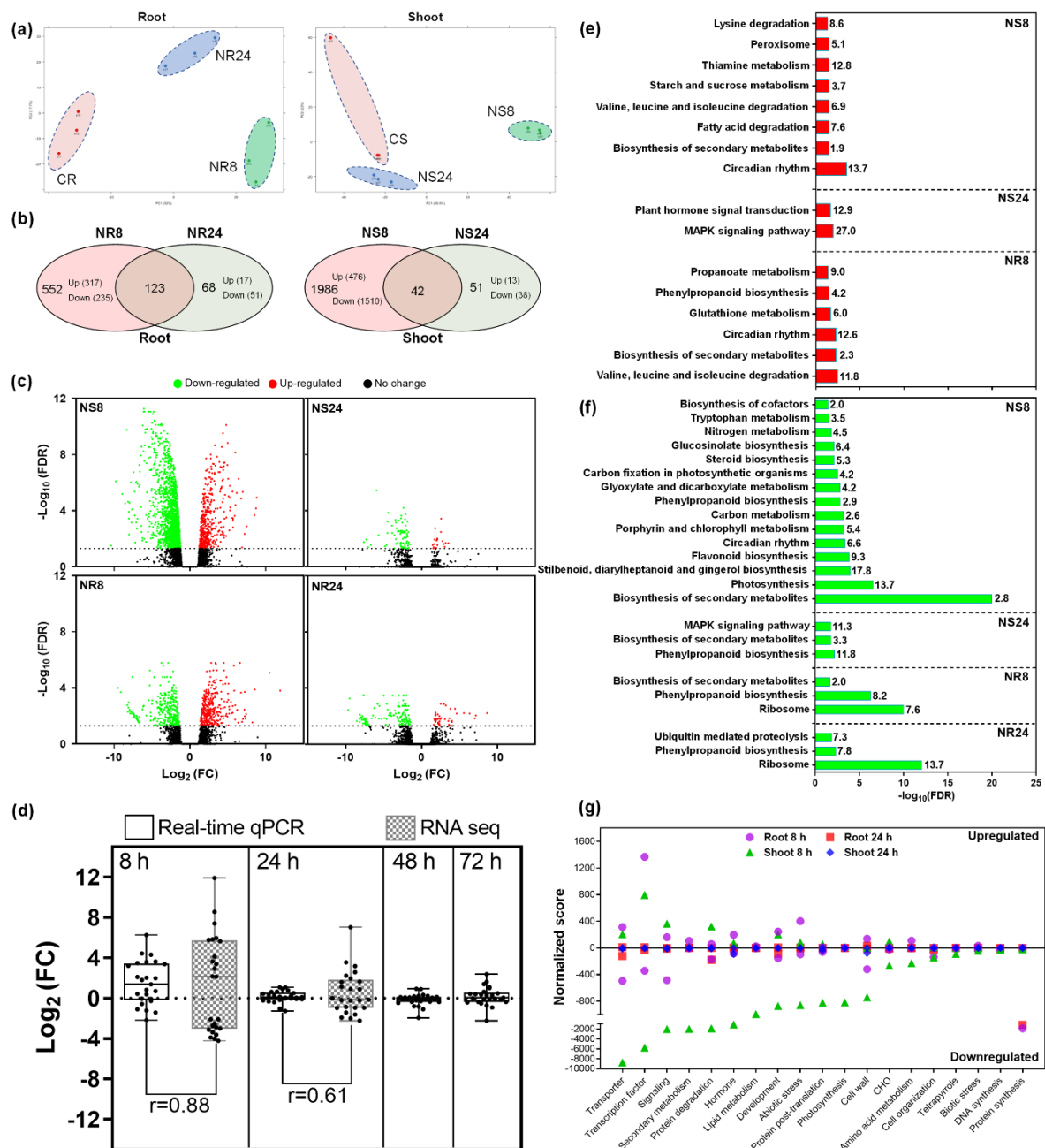


Figure 5: The transcriptome of *S. portulacastrum* seedlings in response to NaCl treatment. The 15-d old hydroponically grown seedlings of *S. portulacastrum* were divided into three sets. In the first set, fresh half strength MS medium was added and root and shoot tissues were then harvested immediately as control (CR-root; CS-shoot). Second and third sets were treated with 250 mM NaCl (in half strength MS medium) and harvested after 8 (NR8-root; NS8-shoot) and 24 h (NR24-root; NS24-shoot) time-point, respectively. Refer Figure S1d for experimental layout. Total RNA was extracted for RNA sequencing by Illumina. Data are means of three independent set of experiments (five seedlings/set) a: PCA clustering of the samples based on normalized transcript levels. Replicate samples are circled. b: Venn diagram highlighting numbers of specific and overlapping DEGs in root and shoot (for details refer Table S1; 2). c: Volcano plot of the distribution of average Log_2 fold changes (compared to control) of DEGs in shoot and root (c). FDR: false detection rate, FC fold change. d: Fold changes of root transcript levels over a 72-h time course of treatment with 250 mM NaCl obtained

with qPCR or RNAseq for 25 selected genes (Table S3a). e: KEGG pathway enrichment analysis of up-regulated DEGs (e). f: KEGG pathway enrichment analysis of down-regulated DEGs (numbers represents fold-enrichment) (f). g: Functional classification of DEGs in *S. portulacastrum* seedlings under different treatment conditions using MapMan. Normalized scores = $\Sigma(\text{Log}_2 \text{ Fold change}) \times \text{Number of genes}/2$).

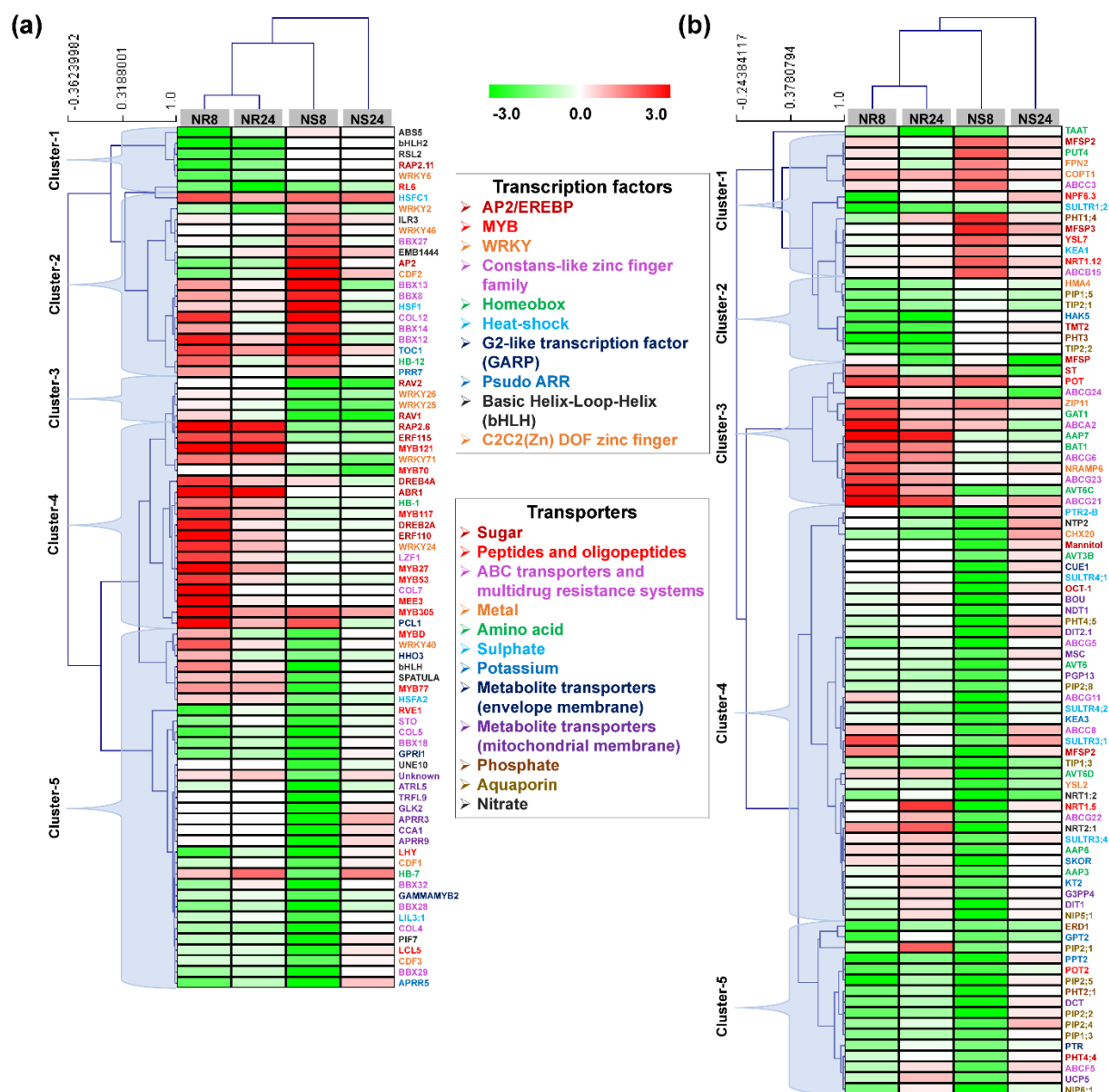


Figure 6: Heat map-based clustering of differentially expressed transcription factors and transporters under NaCl stress. The differentially expressed transporters and transcription factors after 8h or 24 h treatment with 250 mM NaCl (compared to control) were functionally classified using MapMan, independently in root and shoot. Transcript profiles of transcription factors (a) and transporters (b) were analyzed by hierarchical clustering and are displayed as heat maps.

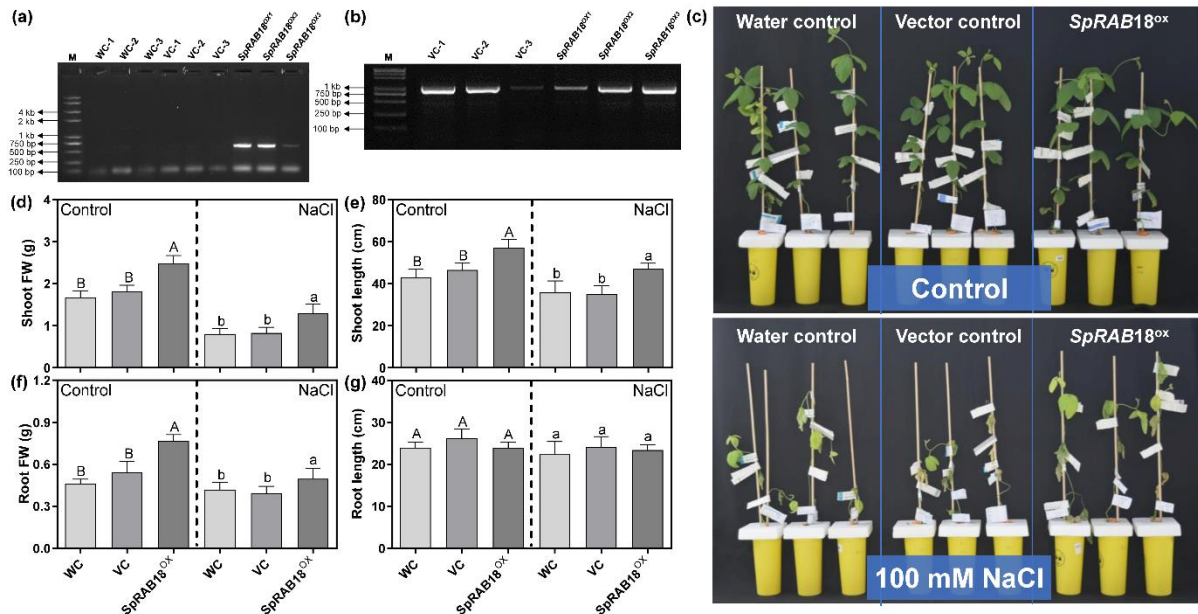


Figure 7: Phenotypes of soybean plants expressing *SpRAB18*. The soybean seedlings were transformed by *Agrobacterium rhizogenes* mediated root transformation. The soybean seedlings were transformed with water (water control), *A. rhizogenes* carrying vector (vector control) and *SpRAB18* construct. The phenotype of soybean was studied by hydroponically growing soybean seedlings for 18 d under half-strength MS media without NaCl and then subjected to either control or 100 mM NaCl. a,b: Agarose gel of PCR products obtained with primers for *SpRAB18* (a) or hygromycin-resistance gene (b) using genomic-DNA from the transformed seedlings as template (For primer details refer Table S3b). At 10 d after treatment, phenotypes were recorded qualitatively (c) and then quantified in terms of shoot fresh weight (d), shoot length (e), root fresh weight (f) and root length (g). Values are means $\pm$ standard error of three independent soybean overexpressed lines. Duncan's multiple range test (DMRT) using SPSS software was performed and different letters on the bar graph indicate significant difference at $p \leq 0.05$.