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Transcript Analysis of Two Spinach Cultivars Reveals the Complexity of Salt Tolerance Mechanisms

Chaoyang Zhao, Devinder Sandhu,* and Jorge F. S. Ferreira

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ABSTRACT: Increasing soil salinization threatens global crop productivity. An understanding of the genetic networks involved in salinity tolerance mechanisms of high-value crops, such as spinach, is lacking. RNA-Seq analysis of leaves and roots of two spinach genotypes, Monstrans Viroflag and Palek, subjected to high-salinity irrigation, revealed that a higher degree of differential gene expression was caused by salinity rather than by genotype. Genotypic comparisons suggested that the low salt tolerance index for root and shoot biomass of Palek, compared to Monstrans Viroflag, was due to the differential expression of genes involved in water/nutrient uptake rather than tissue salt accumulation. Montrans Viroflag displayed a better Cl^- exclusion than Palek and was more efficient in restricting Na^+ from entering its roots, thus protecting leaves from ion toxicity. In addition, differentially expressed genes (DEGs) involved in MAPK signaling, hormonal signaling, and transport revealed salinity- and genotype-specific differences and resulted in the identification of candidate genes that may function to mediate ion influx across cell membranes to maintain osmotic homeostasis when plants are under salt stress. The quantitative reverse transcription assay validated the overall expression trends of the selected RNA-Seq-based DEGs among different spinach samples. Collectively, the assays used in this study highlighted the complexity of the salinity tolerance mechanism and isolated several putative genes with the potential to improve salinity tolerance in spinach.

KEYWORDS: salinity, spinach, RNA-Seq, transcriptome, differential expression

INTRODUCTION

Modern agriculture uses innovative technologies and practices that increase farming efficiency and crop yield to meet the food demands of an ever-growing world population. Agriculture, however, is constrained by many resource logistics and human practices, among which soil salinization is a widespread and important undesired outcome that often reduces plant productivity.[1] Soil salinization may cause the uptake of excessive salt ions by plants, which results in the disruption, or even failure, of cellular homeostasis of water, ions, and other nutritional elements and, consequently, impairs plant growth and development.[2] In addition, salinity stress induces reactive oxygen species (ROS), which increases the rate of cell death and impedes plant growth.[3]

Numerous studies have been carried out to understand the molecular mechanisms underlying plant responses to salinity stress, especially on how salinity tolerance mechanisms have evolved in certain plant species.[4−10] After sensing salt stress, plants activate a complex cellular signaling cascade to regulate biochemical and physiological processes that minimize the detrimental effects imposed by the perceived stress.[11] In plants, these processes include retention of water, maintenance of ion homeostasis, detoxification of radicals, and adjustment of the growth rate.[12,13]

Despite the discovery of these general mechanisms, salt tolerance may vary considerably with genetic traits associated with plant species and varieties. For example, halophytes (salt-tolerant plants) and glycophytes (salt-sensitive plants) exhibit significantly different salinity-coping capabilities,[14] and within

the same plant species, e.g., alfalfa (*Medicago sativa*), different genotypes displayed various degrees of salt tolerance indexed by their biomass changes and ion accumulation under salinity.[7] In addition, studies of salt tolerance mechanisms have been mainly focused on model plant species, e.g., *Arabidopsis thaliana* and *Oryza sativa*, and the knowledge about how nonmodel species sense, adjust themselves, and adapt to salinity at the molecular level is limited.[15,16] Thus, characterizing molecular responses to salinity in poorly understood, nonmodel species becomes imperative in gaining comprehensive insights into the salinity tolerance mechanisms and their evolution in plants. The knowledge gained is expected to be valuable in identifying and selecting new genetic traits for salinity tolerance that will advance plant breeding significantly.

Spinach (*Spinacia oleracea*) is an economically important vegetable crop that is extremely rich in many core nutrients such as vitamins A, C, and K, folate, calcium, iron, and potassium, as well as phytochemicals such as carotenoids and phenolic compounds.[17] In addition, fresh spinach is a good source of antioxidant compounds that may benefit humans.[18] In 2019, ~66.8 thousand acres of spinach were grown in the United States, resulting in the production of 435.7 thousand metric

million tons of fresh spinach, with an estimated yearly value of $526.8 million (https://www.nass.usda.gov/Publications/Todays_Reports/reports/vegean20.pdf).

Spinach is a glycophytic C3 species that has been reported as moderately sensitive to salinity with a soil-paste salinity (EC_e) threshold of 2.0 dS m^{-1}.[19,20] Although this sensitivity has been confirmed by a recent updated review of the literature, specifying that spinach drops 7.6% in fresh yield biomass for every decisiemen per meter above an EC_e of 2.0 dS m^{-1},[21] it has been recently reported that the salinity tolerance of some spinach cultivars is 2.5−3.5 times higher than previously reported. For instance, the cultivars Raccoon and Gazelle cultivated in a greenhouse in a 1:1 mix of loamy soil and sand had biomass losses of 23% (Raccoon) and 45% (Gazelle) at the highest irrigation water salinity (EC_{iw}) of 13.0 dS m^{-1} (EC_e = 5.9 dS m^{-1}), with Gazelle maintaining shoot biomass up to an EC_{iw} of 7.0 dS m^{-1}.[22] However, when the same cultivars were cultivated in sand with a leaching fraction of 0.3, they could tolerate an EC_{iw} of 17.0 dS m^{-1}, an equivalent EC_e of 7.7 dS m^{-1}, without any significant loss of shoot biomass.[23] These authors also showed that both cultivars did not respond to potassium doses >40-fold lower than the potassium recommendation for the crop and had a remarkable capacity to maintain N, P, and K homeostasis regardless of the significant increase in the levels of both Na and Cl in roots and shoots. These recent reports reflect the complexity of salt tolerance mechanisms in spinach that may include genes that control the uptake of salt ions and the maintenance of important ions needed for growth and development.

Given the nutritional and economic significance of spinach and its tolerance to salinity stress,[18,22] we compared two contrasting varieties for their salt tolerance, to investigate the mechanisms underlying salinity tolerance in spinach. On the basis of our preliminary study, where 15 different varieties were evaluated for their salt tolerance, Monstrans Viroflag and Palek were selected as the most salt-tolerant and salt-sensitive, respectively (data not shown). By comparing their growth performance under salinity stress and characterizing their expression profiles, we reveal that spinach acquires salinity tolerance by activating hundreds of genes, many of which are involved in stress-related signaling pathways.

■ MATERIALS AND METHODS

Plant Material and Salt Treatment. Seeds of two spinach varieties, Palek (PI# 220686) and Monstrans Viroflag (PI# 176371), were sowed in water-saturated vermiculite. After germination, plants were kept in vermiculite with light watering until the two-leaf stage. Seedlings were then transplanted into two sand tanks, each of which contained six rows of plants (one row of Palek and one row of Monstrans Viroflag in three replicates). Transplanted seedlings were irrigated with half-strength (basic nutrient) Hoagland's solution [electrical conductivity (EC) = 1.87 dS m^{-1}] prepared with Riverside municipal water (EC = 0.65 dS m^{-1}). This basic nutrient solution contained 0.11 g/L $CaCl_2$, 0.51 g/L KNO_3, 0.07 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.26 g/L Fe Sprint 138, 0.001422 g/L H_3BO_3, 0.002535 g/L $MnSO_4 \cdot H_2O$, 0.000345 g/L $ZnSO_4 \cdot 7H_2O$, 0.000075 g/L $CuSO_4 \cdot 5H_2O$, and 0.001236 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. When plants were fully established (3 weeks after transplanting), they were irrigated with either the basic nutrient solution (EC = 1.87 dS m^{-1}; control) or the basic nutrient solution enriched with sodium and chloride salts (EC = 16 dS m^{-1}; treatment). The salt compounds added were 4.97 g/L NaCl, 1.44 g/L $CaCl_2$, 2.13 g/L $MgCl_2 \cdot 6H_2O$, and 2.98 g/L $NaSO_4$. After 48 h from the first saline irrigation, leaves and roots from one plant in each replicate were collected for RNA extraction.[18] The remaining plants were irrigated for 25 days with the control or treatment solution until

their leaves and roots were harvested for fresh and dry weight measurements.

RNA Extraction and RNA Sequencing. Total RNA of leaf and root samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I (Thermo Scientific, Waltham, MA). RNA quantity and quality were assessed using the Nanophotometer spectrophotometer (IMPLEN) and the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). Messenger RNA molecules were enriched using Poly-T oligo-attached magnetic beads from 1 μg of total RNA, and sequencing libraries were constructed using the NEBNext Ultra RNA library prep kit from Illumina (NEB). RNA sequencing was conducted on the Illumina HiSeq platform to generate 150 bp paired-end reads (Novogene Corp. Inc., Sacramento, CA). Raw reads were subsequently processed to obtain clean reads by trimming adaptors and removing poly-N-containing and low-quality reads. Quality score Q20, Q30, and GC content and sequence duplications of the clean reads were also calculated, and all of the downstream analyses were conducted using the clean data.

Differential Gene Expression Analysis. RNA-Seq reads were first aligned to the annotated spinach genome[24] using HISAT2 to identify the genes differentially expressed between the sequenced spinach samples of different conditions and/or groups. The numbers of reads were mapped to each gene and counted using featureCounts.[25] We then calculated the values of FPKM (number of fragments per kilobase of transcript per million mapped reads) of each gene based on the length of the gene and read counts mapped to this gene. Differential expression analysis on two conditions and/or groups of samples was performed using the DESeq2 R package (version 1.14.1). The resulting p values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate (FDR). Genes with an adjusted p value of <0.05 and $\log_2$(fold change) of >2 determined by DESeq2 were considered as differentially expressed genes (DEGs).

GO Annotation and GO Enrichment Analysis of DEGs. Gene ontology (GO) analysis of DEGs was performed using Blast2GO PRO by its default setting unless explicitly stated.[26] Briefly, the protein sequences of DEGs were first uploaded to search (Blastp-fast) against the NCBI nonredundant (NR) database, and the resulting hits were used to obtain functional labels by mapping to the extensively curated GO annotated proteins (GOA, version 2020.04). Subsequently, GO annotation was performed for all three GO terminologies: biological process, molecular function, and cellular component.

To investigate which gene sets were overrepresented in the DEGs, GO enrichment analysis was implemented at SpinachBase, where GO annotation of the spinach genome was previously completed.[24] DEGs were retrieved to run against the annotated genome in all three ontologies based on an FDR-adjusted p value of 0.05.

KEGG Enrichment Analysis of DEGs. KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.kegg.jp/) is a collection of databases containing genomic, biological pathway, and disease information.[27,28] KEGG pathway enrichment analysis of DEGs was performed by comparing the KEGG pathways assigned to DEGs with those associated with the whole genome background, and the statistical enrichment was tested using the KOBAS software.[29,30] KEGG terms with a p_{adj} of <0.05 were considered as a significant enrichment.

Stress-Related Pathway Analysis of DEGs. To identify DEGs involved in stress-related pathways, specifically plant hormone signal transduction (KEGG: map04075) and calcium signaling (KEGG: map04020), we first searched the protein sequences of DEGs against the KEGG data set (https://www.kegg.jp/) of the Chenopodioideae (Taxonomy ID: 1804623), a subfamily of flowering plants to which spinach belongs, using BlastKOALA.[27] The output, a list of genes assigned with KEGG Orthology (KO) identifiers or K numbers, was then submitted for functional annotation using the KEGG mapping tool "Reconstruct Pathway", to obtain the DEGs associated with plant hormone signal transduction and calcium signaling.

Transporter Analysis of DEGs. The Transporter Classification Database (TCDB) is a classification system for membrane transport proteins (http://www.tcdb.org/).[31] It contains more than 20000 proteins that are classified into 1485 transporter families based on the transporter classification (TC) system.[31] DEG protein sequences were

used to search (BLASTP; E value of $<1 \times 10^{-5}$) against the TCDB proteins, and the TC numbers of the best hits were assigned to each DEG to retrieve the TC family and superfamily information.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Total RNA of leaf and root samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I (New England Biolabs Inc., Ipswich, MA). qRT-PCR was performed using the iTaq Universal SYBR Green One-Step Kit in the Bio-Rad CFX96 System (Bio-Rad Laboratories, Hercules, CA). The volume of each PCR was 10 μL, containing 20 ng of total RNA, each of the primers at a concentration of 0.75 μM (Table S7), 0.125 μL of iScript Reverse Transcriptase, and 5 μL of 2× one-step SYBR Green Reaction mix. Two technical replicates were included for each sample. The PCR conditions were as follows: 50 °C for 10 min, 95 °C for 1 min, and then 40 cycles of 95 °C denaturation for 10 s, 57 °C annealing for 30 s, and 68 °C extension for 30 s. Relative expression values were calculated using the comparative $2^{-\Delta\Delta Ct}$ method.[32] The spinach *Actin* (*Spo18993*) was used as the reference gene to normalize the expression of genes tested. Pairwise one-tailed t test analysis was performed to compare the gene expression difference between different samples. Differences with p values of <0.05 (one-tailed t test) were considered significant.

RESULTS

Salinity Tolerance of Two Spinach Varieties. Two spinach varieties, Monstrans Viroflag and Palek, were irrigated with high-salinity water (EC = 16 dS m^{-1}) and compared to control plants irrigated with low-salinity water (EC = 1.87 dS m^{-1}). The dry weight of leaf and root tissues for each plant replicate was measured and converted into weight per plant. Compared under high-salinity irrigation, Palek had significant reductions in leaf and root dry weights (83.3% and 39.0%, respectively) while Monstrans Viroflag had a leaf dry weight reduction of 56.0% and no significant difference in root dry weight (Figure 1). Notably, root dry weight was not significantly changed in the treatment group compared to the control for Monstrans Viroflag (Figure 1). Thus, Monstrans Viroflag was considered as moderately salt-tolerant and Palek was considered as salt-sensitive in this study.

Effect of Salinity on Shoot and Root Ion Compositions. Both genotypes, Monstrans Viroflag and Palek, displayed increased Na and Cl concentrations in root and shoot tissues under salinity as compared to the control but varied in the extent of that increase (Figure 2). Montstrans Viroflag and Palek had 3.0- and 2.4-fold increases in root-Na concentration, respectively, and 21.4- and 28.6-fold increases in shoot-Na concentration, respectively, under salinity compared to the control (Figure 2). Similarly, Montstrans Viroflag and Palek had 5.0- and 8.2-fold increases in root-Cl concentration, respectively, and 10.6- and 13.8-fold increases in shoot-Cl concentration, respectively, under salinity compared to the control (Figure 2). Although genotypes maintained root-K concentration under salinity, both had a 30% reduction in shoot-K concentration under salinity compared to the control (Figure 2). The Ca concentration significantly decreased in Palek roots, but not shoots, and decreased in Monstrans Viroflag shoots, but not roots, under salinity compated to the control (Figure 2). Both genotypes maintained similar Mg concentrations under control and salinity in both roots and shoots (Figure 2).

**RNA Sequencing and Differential Gene Expression.** RNA sequencing was performed on 24 spinach samples, including three replicates of root and leaf tissues collected from two spinach varieties (Monstrans Viroflag and Palek), each of which was irrigated with high-salinity (treatment) or low-salinity (control) water (Table S1). For an easy description, in
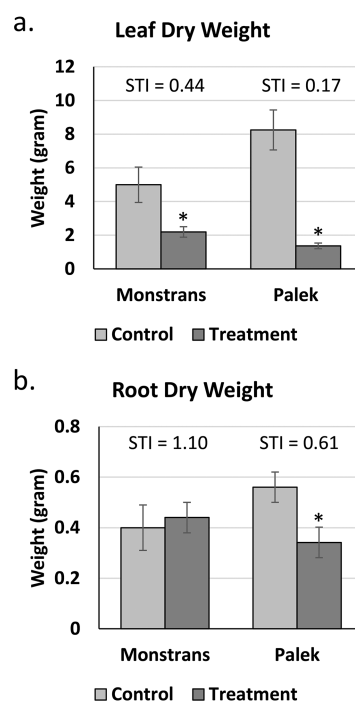
Figure 1. Comparison of biomass reduction between two spinach varieties subjected to salt treatment: (a) Leaf dry weight and (b) root dry weight. The fresh and dry weight of leaf and root tissues were compared between two spinach varieties, Monstrans (Monstrans Viroflag) and Palek, which were irrigated with half-Hoagland's solution with (treatment) or without (control) an increased level of salt. Asterisks on top of bars indicate a significant difference, according to Fisher's LSD test ($p < 0.05$). STI represents the salt tolerance index.

this paper, each spinach sample is signified in a three-letter format, i.e., "M or P.C or T.L or R", where the first letter, M or P, represents the variety Monstrans Viroflag or Palek, respectively, the second letter, C or T, represents control or treatment, respectively, and the third letter, L or R, represents tissue type leaf or root, respectively. A total of 1,489,903,140 raw reads were obtained, which, after the low-quality reads, ambiguous nucleotides, and adapter sequences had been removed, generated 1,441,468,636 high-quality clean reads, consisting of 216.3 gigabases with at least 8.3 gigabases for each of these 24 sequenced samples (Table S1). The repeatability of the RNA-Seq analysis was tested by principal component analysis (PCA), which showed a high level of similarity among biological replicates (Figure S1). The PCA gene expression plot showed distinct clusters for control compared to treatment, salt-sensitive compared to salt-tolerant, and leaf compared to root.

To perform differential gene expression analysis, RNA-Seq reads obtained from each sample were aligned to the spinach genome sequence, yielding an average mapping rate of 86.5% for each sample. Of a total of 25,495 annotated genes encoded by the spinach genome, 12,806 were considered as DEGs, which were differentially expressed in at least one of the comparisons, including the root versus leaf, control versus salt treatment, and Palek versus Monstrans Viroflag (Figure 3). Expression-based gene cluster analysis of these DEGs revealed the presence of two major groups: one including all root samples and the other including all leaf samples (Figure 3a). In the root group, genes from M.T.R and P.T.R formed a subgroup, while genes from P.C.R and M.C.R were clustered separately. In the leaf group,
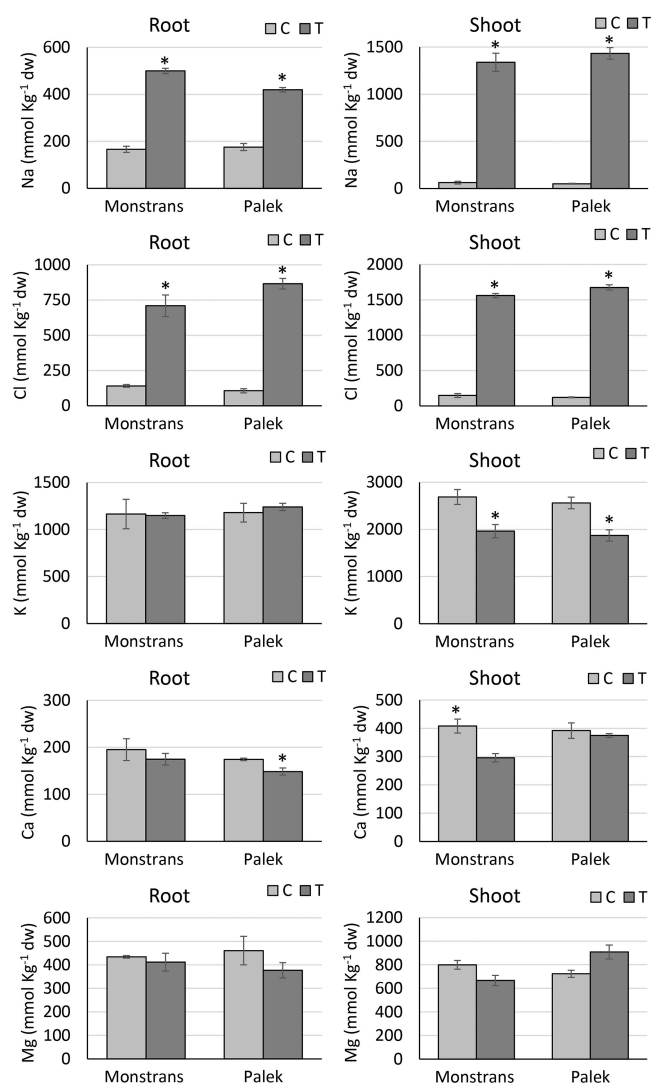
Figure 2. Comparison of tissue concentrations of various ions for two spinach varieties subjected to salt treatment. The light gray bars represent control (C), and the dark gray bars represent salt treatment (T). Asterisks on top of bars indicate a significant difference, according to Fisher's LSD test ($p < 0.05$). Error bars represent the standard error.

genes first formed two subgroups: a control subgroup and a treatment subgroup, which were finally clustered according to plant varieties (Figure 3a).

The control versus salt treatment gene expression analysis determined 1170 DEGs, among which there were 342 in M.T.R versus M.C.R (145 upregulated and 197 downregulated), 161 in M.T.L versus M.C.L (54 upregulated and 107 downregulated), 547 in P.T.R versus P.C.R (153 upregulated and 394 downregulated), and 439 in P.T.L versus P.C.L (139 upregulated and 300 downregulated) (Table S2 and Figure 3b). In comparisons of salt-tolerant (Monstrans Viroflag) versus salt-sensitive (Palek) varieties, 544 DEGs were found, including 245 in M.C.R versus P.C.R (92 upregulated and 153 downregulated), 57 in M.C.L versus P.C.L (26 upregulated and 31 downregulated), 117 M.T.R versus P.T.R (58 upregulated and 59 downregulated), and 195 in M.T.L versus P.T.L (84 upregulated and 111 downregulated) (Table S2 and Figure 3c). An extremely large number (12,577) of DEGs were detected in the leaf versus root comparisons, including 8924 in M.T.L versus M.T.R (4457 upregulated and 4467 down-

regulated), 9178 in M.C.L versus M.C.R (4554 upregulated and 4624 downregulated), 9118 in P.T.L versus P.T.R (4615 upregulated and 4503 downregulated), and 8247 in P.C.L versus P.C.R (4232 upregulated and 4015 downregulated) (Figure 3d).

In four control versus treatment comparisons, there were 160, 86, 348, and 324 DEGs unique to M.T.R versus M.C.R, M.T.L versus M.C.L, P.T.R versus P.C.R, and P.T.L versus P.C..L, respectively, and 15 DEGs common to all four comparisons (Figure 3b).

In four Palek versus Monstrans Viroflag comparisons, there were 208, 34, 5, and 79 DEGs unique to M.C.R versus P.C.R, M.C.L versus P.C.L, M.T.R versus P.T.R, and M.T.L versus P.T.L, respectively, and 2 DEGs common to all four comparisons (Figure 3c).

More than 10,000 DEGs were detected in leaf versus root comparisons, including 5,747 DEGs common to all four comparisons (M.T.L vs M.T.R, M.C.L vs M.C.R, P.T.L vs P.T.R, and P.C.L vs P.C.R) (Figure 3d). A large proportion of these DEGs were likely responsible for the physiological differences between leaf and root tissues rather than being responsive to salt stress. Hence, the functional gene annotations described below were merely on DEGs obtained from the control versus salt treatment and salt-sensitive versus salt-tolerant comparisons.

GO Enrichment Analysis of DEGs. GO enrichment analysis of DEGs was performed by comparing DEG GO annotations against the genomewide gene annotations. In control versus salt treatment comparisons, 28 GO terms were found to be enriched in the M.T.R versus M.C.R comparison that included GO terms such as "carbohydrate metabolic process" (GO:0005975), "response to acid chemical" (GO:0001101), and "manganese ion binding" (GO:0030145), 13 GO terms were enriched in the M.T.L versus M.C.L comparison such as "hydrolase activity" (GO:0016787), "carbohydrate metabolic process" (GO:0005975), and "heme binding" (GO:0020037), 24 GO terms were enriched in P.T.R versus P.C.R such as "catalytic activity" (GO:0003824), "ion binding" (GO:0043167), "response to oxygen-containing compound" (GO:1901700), and "response to endogenous stimulus" (GO:0009719), and 75 GO terms were enriched in P.T.L versus P.C.L such as "metabolic process" (GO:0008152), "cellular process" (GO:0009987), and "structural molecule activity" (GO:0005198) (Figure 4 and Table S3). In the salt-sensitive versus salt-tolerant comparisons, two GO terms were enriched in the M.C.R versus P.C.R comparison, i.e., "salicylic acid-mediated signaling pathway" (GO:0009863) and "cellular response to salicylic acid stimulus" (GO:0071446), no GO terms were found to be enriched in the M.C.L versus P.C.L comparison, one GO term, "β-amyrin synthase activity" (GO:0042300), was enriched in the M.T.R versus P.T.R comparison, and 13 GO terms were enriched in the M.T.L versus P.T.L comparison such as "catalytic activity" (GO:0003824), "hydrolase activity, acting on glycosyl bonds" (GO: 0016798), and "transferase activity, transferring hexosyl groups" (GO:0016758) (Figure 4 and Table S3).

KEGG Enrichment Analysis of DEGs. KEGG enrichment analysis indicated that DEGs in the control versus salt treatment comparison were over-represented in several KEGG pathways compared to the genomewide KEGG annotation (Figure 4). Three pathways, "MAPK signaling pathway—plant" (soe04016), "plant hormone signal transduction" (soe04075), and "nitrogen metabolism" (soe00910), were enriched in the M.T.R versus
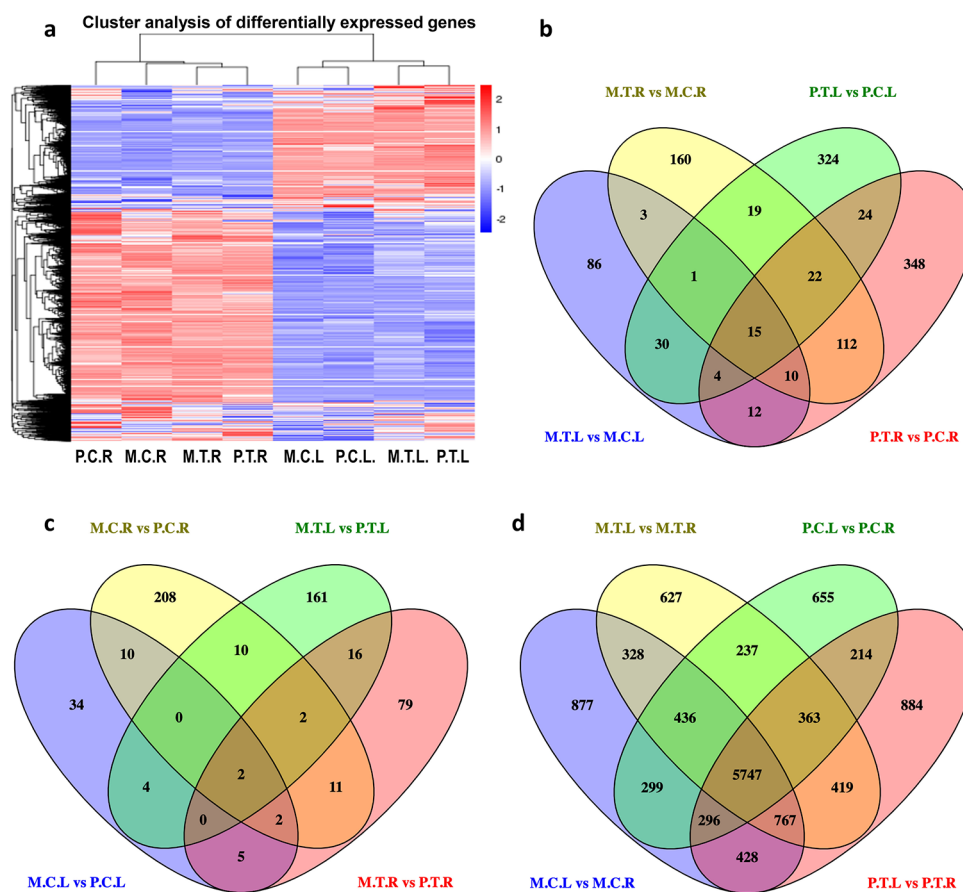
Figure 3. Heat map-based clustering and Venn diagram analysis of differentially expressed genes (DEGs). (a) Heat map and hierarchical clustering of DEGs across all eight samples. (b) Venn diagram showing the number of DEGs across four salt treatment vs control comparisons. (c) Venn diagram showing the number of DEGs across four salt-tolerant vs salt-sensitive comparisons. (d) Venn diagram showing the number of DEGs across four leaf vs root comparisons. Abbreviations: P, Palek; M, Monstrans Viroflag; C, control; T, treatment; R, root; L, leaf.

M.C.R comparison (Table S4). For the P.T.R versus P.C.R comparisons, four pathways were detected to be enriched, i.e., "MAPK signaling pathway—plant" (soe04016), "plant hormone signal transduction" (soe04075), "phenylpropanoid biosynthesis" (soe00940), and "plant—pathogen interaction" (soe04626). Only one pathway, "ribosome" (soe03010), was enriched in the P.T.L versus P.C.L comparison, and no pathway was found to be enriched in the M.T.L versus M.C.L comparison. Interestingly, there was only one KEGG pathway detected to be enriched in the salt-sensitive versus salt-tolerant comparison (Figure 4). Specifically, the pathway "phenylpropanoid biosynthesis" (soe00940) was enriched in the M.T.L versus P.T.L comparison (Table S4).

DEGs Involved in Stress-Related Pathways. To investigate whether spinach genes may respond to salinity stress through regulating stress-related pathways, we retrieved the DEGs that were annotated as genes involved in plant hormone signal transduction (KEGG: map04075) and calcium signaling (KEGG: map04020).

Within the control versus treatment comparisons, there were several DEGs involved in plant hormone signal transduction; however, no DEGs were found to be involved in calcium signaling (Figure 6 and Table S5). For the M.T.R versus M.C.R comparison, 11 DEGs were involved in hormone signal transduction, including 10 for abscisic acid (ABA) biosynthesis (four upregulated and six downregulated) and one for cytokinin signaling (downregulated). For the M.T.L versus M.C.L

comparison, one and two DEGs were involved in indole-3-acetic acid (IAA) signaling and ABA signaling, respectively, with all being downregulated in M.T.L. Twenty DEGs were found to be involved in plant hormone signal transduction in the P.T.R versus P.C.R comparison, including one (downregulated) in salicylic acid (SA), five (downregulated) in jasmonic acid (JA), three (downregulated) in brassinosteroids (BRs), two (one upregulated and one downregulated) in IAA, eight (three upregulated and five downregulated) in ABA, and one (downregulated) in ethylene signal transduction. For the P.T.L versus P.C.L comparison, two DEGs (downregulated) were involved in BRs, three (downregulated) in IAA, and four (one upregulated and three downregulated) in ABA (Figure 6 and Table S5).

For the salt-sensitive versus salt-tolerant comparisons, only eight DEGs in total were detected to be involved in plant hormone signal transduction (Figure 6 and Table S5). However, two DEGs in the salt-sensitive versus salt-tolerant comparisons were for calcium signaling. Both genes were upregulated in M.T.R compared to P.T.R (Figure 6 and Table S5). For DEGs involved in hormone signal transduction, five (all downregulated) were in the M.C.R versus P.C.R comparison, including two for JA and three for BRs, one (upregulated) was in the M.C.L versus P.C.L comparison for SA, and two were in the M.T.L versus P.T.L comparison, including one (upregulated) for BRs and one (downregulated) for ethylene. There
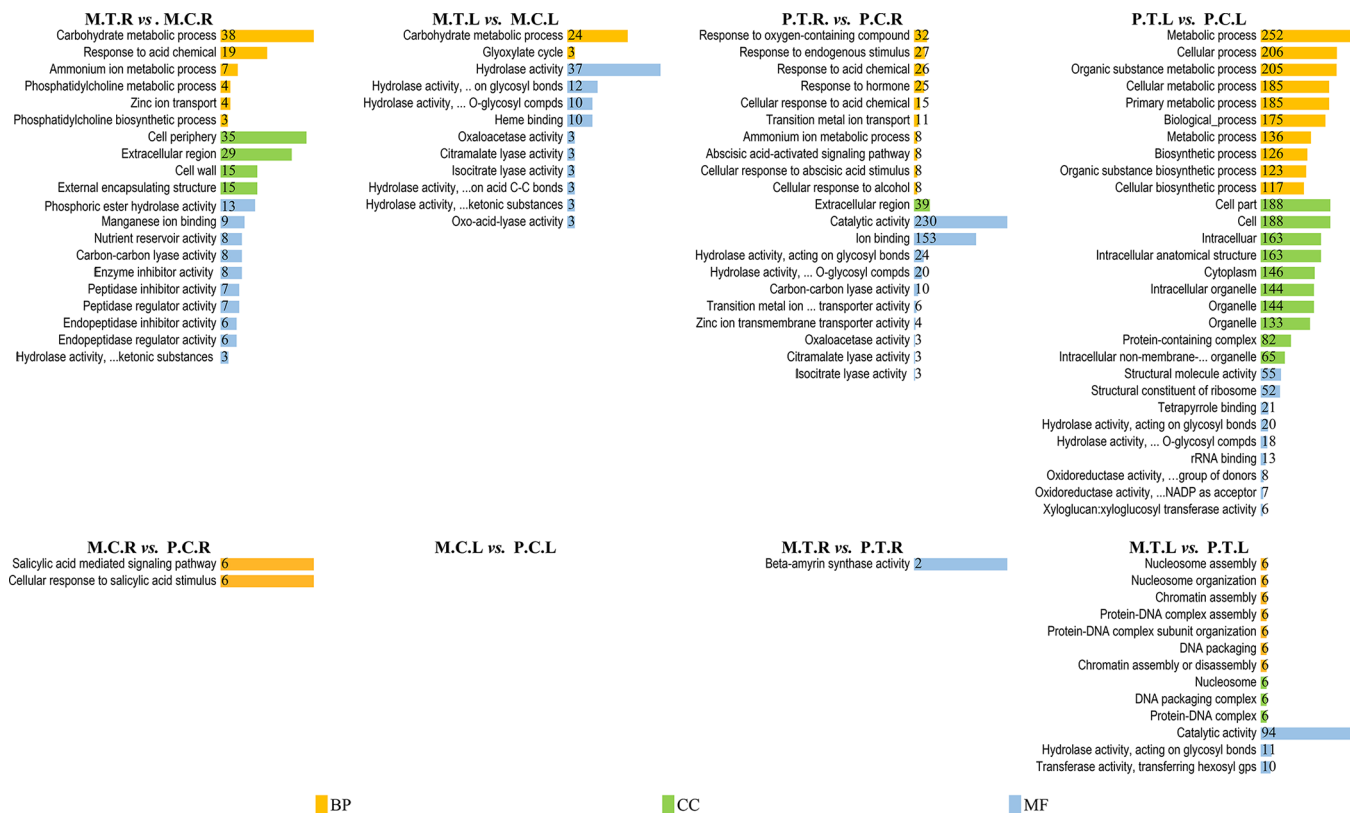
Figure 4. Gene ontology (GO) enrichment analysis of DEGs. Four salt treatment vs control comparisons are shown on top, and four salt-tolerant vs salt-sensitive comparisons are shown at the bottom. For each comparison, the categories of biological process (BP), cellular component (CC), and molecular function (MF) terms are colored yellow, green, and blue, respectively. For each category, only the top 10 GO terms (if >10) are shown, and the numbers in the bars represent gene counts. Abbreviations: P, Palek; M, Monstrans Viroflag; C, control; T, treatment; R, root; L, leaf.

were no DEGs found to be involved in hormone signaling for the M.T.R versus P.T.R comparison (Figure 5 and Table S5).
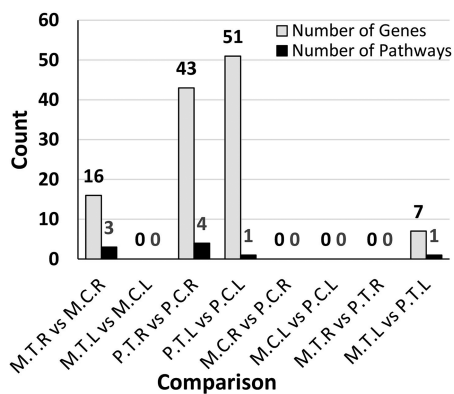
Figure 5. KEGG enrichment analysis of DEGs. For each comparison, the number of enriched genes and the number of enriched KEGG pathways are shown on top of bars. Abbreviations: P, Palek; M, Monstrans Viroflag; C, control; T, treatment; R, root; L, leaf.

DEGs Encoding Transporters. Transporters play important roles in alleviating salt stress by participating in the processes of ion relocation in plants. To understand whether DEGs encode transporters that are involved in ion redistribution, we predicted the transporter-encoding DEGs through sequence homology searches against the Transporter Classification Database (TCDB) and classified these DEGs into superfamilies based on the category of their best hits. Overall,
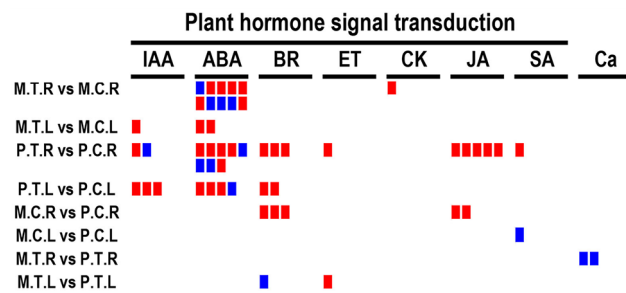
Figure 6. DEGs involved in different plant hormone and calcium signaling pathways. Blue and red bars represent the number of DEGs up- and downregulated, respectively, in the samples shown in the left part of the pairwise comparisons. The blue color represents upregulation, and the red represents downregulation. Abbreviations: P, Palek; M, Monstrans Viroflag; C, control; T, treatment; R, root; L, leaf.

there were more than 100 DEGs encoding transporters in both the control versus treatment and salt-sensitive versus salt-tolerant comparisons.

Within the treatment versus control comparison, DEGs encoded 17 superfamilies of transporters in the M.T.R versus M.C.R comparison, including the major facilitator superfamily (MFS), the leucine-rich repeat-containing domain (LRRD) superfamily, and the amino acid-polyamine-organocation (APC) superfamily (Figure 7 and Table S6). DEGs in the M.T.L versus M.C.L comparison encoded 11 superfamilies of transporters, including MFS, AAA-ATPase, LRRD, and calmodulin/calcineurin/KChIP (CaCa) superfamilies. For the P.T.R
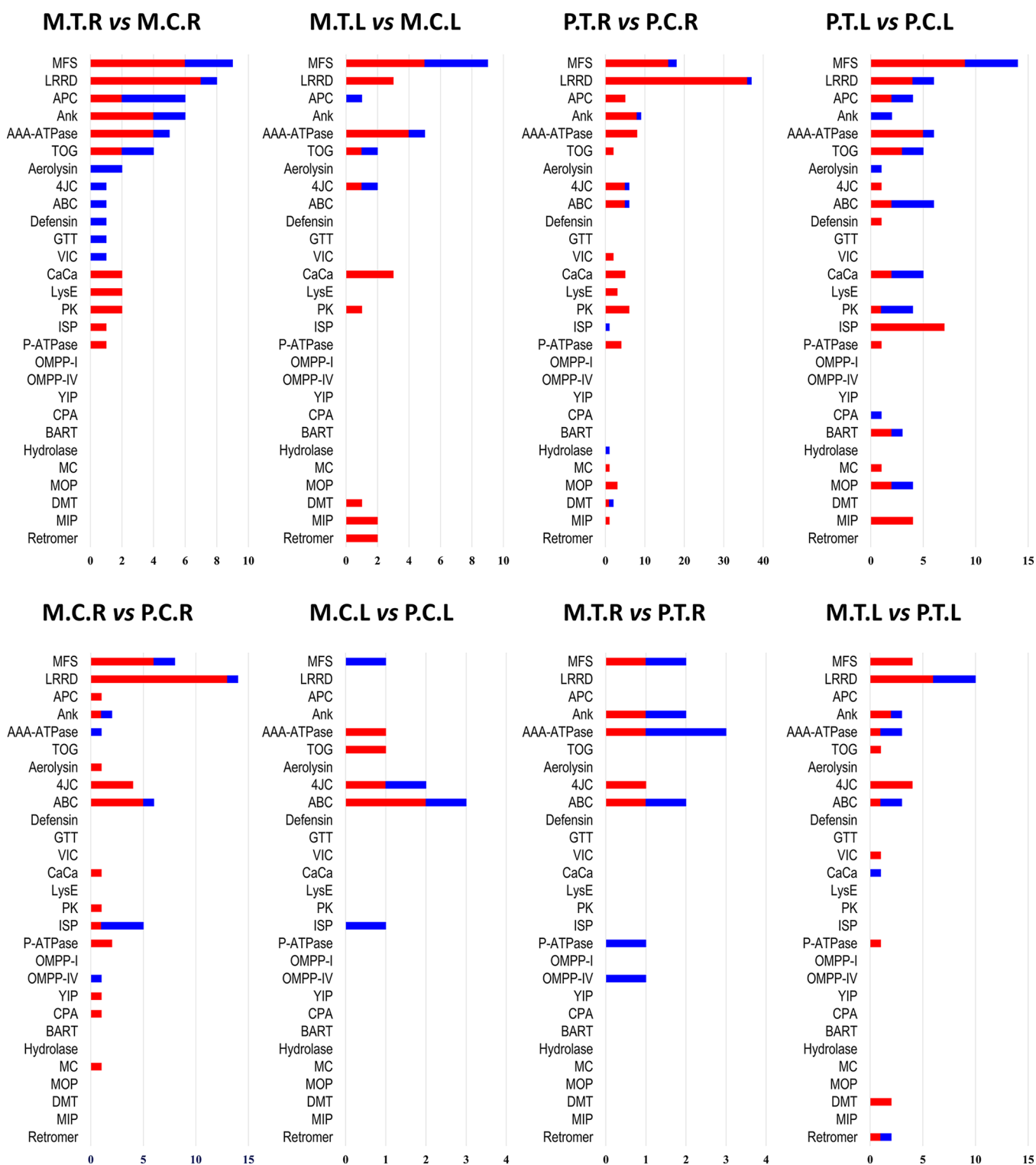
Figure 7. Transporter analysis of DEGs. DEGs of each comparison were classified into transporter superfamilies (*Y*-axis), and the gene counts are shown on the *X*-axis. Four salt treatment versus control comparisons are shown on top, and four salt-tolerant versus salt-sensitive comparisons are shown at the bottom. The blue color represents the upregulated DEGs, and the red color represents downregulated DEGs.

versus P.C.R comparison, 19 superfamilies of transporters were encoded by DEGs, with LRRD, MFS, and ankyrin repeat domain-containing (Ank) as the three largest superfamilies. For the P.T.L versus P.C.L comparison, 19 transporter superfamilies were also predicted, which included MFS and iron−sulfur protein (ISP) as the two largest superfamilies (Figure 7 and Table S6).

Within salt-sensitive versus salt-tolerant comparisons, 16 transporter superfamilies were for the M.C.R versus P.C.R comparison, including LRRD, MFS, and ISP superfamilies. Only six transporter superfamilies were found in the M.C.L versus P.C.L comparison, with only the ATP-binding cassette (ABC) superfamily and tetraspan junctional complex protein (4JC) superfamily containing more than one gene member each. For
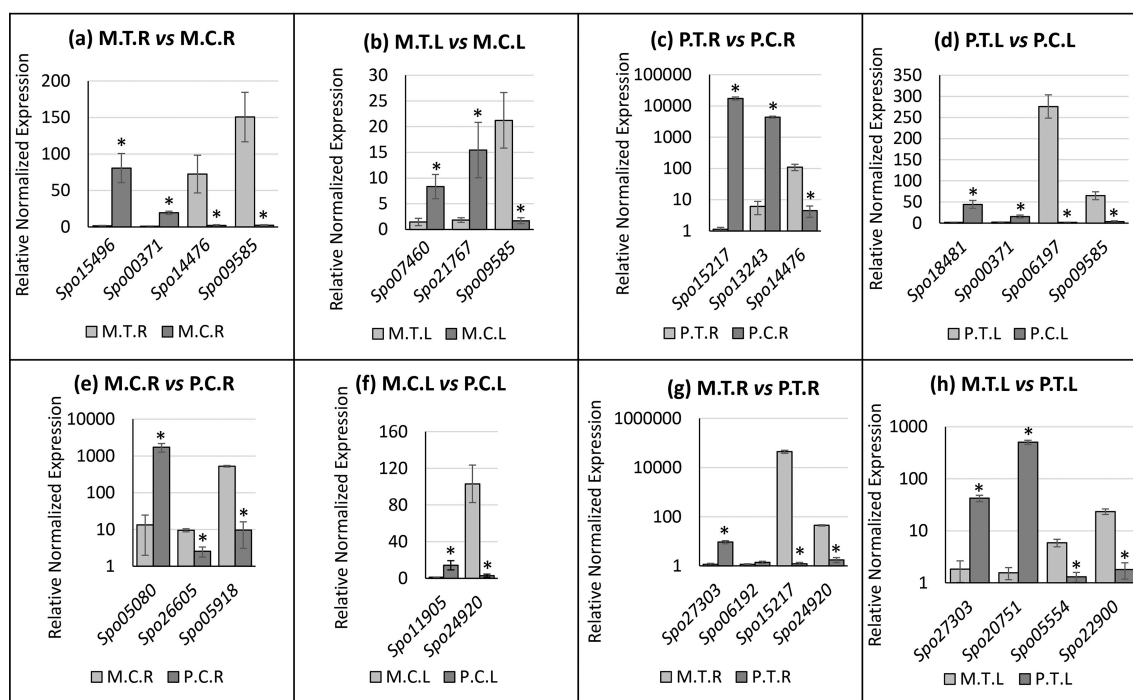
Figure 8. qRT-PCR validation of gene expression differences inferred from RNA-Seq analysis. Relative gene expression levels are shown on the Y-axis, and significant gene expression differences (Fisher's LSD test; $p < 0.05$) are indicated by asterisks shown on top of bars. Four salt treatment vs control comparisons are shown on top, and four salt-tolerant vs salt-sensitive comparisons are shown at the bottom.

the M.T.R versus P.T.R comparison, seven superfamilies were detected, including AAA-ATPase, APC, MFS, and ABC. In the M.T.L versus P.T.L comparison, DEGs encoded 12 superfamilies of transporters such as LRRD, MFS, and 4JC (Figure 7 and Table S6).

Expression Validation of DEGs Using qRT-PCR. To validate the expression of DEGs determined by RNA-Seq analysis, we selected 20 DEGs, based on their relevance to salinity stress and their expression patterns to include at least one upregulated and one downregulated gene in each comparison for the qRT-PCR assay (Table S7). Among these 20 genes, 14 (*Spo05080*, *Spo05554*, *Spo05918*, *Spo06192*, *Spo06197*, *Spo07460*, *Spo11905*, *Spo13243*, *Spo15496*, *Spo18481*, *Spo20751*, *Spo21767*, *Spo22900*, and *Spo26605*) were used to assess a single comparison, five (*Spo00371*, *Spo14476*, *Spo15217*, *Spo24920*, and *Spo27303*) were used to assess two different comparisons, and one gene (*Spo09585*) was used to assess three comparisons (Figure 8). qRT-PCR results demonstrated that these 20 selected genes corresponding to 27 expression comparisons exhibited gene expression patterns similar to those of RNA-Seq results (Figure 8). One exception was the expression of *Spo06192* was significantly upregulated in P.T.R compared to M.T.R in RNA-Seq data; however, such upregulation was not significant according to the qRT-PCR assay (Figure 8). We also compared the gene expression fold change values obtained using these two approaches and found that these values were similar for some genes and different for others. For example, *Spo07460* was 4.3- and 5.7-fold downregulated on the basis of RNA-Seq and qRT-PCR analyses, respectively, in the M.T.L versus M.C.L comparison. In contrast, *Spo24920* in the M.C.L versus P.C.L comparison was shown to be 4.9-fold upregulated according to RNA-Seq analysis but was 35.2-fold upregulated in the qRT-PCR assay (Figure 8 and Table S2). Nevertheless, the consistency between RNA-Seq and

qRT-PCR results in the general trends of gene expression profiles, exemplified in 26 of 27 comparisons tested, suggested the reliability and validity of the data from RNA-Seq experiments conducted in this study.

■ DISCUSSION

Soil salinization poses a significant threat to crop growth and productivity worldwide. In this study, we aimed to discover the genetic basis of salinity tolerance in spinach by comparing the expression profiles of two varieties that demonstrated different degrees of biomass losses when stressed by irrigation water salinity. We performed RNA-Seq analysis on eight spinach samples, including two tissues (roots and leaves) collected from two spinach varieties varying in salinity tolerance, and cultivated under two different irrigation waters (low and high salinity). Previously, salt-tolerant spinach cultivars were reported to survive irrigation water electrical conductivity (EC_{iw}) up to 13 dS m^{-1} (in soil) or 17 dS m^{-1} (in sand) with some, or no, decrease in shoot biomass.[22,23] Here, we report that two spinach varieties, Monstrans Viroflag and Palek, showed contrasting reductions in biomass at an EC_{iw} of 16 dS m^{-1} but survived a high level of salinity (Figure 1) and an increased level of accumulation of shoot Na and Cl. These observations confirm previously published reports that spinach is relatively tolerant to salinity and must have evolved certain salinity tolerance mechanisms absent in many glycophytic plants. One of these reported mechanisms is that spinach can maintain N, P, and K tissue concentrations under high salinity (EC_{iw} = 13.2 dS m^{-1}), even under K deficiency.[22]

Our gene clustering analysis indicated that gene expression was most distinct between plant tissues and was the least distinct between the two varieties studied (Figure 3a). Supportively, the highest numbers of DEGs (12,806) were detected in the leaf versus root comparisons, followed by those detected in control

versus salt treatment comparisons (1,170 DEGs), and the salt-sensitive versus salt-tolerant comparisons (544 DEGs) (Figure 3). Given the tremendously different roles roots and leaves play in plant development and growth, and the fact that roots are the first morphological barrier to salinity, it is not surprising that a vast number of DEGs were found in leaf versus root comparisons, reflecting distinct regulatory programs controlling tissue specificity.[33]

Of the two genotypes, Mostrans Viroflag had relatively high root and leaf biomasses under salinity compared to the control (Figure 1). The STI for roots, based on root dry weight, was 1.10 and 0.61 for Monstrans Viroflag and Palek, respectively. Similarly, the STI for shoot dry weight was 0.44 and 0.17 for Monstrans Viroflag and Palek, respectively (Figure 1). These observations suggest that Monstrans Viroflag is relatively tolerant to salinity compared to Palek. Under increased salinities, the decrease in shoot biomass was primarily due to a reduced number of lateral buds and fewer leaves. In plants, the tillering and dwarf 1 (*TAD1*) gene encodes an E3 ubiquitin ligase protein that suppresses the lateral shoot formation.[35] Of the 13 DEGs encoding E3 ubiquitin ligase, 11 were down-regulated under salinity treatment compared to the control (Table S2). Two genes (*Spo11598* and *Spo18524*) were downregulated in M.T.R compared to M.C.R. One gene (*Spo11258*) was downregulated in M.T.L compared to M.C.L. Five (*Spo11257*, *Spo12872*, *Spo17953*, *Spo18524*, and *Spo20595*) were downregulated in P.T.R compared to P.C.R. Three (*Spo11258*, *Spo20595*, and *Spo25602*) were down-regulated in P.T.L compared to P.C.L (Table S2). Of the five DEGs between Monstrans Viroflag versus Palek comparisons, four were downregulated in Monstrans Viroflag compared to Palek. These observations are in line with the involvement of E3 ubiquitin ligase in negative regulation of lateral shoot formation and suggest that a reduced number of lateral shoots in Palek compared to Monstrans Viroflag may be due to a higher level of expression of TAD1.

For the crops that can tolerate moderate to high salinity, osmotic stress is an essential player for plants to modulate their genetic response to salinity.[7,8] Genotypes with a low rate of transpiration and stomatal conductance tend to evade osmotic stress better than the genotypes with high rate of transpiration and stomatal conductance. Aquaporins are critical during osmotic stress and regulate the transpiration rate and stomatal conductance in plants.[34] In our investigation, two aquaporin genes (*Spo06391* and *Spo06392*) were downregulated in M.T.L and P.T.L compared to M.C.L and P.C.L, respectively (Table S2). Another aquaporin gene, *Spo16823*, was downregulated in P.T.R compared to P.C.R. On the similar lines, two aquaporin homologues, *Spo14763* and *Spo23126*, were downregulated in P.T.L compared to P.C.L (Table S2). Downregulation of aquaporin genes in salinity treatments compared to the control suggests their importance in ion uptake, critical for salinity tolerance.

Ion analysis indicated that both varieties, Monstrans Viroflag and Palek, had an increased level of accumulation of Na in their tissues under salinity. However, Monstrans Viroflag had a slightly higher Na concentration in roots than Palek and slightly lower Na concentration in shoots compared to Palek (Figure 2). These results suggest that Monstrans Viroflag may have a better control mechanism in protecting leaves from ion toxicity by restricting Na⁺ to roots. Montrans Viroflag may also take less Cl⁻ from soil, or it may have a better Cl⁻ exclusion mechanism, as indicated by the lower tissue concentration of Cl in its roots and

shoots compared to Palek (Figure 2). However, some spinach cultivars, when grown in soil under the same salinity (EC_{iw} = 13.8 dS m^{-1}), accumulated almost 2-fold more Na and Cl in shoots,[22] indicating that spinach can tolerate high Na and Cl concentrations in shoots without showing salt toxicity symptoms, albeit reducing shoot biomass, similar to what was observed in this study for Monstrag Viroflag and Palek. For K concentration, both genotypes had similar K-root concentrations under control and salinity treatments, but significantly lower shoot concentrations under salinity (Figure 2). However, despite decreased K-shoot concentrations, plants still had more than the minimum of 20 g of K kg^{-1}, under which plants are considered deficient in K.[36] The results show that Palek had significant decreases in both root and shoot dry weight, while Monstrans Viroflag showed no decrease in root dry weight under the same high salinity of irrigation water. However, their differences in salinity tolerance may not be attributed only to K homeostasis. Biomass is a complex trait that involves several genes controlling photosynthesis, metabolism, and hormones, among other processes associated with plant growth.[37] In the following section, we discuss several genetic responses in spinach associated with salinity tolerance that may have influenced the difference in shoot biomass observed for the cultivars evaluated in this study.

GO analyses indicated that GO terms were more enriched in control versus treatment comparisons than in salt-sensitive versus salt-tolerant comparisons (Figure 4), suggesting that salinity stress-induced changes in transcription exceeded the genetic variations between the two varieties tested. Notably, the most abundant GO term in the category of biological process was "carbohydrate metabolic process" (GO:0005975) for both M.T.R versus M.C.R and M.T.L versus M.C.L comparisons and was "response to oxygen-containing compound" (GO:1901700) and "metabolic process" (GO:0008152) for P.T.R versus P.C.R and P.T.L versus P.C.L, respectively (Figure 4 and Table S3). Salinity tolerance in plants generally involves the regulation of metabolic processes, including carbohydrate metabolic processes.[38] Starch metabolism, for example, plays a vital role in salinity tolerance by providing energy and the release of sugars and other derived metabolites that act as compatible osmolytes to mitigate the damaging effect of salinity stress.[39] On the contrary, "response to oxygen-containing compound" may account for the functions including mediation of ROS removal because abiotic stresses induce a high concentration of ROS, which damages proteins, lipids, DNA, and carbohydrates, and therefore, its concentration must be tightly regulated in plant cells.[40] For the cellular component, the GO term "extracellular region" (GO:0005576) was highly enriched in both M.T.R versus M.C.R and P.T.R versus P.C.R comparisons (Figure 4 and Table S3), which is in line with the importance of the extracellular environment in regulating ion exclusion from the roots.

GO analysis also indicated that among the four salt-sensitive versus salt-tolerant comparisons, the GO term "catalytic activity" (GO:0003824) was mostly enriched in the M.T.L versus P.T.L comparison (Figure 4), which may be, at least partially, responsible for the salinity tolerance difference between Monstrans Viroflag and Palek, given that activities of many enzymes, including antioxidant enzymes, were correlated with salinity tolerance.[4]

KEGG and signaling analyses further supported the idea that more genes were involved in biological pathways, including hormone signaling pathways, induced by salinity stress than

varietal variation in spinach (Figures 5 and 6). Interestingly, in plant roots of the control versus salt treatment comparisons, the most enriched DEGs were involved in the "MAPK signaling pathway" (soe04016) and "plant hormone signal transduction" (soe04075) for both varieties (Figure 5 and Table S4). While MAPK cascades participate in a vast array of cellular processes, including salt stress signaling responses in plants,[41] plants may also adapt to salinity stress by precisely regulating hormone levels.[42] Hence, spinach appeared to activate both MAPK and hormone signal pathways in response to salinity stress. With regard to the latter, ABA signaling was among the most important because more ABA genes were differentially expressed when plants, including both varieties, were salt-stressed than other hormone signaling genes (Figure 6).

Transporters are crucial for relocating excessive ions and maintaining ion hemostasis in plant cells;[43] hence, we also examined the distribution of transporter-encoding DEGs in all of the comparisons. Overall, transporters of MFS and LRRD superfamilies were most abundant in nearly all four control versus salt treatment comparisons (Figure 7). MFS transporters are membrane proteins that facilitate the movement of small solutes across cell membranes;[44] thus, differential expression of MFS transporter genes in salt-stressed spinach in comparison to nonstressed spinach suggested that these transporters were employed to maintain osmotic homeostasis to confer salinity tolerance in spinach. With regard to the LRRD superfamily of transporters detected in this study, they all belonged to the mechanosensitive calcium channel (MCA) family (Table S6), whose members modulated calcium ion influx through the plasma membrane and were shown to play roles in the generation of ROS and hypo-osmotic signaling in rice.[45] Hence, we hypothesize that MCA transporters in spinach also function as calcium ion mediators in response to salinity stress.

The salt overly sensitive (SOS) pathway regulates sodium ion homeostasis in plant cells as a salt stress response.[46] Three spinach SOS genes (*SoSOS1*, *SoSOS2*, and *SoSOS3*) have been recently identified and characterized, showing that their expression was upregulated 72 h after salt treatment (EC_{iw} = 13.8 dS m^{-1}) in leaf tissue, root tissue, or both of spinach var. "Gazelle".[47] Surprisingly, we did not find any of these SOS genes differentially expressed between the control and salt-treated spinach samples, including leaves and roots for both Palek and Monstrans Viroflag. The inconsistency in SOS gene expression patterns between that study and the study presented here could be affected by the applied salt concentration,[48] tissue sampling time,[49] plant variety,[50] or other SOS network regulators.[51] Nevertheless, the RNA-Seq-based gene expression profiles presented in this study were demonstrated to be highly reliable on the basis of these facts. First, we generated an in-depth clean RNA-Seq data set with 8.3−14.4 gigabases per sample replicate in a low error rate (≤0.03%) (Table S1). Second, mapping rates of clean reads to the genome sequence were high (83−95%) for differential gene expression analysis (Table S1). Third, RNA-Seq results and qRT-PCR analysis showed a high level of consistency.

CONCLUSION

This investigation compared root and leaf transcriptomes of two spinach genotypes of contrasting salinity tolerances under control and saline conditions. The objective was to understand the global genomic changes during salinity stress. On the basis of the salt tolerance index for root and shoot dry weight, Monstrans Viroflag was salt-tolerant while Palek was salt-sensitive. The high

sensitivity of Palek under salinity was expressed through the decreased number of lateral buds and fewer leaves. The differences in the performance of the two genotypes were explained by differences in the expression of genes regulating various metabolic processes such as the ones involved in the slightly higher level of accumulation of Na in roots than in leaves of Monstrans Viroflag and the lower level of accumulation of Na and Cl in both tissues of Monstrans Viroflag compared to Palek. Although the tissue K concentration of both cultivars was decreased with salinity, plants still had enough K for growth, suggesting that the decreased biomass was driven by the higher level of accumulation of tissue Na and Cl as the salinity of irrigation water increased. Differentially expressed genes involved in ion transport, hormonal signaling, and calcium signaling showed specific genotypic differences between control and salt treatments. Among the genes involved in hormonal signaling, genes involved in ABA metabolism were the most significant. Although pronounced differences in gene expression were detected in leaves versus roots and between cultivars, the most representative differences in gene expression in both cultivars were detected as a result of salinity. Our global genomic analyses led to the identification of several candidate genes involved in the response to salinity stress. These genes may be further investigated and become instrumental markers in improving salinity stress in future spinach varieties.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.0c00063.

(PDF)

Experimental setup and RNA-Seq summary (Table S1) (XLSX)

Expression comparison and gene ontology annotation of DEGs (Table S2) (XLSX)

GO enrichment analysis of DEGs (Table S3) (XLSX)

KEGG enrichment analysis of DEGs (Table S4) (XLSX)

DEGs involved in plant hormone signal transduction and calcium signaling pathways (Table S5) (XLSX)

DEGs encoding transporters (Table S6) (XLSX)

Quantitative reverse transcription PCR (qRT-PCR) analysis of selected DEGs for expression validation (Table S7) (XLSX)

AUTHOR INFORMATION

Corresponding Author

Devinder Sandhu − *U.S. Salinity Lab, USDA-ARS, Riverside, California 92507, United States;* orcid.org/0000-0003-4193-3408; Email: devinder.sandhu@ars.usda.gov

Authors

Chaoyang Zhao − *U.S. Salinity Lab, USDA-ARS, Riverside, California 92507, United States; College of Natural and Agricultural Sciences, University of California, Riverside, Riverside, California 92521, United States*

Jorge F. S. Ferreira − *U.S. Salinity Lab, USDA-ARS, Riverside, California 92507, United States*

Complete contact information is available at:
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■ ABBREVIATIONS

DEG, differentially expressed gene; FPKM, number of fragments per kilobase of transcript per million mapped reads; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TCDB, transporter classification database

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