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# Divergent Gene Expression Responses to Salinity Stress in 16 Geographically Diverse Spinach Genotypes

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**ABSTRACT:** Salinity stands as a critical abiotic stress factor, posing significant challenges to global agricultural productivity. However, there is no comprehensive study that simultaneously investigates multiple spinach genotypes; integrates assessments of various parameters like biomass yield, ion uptake, and partitioning; and conducts genetic characterization of salinity tolerance mechanisms. To address this gap, we conducted a greenhouse experiment with 16 spinach genotypes, from diverse geographical regions, irrigated with saline waters of 1.87 and 23.3 dS $m^{-1}$. The salt tolerance index for shoot biomass exhibited significant variability among the genotypes, with 'Dikenli', 'Victoria', and 'Cornell ID #148' being the top performers and 'Cornell ID #87', 'Gazelle', and 'Polag Benaresi' being the bottom performers. Under high salinity, on average, plants accumulated 25-fold higher Na and 8.5-fold higher Cl in leaves compared to the control. Leaves accumulated 2.4-fold more Na and Cl than roots under salinity compared to the control. Expression analyses of specific genes in roots and leaves provided insights into $Na^+/Cl^-$ efflux, vacuolar sequestration, root-to-shoot movement, ion homeostasis, and scavenging of reactive oxygen species. Our results demonstrate the importance of screening geographically diverse genotypes and considering multiple traits when selecting genotypes for salt tolerance.

**KEYWORDS:** *salinity, salinity tolerance, spinach, gene expression, abiotic stress, qRT-PCR*



## INTRODUCTION

Spinach (*Spinacia oleracea* L.) is a green leafy vegetable rich in vitamins (A, B2, B6, C, and K), minerals (Ca, Cu, Fe, K, Mg, Mn, P, and Zn), dietary fiber, and antioxidants, with a global market value of over 39 billion dollars.[1] Spinach breeding is currently focused on improving growth and appearance as well as resistance to pests and diseases. However, the issue of salinity tolerance in spinach has not received significant attention in the research community. Increasing water and soil salinity has been reported to reduce spinach yield, and because climate change continues to impact global weather patterns, issues related to salinity are anticipated to intensify. Also, the overuse of fresh groundwater resources is aggravating seawater intrusion, and groundwater salinity, in low-lying coastal arid and semiarid regions worldwide.[2]

Spinach has been reported as a glycophytic plant that is moderately sensitive to salinity with reduction in biomass yield starting at a soil-paste electrical conductivity ($EC_e$) of 2 dS $m^{-1}$.[3] A salinity study conducted on 'Crocodile' cultivar showed a significant reduction in biomass at $EC_{iw}$ = 6.5 dS $m^{-1}$.[4] However, a NaCl concentration of 100 mM [irrigation-water electrical conductivity ($EC_{iw}$) ∼ 10 dS $m^{-1}$] in irrigation water for 17 days did not impair spinach growth.[5] A recent study focusing on the interaction among salinity, water, and nitrogen stresses reported no decrease in biomass up to $EC_{iw}$ = 7 dS $m^{-1}$, while there were significant reductions at 9 and 11 dS $m^{-1}$, perhaps due to reduced leaf area.[6] Our recent research on two spinach cultivars ('Raccoon' and 'Gazelle') showed tolerance to an irrigation-water electrical conductivity ($EC_{iw}$) of 17 dS $m^{-1}$, equivalent to a soil-paste electrical conductivity ($EC_e$) of 7.7 dS $m^{-1}$.[7−9] Based on the germination rate and the coefficient of the velocity of germination, 'Gazelle' was considered more salt-tolerant than 'Raccoon'.[8] These observations indicate that different spinach genotypes show a vast variation in salinity tolerance. However, salinity studies focusing on the simultaneous characterization of various diverse genotypes are missing in spinach.

Advances in plant breeding depend on the accessibility of genetic variability in the gene pools of a plant species. Therefore, continued access to the genetic resources in a crop's region of diversity is crucial to the breeding success of such crop species.[10] Spinach breeding draws on the genetic diversity of three species that form the spinach gene pool. The first is *Spinacia turkestanica* Iljin, which is believed to be the progenitor of cultivated spinach and is mainly found in Central Asia. The second is the wild species *Spinacia tetrandra* Steven ex M. Bieb., which has low pollen viability and is widely distributed in the trans-Caucasus region. The third species is *S. oleracea*, the most widely cultivated and consumed species of spinach. These three spinach species hold promise as potential sources of genes that could confer salinity tolerance. Unfortunately, wild species, such as *S. turkestanica* and *S. tetrandra*, are under-represented in genetic resource collections due to the high costs involved in seed collection expeditions to access wild relatives in their remote natural habitats and the

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stricter legislation preventing access and dissemination of natural indigenous genetic resources.[11] To have a diverse representation, we chose spinach lines from different countries, acquired from USDA-ARS Germplasm Resources Information Network (GRIN), for salinity tolerance evaluation.

In this investigation, we evaluated 16 spinach genotypes from countries far and close to spinach's postulated centers of origin (Iran, Afghanistan, and Pakistan) (Table 1). Genotypes were ranked for root and shoot biomass under control and salinity conditions, for their ion uptake and partitioning. We also associated the variation in these traits with expression differences of various genes involved in salinity tolerance. The information generated here will be critical for selecting genotypes for regions where saline irrigation water or soil salinity is prevalent. Furthermore, these findings will contribute to a deeper understanding of the genetic mechanisms underpinning salinity tolerance in spinach, thereby facilitating the development of more resilient varieties suitable for these challenging environments.

**Table 1. Spinach Genotypes Evaluated for Salinity Tolerance and Response under Greenhouse Conditions**

| genotype | accession | species | country of origin |
|---|---|---|---|
| 6.2 | PI 647856 | *Spinacia oleracea* | Georgia |
| 11 | PI 103063 | *Spinacia oleracea* | China, Beijing Shi |
| CGN 9629 | PI 206753 | *Spinacia oleracea* | Turkey |
| Cornell ID #148 | PI 608712 | *Spinacia tetrandra* | Germany |
| Cornell ID #87 | PI 173131 | *Spinacia oleracea* | Turkey, Malatya |
| Dikenli | PI 171861 | *Spinacia oleracea* | Turkey, Samsun |
| Dikensiz | PI 169668 | *Spinacia oleracea* | Turkey, Izmir |
| Gazelle | Lot #48060 | *Spinacia oleracea* | Unknown (Johny's Seeds) |
| Indures | NSL 68263 | *Spinacia oleracea* | United States, New York |
| Koelz 8366 | PI 163310 | *Spinacia oleracea* | Pakistan |
| Monstrans Viroflag | PI 176371 | *Spinacia oleracea* | Italy |
| New Asia | PI 604778 | *Spinacia oleracea* | Japan, Hokkaido |
| Palek | PI 220686 | *Spinacia oleracea* | Afghanistan |
| Polag Benaresi | PI 163309 | *Spinacia oleracea* | India |
| Victoria | PI 179595 | *Spinacia oleracea* | Belgium |
| Viking | NSL 28218 | *Spinacia oleracea* | Sweden |

## MATERIALS AND METHODS

**Plant Materials and Experimental Setup.** The evaluation of 16 spinach genotypes was carried out in a greenhouse lysimeter system at United State Salinity Laboratory (USDA-ARS), Riverside, CA, U.S.A. Greenhouse conditions were set at a day/night temperature of 25/17 °C under natural illumination. Spinach seeds were acquired from different geographical sources through USDA-ARS GRIN. All accessions were *S. oleracea*, except 'Cornel ID #148', which was *S. tetrandra*. 'Gazelle' has been discontinued by Johny's Seeds, and they could not provide the country of origin of this cultivar (Table 1). Our acquired spinach germplasm comes from different countries, including the ones geographically adjacent to the postulated center of origin of spinach (Iran), such as Pakistan and Afghanistan (Table 1) and then going east to Georgia, Turkey, Italy, Germany, Belgium, and Sweden or west to India, China, and Japan. The modern breeding history of spinach (after1950s) is difficult to trace as breeding companies keep their information confidential.

Seeds were germinated in pots containing vermiculite, and upon reaching the two-leaf stage, the seedlings were carefully transplanted into sand tanks. Each plastic tank, measuring 1.2 m long by 0.6 m wide by 0.5 m deep, housed one row per genotype, with each row consisting of six individual plants. To ensure consistent watering, each grouping of three tanks was connected to an 890 L reservoir situated in the basement of the greenhouse and pumped through poly vinyl chloride pipes to irrigate sand tanks. The excess irrigation water was returned to the reservoirs via drainage by gravity for reuse. Irrigation reservoirs are monitored for water consumption. The system is equipped with automatic irrigation frequency and duration.

All 16 genotypes were accommodated in two adjacent tanks: one tank contained nine genotypes, and the second contained eight. 'Gazelle' was present in both tanks and was used as an internal control for both tanks based on our previous knowledge of its response to irrigation with moderate- to high-salinity waters. The experiment was conducted in a randomized complete block design with three replications and two treatments (control and salinity). The control treatment consisted of half-strength Hoagland's basic nutrient solution containing $CaCl_2$ (0.11), $KNO_3$ (0.51), $KH_2PO_4$ (0.07), $MgSO_4{\cdot}7H_2O$ (0.25), Fe Sprint 138 (0.26), $H_3BO_3$ (0.001422), $MnSO_4{\cdot}H_2O$ (0.002535), $ZnSO_4{\cdot}7H_2O$ (0.000345), $CuSO_4{\cdot}5H_2O$ (0.000075), and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.001236). For the first 4 weeks after transplanting, plants were irrigated with a basic nutrient solution twice a day; after that (at the six-leaf stage), they were either irrigated with a basic nutrient solution ($EC_{iw}$ = 1.87 dS $m^{-1}$) or with a basic nutrient solution with sodium, chloride, and sulfate salts added to reach an $EC_{iw}$ of 23.3 dS $m^{-1}$. The additional salts added to the treatment (in g $L^{-1}$) were NaCl (8.39), $CaCl_2$ (1.66), $MgSO_4{\cdot}7H_2O$ (3.7), $Na_2SO_4$ (4.01), $KNO_3$ (0.51), $NaHCO_3$ (0.0084), and $KH_2PO_4$ (0.034). To avoid osmotic shock, salinity was raised in steps: 8 dS $m^{-1}$ on the first day, 16 dS $m^{-1}$ on the second day, and 23.3 dS $m^{-1}$ on the third day. For RNA isolation, leaf and root samples were harvested in liquid nitrogen 48 h after the final increment in salt treatment and stored at −80 °C. The remaining plants were allowed to grow for two more weeks and then harvested for biomass and ion analysis. Roots and leaves were dried at 70 °C for 96 h, weighed separately, and ground for ion analysis.

**Mineral Analysis.** Leaf and root tissues dried at 70 °C to a constant mass were ground for mineral analysis. Chloride was determined using a mercuric thiocyanate reaction in the presence of ferric nitrate with an AQ300 discrete analyzer.[12] The levels of other macro- (Na, K, Ca, Mg, and $SO_4$) and micronutrients (Zn) were determined from nitric acid digestions using inductively coupled plasma optical emission spectrometry (3300DV, Perkin-Elmer Corp., Waltham, MA, U.S.A.).

**Quantitative Reverse-Transcription PCR Analysis.** Total RNA from leaf and root samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) and treated with DNase I (Thermo Scientific, Waltham, MA, U.S.A.) following the manufacturer's protocol. Quantitative reverse-transcription PCR (qRT-PCR) reactions were performed using the iTaq Universal SYBR Green One-Step Kit in the Bio-Rad CFX96 System (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The PCRs were carried out in 10 μL of total volume, containing 20 ng of total RNA, 0.75 μM of each of the primers (Table S1), 0.125 μL of iScript reverse transcriptase, and 5 μL of 2× one-step SYBR Green Reaction mix. The reactions were conducted in three biological and two technical replicates. The PCR conditions were as follows: 50 °C for 10 min, 95 °C for 1 min, then 40 cycles of 95 °C denaturation for 10 s, 57 °C annealing for 30 s, and 68 °C extension for 30 s. The comparative $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression values.[13] The spinach *ACTIN* (Spov3_chr2.02265), *Actdf* (Spov3_chr6.00169), and *GAPDH* (Sov3_C0001.00042) were used as the reference genes to normalize the expression of the genes tested.

## RESULTS AND DISCUSSION

**Salinity Responses of Spinach Genotypes.** Evaluation of 16 spinach genotypes under control ($EC_{iw}$ = 1.87 dS $m^{-1}$) and high-salinity ($EC_{iw}$ = 23 dS $m^{-1}$) irrigation water treatments showed a wide variation in shoot biomass. Most genotypes showed significant reductions in shoot biomass


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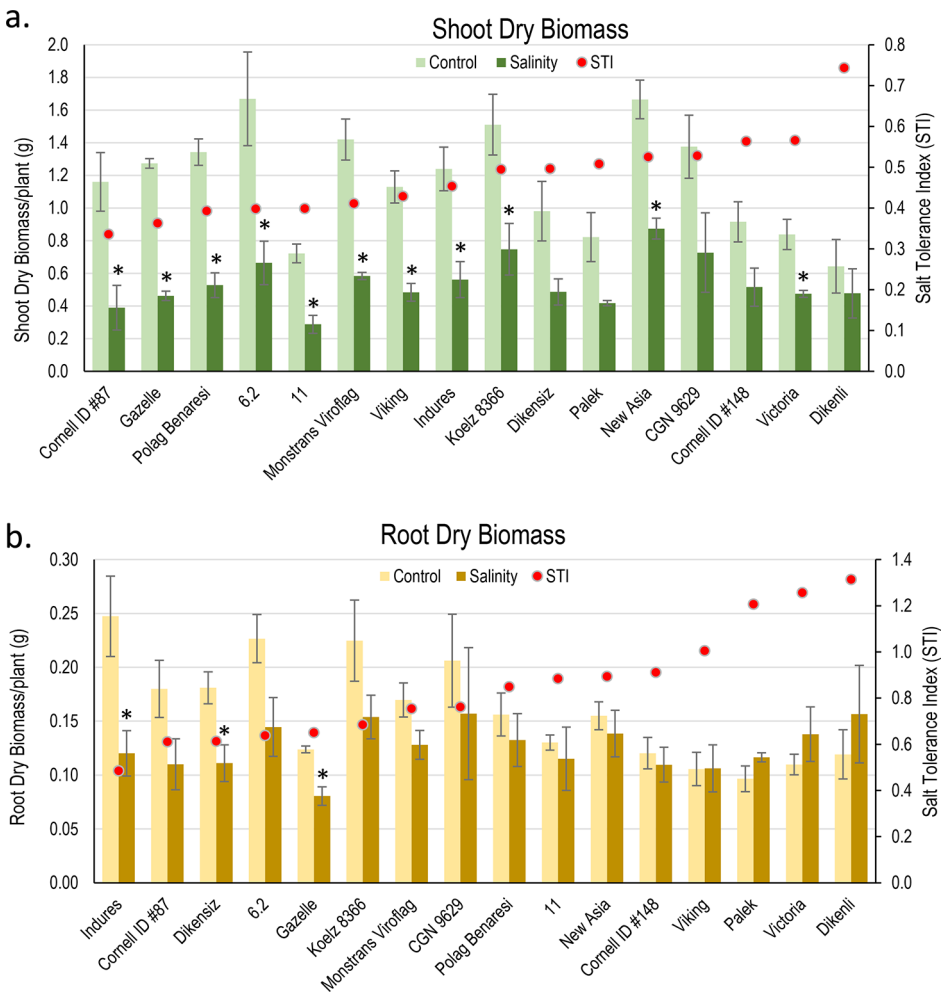


**Figure 1.** Performance of 16 spinach genotypes under control and salinity conditions. (a) Shoot dry biomass. (b) Root dry biomass. Asterisks signify significant differences between the control and saline conditions ($p \leq 0.05$) ($n = 3$). Error bars represent standard errors.

under salinity (Figure 1a). The average dry biomass yield was 1.17 g plant$^{-1}$ under control, whereas it was 0.54 g plant$^{-1}$ under salinity. Top-performing cultivars for shoot biomass under salinity stress (>0.6 g plant$^{-1}$) include 'New Asia' (0.87 g plant$^{-1}$), 'Koelz 8366' (0.75 g plant$^{-1}$), 'CGN 9629' (0.73 g plant$^{-1}$), and '6.2' (0.66 g plant$^{-1}$). These cultivars were also top performers under control conditions with shoot biomasses of close to 1.38 g plant$^{-1}$ or higher (Figure 1a). 'Monstrans Viroflag' was closely behind these top performers. On the other hand, cultivars '11' (0.29 g plant$^{-1}$), 'Cornell ID #87' (0.39 g plant$^{-1}$), and 'Palek' (0.42 g plant$^{-1}$) had the lowest shoot biomass under salinity. For roots, the average dry biomass reduced from 0.16 g plant$^{-1}$ under control to 0.13 g plant$^{-1}$ under salinity. The three top performers, 'CGN 9629', 'Dikenli', and 'Koelz 8366', produced 0.16, 0.16, and 0.15 g plant$^{-1}$, respectively. Conversely, the three bottom performers, 'Gazelle', 'Viking', and 'Cornell ID #148', produced 0.08, 0.11, and 0.11 g plant$^{-1}$, respectively (Figure 1b).

The salt tolerance index (STI), represented by the ratio of shoot or root dry biomass under salinity to the dry biomass under control, is a useful parameter to discriminate vigorous genotypes under control salinity from salt-tolerant ones.[14] Based on the STI for shoot biomass, the top performers were 'Dikenli' (0.74), 'Victoria', (0.57), and 'Cornell ID #148' (0.56) and the bottom performers were 'Cornell ID #87' (0.33), 'Gazelle' (0.36), and 'Polag Benaresi' (0.39) (Figure 1a). 'Gazelle', a comparison genotype tested in previous salinity-tolerance studies, produced 1.27 g plant$^{-1}$ under control and 0.46 g plant$^{-1}$ under salinity with a drop in biomass of 66.67% due to salinity (Figure 1a). The genotypes with the highest STI for root biomass were 'Dikenli' (1.31), 'Victoria' (1.26), and 'Palek' (1.21), and those with the lowest STI were 'Indures' (0.49), 'Cornell ID #87' (0.61), and 'Dikensiz' (0.61) (Figure 1b). Most genotypes showed a considerable reduction in shoot biomass under salinity compared to the control; however, very few genotypes showed a significant reduction in root biomass (Figure 1). These observations suggest that the spinach shoot is more sensitive to salinity than the root. High salt concentrations typically impact roots less than leaves as photosynthates are transported to the roots to help maintain the osmotic balance.[15] In contrast, leaves are more susceptible to salt toxicity and exhibit symptoms earlier than the roots.

**Tissue Mineral Ion Analysis.** Regulation of tissue ion concentrations is one of the most important characteristics under salinity stress, as excessive accumulation of $Na^+$ and $Cl^-$ is toxic to plant cells.[16] The analyzed leaf Na concentration in 16 spinach genotypes under control and salinity ranged from 59.9 to 95.0 mmol kg$^{-1}$ and 1445.4 to 2775.5 mmol kg$^{-1}$, respectively (Figure 2a). All genotypes showed a significant increase in leaf Na concentration under salinity compared to the control. On average, the leaf Na concentration was more than 25-fold higher under salinity than under control. Under


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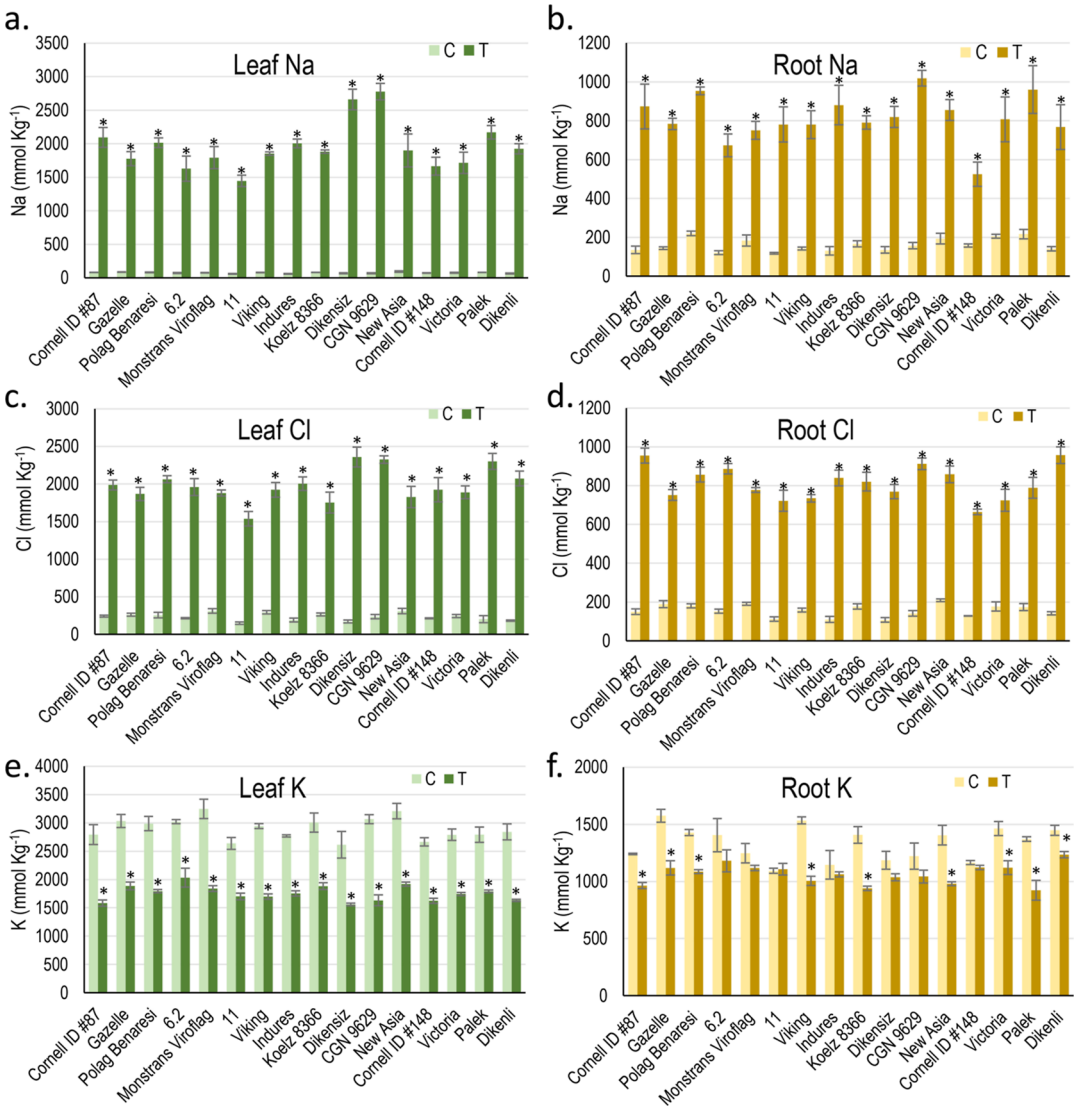


**Figure 2.** Tissue ion concentrations of the 16 spinach genotypes irrigated with control and saline irrigation waters. (a) Leaf Na concentrations. (b) Root Na concentrations. (c) Leaf Cl concentrations. (d) Root Cl concentrations. (e) Leaf K concentrations. (f) Root K concentrations. Asterisks signify significant differences between the control and saline conditions ($p \leq 0.05$) ($n = 3$). Error bars represent standard errors.

salinity, the lowest Na concentration was recorded for '11' (1445.4 mmol $kg^{-1}$), '6.2' (1628.6 mmol $kg^{-1}$), and 'Cornell ID #148' (1666.3 mmol $kg^{-1}$), and the highest was observed for 'CGN 9629' (2775.5 mmol $kg^{-1}$), 'Dikensiz' (2659.6 mmol $kg^{-1}$), and 'Palek' (2169.3 mmol $kg^{-1}$) (Figure 2a).

Root Na concentrations under control and salinity ranged from 118.4 to 220.2 mmol $kg^{-1}$ and 525.1 to 1018.9 mmol $kg^{-1}$, respectively (Figure 2b). The root Na concentration was significantly higher under salinity than under control in all genotypes. On average, in all genotypes, root Na concentration was about fivefold higher under salinity than under control. Three genotypes with the lowest root Na concentrations were 'Cornell ID #148' (525.1 mmol $kg^{-1}$), '6.2' (673.0 mmol $kg^{-1}$), and 'Monstrans Viroflag' (750.2 mmol $kg^{-1}$), and three with the highest Na concentrations were 'CGN 9629' (1018.9 mmol $kg^{-1}$), 'Palek' (960.4 mmol $kg^{-1}$), and 'Polag Benaresi' (954.1 mmol $kg^{-1}$) (Figure 2b).

Under control conditions, roots averaged more than double the Na concentration than leaves (Figure 2a,b). However, leaves stored a 2.4-fold Na concentration under salinity compared to roots. These observations indicate that, under normal conditions, the movement of $Na^+$ from the root to the shoot is regulated by a mechanism that limits the amount of $Na^+$ exported to the leaves. However, when exposed to high levels of salinity, the Na concentration surpasses the threshold under which the plant can control $Na^+$ transport to shoots, resulting in an increased influx of $Na^+$ into leaves. These findings are consistent with previous studies conducted on spinach, which have reported similar observations.[7,8,17] Despite exhibiting medium levels of Na in their roots, 'Victoria' and '11' demonstrated a low concentration of Na in their leaves when exposed to salinity stress (Figure 2a,b). This suggests that these genotypes can regulate the transport of $Na^+$ from roots to leaves more effectively than other genotypes. The ability to regulate the transport of $Na^+$ from roots to shoots is a crucial mechanism employed by various plant species to maintain low Na concentrations in the leaves.[14,18,19] High levels of Na in both the leaves and roots of 'CGN 9629' (Figure 2a,b) suggest an inefficient efflux mechanism for extruding $Na^+$ from roots to the soil, as well as a poor regulatory mechanism for controlling the movement of $Na^+$ from roots to the shoot. Conversely, the low concentration of $Na^+$ observed in both the leaves and roots


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of 'Cornell ID #148'" (Figure 2a,b) may indicate its efficient mechanisms for $Na^+$ extrusion and regulation of root-to-shoot $Na^+$ transport.

Leaf Cl concentration was also significantly higher for all genotypes under salinity than under control (Figure 2c). The average leaf Cl concentration was 234.0 mmol $kg^{-1}$ under control and 1979.5 mmol $kg^{-1}$ under salinity. The three genotypes that accumulated the highest leaf Cl under salinity were 'Dikensiz', 'CGN 9629', and 'Palek' with 2358.7, 2325.3, and 2301.1 mmol $kg^{-1}$, respectively, and the three genotypes that accumulated the lowest leaf Cl were '11', 'Koelz 8366', and 'New Asia' with 1537.0, 1753.5, and 1826.7 mmol $kg^{-1}$, respectively (Figure 2c).

All the genotypes showed significantly higher root Cl concentration under salinity than under control (Figure 2d). Under control, the average leaf Cl concentration was 156.2 mmol $kg^{-1}$, and under salinity, the concentration was 813.6 mmol $kg^{-1}$. The three top root Cl accumulating genotypes under salinity were 'Dikenli', 'Cornell ID #87', and 'CGN 9629' with 957.0, 955.1, and 912.3 mmol $kg^{-1}$, respectively, and the three bottom root Cl accumulating genotypes were 'Cornell ID #148', '11', and 'Victoria' with 664.1, 721.7, and 724.3 mmol $kg^{-1}$, respectively (Figure 2c).

On average, the Cl concentration was 1.5-fold and 2.4-fold higher in leaves than in roots under control and salinity, respectively (Figure 2c,d). The results showed that '11' exhibited low Cl concentrations in leaves and roots (Figure 2c,d), indicating efficient regulation of $Cl^-$ uptake and root-to-shoot movement. In contrast, 'New Asia' and 'Koelz 8366' stored relatively higher concentrations of Cl in their roots while maintaining lower concentrations in their leaves (Figure 2c,d), which suggests that they also have efficient mechanisms to regulate root-to-shoot $Cl^-$ transport.

Leaves are more susceptible to the harmful effects of $Na^+$ and $Cl^-$ than roots because $Na^+$ and $Cl^-$ tend to accumulate to higher concentrations in the shoots than in roots. Roots maintain relatively low levels of $Na^+$ and $Cl^-$ as they can regulate the extrusion of excess ions into the soil.[20,21] Although all spinach genotypes accumulated more Na and Cl under high salinity, plants did not show any visual symptoms of Na or Cl toxicity. The same was observed in previous studies with 'Raccoon' and 'Gazelle'.[8,9]

Interestingly, we observed a strong correlation ($R^2$ = 0.73) between Na and Cl accumulations in different spinach genotypes (Figure 3). It is noteworthy that such a high correlation between leaf Na and Cl accumulation is not commonly observed across plant species, mainly the ones that present mechanisms of either Na or Cl exclusion (or both), such as strawberry and Jerusalem artichoke;[22−24] however, similar correlations have been documented in the case of *Prunus*.[25] It is worth noting that 'CGN 9629', which exhibited significant Na accumulation in both leaves and roots, also displayed elevated levels of Cl in these same plant tissues (Figure 2a−d). Although 'CGN 9629' accumulated considerable levels of Na and Cl in both leaves and roots, it ranked among the highest performers in terms of shoot biomass based on STI (Figure 1a). Our results with these 16 spinach cultivars suggest that the mere accumulation (or exclusion) of Na and Cl in (from) shoots and roots cannot explain the cultivar performance in biomass accumulation under salinity, suggesting that critical components, other than tissue Na/Cl accumulation, may be responsible for the salt tolerance of these cultivars.

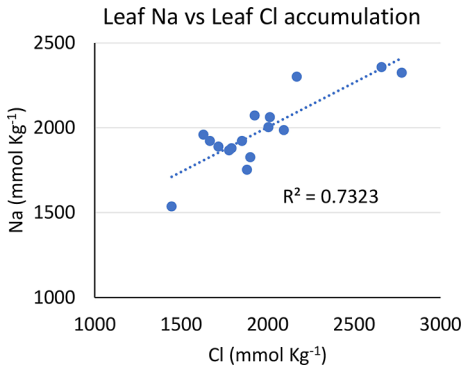


**Figure 3.** Correlation between Na and Cl accumulations in leaves of 16 spinach genotypes under salinity treatment.

Leaf K concentration significantly reduced under salinity compared to the control (Figure 2e). On average, there was a 39.5% reduction in leaf K concentration under salinity compared to the control. Maximum reduction was displayed by 'CGN 9629' (46.8%) followed by 'Monstrans Viroflag' (43.3%) and 'Cornell ID #87' (43.3%). The minimum reduction was displayed by '6.2' (32.8%), followed by '11' (35.6%) and 'Palek' (35.9%) (Figure 2e). For root K concentration, on average, there was a 20.1% reduction in root K concentration under salinity compared to the control (Figure 2f). The three genotypes with the highest reduction in root K concentration under salinity compared to control include 'Viking' (34.5%), 'Koelz 8366' (33.3%), and 'Palek' (32.7%), and three genotypes with minimum reduction include '11' (−1.3%), 'Cornell ID #148' (3.7%), and 'Indures' (7.3%) (Figure 2f). On average, leaves maintained a 2.18-fold higher K concentration compared to roots under control. In contrast, leaves had a 1.65-fold higher K concentration than roots under salinity. On average, there was a 39.5% reduction in leaf K concentration under salinity compared to the control. However, there was only a 21.1% reduction in root K concentration under salinity compared to the control (Figure 2e,f). When exposed to salinity, roots exhibited a smaller decrease in K concentration compared to that observed under control conditions, as compared to the leaves. This finding indicates that roots may possess greater resilience to salinity stress than leaves due to their ability to maintain K levels more effectively.

Analysis of other mineral elements such as Ca, Mg, $SO_4$, and Zn also showed variation in concentration in different genotypes (Table S2). However, there was not much association between root or leaf ion concentrations and shoot biomass STI.

**Expression Analyses.** Expression analysis was performed on the roots and leaves of 16 spinach genotypes using various genes known to play roles in Na or Cl transport. In addition, several other genes were selected based on their upregulation or downregulation under salinity compared to control or their differential expressions between two spinach cultivars in a previous RNA-seq experiment.[17]

*Roots.* For the genes involved in $Na^+$ transport, 'Dikenli', a cultivar with the highest STI for shoot biomass, showed upregulation for *SOS1*, *SOS2*, *NHX1*, *NHX2*, *AKT1*, and *HKT1* (Figure 4). Upregulation of *SOS1* and *SOS2* under salinity suggests that 'Dikenli' actively maintains $Na^+$ efflux from root to soil,[21] thereby maintaining low Na in the root and


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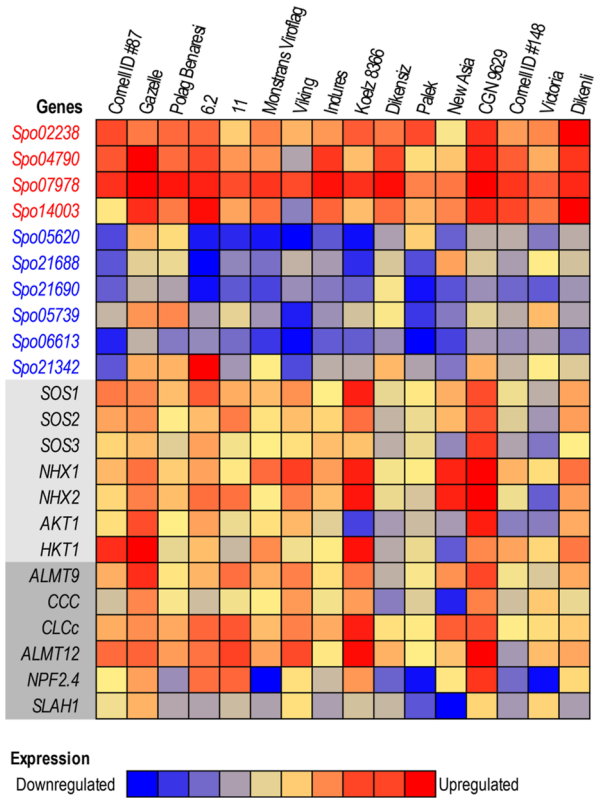


**Figure 4.** Heatmap representing the relative expression of salt stress-related genes in roots of 16 spinach genotypes. The ratio between the expression values under salinity and control was used to calculate the relative expressions and is depicted using different color shades. The gene names in red and blue fonts represent upregulated and downregulated genes, respectively, under salinity compared to the control, from a previous RNA-seq study.[17] Gene names highlighted with light gray represent genes involved in $Na^+$ transport, and genes highlighted in dark gray represent genes involved in $Cl^-$ transport.

eventually low Na in the leaf tissue (Figure 2a,b). This may be one of the reasons for its high STI for shoot biomass. High expressions of *NHX1* and *NHX2* in 'Dikenli' (Figure 4) suggest that it may also have efficient partitioning of Na in the vacuole.[26,27] *AKT1*, a member of the Shaker family of inward rectifying channels that regulates potassium uptake in plants and exhibits predominant expression in the root hairs and root endodermis.[10,28,29] Its upregulation indicates better K homeostasis in 'Dikenli'. 'New Asia', 'CGN 9629', and 'Koelz 8366', the three top performers based on total biomass produced under salinity, had a high upregulation of *NHX1* and *NHX2* under salinity compared to the control (Figure 4), suggesting that Na partitioning in the roots may have led to enhanced performances of these genotypes under salinity. Although most Na transporter genes, including the *SOS* genes, were upregulated in roots under salinity in 'CGN 9629' (Figure 4), it accumulated high concentrations of Na in roots and allowed a high amount of $Na^+$ to reach leaves (Figure 2). These findings suggest that unidentified genes might play a role in the elevated leaf Na concentration observed in 'CGN 9629'. Despite the high leaf Na concentration, the superior relative performance of 'CGN 9629' could be attributed to its enhanced capacity for tissue tolerance.

'Cornell ID #87', a line with the lowest STI for shoot biomass, showed considerable upregulation for *SOS1* and *HKT1* in the roots (Figure 4). Still, it stored relatively high Na concentration in leaves (ranked 4) and roots (ranked 4). 'Cornell ID #87' did not show significant upregulation for the *NHX1* and *NHX2* genes under salinity than control, suggesting that the lack of partitioning of $Na^+$ into the root vacuole may have led to increased leaf Na concentration in this genotype. Interestingly, 'Palek' and 'Victoria' did not show significant induction of any $Na^+$ or $Cl^-$ transport genes in roots under salinity compared to the control (Figure 4). *AKT1* and *HKT1* were downregulated in 'Dikensiz' (Figure 4). These observations explain the relatively high concentrations of sodium found in the leaves of 'Dikensiz' and 'Palek', ranking as the second and third highest, respectively. The high shoot biomass STI of 'Palek' may be justified by some genes involved in leaf tissue tolerance.

Among the $Cl^-$ genes, *ALMT9*, known to sequester $Cl^-$ in vacuoles,[30] showed upregulation in 'Dikenli' roots under salinity compared to the control (Figure 4). On the other hand, *SLAH1*, which is involved in $Cl^-$ loading in the xylem and facilitates its movement from the root to the shoot,[31] was downregulated (Figure 4). The finding that 'Dikenli' had the highest Cl concentration in roots but one of the lowest leaf-to-root chloride ratios is consistent with these observations (Figure 2c,d). *SLAH1* was downregulated in roots under salinity in several lines that showed high shoot biomass STI, including 'New Asia' (Figure 4). Downregulation of *SLAH1* may have contributed toward the relatively low leaf Cl concentration of 'New Asia' (Figure 2c). *NPF2.4*, which also regulates $Cl^-$ loading to xylem,[32] was significantly downregulated in 'Palek' and 'Victoria', two genotypes with high STI for shoot biomass (Figures 1a and 4). On similar lines, 'Cornell ID #87' and 'Gazelle', two genotypes with the lowest shoot biomass STI did not show downregulation of *NPF2.4* and *SLAH1* under salinity compared to the control. Another gene believed to be involved in $Cl^-$ loading to xylem, *ALMT12*, showed relatively high upregulation in salt-sensitive genotypes and no-to-slight upregulation in five top salt-tolerant genotypes (Figure 4). These observations suggest that the induction of *ALMT12* under salinity can be correlated with the salt tolerance of spinach genotypes.

*CCC* is involved in the retrieval of $Cl^-$ from the xylem back into the root to decrease the movement of $Cl^-$ from the root to the shoot.[33,34] Downregulation of *CCC* in 'Dikensiz' under salinity can be associated with its high leaf Cl concentration (Figure 2c). However, although *CCC* was downregulated under salinity in 'New Asia', it still maintained low leaf Cl concentrations, highlighting the complexity of salinity tolerance mechanisms in spinach. Nevertheless, there is a possibility that the upregulation of *CLCc* in 'New Asia' roots under salinity (Figure 4) may have restricted $Cl^-$ movement from the root to the shoot. *CLCc* is known to be involved in the sequestration of chloride ions in the root vacuoles by facilitating their transport from the cytoplasm into the vacuole.[35]

Four upregulated genes (*Spo02238*, *Spo04790*, *Spo07978*, and *Spo14003*) and six downregulated genes (*Spo05620*, *Spo05739*, *Spo06613*, *Spo21342*, *Spo21688*, and *Spo21690*) in roots under salinity compared to control in a previous RNA-seq experiment[17] were also analyzed for their expression in different genotypes (Figure 4). All four upregulated genes were also upregulated in most spinach genotypes in this study, except for *Spo04790* and *Spo14003*, which were downregulated in 'Viking' (Figure 4). *Spo02238*, *Spo04790*, *Spo07978*, and *Spo14003* encode later embryogenesis abundant (LEA)


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protein, protein phosphatase 2C (PP2C)6, tonoplast intrinsic protein (TIP), and temperature-induced lipocalin-1, respectively. LEA proteins enhance salinity tolerance in plants by binding and sequestering excess sodium ions, as well as by providing chaperone-like activity to stabilize and protect other important proteins.[36,37] PP2C6 may reduce the negative effects of salinity stress on plant growth and development by dephosphorylating and inactivating target proteins involved in regulating plant growth.[38] TIPs regulate the transport of small molecules and ions across the tonoplast, maintain osmoregulation, and help to sequester excess sodium ions in the central vacuole, thereby effectively mitigating the adverse effects of salinity stress on plant growth and development.[39] It has been shown that TIL is translocated from the cytoplasm to the chloroplasts under salt stress and can protect the chloroplasts from ion toxicity by maintaining the functionality of the photosynthetic machinery under stress.[40] Our results confirmed that these four genes are sensitive to induction under salinity treatment, advocating their role during salinity stress. It is worth noting that all four genes showed a marked increase in expression in both 'Dikenli' and 'CGN 9629' roots when exposed to salinity (Figure 4). This suggests that these two varieties may rely on distinct mechanisms to cope with salt stress, as reflected in their enhanced performance under such conditions. Six genes selected based on their downregulation under salinity compared to control from a previous RNA-seq study[17] also showed downregulation in the roots of most genotypes in the current study (Figure 4). Three of these genes, *Spo05620*, *Spo21688*, and *Spo21690*, encode germin-like protein (GLP), while the other three, *Spo05739*, *Spo06613*, and *Spo21342*, respectively, encode for a zinc transporter 1 (ZnT1)-like protein, allantoinase isoform X1, and metallothionein-like protein. GLPs have been shown to play a role in salinity stress response in various plant species.[41] *OsGLP*1 was shown to negatively regulate salt tolerance in rice at early stages of growth and development,[42] consistent with the downregulation of three GLP genes (*Spo05620*, *Spo21688*, and *Spo21690*) observed under salinity treatment in our study (Figure 4). ZnTs play a role in the unfolded protein response (UPR) by regulating the levels of zinc in the endoplasmic reticulum (ER), which is required for the proper folding and function of many proteins.[43] Salt stress induces the expression of ZnTs that move zinc from ER to the cytoplasm leading to decreased zinc levels in ER, resulting in the induction of UPR.[43] Activation of UPR initiates stress response, providing salinity tolerance in plants. Upregulation of the gene encoding a ZnT1-like (*Spo05739*) under salinity compared to control in 'Victoria' may indicate an active role of zinc transport in regulating salinity tolerance to this genotype (Figure 4). Allantoinase is an enzyme that hydrolyzes allantoin into allantoate.[44] It has been shown in beet that salinity stress reduces allantoinase, causing an accumulation of allantoin that helps remove reactive oxygen species (ROS) and protect the plant from oxidative damage.[45] The downregulation of allantoinase isoform X1 (*Spo06613*) in response to salinity stress was observed in leaves of most spinach genotypes (Figure 4). This finding suggests that spinach and beet, which are closely related species, may share similar mechanisms for salinity tolerance. Metallothioneins are a family of proteins involved in metal ion homeostasis, critical for scavenging ROS during stress conditions in plants.[46] Despite exhibiting high concentrations of Na and Cl in the shoot, 'CGN 9629' demonstrated comparatively superior performance for shoot biomass STI (Figures 1 and 2). This could be attributed to the upregulation of a root metallothionein-like gene, *Spo21342* (Figure 4).

*Leaves.* *NHX1* and *NHX2* genes are known to play important roles in the sequestration of $Na^+$ in leaf vacuoles.[26,27] While *NHX1* was induced in most spinach genotypes under salinity, it was found to be significantly upregulated in the leaves of 'Indures', 'Dikensiz', 'Victoria', and 'Dikenli' when compared to the control (Figure 5). These observations indicated active sequestering of Na in leaf vacuoles in these genotypes. Although *NHX2* was upregulated in several spinach genotypes under salinity, it was found to be downregulated in some high-performing genotypes such as 'Dikenli', 'Palek', 'Victoria', and 'New Asia' (Figure 5), suggesting that *NHX2* may not play a critical role in the sequestration of Na into leaf vacuoles in these genotypes. The results showed downregulation of both *NHX1* and *NHX2* genes under salinity in 'Palek' (Figure 5), a genotype with high leaf Na concentration under salinity conditions (Figure 2a). However, despite this downregulation, 'Palek' exhibited superior performance in terms of STI for biomass (Figure 2), indicating that tissue tolerance may play a more crucial role in the salinity tolerance mechanism of this genotype.

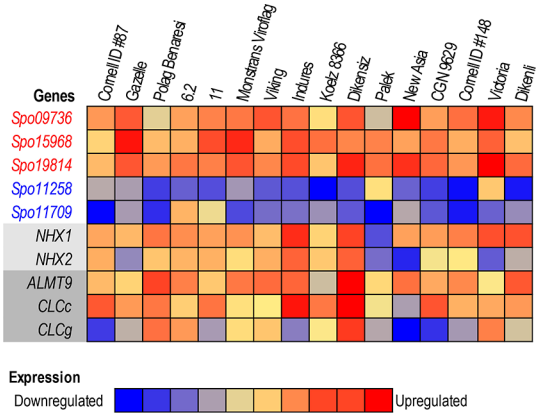


**Figure 5.** Heat map representing the relative expression of salt stress-related genes in leaves of 16 spinach genotypes. The ratio between the expression values under salinity and control was used to calculate the relative expressions and is depicted using different color shades. The gene names in red and blue fonts represent upregulated and downregulated genes, respectively, under salinity compared to control, from a previous RNA-seq study.[17] Gene names highlighted with light gray represent genes involved in $Na^+$ transport, and genes highlighted in dark gray represent genes involved in $Cl^-$ transport.

The genes *ALMT9*, *CLCc*, and *CLCg* are involved in sequestering $Cl^-$ in leaf vacuoles.[33] 'Dikensiz' showed significant upregulation of all three genes under salinity compared to control (Figure 5), suggesting effective sequestration of $Cl^-$ in leaf vacuole. However, 'Dikensiz' did not display efficient regulation of root-to-shoot movement of $Cl^-$, as indicated by its low Cl concentration in roots but high Cl concentration in leaves (Figure 2). This may be the reason for its relatively low STI for biomass (Figure 1). 'Dikenli' showed upregulation of *ALMT9* and *CLCc* but not for *CLCg* (Figure 5). There was a strong upregulation of *CLCc* in 'CGN 9629' and 'Indures' under salinity (Figure 5). Expression analysis led to the classification of different genotypes based on their ability to sequester $Na^+$ and $Cl^-$ in leaf vacuoles. Based on the expression analysis the genotypes 'Dikensiz' and 'Indures'


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were best equipped to sequester $Na^+$ and $Cl^-$ in their leaf vacuoles, protecting cytoplasm from ion toxicity.

From a previous RNA-seq experiment,[17] three highly upregulated genes (*Spo09736*, *Spo15968*, and *Spo19814*) and two significantly downregulated genes (*Spo11258* and *Spo11709*) under salinity compared to control in leaves were further investigated in this study for their expression in different genotypes. *Spo09736* was upregulated under salinity in all genotypes except 'Palek', with the highest upregulation in 'New Asia' and 'Victoria' (Figure 5). *Spo09736* encodes for expansin-like B1 (EXLB1), which is believed to play a critical role in plant responses to salinity stress.[47] Salinity stress can lead to reduced cell expansion and growth inhibition, but EXLB1 can help counteract these effects by loosening the cell wall, increasing its elasticity, and promoting water and nutrient uptake.[47] *Spo15968* was upregulated under salinity in leaves in all 16 genotypes, with a minimum upregulation in 'Cornell ID # 87', a genotype with the lowest STI for biomass (Figure 5). *Spo15968* codes for S-type slow anion channel-associated homologue 2-like (SLAH2-like) protein, which is critical in nitrate transport and is important for ion homeostasis during salinity stress.[48] *Spo19814*, which encodes a probable zinc metallopeptidase, EGY3, was upregulated in all 16 spinach genotypes, with the highest upregulation in 'Victoria', a genotype with the second highest STI for shoot biomass (Figure 5). 'Cornell ID #87' had the second-lowest upregulation under salinity compared to the control. EGY3 enhances salinity tolerance by mediating chloroplastic ROS homeostasis and retrograde signaling.[49] *Spo11258* and *Spo11709* were downregulated in the leaves of most spinach genotypes under salinity compared to the control (Figure 5). *Spo11258* encodes an E3-ubiquitin ligase, which has been shown to negatively regulate drought and salinity responses in soybean by increasing stomata density and aperture through the ABA-signaling pathway.[50] On the other hand *Spo11709* encodes a cysteine-rich receptor-like protein kinase 10 (CRK10). Although biotic and abiotic stress-responsive pathways were constitutively upregulated in the *crk*10 mutant in Arabidopsis,[51] direct involvement of CRK10 in salinity tolerance has not been shown.

In conclusion, based on root and shoot biomass yield under salinity, mineral ion analysis, and expression analyses in the roots and the leaves, we established that the salinity tolerance mechanism in spinach is a complex and multifaceted trait. Our findings have allowed us to classify different genotypes based on their individual component traits regarding salinity tolerance, which showed that different genotypes from diverse geographical origins possess varying degrees of different component traits. This indicates that there may be a possibility of introgressing multiple traits into a single genotype to develop salt-tolerant genotypes. Although the component traits are likely to be regulated by multiple genes, identification and isolation of individual genes could pave the way for developing genotypes that are tolerant to multiple components of the salinity tolerance mechanism.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.3c00149.

Primers used for expression analysis (PDF)

Leaf and root ion (Ca, Mg, $SO_4$, and Zn) concentrations under the control and salinity treatments in 16 spinach genotypes (XLSX)

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### Author Contributions

D.S. and J.F.S.F. conceptualized and designed the experiments; M.P., D.S., and M.W. conducted the salinity evaluation experiments. M.P. and M.W. conducted the gene expression analysis; D.S., M.W., M.P., J.F.S.F., and A.K. analyzed and interpreted the data; D.S. supervised the experiments; and D.S. and J.F.S.F. evaluated and discussed the results. All authors participated in the writing and approval of the manuscript.


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All the data sets supporting this study are included within the article and its Supporting Information.

## ■ ABBREVIATIONS

AKT1, arabidopsis $K^+$ transporter CBL; ALMT, aluminum-activated malate transporter; CCC, cation-chloride cotransporter; CIPK, CBL-interacting protein kinase; CLC, chloride channel; dS $m^{-1}$, desiSiemens per meter; $EC_e$, electrical conductivity of soil paste; $EC_{iw}$, electrical conductivity of irrigation water; HKT, high-affinity $K^+$ transporter; NHX, $Na^+/H^+$ exchanger; NPF, nitrate transporter/peptide transporter family; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RNA-seq, RNA sequencing; ROS, reactive oxygen species; SLAH, slow anion channel-associated homologue; SOS, salt overly sensitive; STI, salt tolerance index

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