Communication

Expression Profile of Selected Genes Involved in Na+ Homeostasis and In Silico miRNA Identification in *Medicago sativa* and *Medicago arborea* under Salinity Stress

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Abstract: The accumulation of ions due to increased salinity in the soil is one of the major abiotic stressors of cultivated plants that negatively affect their productivity. The model plant, *Medicago truncatula*, is the only *Medicago* species that has been extensively studied, whereas research into increased salinity adaptation of two important forage legumes, *M. sativa* and *M. arborea*, has been limited. In the present study, the expression of six genes, namely *SOS1*, *SOS3*, *NHX2*, *AKT*, *AVP* and *HKT1* was monitored to investigate the manner in which sodium ions are blocked and transferred to the various plant parts. In addition, in silico miRNA analysis was performed to identify miRNAs that possibly control the expression of the genes studied. The following treatments were applied: (1) salt stress, with initial treatment of 50 mM NaCl and gradual acclimatization every 10 days, (2) salt shock, with continuous application of 100 mM NaCl concentration and (3) no application of NaCl. Results showed that *M. arborea* appeared to overexpress and activate all available mechanisms of resistance in conditions of increased salinity, while *M. sativa* acted in a more targeted way, overexpressing the *HKT1* and *AKT* genes that contribute to the accumulation of sodium ions, particularly in the root. Regarding miRNA in silico analysis, five miRNAs with significant complementarity to putative target genes, *AKT1*, *AVP* and *SOS3* were identified and served as a first step in investigating miRNA regulatory networks. Further miRNA expression studies will validate these results. Our findings contribute to the understanding of the molecular mechanisms underlying salt-responsiveness in *Medicago* and could be used in the future for generating salt-tolerant genotypes in crop improvement programs.

Keywords: salinity; gene expression; resilient mechanisms; ion transportation; *Medicago* sp.; miRNA; MiR156

1. Introduction

Alfalfa (*Medicago sativa* L.) is the most extensively cultivated perennial forage legume with high ecological importance [1]. Tree medick (*Medicago arborea* L.) is a Mediterranean stress-tolerant leguminous fodder shrub [2] that could play an important role as a genetic resource for the improvement of *Medicago* sp. salt tolerance [3]. Salinity is a major environmental abiotic stress factor in crop productivity worldwide that impacts plant development and yield via two main mechanisms: hyperosmotic pressure, which is caused by low water availability, and ion toxicity (especially Na+), arising from solute imbalances [4]. However, little is known regarding the molecular mechanisms involved in the responses of *Medicago* sp. to salinity except *Medicago truncatula*.

Salt tolerance in higher plants is a quantitative trait that is regulated by polygenes and numerous signaling pathways, and a complex interaction system exists between the genes

and the signaling pathways [5,6]. Genes that contribute to salt tolerance in plants can be categorized into three functional groups: the first contains genes that regulate salt uptake and transport, the second contains genes with osmotic function, and the third contains genes that regulate plant growth under saline soil [3]. The salt overly sensitive (*SOS*) signal transduction pathway is pivotal for discharging Na$^+$ and maintaining ionic balance on a cellular level and confers improved salt tolerance in plants [7]. Several studies reported that the overexpression of *Arabidopsis thaliana SOS1*, *SOS2* and *SOS3* genes enhances salt tolerance in a variety of plant species including *M. sativa* [1]. Previous studies have also indicated that overexpression of the stress-associated gene encoding the compatible solute, the ion transporter *SeNHX1*, results in enhanced tolerance to salinity stress in alfalfa [8]. Na$^+$/H$^+$ antiporters (*NHXs*), which are located on plasma membranes and tonoplast, participate in the maintenance of Na$^+$ homeostasis by transporting Na$^+$ from the cytoplasm into either the extracellular spaces or vacuoles [9]. *AKT* and *HKT1* genes play a major role in the transport of Na$^+$ from shoots to roots, thus preventing leaf damage [10]. *AVP* gene generates a proton gradient for the driving force for Na$^+$/H$^+$ antiporter and other transporters on the vacuolar membranes, leading to increased salt tolerance and osmotic adjustment in plants [11].

A plethora of miRNAs have been reported to contribute to plant stress responses [12,13] and stimulate interest due to their ubiquitous interaction with target genes related to stress tolerance [14,15]. Recent studies have confirmed the existence of highly conserved, yet not functionally characterized miRNAs, that are induced upon several abiotic stressors [16,17]. Research into miRNAs involved in alkali stress is relatively rare. Regardless of the species, the miR156, miR319, miR398, miR172, miR408, miR169 and miR528 families were most frequently regulated by salt/alkali stress responses [16,18]. Regarding the *Medicago* species, further elucidation of the molecular mechanism's underlying responses to salt/alkali stress can be accomplished by identifying miRNAs and their putative target genes.

The present work was carried out following the findings of a previous study [3] concerning the species *M. arborea* and *M. sativa*. An effort has been made to further clarify those findings, suggesting the molecular mechanisms that the parents of the Alborea hybrid utilize to efficiently respond against salinity stress. To the best of our knowledge, there are no reports regarding the response of the Alborea hybrid to salinity, except from our previous studies [3,19] that clearly demonstrated Alborea's sensitivity to this stressor. To that end, the present study focused on salinity-induced changes in six genes, namely *SOS1*, *SOS3*, *NHX2*, *AKT*, *AVP* and *HKT1*, to investigate more extensively the way sodium ions are blocked and transferred to the various plant parts, preventing ion toxicity and ensuring Na$^+$ homeostasis. Additionally, an in-silico analysis was conducted to identify miRNAs that may interact with the genes under study.

2. Results and Discussion

2.1. Gene Expression Levels

Figure 1 displays the expression levels of six salt stress-related genes in *M. sativa* and *M. arborea*. Information and additional data regarding phenotypical and physiological differences among the two species have already been published in previous works [3,19].

From the *SOS* gene family, we studied two genes, namely *SOS1* and *SOS3*, which are directly involved in the regulation of salt tolerance by eliminating Na$^+$ from plant roots. According to our results, *M. arborea* showed generally higher gene expression values than *M. sativa* for all treatments, for both shoots and roots, while *M. sativa* overexpressed the genes more targeted focusing on roots. However, the highest *SOS1* and *SOS3* gene expression values appeared in plants (both roots and shoots) that were subjected to 100 mM NaCl. (Figure 1(a1,b2)). According to Mäser et al. [20], overexpression of *SOS* genes in *M. falcata* and *M. truncatula* shows that Na$^+$ release from cells is an important factor in plant survival under saline conditions, and activation of this mechanism can improve plant resistance.
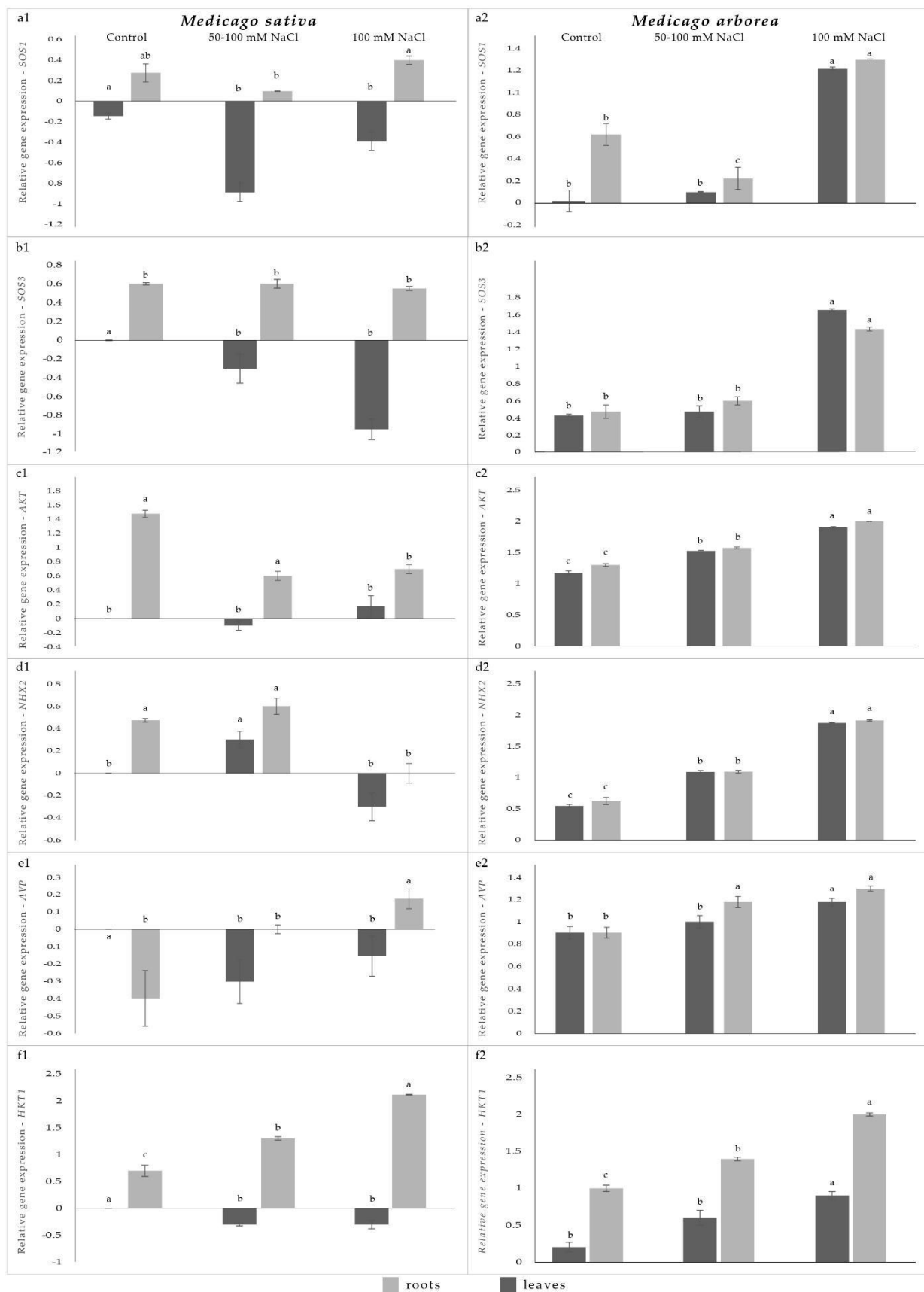
Figure 1. Mean expression values of roots (light grey) and leaves (dark grey) of: (**a1**) *SOS1* gene in *M. sativa*, (**a2**) *SOS1* gene in *M. arborea*, (**b1**) *SOS3* gene in *M. sativa*, (**b2**) *SOS3* gene in *M. arborea*,

(**c1**) *AKT* gene in *M. sativa*, (**c2**) *AKT* gene in *M. arborea*, (**d1**) *NHX2* gene in *M. sativa*, (**d2**) *NHX2* gene in *M. arborea*, (**e1**) *AVP* gene in *M. sativa*, (**e2**) *AVP* gene expression in *M. arborea*, (**f1**) HKT1 gene in *M. sativa*, (**f2**) *HKT1* gene in *M. arborea* in three different salinity treatments. Comparisons between genotypes differ significantly at $p < 0.05$. Means followed by a different letter differ significantly ($p < 0.05$) based on the Tukey analysis. Relative expression ratios were calculated using the $2^{-\Delta\Delta CT}$ method [21]. The first two bars are for control treatment, the next two bars are for the 50–100 mM NaCl treatment and the final two bars are for the 100 mM NaCl treatment.

They also argue that Na$^+$/H$^+$ antiporter *SOS* genes had a role in counteracting root to shoot Na$^+$ transport. *SOS* genes are expressed in the root and leaf vasculature, predominately in the root tips, and have been considered to re-extract Na$^+$ from the root xylem under salt stress via a proposed reversal of the *SOS* transporter. Sandhu [10] highlighted the major role that *AKT* and *HKT1* genes play in the transport of Na$^+$ from shoots to roots. In this study, the two genes were overexpressed in the roots of *M. sativa* while the expression values for both genes in leaves were very low for all treatments (Figure 1(c1,f1)). Specifically for the *HKT1* gene, the most elevated expression level was observed in the roots of plants subjected to 100 mM NaCl. Similarly, ref. [20] highlighted that in *Arabidopsis* the *HKT1* gene was strongly expressed in roots. Inversely, the overexpression of these two genes in *M. arborea* plants was very profound for both leaves and roots of the stressed plants, especially in plants which were subjected to salt shock (Figure 1(c2,f2)). Additionally, it was [10] demonstrated by testing 12 genotypes of *M. sativa* that the two genotypes of highest salinity resistance overexpressed the *AKT* and *HKT1* genes, mainly in roots compared to leaves, suggesting the contribution of these genes to salinity tolerance. Mäser et al. [21] also showed that in the *Arabidopsis thaliana* species, K$^+$ starvation induced *HKT1* expression in root cortex cells and enhanced inward Na$^+$ currents that included *HKT1*-like properties in root cortex cells; therefore, disruption of *HKT1* facilitates the net transfer of Na$^+$ to shoots. Furthermore, *HKT1* could counteract shoot-ward Na$^+$ transport either by sequestering Na$^+$ in the roots and/or preventing it from being loaded into the xylem by re-extracting Na$^+$ that is being loaded into the xylem, or by removing Na$^+$ from leaves and transporting Na$^+$ to roots via the phloem.

The *NHX2* and *AVP* genes are known to play an important role in isolating sodium in the vacuole to protect the cytoplasm from toxic effects. Overexpression of *NHX2* gene in alfalfa transgenic plants [8,22] and the *AVP* gene in Arabidopsis transgenic plants [23,24] improved salinity tolerance by improving intracellular potassium homeostasis. Additionally, several studies in cultivated crops highlighted the importance of *AVP* overexpression for providing increased plant yield under salinity and drought stress conditions [25–27]. In this study, *M. sativa* demonstrated the highest gene expression value for *NHX2* in both roots and leaves for plants subjected to gradually increased salt concentrations (Figure 1(d1)). On the contrary, regarding *AVP* expression profile in *M. sativa*, the highest up-regulation was observed in the roots of plants which were treated with 100 mM NaCl (Figure 1(e1)). In contrast to *M. sativa*, gene expression profiles for all *M. arborea* salt treatments were clearly elevated (Figure 1(d2,e2)).

Considering all gene expression values under increased salinity conditions for both *M. sativa* and *M. arborea*, we can conclude that *M. arborea* seems to overexpress all examined genes in both shoots and roots, while *M. sativa* acts in a more targeted manner, overexpressing specific genes (*HKT1* and *AKT*), which help the accumulation of Na$^+$ more specifically in roots. The same trend was observed in another study [3] in which gene expression related to ion transport was carried out under salinity stress. Both studies agree that *M. sativa* uses a more cost-efficient strategy to respond to salinity by activating specific genes.

2.2. miRNA In Silico Analysis

Given the importance of miRNAs in the regulation of gene expression during plant development and in response to abiotic stresses, we performed in silico analysis by use of the 'psRNATarget' tool, to uncover miRNAs that may target the genes under study

(Table 1). The analysis used miRNA and mRNA sequences from *M. truncatula* since most *Medicago* miRNAs sequences in the miRNA databases have been derived from this model leguminous plant, as well as due to its high genetic relationship with related species of the *Medicago* genus, such as *M. sativa* and *M. arborea*. Significant complementarity was detected between the conserved miRNA *mtr-miR156a* and the mRNA of the gene *MtAVP* (Pyrophospate-energized vacuolar membrane proton pump) in 15 consecutive positions (1–15 bp) from the 5′ end of the miRNA (Table 1). G-U wobbles are allowed in miRNA-target complementarities, and in plants, it seems that complementarity in at least 1–13 consecutive bases from the 5′ end of the miRNA may be associated with actual cleavage of the mRNA of the target gene and further degradation, or translational inhibition. This implies putative binding of *mtr-miR156a* to the *MtAVP* mRNA target and subsequent cleavage or translational inhibition of the transcript [28,29], leading to possible downregulation of the *MtAVP* gene.

Table 1. In silico identification of putative miRNAs targeting *AKT1*, *AVP*, *SOS3* and alignments of miRNAs and their corresponding mRNAs.

miRNA	Target Name and Accession Number	Alignment				Target Description	Inhibition Type
mtr-miR167b-3p	*AKT1* XM_003609192	miRNA	20	CCACUUCGAGGUUGUACUAG	1	*M. truncatula* potassium channel protein LOC11443237 *AKT1* mRNA	Cleavage
				`::::::::::::: ::::.`			
		Target	1354	AAUGAAGCUCCAAC-UGAUU	1372		
mtr-miR156a	*AVP* XM_003609415.3	miRNA	21	ACACGAGAGAGAGAAGACAGU	1	*M. truncatula* pyrophosphate-energized vacuolar membrane proton pump (LOC11438181) *AVP* mRNA	Cleavage
				`:: :::::::::.::::.:`			
		Target	2478	CAUGGCCUCUCUUUUUUGUUA	2498		
mtr-miR2605	*SOS3* XM_003602196.3	miRNA	20	CAUCCAGUAUAUUUGAUUCA	1	*M. truncatula* calcineurin B-like protein 7 (LOC11440434), transcript variant X4, *SOS3* mRNA	Cleavage
				`::::::.::::.: ::.::`			
		Target	631	UUAGGUUGUAUGAUCUGAGG	650		
mtr-miR2670f	*SOS3* XM_003602196.3	miRNA	21	AGGGGUUGGAUUGUCUGGUGA	1	*M. truncatula* calcineurin B-like protein 7 (LOC11440434), transcript variant X4, *SOS3* mRNA	Cleavage
				`..::::..:::..:.:..::`			
		Target	280	AAUUCAAUUUAAUGGGCUGCU	300		
mtr-miR2670g	*SOS3* XM_003602196.3	miRNA	21	AGGGGUUGGAUUGUCUGGUGA	1	*M. truncatula* calcineurin B-like protein 7 (LOC11440434), transcript variant X4, *SOS3* mRNA	Cleavage
				`..::::..:::..:.:..::`			
		Target	280	AAUUCAAUUUAAUGGGCUGCU	300		

Similarly, a significant match was observed between *miR167b* and *MtAKT1* mRNA in 17 consecutives bases from the 5′ end of the miRNA. A gap has been introduced, indicating that there may be a bulge in that position in the miRNA-target dimer structure. *MiR156a* and *miR167b* both belong to well-conserved plant miRNA families and have been implicated in salt responsiveness in a variety of species [30–32]. Based on the above observations, we could speculate that if *miR156a* targets *AVP* mRNA in *M. sativa* salt-stressed leaf tissue, the *AVP* target is downregulated, presumably due to high expression of *miR156a*. On the other hand, in root tissue *miR156a,* it is probably downregulated and, consequently, the *AVP* gene is upregulated (Figure 1). Likewise, if *miR167b* targets *AKT1*, a negative correlation between *miR167b* and *AKT1* messenger RNA levels might be expected in salt-stressed leaves and roots in a manner equivalent to the regulation of the *miRNA156-AVP* putative module. However, these hypotheses await further experimentation and validation.

Thus far, *miR167* and *miR156* have been implicated in the salt response as well as in other abiotic stresses and developmental processes in a variety of species through the targeting and regulation of transcription factor genes, mainly *ARF (AUXIN RESPONSE FACTOR)* and *SQUAMOSA LIKE PROTEIN (SLP)*, respectively [33–38]. Research on miRNA regulation of gene expression has focused on transcription factor target genes, and modules such as *miR156-SPL, miR159-MYB, miR164-NAC, miR167-ARF* and *miR172-AP* have been widely investigated. However, to date, little information exists regarding the engagement of other target genes in these regulatory processes through miRNA-mRNA modules, including the genes under study. An ever-increasing number of miRNAs and putative target genes identified in recent years [33,34] will facilitate the study of other miRNA-target gene pairs and their function in salt-tolerance. Our analysis of *AKT1, AVP* and *SOS3* will provide additional information to these studies and contribute towards this goal. Furthermore, candidate miRNAs targeting *SOS3* seem to be the *miR2605, mir2670f* and *miR2670g*. Nevertheless, there is no information thus far regarding these sequences, and they may represent species-specific miRNAs that need to be studied further.

This work highlights the importance of Na^+ compartmentation and the action of the *SOS1, SOS3, NHX2, AKT, AVP* and *HKT1* genes towards salinity tolerance. MiRNAs are also known to contribute to salinity resilience. Expression studies via qPCR experiments for the identified miRNA to validate their significance will possibly enable their future use into breeding programs.

3. Materials and Methods

3.1. Plant Material and Seed Pretreatment

The *M. sativa* population used in this study was a hybrid of two *M. sativa* parents [3]. Seeds from both *M. sativa* and *M. arborea* were obtained from E. Bingham, *Agronomy* Department, University of Wisconsin-Madison, Madison, WI, USA. The germplasm of *M. arborea* is a collection from Greece [3]. The seeds were scarified with absolute sulfuric acid for 10 min and then rinsed thoroughly with sterile distilled water. Bleach solution (3%) was added for 1.5–2 min and then rinsed thoroughly. The scarified seeds were placed on Petri dishes containing 0.5% agar at 4°C overnight, and then transferred to 20°C (dark) for 3–4 days.

3.2. Growth Conditions and Salt Stress Treatments

From each of the *Medicago* species, 60 seeds were planted, after their germination, in pots of 8.5 cm height and 10 cm in diameter. Each pot contained peat, Kronos N 50–300 mg/L, P_2O_5 80–300 mg/L, K2O 80–300 mg/L, pH 5–6.5, salinity <1.75 g/L and one seed. The pots were then placed in a growth chamber at a constant temperature of 25 °C, and a photoperiod of 16 h of light and 8 h of darkness, where they were regularly watered and fertilized every week. For each treatment, 20 plants in seedling stage were used for each species in a completely randomized design. The following treatments were applied: (1) salt stress, with initial treatment of 50 mM NaCl and gradual step acclimatization 10 days after, with treatment of 100 mM NaCl, (2) salt shock, with continuous application of 100 mM NaCl concentration and (3) no application of NaCl. Plants were watered every five days after salt treatment initiation. Once per week, Hoagland solution [39] was added to the salt solution. The growth chamber experiment lasted 34 days. After the completion of the experiment, whole shoots, excluding cotyledons and the roots, free of pathogens, were cut and immediately transferred into liquid nitrogen in the middle of the light period. When harvested, all plants were in the vegetative stage, and roots did not show nodules.

3.3. RNA Isolation and cDNA Synthesis

For plant RNA isolation, the TRIzol reagent kit (Sigma-Aldrich, St. Louis, MO, USA) was used and RNA concentration was measured by Nanodrop Spectrophotometer (Thermo Scientific NanoDrop™ 1000 Spectrophotometer). For cDNA synthesis, Superscript II enzyme (Invitrogen, Carlsbad, CA, USA) was used. The quality of the cDNA was evaluated

by PCR using an actin gene as the endogenous control reference gene. The primers used in this experiment are based on Sandhu et al., 2017 [10].

3.4. Real-Time PCR Experiments

The StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) machine was used to detect relative transcript levels using SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA). The threshold cycle CT method was used, in which the relative amount of a target gene in the samples can be determined. In our experiment, six target genes were studied (*SOS1, SOS3, NHX2, AKT, AVP* and *HKT1*) and the primers that were used were the same as Sandhu et al., 2017. Primer selection was based on Sandhu et al., 2017 [10]. All reactions were performed in triplicates. Melting curves of the reaction products were generated and fluorescence data were collected at a temperature above the melting temperature of non-specific products. Relative expression levels of the studied genes were calculated according to the $2^{-\Delta\Delta Ct}$ method [20] (for calculating $\Delta\Delta Ct$ value, the control sample of each entry was used). Actin-2 was used as an internal control for normalization.

3.5. Statistical Analysis

The effect of variables on gene expression was tested using the one-way analysis of variance (ANOVA) followed by mean comparisons through Tukey's HSD post hoc test with p-values of $p < 0.05$. Values are expressed as mean $\pm$ SEM (standard error of mean). The statistical analysis was performed using the IBM SPSS Statistics 23 statistical package for Windows (SPSS Inc., Chicago, IL, USA).

3.6. In Silico miRNA Analysis

For the prediction of potential miRNAs that may target and regulate the expression of the genes under study, the "psRNATarget: A Plant Small RNA Target Analysis Server" tool (https://www.zhaolab.org/psRNATarget/-version, 20 December 2022) was employed. The default parameters were used and were as follows: maximum expectation value of 5, penalty for G:U pair of 0.5, penalty for other mismatches of 1, seed region of 2–13 nt, number of mismatches allowed in the seed region of 2.0 and translation inhibition ranges of 10 nt to 11 nt. Under these parameters, and following the analysis of the results, the most significant score which designates the best possibility for miRNA-mRNA pair formation was selected.

References

1. Wang, C.M.; Zhang, J.L.; Liu, X.S.; Li, Z.; Wu, G.Q.; Cai, J.Y.; Flowers, T.J.; Wang, S.M. *Puccinellia tenuiflora* maintains a low Na$^+$ level under salinity by limiting unidirectional Na$^+$ influx resulting in a high selectivity for K$^+$ over Na$^+$. *Plant Cell Environ.* **2009**, *32*, 486–496. [CrossRef] [PubMed]
2. Aisset Amel, M.Z. Effect of salinity and water stress on the germination of *Medicago arborea* L. seeds. *J. Appl. Biol. Sci.* **2016**, *6*, 113–121.

3. Tani, E.; Sarri, E.; Goufa, M.; Asimakopoulou, G.; Psychogiou, M.; Bingham, E.; Skaracis, G.N.; Abraham, E.M. Seedling growth and transcriptional responses to salt shock and stress in *Medicago sativa* L.; *Medicago arborea* L.; and their hybrid (Alborea). *Agronomy* **2018**, *8*, 231. [CrossRef]

4. Volkov, V.; Amtmann, A. *Thellungiella halophila*, a salt-tolerant relative of *Arabidopsis thaliana*, has specific root ion-channel features supporting K$^+$/Na$^+$ homeostasis under salinity stress. *Plant J.* **2006**, *48*, 342–353. [CrossRef]

5. Pandita, D. Role of miRNA technology and miRNAs in abiotic and biotic stress resilience. In *Plant Perspectives to Global Climate Changes*; Aftab, T., Roychoudhury, A., Eds.; Academic Press: Cambridge, MA, USA, 2022; pp. 303–330.

6. Zelm, E.V.; Zhang, Y.; Testerink, C. Salt tolerance mechanisms of plants. *Annu. Rev. Plant Biol.* **2020**, *71*, 403–433. [CrossRef]

7. Zhu, J.K. Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* **2002**, *53*, 247–273. [CrossRef]

8. Zhang, L.Q.; Niu, Y.D.; Huridu, H.; Hao, J.F.; Qi, Z.; Hasi, A. *Salicornia europaea* L. Na$^+$/H$^+$ antiporter gene improves salt tolerance in transgenic alfalfa (*Medicago sativa* L.). *Genet. Mol. Res.* **2014**, *13*, 5350–5360. [CrossRef]

9. Wu, G.-Q.; Wang, J.-L.; Li, S.-J. Genome-wide identification of Na$^+$/H$^+$ antiporter (*NHX*) genes in sugar beet (*Beta vulgaris* L.) and their regulated expression under salt stress. *Genes* **2019**, *10*, 401. [CrossRef]

10. Sandhu, D.; Cornacchione, M.V.; Ferreira, J.F.S.; Suarez, D.L. Variable salinity responses of 12 alfalfa genotypes and comparative expression analyses of salt-response genes. *Sci. Rep.* **2017**, *7*, 42958. [CrossRef] [PubMed]

11. Toranj, S.; Aliabad, K.K.; Abbaspour, H.; Saeedpour, A. Effect of salt stress on the genes expression of the vacuolar H$^+$-pyrophosphatase and Na$^+$/H$^+$ antiporter in *Rubia tinctorum*. *Mol. Biol. Rep.* **2020**, *47*, 235–245. [CrossRef] [PubMed]

12. Sunkar, R.; Chinnusamy, V.; Zhu, J.; Zhu, J.K. Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. *Trends Plant Sci.* **2007**, *12*, 301–309. [CrossRef]

13. Xu, T.; Zhang, L.; Yang, Z.; Wei, Y.; Dong, T. Identification and functional characterization of plant miRNA under salt stress shed light on salinity resistance improvement through miRNA manipulation in crops. *Front. Plant Sci.* **2021**, *12*, 665439. [CrossRef]

14. Zhang, Y.M.; Zhang, H.M.; Liu, Z.H.; Li, H.C.; Guo, X.L.; Li, G.L. The wheat *NHX* antiporter gene *TaNHX2* confers salt tolerance in transgenic alfalfa by increasing the retention capacity of intracellular potassium. *Plant Mol. Biol.* **2015**, *87*, 317–327. [CrossRef]

15. Barciszewska-Pacak, M.; Milanowska, K.; Knop, K.; Bielewicz, D.; Nuc, P.; Plewka, P.; Pacak, A.M.; Vazquez, F.; Karlowski, W.; Jarmolowski, A.; et al. Arabidopsis microRNA expression regulation in a wide range of abiotic stress responses. *Front. Plant Sci.* **2015**, *6*, 410. [CrossRef]

16. Cao, X.; Wu, Z.; Jiang, F.; Zhou, R.; Yang, Z. Identification of chilling stress-responsive tomato microRNAs and their target genes by high-throughput sequencing and degradome analysis. *BMC Genom.* **2014**, *15*, 1130. [CrossRef]

17. Shi, R.; Jiao, W.; Yun, L.; Zhang, Z.; Zhang, X.; Wang, Q.; Li, Y.; Mi, F. Utilization of transcriptome, small RNA, and degradome sequencing to provide insights into drought stress and rewatering treatment in *Medicago ruthenica*. *Front. Plant Sci.* **2021**, *12*, 675903. [CrossRef]

18. Wani, S.H.; Kumar, V.; Khare, T.; Tripathi, P.; Shah, T.; Ramakrishna, C.; Aglawe, S.; Mangrauthia, S.K. miRNA applications for engineering abiotic stress tolerance in plants. *Biologia* **2020**, *75*, 1063–1081. [CrossRef]

19. Sarri, E.; Termentzi, A.; Abraham, E.M.; Papadopoulos, G.K.; Baira, E.; Machera, K.; Loukas, V.; Komaitis, F.; Tani, E. Salinity stress alters the secondary metabolic profile of *M. sativa*, *M. arborea* and their hybrid (Alborea). *Int. J. Mol. Sci.* **2021**, *22*, 4882. [CrossRef] [PubMed]

20. Mäser, P.; Eckelman, B.; Vaidyanathan, R.; Horie, T.; Fairbairn, D.J.; Kubo, M.; Yamagami, M.; Yamaguchi, K.; Nishimura, M.; Uozumi, N.; et al. Altered shoot/root Na$^+$ distribution and bifurcating salt sensitivity in Arabidopsis by genetic disruption of the Na$^+$ transporter *AtHKT1*. *FEBS Lett.* **2002**, *531*, 157–161. [CrossRef] [PubMed]

21. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]

22. Zhang, Y.M.; Liu, Z.H.; Wen, Z.Y.; Zhang, H.M.; Yang, F.; Guo, X.L. The vacuolar Na$^+$-H$^+$ antiport gene *TaNHX2* confers salt tolerance on transgenic alfalfa (*Medicago sativa*). *Funct. Plant Biol.* **2012**, *39*, 708–716. [CrossRef]

23. Gaxiola, R.A.; Li, J.; Undurraga, S.; Dang, L.M.; Allen, G.J.; Alper, S.L.; Fink, G.R. Drought- and salt-tolerant plants result from overexpression of the *AVP1* H$^+$-pump. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 11444–11449. [CrossRef]

24. Sandhu, D.; Kaundal, A.; Acharya, B.R.; Forest, T.; Pudussery, M.V.; Liu, X.; Ferreira, J.F.S.; Suarez, D.L. Linking diverse salinity responses of 14 almond rootstocks with physiological, biochemical, and genetic determinants. *Sci. Rep.* **2020**, *10*, 21087. [CrossRef] [PubMed]

25. Arif, A.; Zafar, Y.; Arif, M.; Blumwald, E. Improved growth, drought tolerance, and ultrastructural evidence of increased turgidity in tobacco plants overexpressing *Arabidopsis* vacuolar pyrophosphatase (*AVP1*). *Mol. Biotechnol.* **2013**, *54*, 379–392. [CrossRef] [PubMed]

26. Yang, H.; Knapp, J.; Koirala, P.; Rajagopal, D.; Peer, W.A.; Silbart, L.K.; Murphy, A.; Gaxiola, R.A. Enhanced phosphorus nutrition in monocots and dicots over-expressing a phosphorus-responsive type I H$^+$-pyrophosphatase. *Plant Biotechnol. J.* **2007**, *5*, 735–745. [CrossRef] [PubMed]

27. Bhaskaran, S.; Savithramma, D.L. Co-expression of Pennisetum glaucum vacuolar Na$^+$/H$^+$ antiporter and *Arabidopsis* H$^+$-pyrophosphatase enhances salt tolerance in transgenic tomato. *J. Exp. Bot.* **2011**, *62*, 5561–5570. [CrossRef] [PubMed]

28. Jones-Rhoades, M.W.; Bartel, D.P. Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell* **2004**, *14*, 787–799. [CrossRef]

29. Mallory, A.C.; Bouché, N. MicroRNA-directed regulation: To cleave or not to cleave. *Trends Plant Sci.* **2008**, *13*, 359–367. [CrossRef] [PubMed]

30. Cao, C.; Long, R.; Zhang, T.; Kang, J.; Wang, Z.; Wang, P.; Sun, H.; Yu, J.; Yang, Q. Genome-wide identification of microRNAs in response to salt/alkali stress in *Medicago truncatula* through high-throughput sequencing. *Int. J. Mol. Sci.* **2018**, *19*, 4076. [CrossRef]

31. Jodder, J.; Das, R.; Sarkar, D.; Bhattacharjee, P.; Kundu, P. Distinct transcriptional and processing regulations control miR167a level in tomato during stress. *RNA Biol.* **2018**, *15*, 130–143. [CrossRef]

32. Ma, J.; Wang, Y.; Li, J. Global identification and analysis of microRNAs involved in salt stress responses in two alfalfa (*Medicago sativa* 'Millennium') lines. *Can. J. Plant Sci.* **2019**, *100*, 445–455. [CrossRef]

33. Fu, R.; Zhang, M.; Zhao, Y.; He, X.; Ding, C.; Wang, S.; Feng, Y.; Song, X.; Li, P.; Wang, B. Identification of salt tolerance-related microRNAs and their targets in maize (*Zea mays* L.) using high-throughput sequencing and degradome analysis. *Front. Plant Sci.* **2017**, *8*, 864. [CrossRef] [PubMed]

34. Islam, W.; Waheed, A.; Naveed, H.; Zeng, F. MicroRNAs mediated plant responses to salt stress. *Cells* **2022**, *11*, 2806. [CrossRef]

35. Kang, T.; Yu, C.Y.; Liu, Y.; Song, W.M.; Bao, Y.; Guo, X.T.; Li, B.; Zhang, H.X. Subtly manipulated expression of ZmmiR156 in tobacco improves drought and salt tolerance without changing the architecture of transgenic plants. *Front. Plant Sci.* **2020**, *10*, 1664. [CrossRef] [PubMed]

36. Khraiwesh, B.; Zhu, J.K.; Zhu, J. Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochim. Biophys. Acta Gene Regul. Mech.* **2012**, *1819*, 137–148. [CrossRef] [PubMed]

37. Sharma, A.; Ruiz-Manriquez, L.M.; Serrano-Cano, F.I.; Reyes-Pérez, P.R.; Tovar Alfaro, C.K.; Barrón Andrade, Y.E.; Paul, S. Identification of microRNAs and their expression in leaf tissues of guava (*Psidium guajava* L.) under salinity stress. *Agronomy* **2020**, *10*, 1920. [CrossRef]

38. Song, C.; Zhang, D.; Zheng, L.; Zhang, J.; Zhang, B.; Luo, W.; Li, Y.; Li, G.; Ma, J.; Han, M. miRNA and degradome sequencing reveal miRNA and their target genes that may mediate shoot growth in spur type mutant "Yanfu 6". *Front. Plant Sci.* **2017**, *8*, 441. [CrossRef]

39. Suh, C. Evaluation of Bioactivity of Phytotoxins from Pathogenic Fungi of *Orobanche* sp. Ph.D. Thesis, Agricultural University of Athens, Athina, Greece, 2011. [CrossRef]