



Article

Transcriptome and GWAS Analyses Reveal Candidate Gene for Root Traits of Alfalfa during Germination under Salt Stress

Fei He ^{1,†}, Tianhui Yang ^{2,†}, Fan Zhang ¹, Xueqian Jiang ¹, Xianyang Li ¹, Ruicai Long ¹, Xue Wang ¹, Ting Gao ², Chuan Wang ², Qingchuan Yang ¹, Lin Chen ^{1,*} and Junmei Kang ^{1,*}

¹ Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China

² Institute of Animal Science, Ningxia Academy of Agricultural and Forestry Sciences, Yinchuan 750002, China

* Correspondence: chenlin@caas.cn (L.C.); kangjunmei@caas.cn (J.K.)

† These authors contributed equally to this work.

Abstract: Alfalfa growth and production in China are negatively impacted by high salt concentrations in soils, especially in regions with limited water supplies. Few reliable genetic markers are currently available for salt tolerance selection. As a result, molecular breeding strategies targeting alfalfa are hindered. Therefore, with the continuous increase in soil salinity in agricultural lands, it is indispensable that a salt-tolerant variety of alfalfa is produced. We collected 220 alfalfa varieties around the world for resequencing and performed genome-wide association studies (GWASs). Alfalfa seeds were germinated in saline water with different concentrations of NaCl, and the phenotypic differences in several key root traits were recorded. In the phenotypic analysis, the breeding status and geographical origin strongly affected the salt tolerance of alfalfa. Forty-nine markers were significantly associated with salt tolerance, and 103 candidate genes were identified based on linkage disequilibrium. A total of 2712 differentially expressed genes were upregulated and 3570 were downregulated based on transcriptomic analyses. Some candidate genes that affected root development in the seed germination stage were identified through the combination of GWASs and transcriptome analyses. These genes could be used for molecular breeding strategies to increase alfalfa's salt tolerance and for further research on salt tolerance in general.

Keywords: alfalfa; GWAS; salt stress; root traits; SNP



Citation: He, F.; Yang, T.; Zhang, F.; Jiang, X.; Li, X.; Long, R.; Wang, X.; Gao, T.; Wang, C.; Yang, Q.; et al. Transcriptome and GWAS Analyses Reveal Candidate Gene for Root Traits of Alfalfa during Germination under Salt Stress. *Int. J. Mol. Sci.* **2023**, *24*, 6271. <https://doi.org/10.3390/ijms24076271>

Academic Editor: Juan Manuel Ruiz Lozano

Received: 20 January 2023

Revised: 18 March 2023

Accepted: 21 March 2023

Published: 27 March 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Salt stress can affect plant productivity, leading to retarded development, with this effect continuing throughout the growth and developmental stages of the plant [1]. One effect of salt stress is reduced water availability and increased osmotic stress imposed on plants, resulting in nutritional disorders and, ultimately, the inhibition of plant growth and photosynthesis [2]. Salt stress in the early stages of plant development can reduce the grain yield and plant protein content, resulting in declines in subsequent plant growth and yields [3,4]. During seed germination, the enzyme activity and water absorption capacity of seeds under salt stress significantly decrease and the germination rate of the seeds rapidly reduces [5]. Therefore, investigating the effect of salt stress in the seed germination stage is important, which is the first step in studying its impact on plant growth and nutrient production [6].

The root system is important in the growth, development, and physiology of crop plants, as well as in their responses to various stresses. The roots absorb water and nutrients from the soil and synthesize plant hormones, making them a necessary synthesis site for plant growth and development [7]. Serving as the interface for plant–soil interactions, the roots also play a key role in responding to environmental changes that can affect important traits, such as salt tolerance [8], drought resistance [9], and resistance to other abiotic stressors [10,11]. Under conditions of salt stress, the first response by plants is elicited by

the roots, the functioning of which depends on their characteristics and structure. Reducing the density of lateral roots and the number of axial roots in maize allows for greater axial root elongation and reduces root system competition among plants, thereby improving the water capture and resistance capabilities of maize plants to adverse environments [12]. Therefore, studying the mechanisms employed by plant roots to resist salt stress is essential for expanding our knowledge of plant salt tolerance, which could be used to develop more salt-tolerant crop varieties for improved agricultural sustainability.

Alfalfa (*Medicago sativa* L.) has the characteristics of high yield and high quality; it can improve the soil of saline–alkali land due to its ability to fix atmospheric nitrogen [13]. Alfalfa is easily affected by high-salinity soils, preventing many existing alfalfa varieties from growing on saline lands [14,15]. Therefore, studying the salt tolerance of alfalfa for the development of animal husbandry is important. A surge in the application of genome-wide association studies (GWASs) to a wide variety of plants has occurred in recent years. Many single-nucleotide polymorphisms (SNPs) related to various resistance traits have been identified, including those that confer abiotic stress in maize and soybean plants [16–18]. Many SNPs associated with salt and drought tolerance [19,20] and water scarcity [21] have been identified in alfalfa through GWASs. However, the genetics and molecular mechanisms underlying the responses to salt stress in alfalfa remain unclear, thus, greatly limiting the targeted improvement of alfalfa varieties [22,23]. The recent publication of several alfalfa cultivar genomes [13,23,24] may provide a theoretical basis for the identification of salt tolerance genes in alfalfa.

This study involved GWASs based on root traits under normal and salt stress conditions by combining GWASs and RNA-seq analyses to investigate the alleles associated with salt tolerance and candidate genes associated with root system development. The findings of this study might provide valuable molecular markers to improve the molecular breeding strategies for alfalfa.

2. Results

2.1. Analysis of Phenotypic Variations and Correlations among Salt-Related Root Traits

The following four traits were measured under different salt concentrations: the number of lateral roots (LRs), root length (RL), root volume (RV), and root diameter (RD). The descriptive statistics of the relative values of root traits under a salt treatment were calculated (Table 1). In most traits, the range of phenotypic variation was relatively large among different alfalfa varieties under different salt concentrations, with medians ranging from 0.88 (for RV_200) to 1.12 (for LR_100). RD_200 varied the most, with values ranging from 0.31 to 5.57 and a mean of 1.72. RL_200 varied the least, with values ranging from 0.64 to 1.58 and a mean of 1.06. The coefficient of variation ranged from 0.32 to 0.86.

Table 1. Phenotypic variation for 4 root traits under 3 different salt concentrations, including lateral roots (LRs), root length (RL), root volume (RV), and root diameter (RD), among 220 accessions.

Trait	Median	Mean	Range	SD	Kurtosis	Skewness	CV
LR_100	1.12	1.31	0.24–3.80	0.78	0.77	1.06	0.59
RV_100	0.96	1.03	0.35–2.52	0.40	1.45	1.21	0.39
RL_100	0.94	1.14	0.21–4.19	0.71	1.12	1.74	0.63
RD_100	1.00	1.03	0.54–1.71	0.24	0.02	0.44	0.43
LR_150	0.91	1.37	0.21–4.32	1.12	0.33	1.20	0.82
RV_150	0.94	1.11	0.22–3.37	0.59	1.08	1.65	0.53
RL_150	0.92	1.02	0.32–2.57	0.50	0.70	1.09	0.49
RD_150	1.02	1.04	0.50–1.72	0.28	−0.60	0.20	0.37
LR_200	0.91	1.21	0.25–4.95	0.99	4.23	1.93	0.81
RV_200	0.88	0.91	0.27–1.93	0.29	1.44	0.76	0.32
RL_200	1.04	1.06	0.64–1.58	0.26	−0.95	0.30	0.44
RD_200	1.11	1.72	0.31–5.57	1.48	0.51	1.30	0.86

In total, four root traits with normal distributions under three salt concentrations were measured (Figure 1). The correlation analysis showed that, for these traits, the largest correlation was between RV_100 and RL_100, reaching 0.73, followed by RV_150 and RL_150, reaching a significance level of 0.70. All other traits displayed significant differences at a salt concentration of 100 mM, excluding RV_100 and RD_100. All four traits showed significant differences at a salt concentration of 150 mM. All other traits exhibited significant differences at a salt concentration of 200 mM, excluding RV_200 and RL_200. The traits LR_200, RL_200, RL_200, and RD_200 exhibited a significant negative correlation. Most of these traits displayed positive correlations under different concentrations of salt treatment.

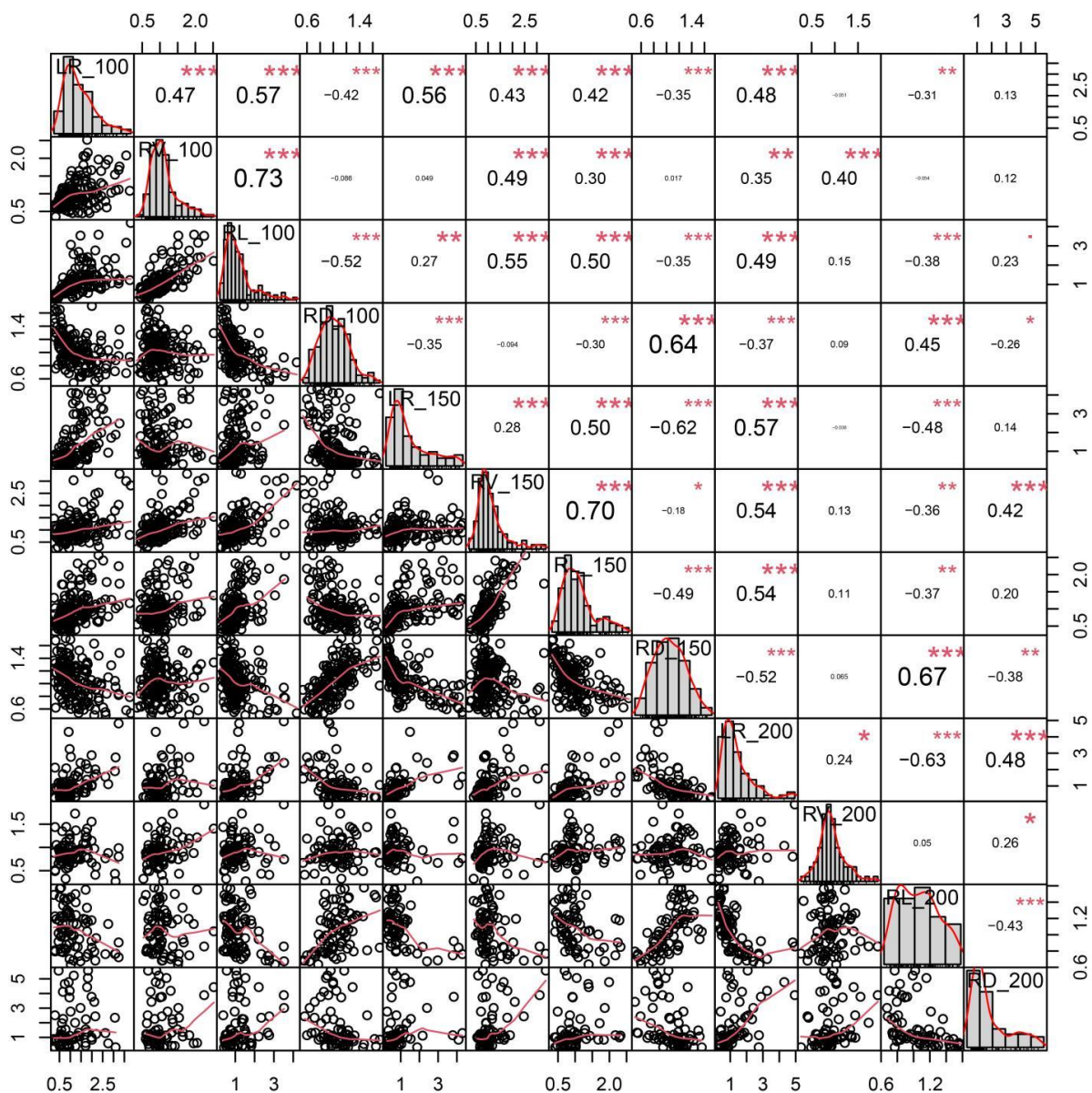


Figure 1. Distributions of and correlations between 12 relative phenotypic traits. The frequency distribution of each trait is shown on a central diagonal in the form of a histogram. Scatter plots between every pair of traits are shown in the areas below the diagonal, and numerical correlation coefficients between every pair of traits are shown in the areas above the diagonal. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

We divided our population into three categories based on their breeding status. The statistical analysis of these 12 salt-tolerance-related traits showed no significant differences, except for the LR_150 phenotypes of the wild and landrace subgroups, as well as of the wild and cultivar subgroups, for the RV_150 phenotypes of landrace and cultivar subgroups, and for the RV_200 phenotypes of wild and cultivar subgroups. The landrace and cultivars exhibited a greater salt tolerance, whereas the wild varieties had a lower salt tolerance in this population (Figure S1). We also divided our population into four categories based on their geographical origin. In these four subgroups, American and Turkish varieties had a better salt tolerance, followed by European and Chinese varieties (Figure S2).

2.2. GWAS and Identification of Candidate Genes for 12 Relevant Phenotypic Traits

A GWAS was conducted on the 12 relative traits (LR_100, RV_100, RL_100, RD_100, LR_150, RV_150, RL_150, RD_150, LR_200, RV_200, RL_200, and RD_200). We identified 49 significant loci (Figure 2 and Table S1). These SNPs were distributed across all chromosomes. They included a maximum of eight SNPs on chromosome three. The fewest number of SNPs, five, was found on chromosomes two and seven. Among the 12 traits, seven significant SNPs associated with RV_150 and nine significant SNPs associated with RL_200 were found. For RV_150, these SNPs were located on six chromosomes, but were not found on chromosomes two and three (Table S1). RL_100 had six significant markers distributed on chromosomes three, four, six, and eight. Only one marker (chr3_3146487) was significantly associated with RD_200.

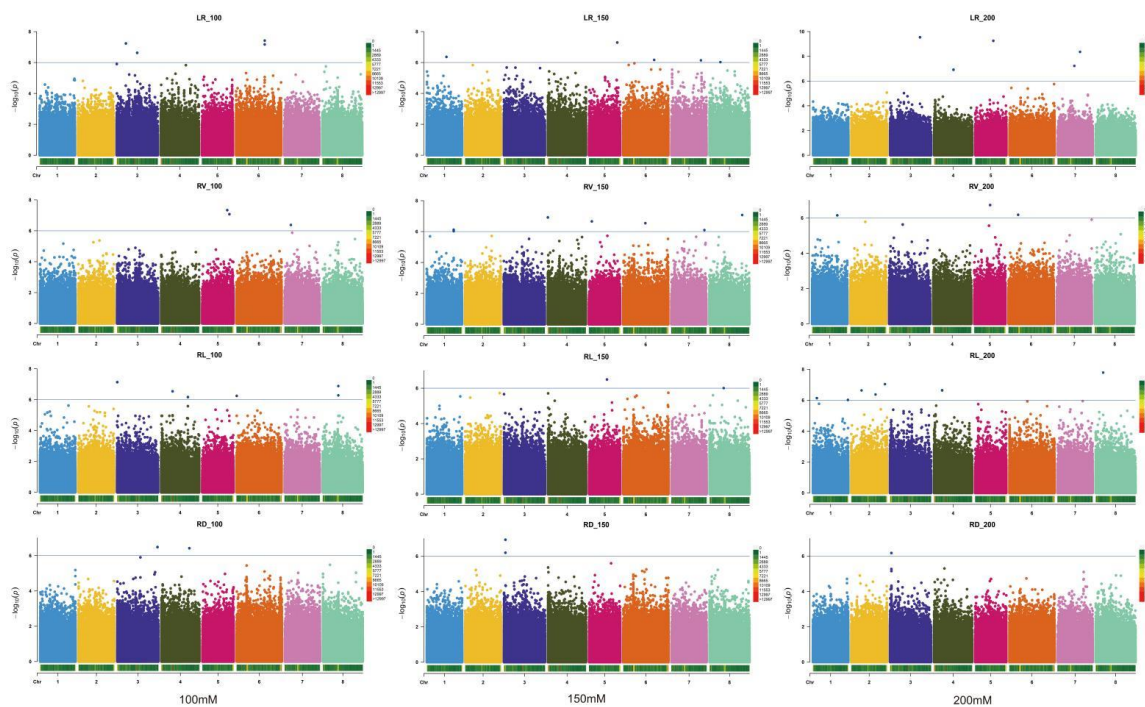


Figure 2. Manhattan plots of marker–trait associations for salt tolerance traits. Significant markers that passed a cutoff (p -value) of 6 are above the dot lines. The GWAS was performed using Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway C software, and the threshold for significantly associated loci was a logarithm of odds score of ≥ 6 (blue line). Different colors represent markers on different chromosomes.

One SNP with the highest threshold for each trait was selected to construct a raincloud plot so as to observe the effects of different alleles on salt tolerance (Figure 3). No homozygous genotypes (A/A) existed for SNPs chr3_381023, chr5_65228093, or chr5_36612531. In addition, no homozygous genotypes (T/T) existed for chr5_41320598, chr8_75617830, chr3_68865982, and chr3_3146487. Therefore, we speculated that the two genotypes

might be correlated with root salt tolerance in alfalfa. Next, we identified all genes within the 40 kb range of 49 significant SNP loci based on linkage disequilibrium (LD) and identified 103 candidate genes related to salt tolerance based on the sequence similarity with *A. thaliana* (Table S2).

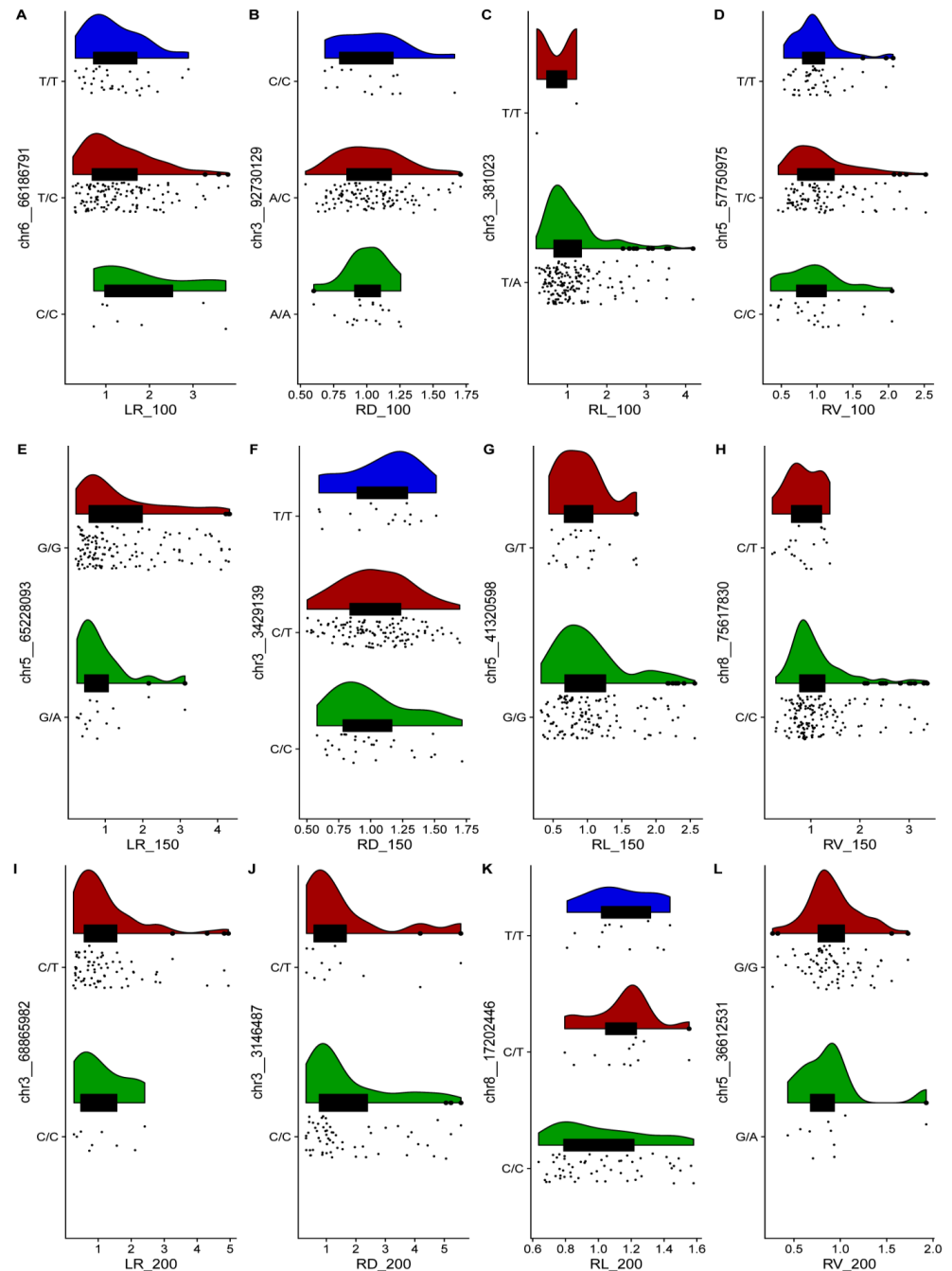


Figure 3. Raincloud plots of the highest distribution of the salt stress of plants with relevant SNP genotypes. The top plots represent the kernel density estimation, the middle plots represent box diagrams, and the bottom plots represent dithering scatter diagrams. Different colors represent different genotypes. (A–D) represents the genotype of Chr6_66186791, Chr3_92730129, Chr6_381023 and Chr6_57750975 at 100mM salt concentrations. (E–H) represents the genotype of chr5_65228093, Chr3_3429139, chr5_41320598 and chr8_75617830 at 150mM salt concentrations. (I–L) represents the genotype of chr3_68865982, chr3_3146487, chr8_17202446 and chr5_36612531 at 200mM salt concentrations.

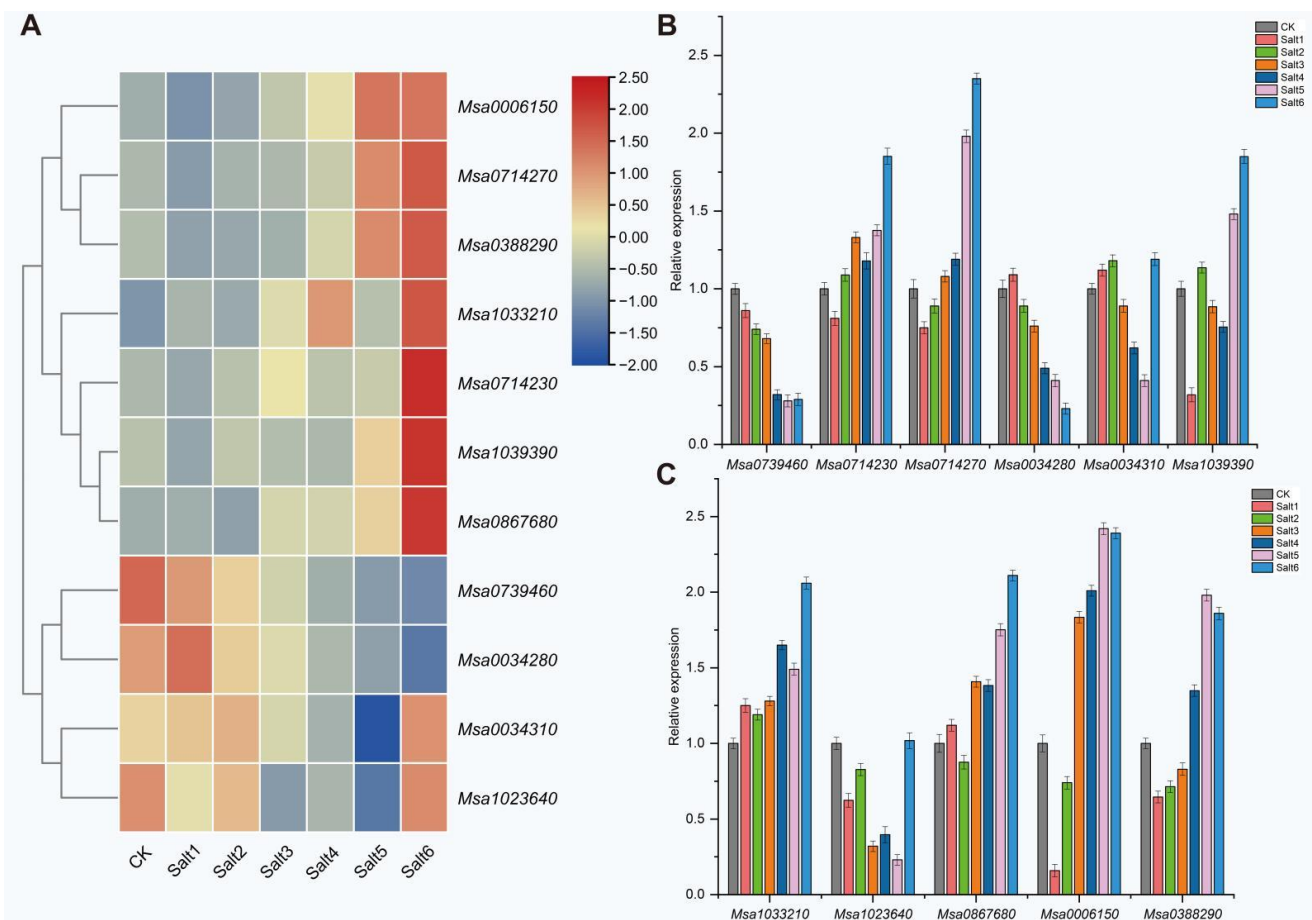


Figure 5. (A) Heatmap of the 11 DEGs that responded to salt stress. The expression levels were normalized by row using the Z-scores algorithm. The color scale on the right of the heatmap refers to the relative expression level, and the color gradient from blue to red presents an increasing expression level. (B) RT-qPCR analysis of *Msa0739460*, *Msa0714230*, *Msa0714270*, *Msa0034280*, *Msa0034310*, and *Msa1039390*. (C) RT-qPCR of *Msa1033210*, *Msa1023640*, *Msa0867680*, *Msa0006150*, and *Msa0388290*. CK was arbitrarily set to 1. Error bars represent the standard deviations of three technical replicates.

3. Discussion

Research shows that seedling vigor under salt stress is an effective selection method for breeding programs [25]. At present, minimal research has been conducted on salt tolerance in alfalfa [19,26,27]. In this study, the genetic basis of the salt tolerance of alfalfa was analyzed using a GWAS, revealing many promising gene targets significant for cultivating new alfalfa varieties in saline and alkaline soils.

Salt stress is a major abiotic factor that restricts plant growth and productivity; it can significantly reduce the fresh and dry weights of aboveground parts [28]. The root system is the first part of the plant to perceive toxic ions in the soil, and can guarantee the subsequent normal growth of plants [29]. Thus, the genetic mechanisms underlying different root traits have recently become a hot research topic. More recently, GWAS-based explorations have identified some genes related to roots that allow plants to acclimate to soil nitrogen [30] and phosphate [31] contents, and that are involved in root growth responses to salt stress [32]. Other studies showed that the root activity reduced, the RL shortened, and the lateral roots of plants became reduced under stress, which, ultimately, affected the overall RL and plant height [33,34]. In the present study, the phenotypic differences at different NaCl concentrations indicated that salt stress significantly inhibited the root growth of alfalfa seeds during germination. Previous studies showed that the RL of barley and maize was significantly reduced under salt stress [35,36], which was consistent with

our results. The formation and spatial distribution of LRs were the most important factors mediating soil exploration by plants. In the present study, we observed that the LRs of germinated seeds decreased significantly after the salt treatment, indicating that the LRs were inhibited, which might further lead to a delayed seedling growth. Further, the current results showed that most traits were highly correlated, suggesting that they might undergo similar molecular regulatory mechanisms under different degrees of salt stress.

The results demonstrated that the landrace and cultivated varieties exhibited a greater salt tolerance compared with the wild varieties, suggesting that some varieties with a good tolerance were selected for breeding during the process of artificial selection. In addition, we found that the varieties from America and Turkey also exhibited a better salt tolerance. This might have been related to the fact that most alfalfa varieties in Asia and the western United States were grown in saline–alkaline soils, because most of the higher-quality farmland was used for planting staple food crops. This results further indicated that selective breeding to improve salt tolerance and geographical origins might affect the allelic diversity in alfalfa. Further, the research could lead to a greater understanding of salt-tolerance-related genes, which could be used to improve salt tolerance in cultivated varieties.

Alfalfa is sensitive to salts in the soil, especially during germination. GWASs are effective in identifying such genes. Yu et al. used GBS technology to identify 36 molecular markers significantly related to salt tolerance using 198 materials under three salt treatments [37]. Liu et al. used natural populations to identify 42 markers related to dry weight and plant height traits that were significantly related to salt tolerance [38]. In this study, 49 significant SNPs with root development were found. However, no consistent loci were detected, indicating that these markers were independently related to their respective characteristics and that different genetic structures and molecular mechanisms were responsible for different root responses in alfalfa under salt stress. Further research on these mechanisms could provide the theoretical basis for cultivating salt-tolerant alfalfa.

Although GWASs can be used to identify the genes involved in abiotic stress responses in a wide range of crops [12,19], unresolved problems, such as false positives, can hinder our understanding of these processes. RNA-seq analysis has also become a tool for detecting gene expression [39]. A large number of DEGs can be obtained from transcriptome data. However, identifying potential key candidates can be challenging [40]. In recent years, GWASs and RNA-seq analyses have been integrated to predict candidate genes for traits related to defoliation [41], drought stress [42], and salt stress in alfalfa [43]. In this study, we identified 11 DEGs in six different growth stages, all of which played a role in the response of alfalfa to salt tolerance. A number of plant hormones play a role in salt stress responses, including jasmonic acid and abscisic acid (ABA) [44]. *AtHAD1* had an inhibitory effect on ABA responses and ABA-mediated tolerance in *A. thaliana*. In this study, we found that *Msa0034310*, encoding HAD (haloacid-dehalogenase-like hydrolase), was significantly correlated with the root development of alfalfa under salt stress, as the expression of *Msa0034310* in roots was upregulated under salt stress. In addition, we found that on chromosome one, the marker chr1__8903020 (*Msa0006150*) encoded betaine aldehyde dehydrogenase, which was necessary for glycine betaine biosynthesis and positively regulated plant responses to stress [45]. Therefore, the overexpression and the knockdown experiments with these genes were required to validate their functions in alfalfa salt stress responses.

In conclusion, we identified 49 SNP markers, distributed across all eight alfalfa chromosomes, through the use of a GWAS. In this study, 103 salt stress response genes were associated with significant markers related to salt tolerance in the roots of alfalfa during germination. Combining RNA-seq data, we identified 11 candidate genes that could be used for gene cloning and functional characterization, thus, helping improve our understanding of salt tolerance in alfalfa.

4. Materials and Methods

4.1. Plant Materials

The association mapping panel consisted of 220 alfalfa (*Medicago sativa* L.) varieties [46]. After the alfalfa seeds were surface sterilized, 250 mM of a NaCl solution was used to treat the seeds. The seeds were grown in a greenhouse at 24 °C (day)/20 °C (night) under a 16 h light/8 h dark photoperiod at a relative humidity of 70–80% for 1 week. The root tips were collected after the NaCl treatment for 0, 0.5, 1, 3, 6, 12, and 24 h, which were renamed CK, Salt1, Salt2, Salt3, Salt4, Salt5, and Salt6, respectively. Three replicates, each of which included five seedlings, were collected for each treatment. The samples were stored at –80 °C prior to the RT-qPCR experiments.

4.2. Salt Stress Treatment and Phenotyping

A total of 20 seeds of each variety were placed in a plastic Petri dish, and 5 mL of the respective NaCl solution (100, 150, and 200 mM) or water (0 mM) was added to each plate [37]. In total, the 100 mM salt concentration consisted of 5.85 g sodium chloride in 1 L of water, the 150 mM salt concentration consisted of 8.775 g sodium chloride in 1 L of water, and the 200 mM salt concentration consisted of 11.7 g sodium chloride in 1 L of water. For both control and salt treatments, all alfalfa seeds were laid out in a randomized complete-block design with three replications. The root traits were examined after the seeds no longer germinated (the tenth day). Four traits were measured at different concentrations: the number of LR, RL, RV, and RD.

4.3. Phenotypic Data Analysis

The SSI (stress susceptibility index) was calculated for each trait to evaluate salt tolerance using the following formula:

$$SSI = \frac{Y_s/Y_n}{M_s/M_n} \quad (1)$$

where Y_s and Y_n are values for the measured traits of the plant under stress and without stress, respectively; M_s and M_n are the mean values of the measured traits across all plants in the given test under stress and without stress conditions, respectively [47]. The phenotypic correlations were analyzed using the TBtools software [48].

4.4. SNP Calling and Genome-Wide Association Studies

The young leaves of the plants were collected, and the total DNA of the leaves was extracted with a Nuclean plant Genomic DNA kit (Kangwei century, Taizhou, China), which was used to construct the sequencing library. Sequencing using the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) to generate 150 bp paired-end reads and approximately 10 Gb sequencing data, with an average Q30 of 85%, was used for each accession [24]. The paired-end sequencing reads were mapped to the assembled Zhongmu-4 genome using the default parameters of BWA-MEM [49]. Approximately 29.6 million SNPs were detected with the BWA SAMtools VarScan pipeline, and then filtered using the vcftools v.0.1.16 software by following the criteria of a missing rate $\leq 10\%$, minimum average reading depth > 5 , and minor allele frequency (MAF) > 0.05 [24]. The obtained high density of SNP data was used for the GWASs. The GWASs were performed using the C language v0.01 version of the Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway software [50]. The threshold for significantly associated loci was a logarithm of odds score ≥ 6 . Manhattan plots were constructed using the R 4.0.3 version software.

4.5. RNA-Seq and Transcriptomic Analysis

Transcriptomic data for alfalfa plants exposed to salt treatments were collected from the NCBI database (SRR7160314–SRR7160315, SRR7160322–SRR7160331, SRR7160339–SRR7160341, SRR7160351–SRR7160352, and SRR7160354–SRR7160357) [51]. The obtained

raw sequencing reads were filtered with the fastp software, and the clean reads were retained. The gene expression levels were calculated using the clean reads mapped on Zhongmu-4 reference haploid genomes with the HISAT2 software [52], and were then normalized to transcripts per kilobase million (TPM) via a script. The average genome comparison rate was 71.03%. The TPM value was used to estimate the gene expression level and the differentially expressed genes were obtained using DESeq with $\text{padj} < 0.05$ and $|\log_2\text{FC}| \geq 1$ [53]. The TBtools software was used for data visualization [48].

4.6. Candidate Gene Analysis and RT-qPCR

Based on the LD, all genes within the 40 kb range of significant sites were identified using the reference genome of Zhongmu-4. The orthologues were identified by comparing them with the *A. thaliana* genome. The candidate genes were identified by combining the GWAS and transcriptomic results. The root tips of the plants were collected and the RNA was extracted using the MiniBEST RNA kit (TaKaRa, Beijing, China), according to the manufacturer's instructions, and washed with the DNA incubation solution provided with the kit to remove the residual DNA in the genome. In total, 60 μL RNA was extracted from each sample and used to construct the cDNA library. A quantitative real-time PCR (qRT-PCR) was constructed using the Taq Pro Universal SYBR qPCR Master Mix kit (vazyme, Nanjing, China) on the CFX96 Touch™ RT-PCR system (BioRad, Los Angeles, CA, USA). Three technical replicates were set for each sample. All primers used in this study are listed in Table S10, and the alfalfa *actin* gene was used for an internal control. The data were quantified with the $2^{-(\Delta\Delta\text{CT})}$ method [54]. The SPSS 26 version software was used for the analysis of variance (ANOVA). Data visualization was presented using the Origin 2019b version software.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms24076271/s1>.

Author Contributions: F.H. and J.K. planned and designed the study and wrote the manuscript; T.Y., X.J. and X.W. prepared the figures and tables; R.L. and F.Z. validated the data; Q.Y., T.G., C.W. and X.L. validated the data and reviewed and edited the manuscript; L.C. and J.K. curated the software and data and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (32071868), the Agricultural Science and Technology Innovation Program (ASTIP-IAS14), the Key Research Project of Ningxia Province for the Alfalfa Breeding Program (2022BBF02029 and 2019NYY203), the Central Public-Interest Scientific Institution Basal Research Fund (no. 2022-YWF-ZYSQ-04), the Agricultural Science and Technology Innovation Program (ASTIP no. CAAS-ZDRW202201), and the Ordos Science and Technology Plan (2022EEDSKJZDZX011).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sakadevan, K.; Nguyen, M.L. Extent, impact, and response to soil and water salinity in arid and semiarid regions. *Adv. Agron.* **2010**, *109*, 55–74.
2. Tang, H.L.; Niu, L.; Wei, J.; Chen, X.Y.; Chen, Y.L. Phosphorus Limitation Improved Salt Tolerance in Maize through Tissue Mass Density Increase, Osmolytes Accumulation, and Na^+ Uptake Inhibition. *Front. Plant Sci.* **2019**, *10*, 856. [[CrossRef](#)] [[PubMed](#)]
3. Manchanda, G.; Garg, N. Salinity and its effects on the functional biology of legumes. *Acta Physiol. Plant.* **2008**, *30*, 595–618. [[CrossRef](#)]
4. Shannon, M.C.; Grieve, C.M. Tolerance of vegetable crops to salinity. *Sci. Hortic. Amst.* **1999**, *78*, 5–38. [[CrossRef](#)]
5. Foolad, M.; Hyman, J.; Lin, G. Relationships between cold- and salt-tolerance during seed germination in tomato, Analysis of response and correlated response to selection. *Plant Breed.* **1999**, *118*, 49–52. [[CrossRef](#)]

6. Yohannes, G.; Kidane, L.; Abraha, B.; Beyene, T. Effect of Salt Stresses on Seed Germination and Early Seedling Growth of *Camelina sativa* L. Momona Ethiop. *J. Sci.* **2020**, *12*, 1–19. [[CrossRef](#)]
7. Zhu, J.; Ingram, P.A.; Benfey, P.N.; Elich, T. From lab to field, new approaches to phenotyping root system architecture. *Curr. Opin. Plant Biol.* **2011**, *14*, 310–317. [[CrossRef](#)]
8. Ma, Y.; Qiu, C.W.; Fan, Y.; Huang, X.; Khan, W.; Wu, F.; Zhou, M.; Wang, Y.; Cao, F. Genome-wide association and transcriptome analysis reveals candidate genes for potassium transport under salinity stress in wheat. *Environ. Exp. Bot.* **2022**, *202*, 105034. [[CrossRef](#)]
9. Uga, Y.; Sugimoto, K.; Ogawa, S.; Rane, J.; Ishitani, M.; Hara, N.; Kitomi, Y.; Inukai, Y.; Ono, K.; Kanno, N.; et al. Control of root system architecture by DEEPER ROOTING 1 increases rice yield under drought conditions. *Nat. Genet.* **2013**, *45*, 1097–1102. [[CrossRef](#)]
10. Landi, P.; Albrecht, B.; Giuliani, M.M.; Sanguineti, M.C. Seedling characteristics in hydroponic culture and field performance of maize genotypes with different resistance to root lodging. *Maydica* **1998**, *43*, 111–116.
11. Bruce, W.; Desbous, P.; Crasta, O.; Folkerts, O. Gene expression profiling of two related maize inbred lines with contrasting root-lodging traits. *J. Exp. Bot.* **2001**, *52*, 459–468.
12. Guo, J.; Li, C.H.; Zhang, X.Q.; Li, Y.X.; Zhang, D.; Shi, Y.; Song, Y.; Li, Y.; Yang, D.; Wang, T. Transcriptome and GWAS analyses reveal candidate gene for seminal root length of maize seedlings under drought stress. *Plant Sci.* **2020**, *292*, 110380. [[CrossRef](#)] [[PubMed](#)]
13. Shen, C.; Du, H.; Chen, Z.; Lu, H.; Zhu, F.; Chen, H.; Meng, X.; Liu, Q.; Liu, P.; Zheng, L.; et al. The chromosome-level genome sequence of the autotetraploid alfalfa and resequencing of core germplasms provide genomic resources for alfalfa research. *Mol. Plant* **2013**, *13*, 1250–1261. [[CrossRef](#)] [[PubMed](#)]
14. Long, R.C.; Yang, Q.C.; Kang, J.M.; Chao, Y.; Wang, P.; Wu, M.; Sun, Y. Molecular cloning and characterization of a novel stress responsive gene in alfalfa. *Biol. Plant.* **2012**, *56*, 43–49. [[CrossRef](#)]
15. Qiao, G.; Zhang, X.; Jiang, J.; Liu, M.; Han, X.; Yang, H.; Zhuo, R. Comparative proteomic analysis of responses to salt stress in Chinese willow (*Salix matsudana* Koidz). *Plant Mol. Biol. Rep.* **2014**, *32*, 814–827. [[CrossRef](#)]
16. Qi, X.; Li, M.W.; Xie, M.; Liu, X.; Ni, M.; Shao, G.; Song, C.; Kay-Yuen Yim, A.; Tao, Y.; Wong, F.L.; et al. Identification of a novel salt tolerance gene in wild soybean by whole-genome sequencing. *Nat. Commun.* **2014**, *5*, 4340. [[CrossRef](#)]
17. Luo, M.; Zhang, Y.; Li, J.; Zhang, P.; Chen, K.; Song, W.; Wang, X.; Yang, J.; Lu, X.; Lu, B.; et al. Molecular dissection of maize seedling salt tolerance using a genome-wide association analysis method. *Plant Biotechnol. J.* **2021**, *19*, 1937–1951. [[CrossRef](#)]
18. Liu, Z.; Li, H.; Gou, Z.; Zhang, Y.; Wang, X.; Ren, H.; Wen, Z.; Kang, B.-K.; Li, Y.; Yu, L.; et al. Genome-wide association study of soybean seed germination under drought stress. *Mol. Genet. Genom.* **2020**, *295*, 661–673. [[CrossRef](#)]
19. He, F.; Wei, C.X.; Zhang, Y.X.; Long, R.; Li, M.; Wang, Z.; Yang, Q.; Kang, J.; Chen, L. Genome-wide association analysis coupled with transcriptome analysis reveals candidate genes related to salt stress in alfalfa (*Medicago sativa* L.). *Front. Plant Sci.* **2022**, *12*, 826584. [[CrossRef](#)]
20. Zhang, T.J.; Yu, L.X.; Zheng, P.; Li, Y.; Rivera, M.; Main, D.; Greene, S.L. Identification of loci associated with drought resistance traits in heterozygous autotetraploid alfalfa (*Medicago sativa* L.) using genome-wide association studies with genotyping by sequencing. *PLoS ONE* **2015**, *10*, e0138931. [[CrossRef](#)]
21. Lin, S.; Medina, C.A.; Boge, B.; Hu, J.; Fransen, S.; Norberg, S.; Yu, L.-X. Identification of genetic loci associated with forage quality in response to water deficit in autotetraploid alfalfa (*Medicago sativa* L.). *BMC Plant Biol.* **2020**, *20*, 303. [[CrossRef](#)]
22. Wang, Z.; Wang, X.; Zhang, H.; Ma, L.; Zhao, H.; Jones, C.S.; Chen, J.; Liu, G. A genome-wide association study approach to the identification of candidate genes underlying agronomic traits in alfalfa (*Medicago sativa* L.). *Plant Biotechnol. J.* **2020**, *18*, 611–613. [[CrossRef](#)]
23. Chen, H.; Zeng, Y.; Yang, Y.; Huang, L.; Qiu, Q. Allele-aware chromosome-level genome assembly and efficient transgene-free genome editing for the autotetraploid cultivated alfalfa. *Nat. Commun.* **2020**, *11*, 2494. [[CrossRef](#)] [[PubMed](#)]
24. Long, R.C.; Zhang, F.; Zhang, Z.W.; Li, M.; Chen, L.; Wang, X.; Liu, W.; Zhang, T.; Yu, L.-X.; He, F.; et al. Genome assembly of alfalfa cultivar zhongmu-4 and identification of SNPs associated with agronomic traits. *Genom. Proteom. Bioinform.* **2022**, *20*, 14–28. [[CrossRef](#)] [[PubMed](#)]
25. Sahi, C.; Singh, A.; Kumar, K.; Blumwald, E.; Grover, A. Salt stress response in rice: Genetics, molecular biology, and comparative genomics. *Funct. Integr. Genom.* **2006**, *6*, 263–284. [[CrossRef](#)] [[PubMed](#)]
26. Zhang, D.; Zhang, Z.; Li, C.; Xing, Y.; Luo, Y.; Wang, X.; Cai, H. Overexpression of MsRCI2D and MsRCI2E Enhances Salt Tolerance in Alfalfa (*Medicago sativa* L.) by Stabilizing Antioxidant Activity and Regulating Ion Homeostasis. *Int. J. Mol. Sci.* **2022**, *23*, 9810. [[CrossRef](#)]
27. Li, J.; Ma, M.; Sun, Y.; Lu, P.; Shi, H.; Guo, Z.; Zhu, H. Comparative Physiological and Transcriptome Profiles Uncover Salt Tolerance Mechanisms in Alfalfa. *Front. Plant Sci.* **2022**, *13*, 931619. [[CrossRef](#)]
28. Shahzadi, A.K.; Bano, H.; Ogbaga, C.C.; Ayyaz, A.; Parveen, R.; Zafar, Z.U.; Athar, H.-U.; Ashraf, M. Coordinated impact of ion exclusion, antioxidants and photosynthetic potential on salt tolerance of ridge gourd [*Luffa acutangula* (L.) Roxb.]. *Plant Physiol. Biochem.* **2021**, *167*, 517–528. [[CrossRef](#)]
29. Pierik, R.; Testerink, C. The art of being flexible: How to escape from shade, salt, and drought. *Plant Physiol.* **2014**, *166*, 5–22. [[CrossRef](#)]

30. Jia, Z.T.; Giehl, R.F.; Meyer, R.C.; Altmann, T.; von Wirén, N. Natural variation of BSK3 tunes brassinosteroid signaling to regulate root foraging under low nitrogen. *Nat. Commun.* **2019**, *10*, 2378. [[CrossRef](#)]
31. Giovannetti, M.; Göschl, C.; Dietzen, C.; Andersen, S.U.; Kopriva, S.; Busch, W. Identification of novel genes involved in phosphate accumulation in *Lotus japonicus* through Genome Wide Association mapping of root system architecture and anion content. *PLoS Genet.* **2019**, *15*, e1008126. [[CrossRef](#)] [[PubMed](#)]
32. Deolu-Ajayi, A.O.; Meyer, A.J.; Haring, M.A.; Julkowska, M.M.; Testerink, C. Genetic loci associated with early root responses to salt stress. *iScience* **2019**, *21*, 458–473. [[CrossRef](#)] [[PubMed](#)]
33. Huang, J.; Zhang, J.H.; Li, W.Z.; Hu, W.; Duan, L.; Feng, Y.; Qiu, H.; Yue, B. Genome-wide association analysis of ten cold tolerance indices at the germination and seeding stages in maize. *J. Integr. Plant Biol.* **2013**, *55*, 735–744. [[CrossRef](#)] [[PubMed](#)]
34. Nagel, K.A.; Kastenholz, B.; Jahnke, S.; van Dusschoten, D.; Aach, T.; Mühlich, M.; Truhn, D.; Scharf, H.; Terjung, S.; Walter, A.; et al. Temperature responses of roots: Impact on growth, root system architecture and implications for phenotyping. *Funct. Plant Biol.* **2009**, *36*, 947–959. [[CrossRef](#)]
35. Osthoff, A.; Baldauf, J.A.; Piepho, H.-P.; Hochholdinger, F. Transcriptomic reprogramming of barley seminal roots by combined water deficit and salt stress. *BMC Genom.* **2019**, *20*, 325. [[CrossRef](#)] [[PubMed](#)]
36. Zhang, M.; Kong, X.; Xu, X.; Li, C.; Tian, H.; Ding, Z. Comparative transcriptome profiling of the maize primary, crown and seminal root in response to salinity stress. *PLoS ONE* **2015**, *10*, e0121222. [[CrossRef](#)]
37. Yu, L.X.; Liu, X.; Boge, W.; Liu, X.-P. Genome-wide association study identifies loci for salt tolerance during germination in autotetraploid alfalfa (*Medicago sativa* L.) using genotyping-by-sequencing. *Front. Plant Sci.* **2016**, *7*, 956. [[CrossRef](#)]
38. Liu, X.P.; Yu, L.X. Genome-wide association mapping of loci associated with plant growth and forage production under salt stress in alfalfa (*Medicago sativa* L.). *Front. Plant Sci.* **2017**, *8*, 853. [[CrossRef](#)]
39. Tai, H.H.; Lu, X.; Opitz, N.; Marcon, C.; Paschold, A.; Lithio, A.; Nettleton, D.; Hochholdinger, F. Transcriptomic and anatomical complexity of primary, seminal, and crown roots highlight root type-specific functional diversity in maize (*Zea mays* L.). *J. Exp. Bot.* **2016**, *67*, 1123–1135. [[CrossRef](#)]
40. Zhang, H.; Zhang, J.; Xu, Q.; Wang, D.; Di, H.; Huang, J.; Yang, X.; Wang, Z.; Zhang, L.; Dong, L.; et al. Identification of candidate tolerance genes to low-temperature during maize germination by GWAS and RNA-seq approaches. *BMC Plant Biol.* **2020**, *20*, 333. [[CrossRef](#)]
41. Cheng, Q.M.; Bai, S.Q.; Ge, G.T.; Li, P.; Liu, L.; Zhang, C.; Jia, Y. Study on differentially expressed genes related to defoliation traits in two alfalfa varieties based on RNA-Seq. *BMC Genom.* **2018**, *19*, 807. [[CrossRef](#)] [[PubMed](#)]
42. Arshad, M.; Gruber, M.Y.; Hannoufa, A. Transcriptome analysis of microRNA156 overexpression alfalfa roots under drought stress. *Sci. Rep.* **2018**, *8*, 9363. [[CrossRef](#)] [[PubMed](#)]
43. Postnikova, O.A.; Shao, J.; Nemchinov, L.G. Analysis of the alfalfa root transcriptome in response to salinity stress. *Plant Cell Physiol.* **2013**, *54*, 1041–1055. [[CrossRef](#)] [[PubMed](#)]
44. Zhao, C.; Zhang, H.; Song, C.; Zhu, J.K.; Shabala, S. Mechanisms of plant responses and adaptation to soil salinity. *Innovation-Amsterdam* **2020**, *1*, 100017. [[CrossRef](#)]
45. Missihoun, T.D.; Willée, E.; Guegan, J.P.; Guegan, J.P.; Berardocco, S.; Shafiq, M.R.; Bouchereau, A.; Bartels, D. Overexpression of ALDH10A8 and ALDH10A9 genes provides insight into their role in glycine betaine synthesis and affects primary metabolism in *Arabidopsis thaliana*. *Plant Cell Physiol.* **2015**, *56*, 1798–1807. [[CrossRef](#)]
46. Chen, L.; He, F.; Long, R.C.; Zhang, F.; Li, M.N.; Wang, Z.; Kang, J.M.; Yang, Q.C. A global alfalfa diversity panel reveals genomic selection signatures in Chinese varieties and genomic associations with root development. *J. Integr. Plant Biol.* **2021**, *63*, 1937–1951. [[CrossRef](#)]
47. Fischer, R.; Maurer, R. Drought resistance in spring wheat cultivars. I. Grain yield responses. *Aust. J. Agric. Res.* **1978**, *29*, 897–912. [[CrossRef](#)]
48. Chen, C.J.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An integrative toolkit developed for interactive analyses of big biological data. *Mol. Plant* **2020**, *13*, 1194–1202. [[CrossRef](#)]
49. Li, H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv* **2013**, arXiv:1303.3997.
50. Huang, M.; Liu, X.; Zhou, Y.; Summers, R.M.; Zhang, Z. BLINK: A package for the next level of genome-wide association studies with both individuals and markers in the millions. *GigaScience* **2019**, *8*, giy154. [[CrossRef](#)]
51. Dong, X.M.; Deng, H.; Ma, W.X.; Qiang, Z.; Liu, Z.P. Genome-wide identification of the MADS-box transcription factor family in autotetraploid cultivated alfalfa (*Medicago sativa* L.) and expression analysis under abiotic stress. *BMC Genom.* **2021**, *22*, 603. [[CrossRef](#)] [[PubMed](#)]
52. Kim, D.; Pertea, G.; Trapnell, C.; Pimentel, H.; Kelley, R.; Salzberg, S.L. TopHat2: Accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* **2013**, *14*, R36. [[CrossRef](#)] [[PubMed](#)]
53. Anders, S.; Huber, W. Differential expression analysis for sequence count data. *Genome Biol.* **2010**, *11*, R106. [[PubMed](#)]
54. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.