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Comparative Transcriptomic Analysis Reveals Transcriptional Differences in the Response of Quinoa to Salt and Alkali Stress Responses

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Abstract: Soil salinization is a global agro-ecological problem and a major factor impeding agricultural development. Planting salt-tolerant plants to improve saline soils offers both ecological and economic benefits. Currently, there are few studies addressing the combined effects of salt and alkali stress. Quinoa is known for its salinity tolerance. However, research has predominantly focused on the effects of salinity stress on quinoa's morphology and physiology, with its molecular mechanisms remaining unclear. To better understand quinoa's response mechanisms to salinity and alkali stress, we employed RNA-seq technology to analyze transcriptomes under these conditions. We identified 1833 differentially expressed genes (DEGs) under salt stress and 2233 DEGs under alkali stress. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations revealed that quinoa responds to salt and alkali stress through similar mechanisms. Both stresses promoted sucrose synthesis, starch synthesis and catabolism, which increased the osmotic potential of quinoa leaves. Additionally, there was a regulation of the down-regulated expression of the abscisic acid receptor PYR/PYL and the up-regulated expression of the serine/threonine protein kinase (PP2C) gene in the ABA signaling pathway. Contrasting with salt tolerance, the mechanism specific to quinoa's alkalinity tolerance involves the up-regulation of the citric acid cycle via an active γ-aminobutyric acid (GABA) branch, enhancing quinoa's energy metabolism. In summary, our transcriptome analysis revealed key regulatory mechanisms in quinoa's response to saline and alkaline stress. This study deepens the understanding of quinoa's stress response mechanisms and provides theoretical references for the biological improvement of salinized soils.

Keywords: quinoa; RNA seq; salt stress; alkali stress; transcriptome

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Citation: Bao, Q.; Wu, Y.; Wang, Y.; Zhang, Y. Comparative Transcriptomic Analysis Reveals Transcriptional Differences in the Response of Quinoa to Salt and Alkali Stress Responses. *Agronomy* **2024**, *14*, 1596. https://doi.org/10.3390/agronomy14071596

Academic Editors: Víctor Manuel Rodríguez and Ainong Shi

Received: 1 June 2024
Revised: 4 July 2024
Accepted: 18 July 2024
Published: 22 July 2024

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1. Introduction

Salinity stress poses a significant threat to plant growth and crop productivity [1]. Over 1 billion hectares of land worldwide are affected by salinization [2], accounting for 10% of the total land area. This issue spans more than 100 countries and regions, and the extent of soil salinization continues to increase due to secondary salinization processes [3]. The Songnen Plain in northeastern China is one of the major saline–alkali soil regions globally [4], covering approximately 3.42 million hectares. This region's soil salinization is characterized by the concurrent occurrence of salinity and alkalinity, leading to the deterioration of soil chemical and physical properties. This simultaneous salinization and alkalinity is unique compared to other regions where soils typically experience either salinization or alkalization alone. In the Songnen Plain, $NaHCO_3$ and Na_2CO_3 are the predominant salts [5]. Salinization in this area results in the destruction of ecosystems and agricultural structures, significantly limiting the variety of food crops that can be planted, reducing crop yields, and restricting agricultural production [6].

Salt stress causes osmotic stress and ionic toxicity, leading to plant metabolic disorders and impaired photosynthesis [7,8]. Alkaline stress primarily stems from the harmful effects of high pH levels on plants, resulting in secondary oxidative stress, which can reduce crop yield and quality [9]. It is generally believed that alkali stress inhibits plant growth more than neutral salt stress [10,11]. To cope with salinity stress, plants have evolved various response strategies. Strongly salt-resistant plants can enhance their osmoregulatory capacity under stress by securing water through a robust root system [12] and synthesizing large amounts of osmotic substances such as proline, betaine, and soluble sugars [13–15]. For instance, cowpea and rice increase the activity of ascorbic acid–glutathione cycle enzymes to boost their antioxidant capacity [16,17].

On the molecular side, transcriptome sequencing technology has been widely used to study abiotic stresses in plants [18,19]. RNA-seq analysis of rice seedlings under Na_2CO_3 stress identified 1780 differentially expressed genes (DEGs), with KEGG enrichment indicating that metabolic pathways, mainly photosynthesis, glyoxylate and dicarboxylic acid metabolism, carbon metabolism, and carotenoid biosynthesis pathways, were affected [20]. Cartagena et al. [21] found that rice transcription factors NAC, WRKY, and MYB were involved in transcriptional regulation under salt stress. Rasouli et al. [22] assessed transcriptome changes in quinoa and spinach guard cells under 250 mmol/L NaCl stress and showed that, compared to spinach, genes involved in abscisic acid signaling and biosynthesis, as well as pectin methylesterase associated with cell wall plasticity, were up-regulated in quinoa. Additionally, quinoa guard cells exhibited increased amino acid, sugar, and protein content.

Quinoa (*Chenopodium quinoa* Willd.), an ancient annual broad-leaved herb native to the Andes Mountains of South America, was a crucial staple for the ancient Inca people [23–26]. Nicknamed the "Mother of Food", quinoa is celebrated for its high protein content and a wealth of essential amino acids, minerals, vitamins, and other nutrients. The United Nations Food and Agriculture Organization has recognized it as the "perfect all-nutrient food for human consumption", promoting its cultivation worldwide [27–29]. Quinoa not only boasts high nutritional value but also exhibits resilience to various abiotic stresses, including drought [30–32], salinity [33–37], cold [38], frost, and barrenness [39]. It is considered one of the most salt-tolerant crops [38], with studies indicating that quinoa is more salt-tolerant than barley [40], maize [41], and wheat [42]. Currently, research on soda saline soils is limited. Given the specificity of soda saline soils in the Songnen Plain of China, it is crucial to study quinoa's salinity tolerance to improve these soils and maintain agrobiodiversity. The completion of quinoa genome sequencing and the application of high-throughput genomics methods have laid a solid foundation for investigating quinoa's stress tolerance [28]. In this study, we utilized RNA-seq for comparative transcriptome analysis, focusing on the molecular factors involved in the response of quinoa seedlings to salt and alkali stress. Our study provides an in-depth understanding of quinoa's regulatory pathways and the response of important genes to salt and alkali stress, offering a theoretical basis for further research and practical applications.

2. Materials and Methods

2.1. Plant Materials

The experimental materials were provided by the Gansu Academy of Agriculture, Lanzhou, China. LL1, *Chenopodium quinoa* Willd., was used for both growth index determination and transcriptomics analysis. Uniform-sized and disease-free quinoa seeds were surface-sterilized and soaked in sterile distilled water for 6 h to promote germination. Subsequently, the seeds were evenly sown in plastic pots with a diameter of 10 cm and a height of 8 cm. The pots were thoroughly watered for the first time. The planted pots were placed in an intelligent light incubator under controlled conditions, including a 14 h light cycle with a light intensity of 6000 Lux, and day/night temperatures were set at 26/20 °C, with a relative humidity of 60 ± 5%. Quinoa seedlings were watered every 2 days, and once a week, they were provided with Hoagland nutrient solution to ensure optimal growth.

When the seedlings reached the four-leaf stage, they were thinned to maintain uniform growth and suitable spacing. Each treatment included 15 pots of seedlings with consistent growth, with 3 seedlings remaining in each pot. At the eight-leaf stage, the seedlings were subjected to salt stress and alkali stress treatments (Table 1). The control group was watered with distilled water. Samples were collected from the inverted three leaves of quinoa after 48 h of stress treatment. These samples were immediately frozen in liquid nitrogen for 15 min and stored in a −80 °C refrigerator. Transcriptome sequencing samples of quinoa were taken by mixing the leaves of multiple seedlings within the same treatment. Each treatment was replicated three times to ensure the robustness of the experimental data.

Table 1. Composition and molar ration of treatments at quinoa seeding stage.

Treatment	Transcriptome	Salt Composition and Molar Ratio				mmol/L
		NaCl	Na_2SO_4	$NaHCO_3$	Na_2CO_3	
CK	TW	0	0	0	0	0
Salt stress	TS	1	1	0	0	300
Alkali stress	TA	0	0	1	1	100

2.2. RNA Extraction and Sequencing

Total RNA extraction was carried out using the TRIzol® Reagent kit (Invitrogen, Carlsbad, CA, USA), and the purity and concentration of the extracted RNA were assessed using NanoPhotometerN50 (Implen, Munich, Germany). Following library construction, preliminary quantification was conducted using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The library was then diluted to 1.5 ng/μL, and the insert size was determined using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). For the accurate quantification of library concentration, qRT-PCR was employed, ensuring that the effective concentration of the libraries exceeded 2 nM to maintain library quality. Each sample was sequenced to yield 8G of sequencing data. The Clean Reads, obtained after quality control, were aligned to the *Chenopodium quinoa* Willd. reference genome to generate Mapped Reads. Sequencing saturation, gene coverage, and the distribution of Reads across different regions of the reference genome were evaluated to assess sequencing quality. The comparison efficiency (Mapped Reads/Clean Reads) was calculated to gauge the utilization of transcriptome data. Pearson's correlation coefficient was utilized to assess the correlation among the nine samples, and the closer the squared correlation coefficient (R^2) value was to 1, the stronger the correlation among the samples. The R^2 of the biological replicates was required to be at least greater than 0.8 in our study, which was considered reliable for the sequencing results. Differential expression analysis between treatment groups was conducted using DESeq, with screening criteria set at false discovery rate (FDR) $\leq$ 0.01 and fold change (FC) $\geq$ 2 for differential genes. Enrichment analysis (KS < 0.001) was performed on the differentially expressed genes with the GO (http://www.geneontology.org/, accessed on 14 January 2024) functional annotations obtained in this study. Additionally, enrichment analysis was conducted on the KEGG (http://www.kegg.jp/kegg/, accessed on 14 January 2024) pathway of differentially expressed genes. The annotation of transcription factors in differentially expressed genes was compared using the plant transcription factor database PlantTFDB (https://planttfdb.gao-lab.org/, accessed on 15 January 2024).

2.3. Quantitative Real Time PCR Analysis

To validate the accuracy of the transcriptome sequencing results, RNA extraction was carried out using the TRIzol® Reagent Kit (Invitrogen, Carlsbad, CA, USA), followed by reverse transcription using the HiScript 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd., Nanjing, China). The internal reference gene ACT-1 (gene56001) was employed to normalize the gene expression level. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ comparative threshold cycling (Ct) method. This method involves comparing the Ct values of the target genes with that of the internal reference

gene and normalizing them against a control sample. The fold change in expression levels between treatment groups can thus be determined accurately.

2.4. Statistical Analysis

SPSS 26.0 software was used for the analysis of variance (ANOVA) between treatment groups, and experimental data are represented as mean ± standard errors. Graphs were drawn using GraphPad Prism 9.4 software. Significant differences were detected using LSD tests ($p < 0.05$). The PPI network was imported from Cytoscape (v3.9.1), where network nodes were calculated using the CytoNCA plugin.

3. Results

3.1. Phenotype and Growth Parameters

Figure 1A shows that compared with the TW, there is a decrease in the seedling height and leaf area of quinoa plants under salt stress and alkali stress. The morphological indicators of quinoa (ANOVA), including fresh weight, leaf area, and water content, decreased significantly after being subjected to salt stress and alkali stress treatments (Figure 1B,E,F), and the height of quinoa seedlings treated with salt stress decreased significantly, but the differences between stress groups were not statistically significant (Figure 1D). In addition, seedling dry weight showed the least impact (Figure 1C). Comparisons among stress groups showed that fresh weight, leaf area and water content were significantly reduced under alkali stress compared to salt stress (Figure 1B,E,F). Therefore, it can be concluded that alkali stress exerts a greater adverse effect on quinoa seedlings relative to salt stress.

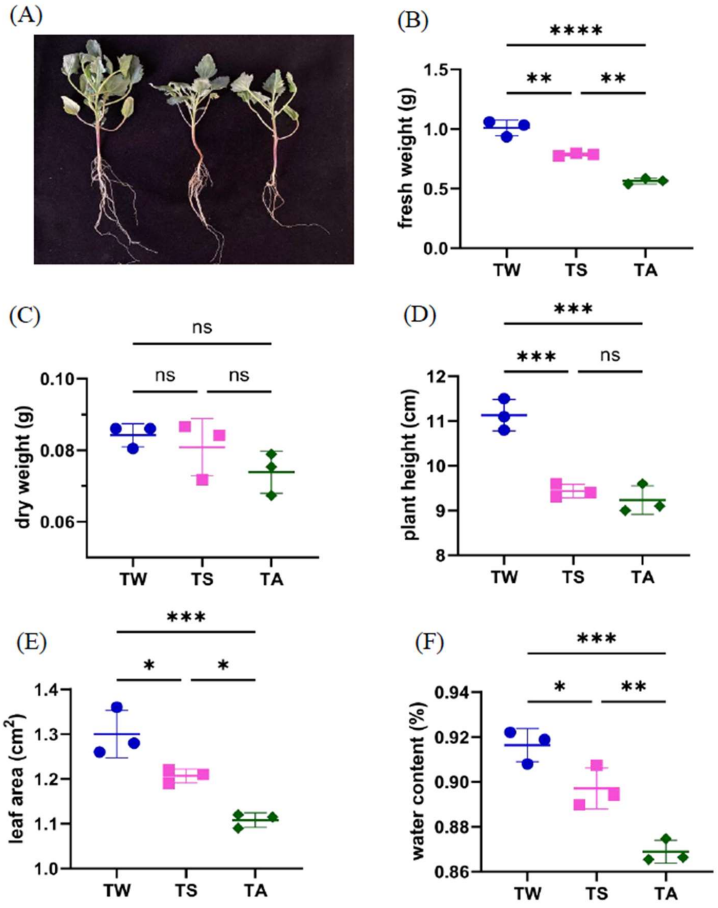


Figure 1. Growth of quinoa seedlings under salt and alkali stress. Gross inspection of salt and alkali stress (**A**), statistical analysis for quinoa fresh weight (**B**), dry weight (**C**), plant height (**D**), leaf area (**E**) and water content (**F**). ****, ***, **, * and ns indicate significant differences ($p < 0.0001$), ($p < 0.001$), ($p < 0.01$), ($p < 0.05$) and no significant.

3.2. Evaluation and Bioinformatics Analysis of DEGs in Salt and Alkali Stress Treatments

To investigate the transcriptomic response of quinoa to salt and alkali stress, we conducted transcriptome analysis on quinoa leaves subjected to these conditions. Samples were categorized into three groups: control (TW), salt stress (TS), and alkali stress (TA), each with three biological replicates. Utilizing the Illumina sequencing platform, we generated approximately 49 Gb of Clean Reads. The number of Clean Reads obtained for TW, TS, and TA were 68,570,707, 67,507,475, and 62,893,398, respectively. The quality of sequencing, indicated by Q30 bases > 93.23% and GC content ranging from 38.87% to 44.11% (Table S1), along with the comparison efficiency with the quinoa genome ranging from 94.43% to 97.85% (Table S2), was satisfactory for subsequent data analysis. Spearman's correlation analysis showed that the correlation coefficients of the three replicates within the treatment group were all greater than 0.8, with strong reproducibility within the group (Figure 2A). Principal component analysis (PCA) showed that PC1, PC2, and PC3 explained 18.94%, 14.43%, and 12.22% of the total variance, respectively, with good sample dispersion and reliable data for transcriptome analysis (Figure 2B). Differentially expressed genes (DEGs) were identified using criteria of a fold change ≥ 2 and a false discovery rate (FDR) < 0.01.

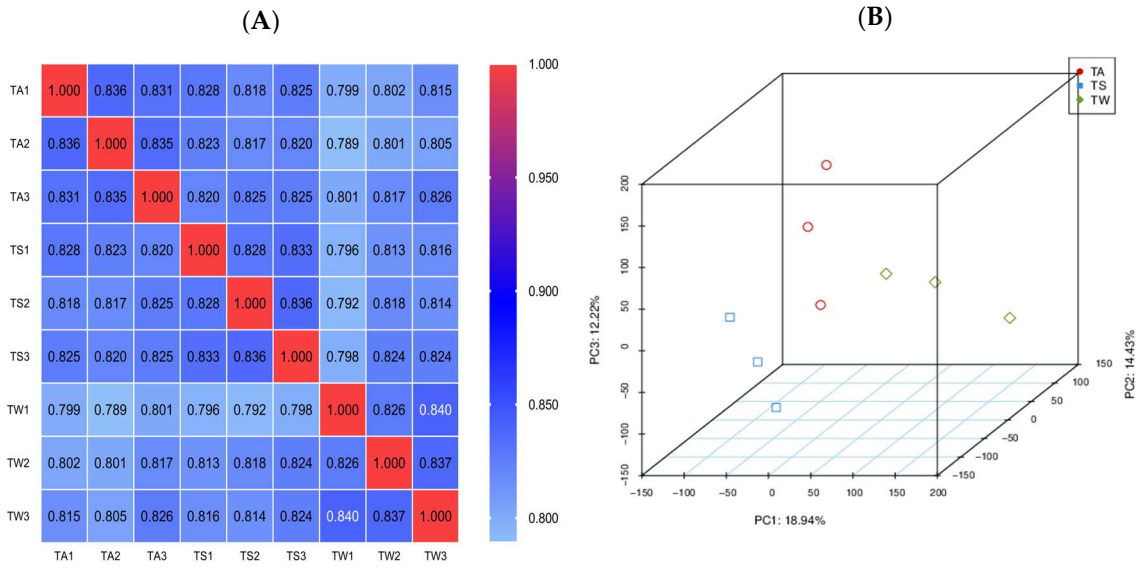


Figure 2. Transcriptome analysis after salt and alkali stress treatment. (**A**) Correlation heat map, (**B**) Principal component analysis (PCA).

3.3. Evaluation and Bioinformatics Analysis of Differentially Expressed Genes (DEGs) in Response to Salt Stress

A total of 1833 DEGs (615 up-regulated and 1218 down-regulated) were detected in the comparison between TS and TW (Figure 3A,D); the top 30 up-regulated and down-regulated genes are presented in Figure 4A.

To elucidate the molecular mechanisms underlying the response to TS in quinoa seedling leaves, we conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. GO enrichment analysis of TS vs. TW treatments (Figure 5A) revealed the enrichment of the GO database with 1285 DEGs (428 up-regulated and 857 down-regulated), Among these, 379 GO terms were significantly enriched in Biological Processes (BPs), 30 GO terms were significantly enriched in Cellular Components (CCs), and 188 GO terms were significantly enriched ($p < 0.05$) in Molecular Functions (MFs). In Biological Processes (BPs), the top three enriched terms were response to organic substance (GO: 0010033, 197 genes), response to chemical (GO: 0042221, 258 genes), and response to chitin (GO: 0010200, 27 genes). In Cellular Components (CCs), the top three enriched terms were in the extracellular region (GO: 0005576, 154 genes), cell periphery (GO: 0071944, 371 genes), plant-type cell wall (GO: 0009505, 65 genes). In Molecular

Function (MFs), the top three enriched terms were as follows: polygalacturonase inhibitor activity (GO: 0090353, 11 genes), hydrolase activity, hydrolyzing O-glycosyl compounds (GO: 0004553, 69 genes), hydrolase activity, and acting on glycosyl bonds (GO: 0016798, 70 genes).

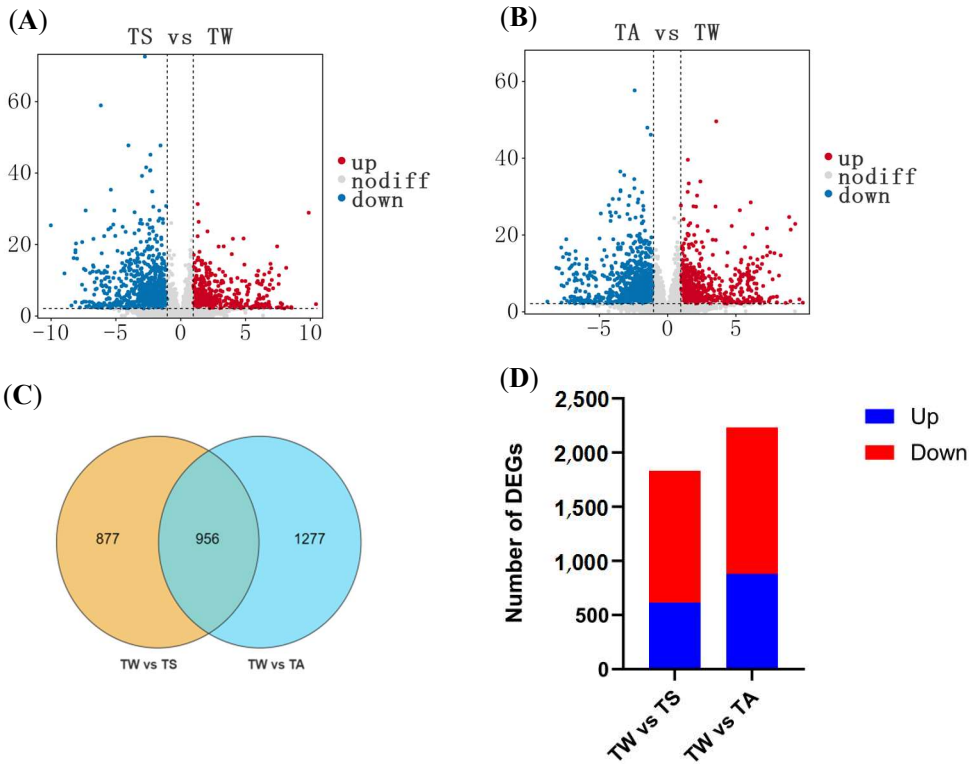


Figure 3. Overview of DEGs. (**A**) and (**B**) Volcano map of transcriptome genes among different treatments. TW vs. TS. Red dots, up-regulated genes; green dots, down-regulated genes; gray dots, genes with no significance. (**C**) Venn diagrams of DEGs among treatments. (**D**) Up-regulation of DEGs and down-regulation of DEGs statistics.

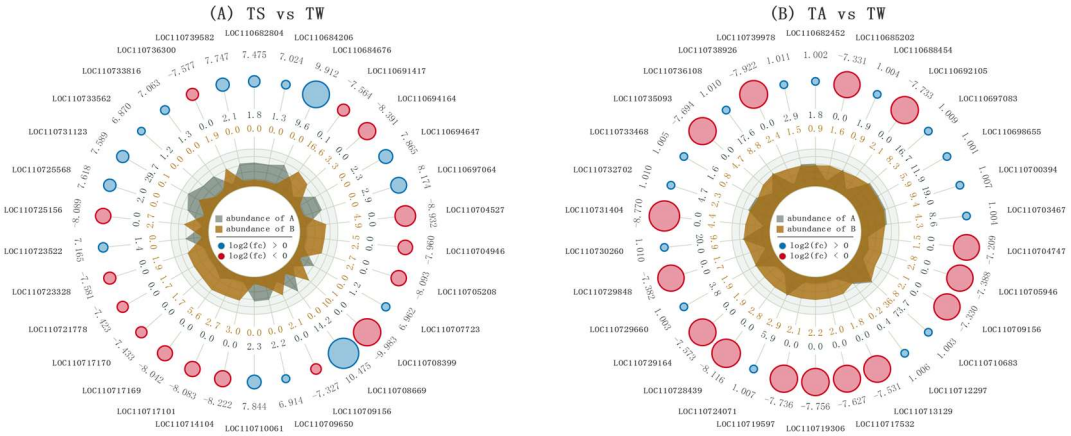


Figure 4. Top 15 up-regulated (blue) and 15 down-regulated (red) genes in salt and alkali stress (**A**,**B**). The first loop indicates the names of 15 top up-regulated and down-regulated genes. The second loop indicates the fold change in salt and alkali treated. The third loop indicates larger circle presented larger fold change. The fourth and fifth loop show the mean FPKM of TS and TA, respectively.

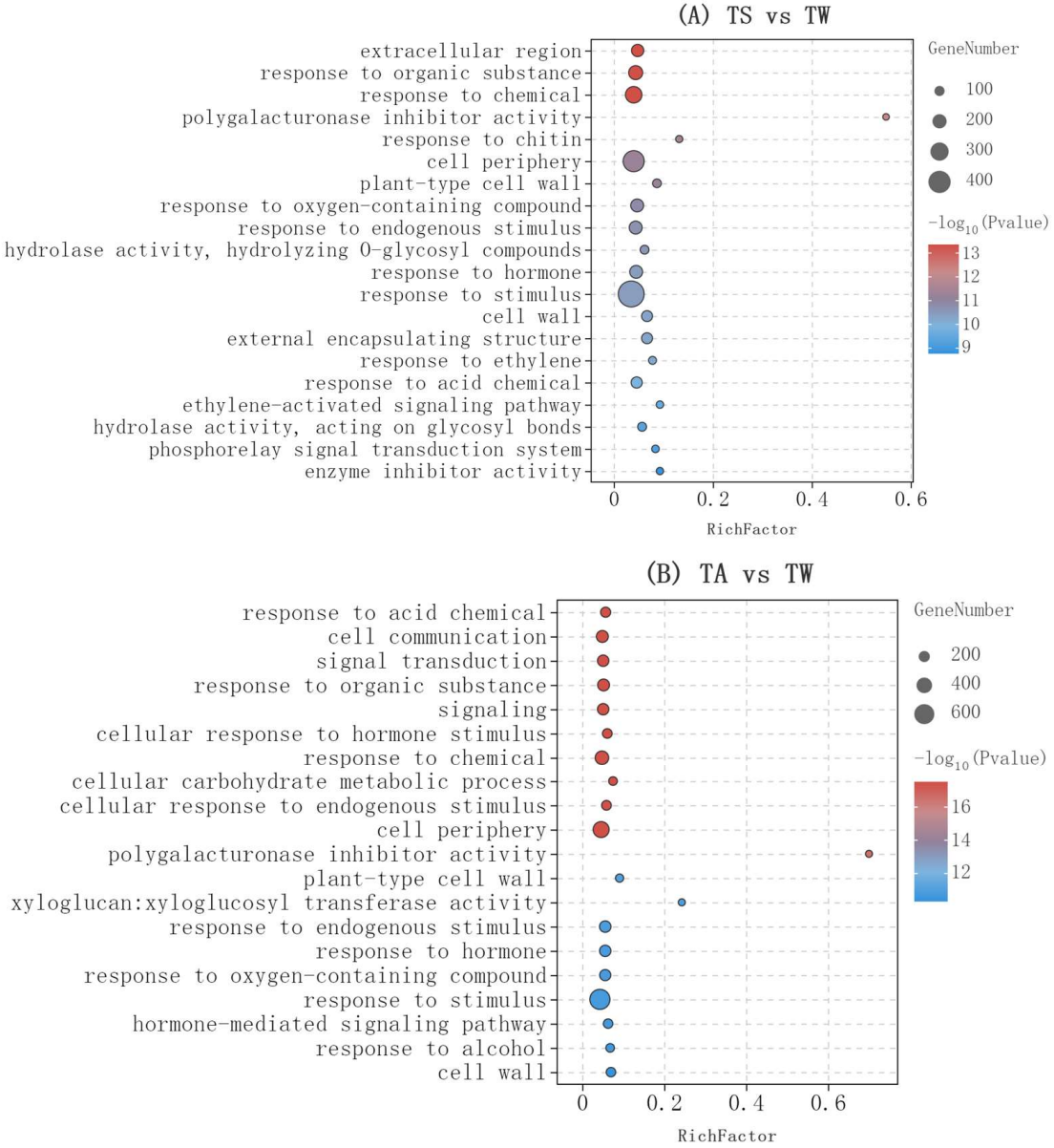


Figure 5. GO enrichment analysis for DEGs in TOP20 pathways (**A**,**B**). The color scale represents the *p*-value, and the dot size represents the number of DEGs mapped in each pathway.

KEGG analysis of differentially expressed genes (DEGs) revealed the enrichment of 237 DEGs (89 up-regulated and 148 down-regulated) in 250 pathways. Among these pathways, a total of 18 were significantly enriched ($p < 0.05$) and associated with plant metabolism (Figure 6A). These enriched pathways primarily included plant hormone signal transduction, starch and sucrose metabolism, MAPK signaling pathway-plant, biosynthesis of secondary metabolites synthesis, phenylpropanoid biosynthesis, alpha-linolenic acid metabolism, plant–pathogen interaction, and the calcium signaling pathway. These pathways provide insights into the molecular mechanisms underlying the response of quinoa to salt stress and offer potential targets for further investigation into plant stress responses.

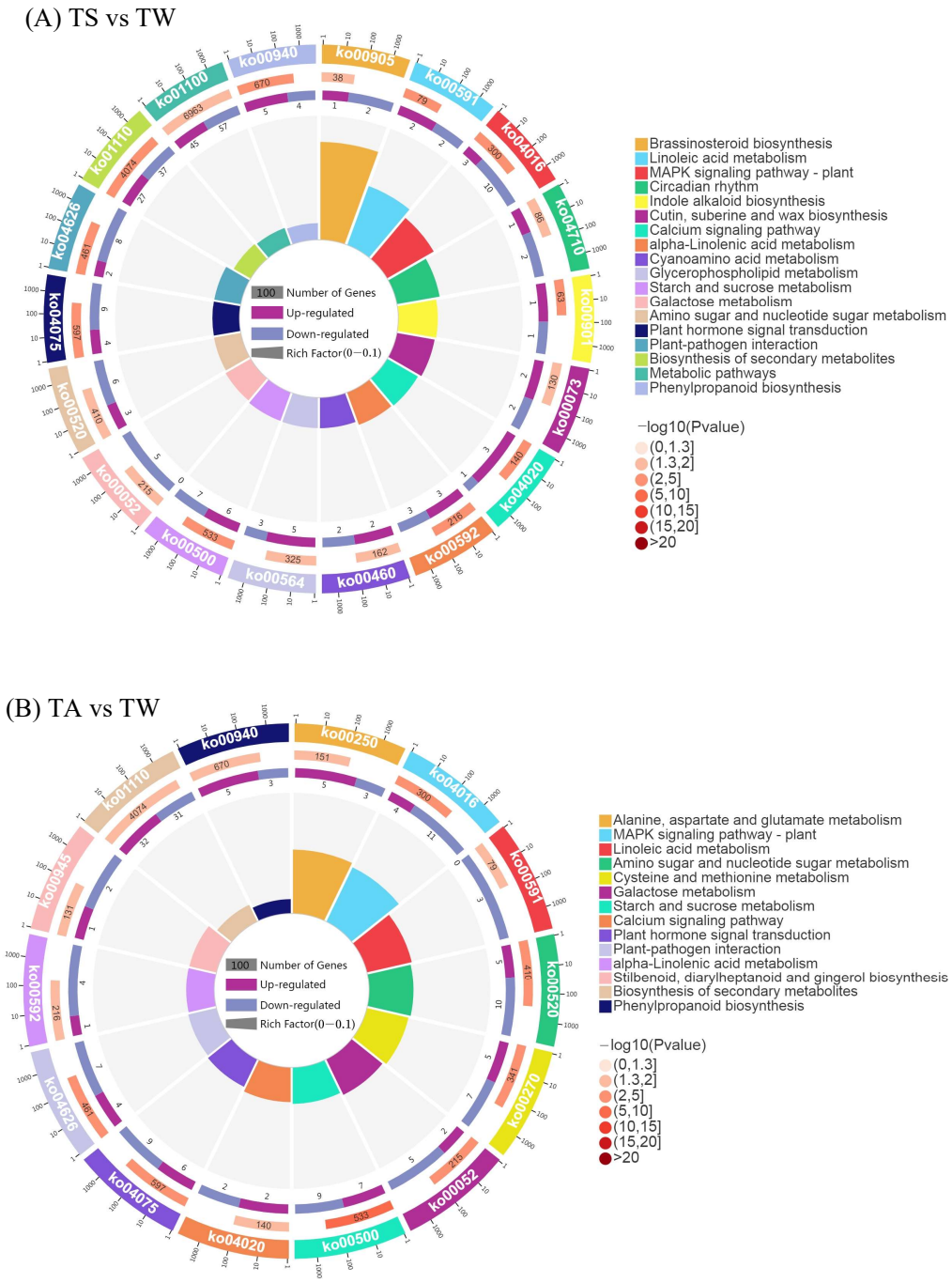


Figure 6. KEGG enrichment analysis for DEGs in salt-alkali treated (**A**,**B**). The first circle shown KEGG ID. The length of the bars in the second circle indicates the number of background genes, the color shade indicates the *p*-value, the third circle indicates the number of up-regulated and down-regulated genes, and the fourth circle indicates the rich-factor.

3.4. Evaluation and Bioinformatics Analysis of Differentially Expressed Genes (DEGs) in Response to Alkali Stress

TA vs. TW detected 2233 DEGs (881 up-regulated and 1352 down-regulated) (Figure 3B,D); the top 30 up-regulated and down-regulated genes were presented in Figure 4B.

GO enrichment analysis of TA vs. TW treatments (Figure 5B) revealed enrichment with 1570 DEGs (625 up-regulated and 945 down-regulated). Among these, 458 GO terms were significantly enriched for Biological Processes (BPs), 37 GO terms were significantly enriched for Cellular Components (CCs), and 167 GO terms were significantly enriched

($p < 0.05$) for Molecular Functions (MFs). In Biological Process (BPs), the top three enriched terms were response to acid chemical (GO: 0001101, 154 genes), cell communication (GO: 0007154, 237 genes), and signal transduction (GO: 0007165, 212 genes). In Cell Components (CCs), the top three enriched terms were cell periphery (GO: 0071944, 419 genes), plant-type cell wall (GO: 0009505, 68 genes), and cell wall (GO: 0005618, 126 genes). In Molecular Function (MFs), the top three enriched terms were polygalacturonase inhibitor activity (GO: 0090353, 14 genes), xyloglucan: xyloglucosyl transferase activity (GO: 0016762, 18 genes), and enzyme inhibitor activity (GO: 0004857, 35 genes).

KEGG analysis of differentially expressed genes (DEGs) revealed that 264 DEGs (134 up-regulated and 154 down-regulated) were enriched into 279 pathways. Among these, a total of 14 pathways were significantly enriched ($p < 0.05$) and related to plant metabolism (Figure 6B). These pathways primarily included starch and sucrose metabolism, plant hormone signal transduction, MAPK signaling pathway-plants, aminosugar and nucleotide sugar metabolism, cysteine and methionine metabolism, plant–pathogen interaction, galactose metabolism, and phenylpropanoid biosynthesis.

3.5. Evaluation and Bioinformatics Analysis of DEGs Response to Salt and Alkali Stress

The comparison between TS and TA stress groups revealed 956 differentially expressed genes (Figure 3C). These genes were classified into four subclasses using k-mean cluster analysis. The number of DEGs in each subclass were 474, 455, 97, and 8, respectively.

The top 20 significantly enriched GO terms were analyzed. Eleven GO terms were co-enriched in both TS and TA, including response to organic matter (GO: 0010033, BP), response to chemicals (GO: 0042221, BP), cell periphery (GO: 0071944, CC), and plant-type cell wall (GO: 0009505, CC). Enriched only in the TS group were the following categories: extracellular region (GO: 0005576, CC), polygalacturonase inhibitor activity (GO: 0090353, MF), response to chitin (GO: 0010200, BP), and hydrolase activity involved in the hydrolysis of O-glycosyl compounds (GO: 0004553, MF). Enriched only in the TA group were signaling hormone classes, including cellular communication (GO: 0007154, BP), signal transduction (GO: 0007165, BP), signaling (GO: 0023052, BP), cellular response to hormonal stimuli (GO: 0032870, BP), and cellular carbohydrate metabolic processes (GO: 0044262, BP). Overall, the TS group exhibited enhanced salt tolerance by maintaining cell wall structure, while the TA group responded to alkali stress through mechanisms involving signal transduction, hormones, and increased carbohydrate metabolic activities.

In the comparison of KEGG pathways between the TS and TA treatment groups, it was observed that the number of up-regulated DEGs was greater in TA compared to TS, while the number of annotated KEGG pathways was higher in TS than in TA. In TS, the annotated pathways included indole alkaloid biosynthesis (ko00901), glycerophospholipid metabolism (ko00564), brassinosteroid biosynthesis (ko00905), cutin, suberine, and wax biosynthesis (ko00073), and cyanoamino acid metabolism (ko00460). On the other hand, in TA, the annotated pathways were cysteine and methionine metabolism (ko00270), stilbenoid, diarylheptanoid, and gingerol biosynthesis (ko00945), and alanine, aspartate, and glutamate metabolism (ko00250).

3.6. PPI Analysis of DEGs Response to Salt and Alkali Stress

To further discover the interactions between genes, we mapped the DEGs to the string database and performed Protein–Protein Interaction (PPI) network analysis to obtain the details of protein interaction network relationships between DEGs. Then, Cytoscape was used to display the analysis results (betweenness centrality analysis node degree, BC $\geq$ 50). The results showed that 65 and 80 proteins were actively involved in the response of quinoa under salt stress and alkali stress treatments, respectively. Also, 35 proteins shared salt stress and alkali stress. The PPI network analysis showed that quinoa was under salt stress and alkali stress, which was dominated by the carbon-hydrate metabolic pathway proteins (trehalose-phosphate synthase, GDP-mannose 3,5-epimerase, etc.), and abscisic

acid signaling pathway proteins (protein phosphatase 2C, etc.) play a key role in protecting quinoa from stress injury.

Proteins under salt stress include heat stress factor (XM-021893349.1), adenylyl-sulfate kinase (XM-021895712.1), and 1,5′-adenylylsulfate reductase (XM-021880798.1) (Figure 7A). Proteins under alkali stress include sucrose synthase (XM-021907521.1), receptor-like protein kinase (XM-021920732.1), beta-fructofuranosidase (XM-021912726.1), superoxide dismutase (XM-021868723.1), and alanine aminotransferase (XM-021906179.1) (Figure 7B).

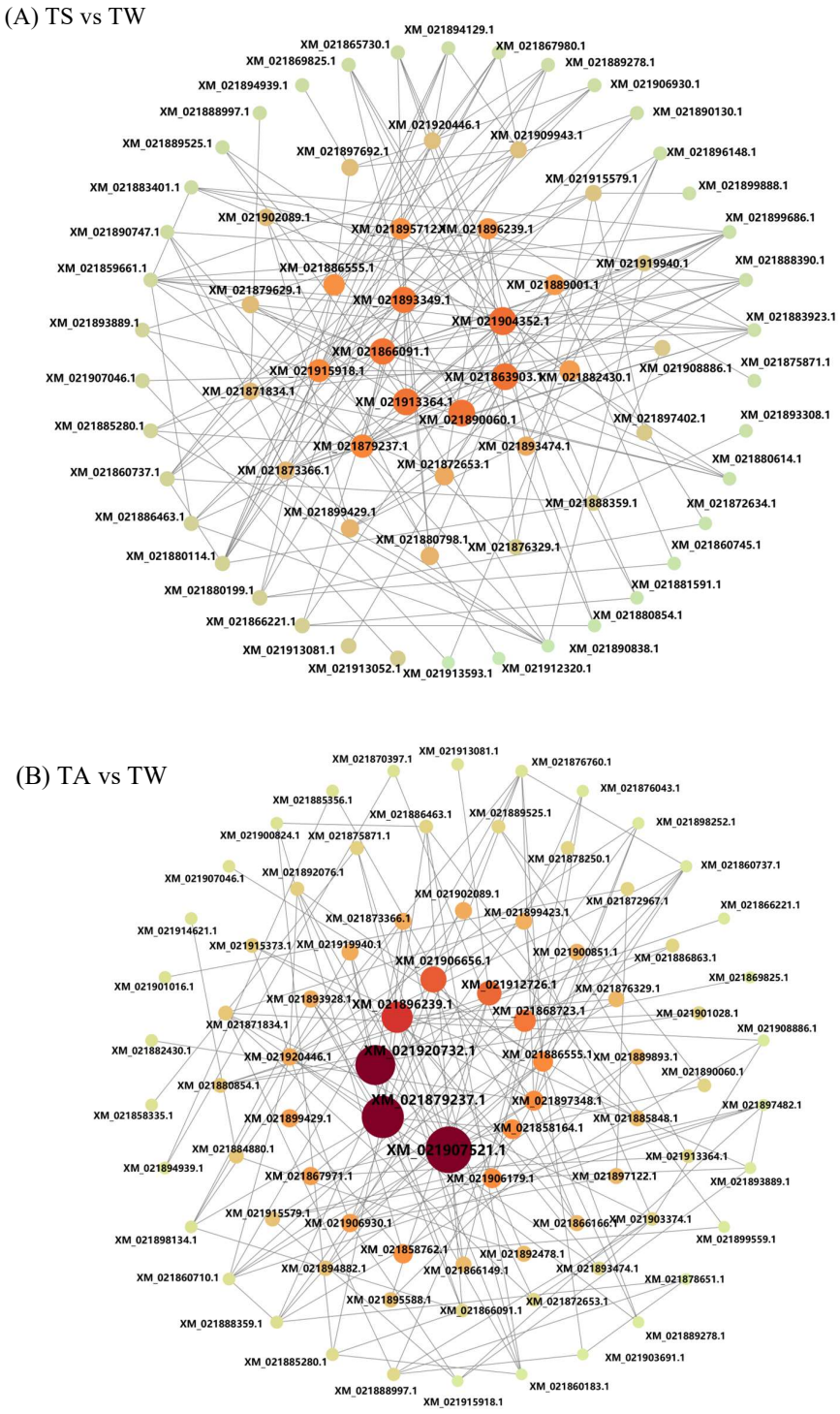


Figure 7. PPI network of the DEGs under salt stress and alkali stress (**A**,**B**). The size of the node indicates the size of the degrees of freedom.

3.7. Validation of RNA-Seq Data by RT-PCRss

To validate the transcriptome sequencing data, we selected ten DEGs for qRT-PCR, which were common in both TS and TA, exhibited high expression, and were significantly enriched in KEGG pathways. These included three genes related to sugar metabolism (TPS, AMY, raffinose synthase), three genes for lipid metabolism (PEAMT, GPAT), three genes for hormone metabolism (AUX/IAA, PYR/PYL, WRKY), and chitinase. ACT-1 (gene56001) was used as an internal control to normalize the gene expression level. The annotation information of DEGs and the list of qRT-PCR primers are presented Table S3. The relative expression levels were transformed into log2Fold Change and compared with the RNA-Seq data. The qRT-PCR results showed a good correlation with the TS (R^2 = 0.962) (Figure 8A,B) and TA (R^2 = 0.959) (Figure 8C,D) of the RNA-Seq data had a strong correlation. In summary, we concluded that the RNA-seq data were reliable and could be used to genetically characterize the response of quinoa seedlings to salt-alkali treatments.

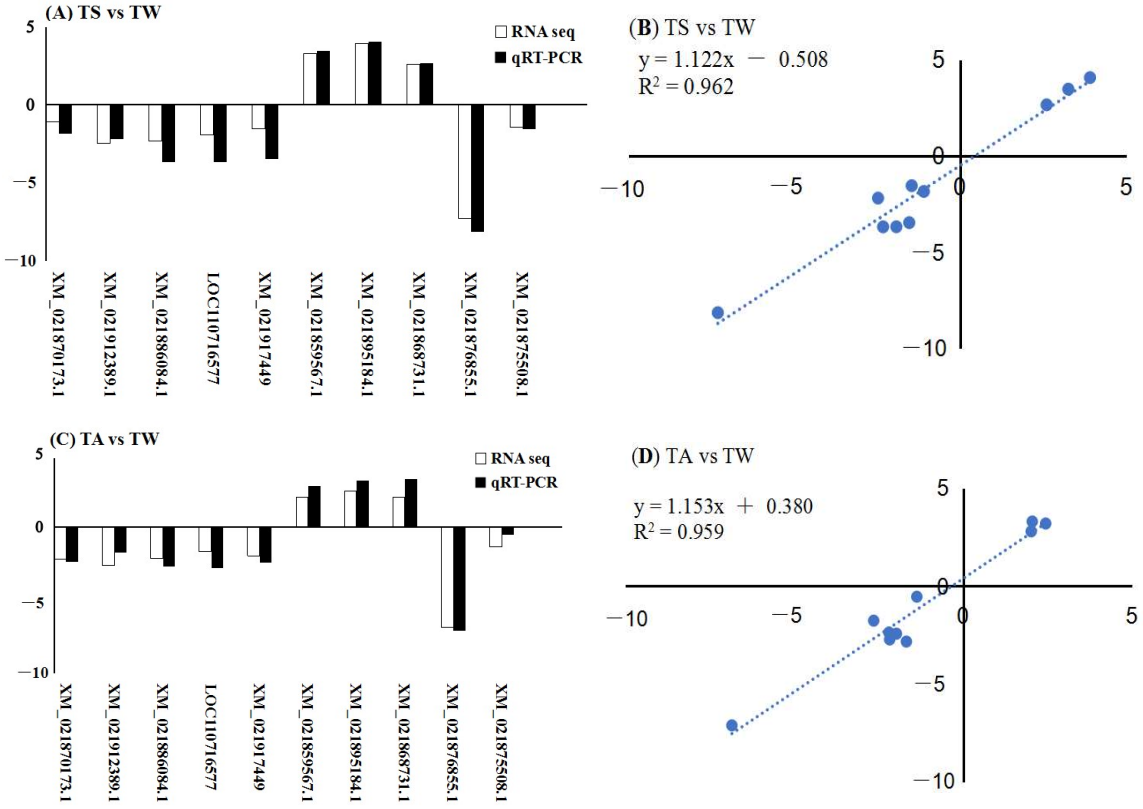


Figure 8. Validation of RNA-seq expression profiles by qRT-PCR (**A**,**C**). RNA-Seq and qRT-PCR Correlation Analysis (**B**,**D**).

4. Discussion

4.1. Carbohydrate Metabolism

Carbohydrate synthesis, catabolism, and interconversion are crucial processes that influence plant growth and its stress response [43]. Plants synthesize sugars through photosynthesis, which serves as the basis for energy storage and material transport. Sugar metabolites play a vital role in regulating osmotic potential, providing energy and carbon sources when photosynthesis is limited under stress. In this study, isoamylase and amylase synthase were found to be up-regulated in both TS and TA, indicating their role in degrading starch into maltose and glucose. This suggests that quinoa can adapt to or resist adversity by regulating its osmoregulatory capacity when facing saline and alkaline stresses, consistent with findings on drought tolerance in tea leaves by Liu et al. [44]. Glu-

cose, fructose, sucrose, and alginate are known to play roles in regulating plant growth, development, and responses to the environment [45].

Sucrose, a major product of photosynthesis, plays a critical role in mediating the accumulation of diphenylalanine, tryptophan, and alkaloids to maintain osmotic potential under saline and alkaline stress [46]. During adversity stress, sucrose is transported to guard cells via the cytoplasmic matrix in chloroplasts [47]. Studies have shown that sucrose degradation regulates osmotic pressure in guard cells and is closely related to stomatal opening and closure [48]. Sucrose synthase (SuS) is involved in both sucrose anabolic and catabolic processes [49,50]. Transcriptomic analysis of quinoa guard cells under salt stress revealed a significant up-regulation of the sucrose synthase (SUS2) gene, suggesting the importance of sucrose in controlling stomatal opening in quinoa [22]. In this study, one and two sucrose synthase (SuS) genes were up-regulated in TS and TA, respectively. The up-regulation of sucrose synthase in quinoa variety LL1 underscores the need for further investigation into its role in stomatal regulation. Fructokinase (scrK) catalyzes the breakdown of fructose to 6-P-fructose, with five DEGs up-regulated in TA but none in TS treatments, indicating that the up-regulation of fructokinase may be a specific mechanism in quinoa's response to alkali stress. Studies in rice have shown that fructokinase plays a crucial role in coping with alkali stress, as evidenced by significantly higher expression levels in salt-tolerant rice compared to salt-sensitive varieties [51]. Transcriptomic analysis revealed the differential expression of numerous genes involved in sugar metabolism (Figure 9). These genes were mainly associated with metabolic processes such as sucrose synthesis, starch polysaccharide synthesis and catabolism. Sugar catabolism metabolites serve as osmoregulatory substances to regulate cellular osmotic potential, improving quinoa's salinity tolerance. Additionally, they replenish energy for quinoa under saline and alkaline stress by fueling the citric acid cycle (TCA) through glycolysis. Our study demonstrated that starch synthesis and catabolism were active, and sucrose anabolism was enhanced under salt stress and alkali stress. There is a higher number of DEGs and up-regulation folds under TA compared to TS, and we suggest that sugar metabolism is more active in quinoa under TA.

4.2. Hormone Signaling

Phytohormones, crucial in the response to salt stress, play significant roles in plant physiology [52,53]. Serine/threonine protein kinase (PP2C), the largest phosphatase family in *Arabidopsis thaliana*, is pivotal in plant growth, development, and adversity signaling pathways. Under salt or alkali stress, ABA binds to PYL receptors, inhibiting PP2C activity. Consequently, free SnRK2 phosphorylates ABA-responsive element binding factors (ABFs), inducing transcription factors' expression to activate downstream resistance genes and promote stomatal closure [54]. The increase in ABA content under salinity stress in *Arabidopsis* triggers SNRK2 protease release upon ABA-PYL binding, facilitating negative ion efflux and stomatal closure [55]. PP2C is generally known to negatively regulate the ABA signaling pathway. The heterologous expression of the PP2C gene in rice and *Arabidopsis* rendered *Arabidopsis* insensitive to exogenous ABA, consistent with *Artemisia annua* findings, indicating PP2C's role as a negative regulator of the ABA signaling pathway [56,57]. Studies on Bitter Ginseng and apple rootstocks revealed the down-regulation of genes encoding ABA receptors and the up-regulation of PP2C genes under salinity stress [58,59], further affirming ABA's involvement in plant responses to salt and alkali stress. In our study (Figure 9), DEGs of PP2C and PYL were significantly up-regulated under TS and TA. Additionally, the ABF gene was up-regulated under TA, suggesting that quinoa's response to saline and alkaline stress may not solely depend on ABA signaling. However, the precise involvement of PP2C in quinoa's response to saline and alkaline stress remains unclear, warranting further investigation.

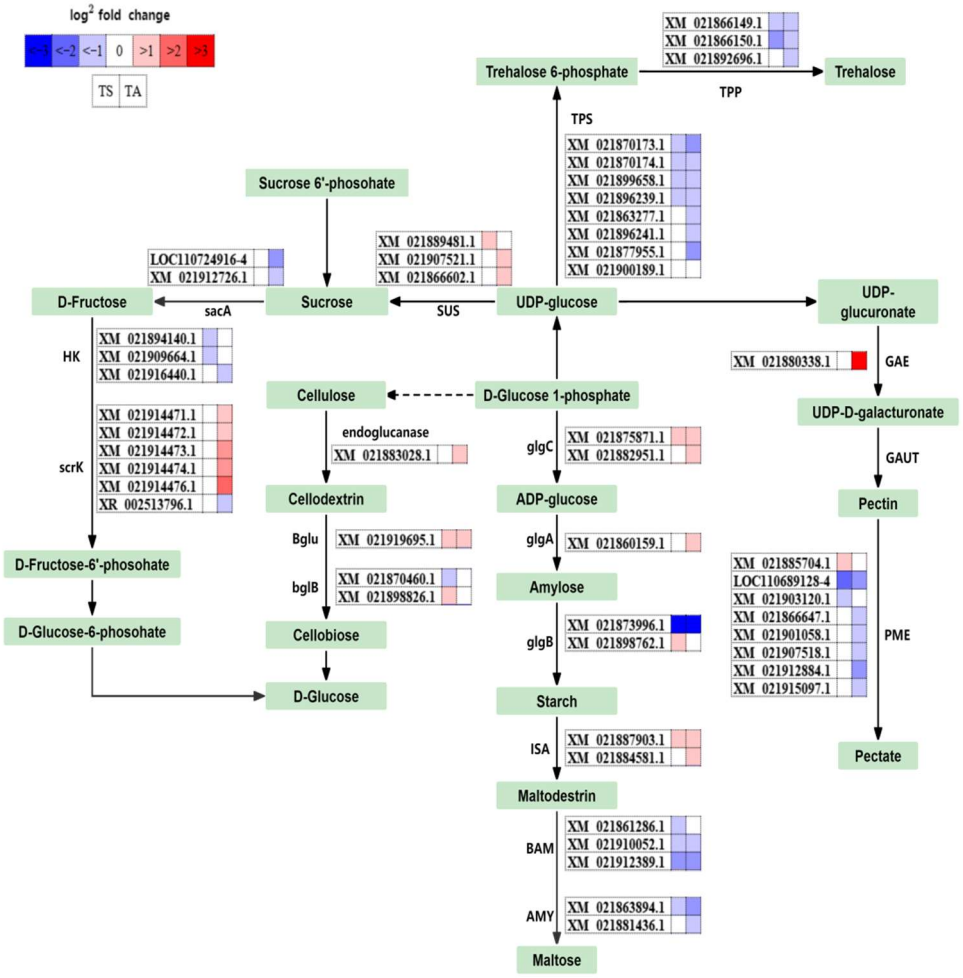


Figure 9. DEGs in Starch and sucrose metabolism pathway. Red blocks represent up-regulation gene expression, while blue represent down-regulation gene expression. Enzyme annotation are as follows: TPP, trehalose-phosphate phosphatase. TPS, alpha-trehalose-phosphate synthase. SUS, sucrose synthase. sacA, beta-fructofuranosidase. scrK, fructokinase-6. HK, hexokinase. ISA, isoamylase. AMY, alpha-amylase. BAM, beta-amylase. bglB, beta-glucosidase. glgB, 1,4-alpha-glucan-branching enzyme. glgC, glucose-1-phosphate adenylyltransferase. GAE, UDP-glucose 4-epimerase. GAUT, galacturonosyltransferase.

The growth hormone-responsive SAUR protein family plays diverse roles in plant growth and development, with suggestions that SAUR regulates polar hormone transport. Recent studies on abiotic stresses like salt and drought have gained attention, with research by Guo Yuan demonstrating that *Arabidopsis*-overexpressing TaSAUR75 exhibited enhanced salt and drought tolerance [60]. Alkali stress (pH 8.0) in *Arabidopsis thaliana* increased PIN2 abundance in the root tip and facilitated growth hormone translocation, while the transcript levels of growth hormone-related genes GH3, ARF5, SAUR36, and IAA were notably elevated in saline-stressed apple plants [61].

In our study, the IAA metabolic pathway was inhibited under TS but promoted under TA, indicating a differential response to these stresses. The up-regulation of SAUR genes under TA (Figure 10A) suggests a potential regulatory mechanism for quinoa's alkali tolerance, as alkali stress during the seedling stage reduced plant height and biomass through the up-regulation of IAA genes.

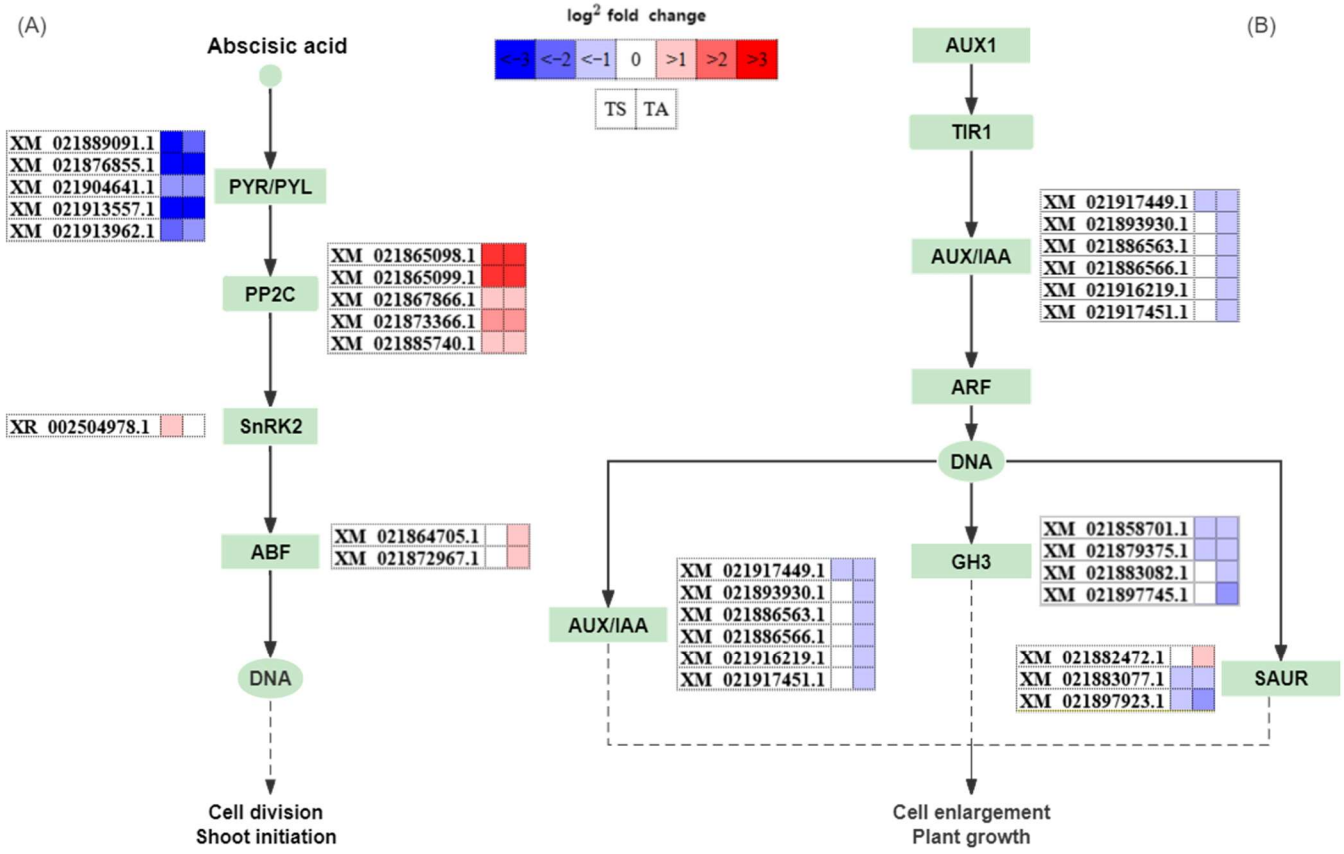


Figure 10. DEGs in hormone metabolism pathway. (**A**) ABA signal transduction pathway. (**B**) IAA signal transduction pathway.

The TGA transcription factor belongs to the D subfamily of the bZIP family, primarily involved in plant defense against diseases and growth and development processes. There have been fewer reports on its involvement in abiotic stresses. For instance, Duan [62] found that overexpressing TGA in *Arabidopsis thaliana* conferred a growth advantage and regulated stomatal movement under drought stress. Similarly, Danxia Ke et al. [63] discovered that the overexpression of GmTGA26 in soybean enhanced salt tolerance. In our study, the expression of TGA transcription factors was down-regulated under TS, while TA led to the up-regulation of TGA transcription factors. However, the specific role of TGA in quinoa's response to alkali stress requires further investigation.

4.3. γ-Aminobutyric Acid Branch

The γ-aminobutyric acid branch (GABA-shunt) is a branch of the TCA cycle, which has been shown to be involved in important biological processes such as plant carbon and nitrogen metabolism [64], signaling [65], and oxidative stress defense [66] under stress. In the plant GABA-shunt, glutamate is catalyzed by glutamate decarboxylase (GAD) to synthesize GABA, which is further catalyzed by the mitochondrial enzymes γ-aminobutyric acid transcarbamylase (GABA-T) and succinate semialdehyde dehydrogenase (SSADH) to produce succinate while providing NADH for respiration, and the GABA-branching pathway in plants enhances the resilience to a variety of biotic and abiotic stresses. The results of Kilian et al. [67]'s study on *Arabidopsis thaliana* under various adversity stresses (temperature, salt, drought, UV, water) showed that GABA-T was up-regulated in *Arabidopsis thaliana* juvenile leaves under salt stress with a prolongation of the stress time. Renault et al. [68] found that *Arabidopsis thaliana* GABA-T deletion mutants had reduced salt tolerance, suggesting that GABA-T genes play an important role. Our study of the alanine, aspartate, and glutamate metabolism (ko00250) pathway was significantly enriched under TA, with the

up-regulated expression of glutamic acid decarboxylase (GAD), γ-aminobutyric acid aminotransferase (GABA-T), alanine aminotransferase (ALT), two aspartate aminotransferases (GOT), and mitochondrially localized alanine-glyoxylate aminotransferase (AGXT). Both GAD and GABA-T are key enzymes in the GABA pathway, and alanine aminotransferase and aspartate aminotransferase catalyze the reaction in which glutamate and aspartate are generated into pyruvate and glycolate, which supplement the intermediates of the TCA cycle. It can be hypothesized that under alkali stress, quinoa can supplement the TCA cycle by generating succinic acid via the GABA bypass, as a way to maintain energy replenishment when quinoa's carbon source is insufficient during stress; the regulation of GABA synthesis control may be a key mechanism for quinoa to improve alkali tolerance.

Epidermal bladder cells (EBCs) play crucial roles in quinoa stem tips and the abaxial surface of leaves and young inflorescences, primarily involved in sodium ion compartmentalization, potassium conservation, and metabolite storage. Among these metabolites, γ-aminobutyric acid (GABA) is predominant, known for its regulation of plant ion content [69]. The number of EBCs tends to increase with rising saline concentrations [70,71], and they significantly enhance sodium compartmentalization in young leaves under 400mmol/L salt stress levels. Research has shown the involvement of EBCs in the molecular mechanisms of salt accumulation in quinoa [72,73], with evidence suggesting a positive correlation between the number of EBCs and salt stress tolerance [74,75]. The artificial removal of EBCs has been found to decrease quinoa's tolerance to salt stress [76,77]. In light of these findings, further investigation is required to determine whether the up-regulated expression of glutamic acid decarboxylase (GAD) and γ-aminobutyric acid aminotransferase (GABA-T) observed in our study is linked to the structure and function of EBCs. This deeper exploration could shed light on the specific mechanisms by which EBCs contribute to quinoa's response to salt stress.

5. Conclusions

In this study, we utilized quinoa variety LL1 to investigate the response to salt and alkali stress by conducting a comparative transcriptome analysis. The aim was to elucidate the molecular mechanisms underlying salt and alkali tolerance in quinoa at the transcriptional level and identify key resistance genes. Our findings suggest that quinoa responds to both salt and alkali stress through similar mechanisms. Specifically, it promotes sucrose and starch synthesis and catabolism, leading to increased osmotic potential in quinoa leaves. Additionally, we observed the down-regulation of the abscisic acid receptor PYR/PYL and up-regulation of the serine/threonine protein kinase (PP2C) gene in ABA signaling metabolism, indicating their involvement in stress response. Furthermore, we found that alkali stress posed a greater challenge to quinoa compared to salt stress. The mechanism underlying alkali resistance in quinoa involves the up-regulation of the citric acid cycle through the activation of the γ-aminobutyric acid branch, thereby enhancing energy metabolism in quinoa compared to salt stress conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14071596/s1, Table S1: Summary of sample sequencing data statistics; Table S2: Comparison efficiency between sample sequencing data and quinoa reference genome; Table S3: Primers used for validating by qRT-PCR.

Author Contributions: Data curation, Methodology, Writing—original draft, Writing—review and editing, Q.B.; Data curation, Software, Writing—original draft, Y.W. (Yang Wu); Investigation, Writing—review and editing, Y.W. (Yang Wang); Y.Z.: Conceptualization, Validation, Writing—review. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Key Research and Development Program of China (2023YFD1600702-03) and Industrialization Research Project of Jilin Provincial Department of Education (JJKH20230522CY).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The Illumina raw sequencing profiles were submitted to the NCBI. BioProject data-base under number PRJNA1117781.

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

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