Physiological and Molecular Responses of *Apocynum venetum* L. (Apocynaceae) on Salt Stress

Lulu Li¹, Jingyang Wang¹, Cheng Qian¹, Cuiping Zhang¹, Haixia Wang², Wei Li¹*, Han Zhao³,* and Yiqian Ju¹,*

¹ College of Landscape Architecture and Forestry, Qingdao Agricultural University, Qingdao 266000, China; yuanlinlilulu@126.com (L.L.)
² College of Grassland Science, Qingdao Agricultural University, Qingdao 266000, China; hxwang@qau.edu.cn
³ Research Institute of Non-Timber Forestry, Chinese Academy of Forestry, Zhengzhou 450000, China
* Correspondence: zhaohan@caf.ac.cn (H.Z.); juyiqian1991@163.com (Y.J.)

Abstract: Soil salinization is a crucial factor that impacts plant distribution and growth. *Apocynum venetum*, an ornamental plant with medicinal value, has shown remarkable salt tolerance. However, the specific mechanisms through which *A. venetum* responds to salt stress are not yet fully understood. To address this gap, we conducted a study where 10-week-old *A. venetum* seedlings were subjected to salt stress by irrigating them with a nutrient solution containing varying concentrations of NaCl (0, 100, 200, and 350 mmol L⁻¹). After the salt stress treatment, various growth indicators (such as plant height, root length, root fresh weight, root dry weight, leaf fresh weight, leaf dry weight, root water content, leaf water content, and root–leaf ratio) as well as physiological indicators (including SOD and CAT activities in both leaves and roots, soluble protein contents in leaves and roots, and chlorophyll and carotene contents in leaves) were determined. In addition, the gene expression profile of roots under salt stress was examined by transcriptome sequencing to explore the mechanism of salt response in *A. venetum*. Our results show that salt stress led to yellowing and wilting of *A. venetum* seedling leaves. Furthermore, the chlorophyll and carotenoid contents in the leaves of the 350 mmol L⁻¹ NaCl-treated group were significantly reduced. Although the leaf and root biomass gradually decreased with an increase in the salt concentration, the root–leaf ratio exhibited a decreasing trend. NaCl stress also caused significant changes in physiological indices in the *A. venetum* leaves and roots. The activities of superoxide dismutase (SOD) and catalase (CAT) increased in both leaves and roots of the 100 mmol L⁻¹ NaCl-treated group. The soluble protein content in both leaves and roots increased under the 200 mmol L⁻¹ NaCl stress. To screen changes in root gene expression, transcriptome sequencing and qRT-PCR were performed. GO and KEGG enrichment analyses revealed that salt stress primarily affects carbohydrate metabolism, MAPK signaling transduction, phytohormone signaling pathways, glyoxylate and dicarboxylate metabolism, and other pathways. This study provides a novel understanding of the growth and physiological response of *A. venetum* leaf and root to NaCl stress, as well as the changes in the transcription levels in *A. venetum* root. The results serve as a reference for future research on salt-tolerant mechanisms and molecular breeding of *A. venetum*.

Keywords: *Apocynum venetum*; NaCl treatment; biomass; physiological response; transcriptome

1. Introduction

Soil salinization is a vital factor affecting plant distribution and growth. In high-salt soil, excessive salt ions reduce the soil-water potential, thereby causing water absorption problems in plants. In plants with a poor water potential, water is drained out from the plants in large quantities, thereby causing osmotic stress to the plants [1]. According to relevant studies, osmotic stress is a type of first-level stress that plants experience under salt stress [2]. The response of osmotic stress is very fast, and its duration is very short.
From the physiology perspective, salt stress is alleviated by osmotic adjustment, antioxidant enzyme response, and exogenous substance exertion. Osmotic stress has a considerable adverse influence on nutrient absorption from the soil for the normal growth of plants and can even cause physiological water shortage. Thus, leaf transpiration is reduced, stomata are closed, and the photosynthetic system is inhibited [3]. Moreover, salt stress can lead to an imbalance of ions and increase the content of superoxide anions in plant cells, causing damage to the cell membrane system and photosynthetic system as well as disorder of mineral nutrient metabolism. This hinders plant growth and development by affecting seed germination, seedling growth, and plant yield [4]. Moreover, excessive Na\(^+\) accumulation in cells causes cytotoxicity and further wilting and necrosis of leaf margins as well as interfering with the absorption of other ions [5–7]. A high Na\(^+\) soil microenvironment resulted in a K\(^+\) deficiency in plants. K\(^+\) plays an indispensable role in maintaining the activity of various antioxidant enzymes and regulating osmotic pressure in plants. However, the K\(^+\) concentration is generally lower in soil than in plants. Therefore, plants need to actively absorb K\(^+\) from the soil to meet their needs. Under salt stress, the intracellular K\(^+\) transporter cannot distinguish between these two ions because K\(^+\) and Na\(^+\) have similar chemical properties. Salt-damaged soil usually contains a high Cl\(^-\) concentration, which is no less harmful to plants than Na\(^+\) [8]. Although Cl\(^-\) has an auxiliary role in promoting chlorophyll synthesis, a high Cl\(^-\) concentration affects photosynthesis, breaks the turgor equilibrium of cells, causes damage to leaves, and eventually causes withering and death of leaves [8].

At the molecular level, salt-stress-related genes are activated, thereby regulating protein synthesis under salt stress. Ca\(^{2+}\) is an important secondary messenger. When plants receive various environmental stimuli, Ca\(^{2+}\) channels on the cell membrane and intracellular calcium reservoir membrane open, resulting in changes in the concentration of Ca\(^{2+}\) in the cytoplasm for the completion of the intracellular and intercellular signal transduction of plants. The calmodulin-binding protein (CaM) and calcium-dependent protein kinases (CDPKs) can also improve salt tolerance [9,10]. The activation of the AtSOS2 gene, encoding a CaM, can accelerate the effect of Na\(^+\) and improve the salt tolerance of Arabidopsis root [11]. The mitogen-activated protein kinase (MAPK) cascade pathway has crucial roles in different stresses and signal transductions in various plants. OsMKK1 and OsMPK4 constitute a signaling pathway that regulates the salt resistance in rice (Oryza sativa) [12]. In alfalfa (Medicago sativa), the phosphorylation/dephosphorylation states of protein kinases (PKs), phosphatases (PPs), and phospholipases (PLs) are involved in response to salt stress [13]. In addition, several disease-course-associated proteins, disease-resistance-responsive proteins, and β-1-3 glucan endo-1,3-beta-glucosidase-like protein are upregulated in rice roots under salt stress [14–16].

Apocynum venetum L. (Apocynaceae) is not only a crucial plant in the ecological environmental control of saline-alkali land but also an ornamental, fiber, and medicinal plant. Considering that A. venetum is a medicinal plant, several reports have investigated the changes in flavonoid content and flavonoid metabolism genes under salt stress in A. venetum leaves. Xu et al. and Chen et al. revealed that salt stress can lead to a decrease in the flavonoid content. In addition, the expression of genes involved in metabolism, phenylpropanoid biosynthesis, flavonoid biosynthesis, and phytohormone signal transduction was also affected under salt stress in A. venetum leaves [17,18]. Moreover, at the seed germination stage, low NaCl concentrations (0–50 mmol·L\(^{-1}\)) can enhance the seed germination rate and high NaCl concentrations (100–300 mmol·L\(^{-1}\)) inhibited this rate. Salt stress also affected physiological processes, such as antioxidant enzyme activity, osmolyte content, and protein content [19]. Previous studies have shown that studies on the salt tolerance mechanism of A. venetum mainly focused on leaves. However, few studies have revealed the differences and relationships between leaves and roots at the physiological level, as well as the root molecular mechanism, under salt stress. What are the differences in the response mechanisms of roots and leaves to salt stress? It is still not clear.
To enhance the understanding of the salt response mechanism under different degrees of salt stress, *A. venetum* seedlings were treated with different NaCl concentrations, and the physiological response of the leaf and root was examined by measuring the growth amount, antioxidant enzyme activity, and soluble protein content. Additionally, salt-tolerance-related genes in the root were screened through transcriptome sequencing to explore their molecular mechanism, providing genetic material for breeding salt-tolerant varieties of *A. venetum*.

2. Materials and Methods

2.1. Plant Materials

*A. venetum* plants were cultivated in the intelligent greenhouse of Qingdao Agricultural University (Qingdao, China, 120°12’ E, 36°20’ N). At the intelligent greenhouse, the potting medium is soil: sand (1:2), supplemented by 1/2 Hoagland nutrient solution once a week. The daytime temperature was controlled at 25 ± 3 °C for 16 h, the night temperature was controlled at 20 ± 3 °C for 8 h and air humidity was controlled at 60–70%. The 10-week-old seedlings (plant is about 20 cm tall) were treated with 1/2 Hoagland nutrient solution containing different concentrations of NaCl (CK (0 mmol L⁻¹), C1 (100 mmol L⁻¹), C2 (200 mmol L⁻¹), and C3 (350 mmol L⁻¹)). The NaCl concentration was increased gradually by 50 mmol L⁻¹ per day, to reduce osmotic shock, until the target concentration was reached, and the target concentration was maintained for 1 week. Each treatment was performed for 10 replicates with 10 plants per replicate, randomly arranged. The mature leaves in the middle of the branch were selected for physiological experiments and transcriptome sequencing.

2.2. Growth Index Measurement

Fresh leaves or roots of the whole plant were cut, the fresh weight (FW) was measured with an analytical balance, then killed green at 105 °C for 10 min, dried at 80 °C to a constant weight, and the dry weight (FD) was measured. The calculation formulas of leaf/root water content and leaf–root radio are as follows:

Leaf (Root) water content (%) = \( \frac{\text{FW} - \text{FD}}{\text{FW}} \times 100\% \)

Leaf–root radio (%) = \( \frac{\text{leaf FD}}{\text{root FD}} \times 100\% \)

2.3. Physiological Index Measurement

2.3.1. Chlorophyll and Carotenoid Contents Determination

The samples (0.3 g) were cut and soaked in 15 mL of 95% ethanol solution in the dark for 12 h to extract chlorophyll and carotenoid. The contents of those pigments were determined through spectrophotometry at 663, 646, and 470 nm [20]. The pigment contents were determined according to Winterman method [21].

\[
\text{Chlorophyll a content (Ca, mg·g}^{-1}) = \left[13.95 \times A_{665} - 6.88 \times A_{649}\right] \frac{V}{W \times 1000}
\]

\[
\text{Chlorophyll b content (Cb, mg·g}^{-1}) = \left[24.96 \times A_{649} - 7.32 \times A_{665}\right] \frac{V}{W \times 1000}
\]

Total chlorophyll content = Ca + Cb

Carotenoid content (Cc, mg·g⁻¹) = \((1000 \times A_{470} - 2.05 \times \text{Ca} - 114.8 \times \text{Cb})/245\)
2.3.2. Soluble Protein Content Determination

The soluble protein contents in the leaves and roots were determined through Coomassie blue staining [22]. The sample powder (0.05 g) was homogenized with 1 mL of 0.05 M phosphate buffer (pH 7.8) and then centrifuged (4 °C, 5000×g, 10 min). Then, the supernatant (1 mL) was mixed with 0.1 g·L⁻¹ Coomassie brilliant blue solution. After 5 min at room temperature, the absorbance of the reaction liquid at 595 nm wave was measured. The soluble protein contents were calculated based on the standard curve and dilution of the bovine serum protein.

2.3.3. SOD and CAT Activity Determination

SOD and CAT enzyme activities were determined using NBT (Nitrotetrazolium Blue Chloride) and ultraviolet absorption of hydrogen peroxide methods [23,24]. For the SOD activity measurement, the sample powder (0.05 g) was mixed with 1 mL of 0.05 M phosphate buffer (pH 7.8) and centrifuged at 10,000×g for 10 min at 4 °C. After centrifugation, the supernatant was added to 3 mL of reaction solution (130 mM Met, 750 µM NBT, 20 µM riboflavin, 100 µM EDTA, 0.05 M phosphate buffer, and pH 7.8), and then the reaction liquid was placed in an artificial climate chamber (at 25 °C and 4000 lux) for 30 min. Distilled water was used instead of supernatant as the control. Finally, the absorbance of the reaction liquid at 560 nm was measured. The SOD activity was calculated as follows:

\[
\text{SOD activity [U·g}^{-1}·\text{min}^{-1}(\text{FW})] = \frac{\text{Ac} - \text{Ae}}{50\%W·T}
\]

Ac is the absorbance of control, Ae is the absorbance of enzyme solution, W is the sample weight (g), and T is the reaction time (min).

For the CAT activity, the sample powder (0.01 g) was mixed with 1 mL of 0.1 M phosphate buffer (pH 7.0) and centrifuged (at 4 °C and 5000×g for 10 min). Then, the supernatant (1 mL) was added to 2 mL of the 0.1 M phosphate buffers with 4 mM H₂O₂. The absorbances of the reaction liquid were detected at 240 nm at 0 min (A₀) and 3 min (A₃min). The CAT activity was expressed as mmol·min⁻¹·mg⁻¹ protein [25], and the formula is as follow:

\[
\text{CAT activity [mmol·mg}^{-1}(\text{FW})·\text{min}^{-1}] = \frac{(A₃\text{min} - A₀)·V_r}{W·T·d·ε}
\]

W is the weight of the sample, V is the volume of CAT enzyme used in the reaction, T is the reaction time (min), V_r is the total volume of the reaction, ε is the extinction coefficient of H₂O₂ (43.6 L·mol⁻¹·cm⁻¹), and d is the color plate thickness.

2.4. RNA Sequencing and Transcript Annotation

The roots were sampled after they were washed with distilled water and dried. Samples for RNA extraction were immediately frozen in liquid nitrogen and then stored at −80 °C with three replicates for each treatment. A Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) was used to isolate the total RNA from the A. venetum roots treated with 0, 100, 200, and 350 mmol·L⁻¹ NaCl. Then, Poly-T oligo magnetic beads (NEB, Ipswich, MA, USA) and the Ribo-ZeroTM Magnetic Kit (Epicentre, Madison, WI, USA) were used to enrich eukaryotic mRNA and prokaryotic mRNA from total RNA, respectively. Then, the samples were fragmented with divalent cations by the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). The first-strand cDNA synthesis was performed by Random hexamer primers and M-MLV reverse transcriptase (RNase H) (Takara, Dalian, China). The cDNA fragments were purified using the QiaQuick PCR extraction kit (Qiagen, Hilden, North Westphalia, Germany) and ligated to Illumina sequencing adapters. Polymerase and exonuclease enzymes were then used to convert any overhanging sequences into blunt ends. Following the 3’ adenylation of these DNA fragments, the fragments were ligated with NEB Next adapters with a hairpin loop structure (NEB, Ipswich, MA, USA). The 150–200 bp cDNA fragment enrichment and PCR amplification were performed on
an AMPure XP system (Beckman Coulter, CA, USA) and a Phusion High-Fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA), respectively. The library quality was assessed by an Agilent Bioanalyzer 2100 instrument (Agilent, Palo Alto, CA, USA). After sample clustering by the cBot Cluster Generation System (Illumina, San Diego, CA, USA) and the TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA), an Illumina novaseq 6000 instrument (Guangzhou, China) was used for PCR amplification and sequencing.

To obtain high-quality clean reads, fastp was used to remove the reads having more than 10% of unknown nucleotides (N) and low-quality reads containing more than 50% of low-quality bases [26]. Trinity was then used for the de novo assembly of cleaned reads [27]. The functions of these transcripts were annotated with the non-redundant protein sequence available on the NCBI (https://www.ncbi.nlm.nih.gov/, 4 July 2022), the Swiss-Prot protein database (http://www.expasy.ch/sprot, 23 January 2022), Clusters of Orthologous Groups (KOG/COG) (https://www.ncbi.nlm.nih.gov/research/cog-project/, 24 January 2022), and Kyoto encyclopedia (KEGG) orthology (http://www.genome.jp/kegg, 20 January 2022) databases.

2.5. Screening of DEGs and qPCR Verification

The unigene expression level was calculated to FPKM (Fragments Per kb per Million reads), and the RNA differential expression analysis relied on DESeq2 [28]. The genes with a false discovery rate (FDR) of <0.05 and a fold change of $\geq 2$ were regarded as differentially expressed genes (DEGs).

To verify the transcript sequencing data, qPCR was performed to detect the expression levels of 10 genes. Total RNA extraction and First-strand cDNA synthesis were conducted by the Plant RNA Extraction Kit (Omega, SAN Antonio, TX, USA) and the TIANScript First Strand cDNA Synthesis Kit (Tiangen, Dalian, China). qPCR was carried out using the ChamQ SYBR Color qPCR Master Mix (Vazyme Biotechnology, Nanjing, China) and the StepOnePlus Real-Time PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA). The qPCR reaction was performed [29]. The relative expression level of genes was calculated using the $2^{-\Delta\Delta Ct}$ method, and the AvEF1$\alpha$ was used as the reference gene. The primer sequences used for qPCR are shown in Table S1.

2.6. Statistical Analysis

The Tukey multiple comparison test ($p < 0.05$) was used to calculate the statistical significance between the different treatment groups by SPSS 18.0 software (SPSS, Chicago, IL, USA). Excel 2013 and GraphPad Prism 8 software were used to segregate data and create graphs. Three biological replicates were maintained for every experimental group.

3. Results

3.1. Effects of Different NaCl Concentrations on A. venetum Growth

Salt stress significantly inhibited the growth and development of A. venetum seedlings. As the salt stress increased, the effects on A. venetum leaves and roots became more obvious (Figure 1A,B). The growth rate of A. venetum seedlings reduced with an increase in the NaCl concentration. The plant height was significantly higher in the control group (22.34 cm) than in each treatment group (Figure 1A,D). The plant height in the 350 mmol L$^{-1}$ treatment group was the lowest, that is, only 16.30 cm, which was 72.9% of the plant height in the control group. As the NaCl concentration increased to more than 200 mmol L$^{-1}$, A. venetum leaves appeared yellow and withered. The salt concentration of 350 mmol L$^{-1}$ also affected root growth, thereby resulting in fewer lateral roots and shorter taproots (Figure 1C,E). This indicated that a high NaCl concentration would inhibit their growth.
Figure 1. Effects of different NaCl concentrations on A. venetum growth. (A). Morphology of plants treated with different NaCl concentrations; (B), characterization of leaves of plants treated with different NaCl concentrations; (C), characterization of roots of plants treated with different NaCl concentrations; (D), plant height under salt stress; and (E), root length under salt stress. Values are the means of three replicates ± standard error (SE). Non-overlapping letters indicate significant differences between different bud stages based on the ANOVA and multiple range test procedures with a confidence level of 95%.
Biomass, which is measured by dry weight and fresh weight, is the most apparent indicator of plant growth. Compared with the control group, the fresh weight and dry weight of *A. venetum* roots decreased significantly in the NaCl-treated groups (Figure 2A,B). As the salt concentration increased, the leaf dry and fresh weights exhibited a decreasing trend (Figure 2C,D). When the NaCl concentration increased to 200 mmol·L\(^{-1}\), the leaf fresh and dry weights were significantly lower than those in the control group (Figure 2C,D). Although no significant difference was observed in the leaf water content, the root water content in the 200 mmol·L\(^{-1}\) NaCl-treated group was significantly lower than that in the control group (Figure 2E,F). Moreover, the leaf–root ratios were significantly decreased after exposure to 350 mmol·L\(^{-1}\) NaCl stress, indicating that organic matter preferentially accumulates in the roots instead of leaves (Figure 2G).

**Figure 2.** Biomass of *A. venetum* under salt stress with different NaCl concentrations. (A), Root fresh weight; (B), root dry weight; (C), leaf fresh weight; (D), leaf dry weight; (E), root water content; (F), leaf water content; and (G), root–leaf ratio. Values are the means of three replicates ± SE. Non-overlapping letters (a–d) indicate significant differences between different bud stages based on the ANOVA and multiple range test procedures, with a confidence level of 95%.

### 3.2. Physiological Changes in *A. venetum* under Salt Stress

Figure 3 shows the physiological changes in *A. venetum* leaves under salt stress. SOD activities in the leaves of the 100 mmol·L\(^{-1}\) and 200 mmol·L\(^{-1}\) NaCl-treated groups were higher than those of the control group (Figure 3A). CAT activities reached the maximum value of 2.92 U·mg\(^{-1}\)·min\(^{-1}\) after treatment with 350 mmol·L\(^{-1}\) NaCl (Figure 3B). These results suggest that salt stress can increase SOD and CAT activities to prevent lipid membrane peroxidation in *A. venetum* leaves. At a low salt concentration (100 mmol·L\(^{-1}\)), the contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid increased, but as the salt concentration increased, these contents decreased gradually (Figure 3D–G). This indicated that the membrane lipid in leaf cells was peroxidized after the 200 mmol·L\(^{-1}\) NaCl treatment.
weight was used for calculating soluble protein, chlorophyll, and carotenoid contents. Values are means of three replicates ± SE. Non-overlapping letters (a–c) indicate significant differences between different bud stages based on ANOVA and multiple range test procedures, with a confidence level of 95%.

In the roots, SOD activity and soluble protein contents were significantly increased after the 200 mmol·L⁻¹ NaCl treatment (Figure 4A,C). Moreover, CAT activity significantly increased after the 100 and 200 mmol·L⁻¹ NaCl treatments (Figure 4B,C).

**Figure 3.** Changes in the physiological indices of *A. venetum* leaves under salt stress with different NaCl concentrations. (A), SOD activity; (B), CAT activity; (C), soluble protein content; (D), chlorophyll a content; (E), chlorophyll b content; (F), total chlorophyll content; and (G), carotene content. Fresh weight was used for calculating soluble protein, chlorophyll, and carotenoid contents. Values are means of three replicates ± SE. Non-overlapping letters (a–c) indicate significant differences between different bud stages based on the ANOVA and multiple range test procedures, with a confidence level of 95%.

3.3. Analysis of Gene Expression in *A. venetum* Roots under Salt Stress

Roots are the first organs in plants to sense and respond to environmental stress, including salt stress. However, studies on plant stress resistance have tended to focus on the parts above the ground. The resistance mechanism of *A. venetum* roots remains unclear.
3.3.1. Transcriptome Sequencing Quality and Correlation Analysis between Samples

Transcriptome sequencing performed on 12 samples revealed that the proportion of the clean base in each sample was >99.60%, Q20 of all groups was >97.00%, and Q30 ranged from 92.88% to 95.48%. The GC content was 42.87–43.25% (Table S2). These results indicated that the sequencing quality was excellent. The pairwise comparative analysis and pierce correlation coefficient analysis of the samples were performed to determine their repeatability and correlation. Figure S1 shows that the correlation index between the three replicates of each treatment was >0.9. This indicates that each treatment group had good repeatability.

3.3.2. Screening and Annotation of DEGs

The read count obtained through sequencing was normalized, and the screened genes with |log2 fold change| ≥ 1 and FDR < 0.05 were considered DEGs. In the CK vs. C1, C1 vs. C2, and C2 vs. C3 combinations, 413, 126, and 875 DEGs were observed (Figure 5). Among the three combinations, the expression of all five genes changed. The C2 vs. C3 combination had the highest number of DEGs. This indicated that the A. venetum plant response was the strongest to 350 mmol·L\(^{-1}\) NaCl stress.

![Figure 5. Venn diagram of the number of DEGs between the NaCl concentration comparisons: CK vs. C1, C1 vs. C2, and C2 vs. C3. CK, roots treated with distilled water; C1, roots treated with 100 mmol·L\(^{-1}\) NaCl solution; C2, roots treated with 200 mmol·L\(^{-1}\) NaCl solution; and C3, roots treated with 350 mmol·L\(^{-1}\) NaCl solution.](image)

The GO enrichment analysis divided the annotated unigenes into 3 categories (biological process, cell composition, and molecular function) and 37 subgroups (Figure 6). In CK vs. C1, DEGs involved in the biological processes were significantly enriched in transmembrane transport of inorganic anions (GO: 0015698); DEGs involved in the molecular function were enriched in oxidoreductase activity (GO: 0016705), transmembrane transport of inorganic anions (GO: 0015103) and iron ion binding (GO: 0005506); DEGs involved in the cell composition were enriched in extracellular regions (GO: 0005576). The KEGG enrichment analysis revealed that genes involved in ABC transporters; pentose and glucuronate interconversions; MAPK signaling pathway; phytohormone signal transduction; phenylpropanoid biosynthesis; nitrogen metabolism; and cutin, suberin, and wax biosynthesis were enriched (Figure 7). These results indicated that at a low salt concentration, the cell wall organization was accelerated, and related genes, such as those related to the antioxidant enzyme system and ion transport, were activated in A. venetum in response to salt stress.
Figure 6. Scatter plot of enriched GO terms in pairwise comparisons. CK, roots treated with distilled water; C1, roots treated with 100 mmol·L\(^{-1}\) NaCl solution; C2, roots treated with 200 mmol·L\(^{-1}\) NaCl solution; and C3, roots treated with 350 mmol·L\(^{-1}\) NaCl solution. The scatter plot of enriched GO terms (\(p < 0.05\)) in the CK vs. C1 comparison (A), in the C1 vs. C2 comparison (B), and the C2 vs. C3 comparison (C). The bubble size indicates the frequency of GO terms in the underlying GO database, and the green, yellow, and blue bubbles represent “cellular components”, “molecular functions” and “biological processes”, respectively.

### GO term Top 20

<table>
<thead>
<tr>
<th>ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0015979</td>
<td>oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen</td>
</tr>
<tr>
<td>GO:0042803</td>
<td>negative regulator of transmembrane transport activity</td>
</tr>
<tr>
<td>GO:0005056</td>
<td>iron ion binding</td>
</tr>
<tr>
<td>GO:0005593</td>
<td>anion transmembrane transporter activity</td>
</tr>
<tr>
<td>GO:0019333</td>
<td>inorganic anion transmembrane transporter activity</td>
</tr>
<tr>
<td>GO:0019881</td>
<td>carbohydrate transport activity</td>
</tr>
<tr>
<td>GO:0016942</td>
<td>anion transport</td>
</tr>
<tr>
<td>GO:0015664</td>
<td>anion transmembrane transporter activity</td>
</tr>
<tr>
<td>GO:0015791</td>
<td>secondary active transport activity</td>
</tr>
<tr>
<td>GO:0015823</td>
<td>organic acid transport activity</td>
</tr>
<tr>
<td>GO:0040494</td>
<td>catalytic activity</td>
</tr>
<tr>
<td>GO:0019080</td>
<td>inorganic ion transport</td>
</tr>
<tr>
<td>GO:0051511</td>
<td>organic ion transport</td>
</tr>
<tr>
<td>GO:0016664</td>
<td>phosphate ion transmembrane transporter activity</td>
</tr>
<tr>
<td>GO:0016080</td>
<td>peptide transmembrane transporter activity</td>
</tr>
<tr>
<td>GO:0043393</td>
<td>hydrolytic activity, basic hydrolysis</td>
</tr>
<tr>
<td>GO:0030337</td>
<td>nucleotide binding</td>
</tr>
<tr>
<td>GO:0045462</td>
<td>carboxylic acid transport</td>
</tr>
<tr>
<td>GO:0030123</td>
<td>disulfide reductase activity</td>
</tr>
<tr>
<td>GO:0043587</td>
<td>enolase transmembrane transporter activity</td>
</tr>
</tbody>
</table>

### Diagrams

A. Scatter plot for CK vs. C1 comparison

B. Scatter plot for C1 vs. C2 comparison

C. Scatter plot for C2 vs. C3 comparison

### Table

<table>
<thead>
<tr>
<th>ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q00550874</td>
<td>mitochondrion</td>
</tr>
<tr>
<td>Q00558712</td>
<td>microtubule</td>
</tr>
<tr>
<td>Q00559111</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00559111</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
<tr>
<td>Q00550875</td>
<td>microtubule-associated complex</td>
</tr>
</tbody>
</table>

### Notes

- The scatter plot of enriched GO terms (\(p < 0.05\)) in the CK vs. C1 comparison (A), in the C1 vs. C2 comparison (B), and the C2 vs. C3 comparison (C). The bubble size indicates the frequency of GO terms in the underlying GO database, and the green, yellow, and blue bubbles represent “cellular components”, “molecular functions” and “biological processes”, respectively.
solution; and C3, roots treated with 350 mmol·L⁻¹ NaCl solution. The scatter plot of enriched GO terms (p < 0.05) in the CK vs. C1 comparison (A), in the C1 vs. C2 comparison (B), and the C2 vs. C3 comparison (C). The bubble size indicates the frequency of GO terms in the underlying GO database, and the green, yellow, and blue bubbles represent "cellular components", "molecular functions" and "biological processes", respectively.

Figure 7. KEGG category enrichment of DEGs in CK vs. C1 (A), C1 vs. C2 (B), and C2 vs. C3 (C). CK, roots treated with distilled water; C1, roots treated with 100 mmol·L⁻¹ NaCl solution; C2, roots treated with 200 mmol·L⁻¹ NaCl solution; and C3, roots treated with 350 mmol·L⁻¹ NaCl solution. The number of genes in each category is equal to the dot size. The dot color represents the q-value.
In C1 vs. C2, the GO enrichment analysis revealed that genes related to the sodium-potassium-exchanging ATPase complex and ethylene receptor activity were enriched in the cell composition and molecular function categories. In addition to genes involved in the MAPK signaling pathway, genes involved in phytohormone signal transduction, and pentose and glucuronate interconversion pathways were enriched in CK vs. C1. The KEGG analysis revealed that genes involved in amino sugar nucleotide sugar metabolism, starch and sucrose metabolism, and N-glycan biosynthesis pathways were enriched. These results indicated that the expression of genes related to carbohydrate metabolism changes under 200 mmol L\(^{-1}\) NaCl stress.

In C2 vs. C3, DEGs enriched in the microtubule belonged to the cell composition category, those enriched in microtubule binding belonged to the molecular function category, and those enriched in microtubule-based movement belonged to the biological progress category. In addition, KEGG enrichment demonstrated that genes involved in the MAPK signaling pathway, phytohormone signal transduction, pentose and glucuronate interconversion pathways, phenylpropanoid biosynthesis, amino sugar nucleotide sugar metabolism, and starch and sucrose metabolism pathways, were enriched in CK vs. C1 or C1 vs. C2. Moreover, as the salt concentration increased, the \(p\)-value of phenylalanine biosynthesis (Ko00940), starch and sucrose metabolism (Ko00500), MAPK signaling pathway (Ko04016), and phytohormone signaling pathway (Ko03440) decreased, so attention had to be paid to these pathways.

To further explore the molecular response of roots under different salt concentrations, the number of upregulated and downregulated genes as well as KEGG functional clustering of CK vs. C1, C1 vs. C2, and C2 vs. C3 were analyzed (Figure S2). Upregulated genes accounted for 67.8% and 59.0% of DEGs in CK vs. C1 and CK vs. C2, respectively, while they only accounted for 36.1% in CK vs. C3. This indicated that most DEGs were downregulated under high salt stress (Figure S2A). In addition, most DEGs involved in the biosynthesis of metabolic pathways (ko01100) and secondary metabolites (ko01110) were upregulated in CK vs. C1 and CK vs. C2 but downregulated in CK vs. C3. This suggested that some metabolic activities of roots are inhibited under high salt stress (Figure S3B–D). Notably, several genes related to DNA replication (ko03030, ko03440) and mismatch repair (ko03430) were enriched and downregulated in CK vs. C3. This showed that high salt stress may affect and regulate the DNA replication process (Figure S3D).

3.3.3. Gene Expression Patterns Determined through STEM Analysis

Gene expression patterns in the main clusters of the four samples (CK, C1, C2, and C3) were determined through STEM analysis. DEGs in CK vs. C1, C1 vs. C2, and C2 vs. C3 were divided into 20 profiles according to the gene expression trend (Figure 8). The genes of profile 0/9/19/11/7 were significantly enriched.

Profile 0 was defined as downregulation. KEGG analysis of the genes in profile 0 revealed enrichment of genes involved in brassinosteroid biosynthesis, DNA replication, and phenylpropanoid biosynthesis (Figure S3A). Profile 19 exhibited a contrary tendency of downregulation. Genes in profile 19 were enriched in the MAPK signaling pathway (Ko04016), and this suggests that the MAPK signaling pathway was activated with an increase in the salt concentration under salt stress (Figure S3C).

3.3.4. Functional Genes Involved in MAPK Signaling and Phytohormone Signaling Pathways

The MAPK module consists of at least three kinases (MAP3K–MAP2K–MAPK) that phosphorylate each other, thereby leading to sequential activation. They play key roles in phytohormone signaling transduction, such as ABA and ET signaling transduction. The expression level of the MAPK gene (MAPKKK18) was upregulated in C1 vs. C2, whereas it was downregulated in C2 vs. C3. This suggested that MAPKKK18 (unigene0024828) may be involved in the response of *A. venetum* roots to salt stress, and MAPK activity may be affected under salt stress (Figure 9).
Salt stress may affect cell wall permeability by affecting the metabolism of some critical cell wall components. These results revealed that pectin, cellulose, xylan, and chitin metabolism were affected. These genes play the main roles in ABA signaling transduction. In the IAA signaling pathway, auxin/IAA mainly encoded genes (unigene0005199, unigene0005634, unigene0005199, and unigene0005199), and auxin/IAA transport, and other salt tolerance genes (unigene0034781) were downregulated in C2 vs. C3. This suggested that MAPKKK18 (unigene0034410) was downregulated in C2 vs. C3. These results revealed that pectin, cellulose, xylan, and chitin metabolism were affected.

Phytohormones are critical for plant response to salt stress. They act by regulating the expression of downstream genes and are widely involved in osmotic regulation, reactive oxygen species (ROS) homeostasis, Na⁺ transport, and other salt-tolerant regulatory processes [30–32]. Figure 9 presents the annotated NaCl stress response unigenes of Arabidopsis that are involved in phytohormone signaling pathways. The expression of two Arabidopsis PYL4 orthologous genes (unigene0005634 and unigene0010673) was downregulated in the CK vs. C1 and C2 vs. C3 groups, whereas that of PP2C51 orthologous genes...
genes (unigene0030989), At2g29380 (HIGHLY ABA-INDUCED PP2C GENE 3, HA13) (unigene0032329), and ABF4 (unigene0030995) was upregulated in the CK vs. C1 group. These genes play the main roles in ABA signaling transduction. In the IAA signaling pathway, the expression of AUX/IAA family genes (unigene0038906, unigene0034050, unigene0034103, and unigene0021989) was downregulated in C2 vs. C3. Aux/IAA mainly promotes early transcription of genes, such as SAUR, by binding to the auxin response factor. Two SAUR genes (unigene0008174 and unigene0034410) were downregulated in the CK vs. C1 and C2 vs. C3 groups. Brassinosteroids induced the expression of TCH4 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 22) and CYCD3 (CYCLIN D3;1) genes. These genes are involved in the regulation of cell wall modification and cell division, respectively. In C2 vs. C3, TCH4 expression was upregulated, whereas CYCD3 expression was downregulated, indicating inhibition of cell division and an improvement in cell wall modification.

The expression of many JA response genes was downregulated in C2 vs. C3, such as JASMONATE-ZIM-DOMAIN PROTEIN (JAZ) (unigene0012760 and unigene0009151) and MYC2 (unigene0015570 and unigene0016974). The expression of the unigene unigene0015748 annotated as ETHYLENE RESPONSE (ETR) and the SA response genes PR1 (unigene0023741 and unigene0039423) was downregulated in C1 vs. C2 but upregulated in C2 vs. C3. These results revealed that phytohormones have crucial and complex roles in the response of A. venetum plants to salt stress.

3.3.5. Functional Genes Involved in Carbohydrate Metabolism

Carbohydrates are essential for plant growth and development, survival, and reproduction. They are also involved in plant resistance to adversity. Pectin, cellulose, xylan, and chitin are vital components of the plant cell wall [32]. Among 13 DEGs in the pectin cleavage pathway, the expression levels of the pectin lyase (PL)-encoding gene (unigene0021815) and two polygalacturonase (PG)-encoding genes (unigene0004333 and unigene0030221) were upregulated, whereas those of five PL-encoding genes (unigene0009989, unigene0021815, unigene0029129, unigene0033462, and unigene0029882) and five pectinesterase (PEM)-encoding genes (unigene0030221, unigene0032313, unigene001194, unigene0034856, and unigene0029476) were downregulated in C2 vs. C3 (Figure 10); the expression level of only one PEM-encoding gene (unigene0004333) was upregulated. In addition, compared with the other transcripts, the expression of four chitinase-encoding genes (unigene0002432, unigene0005199, unigene0031808, and unigene0027708) in C3 was significantly increased. By contrast, cellulose degradation-related genes, including two beta-glucosidase genes (unigene0027388 and unigene0036082), four endoglucanase genes (unigene0023032, unigene0010430, unigene0028942, and unigene0035840), and one glucan endo-1,3-beta-glucosidase gene (unigene0004353) exhibited a downward expression trend as the salt concentration increased (Figure 10). Xylan is a cell wall component that can regulate cell wall permeability and affect the exchange of materials between the cell and the environment. The expression of the unigene0034781 gene that encodes 1,4-beta-D-xylan synthase was significantly decreased in C2 vs. C3. These results revealed that pectin, cellulose, xylan, and chitin metabolism were affected by salt stress. Thus, salt stress may affect cell wall permeability by affecting the metabolism of some critical cell wall components.

Glucose, maltose, trehalose, starch, amino sugar, and nucleoside sugar can be converted into each other and have various regulatory roles in plant salt tolerance that are mediated by regulating intracellular osmotic pressure. Accumulation of trehalose, a low-molecular carbohydrate, can stabilize the cell membrane structure under high osmotic pressure. The expression of the trehalose synthesis gene encoding trehalose-6-phosphate synthase (TPS) was significantly increased in C1, C2, and C3 compared with CK. The expression of several starch hydrolase-encoding genes significantly changed under salt stress. For example, the expression of beta-amylase genes (unigene0007139 and unigene0025461) was upregulated in C1, C2, and C3 compared with CK. The expression of the 4-alpha-glucanotransferase gene (unigene0018331) was also increased in C3.
The expression of several starch hydrolase synthase (TPS) was significantly increased in C1, C2, and C3 compared with CK. The expression of the trehalose synthase gene was consistent with the expression results of transcriptome sequencing. This also indicated that the transcriptome sequencing results were reliable.

3.3.6. Functional Genes Involved in Glyoxylate and Dicarboxylate Metabolism

Malic acid and oxalic acid, which are metabolized by glyoxylic acid and dicarboxylic acid, respectively, in plants are conducive to osmotic accumulation, which then initiates osmotic regulation and ROS clearance [33]. Seven genes involved in malic acid and oxalic acid metabolism were upregulated with C1 and C2 compared with CK (Figure 11). According to these results, these genes affected the salt tolerance of A. venetum by participating in the regulation of glyoxylic acid and dicarboxylic acid metabolism under salt stress.

Figure 10. Unigenes in the carbohydrate metabolism network in response to salt stress. CK, roots treated with distilled water; C1, roots treated with 100 mmol·L\(^{-1}\) NaCl solution; C2, roots treated with 200 mmol·L\(^{-1}\) NaCl solution; and C3, roots treated with 350 mmol·L\(^{-1}\) NaCl solution. Enzyme names, unigene ids and expression patterns are indicated at each step. Red indicates high relative gene expression (FPKM values), and blue indicates low relative gene expression.

Figure 11. Unigenes in the glyoxylate and dicarboxylate metabolism network in response to salt stress. CK, roots treated with distilled water; C1, roots treated with 100 mmol·L\(^{-1}\) NaCl solution; C2, roots treated with 200 mmol·L\(^{-1}\) NaCl solution; and C3, roots treated with 350 mmol·L\(^{-1}\) NaCl solution. Enzyme names, unigene ids, and expression patterns are indicated at each step. Red indicates high relative gene expression (FPKM values), and blue indicates low relative gene expression.
3.3.7. Validation through Fluorescence qRT-PCR Analysis

qRT-PCR of the 10 DEGs revealed that the melting curves were all single peaks, and the specificity of PCR was good. The expression trend of those genes was consistent with the data from transcriptome sequencing (Figure 12 and Figure S4). Therefore, the expression of the screened genes was consistent with the expression results of transcriptome sequencing. This also indicated that the transcriptome sequencing results were reliable.

![Figure 12. Expression profiles of the selected genes according to qRT-PCR data. AvEF1α was used as the internal reference. Each data point represents the mean ± SE (n = 3). Different letters indicate significance at p ≤ 0.05.](image)

4. Discussion

Salt stress directly affects plant morphogenesis and growth, and plants can increase their tolerance and adaptability to stress by regulating their physiological processes and gene expression patterns. A set of physiological and molecular mechanisms have evolved in plants that confer resistance to salt stress, including osmotic regulation, ion regulation, oxidation defense mechanism, phytohormone metabolism and signal transduction, and synthesis and accumulation of secondary metabolites [34,35].

*A. venetum* is an ecologically important species of vegetation in the arid regions of northwest China. It is believed to be a pioneer plant for ameliorating salt-affected soils and conserving water and soil. It is a valuable medicinal plant and an ornamental plant. However, knowledge about the physiological and molecular mechanisms of *A. venetum* salt response mainly focuses on leaves, but the root response mechanism has not been studied.

4.1. Vegetable Growth Was Inhibited under Salt Stress

Under salt stress, plant growth and development are affected, and the effect of salt stress on plant morphology is the most intuitive. In this study, the growth of both *A. venetum* leaves and roots were significantly inhibited under salt stress (Figure 2). Similar to our results, Xu et al. found that plant height decreased significantly under salt stress in *A. venetum* [17]. Photosynthesis is the main method through which plants safely and
efficiently obtain solar energy and convert it into organic matter. Maize-related research has shown that salt stress significantly decreases leaf chlorophyll a and b contents [36]. The chloroplast structure of sweet potato (Dioscorea esculenta) and tomato (Solanum lycopersicum) leaves were damaged by salt stress, leading to a decrease in the chlorophyll content of the leaves [37,38]. The decrease in the photosynthetic pigment content leads to a decrease in the photosynthetic efficiency [39]. In A. venetum, as the salt concentration increased, the photosynthetic pigment content of leaves exhibited a decreasing trend (Figures 1 and 3), suggesting that salt stress also damaged the photosynthetic system and affected biomass accumulation.

Salt stress affects leaf and root growth and changes the process of plant organ growth, especially the root–shoot ratio. Under 350 mmol·L\(^{-1}\) NaCl stress, fresh and dry weights of the leaves and roots decreased significantly, and the leaf–root ratio also decreased. This indicated that A. venetum may protect photosynthetic tissues by driving biomass to underground tissues, thereby increasing root contact with soil and water absorption under high salt stress. Similar to our results, the increase in salt concentration decreased the plant’s fresh and dry weights and increased the root–shoot ratio in Rubus idaeus [40]. A study in Arabidopsis also reported that salt stress can stimulate the formation of lateral roots and advents [41,42].

4.2. Phytohormone Signaling Transduction under Salt Stress

Phytohormones play a crucial role in plant response to salt stress by regulating the expression of downstream response genes. They are extensively involved in stress signal transduction, Na\(^+\) transport, osmotic regulation, ROS homeostasis, and other salt-tolerant regulatory processes in plants [43]. In total, 25 DEGs were enriched in the phytohormone signaling transduction pathway. ABA is a major hormone that promotes dormancy, reduces water loss, inhibits plant growth, induces the expression of stress-related genes, and promotes the adaptation of plants to a stressful environment [44–46]. In this study, the expression of the ABA response gene ABIF4 was significantly upregulated after 100, 200, and 350 mmol·L\(^{-1}\) NaCl treatments (Figure 9). A study reported that ABA can alleviate the effect of saline-alkaline stress on multiple species by inducing quick responses, such as stomatal closure, and long-term responses, including extended growth inhibition, osmotic regulation, accumulation of cuticular wax, senescence, abscission, and dormancy [47–50]. Auxin is crucial for the development of salt-stress-induced root plasticity. Lateral root formation was induced under mild salt stress and inhibited under high salt stress [51]. In A. venetum, the expression of the auxin transporter AUX/IAA-encoding genes was upregulated under 200 mmol·L\(^{-1}\) NaCl stress, indicating that the polar auxin transport possibly reduces auxin accumulation in roots. The expression levels of several hormone signal transduction-related genes as well as GA and BR biosynthesis-related genes were upregulated in A. venetum leaves under salt stress [18]. Those results suggest that various hormone signals play key roles in both the roots and leaves of A. venetum in response to salt stress.

4.3. Protection of the Cell Membrane Lipid System by Antioxidant Enzymes under Salt Stress

Under mild salt stress (100 mmol·L\(^{-1}\) NaCl), no significant change was observed in most physiological indices of A. venetum leaves. However, in roots, the activities of SOD and CAT enzymes were increased. SOD and CAT activities both reached a peak under 200 mmol·L\(^{-1}\) NaCl stress. These enzymes remove excessive ROS and free radicals from cells to avoid severe damage to proteins, membrane lipids, DNA, and other cell components [52,53]. SOD can convert ROS into O\(_2^-\) and generate H\(_2\)O\(_2\), which is then reduced to O\(_2\) and H\(_2\)O by CAT and POD, thereby alleviating plant stress [54]. These results revealed that roots are more sensitive to salt stress than leaves. This indicated that the aforementioned enzymes were activated in A. venetum roots under salt stress. Moreover, at 350 mmol·L\(^{-1}\) NaCl, except for CAT levels in leaves, all other physiological
indices in leaves and roots exhibited a downward trend, indicating that the high NaCl concentration may inhibit the physiological activities of cells. Similar to our results, in *A. venetum* seedlings, SOD activity and protein contents first increased and then decreased, but the peak value appeared in the 150 mmol·L⁻¹ NaCl-treated group instead of the 200 mmol·L⁻¹ NaCl-treated group [19]. The aforementioned results suggested that seeds are more sensitive to salt stress than seedlings.

Unsaturated fatty acids are crucial components of phospholipids in cell membranes and affect the stability of these membranes [55]. In *A. venetum* roots under 100 mmol·L⁻¹ NaCl stress, several genes involved in unsaturated fatty acid biosynthesis (unigene0004075, unigene0017545, and unigene0024765), linoleic acid metabolism (unigene0032198 and unigene0032480), and ascorbate and aldarate metabolism (unigene0017199) are enriched and upregulated (Figure 7A). This suggested that mild salt stress can induce unsaturated fatty acid synthesis. Salt stress can promote the expression of desaturase genes *FAD7* and *FAD8* and regulate the content of unsaturated fatty acids in maize (*Zea mays*) [56]. Studies have found that environmental stress can increase the content of unsaturated fatty acids in plant cells and increase the ratio of unsaturated fatty acids to saturated fatty acids, thus enhancing the fluidity of the membrane [57,58].

4.4. Changes in the Content of the Osmoregulatory Substances under Salt Stress

Two types of osmo-regulatory substances are involved in plant osmo-regulation under salt stress. One type involves inorganic ions that are transported from the external environment into plant cells, such as K⁺ and Cl⁻, and inorganic salts. At a high NaCl concentration in soil, high Na⁺ prevents K⁺ and Ca²⁺ absorption and transport, thus reducing K⁺/Na⁺ and Ca²⁺/Na⁺ in plants and destroying the ion balance in the cytoplasm and various metabolic pathways [3,5].

In *A. venetum* roots, the ion transport system was activated to protect cells from damage under salt stress. In total, the expression of 40 genes related to the transmembrane transport of inorganic ions changed under salt stress. A study reported that Na⁺ concentrations increased in the *A. venetum* leaf, stem, and root under 100–300 mmol·L⁻¹ NaCl stress. K⁺ concentrations increased in the leaf, but no significant change was observed in the root and stem. Several genes involved in the non-selective cation channels were also upregulated in the leaf [17]. Under mild salt stress (CK vs. C1), the expression levels of three nitrogen metabolism-related genes changed (Figure 7). De et al. and Singh et al. investigating *Sorghum bicolor* and tomato, respectively, reported that nitrogen can regulate the opening and closing of ion channels. It can effectively inhibit the uptake of Na⁺ and increase the K⁺ content in aboveground parts [59,60]. In addition, the spatial distribution of Na⁺ in leaves was also affected by nitrogen, and the application of nitrogen to rice and *Brassica napus* separated Na⁺ in leaf edges to avoid the central part from being poisoned [61].

High salt stress destroys the ion and osmotic balance in plant cells, whereas plant roots maintain a normal ion and osmotic balance by accumulating organic acids (malic acid, citric acid, succinic acid, oxalic acid, etc.) [62]. Although organic acids do not directly regulate the osmotic balance in cells, they have a crucial regulatory role in inorganic ion absorption [63,64].

Studies related to soybean, tomato, and corn have reported that citric acid accumulation may be positively correlated with Fe uptake efficiency [65]. Several genes related to malic acid and oxalic acid metabolism were upregulated under salt stress, suggesting that malic acid and oxalic acid from the roots are beneficial for enhancing salt tolerance in *A. venetum*. In addition, unigene0037344 and unigene0032466 encode two aluminum-activated malate transporter proteins, respectively, that are essential for malic acid and Cl⁻ accumulation and transport in plants [66]. However, DEGs related to organic acid metabolism were not significantly enriched in the leaves and seeds [17–19], suggesting that organic acid synthesis is a unique method of roots to respond to salt stress.

The other class of osmoregulatory substances is organic solutes. They are synthesized in cells and include proline, betaine, soluble sugar, soluble protein, etc. [67]. The soluble
protein content in A. venetum leaves and roots increased significantly under 200 mmol·L\(^{-1}\) NaCl stress and the soluble sugar content increased significantly in A. venetum seeds under 150–300 mmol·L\(^{-1}\) NaCl stress [19]. The gene expression analysis revealed changes in the expression of genes involved in polysaccharide hydrolysis and monosaccharide, disaccharide, and oligosaccharide synthesis (Figure 10). This led to an increase in cell osmotic pressure and prevented cell water loss. Trehalose, a nonreducing sugar composed of two glucose units linked in an α,α-1,1-glycosidic linkage, can increase the soluble sugar content of rice and enhance its salt tolerance [68]. TPS is a key rate-limiting enzyme involved in trehalose biosynthesis. The expression of unigene0017271 that encodes TPS in A. venetum was significantly upregulated after 100, 200, and 350 mmol·L\(^{-1}\) NaCl treatments.

4.5. Cell Wall Organization under Salt Stress

The cell wall is the first line of defense against salt stress in plant cells. Cell wall polymers have a complex arrangement, which maintains the mechanical support and structural integrity of cells. The cell wall coordinates changes in cell division and expansion as well as senses changes in the plant growth environment, thereby transmitting signals to and responding to the cell’s interior [69]. Salt stress affects the physical structure and morphology of the plant cell wall by affecting the metabolic processes of critical components, such as cellulose, pectin, xylan, and lignin. The expression levels of pectin, cellulose, and xylan degradation-related genes were upregulated under salt stress, which suggested that salt stress damaged the cell wall integrity by inducing the degradation of cell wall components. Similar to our results, previous studies have reported that the plant cellulose content decreases under salt stress [70].

Pectin is a group of complex polysaccharides that is widely present in plant cell walls and is generally present in a highly methylated form. Because its structure and crosslinking affect cell wall hydration and porosity, plant cell walls are assumed to affect salt tolerance through changes in the pectin structure and content [69]. The carboxyl group of pectin can bind to Na\(^+\), the main toxic ion under salt stress, and prevent its entry into the cytoplasm, thereby maintaining normal cell metabolism and growth [71].

Three pectin methylesterases (PMEs), a crucial pectin-degrading enzyme, encoding genes (unigene0030221, unigene0029476, and unigene0004333), were significantly downregulated under mild salt stress (Figure 10). The change in pectin content is also related to other enzymes. For example, the dysfunction of pectin biosynthetase AtCSLD5 in the Arabidopsis sos6 mutant resulted in the blockage of cell wall pectin synthesis, thereby leading to the aggravation of oxidative stress and an increase in the sensitivity of the mutant to abiotic stress [72].

The response of A. venetum to salt stress is an extremely complex process and involves the regulation of multiple metabolic pathways, such as MAPK signaling and phytohormone signaling transduction, ion balance and transport, antioxidant enzyme activation, osmoregulatory substance metabolism, cell wall organization, etc. Regarding phenotype and physiological indices, the sensitivity of roots and leaves to salt stress varied. Based on the phenotype, physiological indices, and transcriptome data, significant differences were also observed in the response of roots to NaCl stress at different concentrations. Based on the study results, we presented a schematic diagram of the root response to salt stress in A. venetum (Figure 13). Under 100 mmol·L\(^{-1}\) NaCl stress, root cells could acclimate this low-concentration salt stress by activating antioxidant enzymes, regulating MAPK and ABA signaling, maintaining ion balance, maintaining cell membrane and cell wall stability, regulating nitrogen metabolism and organic acid metabolism, etc. When the NaCl concentration reached 200 mmol·L\(^{-1}\), the following changes occurred to defend the adverse environment: MAPK and multiple phytohormones (IAA, CTK, and ABA) signaling regulatory, organic osmotic (soluble protein, fructose, and mannose) regulatory. At NaCl concentrations as high as 350 mmol·L\(^{-1}\), BR, JA, SA, and MAPK signaling was regulated, and cellulose and chitin metabolism as well as DNA replication were affected, which indicated some degree of injury to root cells. This root response process under salt stress...
stress is only a summary and speculation based on the results of the present study, and the specific regulatory mechanism remains to be further investigated.

Figure 13. Summary of root response to salt stress in *A. venetum*.

5. Conclusions

Our results provide a comprehensive characterization of the effect of salt stress on the growth, physiological response, and root gene expression of *A. venetum* plants. Salt stress inhibited the growth of roots and aboveground parts and activated various antioxidant enzymes. The soluble protein content in the leaves increased to varying degrees. Numerous DEGs involved in carbohydrate metabolism, MAPK signaling transduction, phytohormone signaling pathways, glyoxylate and dicarboxylate metabolism, and other pathways were expressed. Here, a molecular mechanism was proposed in which many pathways collectively regulate salt resistance in *A. venetum*. These results revealed the adaptive mechanism of *A. venetum* to saline soils, providing a valuable reference for *A. venetum* plantation in saline regions and a resource for the breeding of salt-tolerant *A. venetum*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9091010/s1, Figure S1: Hierarchical clustering on pairwise correlation coefficients of unigenes expression data from *Apocynum venetum* samples; Figure S2: Comparison of the number of upregulated and downregulated genes in CK vs. C1, CK vs. C2, CK vs. C3, and KEGG functional clustering; A. The number of upregulated and downregulated genes in CK vs. C1, CK vs. C2, CK vs. C3; B. KEGG functional clustering of CK vs. C1; C. KEGG functional clustering of CK vs. C2; D. KEGG functional clustering of CK vs. C3. Figure S3: KEGG category enrichment of genes in significantly enriched profiles.; Figure S4: The expression levels of genes revealed by RNA-seq; Table S1: The sequences of primers used for qPCR; and Table S2: Sample sequencing quality information.

Author Contributions: Conceptualization, H.Z., L.L. and W.L.; resources, H.Z. and H.W.; writing—original draft preparation, L.L. and J.W.; investigation C.Q. and W.L.; writing—review and editing, L.L., C.Z. and Y.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by Qingdao Agricultural University Doctoral Start-Up Fund (663/1122010), Qingdao Agricultural University Doctoral Start-Up Fund (663/1121012), and Qingdao Agricultural University Doctoral Start-Up Fund (663/1122020).

Data Availability Statement: The raw sequence reads of transcriptome have been deposited in China National Genomics Data Center online with the BioProject ID PRJCA019418.
Conflicts of Interest: The authors declare no conflict of interest.

References
19. Li, X.; Li, J.; Su, H.; Sun, P.; Zhang, Z.; Li, M.; Xing, H. Physiological and transcriptional responses of *Apocynum venetum* to salt stress at the seed germination stage. *Int. J. Mol. Sci.* 2023, 24, 3623. [CrossRef] [PubMed]


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.