Effects of arbuscular mycorrhizal fungus on sodium and chloride ion channels of Casuarina glauca under salt stress

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Research Article

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Abstract

Arbuscular mycorrhizal fungi (AMF) can promote the growth and salt tolerance of plants under salt stress. However, the effects of AMF on the distribution of Na^+ and Cl^- and the expression of related genes in plants under salt stress need to be further explored. This study explored the effects of *Rhizophagus irregularis* on plant biomass, the distribution of Na^+ and Cl^-, and the expression of related genes in *Casuarina glauca* under NaCl stress. *R. irregularis* could promote salt dilution of *C. glauca* by increasing biomass and the content of K^+, compartmentalizing Na^+ and Cl^- in vacuoles. These processes were associated with the expression of *CgNHX1*, *CgNHX2-1*, *CgCLCD*, *CgCLCF*, and *CgCLCG.* This phenomenon may explain why *C. glauca* with *R. irregularis* grows better than that without under the same level of NaCl stress.

Introduction

Salt stress is one of the most serious abiotic stresses that the world is facing (Ahmad et al. 2010). Approximately 800 million hectares of land and approximately 20 percent of irrigated land in the world are under some degree of salt stress (Cirilloa et al. 2018; Singh et al. 2020). The ionic toxicity of salt stress can induce osmotic stress in plants, leading to imbalanced nutrition and even plant death (Zhang et al. 2019). NaCl stress is one of the most extensive and damaging salt stresses (Wei et al. 2021; Wei et al. 2020). However, halophytes can grow under high salt stress (Bueno and Cordovilla 2019; Hassan et al. 2017). There are three ways of their salt tolerance under salt stress: 1. salt exclusion, common in pseudohalophytes. Their roots rarely absorb salt or mainly accumulate the absorbed salt in the roots, so the ion concentration in the leaves is significantly lower than that in the roots within a certain concentration range (Matinzadeh et al. 2019; Zhao et al. 2010). 2. salt excretion, common in recretohalophytes. They have salt glands, which can actively secrete the absorbed salt to the surface of the stems and leaves for the rain to wash away (Ding et al. 2010). 3. salt dilution, common in euhalophytes. They reduce salt concentration by rapidly growing cells to absorb water, increasing osmotic adjustment substances, and compartmentalizing salt in vacuoles (Balnokin et al. 2005; Song and Wang 2015). It would be interesting to explore the mechanism of salt tolerance in these plants and then apply the insights gained to improve the yields of crops under salt stress.

Arbuscular mycorrhizal fungi (AMF) form symbiotic relationships with more than 90% of terrestrial plants and 80–90% of vascular plants (Feng et al. 2013). AMF symbionts may be one of the most extensive beneficial interactions between plants and microorganisms (Dowarah et al. 2021), which occur naturally even in salty environments, where they promote the growth of mycorrhizal plants (Diagne et al. 2020; Gavito et al. 2019). Mycorrhization can enhance the absorption of water and nutrients (Wu et al. 2017; Yang et al. 2014), the accumulation of osmotic adjustment substances, and the activity of antioxidant enzymes in plants (Feldmane et al. 2020). Furthermore, mycorrhization can reduce the concentrations of salt ions in plants under salt stress (Chebaane et al. 2020; Selvakumar et al. 2018). Most studies in this area have focused on the promotion of mycorrhization on plant growth under salt stress (Lenoir et al. 2016; Vives-Peris et al. 2020).

Casuarina glauca is native to Australia, Southeast Asia, and the Pacific islands and was introduced to the southeast coastal areas of China in the 1950s (Shan et al. 2018). Due to its tolerance to drought, infertile soil, salt, and sandstorms, *C. glauca* has become one of the main forest tree species in coastal zones by playing an irreplaceable role in windbreak and sand fixation. Hence, it helps to improve the ecological environment by providing materials (Djighaly et al. 2018; Ye et al. 2019). As a coastal shelterbelt species, *C. glauca* is mainly exposed to Na^+ and Cl^- toxicity (Fan et al. 2018; Vikashini et al. 2018). Djighaly et al. (2018) found that *C. glauca* was more tolerant to NaCl stress than *C. equisetifolia*, and inoculation with AMF could improve the uptake of nutrients (such as N and P) and water of *C. glauca* under NaCl stress. The results of Djighaly et al. (2020) indicated that inoculation with AMF increased the growth of *C. glauca* under NaCl stress, with a positive effect on the diversity of herbaceous vegetation around it, which could be used to reduce the NaCl concentration in the soil. Our previous study also found that AMF inoculation under NaCl stress played an important role in promoting growth, regulating ion balance, and changing the activity of antioxidant enzymes in *C. glauca* (Wang et al. 2022). Besides these ways, NHXs (Na^+/H^+ exchangers) and CLCs (the chloride channels), related to Na^+ and Cl^- transport, are also related to *C. glauca*'s NaCl tolerance (Ayadi et al. 2020; Subba et al. 2020).

NHXs play important roles in the regulation of ion homeostasis and cell growth (Gradogna et al. 2020). They exchange H^+ for Na^+ or K^+, thereby regulating monovalent cation homeostasis (Bassil et al. 2019). *Arabidopsis thaliana* contains 8 NHX isoforms (Cui et al. 2020). NHX1 to NHX4, which are located in the tonoplast, are associated with growth and vacuolar K^+ homeostasis (Zhang et al. 2020). NHX5 and NHX6, which are located in the Golgi and trans-Golgi networks (TGNs), are thought to facilitate Na^+ (K^+)/H^+ exchange (Zhu et al. 2018), auxin homeostasis, and growth (Lv et al. 2020). NHX7 (salt overly sensitive 1, SOS1) and NHX8, which are located in the plasma membrane, are involved in Na^+ (Li^+)/H^+ exchange, respectively transporting Na^+ and Li^+ out of the cell (Sze and Chanroj 2018).

CLC protein family, with its dual functions of NO^{3-} and Cl^- transport, plays important role in salt tolerance and nutrient absorption (Subba et al. 2020). *A. thaliana* contains 7 CLC isoforms (Liu et al. 2020). CLCA and CLCB, which are located in the tonoplast, are associated with NO^{3-}/H^+ exchange (Wei et al. 2015). As a Cl^- channel located in the tonoplast, CLCC plays an important role in regulating stomatal movement, anion homeostasis, and salt tolerance (Jossier et al. 2010). CLCD and CLCF are localized in Golgi membranes (Liu et al. 2020). CLCD is associated with root growth as a Cl^- channel gene (Fecht-Bartenbach et al. 2007). CLCE, which targets the thylakoid membranes in chloroplasts, is related to photosynthesis (Nedelyaeva et al. 2020). Some authors have proposed that CLCF has the same function as CLCE (Subba et al. 2020), whereas others consider CLCF to be more similar to CLCD (Angeli et al. 2009). CLCG, located in the tonoplast, is considered to have the same function with CLCC (Nguyen et al. 2016).

Most studies on AMF and salt stress have focused on the distribution of Na^+ and the expression of related genes. The effects of AMF on the distribution of both Na^+ and Cl^-, and the expression of related genes need to be further explored. This study has three aims:

1. Explore the influence of mycorrhization on the growth and distribution of Na^+ and Cl^-

2. Explore the influence of mycorrhization on the gene expression of the *CgNHX* and *CgCLC* families

3. Explore whether these genes are related to the distribution of Na^+ and Cl^-.

Materials And Methods

Experimental Design and Materials

This experiment was a two-factor experiment, one of which was NaCl stress, applying 0 mM NaCl and 600 mM NaCl, respectively. Another factor was inoculation with AMF, respectively no AMF and inoculation with *Rhizophagus irregularis*. There were three replicates for each treatment, and each replicate consisted of a mix of 12 seedlings.

The experimental materials were prepared in the same way as the previous research (Wang et al. 2022). *C. glauca* seeds were sterilized and germinated into seedlings, and then the best growing plant was selected from the seedlings to prepare a batch of tissue culture seedlings, which were used as experimental materials. *R. irregularis* was multiplied by *Lycopersicon esculentum* Mill and then triturated, and 40 g of inoculum was added to each pot for *R. irregularis* inoculation treatment, and 40 g of sterilized inoculum was added to each pot for no AMF treatment. The growth medium and conditions used for seedlings were consistent with previous research (Wang et al. 2022).

Plant Harvest

First measure the stomatal conductance (Gs) and transpiration rate (Tr) with Li-6400 portable open flow gas-exchange system (Li-Cor Inc., Lincoln, NE, United States) at 8:30 to 11:30 a.m. The method and parameter setting refer to the research of Sheng et al (2008).

After shoots weighed the total fresh weight, one part was used to calculate the water content, one part was used for elemental concentration determination, and one part was used to extract RNA. After weighing the total fresh weight of roots, the grouping and uses were the same as those of shoots, except that a part needs to be separated out to measure the AMF colonization.

Mycorrhizal Colonization

Refer to Phillips and Hayman (1970), Giovannetti and Mosse (1980) for the determination method of AMF colonization.

Plant Biomass and Water Content

The total fresh weight of shoots and roots was obtained by weighing at the time of harvest. The plant height was measured with a tape measure, and the ground diameter was measured with a digital caliper (Hengliang, Shanghai, China). The part of the sample used to measure the water content was weighed

fresh, and then dried at 65°C after fixation at 105°C to measure the dry weight. The water content was calculated using the dry and fresh weights. Total dry weight was calculated using water content and total fresh weight.

Na^+, K^+, and Cl^- Status

After being ground, the part of the sample used to measure the Na^+ and K^+ concentration was extracted with HNO_3 and a microwave digestion instrument (Milestone ETHOS, Milan, Italy). The elements were measured by atomic absorption spectrophotometer (PerkinElmer, Waltham, Massachusetts, USA). The measurement method of Cl^- concentration referred to Matsushita and Matoh (1991) and Zhan et al. (2004). The contents of the element were calculated by the concentration and total dry weight. The extraction method of elements in the soil referred to Bao (2000). The method for measuring elements in soil was the same as above. The transfer factor (TF) of Na^+ and Cl^- was obtained by dividing the shoot content by the root content.

RNA Extraction, Complementary DNA (cDNA) Synthesis, and Determination of Gene Expression via Quantitative Real–Time PCR (qRT–PCR)

RNA extraction used the E.Z.N.A Plant RNA Kit R6827-01 (Omega Bio-Tek, Norcross, GA, USA). RNA quality check used 1% agarose gel electrophoresis and a NanoDrop 2000 instrument (Thermo Scientific, Pittsburgh, PA, United States). Reverse transcription of RNA used TIANScript RT Kit (TIANGEN Bio, Beijing, China) to obtain cDNA. The sequences of *CgNHXs* and *CgCLCs* were obtained from the assembly of the *C. glauca* transcriptome sequenced by Illumina HiSeq technology (PRJNA690646). The full-length gene was cloned using the SMARTer® RACE 5'/3' Kit (Takara Bio, Beijing, China). The primers used for qRT–PCR were designed with the NCBI Primer-BLAST tool (Table S1). qRT–PCR was conducted using a CF96X real-time PCR system (Bio-Rad, Hercules, CA, USA). The specific reaction system and reaction steps were consistent with the previous research (Wang et al. 2022). Two technical replicates were required for each sample. The relative quantity of transcripts was determined using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Statistical analyses

SPSS 26 statistical software (SPSS Inc., Chicago, IL, United States) was used for statistical analyses. All data were subjected to one-way ANOVA and post hoc comparisons (Tukey's test, $P < 0.05$, n = 3). Figures were constructed with Origin 2020 (Origin Lab, Northampton, Massachusetts, USA.). The phylogenetic tree was plotted using ITOL (https://itol.embl.de/itol.cgi).

Results

Impact of *R. irregularis* on Mycorrhizal Colonization and Plant Biomass under NaCl Stress

Figure 1 showed the colonization of arbuscule, hypha, vesicle, and spore in mycorrhizal *C. glauca* under NaCl stress. The results showed that with the change in NaCl concentration from 0 to 600 mM, arbuscular colonization decreased from 39–14%, vesicle and spore colonization increased from 34–57%, and hyphal colonization exhibited no significant change.

Under NaCl stress, the biomass of *C. glauca* differed between plants with *R. irregularis* inoculation and those without. Figure 2 showed that NaCl stress inhibited the growth of *C. glauca* and *R. irregularis* inoculation alleviated this inhibition (Fig. 2a). NaCl stress reduced the fresh weight, ground diameter, and height of *C. glauca* (Fig. 2b, c, d). The inoculation with *R. irregularis* could promote the growth of *C. glauca* regardless of the presence of NaCl stress, specifically by increasing the fresh weight, plant height and ground diameter of *C. glauca* (Fig. 2b, c, d). These results showed that *R. irregularis* inoculation could promote the growth and salt tolerance of *C. glauca* under NaCl stress.

Effect of *R. irregularis* on Na^+, K^+, and Cl^- Status under NaCl Stress

The effects of *R. irregularis* inoculation on the Na^+ and Cl^- contents under NaCl stress were consistent, and both were increased. Therefore, the contents in the soil were reduced (Fig. 3a, b, c, and d). The Na^+ and Cl^- concentrations of *C. glauca* were increased by NaCl stress. Instead, under NaCl stress, inoculation with *R. irregularis* decreased the Na^+ and Cl^- concentrations of shoots and roots (Fig. 3e, f). The K^+ content of *C. glauca* was reduced by NaCl stress. But inoculation with *R. irregularis* increased the K^+ content of shoots and roots under NaCl stress (Fig. 3g). The Na^+/K^+ value of *C. glauca* was increased by NaCl stress. Nevertheless, under NaCl stress, *R. irregularis* inoculation decreased the Na^+/K^+ value of shoots and roots by 8% and 24%, respectively (Fig. 3h).

The effects of NaCl stress on the TF of Na^+ and Cl^- were different, increased the TF of Na^+ but decreased that of Cl^-. But the effects of *R. irregularis* inoculation on the Na^+ and Cl^- TF under NaCl stress were consistent, and both were increased (Fig. 4).

Effect of *R. irregularis* on Gs and Tr under NaCl Stress

In order to explore whether the increase of TF of Na^+ and Cl^- caused by *R. irregularis* inoculation is related to Gs and Tr, we explored the effect of *R. irregularis* on Gs and Tr under NaCl Stress, and the results showed that NaCl stress decreased Gs and Tr. Under NaCl stress, compared with no inoculation, inoculation with *R. irregularis* increased Gs and Tr by 31% and 26%, respectively. (Fig. 5).

Effect of *R. irregularis* on Expression of *CgNHXs* and *CgCLCs* under NaCl Stress

To explore whether the effect of *R. irregularis* inoculation on Na^+, K^+, and Cl^- Status is related to Na^+ and Cl^- transport-related genes, we determined the expression of *CgNHXs* and *CgCLCs*.

Fig. 6 is a phylogenetic tree of NHX and CLC protein translated from cloned genes.

The patterns of *CgNHX* expression differed between shoots and roots. In shoots, the expression of *CgNHX1* and *CgNHX2-1* was upregulated by *R. irregularis* inoculation. The expression of *CgNHX2-2* and *CgNHX6* was upregulated by NaCl stress but downregulated by *R. irregularis* inoculation under NaCl stress. In addition, the expression of *CgNHX7* was downregulated by NaCl stress. In roots, the expression of *CgNHX1*, *CgNHX2-1*, and *CgNHX2-2* was upregulated by *R. irregularis* inoculation under NaCl stress. The expression of *CgNHX6* was upregulated by NaCl stress. Under NaCl stress, the expression of *CgNHX7* was upregulated by *R. irregularis* inoculation (Fig. 7).

The patterns of *CgCLC* expression differed between shoots and roots. In shoots, the expression of *CgCLCB* was downregulated by NaCl stress. The expression of *CgCLCC-1* was upregulated by NaCl stress but downregulated by *R. irregularis* inoculation under NaCl stress. In addition, the expression of *CgCLCD*, *CgCLCF*, and *CgCLCG* was upregulated by *R. irregularis* inoculation under NaCl stress. In roots, the expression of *CgCLCB* and *CgCLCD* was downregulated by NaCl stress, whereas the expression of *CgCLCC-1*, *CgCLCF*, and *CgCLCG* was upregulated by NaCl stress. *R. irregularis* inoculation downregulated the expression of *CgCLCC-1* but upregulated *CgCLCF* and *CgCLCG* under NaCl stress (Fig. 7).

Correlation Analysis of Gene Expression and Physiological Indicators of *C. glauca* under NaCl Stress

In order to explore the correlation between the effect of *R. irregularis* inoculation on physiological indicators and the Na^+, Cl^- transport-related genes under NaCl stress, we did a correlation analysis between them under salt stress.

Just the opposite of *CgNHX6*, the correlation analysis results of the expression of *CgNHX1* in shoots and roots and *CgNHX2-2* in roots were all positively correlated with mycorrhizal colonization, biomass, and the content of Na^+, K^+. Except that the expression of *CgNHX1* in shoots was negatively correlated with the concentration of Na^+, and that in roots was negatively correlated with Na^+/K^+. The expression of *CgNHX2-1* in shoots was positively correlated with mycorrhizal colonization and the content of K^+, and that in roots was only positively correlated with mycorrhizal colonization. The expression of *CgNHX7* in roots was positively correlated with mycorrhizal colonization and biomass, but was negatively correlated with the concentration of Na^+, the content of Na^+ in soil, and Na^+/K^+ (Fig. 8).

Correlation analysis showed that the expression of *CgCLCB* in shoots was positively correlated with the concentration of Cl^- and negatively correlated with the TF of Cl^-. However, the expression of *CgCLCC-1* in shoots was positively correlated with the concentration of Cl^- and negatively correlated with other indicators, which was just the opposite of the expression of *CgCLCD* in shoots. The expression of *CgCLCC-1* in roots was negatively correlated with mycorrhizal colonization, fresh weight, and the content of Cl^-. Both the expression of *CgCLCF* in shoots and roots were positively correlated with fresh weight and the content of Cl^-. In addition, the expression of *CgCLCF* in roots was also positively correlated with mycorrhizal colonization. The correlation analysis results of *CgCLCF* in roots were consistent with those of *CgCLCG* in roots (Fig. 8).

Discussion

Effects of *R. irregularis* Inoculation and NaCl Stress on Colonization and Biomass

Salt stress not only inhibited plant growth but also inhibited AMF growth in the same way (Bharti and Garg 2019; Liu et al. 2018). Soil salinity had a negative correlation with the intensity of mycorrhizal colonization on the one hand and a positive correlation with spore density on the other hand. We believed that these results reflected the assistance of *R. irregularis* to plants in resisting NaCl stress (Chebaane et al. 2020). Sporulation was considered a resistance behavior exhibited by mycorrhization against high salt concentrations (Selvakumar et al. 2018).

The most significant and common effect of salt on plants was growth inhibition. Because in order to survive, plants needed to absorb water and ions to maintain osmotic pressure, synthesize osmotic adjustment substances, and compartmentalize harmful ions. These processes required energy, resulting in less energy required for growth and lower biomass (Dell'Aversana et al. 2021; Romero-Munar et al. 2019). AMF could increase energy by enhancing the ability of plants to absorb water, synthesize osmotic adjustment substances, and compartmentalize harmful ions, thereby promoting plant growth (Lin et al. 2021; Santander et al. 2021). Additionally, we regarded the significant increase of *C. glauca* biomass caused by *R. irregularis* inoculation as an important indicator of the ability of plants to tolerate salinity stress (Parvin et al. 2020; Santander et al. 2021).

Effects of *R. irregularis* Inoculation and NaCl stress on Na^+, K^+, and Cl^- Uptake

Many studies only focused on the tolerance mechanism of Na^+ under salt stress, thinking that Na^+ can represent NaCl. However, our results showed that the change trends of Na^+ and Cl^- TF were different under NaCl stress, reflecting the different salt tolerance mechanisms of the two. For Na^+, the Na^+ TF increased under NaCl stress, which meant that the tolerance mechanism of Na^+ in *C. glauca* might be salt accumulation, and a large amount of Na^+ was accumulated in shoots (Ma et al. 2019; Rozentsvet et al. 2018). The shoots were then protected by salt dilution, increasing tissue water content, or compartmentalizing Na^+ to the vacuole (Song and Wang 2015; Wang et al. 2012). But for Cl^-, the TF of Cl^- was significantly decreased under NaCl stress. At the same time, the Cl^- content in shoots was still larger than that in roots. This meant that under the stress of 600 mM NaCl, the tolerance mechanism of *C. glauca* to Cl^- might convert from salt accumulation to salt exclusion (Krishnamurthy et al. 2014). It might because that Cl^- is more toxic to shoots than Na^+ for *C. glauca*, or the concentration of Cl^- in shoots has reached the tolerance threshold, but Na^+ has not (Munns and Teste 2008; Scagel et al. 2017).

Although the mechanisms of *C. glauca* tolerance to Na^+ and Cl^- were different, the mitigation mechanisms of *R. irregularis* inoculation to their stress were similar. The upward transport of Na^+ and Cl^- improved by AMF was caused by transpiration flow in plants under salt stress (Nada et al. 2021). The transpiration flow was caused by the increases in Gs and Tr, which were attributed to *R. irregularis* inoculation. AMF could promote salt dilution of halophytes by increasing plant biomass and content of

osmotic adjustment substances, compartmentalizing Na^+ and Cl^- (Jia et al. 2019; Pan et al. 2020). Due to the increase in biomass caused by *R. irregularis* inoculation, mycorrhizal *C. glauca* could maintain high contents and low concentrations of Na^+ and Cl^- at the same time. It's a common feature of AMF-associated plants under salt stress (Djighaly et al. 2018; Talaat and Shawky 2011). Compared with nonmycorrhizal *C. glauca*, mycorrhizal *C. glauca* absorbed larger amounts of Na^+ and Cl^- from the soil but maintained lower concentrations of Na^+ and Cl^- to protect themselves from excessive damage. Meanwhile, mycorrhizal plants had lower Na^+/K^+ to regulate the dynamic equilibrium of ions and improve salt tolerance (Han et al. 2021; Kapoor et al. 2019). Therefore, even if increased Gs and Tr promoted the upward transport of Na^+ and Cl^-, the mycorrhizal *C. glauca* does not suffer severe ion damage.

Effects of *R. irregularis* Inoculation and NaCl stress on Gene Expression

Roots were the first part to be stressed during salt stress. The AM symbiosis increased saline stress tolerance of *Robinia pseudoacacia* through the upregulation of *SOS1/NHX7* in roots, which enhanced the exclusion of Na^+ from root cells (Chen et al. 2017). It indicated that *R. irregularis* inoculation promoted Na^+ efflux from roots and prevented Na^+ poisoning. Meanwhile, *R. irregularis* inoculation decreased the Na^+ content in the soil and increased TF of Na^+, implying that most of the Na^+ efflux in roots induced by *CgNHX7* upregulated by *R. irregularis* inoculation was transferred to the shoots rather than the soil. Meanwhile, under NaCl stress, the expression of *CgNHX7* was decreased in shoots but increased in roots. It's consistent with our previous notion that *R. irregularis* inoculation might promote the salt accumulation of Na^+ in shoots. We mentioned earlier that AMF could promote salt dilution of halophytes by increasing plant biomass and content of osmotic adjustment substances, compartmentalizing Na^+. So, were these processes associated with *CgNHXs*? As vacuolar *NHXs*, *NHX1* and *NHX2* could promote the transport of Na^+ to vacuoles (Santander et al. 2021). Hence, the upregulation of *CgNHX1* and *CgNHX2-1* caused by *R. irregularis* inoculation might have alleviated the inhibitory effects of Na^+ by transporting it to vacuoles. Furthermore, similar to our correlation analysis results, *NHX1* and *NHX2* had the same functions in growth-promoting and the stabilization of K^+ in vacuoles (Bassil et al. 2011; 2019). Therefore, the upregulation of *CgNHX1* and *CgNHX2-1* by *R. irregularis* inoculation might not only increase the biomass of mycorrhizal *C. glauca* to reduce Na^+ toxicity by bio-dilution but also stabilize the cellular environment by increasing the K^+ content in shoots and roots.

So which *CgCLCs* did mycorrhizal *C. glauca* use for salt dilution? The expression of *CgCLCC-1* in *C. glauca* under NaCl stress was higher than in those without NaCl stress treatment. It's a protection mechanism for plants with high Cl^- toxicity by upregulating the expression of *CLCC* to transport Cl^- into vacuoles (Hu et al. 2017). *AtCLCG* was involved in salt tolerance by altering Cl^- homeostasis in mesophyll cells and Cl^- compartmentalization into the vacuole. Its gene expression is coordinated with the expression of *AtCLCC* in guard cells to regulate stomata (Nguyen et al. 2016). Although *R. irregularis* inoculation downregulated *CgCLCC-1* under salt stress, its upregulated *CgCLCG* might promote the transfer of Cl^- into mesophyll cell vacuoles, thereby improving mycorrhizal *C. glauca* with the tolerance of

NaCl stress, promoting its growth. *CLCD*, as an anion transporter involved in compartments and trafficking, might be related to growth and Cl^- transfer into the trans-Golgi network (Subba et al. 2020). The function of *CgCLCF* might be similar to that of *CgCLCD*, which was correlated with Cl^- transport and growth. The increase in biomass and Cl^- compartmentalization caused by *R. irregularis* inoculation might have been related to *CgCLCD* and *CgCLCF*.

Conclusions

Under NaCl stress, the tolerance mechanisms of *C. glauca* to Na^+ and Cl^- were different. For Na^+, salt accumulation was adopted, and Na^+ absorbed from the soil was transferred from roots to shoots. This process was promoted by *R. irregularis* inoculation and was related to Gs, Tr, and *CgNHX7*. For Cl^-, it's converted from salt accumulation to salt exclusion, and Cl^- absorbed from soil was no longer transferred to shoots in large quantities but started to accumulate in roots. However, the mechanism of *R. irregularis* inoculation alleviating Na^+ and Cl^- stress was similar, by promoting growth, increasing osmotic adjustment substances, and ionic compartmentalization. This resulted in that mycorrhizal *C. glauca* could not only absorb large amounts of Na^+ and Cl^- from the soil to remediate it, but also mitigating Na^+ and Cl^- stress. The process of *R. irregularis* inoculation alleviating Na^+ and Cl^-stress was related to *CgNHX1*, *CgNHX2-1*, and *CgCLCD*, *CgCLCF*, *CgCLCG*. Future studies can focus on the specific effects of *R. irregularis* inoculation on these genes and the transfer pathway of Na^+ and Cl^- in *C. glauca* under NaCl exposure.

Declarations

Conflict of interest The authors declare no competing interests.

Author Contributions Conceptualization, Y.W.; methodology and investigation, Y.W. and F.D.; data curation, Y.W. and F.D.; writing-original draft preparation, Y.W and T.X.; writing-review and editing, T.X.; supervision and project administration, M.T. and H. C.; funding acquisition, M.T. All authors have read and agreed to the published version of the manuscript.

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Figures

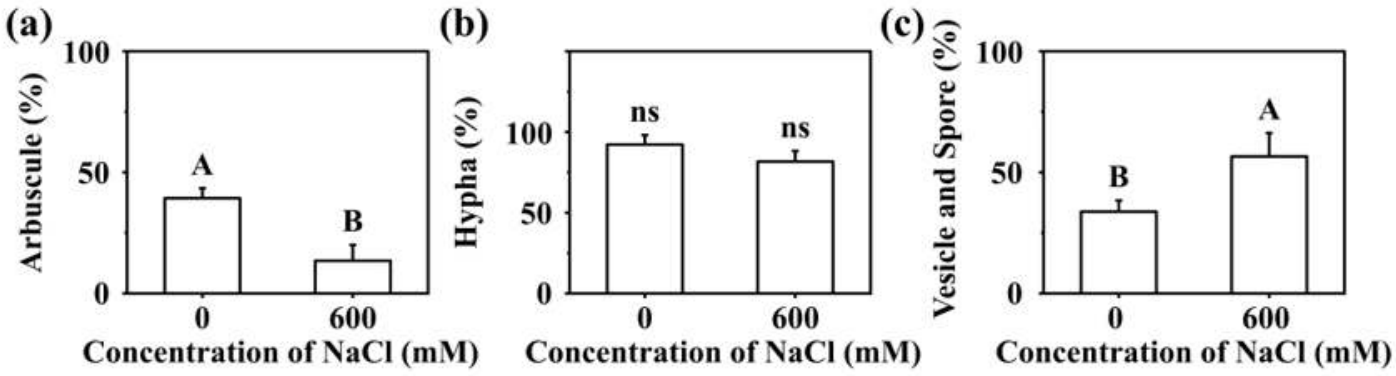

Figure 1

R. irregularis colonization rates of *C. glauca* under different treatments. 0, no NaCl stress; 600, 600 mM NaCl stress. The data are the means ± standard errors (n = 3). Different capital letters indicate significant differences among the means by Tukey's test ($P < 0.05$), "ns" indicates no significant difference ($P \geq 0.05$).

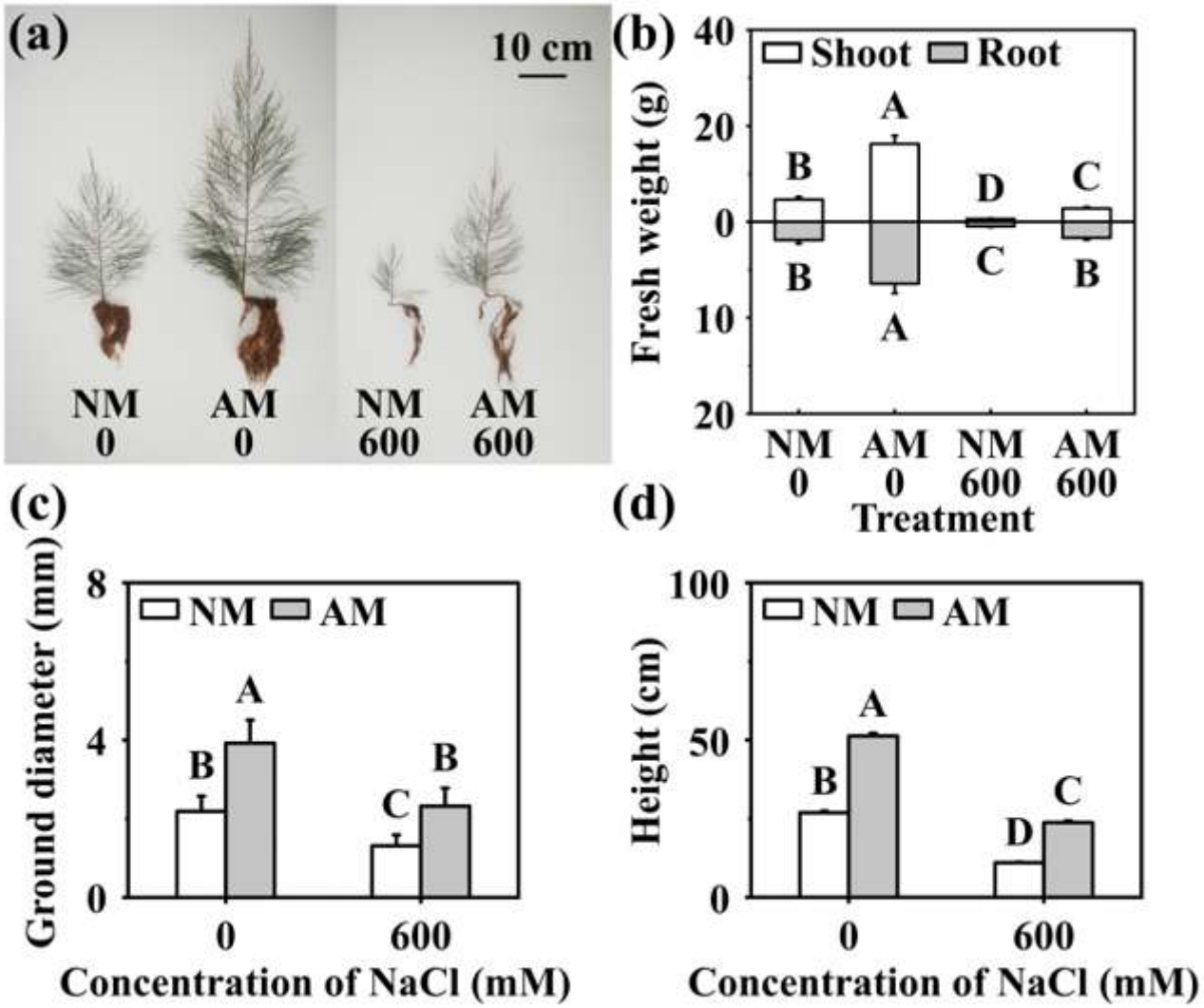

Figure 2

Growth phenotype (a), fresh weight (b), ground diameter (c), and height (d) of *C. glauca* under different treatments. NM, nonmycorrhizal; AM, inoculated with *R. irregularis*; 0, no NaCl stress; 600, 600 mM NaCl stress. The data are the means ± standard error (n = 3). Different capital letters indicate significant differences among the means by Tukey's test ($P < 0.05$).

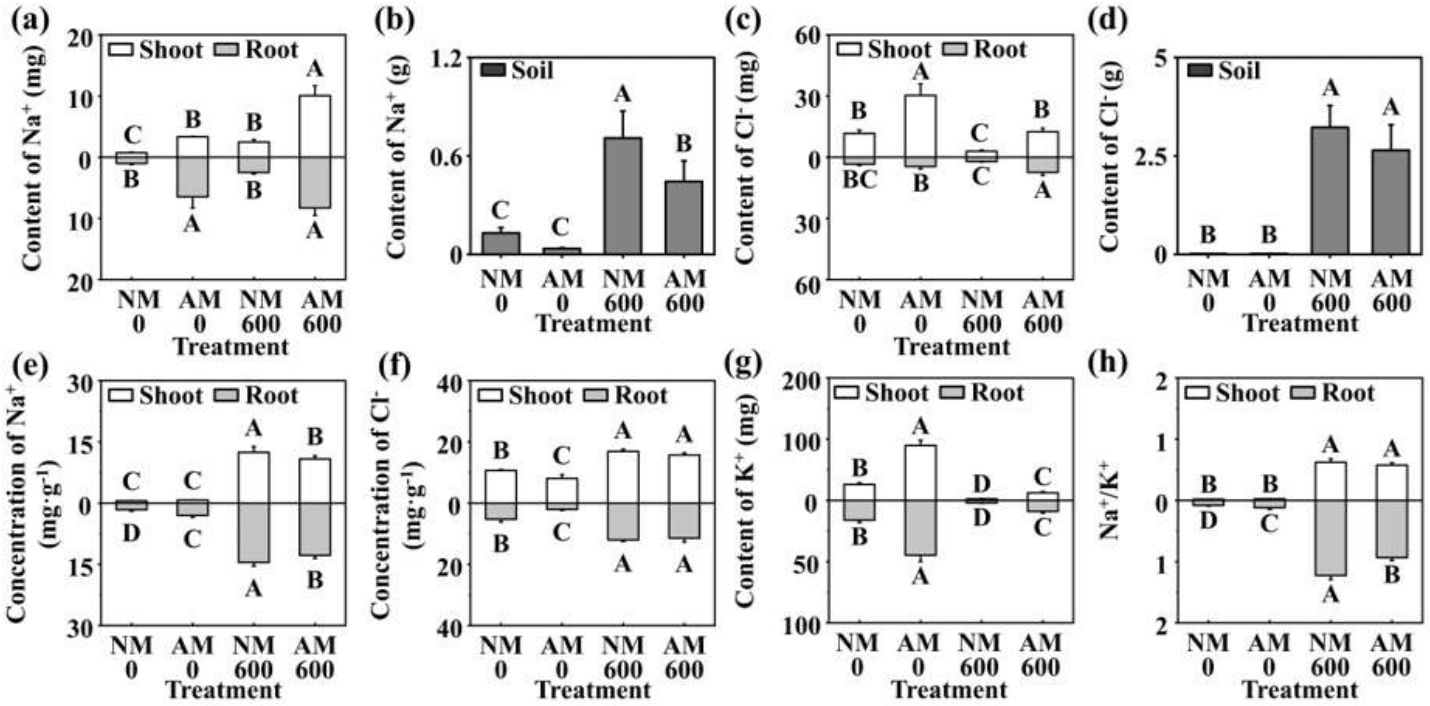

Figure 3

The contents of Na^+ (A) and Cl^- (C) in *C. glauca*, and soil (B and D); the concentrations of Na^+ (E) and Cl^- (F); K^+ content (G); Na^+/K^+ (H) under different treatments. NM, nonmycorrhizal; AM, inoculated with *R. irregularis*; 0, no NaCl stress; 600, 600 mM NaCl stress. The data are the means ± standard errors (n = 3). Different capital letters indicate significant differences among the means by Tukey's test ($P < 0.05$).

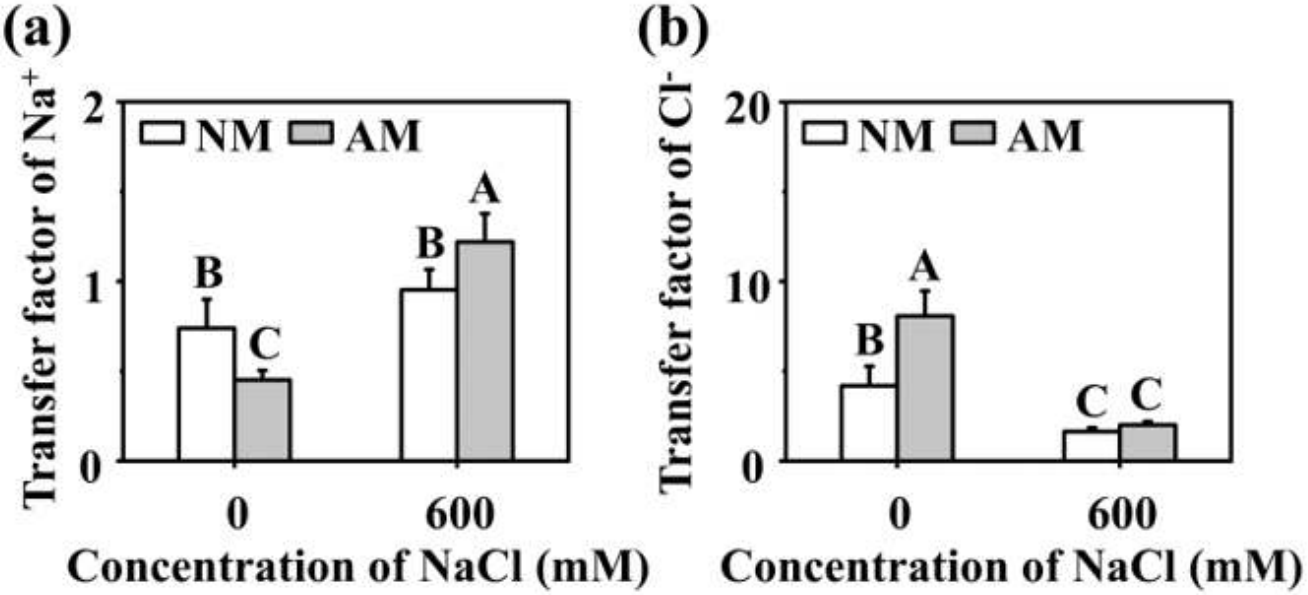

Figure 4

The transfer factor (TF) of Na^+ and Cl^- in *C. glauca* under different treatments. NM, nonmycorrhizal; AM, inoculated with *R. irregularis*; 0, no NaCl stress; 600, 600 mM NaCl stress. The data are the means ± standard errors (n = 3). Different capital letters indicate significant differences among the means by Tukey's test ($P < 0.05$).

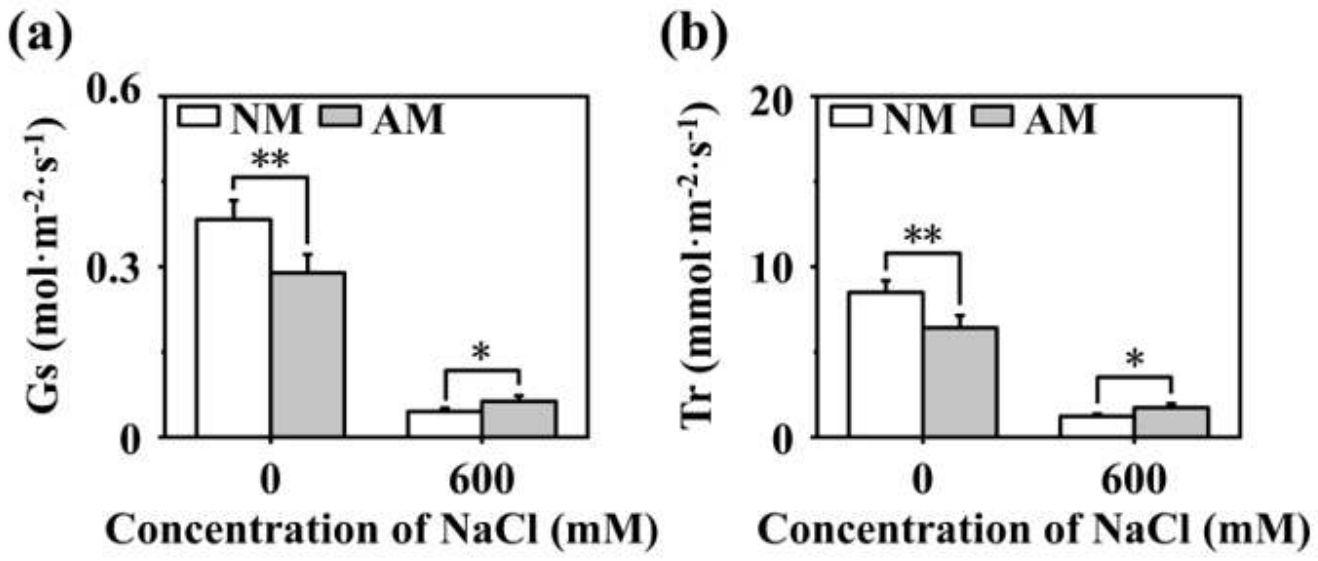

Figure 5

The stomatal conductance (Gs), and transpiration rate (Tr) in leaves of *C. glauca* in different treatments. NM, nonmycorrhizal; AM, inoculated with *R. irregularis*; 0, no NaCl stress; 600, 600 mM NaCl stress. The data are the means ± standard errors (n = 3). "*" indicates significant differences among the means by one-way ANOVA ($P < 0.05$), "**" indicates significant differences among the means by one-way ANOVA ($P < 0.01$).

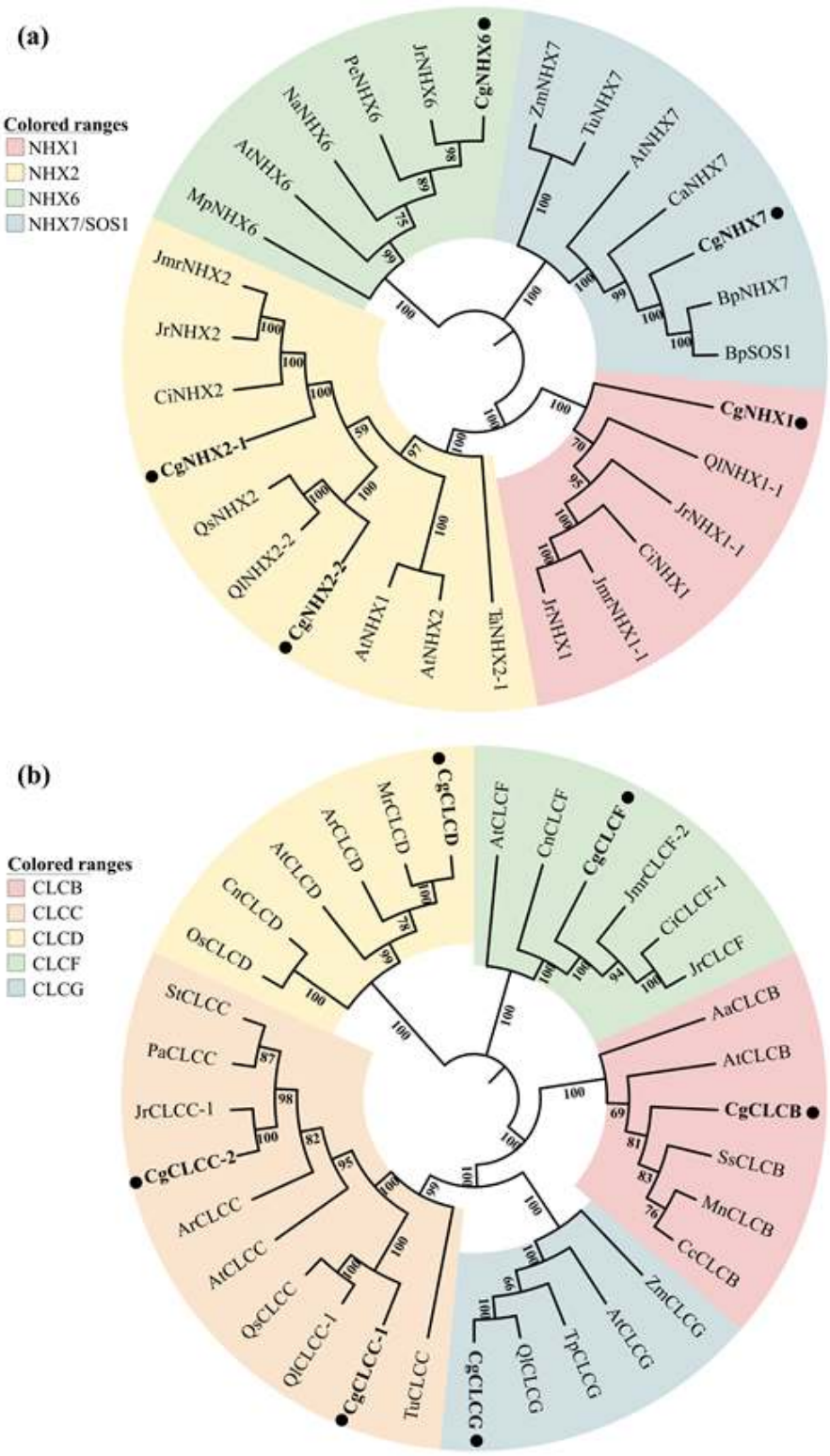

Figure 6

Phylogenetictree of NHX (A) and CLC (B) protein translated from cloned genes. The NCBI number of the cloned gene is as follows: ON246321 (*CgNHX1*), ON246322 (*CgNHX2-1*), ON246323 (*CgNHX2-2*), ON246324 (*CgNHX6*), ON246325 (*CgNHX7*), ON206670 (*CgCLCB*), ON246326 (*CgCLCC-1*), ON246327 (*CgCLCC-2*), ON246328 (*CgCLCD*), ON246329 (*CgCLCF*), ON246330 (*CgCLCG*). The sequences of *Actinidia rufa*, *Arabidopsis thaliana*, *Artemisia annua*, *Betula platyphylla*, *Carya illinoinensis*, *Citrus*

clementina, *Cocos nucifera*, *Cucurbita argyrosperma*, *Juglans microcarpa* × *Juglans regia*, *Juglans regia*, *Morella rubra*, *Morus notabilis*, *Mucuna pruriens*, *Nicotiana attenuate*, *Oryza sativa*, *Populus euphratica*, *Prosopis alba*, *Quercus lobata*, *Quercus suber*, *Senna tora*, *Spatholobus suberectus*, *Trifolium pratense*, *Triticum aestivum*, *Triticum Urartu*, *Zea mays* used for phylogenetic tree building were all from NCBI.

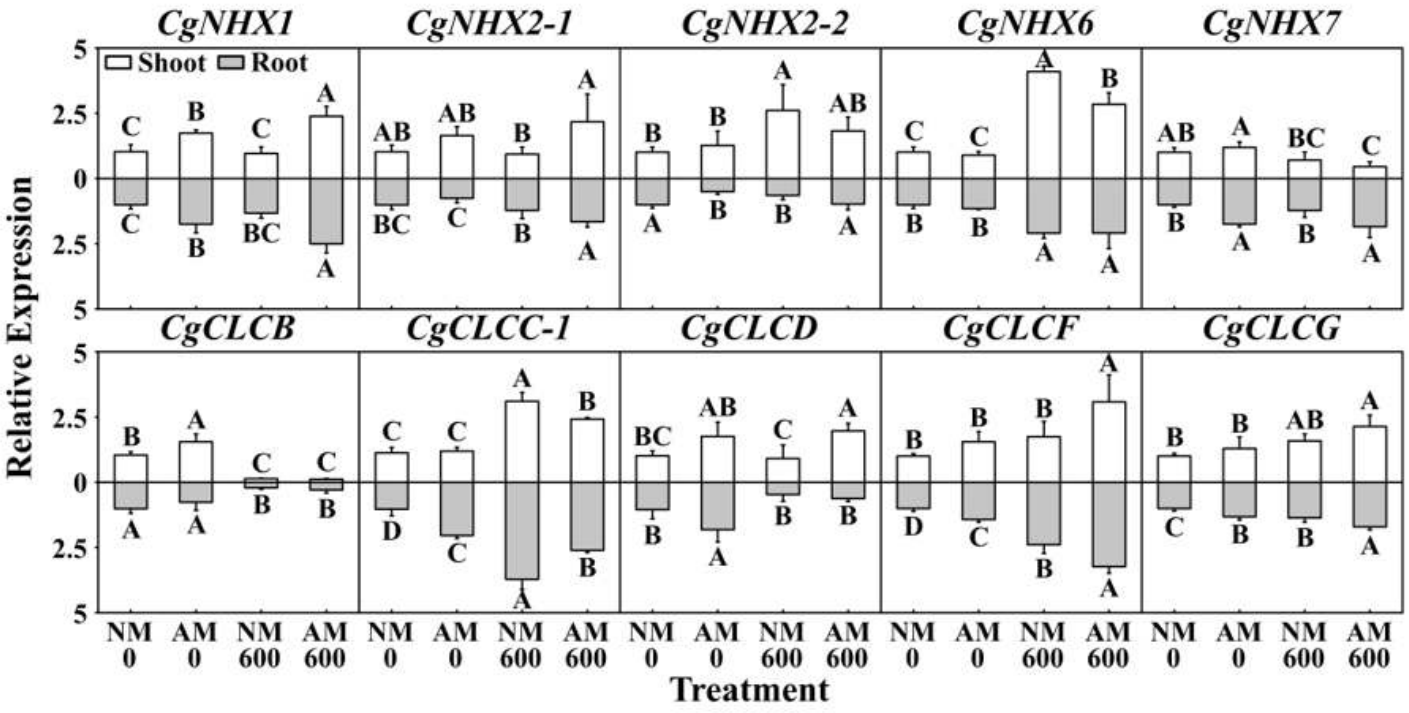

Figure 7

Effects of NaCl stress and *R. irregularis* inoculation on the expression of *CgNHXs* and *CgCLCs* in shoots and roots. NM, nonmycorrhizal; AM, inoculated with *R. irregularis*; 0, no NaCl stress; 600, 600 mM NaCl stress. Different letters indicate significant differences among the means by Tukey's test ($P < 0.05$).

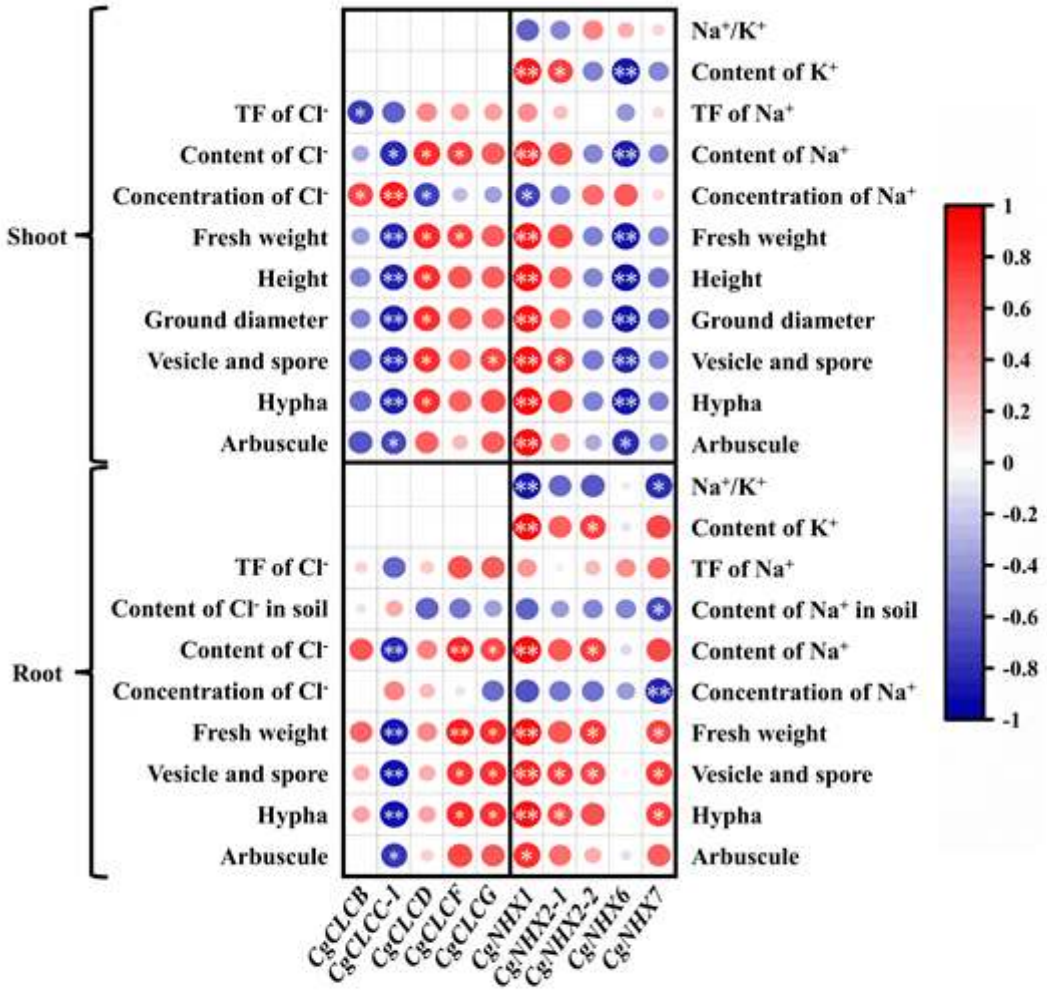

Figure 8

Correlation coefficients of gene expression and physiological indices of *C. glauca* under NaCl stress. "*" indicates a significant correlation coefficient at $P < 0.05$, "**" indicates a significant correlation coefficient at $P < 0.01$.

Supplementary Files

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