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EXPRESSION ANALYSIS OF GENES ENCODING SALT INDUCED TRANSPORT PROTEINS IN TWO CONTRASTING RICE CULTIVARS WITH DIFFERENT SALT STRESS TOLERANCE

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ABSTRACT

Soil salinization is a serious global problem that impedes the growth and development of numerous agricultural crops worldwide. Plants have evolved a diversity of adaptive mechanisms for coping with salt stress. Among the known mechanisms, the ability of plants to maintain intracellular ions and osmotic homeostasis via exclusion and compartmentalization of salt is highly correlated with high salt stress tolerance. Several transport proteins, such as high-affinity K^+ transporter 1 (HKT1), high affinity K^+/Na^+ transporter 10 (HAK10), salt overly sensitive 1 (SOS1), and sodium/hydrogen exchanger 1 (NHX1), have been identified to be associated with the exclusion and compartmentalization of salt. In this study, an investigation was conducted to evaluate the expression of genes encoding SOS1, HKT1, HAK10, and NHX1 transporters in the leaf and root tissues of two contrasting rice cultivars, salt tolerant DP and salt sensitive IR28, under salt stress of 150 mM NaCl by RT-qPCR approach. RT-qPCR data revealed that the expression of *HKT1*, *HAK10*, *SOS1*, and *NXH1* were upregulated at a higher level in the DP cultivar than in the IR28 cultivar in response to salt stress treatment. Our findings also suggest that the DP rice cultivar acquires a higher level of salt tolerance than the IR28 cultivar, at least a part due to a greater degree of Na^+ exclusion and compartmentalization mechanisms provided by HKT1, HAK10, SOS1, and NXH1 transporters.

Keywords: *Oryza sativa*, Doc Phung, IR28, salt stress, transport proteins, gene expression.

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INTRODUCTION

There are more than 100 major crops presently cultivatedworldwide (FAO, 2018; Shahid et al., 2018). Rice is one of the major food crops, providing food for more than 65% of the world's population. Asia is the world's largest rice-producing region, accounting for more than 90% of the world's production. In Vietnam, rice is mainly cultivated in delta areas, such as the Northern Delta and the Mekong River Delta. However, the preservation of rice cultivation areas and ensuring high crop yields are hampered by numerous abiotic stresses, especially salt stress.

Soil salinization is an ongoing and long-term process that is the result of excessive accumulation of dissolved salts (mainly Na^+ and Cl^- ions) in the soil. Soil salinization is caused by extreme climatic conditions (flood tides, drought), climate change (polar ice caps melting), and human farming practices (excess fertilizer use, poor irrigation, and drainage systems) (Hossain, 2019; Munns & Tester, 2008; Shahid et al., 2018). Saline soil has been reported to affect the sustainable agricultural development of over 100 countries across all continents (Hossain, 2019; Shahid et al., 2018). According to a report by the Food and Agriculture Organization of the United Nations (FAO) in 2018, Asia currently has the largest area of saline soil in the world (194.7 million hectares; ha), followed by America with 77 million ha, Africa with 53.5 million ha, Australia with 17.6 million ha, and Europe with 7.8 million ha (FAO, 2018; Shahid et al., 2018). Saline soils have incurred more than 12 billion USD in agricultural losses worldwide (Kamran et al., 2020).

Saline soils comprehensively affect the normal growth and development of rice plants at the physiological, biochemical, and molecular levels (Fogliatto et al., 2019; Neves et al., 2010; Zhang et al., 2018). At the physiological and biochemical levels, high salt concentrations in the cells of rice plants cause ionic imbalance and osmotic pressure, limiting their capacity to absorb water, nutrients and other essential mineral salts from the soil for normal plant growth and development (Isayenkov & Maathuis, 2019; Lotkowska et al., 2015). At the molecular level, Na^+ and Cl^- ions inhibit the expression of genes involved in carbon assimilation in rice (Sharwood et al., 2014; Sui et al., 2015). As a result, all salt-stressed rice plants exhibit stunted growth, fewer tillers, and decreased yield. Plants have developed a number of adaptive mechanisms to cope with salt stress (Munns & Tester, 2008). One of the most effective salt stress responses in plants is the exclusion and/or compartmentalization of salt ions in order to balance intracellular osmotic homeostasis and maintain plant growth and development (Ali et al., 2019; Muchate et al., 2016; Munns & Tester, 2008).

In this study, we aim to investigate the expression level of genes encoding SOS1, HKT1, HAK10, and NHX1 transport proteins in the leaf and root tissues of two rice cultivars with different salt stress tolerances including DP and IR28, under standard and salt stress (150 mM NaCl) conditions by RT-qPCR. Our findings provide a molecular-level understanding of the salt stress response mechanisms and will be beneficial for future generations of plants with a higher salt stress tolerance.

MATERIALS AND METHODS

Plant materials

The rice cultivars used in this study included salt-tolerant, Doc Phung (DP), and salt-intolerant, IR28 from the Faculty of Agriculture, Can Tho University (Farooq et al., 2021; Nguyen et al., 2023).

Fifty rice grains from each rice variety, IR28 and DP, were de-husked and surface sterilized by commercial javel bleach (the ratio of javel: sterile distilled water is 1:6, Javel, Vietnam) for 10 minutes, shaking at 100 rpm. The rice seeds were washed with sterile distilled water for 3–4 times. After surface sterilization, the rice seeds are air dried and germinated on 1/2 MS medium (2.2 g/L MS basal powder (Merck, USA), 20 g/L sucrose (Merck, USA), 7 g/L phytagel (Merck, USA)) at 16h/8h light/dark illumination, 70% humidity. After 6 days of cultivation, rice

plants were divided into two experimental batches. One batch was grown on MS medium (control), and one batch was grown on MS medium supplemented with 150 mM NaCl. All rice plants were cultivated under the same conditions as above for another 8 days.

The 14-day-old rice plants were used to evaluate the morphological characteristics of DP and IR28 rice cultivars under control and salt stress conditions, including plant height, root length, and fresh and dry weights. Statistical analysis was performed by comparing the data of the samples under stress (150 mM NaCl) and control conditions using the T-test method. Statistical significance was indicated by an asterisk (*) symbol with the *P* value ≤ 0.05. All experiments were conducted with at least three replications.

Extraction of RNA and synthesis of cDNA

Leaf and root samples were homogenized in liquid nitrogen by ceramic mortar and pestle. Approximately 100 mg of the sample was mixed with 1 mL of TRIzol® (Invitrogen, USA) in a 1.5 mL tube and incubated at room temperature for 10 minutes before being centrifuged at 13,000 rpm for 10 minutes at 4 ºC. The supernatants were transferred to fresh tubes and vigorously mixed with 0.2 mL chloroform. The sample tubes were incubated at room temperature for 3 minutes and centrifuged at 13,000 rpm for 10 minutes at 4 ºC. The supernatants were transferred to fresh tubes, mixed with 0.5 mL isopropanol, and incubated at -20 ºC overnight. The next day, sample tubes were centrifuged at 13000 rpm for 20 minutes at 4 ºC to obtain total RNA. RNA pellets were washed with 1 ml of 75% ethanol for 3 times and dissolved in nuclease-free water. The total RNA samples were treated with DNase I (2 U; NEB, Vietnam), incubated for 30 minutes at 37 ºC. Then, the total RNA samples were purified using 3M sodium acetate (NaOAc; Sigma Aldrich, USA) according to the formula of 1/10X (v/v) NaOAc, 4X ethanol 100%, and incubated at -20 ºC for 1 hour. RNA samples were collected by centrifugation at 13,000 rpm for 20 minutes at 4 ºC. RNA pellets were washed with 1 mL of 75% ethanol for 3 times and dissolved in nuclease-free water. The concentration and quality of RNA samples were measured using a Nanodrop™ spectrophotometer (Thermo Fisher Scientific, USA).

One microgram (1.0 g) of total RNA was used as a template for cDNA synthesis. cDNA was synthesized using the NEB reverse transcriptase and an oligod$T_{(23)}$VN primer (NEB, Vietnam) according to the manufacturer's instructions. The synthesized cDNA samples were then stored at -20 ºC.

Reverse transcription-quantitative polymerase chain reaction analysis

Table 1. List of primers used for RT-qPCR analysis

Genes	Gene ID	Position	Primer (5'-3')	Amplicon
HKT1	*LOC4341971*	Exon 2	F: CTGAAGCCAAGCAACCCAGA R: TTCGATGGTGATGAGGCTGG	96 bp
HAK10	*LOC4341573*	Exon 3	F: GTGGGGTTCCTTTTTGCACC R: CGCTCGATAGACATTGGGCT	102 bp
NHX1	*LOC4337811*	Exon 1	F: GGCCATCTCGCTTGAATCTG R: CTCCCAAATCCAGCCCCATC	82 bp
SOS1	*LOC4352928*	Exon 22	F: ATCAGGTGGAGGCTAGAGCA R: TGACGCACTCCTTTGCAGAT	83 bp
ACT	*LOC4333919*	Exon 2	F: CAGCCACACTGTCCCCATCTA R: AGCAAGGTCGAGACGAAGGA	67 bp

Reverse transcription-quantitative polymerase chain reaction **(**RT-qPCR**)** analysis was performed on a Rotor-Gene® Q machine (Qiagen, USA) using the NEB qPCR master mix. Each reaction (10 µL) contained 1X qPCR master mix, 1.0 mM of each primer and

50 ng of cDNA sample. The amplification cycle was set up as follows: 1 cycle of 95 ºC for 10 minutes, followed by 40 cycles of 95 ºC for 15 seconds and 60 ºC for 40 seconds. *Actin* was used as a reference gene, and the primers used for RT-qPCR analysis are listed in Table 1. RT-qPCR analysis was performed with at least 3 replicates. The RT-qPCR data were analyzed using Livak's $\Delta\Delta^{Ct}$ method (Livak & Schmittgen, 2001). The statistical significance was calculated by the T-test method.

RESULTS AND DISCUSSION

Evaluation of morphological parameters of two rice varieties under salt stress conditions

Salt stress of 150 mM NaCl had a negative impact on the growth and development of both rice cultivars (Figs. 1a, b). However, a stronger impact was found on the salt sensitive IR28 rice cultivar in comparison to the salt tolerant DP rice cultivar.

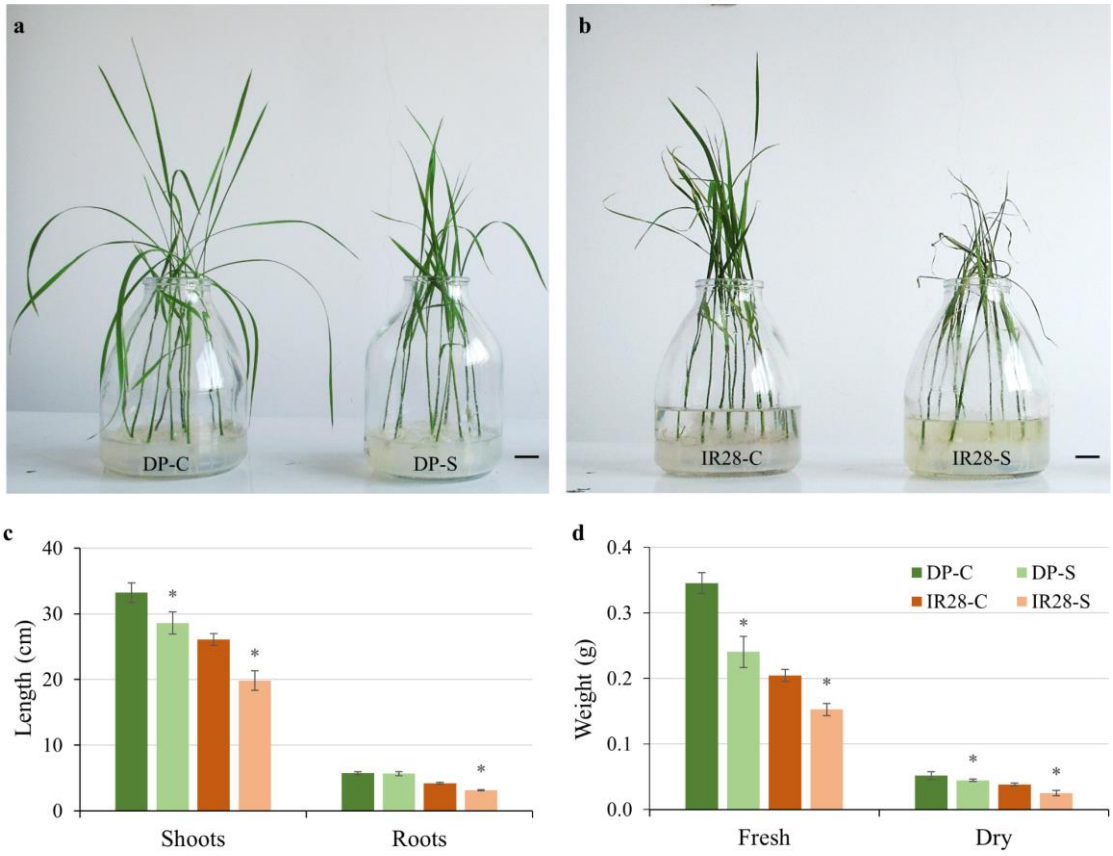

Figure 1. Morphological characteristics of DP and IR28 rice cultivars under control (-C) and salt stress conditions (-S). DP (a) and IR28 (b) rice cultivars cultured in MS and MS media supplemented with 150 mM NaCl. Length of shoots and roots (c), and fresh and dry weights (d) of the two rice varieties under two experimental conditions. Bar = 5 cm. Statistical differences between corresponding samples under control and salt-stress conditions were calculated by the T-test method with the *p-value ≤ 0.05

In particular, when comparing the shoot and root lengths of the two rice varieties, the DP rice cultivar showed a shoot length of 33.2 ± 1.5 cm and a root length of 5.7 ± 0.3 cm in the control condition. Under salt stress, the shoot and root lengths were slightly shorter, reduced to 28.6 ± 1.7 cm and 5.6 ± 0.3 cm, respectively (Fig. 1c). In contrast, there was a

significant decrease in the length of the shoot and root of the IR28 rice cultivar under control and salt stress conditions. Shoot and root lengths decreased from 26.1 ± 0.9 cm and 4.2 ± 0.2 cm in the control condition to 19.8 ± 1.5 cm and 3.1 ± 0.1 cm under salt stress, respectively (Fig. 1c).

Salt also directly affects the fresh and dry weights of the two rice cultivars (Fig. 1d). The results of whole plant weight measurements of the two rice cultivars were closely related to the length of the plants. The fresh and dry weights were 0.35 ± 0.016 g and 0.051 ± 0.024 g in DP-C; 0.24 ± 0.002 g and 0.045 ± 0.002 g in DP-S; 0.2 ± 0.01 g and 0.038 ± 0.002 g in IR28-C, and; 0.15 ± 0.01 g and 0.025 ± 0.004 g in IR28-S, respectively. Together, these results strongly confirmed that DP is a salt tolerant cultivar and IR28 is a salt sensitive cultivar (Fig. 1).

Assessment of the specificity of designed primers

Prior to performing RT-qPCR, the specificity of primers was checked by RT-PCR and melting curves. Figure 2a shows that all designed primers returned a clear band at the expected size. Particularly, the RT-PCR amplification bands of *HKT1*, *HAK10*, *NXH1*, *SOS1*, and *ACT* were found to be approximately 96, 102, 82, 83, and 67 bp, respectively. A similar result was also observed from the analysis of melting curves generated from RT-qPCR runs (Fig. 2b). Melting curve analysis revealed that a single peak was obtained from each set of primers. Taken together, these results proved that the primer pairs selected for use in this study are highly specific for the chosen target genes and highly reliable for RT-qPCR analysis.

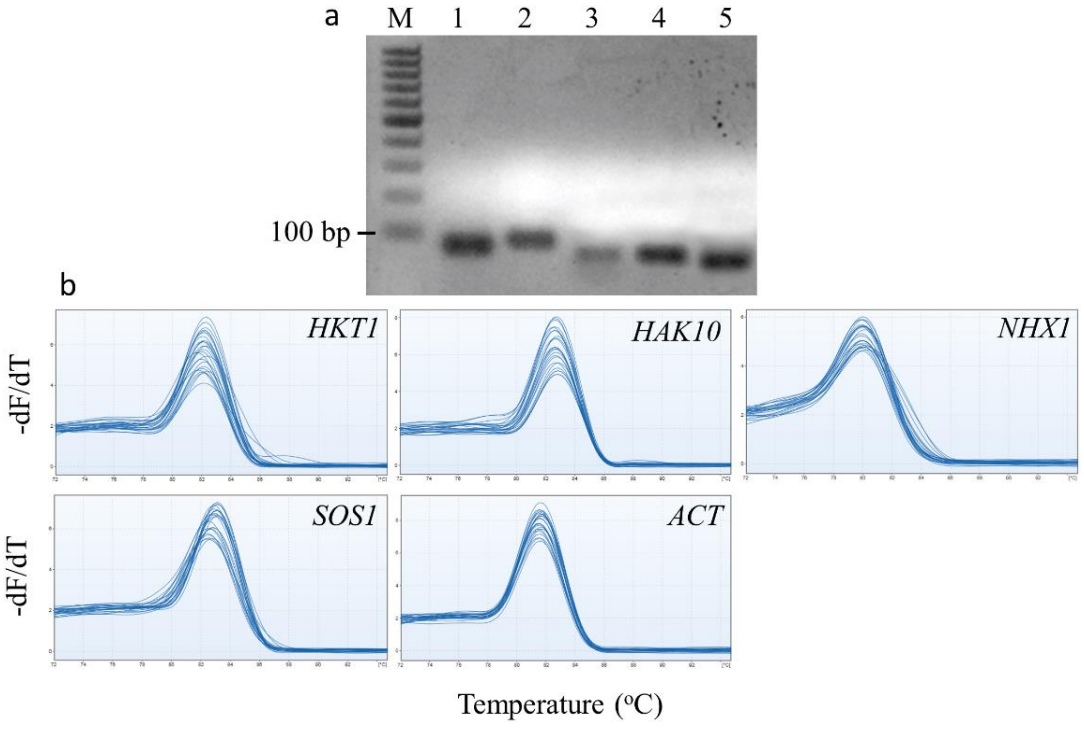

Figure 2. Specificity of each primer pair used for RT-qPCR analysis. a. Agarose gel analysis of RT-PCR generated amplicons for each of the target genes. M, marker; 1, *HKT1*; 2, *HAK10*; 3, *NXH1*; 4, *SOS1*; 5, *ACT*. b. Melt curve analysis of the five primer pairs across shoot and root tissues of DP and IR28 under standard and salt stress conditions. The -(dF/dT) value represents the raw fluorescence (F) versus temperature (T) values

Expression of transport proteins in DP and IR28 rice cultivars under salt stress

Expression of genes encoding transport proteins, HKT1, HAK10, SOS1, and NHX1, was investigated across leaf and root tissues of 14-day-old DP and IR28 rice cultivars under two experimental conditions (control and salt stress of 150 mM NaCl). At a glance, it is clear that all four genes accessed were significantly upregulated in the DP rice cultivar in response to salt stress. Under salt stress, the expression of *HKT1* and *NHX1* increased in both leaf (5.0- and 4.0-folds, respectively) and root (5.8- and 7.0-folds, respectively) tissues of DP rice cultivar (Figs. 3a, d). *HAK10* has been found to only be significantly expressed in leaf tissues, increasing by 5.7-folds, while *SOS1* showed approximately 9.5-fold upregulation in root tissues in response to salt stress (Figs. 3b, c). Our findings were consistent with the expression of *HKT1, HAK10, SOS1,* and *NHX1* genes in the salt tolerant DP rice cultivar. Previously studies reported that *HKT1, HAK10, SOS1,* and *NHX1* transcriptions were upregulated in the root and shoot of salt tolerant Pokkali and O. sativa japonica (cv. Nipponbare & Matsume) under salt stress (Farooq et al., 2021; Wang et al., 2012; Wang et al., 2022). Particularly, the expression of *HKT1, SOS1,* and *NHX1* was upregulated up to 17.0-, 4.0-, and 7.0-fold in shoot tissues under 150 mM NaCl conditions, respectively. In the salt-stressed root tissues, the expression of *HKT1* and *NHX1* was increased by 5.0- and 4.5-fold, respectively (Farooq et al., 2021). The *HAK*s have been found to significantly increase by 8.0- to 11.0-fold in root tissues in response to salt stress (Wang et al., 2022). They are genes encoding transport proteins that function as Na^+/H^+ exchangers and antiporters that are activated under salt stress. The upregulation in the expression of these genes is believed to confer salt stress on rice plants via regulation of Na^+ accumulation and maintaining intracellular ion homeostasis (Farooq et al., 2021; Wang et al., 2012; Wang et al., 2022). Similar findings were also reported for other plant species, including *Arabidopsis*, tomato, mung bean, and potato (Jaime-Pérez et al., 2017; Kumar et al., 2017; Rolly et al., 2020; Wang et al., 2019). In contrast, an opposite trend has been found in the expression of the four genes accessed in the IR28 rice cultivar in response to salt stress. Particularly, expression of *HAK10* and *SOS1* has been found to have no statistical differences across the leaf and root tissues of the IR28 rice cultivar in response to salt stress (Figs. 3b, c). *HKT1* and *NHX1* have been found to be only significantly expressed in leaf (1.8-fold) and root tissues (1.8-fold), respectively (Figs. 3a, d). The expression of these genes was either lowly active or lacking in leaf and root tissues of the IR28 rice cultivar under salt stress, therefore resulting in the failure of tolerance to salt stress (Farooq et al., 2021; Imran et al., 2020).

According to gene expression data, it is strongly suggested that the transport proteins encoded by *SOS1, HKT1, HAK10* and, *NHX1* are involved in the Na^+ ion detoxification process of DP rice cultivars via compartmentalization and exclusion pathways (Ali et al., 2019; Farooq et al., 2021). Under salt stress, Na^+ ions in the soil enter the roots via non-selective cation channels (NSCCs). In the compartmentalization pathway, the incoming Na^+ ions are transported to the xylem by the antiporter SOS1 and to the mature leaves by HAK10 and HKT1. In root and leaf cells, Na^+ ions are stored in the large central vacuoles by the tonoplast localized antiporter NHX1 to maintain intracellular ionic homeostasis. In the exclusion pathway, Na^+ ions from the leaf and shoot cells are translocated back to the roots across xylem vessels via HKT1 before they are extruded to the soil by the antiporter SOS1, thereby restricting the effects of Na^+ ions on photosynthetic tissues.

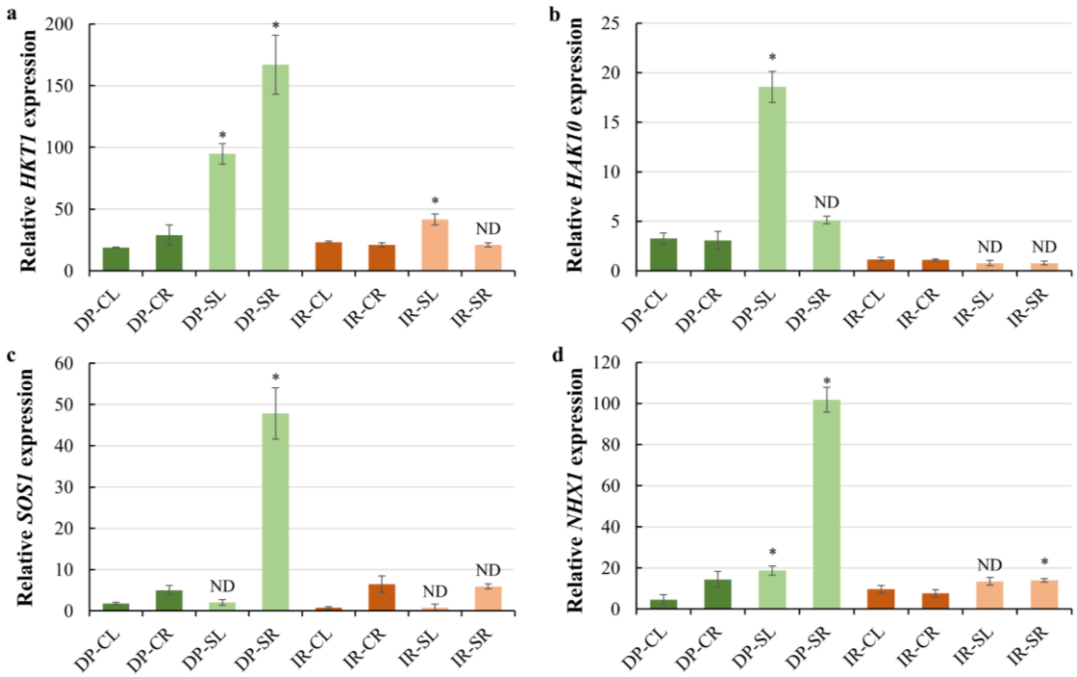

Figure 3. Expression analysis of genes encoding transport proteins, (a) high-affinity K^+ transporter 1 (HKT1), (b) high affinity K^+/Na^+ transporter 10 (HAK10), (c) salt overly sensitive 1 (SOS1), and (d) sodium/hydrogen exchanger 1 (NHX1). Statistical differences between corresponding samples under control and salt-stress conditions were calculated by the T-test method with the *p-value ≤ 0.05. ND: No statistical difference

CONCLUSION

In this study, we have investigated the expression of genes encoding four transport proteins, HKT1, HAK10, SOS1, and NHX1, in leaf and root tissues of two contrasting rice cultivars with different salt stress tolerances. Our findings reveal that the expression of these genes is highly upregulated in the DP cultivar while it is poorly expressed in the IR28 cultivar, suggesting that HKT1, HAK10, SOS1, and NHX1 transporters are involved in the adaptive mechanisms of the DP cultivar in response to salt stress.

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