CHILEAN JOURNAL of AGRICULTURAL RESEARCH

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RESEARCH ARTICLE

Na^+ exclusion and selective transport of K^+ over Na^+ provided salt-adaptive mechanism in introgression lines of rice 'RD6'

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Received: 26 April 2024; Accepted: 24 July 2024, doi:10.4067/S0718-58392024000600782

ABSTRACT

Salinity stress immensely inhibits rice (*Oryza sativa* L.) growth, development, and productivity. Hence, two rice introgression lines, Morkho60-2 and BC_4F_4 132-12-61, were previously developed from 'RD6' rice via marker-assisted backcrossing and their adaptive salt-tolerant mechanisms were characterized in this study. Their seedling physiological and transcriptional responses to salinity stress were observed in comparison to the parental 'RD6' and 'Pokkali'. The salt stress responses were assessed under 150 mM NaCl treatment for 9 d. Interestingly, like 'Pokkali', salinity did not affect the growth parameters of the new rice genotypes. 'Pokkali', Morkho60-2, and BC_4F_4 132-12-61 also showed lower shoot Na^+ content (0.91-1.05 *vs*. 2.83 mg g^{-1} DW), and higher selective transport of K^+ over Na^+ (i.e., ST value of 6.57-10.98 *vs*. 2.65) than RD6 genotype under salinity stress. Accordingly, the partial least squares-discriminant analysis of all physiological parameters suggested shoot Na^+ accumulation and ST value as the key discriminating parameters between 'RD6' and the new genotypes. Transcriptional responses of Na^+ homeostasis-related genes further supported the findings. In comparison to the salt susceptible 'RD6' rice, Na^+ vacuolar compartmentalization was suggested in Morkho60-2 roots due to the higher abundance of *OsNHX1* (i.e., relative expression of 2.65 *vs*. 1.59) and *OsNHX2* (2.42 *vs*. 1.29) transcripts. Morkho60-2 also displayed a significant upregulation of root *OsSOS1* (2.02 *vs*. 1.18), which may contribute to root Na^+ exclusion. Therefore, we suggest that selective transport of K^+ over Na^+ and Na^+ exclusion enhanced ion homeostasis in the newly improved rice genotypes, allowing the seedlings to adapt to the saline condition.

Key words: Na^+ exclusion, *Oryza sativa*, rice, salinity stress, salt tolerance, selective ion transport.

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple crop that is enriched with fiber, energy, minerals, and vitamins (Sen et al., 2020) and is thus consumed worldwide. However, rice is the most susceptible cereal crop to saline soil especially at its seedling stage (Munns and Tester, 2008; Quan et al., 2018). In particular, soil with electrical conductivity (EC) of 4 dS m^{-1} or more, which is sufficient to inhibit rice growth and yield production, is deemed saline soil (Munns and Tester, 2008). The total area of salt-affected soils worldwide equals 17 million km^2 (Negacz et al., 2022). Nonetheless, The major areas of rice cultivation are located in Asia including China, India, Pakistan, Thailand, Indonesia, Myanmar, and Japan, which are facing concerns pertaining to soil salinization, especially in the irrigated zones (Theerawitaya et al., 2020). Therefore, rice growth, development, and productivity are threatened due to both its salt-susceptibility and the wide spreading of soil salinization.

Salinity exposure leads to salt stress symptoms in developing plants. Generally, the early stage of salinity exposure includes the osmotic stress followed by the ionic stress (Munns and Tester, 2008; Hasegawa, 2013).

The high concentration of salt in the root zone lowers a water potential, which, in turn, reduces the water uptake and intracellular turgor pressure, resulting in cell expansion reduction (Hasegawa, 2013). The uptake of salt by root cells is primarily the inlet of sodium ion (Na^+) and chloride ion (Cl^-) (Solis et al., 2020; Theerawitaya et al., 2021). This Na^+ accumulation destabilizes the membrane and negatively affects normal cell functions, molecular responses, cellular metabolism, and nutrient balance (Hasegawa, 2013). The Na^+ homeostasis is a crucial process in the salinity adaptation of the rice plants to prevent the toxicity from ionic imbalance (Solis et al., 2020; Theerawitaya et al., 2021). To restrict cellular Na^+ level, rice plants typically compartmentalize Na^+ to vacuole and exclude Na^+ back to the soil (Parihar et al., 2015). These processes involve the function of several protein transporters. The high affinity K^+ transporter (HKT) is a plasma membrane transporter, which is classified into class I and class II subfamilies (Kobayashi et al., 2017). OsHKT1;5 is a class I HKT, which exhibits Na^+-selective transport with poor K^+ permeability (Kobayashi et al., 2017; Theerawitaya et al., 2021). It functions by retrieving Na^+ from the xylem to prevent the upward transportation, uploading Na^+ in the leaf sheath, and excluding Na^+ in the phloem to keep Na^+ in older leaves (Ren et al., 2005; Chakraborty et al., 2020; Theerawitaya et al., 2021). In contrary, OsHKT2;1, a class II HKT, plays the role of a Na^+ transporter that also exhibits the co-transport of K^+-Na^+, and is associated with shoot Na^+ accumulation in several rice genotypes (Kobayashi et al., 2017; Hartley et al., 2020). Moreover, the Na^+ compartmentalization involves the tonoplast antiporter or Na^+-H^+ antiporter (NHX). Five homologous genes (i.e., *OsNHX1*, *OsNHX2*, *OsNHX3*, *OsNHX4*, and *OsNHX5*) have been identified in rice (Fukuda et al., 2011). The NHX antiporter maintains the cytoplasmic Na^+ concentration and enhances the osmotic adjustment and water absorption by sequestration of excess Na^+ into the vacuole (Fukuda et al., 2011). Its function in reducing cytoplasmic Na^+ concentration is hence a key mechanism of tissue tolerance (Munns et al., 2016). In addition, the mechanism of Na^+ exclusion to extracellular space is also associated with the salt overly sensitive (SOS) signaling pathway (Ji et al., 2013; Chakraborty et al., 2020). The presence of salt induces the Ca^{2+} signal by activating the SOS2-SOS2 complex that further triggers SOS1, a plasma membrane Na^+-H^+ antiporter (Munns and Tester, 2008). Furthermore, the previous study reported that OsSOS1 was associated with the Na^+ loading to xylem sap and long-distance transportation from root to shoot tissue (Shi et al., 2002).

The 'RD6' rice is the most cultivated glutinous rice in Thailand due to its softness and pleasant aroma. Despite being one of the most economically important cultivars, it is both susceptible to blast disease and is hypersensitive to salt (Wongsaprom et al., 2010; Thanasilungura et al., 2020). To tackle its salt susceptibility, Thanasilungura et al. (2020) introduced a new 'RD6' rice line with improved salt-tolerant characteristics. 'Pokkali', a standard salt-tolerant genotype, was used as a donor of salt-tolerance quantitative trait loci (QTL) (*Saltol* QTL) located on rice chromosome 1. The *Saltol* QTL was previously shown to account for low Na^+ absorption, high K^+ absorption, and low Na^+/K^+ ratio in rice shoot tissue, which promoted seedling stage salinity tolerance (Thomson et al., 2010; Waziri et al., 2016). The *Saltol* QTL was integrated into the 'RD6' genome via marker-assisted backcrossing (MAB). The multiple backcrosses with 'RD6' leads to the generation of BC_4F_4 lines, which stably carry the *Saltol* QTL (Thanasilungura et al., 2020). The Morkho60-2 and BC_4F_4 132-12-61 were selected from the breeding program due to the presence of *Saltol* QTL and salinity-tolerant characteristics, while also maintaining the desirable agronomic traits of the original 'RD6' rice genotype.

Although the capability of these newly improved 'RD6' lines to tolerate salinity stress is determined, their adaptive mechanisms to salinity stress have not been investigated. In this study, we comprehensively compared the physiological and molecular responses (i.e., *OsHKTs*, *OsNHXs*, and *OsSOS* gene expression) of Morkho60-2 and BC_4F_4 132-12-61 seedlings to the original 'RD6' and 'Pokkali' under salinity stress. Our hypothesis is that the introgressed *Saltol* QTL and their corresponding molecular and physiological mechanisms allow the improved rice lines to better adapt to salinity stress.

MATERIAL AND METHODS

Plant materials and salinity stress treatment

Morkho60-2 and BC_4F_4 132-12-61 rice (*Oryza sativa* L.) lines were developed through marker-assisted backcrossing. The two genotypes were received from the crossing between the RGD07005-12-165-1 ('RD6' near-isogenic line carrying four blast resistance QTL) and 'Pokkali' (*SalTol* QTL donor). The first filial generation (F_1) were backcrossed with the RGD07005-12-165-1 until received the Morkho60-2 and BC_4F_4 132-12-61 genotypes, which displayed the agronomic trait of 'RD6' and the salt tolerance characters of 'Pokkali' (Thanasilungura et al., 2020). Four studied rice genotypes,

including Pokkali, RD6, Morkho60-2, and BC_4F_4 132-12-61, were obtained from the Rice Breeding Program at the Department of Agronomy, Faculty of Agronomy, Khon Kaen University, Thailand. Rice seeds were soaked in 0.6% (v/v) sodium hypochlorite for 10 min and then rinsed with tap water. Seeds were germinated in petri dish with moist paper towel for 8 d and seedlings were then transplanted into 12.7 cm plastic pot containing potting soil (Din-Wieang-Kow, Khon Kaen, Thailand). Each of the pots were placed in bowl of water with normal tap water (EC0-1, pH7) and placed under natural illumination, temperature, and humidity in the greenhouse of Department of Biology, Khon Kaen University, Thailand. The greenhouse humidity and temperature were monitored daily using the thermo-hygrometer (model 11307, DeltaTrak, Pleasanton, California, USA). The pots were placed in completely randomized design with four biological replicates of each genotype. The salinity stress treatment was initiated at 21 d post transplanting with 150 mM NaCl solution application to reach the electrical conductivity (EC) level of 15 dS m^{-1} (EC15 group). The control group was treated with normal tap water and the EC value was measured and maintained at 0 dS m^{-1} (EC0 group). The EC values of all pots were monitored and maintained throughout the experimental period.

Plant growth measurement

After 9 d salinity treatment, seedlings were harvested and measured for length and fresh weight (FW) of both shoot and root tissues. The shoot and root tissue were then oven-dried (Binder, Redline, Germany) at 80 °C for 3 d and dry weight (DW) was determined.

Relative water content

The leaf tissue was harvested at 0, 3, 6, and 9 d and the relative water content (RWC) was measured immediately. The third leaf from the soil level of about 0.1 g was cut into 1 cm long pieces and the FW was recorded. They were then put in a petri dish containing 5 mL deionized water for 12 h in the dark and the turgid weight (TW) was recorded. The leaf sample was then oven-dried (Binder, Redline, Germany) at 80 °C for 3 d and the DW was recorded. The leaf RWC was calculated using the following equation:

$$\text{RWC}\ (\%) = \frac{(\text{FW} - \text{DW})}{(\text{TW} - \text{DW})} \times 100$$

Photosynthetic pigment content

Photosynthetic pigments were analyzed on the leaf tissue according to the method of Wellburn (1994). The fresh second leaf from the soil level of about 30 mg was harvested at 0, 3, 6, and 9 d salinity treatment. The pigments were extracted with 5 mL 80% (v/v) acetone for 72 h in the dark at room temperature. The absorbance of chlorophyll *a*, chlorophyll *b*, and carotenoid was recorded at 663, 646, and 470 nm respectively using a spectrophotometer (V-530, Jasco, Tokyo, Japan). The pigment content was calculated using the following equations:

$$\text{Chlorophyll } a\ (\text{Chl a})(\text{mg g}^{-1}\ \text{FW}) = \frac{(12.21\text{A}_{663} - 2.81\text{A}_{646}) \times \text{V}}{(1000 \times \text{FW})}$$

$$\text{Chlorophyll } b\ (\text{Chl b})(\text{mg g}^{-1}\ \text{FW}) = \frac{(20.13\text{A}_{646} - 5.03\text{A}_{663}) \times \text{V}}{(1000 \times \text{FW})}$$

$$\text{Total chlorohyll content}\ (\text{mg g}^{-1}\ \text{FW}) = \text{Chl a} + \text{Chl b}$$

$$\text{Carotenoids content}\ (\text{mg g}^{-1}\ \text{FW}) = \frac{\frac{(1000\text{A}_{470} - 3.27\text{Chl a} - 104\text{Chl b})}{198} \times \text{V}}{(1000 \times \text{FW})}$$

where W is weight of leaf sample (g) and V is total volume (mL).

Electrolyte leakage

Electrolyte leakage (EL) was measured on the leaf tissue based on the method of Baninasab and Ghobadi (2011). The fresh first leaf from the soil level was harvested at 0, 3, 6, and 9 d salinity treatment. The leaf was cut into six pieces and placed in a test tube with 10 mL deionized water for 24 h in the dark at room temperature. The EC1 was recorded using an electrical conductivity meter (EC20, Apera Instruments, Columbus, Ohio, USA). The test tube was then placed in a water bath at 100 °C for 20 min. EC2 was then recorded, and the EL was calculated using the following equation:

$$\text{EL}\ (\%) = \frac{\text{EC1}}{\text{EC2}} \times 100$$

Na^+ and K^+ content

The above ground and root tissues of each genotype were harvested after 9 d salinity treatment and oven-dried (Redline, Binder, Tuttlingen, Germany) at 80 °C for 3 d. The dried tissues were ground into powder. About 0.5 g sample powder were digested using triacid solvent containing $HNO_3/HClO_4/H_2SO_4$ (5/2/1, v/v/v). The concentration of Na^+ and K^+ in the sample was determined compared to the standard curve by flame photometer (PFP7, Jenway, UK).

Selective transport (ST) of K^+ over Na^+ value

The Na^+ and K^+ contents in the shoot and root tissues of the rice plant were used to calculate the selective transport of K^+ over Na^+ value (ST value) by using the following equation:

$$\text{ST value} = \frac{K^+/Na^+ \text{ in shoot tissue}}{K^+/Na^+ \text{ in root tissue}}$$

where the higher ST value indicates the higher selective transport of K^+ from the root over Na^+ to the upper part of the plant (Wang et al., 2005).

Total RNA extraction and qRT-PCR analysis

The 9-d salt treated root and leaf tissues were harvested for total RNA extraction following the procedures of Rneasy Plant Mini Kit (QIAGEN, Hilden, Germany). The 0.5 µg total RNA was then converted to cDNA by using ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO, Osaka, Japan). The quantitative real-time RT-PCR analysis was performed using THUNDERBIRD SYBR qPCR mix (TOYOBO) with LightCycler 480 II system (Roche, Basel, Switzerland). The relative expression of Na^+ homeostasis-related genes was calculated with the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with three biological replicates and three technical replicates of each genotype and treatment. The Na^+ homeostasis-related genes include *OsHKT1;5* (GenBank accession nr XM_015776467), *OsHKT2;1* (XM_015785756), *OsNHX1* (AB021878), *OsNHX2* (XM_015762552), *OsNHX3* (AB531433), and *OsSOS1* (AY785147). The internal control genes were *OsUBC* (XM_015769893) and *OseEF-1α* (XM_015774317). The primer sequences are provided in Table 1.

Table 1. The sequences of PCR primers used in the study.

Genes (accession nr)	Protein names		Primer sequences (5'-3')
OsNHX1 (AB021878)	Na^+/H^+ exchanger 1	F	CATTGATCAGGCTGCTGCTA
		R	CTTGCATGCTTGTCAGGAGA
OsNHX2 (XM_015762552)	Na^+/H^+ exchanger 2	F	TTGGATATGGAGAAGTGGAA
		R	TAGTGGGAAAACAAAAGCAG
OsNHX3 (AB531433)	Na^+/H^+ exchanger 3	F	ACCAAGACGAAACACCCCTAC
		R	AGCAACCCAGCAACTACTCC
OsHKT1;5 (XM_015776467)	High-affinity K^+ transporter 1;5	F	CCTGCCACCTTACACCACTT
		R	AGCTTCTGCCATATGCTGCT
OsHKT2;1 (XM_015785756)	High-affinity K^+ transporter 2;1	F	CTTTCCAGCCTCATCACCAT
		R	TGACCTTGTCCCCTGAAAAC
OsSOS1 (AY785147)	Salt overly sensitive 1	F	TCTGCAAAGGAGTGCGTCAT
		R	TCATGCTCCCGTACATGCTC
OsUBC (XM_015769893)	Ubiquitin-conjugating enzyme E2	F	CTGAGCAATACCCTCTCCTT
		R	TCTCACCTGTCTTGAAATGC
OsEF-1α (XM_015774317)	Eukaryotic elongation factor 1-alpha	F	GGAGAAGACGCACATCAACA
		R	GGCTTCCTTCTCGAACCTCT

Statistical analysis

The one-way ANOVA was used to evaluate the difference between treatments and genotypes ($p < 0.05$). Duncan's multiple range test was performed when significant differences were found. The univariate analysis was calculated using SPSS Statistics v.26 (IBM, Armonk, New York, USA). For multivariate analysis, prior to the partial least squares-discriminant analysis (PLS-DA), the data was processed by the generalized log10 transformation and mean centering. The discriminative variables that attributed to observed variations between different genotypes were identified by

the PLS-DA. The data processing and PLS-DA from the ropls package (Thévenot et al., 2015) were performed as implemented in the Metabox 2.0 (https://github.com/kwanjeeraw/metabox2). Both score and loading outputs from the PLS-DA were presented by a biplot, in which the sample group separation and the associated variables can simultaneously be visualized. A variable with an absolute loading> 0.3 was considered as a significant variable that contributed to differentiate the sample groups on a particular principal component (PC) (Berrier et al., 2020).

RESULTS

Effect of salinity stress on seedling growth performance

The growth performance of all rice genotypes was observed after 9 d salinity stress (Figure 1). Morkho60-2, and BC_4F_4 132-12-61 showed nonsignificant difference in length, fresh weight, and dry weight of both shoot and root tissues under salt stress when compared to the control group (Figures 1a-1f). Their growth responses displayed a similar pattern to those observed from Pokkali, a salt-tolerant genotype (Figures 1a-1f). However, the RD6 genotype showed a significant reduction in length and fresh weight of the shoot tissue, and in dry weight of both shoot and root tissues (Figures 1a, 1c, 1e, and 1f).

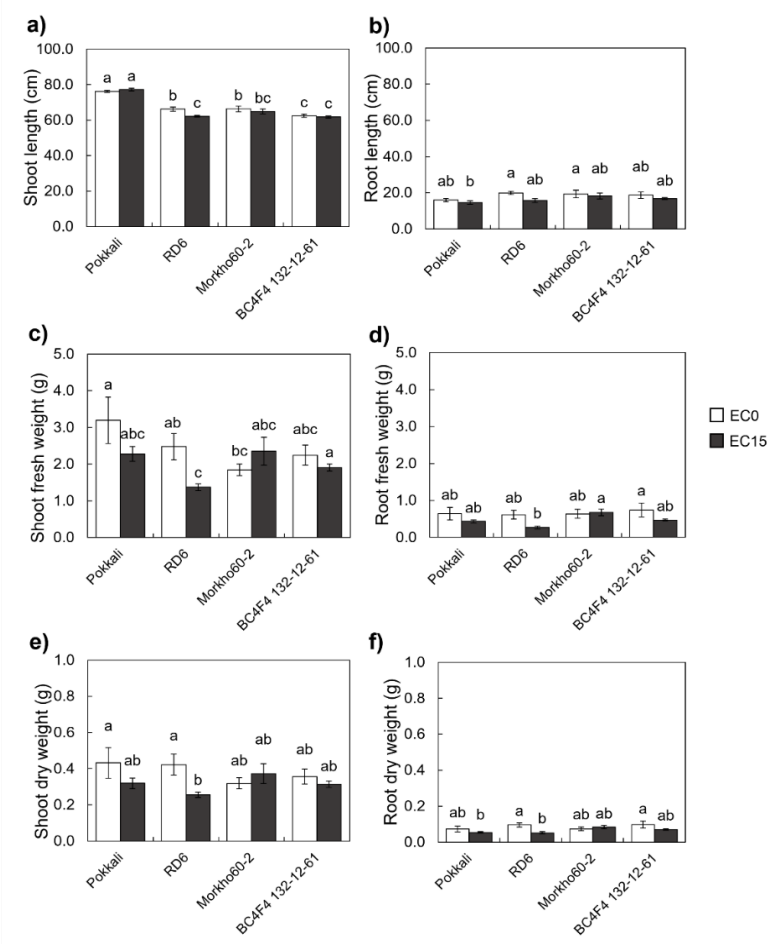

Figure 1. Growth performance of the four rice genotypes under salinity stress. Shoot length (a), root length (b), shoot fresh weight (c), root fresh weight (d), shoot dry weight (e), and root dry weight (f) were measured after 9 d of 150 mM NaCl treatment. Data are shown as means ± SEM of four biological replicates. The different letters display significant difference by Duncan's multiple range test ($p < 0.05$).

Effect of salinity stress on leaf water status and electrolyte leakage

The relative water content (RWC) was measured to observe the leaf water status of the seedlings under salinity stress (Table 2). Over 9 d stress treatment, 'Pokkali' and BC_4F_4 132-12-61 showed an increase in leaf RWC in both treatment and control groups (Table 2). Conversely, nonsignificant changes in RWC were observed in both groups of 'RD6' (Table 2). Interestingly, we only observed a distinct increase in RWC in the treatment group of Morkho60-2 (Table 2). This suggested that salinity stress may enhance the leaf water status in the newly developed Morkho60-2 seedlings. Moreover, the leaf electrolyte leakage (EL) was assessed to investigate the leaf cell membrane stability under salinity stress. The leaf EL of 'RD6' and Morkho60-2 genotypes were significantly affected by salinity stress at 6 d stress exposure compared to the control group (Table 2).

Table 2. Leaf relative water content and electrolyte leakage after salinity stress treatment. Data are shown as means ± SEM of four biological replicates. Different letters display significant difference between the timepoint of the same treatment by Duncan's multiple range test ($p < 0.05$); *significant difference between treatment of the same timepoint by Student's *t*-test ($p < 0.05$).

Genotypes	Treatment	Relative water content				Electrolyte leakage			
		0 d	3 d	6 d	9 d	0 d	3 d	6 d	9 d
		%				%			
Pokkali	EC0	73.76 ± 0.94^{c}	91.51 ± 0.54^{a}	90.44 ±0.81^{ab}	88.76 ± 0.83^{b}	7.62 ± 0.49^{a}	5.62 ± 0.13^{b}	4.83 ± 0.23^{b}	5.00 ± 0.23^{b}
	EC15	74.67 ± 3.23^{b}	90.55 ± 0.45^{a}	87.06 ± 2.49^{a}	89.77 ± 0.26^{a}	6.73 ± 0.33^{a}	5.76 ± 0.23^{b}	4.79 ± 0.31^{c}	5.53 ± 0.12^{bc}
RD6	EC0	84.80 ± 1.37^{a}	88.37 ± 0.69^{a}	84.74 ± 1.66^{a}	86.61 ± 0.37^{a}	10.66 ± 0.75^{a}	5.73 ± 0.44^{b}	6.07 ± 0.17^{b*}	6.69 ± 0.20^{b}
	EC15	85.09 ± 1.89^{a}	89.48 ± 1.13^{a}	88.11 ± 1.39^{a}	88.48 ± 1.18^{a}	10.03 ± 1.25^{a}	6.89 ± 0.57^{b}	6.91 ± 0.14^{b*}	6.61 ± 3.30^{b}
Morkho60-2	EC0	86.59 ± 1.67^{a}	89.25 ± 1.08^{a}	85.48 ± 0.75^{a}	89.05 ± 0.55^{a}	8.44 ± 1.35^{a}	5.52 ± 0.35^{b}	5.59 ± 0.32^{b*}	7.61 ± 0.42^{ab}
	EC15	82.00 ± 3.26^{b}	87.18 ± 0.94^{a}	88.93 ± 0.59^{a}	89.35 ± 1.04^{a}	8.00 ± 0.48^{ab}	6.69 ± 0.34^{b}	6.80 ± 0.34^{b*}	8.56 ± 0.54^{a}
BC_4F_4 132-12-61	EC0	79.21 ± 0.62^{b}	87.03 ± 1.72^{a}	86.47 ± 0.96^{a}	87.38 ± 0.57^{a}	9.89 ± 1.50^{a*}	6.75 ± 0.19^{b}	6.01 ± 0.22^{b}	7.58 ± 0.37^{b}
	EC15	77.89 ± 1.97^{b}	87.97 ± 0.08^{a}	87.08 ± 1.03^{a}	85.57 ± 1.31^{a}	8.56 ± 0.64^{a*}	6.83 ± 0.22^{a}	5.70 ± 0.65^{a}	6.68 ± 0.72^{a}

Effect of salinity stress on Na^+ accumulation and selective transport of K^+ over Na^+

The accumulation of Na^+ after 9 d salinity stress was assessed in different parts of the plant (Figure 2). The Na^+ content and Na^+/K^+ ratio were significantly higher in the shoot of 'RD6' when compared to the control group (Figures 2a and 2e). Conversely, like 'Pokkali', the 'RD6' introgression lines (i.e., Morkho60-2 and BC_4F_4 132-12-61) showed nonsignificant differences in the shoot Na^+ content and Na^+/K^+ ratio between the control and treatment groups (Figures 2a and 2e). A significantly increased root Na^+ content was observed in the salt-stressed plants of all genotypes when compared to the control group, but 'Pokkali' seedlings showed the highest root Na^+ content among them (Figure 2b). Accordingly, 'Pokkali' also showed the highest ST value followed by BC_4F_4 132-12-61, Morkho60-2, and 'RD6', respectively (Figure 2g) In addition, the shoot K^+ content was highest in 'Pokkali' seedlings; however, there were no differences between treatment and control groups of all genotypes (Figure 2c). Interestingly, the root K^+ content of Morkho60-2 was higher than BC_4F_4 132-12-61 and 'RD6' under salinity stress (Figure 2d), but its root Na^+/K^+ ratio was lowest among all genotypes (Figure 2f). These results suggested that the ability to transport K^+ over Na^+ from root to the upper parts of the plant, and the ability to maintain low Na^+ accumulation was highest in 'Pokkali' followed by Morkho60-2, BC_4F_4 132-12-61, and 'RD6', respectively.

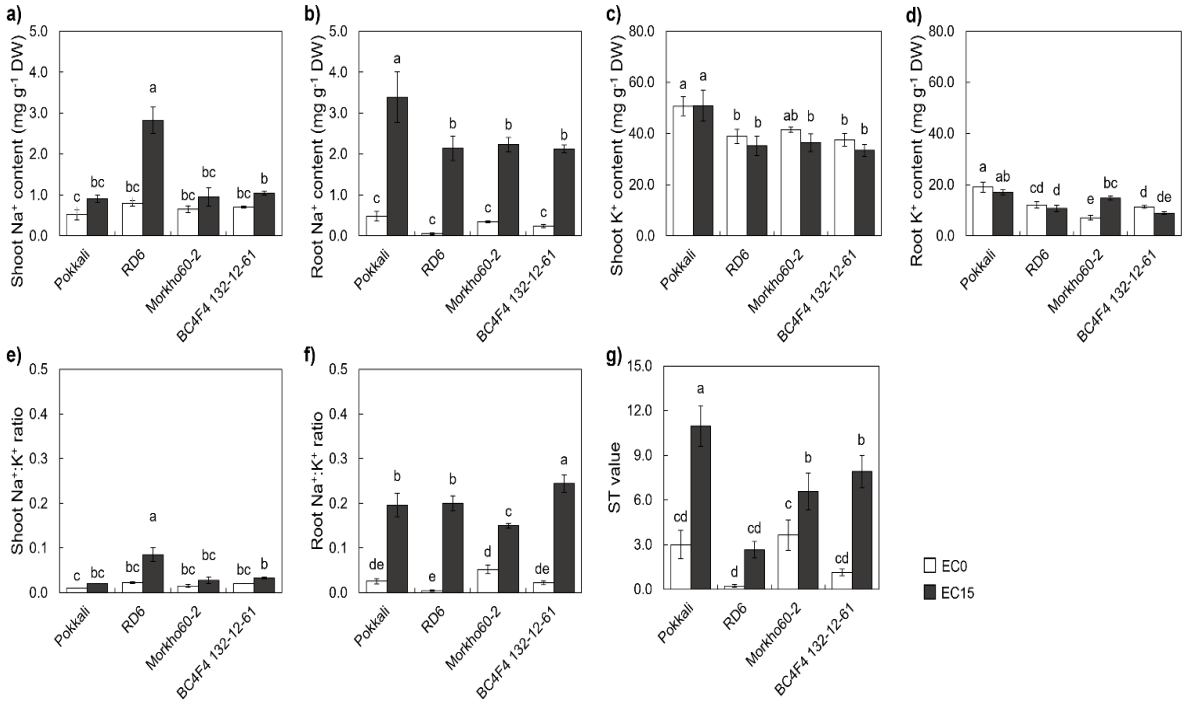

Figure 2. Accumulation of Na^+ and K^+ in shoot and root tissues of the four rice genotypes under salinity stress. The Na^+ content (a-b), K^+ content (c-d), Na^+/K^+ ratio (e-f), and selective transportation (ST value) for K^+ over Na^+ (g) were measured after 9 d of 150 mM NaCl treatment. Data are shown as means ± SEM of four biological replicates. The different letters displayed significant difference by Duncan's multiple range test ($p < 0.05$).

Effect of salinity treatment on photosynthetic pigments

The photosynthetic pigment content is one of the biochemical indicators of salt tolerance in different plant species (Gong et al., 2018). In this study, chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid were measured over the period of salinity stress treatment (Figure 3). Pokkali, a salt-tolerant cultivar, maintained photosynthetic pigment content after 3 d salt stress and showed enhanced pigment content at 6 and 9 d salinity stress (Figures 3a-3d). Interestingly, Morkho60-2 displayed higher chlorophyll *a* and total chlorophyll content after 6 d salinity stress treatment when compared to the control group (Figures 3a and 3c); however, its chlorophyll *a*, chlorophyll *b*, and total chlorophyll content decreased after 9 d salinity stress (Figures 3a-3c). BC_4F_4 132-12-61 also showed a significant reduction in the photosynthetic pigments after 9 d salinity stress compared to day 0 (Figures 3a-3d). Interestingly, 'RD6' did not display a significant decrease in all pigment contents over the period of the salinity treatment (Figures 3a-3d).

Variations among the parental and improved rice genotypes and the associated variables

The PLS-DA was performed to examine variations among the rice genotypes based on growth and physiological characteristics. The PLS-DA biplot revealed that the control (EC0) and treatment (EC15) groups of all genotypes were clearly separated. This separation was influenced by the root Na^+/K^+ ratio (RNaK_ratio) and root Na^+ content (RNa) (Figure 4). Under salinity stress, Morkho60-2 and BC_4F_4 132-12-61 were positioned between their parental genotypes, 'Pokkali' and 'RD6'; however, Morkho60-2 was closer to 'Pokkali' under the salt stress (Figure 4). This could imply an inheritance of salinity growth and physiological responses from the 'Pokkali' parental genotypes. In this case, the ST value, shoot Na^+/K^+ ratio (SNaK_ratio), and shoot Na^+ content (SNa) had the strong loading degree on genotypic separation (Figure 4). Notably, all discriminating variables are involved with the ability of the seedlings to manage their Na^+ transportation,

accumulation, or compartmentalization. Hence, it is suggested that the efficacy in managing shoot Na^+ accumulation may be the key stress adaptive mechanisms of the newly improved 'RD6' genotypes. Therefore, we further investigated the transcriptional changes in Na^+ homeostasis-related genes of both root and shoot tissues in response to salinity stress.

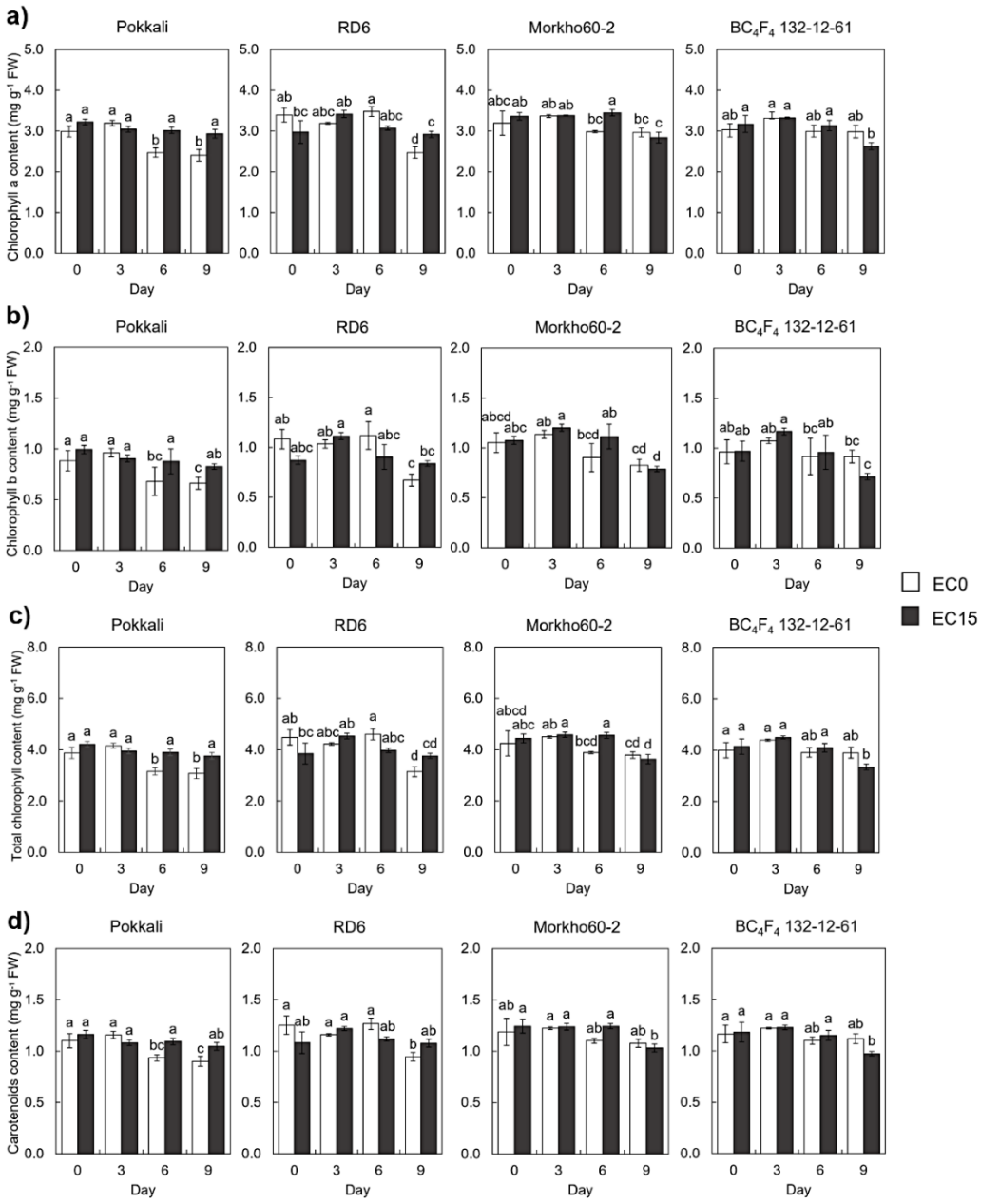

Figure 3. Photosynthetic pigment content of the four rice genotypes under salinity stress. Chlorophyll *a* (a), chlorophyll *b* (b), total chlorophyll (c), and carotenoid (d) were measured after 0, 3, 6, and 9 d of 150 mM NaCl treatment. Data are shown as means ± SEM of four biological replicates. The different letters displayed significant difference between the timepoint and the treatment by Duncan's multiple range test ($p < 0.05$).

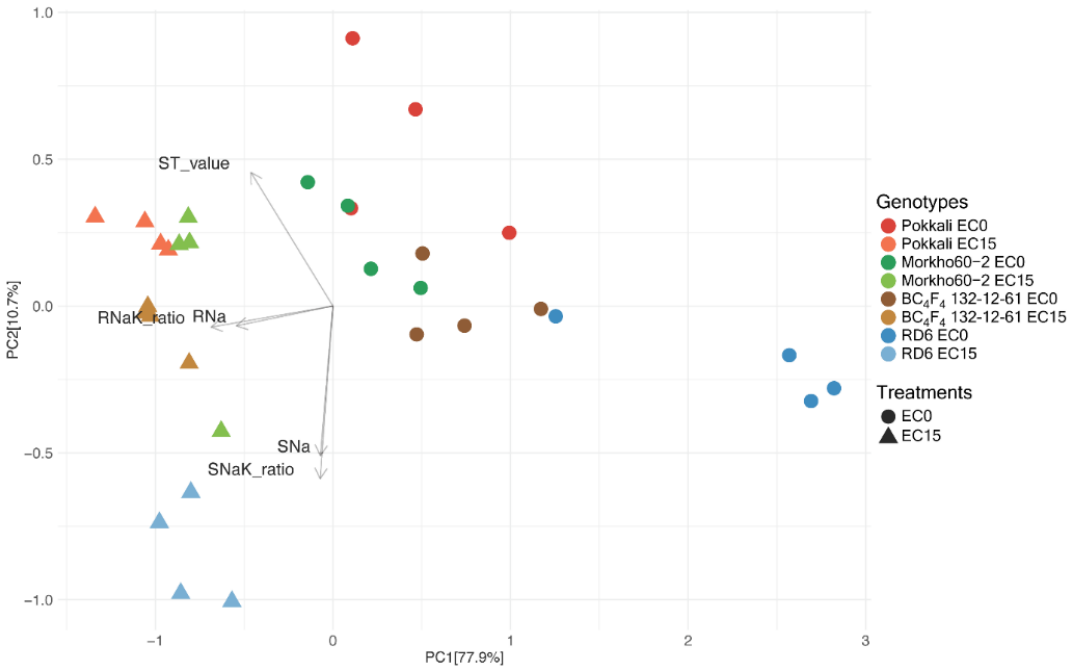

Figure 4. PLS-DA biplot of different rice genotypes. The separation between treatments on PC1 was based on RNaK_ratio and RNa. Here, 77.9% of variance were explained by the first component. Meanwhile, the genotype variability on PC2 was influenced by ST_ value, SNaK_ratio, and SNa. The second component described 10.7% of data variance. RNaK_ratio: Root Na^+/K^+ ratio; RNa: root Na^+ content; ST_ value: selective transportation value; SNaK_ratio: shoot Na^+/K^+ ratio; SNa: shoot Na^+ content.

Effect of salinity stress on the transcriptional response of the Na^+ homeostasis-related genes

The transcriptional responses of Na^+ homeostasis-related genes including *OsHKT1;5*, *OsHKT2;1*, *OsNHX1*, *OsNHX2*, *OsNHX3*, and *OsSOS1* in the leaf and root tissues of all genotypes were analyzed at 9 d salinity stress (Figure 5). The relative expression level of *OsHKT1;5* was highly upregulated in both leaf and root tissues of 'RD6' seedlings (Figure 5A). On the other hand, the expression patterns of *OsHKT1;5* in Morkho60-2 and BC_4F_4 132-12-61 were similar to 'Pokkali' in both tissue types, in which the gene was slightly upregulated in the root but downregulated in the leaf tissue (Figure 5A). In all genotypes, the root *OsHKT2;1* transcripts were suppressed by salt, but they were increased in the leaf tissue (Figure 5B). Moreover, *OsNHX1* relative expression was not significantly different among genotypes in both tissues (Figure 5C). However, it was marginally upregulated in the root tissue of Morkho60-2 and 'RD6' seedlings (Figure 5C). The relative expression pattern of the *OsNHX2* gene of 'Pokkali' was different from the other genotypes, in which it was slightly upregulated in the leaf tissue and marginally downregulated in the root tissue (Figure 5D). On the other hand, the transcripts of *OsNHX2* were highly abundant in Morkho60-2 roots under salt stress (Figure 5D). Despite nonsignificant difference among genotypes, the expression level of leaf *OsNHX3* was lessened by salt in all genotypes, except for BC_4F_4 132-12-61 (Figure 5E). Likewise, this gene was downregulated in the root tissue of the studied genotypes, except for 'RD6' (Figure 5E). For *OsSOS1*, its slight upregulation was only observed in the leaf tissue of 'RD6'. Interestingly, *OsSOS1* was significantly upregulated in the root tissue of Morkho60-2 compared to other genotypes (Figure 5F).

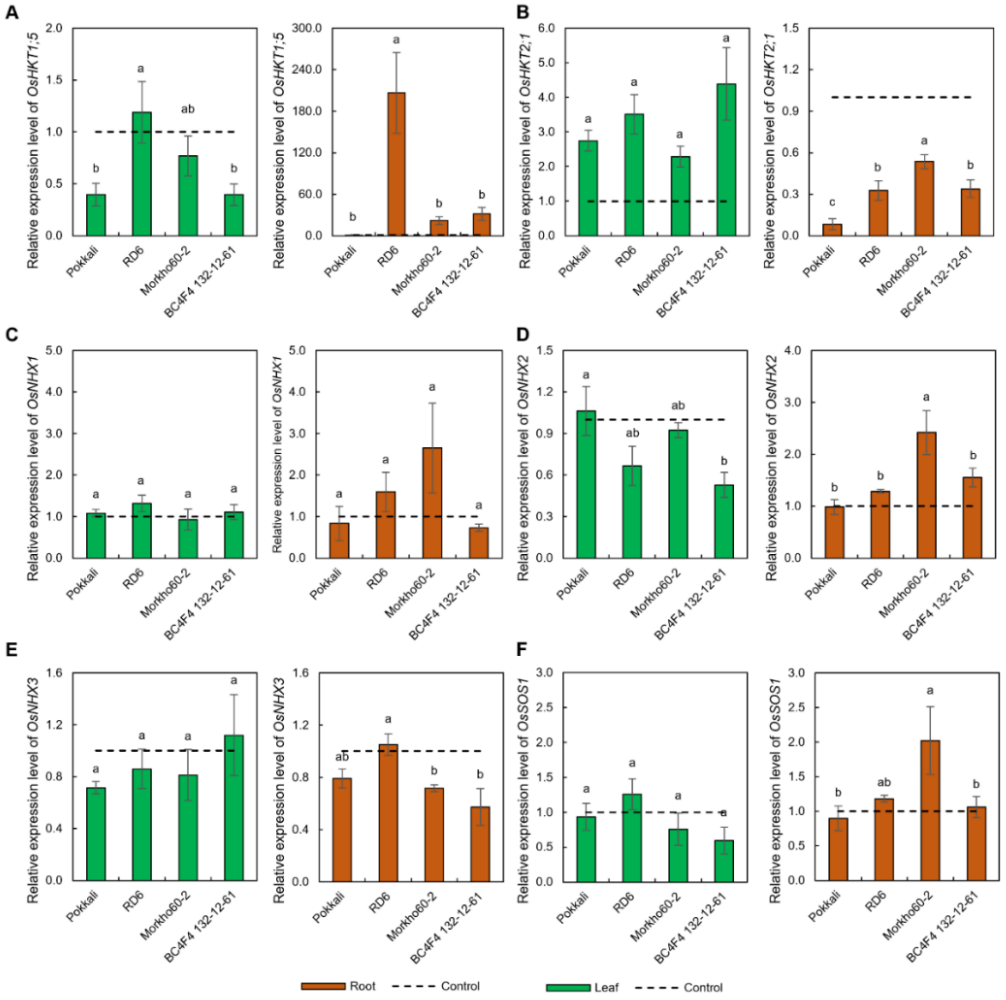

Figure 5. Transcriptional analysis of Na^+ homeostasis-related genes in the leaf and root tissues of different rice genotypes under salinity stress. The relative expression levels of *OsHKT1;5* (A), *OsHKT2;1* (B), *OsNHX1* (C), *OsNHX2* (D), *OsNHX3* (E), and *OsSOS1* (F) in the leaf and root tissues were assessed after 9 d of 150 mM NaCl stress. Data are shown as means ± SEM of three biological replicates. The different letters displayed significant difference by Duncan's multiple range test ($p < 0.05$).

DISCUSSION

In this study, the seedlings' physiological responses and transcriptional changes of Na^+ homeostasis-related genes of the 'RD6' introgression lines were characterized to unravel their putative adaptive mechanisms under salt stress. Our study showed that the growth and biomass of the original 'RD6' seedlings were heavily affected by salt stress (Figure 1). The reduction in growth was presumably due to the accumulation of shoot cytosolic Na^+, and thereby the high ratio of shoot Na^+/K^+ (Figures 2a and 2e). The over accumulation of the Na^+ could impair cell membrane stability, which further disturbed normal cell metabolic process and resulted in growth reduction under salinity stress (Parihar et al., 2015). In 'Sakha 102' Egyptian rice, the high level of Na^+ in the leaf and root tissues strongly affected its biomass (Mekawy et al., 2015). On the other hand, the seedlings' growth performance of the newly improved 'RD6' rice genotypes, Morkho60-2 and BC_4F_4 132-12-61, and the standard salt-tolerant cultivar, Pokkali, was not significantly affected by salt stress (Figure 1).

Moreover, the overaccumulation of shoot Na^+ could induce the instability of cell membrane and introduce the leakage of cellular electrolytes. Accordingly, the higher leaf electrolyte leakage (EL) was observed in 'RD6' and Morkho60-2 genotypes at 6 d stress exposure compared to the control group (Table 2). This higher EL might be responsible for the growth and biomass reduction in the 'RD6' genotype (Figure 1). Moreover, the selective transport of K^+ over Na^+ (ST value) from the root tissue to the upperpart of the plant could provide K^+ ions to maintain electrolyte and osmotic balance in the cells (Zhang et al., 2018). Our results showed that the ST value was most prominent in 'Pokkali', followed by the two newly improved 'RD6' genotypes, Morkho60-2 and BC_4F_4 132-12-61, respectively (Figure 2g). This could contribute to their ability to maintain the EL level upon salt exposure (Table 2) and suggests their salt acclimation strategy via the maintenance of membrane stability (Ueda et al., 2013; Mekawy et al., 2015; Mishra et al., 2021). In contrast, the ST value was lowest in 'RD6' seedlings (Figure 2g). The low preferential transport of K^+ over Na^+ from root to the upper tissues may result in the increased shoot cytosolic Na^+ concentration observed in this genotype (Figure 2a) (Chakraborty et al., 2020). In addition, salinity stress typically induces photosynthesis reduction in rice seedlings (Nounjan et al., 2016; Chakraborty et al., 2020). Photosynthesis is a primary energy production process with chlorophyll *a* being a key pigment in the reaction center, while chlorophyll *b* and carotenoid play a crucial role in membrane protection from oxidative damage (Gong et al., 2018). The changes in chlorophyll content under salinity stress varies depending on rice varieties. For instances, the Korean rice 'Nagdong' showed an increased chlorophyll content, while that of 'CheongCheong' remained unchanged in response to 150 mM NaCl stress (Farooq et al., 2021). Here, 'Pokkali' seedlings could maintain photosynthetic pigments at all timepoints with a slight increase in pigment content at 6 and 9 d salinity stress (Figures 3a-3d). Notably, Morkho60-2 also displayed higher photosynthetic pigment content compared to the control group when exposed to salt for 6 d (Figures 3a and 3c). It is suggested that the production of photosynthetic pigments may contribute to better growth performance in 'Pokkali' and Morkho60-2 genotypes (Figure 1). Moreover, the high level of carotenoid content in 'Pokkali' at 6 and 9 d stress exposure might protect the membrane from oxidative damage (Gong et al., 2018), allowing it to maintain EL level (Table 2).

When considering all observed physiological parameters using PLS-DA (Figure 4), it is suggested that the salt adaptive mechanisms of the 'RD6' introgression lines were attributed to their ability to maintain Na^+ homeostasis. Similar observation was seen in the RGD1 and RGD4 rice lines, which are the 'KDML105' improved rice lines carrying the salt-tolerant *SKC1* gene. Both RGD1 and RGD4 rices showed efficient maintenance of ion homeostasis and lower EL compared to 'KDML105' (Pamuta et al., 2022). We therefore analyzed transcriptional responses of the Na^+ homeostasis-related genes (Figure 5). The OsHKT1;5 transporter is localized in the roots and leaf sheath, where it mediates Na^+ retrieval from the xylem into xylem parenchymal cells to avoid Na^+ overaccumulation in young leaf and excludes Na^+ in the phloem to keep the ions in older leaf (Munns and Tester, 2008; Mekawy et al., 2015; Kobayashi et al., 2017). Surprisingly, the 'RD6' seedlings displayed a high abundance of *OsHKT1;5* transcripts in both leaf and root tissues (Figure 5A); however, the shoot Na^+ overaccumulation was still observed in this genotype (Figures 3a and 3e). The significant accumulation of Na^+ in the shoot tissue of 'RD6' may result from the Na^+ ions that were excluded into the leaf sheaths to protect the young leaf from ion toxicity (Kobayashi et al., 2017). In addition, the *OsHKT1;5* allelic difference was previously demonstrated between the salt-tolerant and salt-sensitive rice genotypes, which allowed the tolerant variety to exclude the Na^+ ions from the xylem vessel more efficiently (Ren et al., 2005). This may explain the discrepency between the *OsHKT1;5* transcript level and the Na^+ accumulation observed in this study.

Besides, the OsHKT2;1 transporter is known to mediate Na^+ influx and prevent Na^+ overaccumulation in rice plants under salt stress (Horie et al., 2007). Here, the expression of *OsHKT2;1* was downregulated in root tissue and upregulated in leaf tissue of all genotypes (Figure 5B). Horie et al. (2007) and Theerawitaya et al. (2020) stated that OsHKT2;1 transporter plays a crucial role in restricting Na^+ influx from the xylem among rice cultivars. Therefore, the lack of its transcripts in the root tissues might be responsible for the increased root Na^+ seen in all genotypes (Figures 5b and 3b). In addition, *OsNHX1* relative expression was marginally upregulated in the root tissue of Morkho60-2 and 'RD6' seedlings (Figure 5C). Its upregulation suggested the cytosolic Na^+ compartmentation into the vacuoles, which could allow the seedlings to maintain cell turgor pressure, cell volume, and overall plant water status under salinity stress (Table 2) (Munns and Tester, 2008; Hasegawa, 2013). In addition to *OsNHX1*, increased *OsNHX2* transcripts was also found in the root of Morkho60-2 (Figure 5D), but their expression was slightly downregulated in its leaf (Figure 5C and 5D). This may suggest the high Na^+ compartmentalization activity in the roots, which left lower amount for Na^+ loading to xylem and long-distance

transportation to the upper parts of the plant (Shi et al., 2002). Hence, Na^+ compartmentalization may stabilize root growth performance of Morkho60-2 under salinity stress (Figure 1). Accordingly, the hight ST value, which indicated the strong selective for K^+ over Na^+ from the root to shoot tissues, was observed in of Morkho60-2 (Chakraborty et al., 2020).

In addition to the HKT and NHX transporters, OsSOS1 transporter also plays a crucial role in Na^+ homeostasis. Its function was to exclude Na^+ from root epidermal cells back to the soil, and also accompany the long-distance transportation of Na^+ from root to above ground tissue (Shi et al., 2002). The highly abundance of *OsSOS1* transcripts in the root tissue of Morkho60-2 suggested exclusion of toxic Na^+ from cytosolic to an apoplastic space (Figure 5F) (Ji et al., 2013; Zhang et al., 2018). Similarly, 'Nipponbare' rice grown under 150 mM NaCl stress showed elevated expression of *OsSOS1* in root tissue accompanying by lower root Na^+ concentration (Fu et al., 2018). This root Na^+ efflux via SOS pathway might also be applied to Morkho60-2 in this study. Consequently, the Na^+ content in the shoot tissue of Morkho60-2 was only slightly enhanced under salt stress (Figure 2a). Presumably, the lower Na^+ in shoot tissue of Morkho60-2 may influence the downregulation of *OsHKT1;5* in the leaf tissue (Figures 5A and 7) since there was less requirement for its action compared to the RD6 genotypes. Our observations suggested the role of extruding Na^+ to soil solution of Morkho60-2 as one of its salt adaptive strategies (Shi et al., 2002; Martínez-Atienza et al., 2006; Ma et al., 2013).

The differential expression of Na^+ homeostasis-related genes in response to salt stress influences the distinct Na^+ level between the original 'RD6' and newly improved genotypes, in particular Morkho60-2. Even though the shoot Na^+/K^+ ratios of the 'RD6' introgression lines were comparable, Morkho60-2 had lower root Na^+/K^+ ratio than BC_4F_4 132-12-61. Besides, although the ST value of both Morkho60-2 and BC_4F_4 132-12-61 resembled that of 'Pokkali' (Figure 2g), Morkho60-2 seedlings accumulated more K^+ in root tissue than BC_4F_4 132-12-61 (Figure 2d). Lesser K^+ retention could induce salt stress susceptibility in rice (Chakraborty et al., 2020). Therefore, it is suggested that Morkho60-2 could maintain the Na^+/K^+ homeostasis in response to salinity conditions better than the BC_4F_4 132-12-61 at seedling stage. As summarized in Figure 6, Morkho60-2 showed a better adaptation to salinity stress than the original 'RD6' genotype in terms of the selective transport for K^+ over Na^+, which could contribute to its unaffected growth under salt stress. The Na^+/K^+ ratio in root tissue presented a large impact on the differential responsive and adaptive pattern to salinity stress in this study (Figures 4 and 6). However, it cannot be excluded that the high level of K^+ acquisition in root tissue of Morkho60-2 could be attributed to *OsAKT1.* This gene is also located on the chromosome 1 as part of the quantitative trait loci (QTL) controlling K^+ concentration and the Na^+/K^+ ratio in salt-stressed plants (Mekawy et al., 2015).

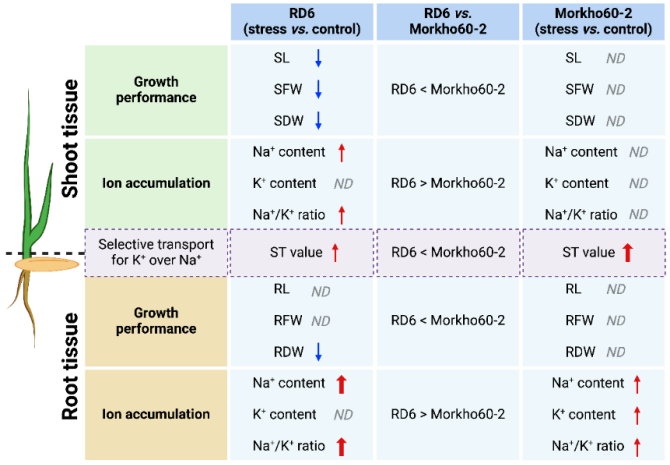

Figure 6. Comparative responses of key physiological parameters between 'RD6' and Morkho60-2 rices under salinity stress. The growth performance, ion accumulation, and selective transport for K^+ over Na^+ (ST value) were displayed. The red arrows display increasing level, while the blue arrows display decreasing level. The difference in arrow size indicates the magnitude of responses, and ND displays nonsignificant changes under salinity stress. SL: Shoot length; SFW: shoot fresh weight; SDW: shoot dry weight; RL: root length; RFW: root fresh weight; RDW: root dry weight.

The overall comparative transcriptional responses of Morkho60-2 *vs*. 'RD6' were also summarized in Figure 7. The abundance of *OsSOS1* transcripts in root tissue of Morkho60-2 (Figures 5F and 7) could contribute to the root epidermal Na^+ exclusion via the discharge of toxic apoplastic Na^+. The less availability of the root Na^+ ions would lead to lower transport to the upper parts of the plants. Furthermore, the Na^+ over accumulation induced the Na^+ compartmentalization into vacuole through the upregulation of OsNHX transporter in both tissues (Figure 7). Although Morkho60-2 and 'RD6' showed the comparable expression of root *OsHKT*, the vacuolar compartmentalization of Na^+ via *OsNHX1* and *OsNHX2* could be higher in Morkho60-2 due to their higher magnitude of transcription (Figure 7).

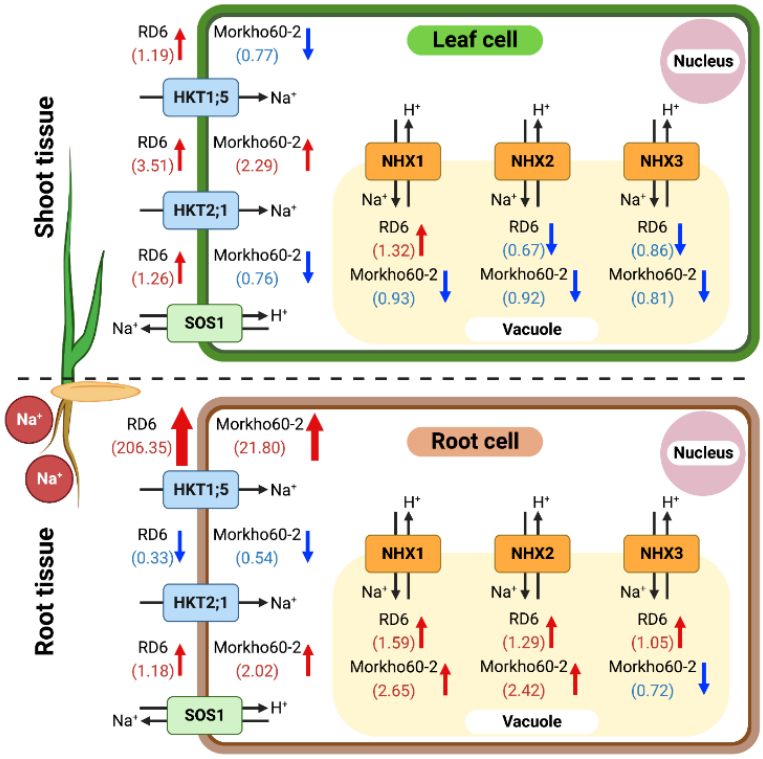

Figure 7. Comparative expression levels of Na^+ homeostasis-related genes in the leaf and root tissues of 'RD6' and Morkho60-2 rices under salinity stress. The red arrows display the upregulation of genes, while the blue arrows display the downregulation of genes under salinity stress. The value accompanying the arrow is relative expression level of each gene under salinity stress in comparison to the control. NHX: Na^+-H^+ antiporter; HKT: high-affinity K^+ transporter; SOS: Na^+-H^+ antiporter Salt Overly Sensitive.

CONCLUSIONS

The role of *Saltol* quantitative trait loci (QTL) that was introgressed into the newly improved 'RD6' rice genomes was characterized. It enhanced salt tolerance characteristics of the newly improved rice lines, in particular Morkho60-2, by maintaining Na^+/K^+ homeostasis under salinity stress. The root Na^+ exclusion, strongly selective transport for K^+ over Na^+ from the root to the upper parts of the plant, along with the vacuolar compartmentalization could effectively maintain Na^+ content and Na^+/K^+ ratio in the shoot tissue of Morkho60-2 in comparison to the 'RD6' genotype. These abilities were differentially influenced by transcriptional response of Na^+ homeostasis-related genes, in particular *OsNHXs* and *OsSOS1*, among the different rice seedlings.

Author contribution

Conceptualization: M.T. Methodology: N.S., K.W., A.K., M.T. Software: K.W. Validation: N.S., A.K. Formal analysis: N.S., K.W. Investigation: N.S., A.K. Resources: J.S., M.T. Data curation: N.S., A.K. Writing-original draft: N.S. Writing-review & editing: M.T., K.W. Visualization: N.S., K.W. Supervision: J.S. Project administration: M.T. Funding acquisition: M.T. All co-authors reviewed the final version and approved the manuscript before submission.

Acknowledgement

This research was funded by the Coordinating Center for Thai Government Science and Technology Scholarship Students (CSTS), National Science and Technology Development Agency (NSTDA), and the National Research Council of Thailand (NRCT) through the Senior Research Scholar Project of Prof. Dr. Piyada Theerakulpisut [Grant number NRCT813/2563]. N.S. is grateful for the support from the Research Support Scholarship, Graduate School of Khon Kean University. We thank the central laboratory, Faculty of Science, Khon Kaen University for providing scientific instruments used in this research.

References

Baninasab, B., Ghobadi, C. 2011. Influence of paclobutrazol and application methods on high-temperature stress injury in cucumber seedlings. Journal of Plant Growth Regulation 30:213-219.

Berrier, K.L., Prebihalo, S.E., Synovec, R.E. 2020. Chapter 7 - Advanced data handling in comprehensive two-dimensional gas chromatography. p. 229-268. In Snow, N.H. (ed.) Separation science and technology. Vol. 12. Academic Press, Cambridge, Massachusetts, USA.

Chakraborty, K., Mondal, S., Ray, S., Samal, P., Pradhan, B., Chattopadhyay, K., et al. 2020. Tissue tolerance coupled with ionic discrimination can potentially minimize the energy cost of salinity tolerance in rice. Frontiers in Plant Science 11:265.

Farooq, M., Park, J.-R., Jang, Y.-H., Kim, E.-G., Kim, K.-M. 2021. Rice cultivars under salt stress show differential expression of genes related to the regulation of Na^+/K^+ balance. Frontiers in Plant Science 12:680131.

Fu, L., Shen, Q., Kuang, L., Yu, J., Wu, D., Zhang, G. 2018. Metabolite profiling and gene expression of Na/K transporter analyses reveal mechanisms of the difference in salt tolerance between barley and rice. Plant Physiology and Biochemistry 130:248-257.

Fukuda, A., Nakamura, A., Hara, N., Toki, S., Tanaka, Y. 2011. Molecular and functional analyses of rice NHX-type Na^+/H^+ antiporter genes. Planta 233:175-188.

Gong, D., Wang, G., Si, W., Zhou, Y., Liu, Z., Jia, J. 2018. Effects of salt stress on photosynthetic pigments and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase in *Kalidium foliatum*. Russian Journal of Plant Physiology 65:98-103.

Hartley, T.N., Thomas, A.S., Maathuis, F.J. 2020. A role for the OsHKT 2; 1 sodium transporter in potassium use efficiency in rice. Journal of Experimental Botany 71(2):699-706.

Hasegawa, P.M. 2013. Sodium (Na^+) homeostasis and salt tolerance of plants. Environmental and Experimental Botany 92:19-31.

Horie, T., Costa, A., Kim, T.H., Han, M.J., Horie, R., Leung, H.Y., et al. 2007. Rice OsHKT2; 1 transporter mediates large Na^+ influx component into K^+-starved roots for growth. The EMBO Journal 26(12):3003-3014.

Ji, H., Pardo, J.M., Batelli, G., van Oosten, M.J., Bressan, R.A., Li, X. 2013. The salt overly sensitive (SOS) pathway: Established and emerging roles. Molecular Plant 6(2):275-286. doi:10.1093/mp/sst017.

Kobayashi, N.I., Yamaji, N., Yamamoto, H., Okubo, K., Ueno, H., Costa, A., et al. 2017. OsHKT1; 5 mediates Na^+ exclusion in the vasculature to protect leaf blades and reproductive tissues from salt toxicity in rice. The Plant Journal 91(4):657-670.

Livak, K.J., Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402-408.

Ma, Q., Li, Y.-X., Yuan, H.-J., Hu, J., Wei, L., Bao, A.-K., et al. 2013. ZxSOS1 is essential for long-distance transport and spatial distribution of Na^+ and K^+ in the xerophyte *Zygophyllum xanthoxylum*. Plant and Soil 374(1-2):661-676. doi:10.1007/s11104-013-1891-x.

Martínez-Atienza, J., Jiang, X., Garciadeblas, B., Mendoza, I., Zhu, J.-K., Pardo, J.M., Quintero, F.J. 2006. Conservation of the salt overly sensitive pathway in rice. Plant Physiology 143(2):1001-1012. doi:10.1104/pp.106.092635.

Mekawy, A.M.M., Assaha, D.V., Yahagi, H., Tada, Y., Ueda, A., Saneoka, H. 2015. Growth, physiological adaptation, and gene expression analysis of two Egyptian rice cultivars under salt stress. Plant Physiology and Biochemistry 87:17-25.

Mishra, M., Wungrampha, S., Kumar, G., Singla-Pareek, S.L., Pareek, A. 2021. How do rice seedlings of landrace Pokkali survive in saline fields after transplantation? Physiology, biochemistry, and photosynthesis. Photosynthesis Research 150(1):117-135. doi:10.1007/s11120-020-00771-6.

Munns, R., James, R.A., Gilliham, M., Flowers, T.J., Colmer, T.D. 2016. Tissue tolerance: An essential but elusive trait for salt-tolerant crops. Functional Plant Biology 43(12):1103-1113.

Munns, R., Tester, M. 2008. Mechanisms of salinity tolerance. Annual Review of Plant Biology 59:651-681.

Negacz, K., Malek, Ž., de Vos, A., Vellinga, P. 2022. Saline soils worldwide: Identifying the most promising areas for saline agriculture. Journal of Arid Environments 203:104775. doi:10.1016/j.jaridenv.2022.104775.

Nounjan, N., Siangliw, J.L., Toojinda, T., Chadchawan, S., Theerakulpisut, P. 2016. Salt-responsive mechanisms in chromosome segment substitution lines of rice (*Oryza sativa* L. cv. KDML105). Plant Physiology and Biochemistry 103:96-105.

Pamuta, D., Siangliw, M., Sanitchon, J., Pengrat, J., Siangliw, J.L., Toojinda, T., et al. 2022. Physio-biochemical traits in improved KDML105 rice (*Oryza sativa* L.) lines containing drought and salt tolerance gene under drought and salt stress.Chilean Journal of Agricultural Research 82:97-110. doi:10.4067/S0718-58392022000100097.

Parihar, P., Singh, S., Singh, R., Singh, V.P., Prasad, S.M. 2015. Effect of salinity stress on plants and its tolerance strategies: A review. Environmental Science and Pollution Research 22(6):4056-4075. doi:10.1007/s11356-014-3739-1.

Quan, R., Wang, J., Hui, J., Bai, H., Lyu, X., Zhu, Y., et al. 2018. Improvement of salt tolerance using wild rice genes. Frontiers in Plant Science 8:2269.

Ren, Z.-H., Gao, J.-P., Li, L.-G., Cai, X.-L., Huang, W., Chao, D.-Y., et al. 2005. A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nature Genetics 37(10):1141-1146.

Sen, S., Chakraborty, R., Kalita, P. 2020. Rice - not just a staple food: A comprehensive review on its phytochemicals and therapeutic potential. Trends in Food Science & Technology 97:265-285. doi:10.1016/j.tifs.2020.01.022.

Shi, H., Quintero, F.J., Pardo, J.M., Zhu, J.K. 2002. The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. Plant Cell 14(2):465-477. doi:10.1105/tpc.010371.

Solis, C.A., Yong, M.T., Vinarao, R., Jena, K., Holford, P., Shabala, L., et al. 2020. Back to the wild: On a quest for donors toward salinity tolerant rice. Frontiers in Plant Science 11:323. doi:10.3389/fpls.2020.00323.

Thanasilungura, K., Kranto, S., Monkham, T., Chankaew, S., Sanitchon, J. 2020. Improvement of a RD6 rice variety for blast resistance and salt tolerance through marker-assisted backcrossing. Agronomy 10(8):1118.

Theerawitaya, C., Samphumphuang, T., Tisarum, R., Siangliw, M., Cha-um, S., Takabe, T., et al. 2021. Transcriptional expression of Na^+ homeostasis-related genes and physiological responses of rice seedlings under salt stress. Journal of Plant Biochemistry and Biotechnology 30(1):81-91. doi:10.1007/s13562-020-00573-w.

Theerawitaya, C., Tisarum, R., Samphumphuang, T., Takabe, T., Cha-Um, S. 2020. Expression levels of the Na^+/K^+ transporter OsHKT2; 1 and vacuolar Na^+/H^+ exchanger OsNHX1, Na enrichment, maintaining the photosynthetic abilities and growth performances of indica rice seedlings under salt stress. Physiology and Molecular Biology of Plants 26:513-523.

Thévenot, E.A., Roux, A., Xu, Y., Ezan, E., Junot, C. 2015. Analysis of the human adult urinary metabolome variations with age, body mass index, and gender by implementing a comprehensive workflow for univariate and OPLS statistical analyses. Journal of Proteome Research 14(8):3322-3335.

Thomson, M.J., de Ocampo, M., Egdane, J., Rahman, M.A., Sajise, A.G., Adorada, D.L., et al. 2010. Characterizing the *Saltol* quantitative trait locus for salinity tolerance in rice. Rice 3(2):148-160.

Ueda, A., Yahagi, H., Fujikawa, Y., Nagaoka, T., Esaka, M., Calcaño, M., et al. 2013. Comparative physiological analysis of salinity tolerance in rice. Soil Science and Plant Nutrition 59(6):896-903.

Wang, S., Zhao, G., Gao, Y., Tang, Z., Zhang, C. 2005. *Puccinellia tenuiflora* exhibits stronger selectivity for K^+ over Na^+ than wheat. Journal of Plant Nutrition 27(10):1841-1857.

Waziri, A., Kumar, P., Purty, R. 2016. *Saltol* QTL and their role in salinity tolerance in rice. Austin Journal of Biotechnology and Bioengineering 3(3):1067.

Wellburn, A.R. 1994. The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. Journal of Plant Physiology 144(3):307-313.

Wongsaprom, C., Sirithunya, P., Vanavichit, A., Pantuwan, G., Jongdee, B., Sidhiwong, N., et al. 2010. Two introgressed quantitative trait loci confer a broad-spectrum resistance to blast disease in the genetic background of the cultivar RD6 a Thai glutinous jasmine rice. Field Crops Research 119(2-3):245-251.

Zhang, Y., Fang, J., Wu, X., Dong, L. 2018. Na^+/K^+ balance and transport regulatory mechanisms in weedy and cultivated rice (*Oryza sativa* L.) under salt stress. BMC Plant Biology 18(1):1-14.