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Contribution of two different Na^+ transport systems to acquired salinity tolerance in rice

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ABSTRACT

To elucidate the mechanisms of salt acclimation, physiological parameters of 70 rice varieties were compared under control and salt stress conditions after the acclimation treatment. The results indicated that some rice varieties had the ability to acclimatize to salt stress, exhibiting improved growth following the acclimation treatment under subsequent salinity stress compared to those without acclimation treatment. Conversely, some varieties exhibited reduced growth both with and without acclimation treatment under subsequent salinity stress. Acclimatized varieties had differential patterns of Na^+ accumulation in the leaf blades because some varieties reduced Na^+ accumulation under salinity stress, whereas others did not. Under salt stress, the acclimatized varieties with low Na^+ accumulation in the leaf blades highly induced the expression of the *OsHKT1;5* gene in the roots, which may contribute to Na^+ exclusion from the shoots. On the other hand, the acclimatized varieties with high Na^+ accumulation in the leaf blades exhibited higher induction of the *OsNHX1* gene, whose gene product participates in the compartmentalization of Na^+ into vacuoles. Thus, rice develops different mechanisms of salinity acclimation using two Na^+ transport systems, and active regulation of Na^+ transport at the transcription level may be involved in the salt acclimation process and enhance salinity tolerance.

1. Introduction

Every year, millions of hectares osf irrigated land are taken out of production because of high salinity, representing hundreds of millions of lost dollars for the agricultural industry. Meanwhile, an ever-increasing global population along with the decrease in arable lands because of high salinity have necessitated the elucidation of salt tolerance mechanisms and improvement of salt-tolerant varieties to decrease the gap in demand and supply. Salinized soils are generated by the accumulation of excess salts, mainly Na^+ and Cl^+ [1,2]. Inland salinity occurs in arid and semi-arid areas by the accumulation of salts on the surface of soils through higher rates of evaporation or improper management of irrigation and drainage. Seawater contamination also increases NaCl concentrations in field soils. Accumulated salts in soils generate an external osmotic potential that reduces the influx of water required for the plants and may also reduce water in plants. Excess Na^+ transported through the transpiration stream disturbs cellular metabolic and physiological functions, such as enzymatic activities, photosynthesis, K^+ homeostasis, and causes a K^+/Na^+ imbalance, resulting in limited growth, thereby jeopardizing yield [3–7]. Thus, plant growth is retarded by both osmotic stress and ionic stress under salinity stress.

To overcome high salinity stress, some plant species have developed several mechanisms to reduce the harmful effects caused by salinity stress. Accumulation of osmoprotectants, such as proline and glycine betaine, is one well-known adaptive mechanism used to adjust cellular osmotic potential in higher plants [8]. Transport systems also participate in adequate distribution of these osmoprotectants at tissue levels [9,10]. To avoid excess Na^+ toxicity, some Na^+ transport systems play a pivotal role in Na^+ exclusion at the cellular and tissue levels [8,11,12]. SOS1, a plasma membrane localized Na^+/H^+ antiporter has been implicated in Na^+ exclusion from the roots. Physiological evidence indicates that NHX, a tonoplast-localized Na^+/H^+ antiporter,

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compartmentalizes excess cytosolic Na^+ into the vacuoles, thereby mediating osmotic adjustment and maintaining K^+, K^+/Na^+, and pH homeostasis of the cytoplasm under saline conditions [13,14]. The high-affinity K^+ transporter family proteins, including OsHKT1;5, which is the ortholog of *Arabidopsis thaliana* AtHKT1;1, has been identified in retrieving Na^+ from the xylem stream to xylem parenchyma cells; thus, limiting Na^+ transport to the shoot and maintaining shoot K^+ homeostasis under salt stress [15]. OsHKT1;4, which retrieves Na^+ from the sheaths, also participates in Na^+ exclusion from leaf blades [5,16]. Coordinated regulation of these Na^+ transporters would be essential for salinity tolerance in plants.

Even though, high salinity causes detrimental effects to plants in many ways, studies have been reported that pre-exposure to low salinity for a certain period of time enhanced tolerance to subsequent high salinity in many herbaceous plants [17–21]. This phenomenon is called acclimation or acquired tolerance. To strengthen acclimation-induced tolerance, mild stress treatment or chemicals can be used to modulate physiological adaptations in plants [20,22]. NaCl induced salt acclimation benefits plants in different ways, such as enhanced growth rate, survival, seed production, and root K^+, but reduced root Na^+ under lethal salinity conditions. During a period of low salinity treatment, plants may receive acclimation signals to trigger responses to acquire salinity tolerance. However, molecular mechanisms that enable lower salinity to enhance salt tolerance have not yet been revealed. Therefore, the primary objective of this study was to elucidate the molecular and physiological mechanisms of salt acclimation by comparing varietal responses, physiological parameters, and expression profiles of the genes that encode Na^+ transport proteins.

To achieve this objective, rice was used as a model crop because rice is the major food crop for more than half of the population of the world, and rice is considered sensitive to salinity among the cereals. Through the screening of divergent rice varieties, we found that there are varietal differences in salt acclimation ability in rice. Seventy varieties screened were classified into three groups: non-acclimatized varieties, acclimatized varieties with higher Na^+ accumulation, and acclimatized varieties with lower Na^+ accumulation. Functional characterization revealed that rice has two different mechanisms of salt acclimation, which depend on the operation of the Na^+ transport systems at the cellular and tissue levels.

2. Materials and methods

2.1. Plant materials, growth conditions, acclimation and salinity treatments

Seeds were initially heat-sterilized at 60 °C for 10 min in a water bath and then surface sterilized using 5% (v/v) sodium hypochlorite solution for 30 min. The seeds were then thoroughly rinsed with distilled water and incubated at 28 °C for 48 h for germination in tap water. The germinated seeds were transferred onto a nylon mesh floating on 20 L tap water for the following 3 days. Water was then replaced with Kimura-B nutrient solution (0.36 mM $(NH_4)_2SO_4$, 0.54 mM $MgSO_4{\cdot}7H_2O$, 0.18 mM KNO_3, 0.36 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.18 mM KH_2PO_4, 19 μM Fe-EDTA, 48.7 μM H_3BO_3, 9 μM $MnSO_4{\cdot}5H_2O$, 0.3 μM $CuSO_4{\cdot}5H_2O$, 0.7 μM $ZnSO_4{\cdot}7H_2O$ and 0.7 μM $Na_2MoO_4{\cdot}2H_2O$). The solution was changed every 3 days and the pH was maintained daily between 5.0 − 5.5. Seedlings were grown in a glasshouse at Hiroshima University.

Three sets of four seedlings from each variety were maintained throughout the experiment. One set received only nutrient solution (control). In the second set, 1-week-old seedlings grown in nutrient solution were pretreated with 1 mM NaCl (salinity/acclimation) for 2 weeks and then transferred to a nutrient solution containing 50 mM NaCl for the next 2 weeks. In the third set, hydroponically grown plants were directly subjected to 50 mM NaCl (salinity) during the third week of growth and maintained for the next 2 weeks. Seedlings were harvested at 21 days (end of pretreatment) and 36 days (end of salinity treatment) (Fig. 1).

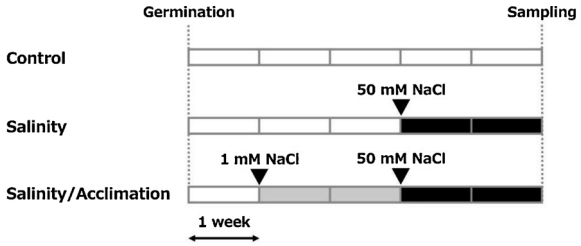

Fig. 1. Duration of salinity stress and acclimation treatments used in this study. After 1 week of germination, 1 mM NaCl was applied to rice seedlings for 2 weeks as an acclimation treatment. After 3 weeks of germination, 50 mM NaCl stress was imposed on rice seedlings both with and without acclimation treatment.

2.2. Measurement of growth parameters

Four seedlings from each treatment were divided into leaves, stems and roots, and their fresh weight (FW) was measured. For the dry weight (DW) determination, all three separated tissues were oven-dried at 70 ℃ for 72 h prior to being weighed. The fully expanded, third leaf from the top of each plant was used for leaf area (LA) measurements using an LA meter (Model: LI-3100C).

To determine the relative water content (RWC), the middle portion of the second topmost leaf was cut into 6–8 cm^2 pieces and placed in a pre-weighed (W1) airtight glass vial containing 10 mL of deionized water. After sampling, the sample vial was weighed (W2) immediately. The leaf samples were soaked in deionized water for 24 h under light and were then placed on paper towels to remove excess water prior to measuring turgid weight (W3). The samples were then oven-dried at 70 ˚C for the next 3 days and DW was obtained (W4). The RWC was estimated using the following formula: $RWC\ (\%) = ((W2 - W1) - W4)/(W3 - W4) \times 100$.

2.3. Measurement of electrolyte leakage ratio and malondialdehyde

To determine the electrolyte leakage ratio (ELR), 0.5 g of leaf samples were cut into 2 cm^2 pieces and soaked in a 50 mL tube containing 30 mL of deionized water. The tubes were gently shaken overnight, and the electrical conductivity (EC) of the solution was measured with a calibrated EC meter (EC1). The tubes were then autoclaved (121 ˚C, 20 min). After cooling, the electrical conductivity was again measured (EC2). The ELR was calculated using the following formula: $ELR\ (\%) = (EC1/EC2) \times 100$.

To measure the concentration of malondialdehyde (MDA), fresh leaf samples (approximately 0.1 g) were homogenized in an extraction buffer (10 mM HEPES, 15% (w/v) TCA, 0.25 N HCl, 0.375% (w/v) 2-thioberbituric acid, 0.04% butylated hydroxytoluene, 2% ethanol, pH 7.0) and were then incubated at 95℃ for 30 min in a water bath. The samples were then cooled on ice and centrifuged at 10,000 × *g* for 10 min [23]. MDA concentration was calculated from the difference in absorbance at 532 and 600 nm using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$ [24].

2.4. Measurement of Na^+ and K^+ concentration

Oven-dried leaf, sheath, and root samples were used to determine the Na^+ and K^+ concentrations. Briefly, samples were gently agitated in 1 N HCl overnight. Then Na^+ and K^+ readings were taken using a flame photometer (ANA-135; Tokyo Photoelectric, Tokyo, Japan) and the Na^+ and K^+ concentrations were estimated using standard curves [25].

2.5. Expression analysis of genes encoding Na^+ transport proteins

Total RNA was extracted from the leaves and roots of the control, pretreated, and non-pretreated plants using an RNA Extraction Mini Kit (RBC Bioscience). The concentration and purity of the RNA were measured using a Nano Drop 1000 (Thermo Fisher Scientific) at A_{260} and A_{280}. After digestion with DNase I, 1 μg of total RNA was reverse transcribed to cDNA at 37 °C for 15 min and then reverse transcriptase was denatured at 98 °C for 5 min. The Thunderbird SYBR qPCR Mix (Toyobo) and an ABI StepOne system (Applied Biosystems) were used to conduct a quantitative polymerase chain reaction (qPCR) [26]. RT-qPCR was performed using the following profile: initial denaturation at 95 °C for 1 min, followed by denaturation at 95 °C for 15 s, and an extension at 60 °C for 1 min. Forty cycles were performed before the melting curve analysis, and the melting curve was used to verify the PCR products by changing the temperature from 60 °C to 95 °C. *Os25SrRNA* was used as an internal control to normalize the relative expression of each gene among the samples. The primer sequences are listed in Supplementary Table 1. The relative abundance of gene transcripts was calculated using the comparative $2^{-\Delta\Delta CT}$ method [27].

2.6. Statistical analysis

Statistical analysis of the data obtained was processed using a *t*-test and one-way ANOVA, and differences between the treatment means ($n = 4$) were compared using Tukey's multiple comparison test with the software SPSS, version 21 (IBM Inc., USA). Differences between the treatments were considered significant at $p < 0.05$.

3. Results

3.1. Screening of salt acclimatized varieties under salinity stress

After 2 weeks of 1 mM NaCl pretreatment, rice seedlings grown under control and acclimation conditions did not show any significant differences in shoot and root biomass production (data not shown). This indicated that the 1 mM NaCl pretreatment did not impose salinity stress on the rice seedlings. Then, 70 rice varieties were tested to examine whether the rice had acclimation ability to salinity stress. After 2 weeks of salinity stress treatment, 29 rice varieties showed significant decreases in both shoot DW and length, indicating that these varieties were susceptible to salinity stress (Supplementary Tables 2 and 3). However, out of 29 rice varieties, 18 varieties showed improved their growth under salinity stress when rice seedlings were pretreated with 1 mM NaCl beforehand for 2 weeks. In comparison to control conditions, these acclimatized varieties showed a 38% decrease in shoot DW under salinity stress, but no significant differences under salinity stress with acclimation treatment (Fig. 2A). On the other hand, 11 non-acclimatized varieties decreased shoot DW by 44% under salinity stress and by 39% under salinity stress with acclimation treatment (Fig. 2A). Acclimation treatment was effective in improving shoot length in both the acclimatized and non-acclimatized varieties (Fig. 2B). Thus, varietal differences in acclimation ability can be clearly seen in dry matter production under salinity stress.

Restriction of Na^+ accumulation in plant organs is critically important for salinity tolerance in glycophytes. Fig. 3 shows Na^+ accumulation patterns in the 10 selected acclimatized varieties (Tima, Vandaran, Rambhog, Naba, ARC 7291, Khao Nok, Hakphaynhay, Bingala, Dianyu 1, Khau Tan Chiem) and 5 non-acclimatized varieties (Jena035, IR 58, Deejaiohualuo, Kalo Dhan, Radin Gol Sesat) under control and salinity stress conditions. Salinity stress promoted Na^+ accumulation in the leaf blades of all varieties tested (Fig. 3). Interestingly, the acclimation treatment reduced Na^+ concentrations in the leaf blades of five acclimatized varieties (Tima, Vandaran, Rambhog, Naba, ARC 7291) under salinity stress conditions, whereas another five acclimatized varieties (Khao Nok, Hakphaynhay, Bingala, Dianyu 1, Khau Tan Chiem) and five non-acclimatized varieties did not reduce Na^+ concentrations in the leaf blades. These findings suggest that acquired salinity tolerance in rice relies on two different mechanisms: the mechanism of Na^+ exclusion from shoots, as seen in the acclimatized varieties (Tima, Vandaran, Rambhog, Naba, ARC 7291) having lower Na^+ concentrations in the leaf blades, and the mechanism of tissue tolerance as seen in the acclimatized varieties (Khao Nok, Hakphaynhay, Bingala, Dianyu 1, Khau Tan Chiem) having higher Na^+ concentrations in the leaf blades under salinity stress conditions.

3.2. Comparative molecular physiological analysis of salt acclimation mechanisms

To further elucidate the mechanisms of salt acclimation in rice, three rice varieties (Rambhog, Bingala, Jena035) were cultivated. Rambhog, one of the acclimatized varieties with lower Na^+ concentrations in the leaf blades under salinity stress, improved DW of shoots and roots and total LA with acclimation treatment under salinity stress (Fig. 4A, B). The same tendency was also observed in Bingala, one of the acclimatized varieties with higher Na^+ concentrations in the leaf blades under salinity stress (Fig. 4A, B). However, Jena035, the non-acclimatized variety, reduced both the DW of shoots and roots and total LA under salinity stress conditions, and acclimation treatment did not affect these parameters (Fig. 4A, B).

The physiological status of Rambhog, Bingala, and Jena035 was evaluated under salinity stress conditions (Fig. 5). Generally, salinity

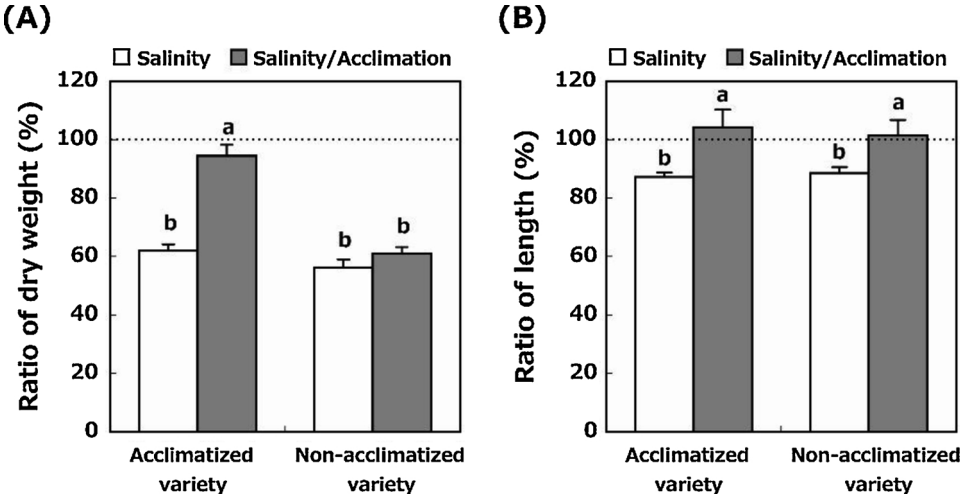

Fig. 2. The ratio of (A) shoot dry weight and (B) shoot length in 18 acclimatized varieties and 11 non-acclimated rice varieties grown under salinity stress conditions. The ratio was calculated for shoot dry weight and shoot length under salinity stress following control conditions (salinity) and salinity stress following acclimation (salinity/acclimation). Data show the means of quadruplicates ± SE. The same letters indicate no significant differences at $p < 0.05$.

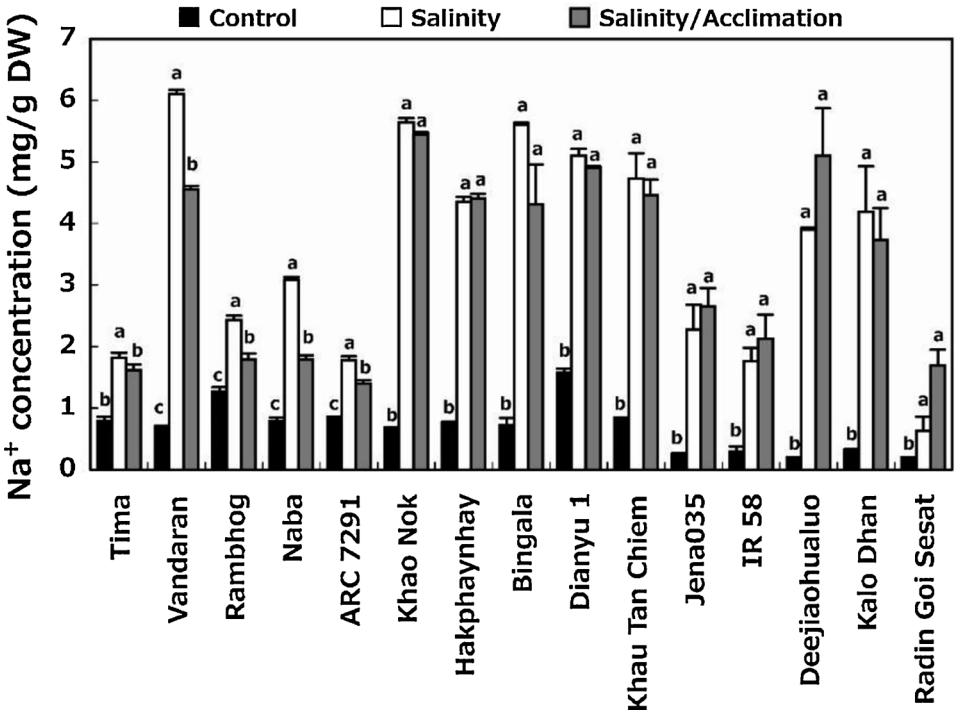

Fig. 3. Na^+ concentrations in the leaf blades of rice varieties grown under control and salinity stress conditions. Data show the means of quadruplicates ± SE. The same letters indicate no significant differences at $p < 0.05$.

stress is known to impose dehydration, membrane damage, and lipid peroxidation in plants; therefore, these can be physiological indicators of salinity tolerance. RWC was measured to estimate the amount of water loss under salinity stress conditions. Salinity stress decreased RWC in all varieties tested; however, acclimation treatment improved RWC in salt acclimatized Rambhog seedlings (Fig. 5A). The cell membrane stability under salinity conditions was measured using the ELR. The results indicated that the two acclimatized varieties exhibited lower electrolyte leakage than did the non-acclimatized varieties under salinity stress (Fig. 5B). Oxidative damage was measured through the production of MDA in the leaf tissues. The non-acclimatized variety produced higher concentrations of MDA compared to acclimatized varieties under salinity stress, indicating that these varieties suffered from oxidative damage caused by salinity stress (Fig. 5C).

3.3. Effect of salt acclimation on Na^+ and K^+ accumulation in different organs

After 2 weeks of acclimation to 1 mM NaCl, acclimatized seedlings of all varieties accumulated increased amounts of Na^+ compared to that of the control plants in all examined tissues (Fig. 6A−C). However, at the end of the salinity stress treatment, the three varieties showed differential patterns of Na^+ accumulation in the roots, sheaths, and leaf blades. In Rambhog, 1 mM NaCl acclimation led to a reduction in Na^+ accumulation in the acclimatized seedlings relative to that of the non-acclimatized ones in all examined organs under salinity stress

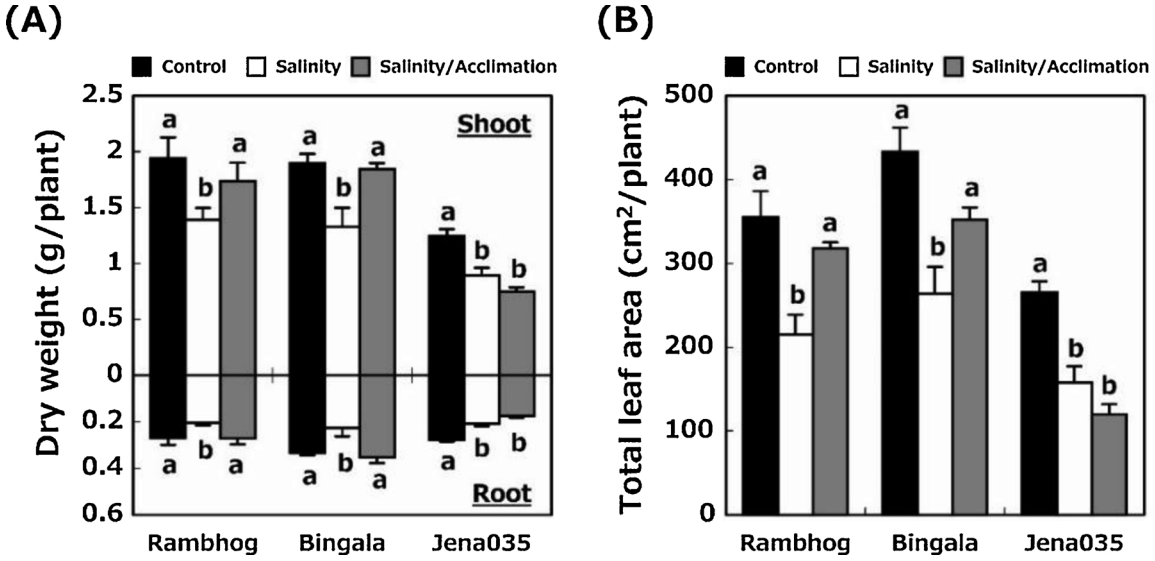

Fig. 4. (A) Shoot and root dry weights and (B) total leaf area in Rambhog, Bingala, and Jena035 under control and salinity stress conditions. Data represent the means of quadruplicates ± SE. The same letters indicate no significant differences at $p < 0.05$.

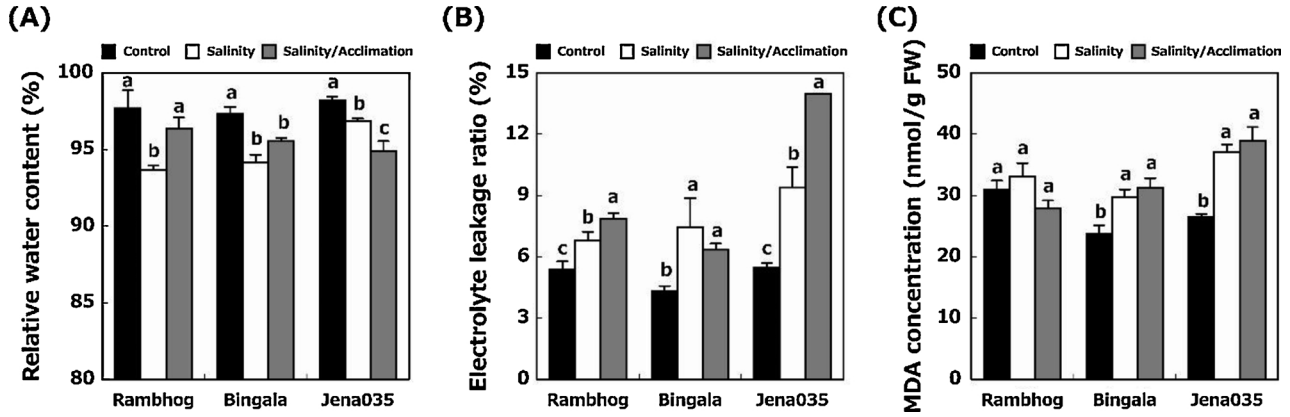

Fig. 5. (A) Relative water content, (B) electrolyte leakage ratio, and (C) MDA concentration (B) of Rambhog, Bingala, and Jena035 under control and salinity stress conditions. Data represent the means of quadruplicates ± SE. The same letters indicate no significant differences at $p < 0.05$.

conditions. On the other hand, 1 mM NaCl acclimation reduced the Na^+ accumulation in roots and sheaths in Bingala under the 50 mM NaCl salinity conditions (Fig. 6A, B), whereas it did not reduce leaf blade Na^+ accumulation (Fig. 6C). In the non-acclimatized variety, Jena035, 1 mM NaCl acclimation treatment did not reduce the Na^+ accumulation in any of the organs of acclimatized plants over non-acclimatized plants under 50 mM NaCl salinity (Fig. 6A − C). Hereafter, Rambhog is denoted 'acclimatized variety with low Na^+ accumulation in the leaf blades', while Bingala is denoted 'acclimatized variety with high Na^+ accumulation in the leaf blades'.

Two weeks of 1 mM NaCl acclimation treatment did not affect the K^+ concentrations in all examined organs of Rambhog, whereas the K^+ concentrations were decreased in the roots and leaf blades of Bingala and in the sheaths and leaf blades of Jena035 (Fig. 7A − C). Salinity stress significantly reduced the K^+ concentrations in the roots, sheaths, and leaf blades of the three varieties. However, in both types of acclimatized varieties, Rambhog and Bingala, K^+ accumulation in the roots and leaf blades were improved under salinity stress by the acclimation to 1 mM NaCl. Acclimation treatment did not change K^+ accumulation in the non-acclimatized variety Jena035 under salinity stress, which may be one of the reasons why this variety could not acquire improved salinity tolerance with acclimation treatment.

3.4. Relative expression of genes encoding Na^+ transporter in salt acclimation process

For salt tolerance, regulation of Na^+ transport to the shoot is crucial for maintaining regular metabolic processes [28]. Therefore, expression profiles of genes encoding Na^+ transporters were studied to determine the mechanisms of Na^+ transporters in the salt acclimation process. In our study, two selected genes (*OsHKT1;5* and *OsNHX1*), which are widely considered to be the key components of salt tolerance mechanisms, were analyzed in both leaf blades and roots of the three selected varieties.

OsHKT1;5 is a Na^+ transporter localized in the root xylem, which retrieves Na^+ from the xylem stream to xylem parenchyma cells, thereby reducing Na^+ accumulation in the shoots. Quantitative RT-PCR analysis showed that acclimation treatment with 1 mM NaCl induced expression of the *OsHKT1;5* gene by 1.3-fold in the roots of Rambhog, but not in those of Bingala and Jena035 (Fig. 8A). Under salt stress, expression of the *OsHKT1;5* gene was highly induced by 31-fold in Rambhog, but not in Bingala and Jena035. Overall, acclimation treatment enhanced the induction of *OsHKT1;5* expression by 60-fold in Rambhog, 7.1-fold in Bingala, and 2.5-fold in Jena035.

Expression of the *OsNHX1* gene in the leaf blades was also studied to assess the contribution of Na^+ compartmentalization into vacuoles to salt tolerance. Acclimation treatment slightly induced the expression of the *OsNHX1* gene in Rambhog, but not in Bingala and Jena035 (Fig. 8B). Under salt stress, *OsNHX1* expression was induced by 1.8-fold and acclimation treatment enhanced it by 4.6-fold in Bingala. Acclimation treatment was also effective for enhanced expression of the *OsNHX1* gene in Rambhog and Jena035. Salt stress did not affect the expression of *OsNHX1* in the roots (data not shown).

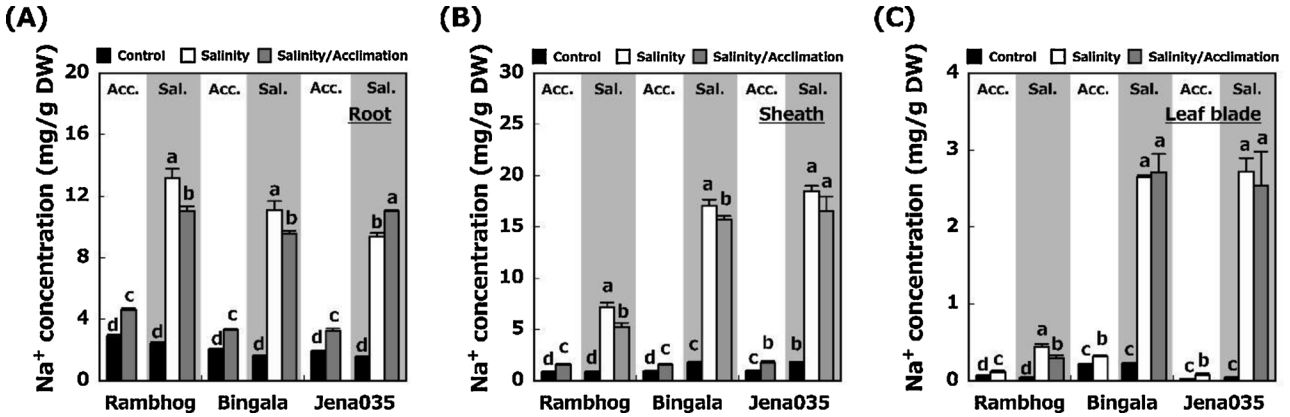

Fig. 6. Na^+ concentration in the (A) roots, (B) sheaths, and (C) leaf blades of Rambhog, Bingala, and Jena035 under control and salinity stress conditions. Na^+ concentration was determined using each organ of control seedlings and acclimated seedlings at the end of the acclimation period (Acc.) and that of control seedlings, salinized seedlings, and seedlings salinized after acclimation to salinity stress (Sal.). Data represent the means of quadruplicates ± SE. The same letters indicate no significant differences at $p < 0.05$.

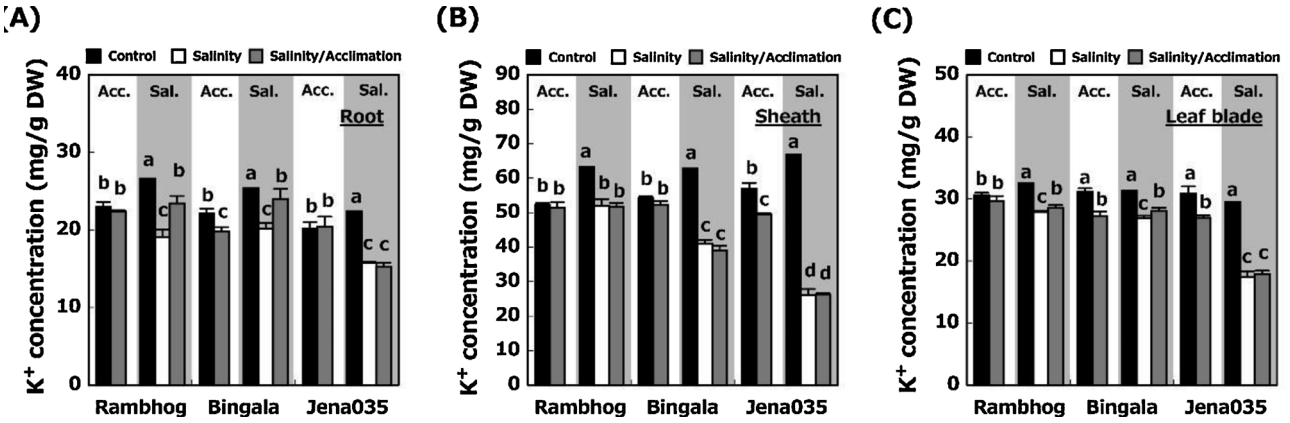

Fig. 7. K^+ concentration in the (A) roots, (B) sheaths, and (C) leaf blades of Rambhog, Bingala, and Jena035 under control and salinity stress conditions. K^+ concentration was determined using each organ of control seedlings and acclimated seedlings at the end of the acclimation period (Acc.) and that of control seedlings, salinized seedlings, and seedlings salinized after acclimation (Sal.). Data represent the means of quadruplicates ± SE. The same letters indicate no significant differences at $p < 0.05$.

4. Discussion

Plants exposed to lower levels of stress enhanced tolerance to subsequent higher levels of stress; this phenomenon is known as acquired tolerance or acclimation. Salt acclimation can be achieved through seed priming [22,29] or by adapting the seedlings to a lower stress level for a certain period of time [18,20,21]. However, the mechanisms of acclimation to salt stress are not clearly understood. In this study, we attempted to elucidate the salt acclimation ability of rice varieties through screening, physiological mechanisms, and gene expression studies. The acclimation process in rice primarily relies on the interactions between the variety and its response to pretreatment (Fig. 2A, B). Of the 70 rice varieties screened, 18 varieties improved biomass both in dry mass production and shoot length by acclimation pretreatment prior to salt stress. However, the acclimation treatment was effective in improving shoot length, but not dry mass production of 11 varieties, suggesting that shoot length cannot be an indicator of the selection of acclimatized varieties. Acclimatized varieties exhibited two different patterns of Na^+ accumulation in the leaf blades (Fig. 3). A group of low Na^+accumulating varieties, including Rambhog, Tima, Vandaran, Naba, and ARC 7291, increased Na^+ accumulation in the leaf blades under salt stress, whereas acclimatized seedlings of these varieties reduced Na^+ accumulation. The other group was the high Na^+accumulating varieties, including Bingala, Khao Nok, Hakphaynhay, Dianyu 1, and Khau Tan Chiem, which did not reduce Na^+ accumulation under salt stress by acclimation treatment. These findings imply that the rice varieties have two different acclimation mechanisms to salt stress through the governance of the Na^+ transport systems.

4.1. Mechanism of Na^+ exclusion from the shoots in the salt acclimation process

OsHKT1;5 retrieves Na^+ from the xylem to the xylem parenchyma cells, resulting in the reduction of Na^+ transport to the shoots [15] and also maintains shoot K^+ homeostasis under salt stress [8]. Therefore, the role of OsHKT1;5 is critically important for regulation of Na^+ accumulation in shoots. Varietal differences were found in regulation of the *OsHKT1;5* expression under salt stress. For example, under moderate salt stress conditions, induced expression of the *OsHKT1;5* gene was observed in the roots of the salt-tolerant variety Pokkali, but not in the salt-sensitive cultivar IR 29 [30]. The same tendency was also found in the salt-tolerant cultivar Egyptian Yasmin, which showed induced expression of the *OsHKT1;5* gene in the roots; however, it was repressed in the salt-sensitive cultivar Sakha102 [31]. In our study, one of the low Na^+ acclimatized varieties, Rambhog, significantly enhanced expression of the *OsHKT1;5* gene in the roots when the seedlings were salt acclimatized prior to salt stress treatment (Fig. 8A). Because acclimatized Rambhog seedlings improved growth and reduced Na^+ accumulation in the leaf blades relative to those without acclimation treatment under salt stress, the Na^+ exclusion mechanism in the salt acclimation process is mainly governed by OsHKT1;5. These findings implied that the magnitude of induction of *OsHKT1;5* expression in the roots may be related to salt tolerance through the Na^+ exclusion mechanism.

Acclimation treatment was also effective in enhancing expression of

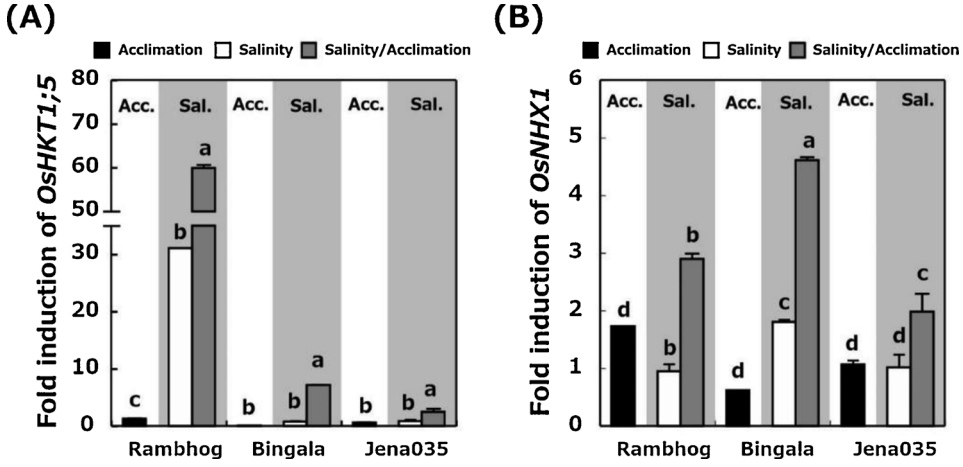

Fig. 8. Relative expression of the genes encoding Na^+ transporter proteins (A) OsHKT1;5 in the roots and (B) OsNHX1 in the leaf blades of Rambhog, Bingala, and Jena035 under control and salinity stress conditions. Fold induction was estimated by the comparative $2^{-\Delta\Delta CT}$ method at the end of the acclimation period (Acc.) and at the end of salinity stress (Sal.). Data represent the means of two independent experiments ± SD.

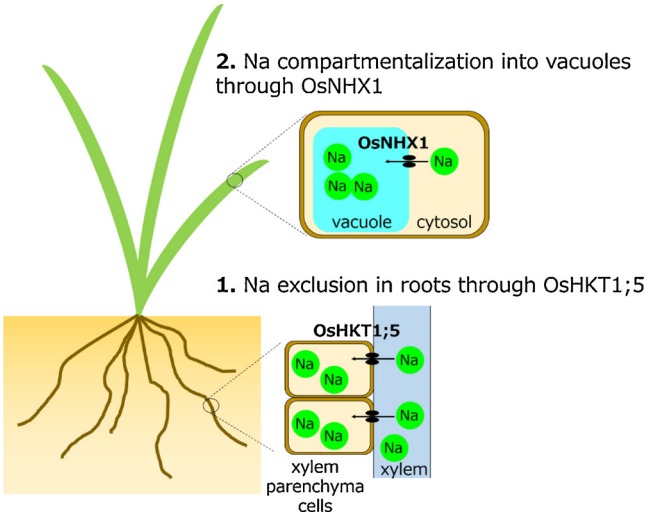

Fig. 9. Mechanisms of acquired salinity tolerance in acclimatized rice varieties identified in this study and classified into two groups: acclimation with lower Na^+ accumulation in the leaf blades and acclimation with higher Na^+ accumulation in the leaf blades under salinity stress. The mechanism of Na^+ exclusion from shoots is activated by acclimation treatments in acclimatized varieties with lower Na^+ accumulation in the leaf blades through induction of the OsHKT1;5 gene in roots. Because active OsHKT1;5 retrieves Na^+ from the transpiration stream in the xylem to xylem parenchyma cells, Na^+ concentration is maintained at lower levels in the leaf blades of acclimatized varieties under salinity stress. The mechanism of Na^+ compartmentalization into vacuoles of the leaf blades is activated by acclimation treatments in acclimatized varieties with higher Na^+ accumulation in the leaf blades through induction of the OsNHX1 gene in the leaf blades. Because active OsNHX1 compartmentalizes Na^+ into vacuoles of the leaf blades, acclimatized varieties confer salinity tolerance even though Na^+ concentrations in the leaf blades reach higher levels under salinity stress.

the *OsHKT1;5* gene in the roots of Bingala and Jena035 under salt stress, although these varieties did not induce expression of the *OsHKT1;5* gene in the roots without acclimation treatment. The mechanisms of acclimation-induced expression of the *OsHKT1;5* gene are not yet understood; however, one possibility is that acclimation may activate an unidentified transcriptional regulator to boost transcription of the *OsHKT1;5* gene under salt stress.

4.2. Mechanism of tissue tolerance in the salt acclimation process

Tissue tolerance is achieved by the effective handling of accumulated Na^+ without detrimental effects on the cellular metabolism in the cytosol [32]. This could be achieved by the tonoplast-localized Na^+/H^+ antiporter NHX1 [2]. In *Arabidopsis thaliana,* salinity tolerance is correlated with higher transcription levels of *AtNHX1* in leaf tissues [33]. In addition, overexpression of *AtNHX1* in tomatoes has improved salt tolerance, despite the high accumulation of Na^+ in leaves [34]. However, some recent studies have shown that NHX proteins are also involved in K^+ homeostasis to enhance salt tolerance [35,36]. To study the relevance of NHX function in acquired salt tolerance of rice, expression profiles of the *OsNHX1* gene in the roots and leaf blades were examined. Salt stress did not affect the expression of *OsNHX1* in the roots of the three rice varieties, implying that the contribution of OsNHX1-mediated Na^+ compartmentalization into vacuoles to salt tolerance was not significant. On the other hand, salt stress significantly induced the expression of the *OsNHX1* gene in the leaf blades of Bingala after acclimation treatment, and the magnitude of induction was much higher in acclimatized seedlings (by 4.6-fold) than in non-acclimatized seedlings (by 1.8-fold). Upon acclimation treatment, Bingala showed better growth under salt stress (Fig. 4A, B), although higher Na^+ accumulation was observed in both acclimatized and non-acclimatized seedlings (Fig. 6C). These results suggested that highly induced expression of the *OsNHX1* gene may participate in improving tissue tolerance to high Na^+ accumulation in the leaf blades under salt stress. Increased expression of the *OsNHX1* gene was also found in Rambhog and Jena035 with acclimation treatment under salt stress. Similar to the expression pattern of the *OsHKT1;5* gene, rice seedlings may induce expression of the genes encoding Na^+ transporters by acclimation treatment under salt stress.

Physiological responses of acclimation to salt stress vary among plant species. Acclimation treatment reduced Na^+ accumulation [20] and prevented K^+ leakage [37] in the leaves of pea plants under salt stress. Pretreatment of the sublethal level of NaCl on rice seedlings also reduced Na^+ accumulation in the leaves [18]. However, wheat did not exhibit reduced Na^+ concentration in the leaves, although acclimation treatment was effective in the improvement of some physiological parameters [17]. The relevance of the function of SOS1, a plasma membrane-localized Na^+/H^+ antiporter, to the acclimation process was also discussed through the screening of *Arabidopsis thaliana* accessions [38,39]. Although acclimation mechanisms seem to be divergent among plant species, regulation of Na^+ accumulation through Na^+ transporters may play a central role in the acclimation mechanism of salt-sensitive plant species.

5. Conclusion

This study demonstrated that rice varieties have the ability to acclimate to low NaCl stress and improve growth under subsequent salt stress. Salt acclimation ability primarily depends on varietal variation and their response to pretreatment. The salt acclimation process led to two distinct patterns of Na^+ accumulation: low and high leaf Na^+ accumulation. Pretreated plants of acclimatized varieties showed enhanced salt tolerance compared to non-acclimatized varieties because of their ability to restrict and effectively compartmentalize the leaf Na^+ under salt stress. Differences in the mechanisms of salt acclimation between varieties may be partly explained by the distinct regulation of gene expression of two key Na^+ transport proteins (Fig. 9). This was evidenced in the induced expression of the *OsHKT1;5* gene in pretreated acclimatized variety plants, which had low Na^+ accumulation by excluding Na^+ from the shoots, whereas the *OsNHX1* gene mainly functions in the acclimatized variety with high Na^+ accumulation by the efficient compartmentalization of Na^+.

Declaration of Competing Interest

The authors declare there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2020.110517.

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