Plant Science 287 (2019) 110171

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Characterization of Na^+ exclusion mechanism in rice under saline-alkaline stress conditions

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ARTICLE INFO

Keywords:
Na^+ exclusion
Na^+ influx
OsHKT1;5
OsSOS1
Saline-alkaline tolerance

ABSTRACT

This study was designed to elucidate the physiological responses of two rice genotypes to different pH levels under high saline stress. A salt-tolerant cultivar, FL478, and a salt-sensitive cultivar, IR29, were exposed to saline-alkaline solutions supplemented with 50 mM Na at pH 9 (severe), pH 8 (moderate), and pH 7 (mild) for three weeks. The results indicated that FL478 is relatively saline-alkaline tolerant compared to IR29, and this was evident from its higher dry mass production, lower Na^+ concentration in the leaf blades, and maintenance of water balance under both mild and moderate saline-alkaline stress conditions. In both cultivars, Na^+ concentrations in the leaf blades were considerably higher at pH 8 than at pH 7, indicating that high alkaline stress promoted Na^+ accumulation under highly saline conditions. FL478 plants had lower Na^+/K^+ ratios in leaf blades and leaf sheaths than IR29 plants under saline-alkaline stress at both pH 7 and pH 8. To understand the mechanisms behind the difference in saline-alkaline tolerance between the two rice genotypes, transcript levels of the genes encoding Na^+ transport proteins were analyzed. In response to mild and moderate saline-alkaline stress conditions, salt-tolerant FL478 had highly induced expression of the *OsHKT1;5* gene in the roots, corresponding to lower Na^+ accumulation in the leaf blades. Induction of high expression of the *OsSOS1* gene in the roots of FL478 implied that Na may be effectively exported from cytosols to apoplasts in the roots resulting in sequestration of Na^+ to outside of the roots and loading Na^+ in xylem transpiration stream. On the other hand, the salt-sensitive IR29 had lower expression of the genes related to Na^+ transporters, such as the *OsHKT1;5* gene and the *OsSOS1* gene, in the roots, leading to higher Na^+ accumulation in the shoots. Expression of the determinant genes for alkaline tolerance, such as K^+ and Fe acquisition and acidification of the rhizosphere was highly induced in FL478, but not in IR29. Thus, molecular analysis suggested that genes encoding Na^+ transport proteins are involved in regulating Na^+ transport under saline-alkaline stress in both salt-tolerant and salt-sensitive rice cultivars, and this is useful information for improving saline-alkaline tolerance traits of rice in the future.

1. Introduction

Salinity is a global problem in crop cultivation. Millions of hectares of both irrigated and non-irrigated agricultural land are affected by high salt accumulation [1]. In nature, soil salinization often co-occurs with alkalization, especially in low precipitation areas. According to a report published by FAO/UNESCO, it is estimated that 831 million hectares of land is affected by saline-alkaline stress [2]. Saline-alkaline soils are characterized by both high concentration of Na^+ and high pH, which cause more complex stress effects on plants than neutral saline soils [3].

Na^+ is the main ion found in salt-affected soils, but it is not an essential element for the crop plants. The negative effects of salt-affected soils on plant growth can be described in two distinct phases. During the first phase, water uptake by roots is inhibited due to osmotic stress, leading to cell dehydration, and significant reduction in the rate of shoot growth [4]. Ionic stress can be thought of as a second phase response, which becomes gradually more severe as excessive Na^+ accumulates through disturbing ion homeostasis in plant cells [5]. In addition, soil pH is an important factor in the regulation of plant growth. The pH of saline-alkaline soils is often greater than 8.0, which is unsuitable for plant growth and development. Several studies have reported that the detrimental effects of saline-alkaline stress are more obvious than those of saline stress [6,7]. Thus, plants growing in saline-alkaline soils have to cope with both physiological drought and Na^+ toxicity in addition to the cellular damages induced by high pH.

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https://doi.org/10.1016/j.plantsci.2019.110171
Received 10 March 2019; Received in revised form 22 May 2019; Accepted 12 June 2019
Available online 16 June 2019
0168-9452/

Rice is one of the most important human crops in the world. It is classified as a glycophyte and its physiological traits are poor when exposed to excessive Na^+ in the soil compared to other crops [5]. Genetic studies by Sahi et al. reported that rice genotypes differ in their sensitivity to saline stress due to additive gene effects [8]. Some rice genotypes have been well documented to be tolerant to saline stress, including FL478, the recombinant inbred line created using the salt-tolerant Pokkali cultivar and salt-sensitive IR29 [9]. To date, extensive studies have focused on the physiological and molecular biological mechanisms behind saline stress in plants [10,11]. However, few studies have been conducted to examine saline-alkaline stress.

Several genes involved in salt tolerance in rice have been studied. The genes encoding Na^+ transport proteins such as SOS and HKT are considered to be important factors in the control of Na^+ accumulation in plant cells under saline stress [12–14]. SOS1 (Salt Overly Sensitive 1) encodes Na^+/H^+ antiporters which are localized in plasma membranes and are responsible for the transportation of Na^+ from the cytosol to the apoplast [15,16]. A study by Shi et al. on Arabidopsis showed that SOS1 could function in both Na^+ loading into and retrieval from the xylem; under mild saline stress at 25 mM NaCl, SOS1 may mediate active loading of Na^+ to the xylem, whereas, at high salinity (100 mM NaCl), expression of SOS1 was induced and was responsible for the retrieval of Na^+ from the xylem [17]. HKT transporters are categorized into K^+/Na^+ uniporters or K^+-Na^+ symporters [18–20]. In addition, recent functional analysis showed that OsMGT1 in rice is required to confer salt tolerance via the enhancement of the transport activity of OsHKT1;5 [21]. In recent decades, a few rice genotypes have been analyzed for their combined saline and alkaline responses, and the tolerance mechanisms behind both stresses have not been well-understood. Therefore, elucidating the molecular and physiological mechanisms by which rice genotypes respond and adapt to saline-alkaline stress are crucial.

The objective of this study was to investigate differences in both physiological and molecular responses of two rice genotypes, salt-tolerant FL478 and salt-sensitive IR29, to a combination of saline stress at 50 mM Na and high alkaline stress at either pH 9 (severe), pH 8 (moderate), or pH 7 (mild). Molecular analysis revealed that the saline-alkaline tolerance of salt-tolerant FL478 is associated with inducing expression of the *OsHKT1;5* gene and alkaline-responsive genes in the roots under saline-alkaline conditions. These findings suggest that saline-alkaline tolerance in rice plants is correlated with the expression of Na^+ transporters.

2. Materials and methods

2.1. Plant materials and growth conditions

Two rice (*Oryza sativa* L.) genotypes, FL478 (salt-tolerant) and IR29 (salt-sensitive), were used in this study. FL478 is known as a salt-tolerant recombinant inbred line, IR66946-3R-178-1-1 [9], and was developed by crossing a salt-tolerant indica landrace, Pokkali, with a salt-sensitive indica genotype, IR29 [22,23]. After incubation in tap water at 60 °C for 10 min., seeds of each genotype were surface-sterilized with 5% (v/v) sodium hypochlorite solution for 30 min. and were then thoroughly rinsed with distilled water. Seeds were subsequently soaked in tap water for 24 h at 30 °C. The germinated seeds were transferred onto a nylons mesh floating in 2-L plastic pots containing tap water for one week. Then, the uniform seedlings were selected and grown in half-strength slightly modified Kimura B nutrient solution containing the following macronutrients (mM): 0.18 $(NH_4)_2SO_4$, 0.27 $MgSO_4{\cdot}7H_2O$, 0.09 KNO_3, 0.18 $Ca(NO_3)_2{\cdot}4H_2O$ and 0.09 KH_2PO_4 and the following micronutrients (μM): 28 $FeSO_4{\cdot}7H_2O$, which was used instead of Fe-EDTA, 9 $MnSO_4{\cdot}5H_2O$, 48 H_3BO_3, 9 $Na_2MoO_4{\cdot}2H_2O$, 0.7 $ZnSO_4{\cdot}7H_2O$ and 0.3 $CuSO_4{\cdot}5H_2O$ [24]. This hydroponic experiment was carried out in a growth chamber maintained at 28/25 °C (16 h light period/ 8 h dark period) under a photosynthetic photon flux density of 400/0 μmol m^{-2} s^{-1} (day/night) at relative humidity of 70%. At day 21, the 4–5 leaf stage rice seedlings were transferred to either half-strength Kimura B nutrient solution (control: pH 5.0–5.5) or to a saline-alkaline nutrient solution supplemented with 50 mM Na at a pH of either 9 (severe), pH 8 (moderate), or pH 7 (mild) for three weeks. The components of the saline-alkaline treatments used were listed in Table 1. All treatments were performed with four replicates. The pH of the nutrient solution was measured daily using a pH meter (AS700 Type) and was regulated with either 2 N HCl or 2 N KOH throughout the growth period. The nutrient solution was renewed every four days, and water lost by evaporation was compensated for by daily addition of tap water.

Table 1
Chemicals used for saline-alkaline treatments.

Treatments	Supplements	pH
Control	–	5.0–5.5
Mild saline-alkaline stress (pH 7 + 50 mM Na)	1 mM $NaHCO_3$ + 49 mM NaCl	7.0
Moderate saline-alkaline stress (pH 8 + 50 mM Na)	8 mM $NaHCO_3$ + 42 mM NaCl	8.0
Severe saline-alkaline stress (pH 9 + 50 mM Na)	45 mM $NaHCO_3$ + 2.5 mM Na_2CO_3	9.0
Mild alkaline stress (pH 7 + 5 mM K)	1 mM $KHCO_3$ + 4 mM KCl	7.0
Moderate alkaline stress (pH 8 + 5 mM K)	4.75 mM $KHCO_3$ + 0.25 mM KCl	8.0

2.2. Physiological parameters

After three weeks of saline-alkaline treatment, the fresh weight (FW) of the 42-day-old seedlings was measured following the separation of leaf blades, leaf sheaths and roots. To determine dry weight (DW), leaf blades, leaf sheaths and roots were dried at 70 °C for three days prior to being weighed. The water contents in the leaf blades was calculated using the equation (FW-DW)/FW.

2.3. Determination of Na^+ and K^+ concentrations

The Na^+ and K^+ concentrations in leaf blades, leaf sheaths, and roots were measured using a flame photometer (ANA-135; Tokyo Photoelectric, Tokyo, Japan) as described previously [25]. Dried samples were gently agitated in 1 N HCl overnight, and the concentrations of Na^+ and K^+ ions were estimated from the Na^+ and K^+ standard curves.

2.4. Expression analysis of the genes encoding Na^+ transport proteins

Total RNA was extracted from leaf sheaths and roots of control and saline-alkaline stressed FL478 (salt-tolerant) and IR29 (salt-sensitive) cultivars using a TRIzol reagent. After digestion with DNaseI, total RNA (0.5 μg) was reverse-transcribed to cDNA using a ReverTra Ace qPCR RT kit, according to the manufacturer's protocol (Toyobo, Osaka, Japan). Quantitative polymerase chain reaction was performed using a TUNDERBIRD SYBR qPCR Mix and ABI StepOne System (Applied Biosystems, CA) as previously described [26]. The reaction mixture contained 7.5 μl of THUNDERBIRD SYBR qPCR Mix, 0.3 μl of 50 × ROX reference dye, 1.5 μl of forward primer, 1.5 μl of reverse primer, 1 μl of cDNA, and 3.2 μl of RNase-free water. Quantitative RT-PCR was performed using the following profile: an initial incubation at 95 °C for 1 min, followed by 45 cycles of denaturation at 95 °C for 15 s and extension at 60 °C for 60 s. Relative expression levels of the gene transcripts were calculated using the comparative $2^{-\Delta\Delta CT}$ method [27] with the *Os25SrRNA* gene as an internal control [28]. Data shown are the average of two technical replicates using RNA extracted from the pooled tissues of four seedlings. The sequences of the primers used are listed in the Supplemental Table 1.

2.5. Statistical analysis

The collected data were subjected to One-Way Analysis of Variance (ANOVA) using the SPSS statistics package, version 22 (IBM Inc., USA), and the means (n = 4) were separated using Duncan's multiple range test at $P < 0.05$.

3. Results

3.1. Effect of saline-alkaline stress on physiological parameters

To compare the tolerance of FL478 (salt-tolerant) and IR29 (salt-sensitive) plants to saline-alkaline stress, three-week-old seedlings of the two rice cultivars were exposed to nutrient solutions supplemented with 50 mM Na at either pH 9 (severe), pH 8 (moderate), or pH 7 (mild) for three weeks. After one week of treatment, no differences in the growth of salt-sensitive IR29 were visually observed when it was grown under mild or moderate saline-alkaline stress (50 mM Na + pH 7 or pH 8). Upon exposure of the two rice cultivars to severe saline-alkaline stress (50 mM Na + pH 9), wilting and death of some seedlings were observed in both rice cultivars within one week, and all rice seedlings were completely dead within two weeks. In addition, under mild and moderate saline-alkaline stress (50 mM Na + pH 7 or pH 8), smaller leaf blades were observed in the salt-tolerant FL478 compared to the control (data not shown). The dry weight (DW) of both rice cultivars was affected by saline-alkaline stress treatments (Fig. 1). Under moderate saline-alkaline stress (50 mM Na + pH 8), the shoot DW of salt-sensitive IR29 plants drastically decreased by 69.3% in comparison with the salt-tolerant FL478 (52.8%). In addition, FL478 showed slight decreases in shoot DW (29.9%) compared to IR29 (76.0%) under mild stress (50 mM Na + pH 7). The root DW of each rice cultivar was not significantly affected by saline-alkaline stress at either pH 7 or pH 8. Additionally, the results showed that mild saline-alkaline stress resulted in decreased shoot and root lengths in FL478 and IR29. However, the root lengths of both rice cultivars increased when moderate saline-alkaline stress was applied, while their shoot length decreased under the same condition (Fig. 2).

To estimate the amount of water loss under saline-alkaline stress conditions, water content (WC) was measured using leaf tissues. The results indicated that salt-tolerant FL478 exhibited a greater potential to maintain tissue water than salt-sensitive IR29. Under mild and moderate saline-alkaline stress conditions (50 mM Na + pH 7 or pH 8), the WC of IR29 plants decreased from 75.1% in control plants to 70.5% and 67.3% at pH 7 and pH 8, respectively. However, there was no significant difference in the WC of FL478 plants between mild (75.1%) and moderate (75.3%) saline-alkaline stress conditions (Fig. 3). These results indicated that salt-tolerant FL478 maintained a better physiological status than salt-sensitive IR29 under conditions of both mild and moderate saline-alkaline stress.

3.2. Effects of saline-alkaline stress on Na^+ and K^+ accumulation in different organs

In both rice cultivars, saline-alkaline stress led to increased Na^+ concentration in all organs examined (Fig. 4). Under mild and moderate (50 mM Na + pH 7 or pH 8) saline-alkaline stress conditions, salt-tolerant FL478 accumulated less Na^+ in the leaf blades than salt-sensitive IR29 (Fig. 4A). In the leaf sheaths, there was no significant differences in Na^+ concentration between the two rice cultivars when moderate saline-alkaline stress (50 mM Na + pH 8) was applied. However, under mild saline-alkaline stress (50 mM Na + pH 7), Na^+ concentration was lower in the leaf sheaths of FL478 compared to IR29 (Fig. 4B). Notably, under both mild and moderate saline-alkaline stress conditions, FL478 accumulated a higher concentration of Na^+ in the roots than IR29 (Fig. 4C). These findings suggest that saline tolerance mechanisms such as Na^+ exclusion in the leaf blades (low Na^+ accumulation) and Na^+ compartmentalization in the roots (high Na^+ accumulation) could confer saline-alkaline tolerance in FL478.

Saline-alkaline stress at either pH 7 or pH 8 significantly decreased the K^+ concentration in the leaf sheaths and roots of both rice cultivars compared to the control (Fig. 5B and C). In the leaf blades, there was no significant difference in K^+ concentration in FL478 between mild and moderate saline-alkaline stress conditions, but IR29 accumulated the least amount of K^+ in the leaf blades (21.7 mg/g DW) under moderate saline-alkaline stress compared to other treatments (Fig. 5A).

Maintaining minimal shoot Na^+/K^+ ratios is an important stress tolerance trait in some halophytes and tolerant glycophytes [5,29]. This study found that, under mild and moderate saline-alkaline stress conditions, salt-tolerant FL478 maintained much lower Na^+/K^+ ratio in the leaf blades and leaf sheaths than the salt-sensitive IR29 (Table 2).

3.3. Differential expression of the genes encoding Na^+ transport proteins in response to saline-alkaline stress

To determine the mechanisms underlying differential Na^+ accumulation in the salt-tolerant FL478 and the salt-sensitive IR29, expression profiles of the genes encoding Na^+ transport proteins were analyzed. Several genes involved in salt tolerance have been identified in rice. OsHKT1;5 plays a major role in the transport of Na^+ from the xylem sap into the surrounding xylem parenchyma cells, thereby protecting the leaves from Na^+ toxicity [30]. In the present study, quantitative RT-PCR analyses showed that expression of the *OsHKT1;5* gene was more induced in the roots of the salt-tolerant FL478 than in the salt-sensitive IR29 under both mild and moderate saline-alkaline stress conditions, which suggests that Na^+ transport from xylem sap to xylem parenchyma was active in the roots of FL478 but not in IR29 (Fig. 6A).

In addition, OsMGT1 is a plasma membrane-localized Mg^{2+} transporter involved in salt tolerance in rice through its enhancement of OsHKT1;5 activity [21]. Our study found that, under control and saline-alkaline stress conditions (mild and moderate), *OsMGT1* expression was not detected in the roots of either rice cultivar (data not shown), which

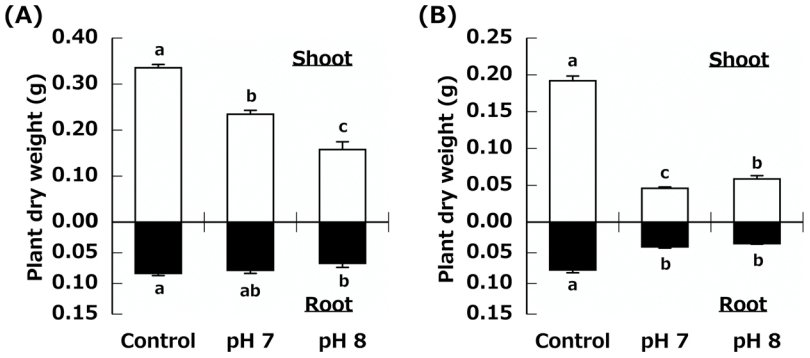

Fig. 1. Plant dry weight (DW) of rice cultivars (A) FL478 (salt-tolerant) (B) IR29 (salt-sensitive) under control and saline-alkaline stress conditions (Severe: 50 mM Na + pH 9, Moderate: 50 mM Na + pH 8, and Mild: 50 mM Na + pH 7) for three weeks. Data represent the means of four replicates ± SE. The same letters indicate no significant difference ($P < 0.05$).

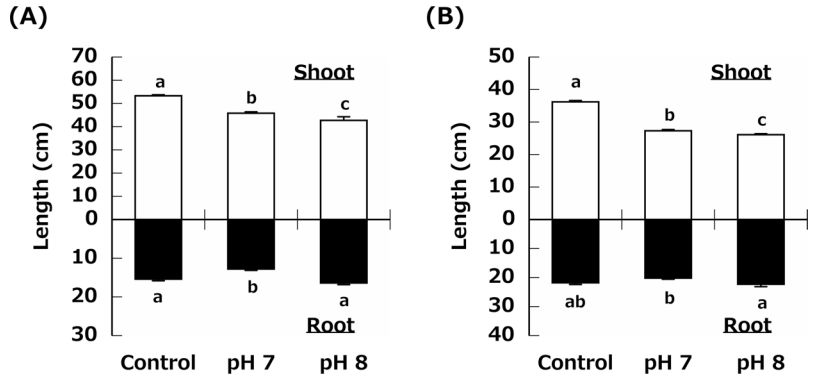

Fig. 2. Effects of saline-alkaline stress on length of two rice cultivars (A) FL478: salt-tolerant and (B) IR29: salt-sensitive. Lengths of shoot and root were measured under control conditions and after three weeks of saline-alkaline conditions. Values are means of four replicates ± standard error.

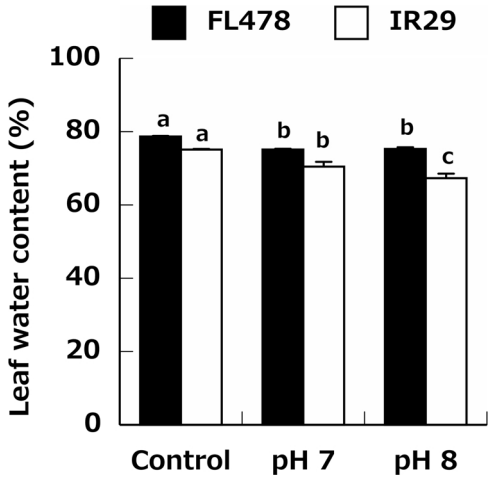

Fig. 3. Effects of saline-alkaline stress on water content of two rice cultivars. Leaf water content was measured under control and after three weeks of saline-alkaline conditions. Values are means of four replicates ± standard error.

suggests that saline-alkaline stress-induced *OsHKT1;5* expression in the roots was not related to the activity of the *OsMGT1* gene.

OsHKT1;4, is known as an alternative candidate for Na^+ exclusion, which is effective in the leaf sheaths, thereby protecting leaf blades from Na^+ toxicity. In response to moderate saline-alkaline stress, 0.20-fold repression of *OsHKT1;4* expression was observed in the leaf sheaths of salt-tolerant FL478, and 0.10-fold repression was measured under mild saline-alkaline stress. While, in the leaf sheaths of salt-sensitive IR29, *OsHKT1;4* expression did not change (0.01-fold) in response to mild or moderate saline-alkaline stress (Fig. 6B).

The Na^+/H^+ antiporter (*SOS1*), localized in the plasma membrane, is considered a general regulator of Na^+ export from the cytosol [31]. Mild saline-alkaline stress (50 mM Na + pH 7) induced expression of the *OsSOS1* gene by 24.8-fold in FL478 roots, but its expression was repressed 0.6-fold in IR29 roots, which suggests that OsSOS1 mediated Na^+ extrusion from the cytosol may not have been active in IR29 when mild saline-alkaline stress was applied. However, when exposed to moderate saline-alkaline stress, expression of the *OsSOS1* gene was greatly induced in the roots of FL478 (44.6-fold) and slightly induced in IR29 (2.2-fold) roots. This result suggests that the salt tolerance mechanisms governed by OsSOS1 in the roots of both rice cultivars are activated in responses to moderate saline-alkaline stress (Fig. 6C).

3.4. Effects of alkaline stress on expression of the genes encoding Na^+ transporters

To further explore the relationship between the genes encoding Na^+ transporters and sole high pH stress, 21-day-old seedlings of two rice cultivars were grown under alkaline conditions without high saline stress at either pH 7 (mild) and moderate (pH 8) for three weeks. Upon exposure of the two rice cultivars to alkaline stress, expression of the *OsHKT1;5* gene was induced greatly in the roots of FL478 by 43.4-fold under moderate alkaline stress (pH 8 + 5 mM K) and by 12.5-fold under mild alkaline stress (pH 7 + 5 mM K) (Fig. 6A). However, expression of the *OsHKT1;5* gene was not highly inducible in the roots of IR29 under both mild and moderate alkaline stresses. In response to alkaline stress at either pH 7 (mild) or pH 8 (moderate), expression of the *OsHKT1;4* gene was repressed in the leaf sheath of FL478 and not affected in that of IR29 (Fig. 6B). Expression patterns of the *OsSOS1* gene in the roots of both rice cultivars were also analyzed (Fig. 6C). Repression of *OsSOS1* expression was observed in the roots of salt-tolerant FL478 under both mild and moderate alkaline stress conditions by 0.2-fold and 0.3-fold, respectively. In contrast, induced expression of the *OsSOS1* gene in the roots of salt-sensitive IR29 was observed under mild alkaline stress (pH 7 + 5 mM K). Induction of *OsSOS1* expression was higher (5.8-fold) mild alkaline stress than under moderate alkaline stress (3.1-fold) in the roots of IR29.

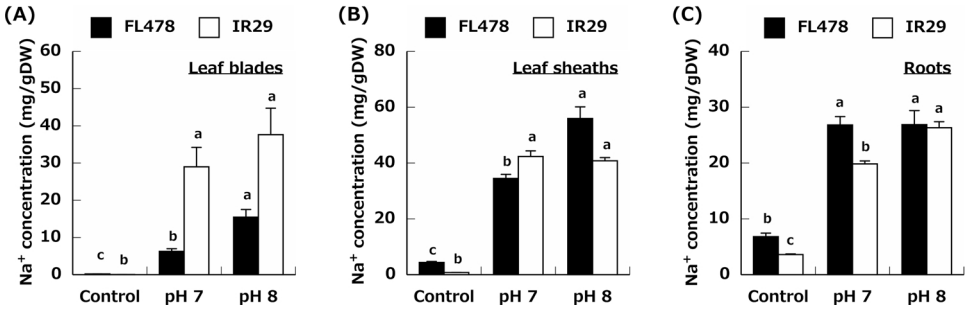

Fig. 4. Na^+ concentrations in (A) leaf blades, (B) leaf sheaths, and (C) roots under control and saline-alkaline conditions. Values are means of three replicates ± standard error.

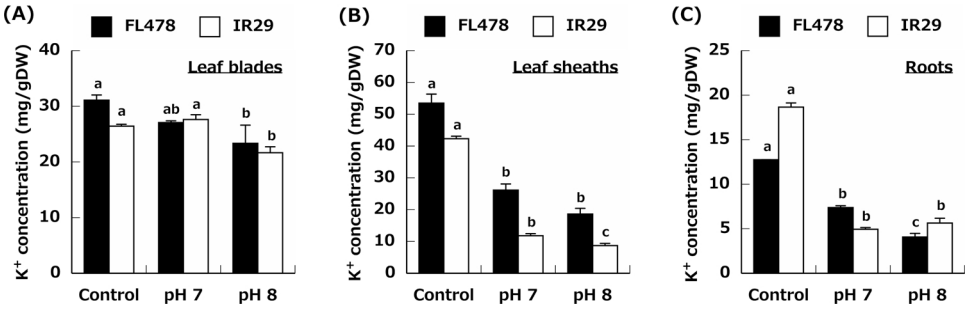

Fig. 5. K^+ concentrations in (A) leaf blades, (B) leaf sheaths, and (C) roots under control and saline-alkaline conditions. Values are means of three replicates ± standard error.

3.5. Transcriptomic responses of alkaline-responsive genes in response to saline-alkaline stress

To further study the mechanisms underlying saline-alkaline stress tolerance in rice, transcription levels of alkaline-responsive genes were analyzed (Fig. 7). Several genes related to K^+ transport such as low affinity K^+ transporter 1 (AKT1) and some members of the HAK/KUP/KT transporters have also been studied in alkaline tolerance in rice [32]. Mild saline-alkaline stress (pH 7 + 50 mM Na) induced expression of the *OsAKT1*, *OsHAK7* and *OsHAK10* genes highly in the roots of FL478 compared to these expressions under moderate saline-alkaline stress (pH 8 + 50 mM Na), while in the roots of IR29, expression of these genes were repressed in response to both mild and moderate saline-alkaline stress conditions (Fig. 7A, B, C). Transcripts of the *OsHAK16* gene in the roots of FL478 was highly detectable under both mild and moderate saline-alkaline stresses, whereas its expression was not detected in IR29 under all conditions tested (data not shown). These results suggested that FL478 activate these K^+ transport system to acquire K^+ more efficiency under saline-alkaline stress.

Expression patterns of Fe deficiency-responsive genes were also investigated, including *OsNAS1*, *OsNAS2*, *OsYSL15*, *OsIRT1* and *OsIRO2* because, in the saline-alkaline soils, Fe is often converted to insoluble forms (hydroxides and oxides) which are unusable for plant growth. Expression of the *OsNAS1* gene showed similar trend to that of the *OsNAS2* gene in the roots of both FL478 and IR29 (Fig. 7D, E). A significantly greater induction of those genes expression was observed under mild saline-alkaline stress (pH 7 + 50 mM Na) than moderate saline-alkaline stress (pH 8 + 50 mM Na). In addition, expression levels of the *OsNAS1* and *OsNAS2* genes were significantly higher in the roots of FL478 than that of IR29 (Fig. 7D, E). Expression level of the *OsIRT1* and *OsIRO2* gene in FL478 roots was greater than that in IR29 roots under both mild and saline-alkaline stress conditions (Fig. 7F, G). Whereas transcripts of the *OsYSL15* genes were not detectable in the roots of two rice cultivars under stress conditions (data not shown). These results imply that the saline-alkaline tolerance of FL478 might also involve with the up-regulation of Fe deficiency-related genes.

Three H^+-ATPase-encoding genes, including *Os03g0689300*, *Os12g0638700* and *Os03g0100800* have been reported to play an important role for saline-alkaline tolerance in rice by mediating proton secretion through the roots [33,34]. Our study found that, in comparison with normal pH condition (pH 5.0–5.5), expression levels of the three genes were not detected in the roots of salt-sensitive IR29 under both mild (pH 7 + 50 mM Na) and moderate (pH 8 + 50 mM Na) saline-alkaline stress conditions (data not shown). In contrast, in the roots of salt-tolerant FL478, expression of the plasma membrane H^+-ATPase genes showed a distinct pattern; a higher level of the *Os03g0689300* gene transcript was observed in both mild (33.0-fold) and moderate (5.1-fold) saline-alkaline stress conditions (pH 7 and pH 8 + 50 mM Na) (Fig. 7H) and an increase in expression of the *Os03g0100800* gene was observed in the roots of FL478 under mild saline-alkaline stress (3.1-fold), but reduced under moderate saline-alkaline stress (0.5-fold) (Fig. 7I). These results indicated that in the roots of salt-tolerant FL478, function of the H^+-ATPase might be active through transcriptomic regulation under various saline-alkaline stress conditions, but not in the salt-sensitive rice cultivar.

4. Discussion

FL478 is known as a salt-tolerant rice genotype, which was developed by IRRI and was created by crossing Pokkali and IR29 genotypes. Thomson et al. reported that FL478 has seedlingstage salt tolerance up to 18 dS/m [35]. However, the molecular physiological mechanisms of saline-alkaline tolerance in FL478 plants remain largely unknown. In the present study, the effects of severe (50 mM Na + pH 9), moderate (50 mM Na + pH 8), and mild (50 mM Na + pH 7) saline-alkaline stress conditions on salt-tolerant FL478 and a salt-sensitive, IR29 were evaluated. FL478 plants were more tolerant to mild or moderate saline-alkaline stress than IR29 plants, as shown by lower reductions in the biomass production of shoots and roots (Fig. 1). The greater dry matter production of FL478 plants may be caused by their higher leaf water content and shoot height compared to IR29 plants under both mild and moderate saline-alkaline stress conditions. Results further showed that FL478 plants had lower leaf blade and leaf sheath Na^+/K^+ ratios than IR29 in both mild and moderate saline-alkaline stress conditions. One important finding in this research is that FL478 plants can maintain lower Na^+ concentrations in the leaf blades and higher Na^+ concentrations in the roots than IR29 plants under high saline-alkaline stress at either pH 7 or pH 8; these finding suggest that FL478 is not

Table 2
Na^+/K^+ ratio in the leaf blades, leaf sheaths and roots under mild and moderate saline-alkaline stress conditions. Values are the mean of three replicates ± standard error.

Treatments	Cultivars	Leaf blades	Leaf sheaths	Roots
Control	FL478	0.01 ± 0.00	0.08 ± 0.01	0.53 ± 0.05
	IR29	0.00 ± 0.00	0.02 ± 0.00	0.19 ± 0.01
Mild saline-alkaline stress	FL478	0.23 ± 0.02	1.34 ± 0.14	3.63 ± 0.13
(50 mM Na + pH 7)	IR29	1.05 ± 0.18	3.62 ± 0.35	4.02 ± 0.21
Moderate saline-alkaline stress	FL478	0.68 ± 0.09	3.12 ± 0.57	6.76 ± 0.91
(50 mM + pH 8)	IR29	1.74 ± 0.33	4.77 ± 0.53	4.73 ± 0.38

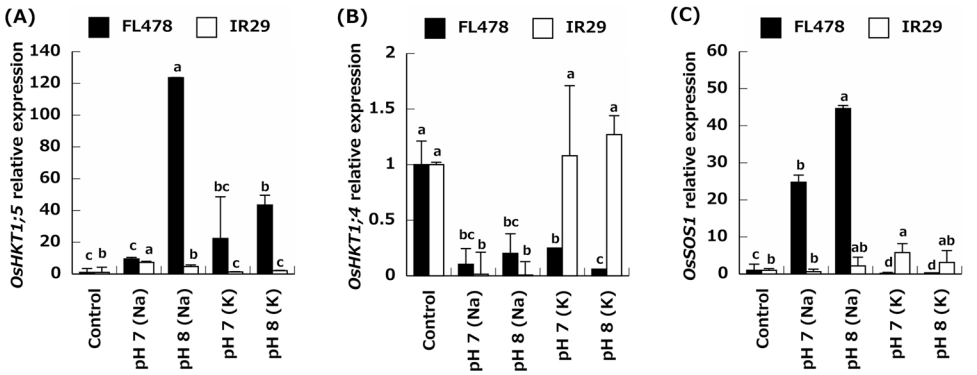

Fig. 6. Relative expressions of genes encoding Na^+ transport proteins. (A) *OsHKT1;5* in the roots, (B) *OsHKT1;4* in the leaf sheaths, and (C) *OsSOS1* in the roots of rice seedlings of the cultivars FL478 and IR29 grown under control and saline-alkaline stress conditions for three weeks. Data represent the means of two independent experiments.

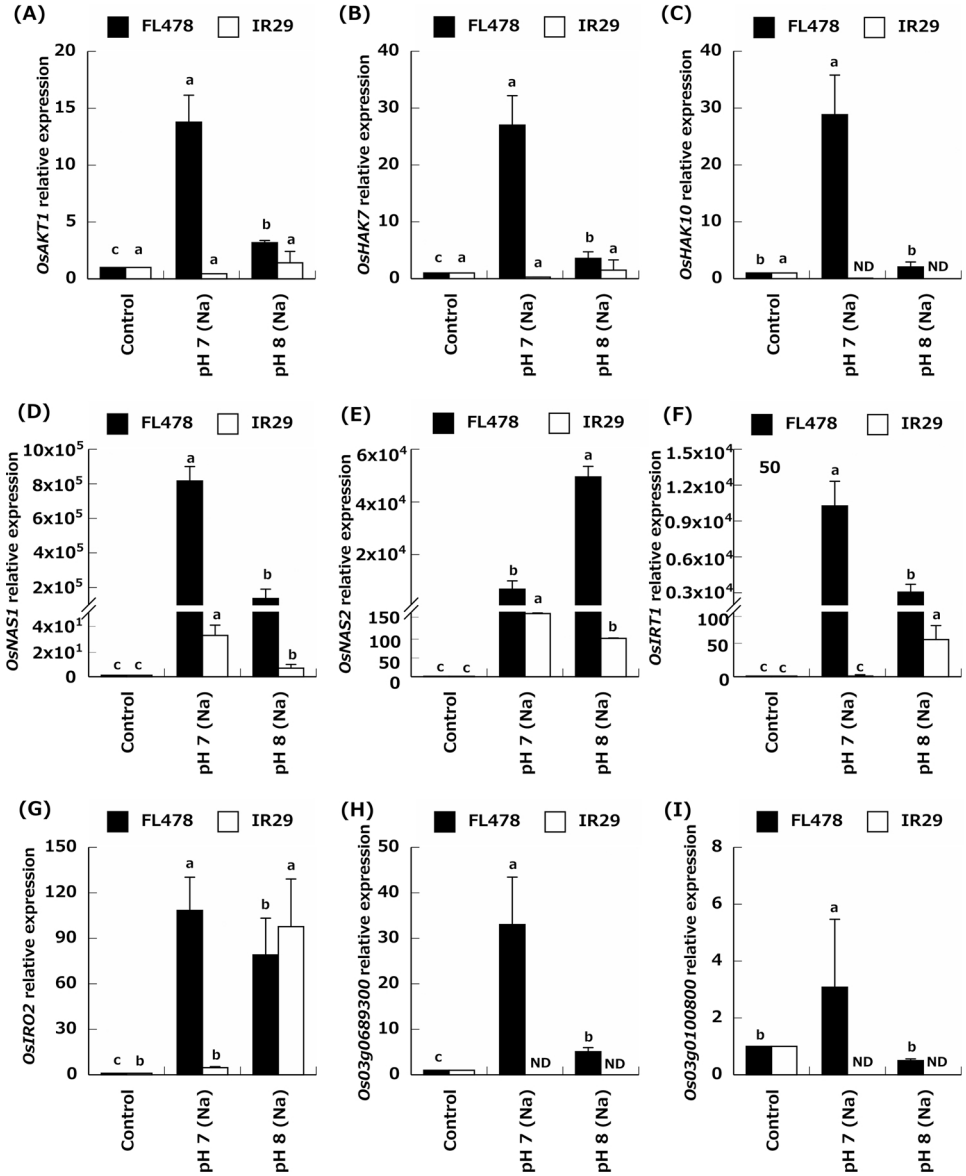

Fig. 7. Relative expressions of alkaline-responsive genes. (A) *OsAKT1*, (B) *OsHKA7*, (C) *OsHKA10,* (D) *OsNAS1,* (E) *OsNAS2,* (F) *OsIRT1,* (G) *OsIRO2,* (H) *Os03g0689300,* and (I) *Os03g0100800* in the roots of rice seedlings of the cultivars FL478 and IR29 grown under control and saline-alkaline stress conditions for three weeks. Data represent the means of two independent experiments.

only tolerant to saline stress but that it is also capable of growing under different pH levels of saline-alkaline environments.

In general, saline-alkaline stress is more complex than neutral-saline stress because plants suffer from both Na^+ toxicity and cellular damages induced by high pH. Several studies have reported that salinity tolerant plants often maintain a low Na^+ and high K^+ concentration in their shoots [5,29,36]. Thus, maintenance of a low Na^+/K^+ ratio in the shoots is considered an indicator of potential salt

tolerance in rice [37]. In the present study, FL478 plants showed better growth performance than IR29 plants, by maintaining a lower Na^+/K^+ ratio in the leaf blades and leaf sheaths when grown under both mild and moderate saline-alkaline stress conditions (Table 2). This suggests that the tolerance to saline-alkaline stress in FL478 plants is achieved by reducing Na^+ accumulation in the leaf cells. To understand the mechanisms in restricted Na^+ transport to the leaf blades in FL478, expressions of the *OsHKT1;5* gene were analyzed (Fig. 6A). OsHKT1;5 in rice is well characterized as a key factor in salinity tolerance as its protein retrieves Na^+ from the xylem and transports it to xylem parenchyma cells. In response to mild and moderate saline-alkaline stress, FL478 markedly induced the expression of the *OsHKT1;5* gene in the roots, but IR29 induced it only slightly in the roots. This result is in agreement with the finding of Walia et al. (2017), which an increase in transcripts of the *OsHKT1;5* gene was observed in the tolerant genotype Pokkali but was reduced in the sensitive genotype IR29 [38]. Therefore, differences of Na^+ accumulation observed between FL478 and IR29 can be explained by restriction of Na^+ transport to the leaf blades through OsHKT1;5 under saline-alkaline stress conditions. However, FL478 accumulated more Na^+ under moderate saline-alkaline stress than mild alkaline stress (Fig. 4A, 4B), although expression of the *OsHKT1;5* gene was highly induced under moderate saline-alkaline stress (Fig. 6A). Over-accumulation of Na^+ in the leaf blades and sheaths under moderate saline-alkaline stress may be due to excess amount of Na^+ absorbed by the roots whose concentration is beyond the Na^+ retrieving capacity by OsHKT1;5 in roots. Chen et al. suggested that the Mg transporter OsMGT1 is required for salt tolerance in rice as it regulates the transport activity of OsHKT1;5, but its expression was not induced by low external NaCl concentration (less than 10 mM NaCl) [21]. In the present study 50 mM Na^+ was used to create the saline-alkaline stress at either pH 7 or pH 8, and control seedlings received on Na^+. The expression profile of the *OsMGT1* gene in the roots of both rice genotypes showed that its expression was not affected by any stress condition including the control (data not shown). This finding suggests that OsMGT1 may not be able to enhance the activity of OsHKT1;5 under saline-alkaline stress conditions.

The presence of low Na^+ accumulation in the leaf cells of rice can be also driven by the *OsHKT1;4* gene, which is mainly localized in the leaf sheaths and produces a protein which functions as a Na^+ excluder by retrieving Na^+ from the xylem [39]. Therefore, the expression profiles of the *OsHKT1;4* gene in both FL478 and IR29 were also investigated in this study. Under mild (50 mM Na + pH 7) and moderate (50 mM Na + pH 8) saline-alkaline conditions, expression of the *OsHKT1;4* gene was repressed in the leaf sheaths of both rice genotypes (Fig. 6B). This indicates that Na^+ retrieval in sheaths mediated by OsHKT1;4 did not contribute to restriction of Na^+ accumulation in the leaves of either genotype when saline-alkaline stress was applied.

The rice OsSOS1 transporter has been isolated based on its homology to AtSOS1 in Arabidopsis [15,17]. Both the *OsSOS1* and *AtSOS1* genes encode a plasma membrane-localized Na^+/H^+ antiporter and, *OsSOS1* expression was upregulated by saline stress [17]. One of the OsSOS1 functions in salinity tolerance is to extrude Na^+ to the outside of cells, leading to reduced Na^+ accumulation in the root cells and load Na^+ into the xylem, leading to increased Na^+ accumulation in shoots through long-distance root-shoot transport system [31]. The latter function likely works in the roots of salt-tolerant FL478 under moderate saline-alkaline stress (pH 8 + 50 mM Na) because high Na^+ concentrations in both leaf blades and leaf sheaths were observed (Fig. 4A and B). On the other hand, Na^+ extrusion to the outside of roots by OsSOS1 might be not active under saline-alkaline stress conditions because plasma membrane-localized Na^+/H^+ antiporters are driven by H^+ gradient across membranes. Thus, it is possible that saline-alkaline stress at pH 8 (moderate), the mechanism of Na^+ loading by OsSOS1 is superior than the Na^+ exclusion mechanism governed by OsHKT1;5 in the roots of FL478, whereas at pH 7 (mild), OsSOS1 and OsHKT1;5 may contribute to the low Na^+ accumulation in the shoots, thereby activated in both sequestration of Na^+ to outside of the roots and retrieving Na^+ from the transpiration stream in xylem.

To gain more understanding with the relationship of Na^+ transporters and alkaline stress in both rice cultivars, expressions of the *OsSOS1* and *OsHKT1;5* genes in the roots were analyzed. Our study revealed that under both mild (pH 7 + 5 mM K) and moderate (pH 8 + 5 mM K) alkaline stress conditions (without high saline stress), the roots of salt-sensitive IR29 were able to activated transcription of the *OsSOS1* and *OsHKT1;5* genes, but in the presence of both saline and alkaline stress conditions; the expression of *OsSOS1* gene was down-regulated, this implies that reduced expression of the *OsSOS1* gene in IR29 roots was caused by Na^+ toxicity rather than high pH stress, whereas the up-regulation of the *OsHKT1;*5 expression is caused by saline-alkaline stress conditions rather than alkaline stress conditions without Na^+ toxicity. However, expression pattern of the *OsSOS1* gene in the roots of salt-tolerant FL478 showed the opposite trend with that observed in IR29 as mentioned above. The increased expression of the *OsSOS1* gene in FL478 roots was found under saline-alkaline stress conditions, but not under alkaline stress conditions without Na^+ toxicity. In addition, in response to saline-alkaline and alkaline stress at either pH 7 (mild) or pH 8 (moderate), expression of the *OsHKT1;4* gene showed no significant changes or reduction in the leaf sheaths of FL478 and IR29. Mechanisms of Na^+ exclusion mediated by OsHKT1;4 was unlikely important in tolerances to these stresses in both rice varieties.

Under high pH conditions, the availability of Fe is quite limited for plants use due to precipitation of Fe. It has been well documented that higher plants use two major Fe uptake strategies (Strategies I; Fe reduction and Strategies II; Fe chelation) to acquire more Fe under this stress condition [40,41]. In graminaceous plants including rice, Fe acquisition has been operated by only Strategy II, which can release mugineic acid family phytosiderophores to uptake Fe^{3+} from the alkaline soils [41]. In the current study, both mild and saline-alkaline stress conditions were markedly enhanced expression of the *OsNAS1*, *OsNAS2*, *OsYSL15* and *OsIRT1* genes in the roots of salt-tolerant FL478 rather than in the salt-sensitive IR29, except for *OsIRO2*, which exhibited a higher expression level in the roots of salt-sensitive IR29 (97.7-fold) than in FL478 roots (79.1-fold) under moderate saline-alkaline stress (pH 8 + 50 mM Na) (Fig. 7D, E, F, G). This finding suggested that, under saline-alkaline stress conditions, Fe utilization in the roots of salt-tolerant FL478 via the activities of Fe deficiency-related genes may be stronger than in the roots of salt-sensitive IR29. However, to clearly understand the relationship between those gene expressions and Fe utilization, the concentrations of Fe in each plant issue should be further investigated.

Root proton-secretion via the activity of plasma membrane H^+-ATPase is considered to be an adaptation of plants to alkaline stress. The present study found that, under mild and moderate saline-alkaline stress conditions (pH 7 and pH 8 + 50 mM Na), expression levels of the H^+-ATPase-encoding genes such as *Os03g0689300* and *Os03g0100800* were up-regulated in the roots of FL478 than in IR29 (Fig. 7H, I). In addition, this result is in agreement with the findings of Li et al., which suggest that under both saline-alkaline and Fe deficiency conditions, three H^+-ATPase-encoding genes (*Os03g0689300*, *Os12g0638700* and *Os03g0100800*) were highly up-regulated in the roots of saline-alkaline tolerant rice variety relative to the sensitive one [33]. These findings indicated that the salt-tolerant FL478 might able to release more H^+ to acidify the rhizosphere and maintaining the root elongation under saline-alkaline conditions rather than the salt-sensitive IR29 rice cultivar. Acidification of the rhizosphere may be beneficial for both activation of OsSOS1 to extrude Na^+ to outside of the cells and acquirement of Fe, thereby improving rice growth under high pH conditions.

K^+ is an essential macronutrient for plant growth and development. The vital roles of K^+ in plant cells under abiotic stress conditions are related to osmoregulation. In rice, several members of the HAK/KUP/KT transporters have also been implicated in salt tolerant, for example; Na^+/K^+ homeostasis in the plant cells was found to be involve with the

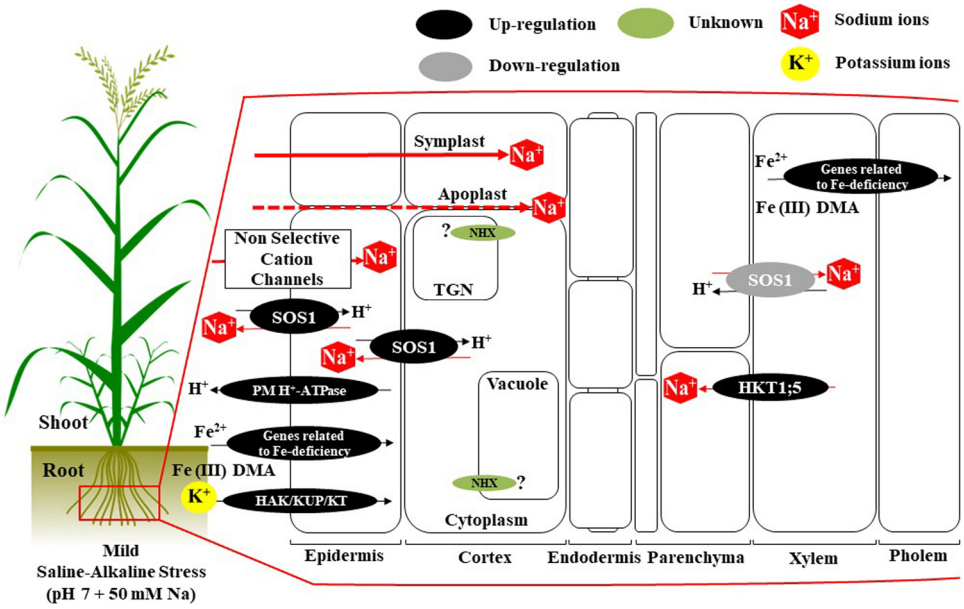

Fig. 8. Proposed models on the mechanisms of saline-alkaline tolerance in rice.

expression *OsHAK1*, *OsHAK5* and *OsHAK21* genes [42]. In response to saline-alkaline stress conditions at either pH 7 (mild) and pH 8 (moderate), expression of the *OsAKT1*, *OsHAK7*, *OsHAK10* and *OsHAK17* gene were markedly enhanced in the roots of salt-tolerant FL478 rather than in IR29, this implies that K^+ accumulation in FL478 roots under both mild and moderate saline-alkaline stress condition was strongly driven by the activities of those genes as seen in Fig. 4A.

5. Conclusion

This study demonstrated that salt-tolerant FL478, was more tolerant to saline-alkaline stress at both pH 7 (mild) and pH 8 (moderate) than the salt-sensitive rice genotype IR29. FL478 plants accumulate less Na^+ in the leaf blades than IR29 plants due to higher expression of the *OsHKT1;5* gene in the roots. This allows FL478 plants to reduce Na^+ accumulation in their leaf blades under the conditions of mild (pH 7) and moderate (pH 8) saline-alkaline stress. FL478 also induced expression of the genes for K^+ acquisition, Fe acquisition, and acidification of the rhizosphere that may participate in saline-alkaline tolerance (Fig. 8). In rice, the mechanisms of saline-alkaline tolerance related to the function of genes encoding Na^+ transport proteins and alkaline-responsive genes have not been fully-understood compared to saline tolerance. Further studies into this area is valuable for identifying the molecular physiological mechanisms associated with responses of rice to saline-alkaline stress.

Acknowledgement

This research was supported by JSPS KAKENHI Grant Numbers 15KK0283 and 16K07643.

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.2019.110171.

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