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SALINITY STRESS

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A salinity-tolerant japonica cultivar has Na^+ exclusion mechanism at leaf sheaths through the function of a Na^+ transporter OsHKT1;4 under salinity stress

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Abstract

This study was designed to investigate differences in patterns of physiological responses to salinity stress among five japonica rice cultivars in comparison with FL478 as a salinity tolerance check. Five japonica cultivars were screened for salinity tolerance at seedling stage based on visual symptoms of salt injury index and physiological parameters including dry matter production, electrolyte leakage ratio and ion concentration. The results indicated that cultivars Ouukan383 and FL478 were relatively more salinity tolerant than other cultivars and that Kanniho was the most salinity-sensitive cultivar. Ouukan383 showed higher leaf water content and lower electrolyte leakage ratio under salinity stress. Notably, under salinity stress, Na^+ concentration in the leaf blades was much lower in Ouukan383 than in FL478, but was much higher in Kanniho. To understand the basis for these differences, we studied transcript levels of the genes encoding Na^+ transport proteins in different tissues. In response to salinity stress, Ouukan383 had highly induced expression of the *OsHKT1;4* gene in the leaf sheaths, corresponding to higher Na^+ accumulation in the leaf sheaths and lower Na^+ accumulation in the leaf blades. On the other hand, the sensitive cultivar, Kanniho, had highly induced expression of the *OsSOS1* and *OsNHX1* genes in the leaf blades, whose gene products are responsible for Na^+ efflux outside cells and Na^+ compartmentalization into vacuoles. Thus, the salinity-tolerant and salinity-sensitive cultivars differed in their responses to Na^+ fluxes in plant cells using different transport systems in each tissue type. The salinity-tolerant japonica cultivar, Ouukan383, has an effective Na^+ exclusion mechanism at the leaf sheaths to prevent Na^+ accumulation in the leaf blades. Such a mechanism, in combination with other genetic traits (e.g. Na^+ exclusion at the roots mediated by OsHKT1;5), offers the potential to improve salinity tolerance in rice.

KEYWORDS
Na^+ exclusion, OsHKT1;4, OsHKT1;5, OsNHX1, OsSOS1, salinity tolerance

1 | INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereals in the tropics and subtropics, but is sensitive to salinity (Flowers, Garcia, Koyama, & Yeo, 1997). Salinity stress is caused by excessive uptake of toxic ions from soils, which decreases plant growth and yield (Boyer, 1982). Grain yield of rice plants is reduced by 70% to 100% of its maximum yield performance due to salinity (Heenan, Lewin, &

McCatery, 1988). Challenges to increasing food production could be overcome by reducing the effects of salt stress on rice plants. Therefore, it is essential to understand the mechanisms that could make rice plants more salinity tolerant.

Under salinity stress, plants must cope with two major stresses, osmotic and ionic stresses. The former stress is immediately induced with a rise in salt concentrations outside the roots, which leads to inhibition of water uptake, cell expansion and lateral bud development (Munns & Tester, 2008). The latter stress develops later when toxic ions, such as Na^+, accumulate in plants over the threshold particularly in leaves, which leads to an increase in leaf mortality due to chlorosis and necrosis and a decrease in the activity of essential cellular metabolisms including photosynthesis (Glenn, Brown, & Blumwald, 1999; Ueda, Kanechi, Uno, & Inagaki, 2003; Yeo & Flowers, 1986). Recent physiological and molecular genetic studies have shed more light on the protection mechanisms that rice plants use to cope with detrimental effects of salinity stress (Blumwald, 2000; Horie, Hauser, & Schroeder, 2009; Pardo, Cubero, Leidi, & Quintero, 2006; Zhu, 2002).

The transmembrane movement of Na^+ and K^+ in plants is mediated by several types of transporters and/or channels (Yao et al., 2010), and many transporters have been implicated in leaf Na^+ exclusion. These include members of the high-affinity K^+ transporters (*HKTs*), including *Arabidopsis thaliana HKT* (*AtHKT1;1*) and its ortholog in rice (*OsHKT1;5*), which retrieve Na^+ from the xylem to the surrounding parenchyma cells (Horie et al., 2009; Ren et al., 2005). However, other *HKTs* such as *OsHKT2;1* (*OsHKT1*) and *OsHKT2;4* are expressed in the outer part of the root and in the root hairs and may provide entry points for Na^+ into plant roots from the soil (Lan et al., 2010; Schachtman & Schroeder, 1994). Plasma membrane protein 3 (*PMP3*) is a small hydrophobic peptide that plays a role in shoot Na^+ exclusion by preventing excess Na^+ entry into the plant roots (Inada, Ueda, Shi, & Takabe, 2005; Nylander et al., 2001). In addition, the SOS1 antiporter has been shown to be localized at the plasma membrane of Arabidopsis, where it catalyses Na^+/H^+ exchange (Shi, Quintero, Pardo, & Zhu, 2002). The preferential expression of *SOS1* in cells surrounding the vasculature throughout the plant, as demonstrated by the *GUS* reporter gene, suggests that this transporter plays a role in long-distance Na^+ transport in plants, as Na^+ is transported from the root to the shoot via the xylem. In addition, the *O. sativa SOS1* (*OsSOS1*) has been shown to complement the function of *SOS1* in the *sos1* mutant of Arabidopsis, indicating the conservation of the salt-sensitive pathway in rice (Martinez-Atienza et al., 2007). In addition to Na^+ exclusion, plants may avoid toxic Na^+ accumulation in the cytosol by sequestering excess Na^+ into vacuoles, which is a process mediated by the Na^+/H^+ antiporter (*NHX1*) localized in vacuolar membranes (Venema, Quintero, Pardo, & Donaire, 2002). However, these transporters only function to counteract the activities of other transporters that are known to induce Na^+ influx into roots. This may occur through cyclic nucleotide-gated channels (CNGCs), which are considered the dominant pathways of Na^+ influx in many plants (Roberts & Tester, 1997). Potential relevance of the aquaporin for Na^+ entry into roots is also discussed (Byrt et al., 2017). A model of synergistic modulation of Na^+ homoeostasis through Na^+ transporters was proposed in a halophytic grass (Zhang et al., 2017). Besides these transporters, transcription factors also participate in regulation of Na^+ accumulation through transcriptional activation (Almeida, Gregorio, Oliveira, & Saibo, 2017).

Although rice, one of the major food crops mainly in Asia, is highly sensitive to salinity stress, there are marked differences in salinity tolerance among rice cultivars (Lee, Choi, Ko, Kim, & Gregorio, 2003; Munns & Termaat, 1986; Yeo & Flowers, 1986). Comparison of varietal differences in rice plants will be helpful to identify the mechanisms of salinity tolerance. Such varietal differences include differences in parameters such as plant growth rate in length and weight, plant survival and plant physiological features (Mekawy et al., 2015; Ueda et al., 2013; Yeo, Yeo, Flowers, & Flowers, 1990) in addition to ion exclusion capacity (Noble & Rogers, 1992). However, salinity-tolerant cultivars were found in the indica subspecies including the aromatic and aus alleles (Platten, Egdane, & Ismail, 2013), and these have similar salinity tolerance mechanisms with Na^+ exclusion from shoots. On the other hand, salinity-tolerant cultivars have been rarely identified in the japonica subspecies. In this study, we screened japonica cultivars and investigated the mechanisms of salinity tolerance. A highly salinity-tolerant japonica cultivar was identified. This cultivar was found to have effective Na^+ exclusion mechanisms at the leaf sheaths. Such a mechanism may be essential for maintenance of lower Na^+ concentrations in the leaf blades.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

Five japonica rice cultivars (Fukuizumi, Midorimai, Ouukan383, Shitan and Kanniho) and one indica salinity-tolerant cultivar (FL478) were used in this study (Walia et al., 2005). FL478 is one of the recombinant inbred lines created using the salinity-tolerant landrace Pokkali and has salinity tolerance comparable to Pokkali (Walia et al., 2005). After incubation in tap water at 60°C for 10 mins, seeds of each cultivar were surface-sterilized with 5% (v/v) sodium hypochlorite solution for 30 mins and then imbibed at 30°C for 24 hrs. The seeds were transferred onto a nylon mesh floating in 2 L (two liters) plastic pots containing tap water in a growth chamber at 28°C for 2 days. Uniformly germinated seeds were grown in half-strength Kimura B solution. Seedlings were grown in a growth chamber at 28/25°C (16 hrs light period/8 hrs 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 16, the nutrient solution was replaced with salinized nutrient solution initially at 25 mM. Thereafter, the concentration of salinity was increased to 50, 75 and 100 mM over 3 days, respectively. Nutrient solution without NaCl addition (0 mM NaCl) was used as a control for comparisons. The pH of nutrient solutions was maintained between 5.0 and 5.5 with 2 N HCl or 2 N NaOH throughout the growth period. The nutrient solution was

renewed every 3 days, and water lost by evapotranspiration was compensated for by daily addition of tap water. Salt stress symptoms were evaluated according to the standard evaluation system (SES) used at the International Rice Research Institute (IRRI) with some modifications (Gregorio, Senathira, & Mendaza, 1997). For visual damages, seedlings were scored as follows: 1 (highly tolerant), 3 (tolerant), 5 (moderate), 7 (sensitive) and 9 (highly sensitive). The experiment was arranged in a completely randomized design (CRD) with four replications.

2.2 | Physiological parameters

After 12 days of salinity treatment, the fresh weight (FW) of 28-day-old seedlings was measured following the separation of leaves, sheaths and roots. For dry weight (DW) determination, leaves, sheaths and roots were dried at 70°C for 3 days prior to being weighed. The water content in the leaf blades was calculated using the equation (FW-DW)/FW.

To determine electrolyte leakage ratio (ELR), the second leaves from the top of the plants were cut into 5 mm length and placed in test tubes containing 30 ml of deionized water. The tubes were covered with plastic caps. The initial electrical conductivity of the medium (EC1) was measured using an electrical conductivity meter (CM-31P, Kyoto Electronics, Kyoto, Japan). The samples were autoclaved afterwards at 121°C for 20 mins to completely deactivate the tissues and release all electrolytes. Samples were then cooled to 25°C, and the final electrical conductivity (EC2) was measured. The ELR was calculated as the ratio of the conductivity before autoclaving to the conductivity after autoclaving using the following formula: ELR (%) = (EC1/EC2) × 100.

Proline concentration was determined according to the method of Bates et al. (1973). Fresh leaves (200 mg) were ground in a mortar with liquid nitrogen. The homogenated powder was mixed with 5 ml of 3% sulfosalicylic acid (w/v). After 10 mins of centrifugation at 10,000 *g* at 4°C, 2 ml of supernatant was transferred to a mixture containing 2 ml acetic acid and 2 ml ninhydrin reagent (1.25 g ninhydrin in 30 ml of acetic acid and 10 ml 12 M phosphoric acid) and incubated at 100°C for 1 hr. The reaction was terminated by placing the container of the mixture in an ice bath. The reaction mixture was vigorously mixed with 4 ml toluene. After warming at 25°C, the chromophore was determined at 520 nm in a UV-spectrophotometer (UV-1850, Hitachi, Japan). L-proline was used as a standard.

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

The concentrations of Na^+ and K^+ were determined by extracting 10 mg dry matter in 10 ml of 1 N HCl with shaking for 1 day. The extracts of the third leaves from the top of the plants, sheaths and roots were determined by a flame spectrophotometer (ANA-135; Tokyo Photoelectric, Tokyo, Japan). Ion concentrations in each sample were estimated using the Na^+ and K^+ standard curves. Na^+ distribution was calculated as the ratio of Na^+ accumulated in each tissue to that in a whole seedling.

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

Total RNA was extracted from the leaves, leaf sheaths and roots of the control and the salinity stressed Ouukan383 and Kanniho cultivars using a TRIzol reagent. After digestion with DNaseI, total RNA (1 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 THUNDERBIRD SYBR qPCR Mix and an ABI StepOne System (Applied Biosystems, CA) as previously described (Ueda et al., 2013). The reaction mixture contained 10 μl of THUNDERBIRD SYBR qPCR Mix, 0.4 μl of 50 × ROX reference dye, 2 μl of forward primer, 2 μl of reverse primer, 1 μl of cDNA and 4.6 μl of sterile water. Quantitative RT-PCR was performed using the following profile: an initial incubation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s and extension at 60°C for 60 s. Relative expression level of the gene transcripts was calculated with the comparative $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) using the *Os25SrRNA* gene as an internal control (Jain, Nijhawan, Tyagi, & Khurana, 2006). Data showed the average of two technical replicates using RNA extracted from the tissues pooled using four seedlings. The sequences of the primers used are listed in Table 1.

3 | RESULTS

3.1 | Screening for salinity tolerance at seedling stage

In this study, we screened salinity-tolerant rice cultivars using the different screening index such as growth performance, ion accumulation, water content, electrolyte leakage ratio and proline

TABLE 1 Primers used for quantitative real-time RT-PCR

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)	References
OsHKT1;4	GTCGAAGTTGTCAGTGCATATGG	TGAGCCTCCCAAAGAACATCAC	Suzuki et al. (2016)
OsHKT1;5	TGCATTCATCACTGAGAGGAG	GGTGCAGTTTCTGCAACCTC	Ueda et al. (2013)
OsSOS1	ATACTGAGTGGGGTTGTTATTGC	AAAGGTAAATTTCAAAAGGTACATGG	Mekawy et al. (2015)
OsNHX1	AATGATCACCAGCACCATCA	AAGGCTCAGAGGTGACAGGA	Mekawy et al. (2015)
Os25SrRNA	AAGGCCGAAGAGGAGAAAGGT	CGTCCCTTAGGATCGGCTTAC	Jain et al. (2006)

TABLE 2 Salinity tolerance rating of six rice cultivars based on the modified standard evaluation score (SES) of visual salt injury at seedling stage after 12 days of salinity stress

Cultivar	SES	Degree of salinity tolerance
Fukuizumi	6.75 ± 0.25	Sensitive
Midorimai	5.75 ± 0.48	Moderately tolerant
Ouukan383	3.50 ± 0.29	Tolerant
Shitan	6.75 ± 0.48	Sensitive
Kanniho	7.50 ± 0.29	Highly sensitive
FL478	3.50 ± 0.29	Tolerant

Values are the mean of four replicates ± standard error.

accumulation. The standard evaluating system (SES) for rating the visual symptoms of salt toxicity established at IRRI (Gregorio et al., 1997) was used with modifications to discriminate the sensitive cultivars from the tolerant and moderately tolerant cultivars. To evaluate the degree of salinity tolerance in five japonica cultivars, the indica cultivar FL478 was used as a salinity-tolerant check. Salinization started when seedlings were 16 days old and had three to four true leaves. After 3 days in salinized solution (25 mM NaCl), initial signs of salt stress damage were observed in the oldest leaves, which started to desiccate and roll inwards, especially in the highly sensitive cultivar Kanniho. When salinity concentration was increased to 100 mM, signs of salt stress damage also appeared in the sensitive and moderately tolerant cultivars such as Fukuizumi, Shitan and Midorimai. At day three after 100 mM NaCl treatment, most leaves of Kanniho and most other cultivars had died, with only the youngest leaves of some plants remaining green. Scoring was performed on day three after salinization at 100 mM and a total of 12 days of salinization, when four categories of tolerance could be visually distinguished (Table 2). The tolerant check, FL478, showed the lowest SES score (3.50) because these seedlings looked nearly normal. Out of the five japonica cultivars, Ouukan383 showed the lowest score similar to FL478 under salinity stress (Table 2). In both Ouukan383 and FL478, only the oldest leaves were wilted and rolled and younger leaves remained green and healthy under salinity stress. On the other hand, most plants of Kanniho had died, with only the youngest leaves of some plants remaining green. These, therefore, were scored 7.50 (highly sensitive). Midorimai exhibited growth retardation and most of its lower leaves rolled, some oldest leaves dried, and only the two youngest leaves remained green and elongated, and was thus scored 5.75 (moderately tolerant). For Fukuizumi and Shitan, most leaves also dried, most plants stopped growing, and some plants were dying. These were scored 6.75 (sensitive). The difference between the sensitive cultivars, Fukuizumi and Shitan, and the moderately tolerant cultivar, Midorimai, was clearly observed on day 12 after salinization as sensitive cultivars showed severe damages on leaf blades. According to visual symptoms under salinity stress, Ouukan383 and Kanniho were chosen as salinity-tolerant and salinity-sensitive cultivars, respectively.

3.2 | Effects of salinity stress on biomass production

The effects of salinity stress on biomass production of seedlings of six rice cultivars are shown in Figure 1. Salt treatment resulted in severe decreases of shoot and root lengths in Fukuizumi, Midorimai, Shitan and Kanniho, but a slight decrease in FL478 (Figure 1a). There were no significant decreases in shoot and root lengths in Ouukan383 under salinity stress. Compared with the control plants, decreases of dry weight in Midorimai, Fukuizumi, Shitan and Kanniho were more severe than Ouukan383 and FL478 under salinity stress (Figure 1b). Kanniho, in particular, exhibited reduced shoot and root dry weights by 23% and 44%, respectively (Figure 1b). Two tolerant cultivars, Ouukan383 and FL478, showed slight decreases in dry weights of shoots and roots. These observations suggested that Ouukan383 and FL478 are highly salinity tolerant relative to the other four cultivars.

3.3 | Effect of salinity stress on physiological parameters

Rice loses water in the tissues due to osmotic imbalance under high salinity. Therefore, measurement of leaf water content can be one of

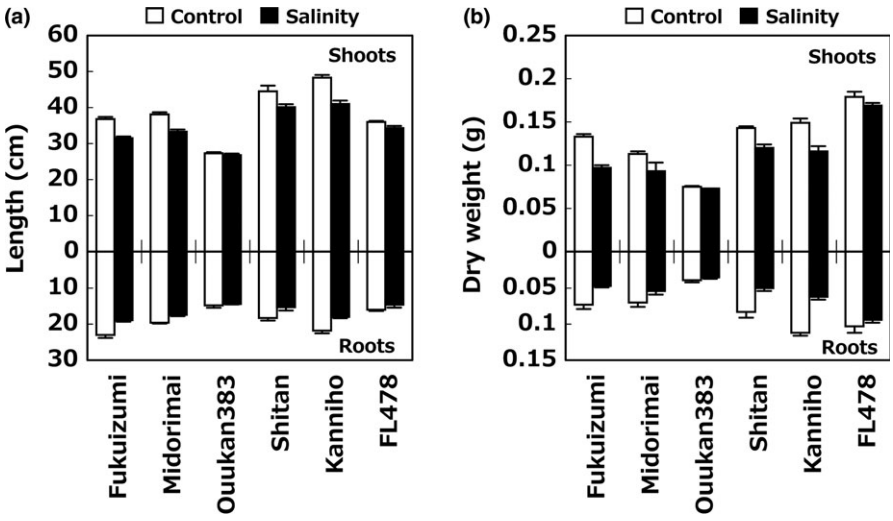

FIGURE 1 Effects of salinity stress on the growth of six rice cultivars. (a) Length and (b) dry weight of shoots and roots were measured under control and 12 days of salinity conditions. Values are means of four replicates ± standard error

the indicators for the screening of salinity-tolerant cultivars. As shown in Figure 2a, salinity stress significantly reduced water content in the leaf blades of the salinity-sensitive cultivars, Kanniho, Shitan, Fukuizumi and Midorimai, but not in the salinity-tolerant cultivars, Ouukan383 and FL478. Membrane integrity was monitored by electrolyte leakage ratio (ELR). Under the control condition, ELR of the leaf tissues of different cultivars ranged from 1.7% to 3.5% (Figure 2b). With salinity treatment, cell membranes in leaves of the most cultivars lost integrity resulting in increase in ELR. The relationship between ELR and salinity tolerance was clearer when comparing ELR of the tissues treated with salinity at 100 mM. Membrane of the sensitive cultivar Fukuizumi tended to be most severely affected by the salinity treatment at 100 mM as shown by the 4.6-fold increase in ELR relative to control seedlings, followed by Shitan and Kanniho showing 3.6- and 3.1-fold increases, respectively. ELR of moderately tolerant and tolerant cultivars was three times lower than that of control seedlings (Figure 2b). Leaves of the tolerant cultivars under non-stressed conditions accumulated relatively lower concentration of free proline (Figure 2c). In response to NaCl treatment, rice leaves accumulated higher concentrations of proline. The cultivar Kanniho accumulated the highest concentration of proline, with Ouukan383 accumulating the lowest. The increase in proline accumulation in Midorimai and FL478 was intermediate, but Ouukan383 showed a slight increase. Overall, higher concentrations of proline were observed in the sensitive cultivars, suggesting that these cultivars suffered from negative impacts of salinity stress.

3.4 | Effects of salinity stress on Na^+ and K^+ accumulation in different tissues

In six cultivars, salinity treatment led to increased Na^+ accumulation in all tissues examined. In the salinized roots, Na^+ concentration was increased in all cultivars (Figure 3a). The tolerant and moderately tolerant cultivars, such as Midorimai, Ouukan383 and FL478, accumulated much more Na^+ in roots than sensitive cultivars. In the leaf sheaths, the highest Na^+ increase was observed in Ouukan383. However, Na^+ concentration in FL478 was the lowest (Figure 3b). Remarkable differences in the Na^+ concentrations of the leaf blades were observed between the tolerant and sensitive cultivars. Ouukan383 and FL478 accumulated Na^+ in the leaf blades reaching 7.0 and 26.2 mg/g DW, respectively. However, four of the sensitive cultivars accumulated Na^+ at the range from 37.7 to 41.9 mg/g DW (Figure 3c). These findings suggest that the japonica cultivar Ouukan383 has a very effective mechanism for Na^+ exclusion from the leaf blades than FL478, which is a well-known Na^+ excluder from the leaf blades.

Salinity stress significantly decreased the K^+ concentration in the roots of the six cultivars (Figure 4a). K^+ concentration was increased in the leaf sheaths of all cultivars (Figure 4b). Notably, Ouukan383 showed higher K^+ concentration in the leaf blades and it was not affected by salinity stress (Figure 4c). As observed in the leaf blades of the sensitive cultivars, the increase in Na^+ accumulation and decrease in K^+ accumulation resulted in the increase in Na^+/K^+ ratio in response to NaCl and the ratio was negatively related to the degree of salt tolerance (Table 3). Salinity-tolerant cultivars showed lower Na^+/K^+ ratio (0.29 in Ouukan383 and 1.93 in FL478) in the leaf blades under salinity stress, and thus, maintenance of lower Na^+/K^+ ratio is likely one of the key traits for salinity tolerance in rice.

Na distribution in each tissue was estimated by calculating the ratio of Na^+ accumulation in each tissue to that of a whole seedling (Figure 5). The salinity-tolerant Ouukan383 accumulated Na^+ in the sheaths and roots, but restricted Na^+ entry in the leaf blades as only 28% of Na^+ absorbed was accumulated in the leaf blades. On the other hand, the salinity-sensitive Kanniho accumulated 70% of Na^+ absorbed in the leaf blades, indicating that this cultivar does not

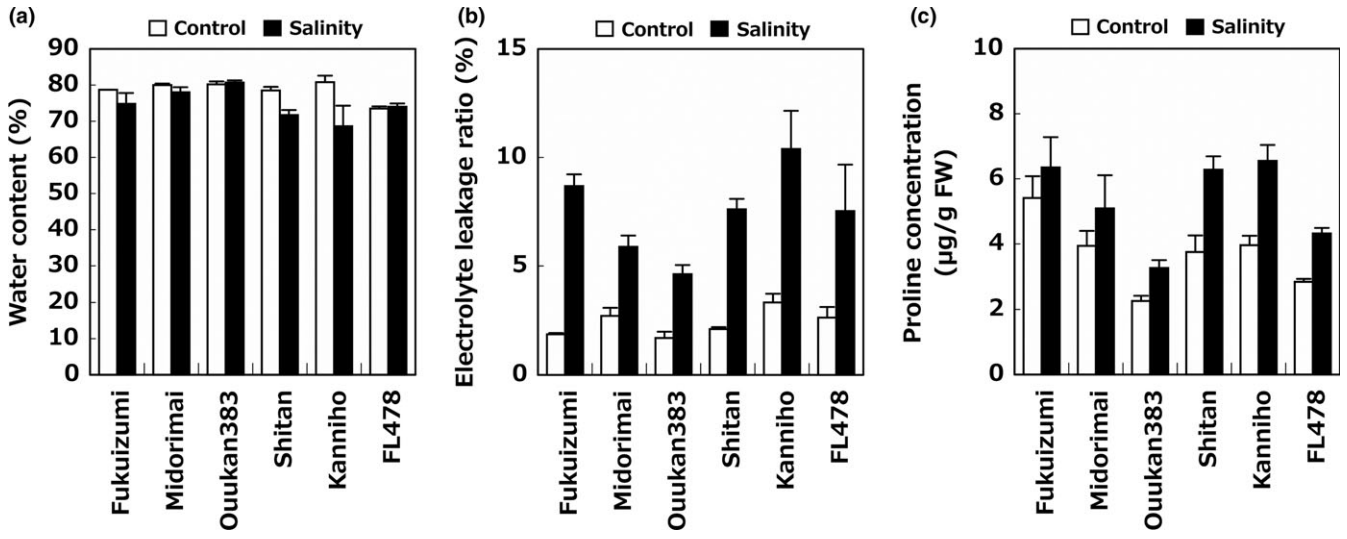

FIGURE 2 Effects of salinity stress on the physiological parameters of six rice cultivars. (a) Water content, (b) electrolyte leakage ratio and (c) proline concentration in the leaf blades were measured under control and 14 days of salinity conditions. Values are means of four replicates ± standard error

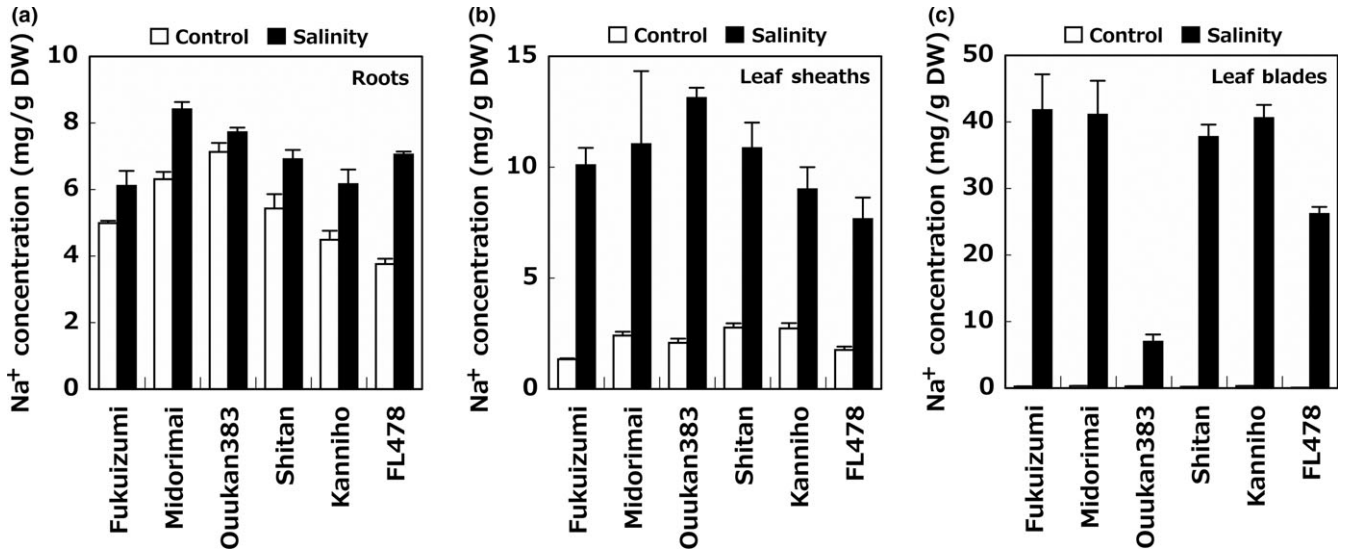

FIGURE 3 Na^+ concentrations in the (a) roots, (b) leaf sheaths and (c) leaf blades under control and salinity conditions. Values are means of four replicates ± standard error

have an effective mechanism of Na^+ exclusion from the leaf blades (Figure 5).

3.5 | Differential expression of the genes encoding Na^+ transport proteins in response to salinity stress

To determine the mechanisms underlying differential Na^+ accumulation in the salinity-tolerant Ouukan383 and the highly salinity-sensitive Kanniho, expression profiles of the genes encoding Na^+ transport proteins were analysed. A Na^+ transporter, OsHKT1;5, participates in the mechanisms of Na^+ exclusion from shoots through retrieving Na^+ from xylem to xylem parenchyma cells in roots (Ren et al., 2005). Therefore, OsHKT1;5 is one of the key regulators restricting Na^+ accumulation in shoots (Assaha, Mekawy, Ueda, & Saneoka, 2015). In this study, quantitative RT-PCR analyses showed that salinity stress induced expression of the *OsHKT1;5* gene by 2.0-fold in the roots of Ouukan383 (Figure 6a), which may cause reduced Na^+ accumulation in the leaf blades under salinity stress

TABLE 3 Na^+/K^+ ratio in the roots, leaf sheaths and leaf blades under salinity stress

Cultivar	Roots	Leaf sheaths	Leaf blades
Fukuizumi	1.05 ± 0.05	1.10 ± 0.07	2.87 ± 0.27
Midorimai	1.72 ± 0.09	0.77 ± 0.13	3.24 ± 0.22
Ouukan383	1.58 ± 0.10	1.06 ± 0.07	0.29 ± 0.06
Shitan	1.73 ± 0.10	1.17 ± 0.05	3.78 ± 0.35
Kanniho	1.53 ± 0.09	0.90 ± 0.05	3.10 ± 0.23
FL478	2.02 ± 0.05	1.14 ± 0.20	1.93 ± 0.12

Values are the mean of four replicates ± standard error.

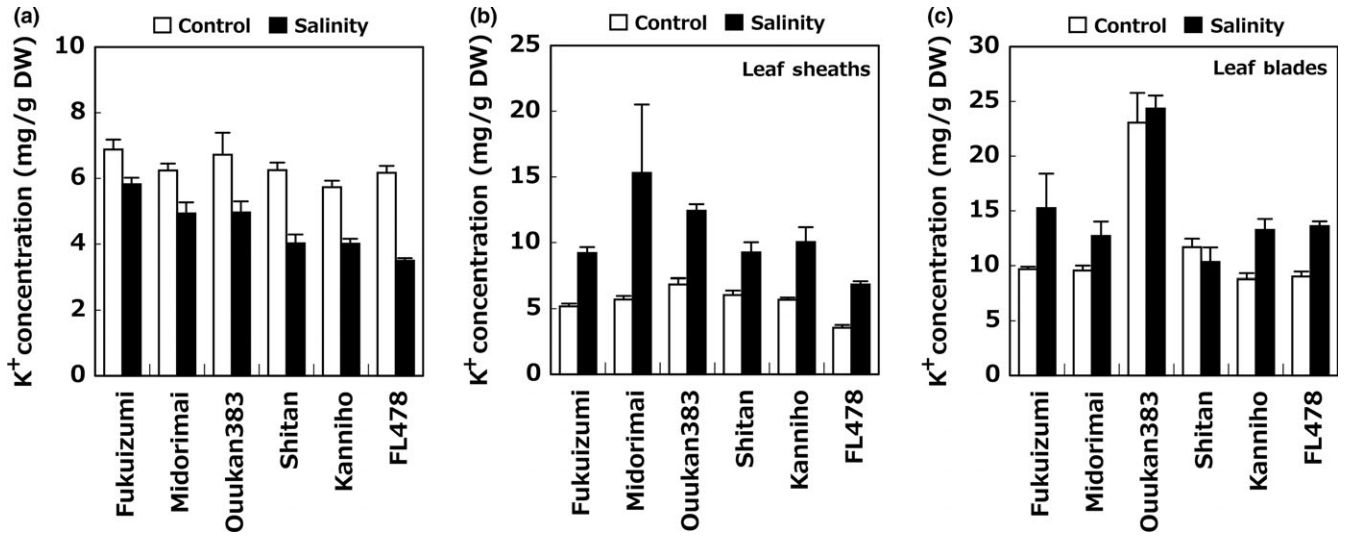

FIGURE 4 K^+ concentrations in the (a) roots, (b) leaf sheaths and (c) leaf blades under control and salinity conditions. Values are means of four replicates ± standard error

(Figure 3c). However, expression of the *OsHKT1;5* gene was not induced in Kanniho under salinity stress (Figure 5a). Another Na^+ transporter, OsHKT1;4, is an alternative candidate for Na^+ exclusion, which is effective in the leaf sheaths, thereby reducing Na^+ accumulation in the leaf blades. Salinity treatment induced expression of the *OsHKT1;4* gene by 4.7-fold in Ouukan383, but it was repressed by 0.1-fold in Kanniho. Thus, Na^+ exclusion mechanisms through the functions of OsHKT1;4 and OsHKT1;5 are active in the salinity-tolerant Ouukan383, but not in the salinity-sensitive Kanniho.

The Na^+/H^+ antiporter (*SOS1*), localized in the plasma membrane, is considered a general regulator of Na^+ export from cytosol (Shi et al., 2002). Our results indicated that there was a higher level of induced expression of the *OsSOS1* gene in the Kanniho roots (2.8-fold) (Figure 6a), which might be responsible for relatively low Na^+ accumulation in its roots under salt stress. However, the *OsSOS1* expression in the Ouukan383 roots was not induced (1.5-fold). Expression of the *OsSOS1* gene in the leaf sheaths was induced in Ouukan383 (3.2-fold), but not in Kanniho (0.9-fold) under salinity stress (Figure 6b). In the leaf blades, induced expression of the *OsSOS1* gene was observed in the Kanniho leaves (5.8-fold) under salinity stress conditions (Figure 6c), which suggests that OsSOS1 mediated Na^+ extrusion from the cytosol may not be active in Ouukan383.

The Na^+/H^+ antiporter plays an important role in tolerance to salt stress by exchanging Na^+ and H^+ across the plasma or vacuolar membranes. The tonoplast Na^+/H^+ antiporter, which was found in several plant species transports Na^+ from the cytoplasm into vacuoles, thereby increasing the cytoplasmic K^+/Na^+ ratio and protecting cells from sodium toxicity (Ballesteros, Blumwald, Donaire, & Belver, 1997; Barkla, Charuk, Cragoe, & Blumwald, 1990; Fukuda, Nakamura, & Tanaka, 1999; Gaxiola et al., 1999). While the high-salinity-induced expression of the *OsNHX1* gene in the leaves of Kanniho (70.5-fold) (Figure 6c) might be responsible for increased Na^+ accumulation in the leaf vacuoles under salt stress (Figure 3a),

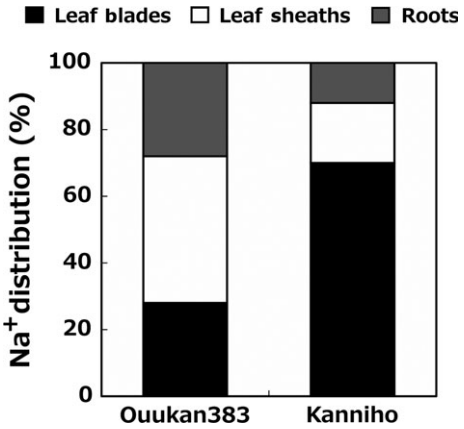

FIGURE 5 Distribution of Na^+ accumulation in the roots, leaf sheaths and leaf blades of the salinity-tolerant Ouukan383 and the salinity-sensitive Kanniho under control and salinity conditions. Na^+ distribution was evaluated by the ratio of the amount of Na^+ in each tissue to that in the whole seedling

the gene expression was not induced in the Ouukan383 leaves (0.7-fold). This difference in the *OsNHX1* induction might be due to higher Na^+ accumulation in the leaves of Kanniho (Figure 3a). Under salinity stress, expression of the *OsNHX1* gene was induced highly in the leaf sheaths of Kanniho (5.4-fold) and slightly in those of Ouukan383 (2.0-fold) (Figure 6b). In the roots, the *OsNHX1* expression was slightly changed in response to salinity stress in Kanniho (1.4-fold), but it was induced in Ouukan383 (5.1-fold) (Figure 6a).

4 | DISCUSSION

In comparison with a salinity-tolerant cultivar FL478, five japonica rice cultivars (Fukuizumi, Midorimai, Ouukan383, Shitan and Kanniho) were used in this study to elucidate their mode of adaptations to salinity stress through physiological and transcriptional analysis. The screening index used in this study would be useful to identify salinity-tolerant varieties by investigating differences in physiological characteristics in rice. The six cultivars showed differential responses to salinity, and Ouukan383 and FL478 appeared to be more tolerant than the other cultivars. These two cultivars exhibited lower Na^+ concentrations in the leaf blades, higher leaf water content and lower electrolyte leakage ratio under salinity stress. Notably, Ouukan383 accumulated much less amount of Na^+ in the leaf blades than FL478, although Na^+ concentration in the leaf sheaths was much higher in Ouukan383 than in FL478. These tolerant cultivars showed to have different mechanisms of Na^+ exclusion from the leaf blades.

Among salinity-tolerant traits in glycophytes, the most significant plant adaptation to salinity is the ability to restrict the transport and accumulation of Na^+ in the leaf blades (Assaha, Mekawy, et al., 2017; Munns & Tester, 2008; Ueda et al., 2013). Thus, rice cultivars such as Ouukan383 and FL478 that exhibited lower Na^+ concentration in the leaf blades would be better adapted to salinity stress (Figure 3c). This restricted transport of Na^+ to the leaf blades is often accompanied by a reduced Na^+/K^+ ratio, which is relevant for the sustainability of normal metabolic functions (Tester & Davenport, 2003). To understand the mechanisms underlying limited Na^+ transport to the leaf blades in Ouukan383, we analysed the expression of the *OsHKT* genes (Figure 6a). One of the well-characterized HKTs is OsHKT1;5, a Na^+ transporter, which functions in Na^+ retrieval from xylem to xylem parenchyma cells. Thus, active contribution of Na^+ retrieval by OsHKT1;5 in roots results in reduced Na^+ accumulation in the aerial parts of rice seedlings (Ren et al., 2005). FL478, a salinity-tolerant rice cultivar, is known to operate Na^+ exclusion mechanisms governed by OsHKT1;5 in the roots, which is pivotal to reduced Na^+ concentration in the leaf blades than other sensitive cultivars (Figure 3c). To assess whether the salinity-tolerant Ouukan383 has the same mechanism, quantitative RT-PCR analysis was conducted to study differences in the expression of the *OsHKT1;5* gene. Under salinity stress conditions, the expression of the *OsHKT1;5* gene was induced in the roots of Ouukan383, but was repressed in the roots of Kanniho. This finding indicates that

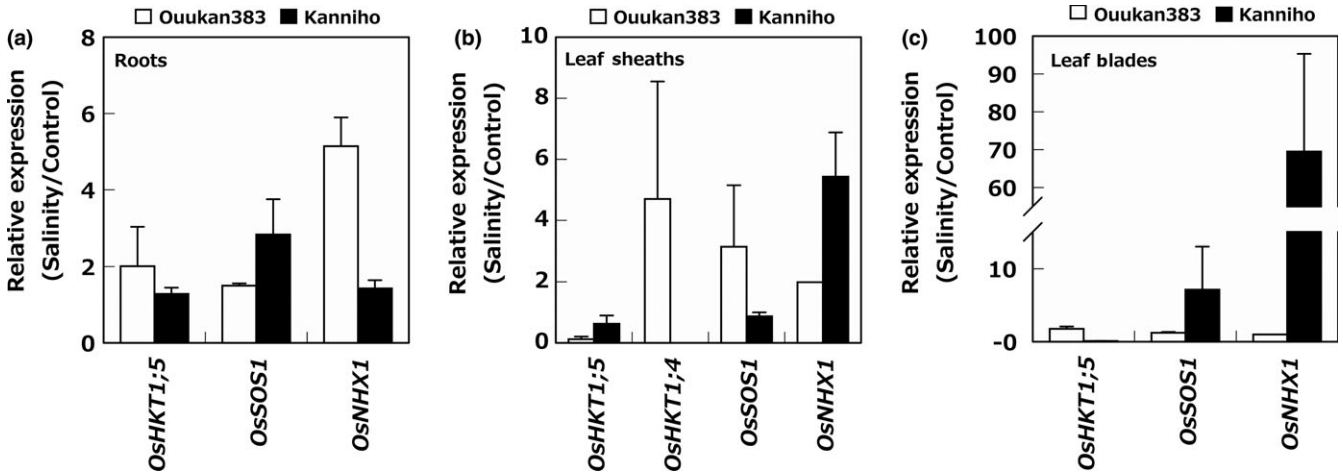

FIGURE 6 Relative expression of the genes encoding Na^+ transport proteins under control and salinity conditions. Expression of the *OsHKT1;5*, *OsHKT1;4*, *OsSOS1* and *OsNHX1* genes was examined using a quantitative RT-PCR in the (a) roots, (b) leaf sheaths and (c) leaf blades of the salinity-tolerant Ouukan383 and the salinity-sensitive Kanniho. Values are means of two independent experiments ± standard deviation

Ouukan383 may have better ability to restrict Na^+ accumulation by OsHKT1;5 in the leaf sheaths and roots than Kanniho. Although Ouukan383 showed the highest degree of salinity tolerance and the lowest Na^+ concentration in the leaf blades among the cultivars tested, this cultivar accumulated higher concentration of Na^+ in the leaf sheaths (Figure 3b). Relevance of the alternative mechanism in the restriction of Na^+ transport in the leaf blades was also assessed in both Ouukan383 and Kanniho by studying the expression of the *OsHKT1;4* gene, whose gene product participates in Na^+ retrieval from xylem in the leaf sheaths (Cotsaftis, Plett, Shirley, Tester, & Hrmova, 2012). The results demonstrated that expression of the *OsHKT1;4* gene was markedly induced by salinity stress in the leaf sheaths of Ouukan383, but it was repressed in those of Kanniho (Figure 6b). Thus, genetic variations underlying japonica cultivars may affect expression of the *OsHKT1;4* gene. Salinity-tolerant Ouukan383 has much effective mechanisms to retrieve Na^+ in the leaf sheaths through the function of OsHKT1;4, which results in higher Na^+ accumulation in the leaf sheaths, but lower Na^+ accumulation in the leaf blades.

Differential expressions of the *OsNHX1* and *OsSOS1* genes were observed in Ouukan383 and Kanniho under salinity stress (Figure 6). Proton-coupled Na^+ transport system plays key roles in salinity tolerance of higher plants (Assaha, Ueda, Saneoka, Al-Yahyai, & Yaish, 2017). SOS-type Na^+/H^+ antiporters, localized in the plasma membranes, facilitate Na^+ export to the outside of cells, and NHX-type Na^+/H^+ antiporters, localized in the tonoplasts, compartmentalize Na^+ into vacuoles (Fukuda et al., 2004; Shi et al., 2002). Ouukan383 induced expression of the *OsNHX1* gene in the roots and leaf sheaths in response to salinity stress. This cultivar accumulated relatively higher concentration of Na^+ in the roots and leaf sheaths. Probably, salinity-tolerant behaviours in Ouukan383 have been achieved through the function of OsNHX1, which is responsible for compartmentalization of Na^+ into vacuoles (Fukuda et al., 2004). The bifunctional roles of SOS-type Na^+/H^+ antiporters in the roots are proposed as the Na^+ excluder from cytosol to apoplast and gate of Na^+ loading into xylem (Assaha, Ueda, et al., 2017). These functions likely work in reducing Na^+ concentration in the roots and increasing Na^+ concentration in the shoots as Kanniho accumulated lesser amount of Na^+ in the roots, but greater amount of Na^+ in the leaf blades. Because Kanniho does not have Na^+ retrieval mechanisms through OsHKT1;5 and OsHKT1;4 in the roots and leaf sheaths, too much Na^+ absorbed in the roots under salinity conditions was transported to the leaf blades. Induced expression of the *OsSOS1* gene was also found in the leaf sheaths of Ouukan383 under salinity stress (Figure 6b). Because Na^+ concentration was not very high in the leaf blades of Ouukan383, OsSOS1 may work in Na^+ exclusion from the cells in the leaf sheaths rather than Na^+ loading into xylem. It appeared reasonable that Kanniho induced high expression of both the *OsSOS1* and *OsNHX1* genes in the leaf blades to export Na^+ outside of the cells and compartmentalize Na^+ into vacuoles because Na^+ concentration in Kanniho reached more than 40 mg/gDW, which is 5.8 times higher than that in Ouukan383. Functions of OsSOS1 and OsNHX1 proteins are recognized as key determinants of salinity tolerance in higher plants (Assaha, Ueda, et al., 2017). Nevertheless, growth of Kanniho was retarded under salinity stress (Figure 1). One possible explanation is that the ability of Na^+ compartmentalization into vacuoles is restricted by the storage capacity of vacuoles. Therefore, transcriptional activation of the *OsNHX1* gene does not effectively contribute to avoid cellular Na^+ toxicity once excess Na^+ is accumulated in the cells of leaf blades of Kanniho. These implied that Na^+ exclusion mechanisms from the leaf blades governed by OsHKT1;4 and/or OsHKT1;5 are superior to mechanisms of Na^+ extrusion by OsSOS1 and Na^+ compartmentalization into vacuoles by OsNHX1 in salinity tolerance of rice.

The other favourable trait of salinity tolerance observed in Ouukan383 was the maintenance of higher K^+ concentration in the leaf blades under both control and salinity conditions. Maintenance of higher K^+ concentrations, and thus lower Na^+/K^+ ratio in the tissues,

is detrimental in salinity tolerance of glycophytes because accumulation of Na^+ in the cytosol disrupts K^+-dependent biochemical reactions that are essential for plant growth. The mechanisms of maintenance of higher K^+ concentrations have not yet been clearly established, although this may be the other factor for the salinity tolerance of Ouukan383. Salinity-tolerant cultivars showed better physiological status under salinity stress (Mekawy et al., 2015; Ueda et al., 2013). Previously, Lutts, Kinet, and Bouharmont (1996) found that electrolyte leakage ratio increased with increasing NaCl concentrations in the medium, and this ratio was higher in the salinity-sensitive cultivars. Because alteration in membrane permeability is one of the first symptoms of salinity-induced senescence, this assay is useful for screening of salinity-tolerant rice cultivars (Theerakulpisut, Bunnag, & Kong-ngern, 2005). Our results also showed that the sensitive cultivar Kanniho had the highest ratio of electrolyte leakage, suggesting that this cultivar suffered from increased membrane damages under salinity stress. Proline has been widely considered to be a compatible solute that accumulates in plants in response to a wide variety of environmental stresses and confers stress tolerance by contributing to osmoregulation and protecting proteins and membranes in conditions of low water potential (Ueda, Shi, Shimada, Miyake, & Takabe, 2008; Ueda, Yamamoto-Yamane, & Takabe, 2007). For example, overproduction of proline with the *P5CS* (Δ-pyrroline-5-carboxylate synthetase) gene enhanced root biomass and flower development under salinity stress in tobacco (Kishor, Hong, Miao, Hu, & Verma, 1995) and rice (Zhu et al., 1998). In our study, the sensitive cultivars accumulated much higher concentrations of proline than the tolerant cultivars under salinity stress. Thus, the negative relationship between proline and salinity tolerance was observed among the six rice cultivars (R^2 = .92 for proline concentration vs SES scores). On the other hand, proline accumulation and Na^+ concentration in the leaf blades showed positive correlation under salinity stress (R^2 = 0.80 for proline concentration vs Na^+ concentration in the leaf blades). Because salinity-tolerant cultivars accumulated lower concentrations of Na^+ in the leaf blades through Na^+ exclusion mechanisms by OsHKTs, they did not accumulate proline at higher concentrations. This implies that proline accumulation in rice may be stimulated by Na^+ accumulation, but not by osmotic stress under salinity stress. These findings indicate that proline accumulation may not be suitable for screening of salinity-tolerant cultivars that have Na^+ exclusion mechanisms from the leaf blades.

5 | CONCLUSION

In the present study, we demonstrated for the first time that the japonica cultivar Ouukan383 is a salinity-tolerant cultivar comparable to the tolerant indica cultivar FL478. Some of salinity-tolerant indica cultivar including FL478 use OsHKT1;5 to restrict Na^+ accumulation in shoots (Platten et al., 2013). On the other hand, Ouukan383 accumulated lesser amount of Na^+ in the leaf blades than FL478, suggesting that this cultivar may have different mechanisms of Na^+ exclusion from the leaf blades. Further investigation on Na^+ exclusion mechanisms through the function of OsHKT1;4 would be helpful for pyramiding the genetic traits to improve salinity tolerance in rice.

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