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Na^+ exclusion mechanism in the roots through the function of *OsHKT1;5* confers improved tolerance to salt stress in the salt-tolerant developed rice lines

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ABSTRACT: To explore the mechanisms underlying salt tolerance in rice, the physiological parameters of rice planted for 12 days under salt stress were examined. Under the salt stress and the use of hydroponic cultures and soil-based pot screening methods, the two salt-tolerant developed rice lines, M-1 and M-3, were found to be more salt-tolerant than the Pathum Thani 1 (PTT1) cultivar owing to the lower Na^+ accumulation in their leaf blades. Growth and physiological parameters (shoot and root dry weights, electrolyte leakage ratio, leaf water content, and chlorophyll concentration) of both mutant lines were unchanged upon exposure to salt stress, but changes were found in the PTT1. In addition, all the examined tissues under salt stress conditions of the mutant lines showed lower Na^+/K^+ ratios. In response to salt stress in both screening methods, the *OsHKT1;5* expression in the roots of the M-1 and M-3 lines had greatly increased, and the Na^+ accumulation in their shoots was decreased. However, the *OsNHX1* expression in the leaf sheaths and the roots of PTT1 was highly upregulated by the salt stress compared with the two mutant lines, suggesting that the NHX antiporter of PTT1 did not effectively transport the Na^+ into the vacuoles, contributing to a high Na^+ accumulation in the leaf blades, which might be related to the repression of the *OsHKT1;5* gene in its roots. Molecular analysis suggested that the Na^+ retrieval mechanisms via *OsHKT1;5* might enhance the salt stress tolerance of the mutant lines by preventing Na^+ accumulation in their aerial parts, whereas Na^+ exclusion via *OsNHX1* may respond to elevated Na^+ sequestration in the vacuoles.

KEYWORDS: hydroponic cultures, Na^+ accumulation, *OsHKT1;5*, *OsNHX1*, soil-based pot

INTRODUCTION

Soil salinity is one of the yield constraining factors in rice farming [1]. Arunin and Pongwichian [2] reported that salt-affected soils in Thailand make up nearly 2.3 million hectares, most of which are distributed in the Northeastern region of Thailand and formed via geochemical processes. Thailand is an agricultural-based country and one of the world noteworthy rice producers; however, the rice production areas are alarmingly affected by the increasing salinity [2]. To improve rice production, characteristics under drought stress conditions of several Thai rice varieties have been studied [3], and development of new salt-tolerant rice cultivars is being undertaken to solve the soil's salinity problem [4].

Plants experience the salt stress due to the slow accumulation of Na^+ over time. Two important mechanisms for decreasing the salt stress in higher plants are Na^+ and Cl^- homeostasis. High Na^+ concentration is involved in the decrease in development and yield of rice seedlings [5]. The mechanisms of Na^+ transport and Na^+ tolerance in higher plants have also been studied, and great molecular details are known [6]. The salt tolerance of plants might rely on the high-affinity K^+ transporter (HKT) intervening in Na^+-specific transport or Na^+-K^+ transport and playing a key role in the control of Na^+ homeostasis [7, 8]. In rice, *OsHKT1;5* (*SKC1*) controls K^+/Na^+ selectivity and maintains high K^+ and low Na^+ contents under salt stress by regulating the removal of Na^+ from the root xylems [9]. Another member of the *HKT* gene,

OsHKT1;4, uses the Na^+ extrusion mechanism at leaf sheaths to control the sheath to blade transfer of Na^+ under salinity stress [10]. Several members of rice *NHX* gene involving in the compartmentalization of Na^+ into plant vacuoles have been identified [11, 12]. For example, overexpression of NHX1 (a Na^+, K^+/H^+ exchanger) enhances Na^+ toxicity tolerance in various plant species, including rice (*Oryza sativa*), by the vacuolar Na^+ sequestration of the plants under salt stress condition [13].

Hydroponic culture and soil-based pot experiments have been used to study the improvement of salt tolerance in bread wheat [14]. However, the responses of plants to salinity stress differ between hydroponic and soil-based pot systems [15]. The salt tolerance mechanisms under the two systems in rice are still poorly understood. The aims of the present study were: (i) to examine the differences in salt tolerance ability between the hydroponic culture and the soil-based pot experiments; (ii) to illustrate the differences in the salt tolerance mechanisms of the mutant lines and the wild type cultivar by comparing the physiological responses; and (iii) to investigate the under salt stress expressions of some genes encoding Na^+ transport proteins, namely, *OsNHX1*, *OsHKT1;4*, and *OsHKT1;5*. The underlying goal was to differentiate between the responses of tolerant mutant lines and susceptible wild type cultivar at the seedling stage of growth using different screening methods.

MATERIALS AND METHODS

Plant materials

M_1 seeds were derived from high-quality seeds of *Oryza sativa* L. 'PTT1' irradiated with acute gamma irradiation. The genetically fixed M_5 salt-tolerant mutant lines, M-1 and M-3, were selected in 2019. Pokkali, a well-known salt tolerant cultivar, was used as a positive control, and PTT1, a highly susceptible to salt cultivar, was used as the negative control.

Screening methods for salt tolerance

The experiments were conducted in a glasshouse during the 2019 summer months from August to September. The average temperature was 25.2 °C at night and 30.4 °C during the day. The experiments were arranged in a completely randomized design with four replications.

Hydroponic culture: Uniformly selected seeds of each cultivar/line were incubated in tap water at 60 °C for 10 min, surface sterilized in 5% (v/v) sodium hypochlorite solution for 30 min, and then imbibed in tap water at 30 °C for 24 h. The seeds were transferred onto a nylon mesh floating in 20 l plastic tanks containing tap water. When the seedlings were four days old, the tap water was replaced with nutrient solution, the half-strength Kimura B solution [16]. When the seedlings were 10 days old, they were transplanted to PVC tubes, one seedling per tube, at 5 cm deep. The tubes were 2.5 cm in diameter, filled with sponge, and placed in 170 l plastic tanks containing nutrient solution. At 21 days after the transplant, the nutrient solution was replaced with a salt (NaCl) nutrient solution, initially at 25 mM. The NaCl concentration was increased to 50, 75, and 100 mM over 3 days. Supplement arrangement with 0 mM NaCl was used as the control. The pH of the nutrient solution throughout the growing period was maintained between 5.0 to 5.5, using 2 N HCl or 2 N KOH; and the water lost by evapotranspiration was compensated for by the daily addition of tap water. The nutrient solution was renewed every 3 days.

Soil-based pot culture: Seeds were surface sterilized as previously described for the hydroponic culture. After 3 days, the seeds were transferred and grown in 6.5 cm-in-diameter plastic pots containing 200 g of paddy soil, six plants per pot, and at 7 cm deep. Before adding the soil, a hole was punched in the center of the bottom of the individual pots, and a plastic net was placed over the hole. One set of all cultivars/lines were maintained in 50 l plastic tanks containing half-strength Kimura B solution. Similarly, the nutrient solution was replaced with salt nutrient solution on day 21 as described for the hydroponic culture. After 12 days of salt stress, the damaged leaves of the plants were evaluated and scored using the modified standard evaluation system (SES) protocol developed by the International Rice Research Institute (IRRI) [10].

Physiological analyses

For the fresh weight (FW) of plant tissues, leaves, sheaths, and roots of the 33-day-old seedlings were separated and individually measured. For the dry weight (DW), all the tissues (the leaves, the sheaths, and the roots) were dried in a hot air oven (KATO-RIKI MFG. CO., LTD, KRS-LB) at 70 °C for 3 days and then weighed. The percentage water content (WC) was calculated from the FW and the DW by the adopted formula: $WC\,(\%) = 100 \times (FW—DW)/FW$ [17].

The leaf electrolyte leakage ratio (ELR) was

examined and calculated following the method outlined by Elsawy et al [18], with slight modifications. The proline concentration of the fresh leaf blades was measured using the ninhydrin reaction rapid method developed by Bates et al [19]. The chlorophyll content was determined in the third leaves from the top of the plants using organic solvent dimethylformamide. Chlorophyll a and b contents were calculated using the method of Porra et al [20]. The sodium and potassium concentrations in the plant tissues (leaf blades, leaf sheaths, and roots) were analyzed using a flame photometer (ANA-135; Tokyo Photoelectric, Tokyo, Japan). The Na^+ distribution in the whole plant was calculated as previously described by Wangsawang et al [10].

Expression analysis of the genes encoding Na^+ transport proteins

RNA was isolated from the leaf sheaths and roots of M-1, M-3, PTT1, and Pokkali plants grown under the control and the salt stress conditions using TRIZOL reagent. The RNA was quantified using a Nanodrop® spectrophotometer ND-1000 (Thermo Fisher Scientific Inc.). DNaseI-treated RNA sample (1 μg) was reverse-transcribed to cDNA using a ReverTra Ace qPCR RT kit, according to the manufacturer's protocol (Toyobo, Osaka, Japan). A quantitative polymerase chain reaction was performed using THUNDERBIRD SYBR qPCR Mix and an ABI StepOne System (Applied Biosystems, CA), following the procedure previously described by Ueda et al [21]. Quantitative RT-PCR was performed using the method described by Chuamnakthong et al [17]. The relative expressions of all target genes, *OsNHX1* [22], *OsHKT1;4* [23], and *OsHKT1;5* [21], were normalized to the expression of the *OsUBC* gene (internal control) [24] and then calculated using the comparative $2^{-\Delta\Delta CT}$ method [25]. The sequences of primers used in the present study are listed in Table S1.

Statistical analysis

Statistical analyses were undertaken using the SPSS version 21 software package (IBM Inc., USA). Data were analyzed using one-way analysis of variance and the means ($n = 4$) were separated using Duncan's multiple range test (DMRT). Association among characters were examined by simple correlation analysis. All tests were subjected to a 95% confidence limit.

RESULTS

Seedling scoring for salt tolerance

In the present study, seedling scoring was performed after 12 days of salt stress, and the degrees of salt tolerance of seedlings grown in hydroponic cultures and in soil-based pots were visually distinguished (Table S2). The positive control seedlings of Pokkali cultivar had the SES score of 3.5 in both the hydroponic culture and soil-based pot. The M-1 and M-3 lines showed similar tolerance to salt stress to the Pokkali, with the scores of 3.5 and 4.0, respectively, in hydroponic cultures; and 2.5 and 2.0, respectively, in soil-based pots (Table S2). Contrarily, most of the PTT1 plants died, or some still had the green youngest leaves. Thus, PTT1 plants were considered highly sensitive, with the scores of 8.5 and 8.0 in the hydroponic culture and in the soil-based pot screening methods, respectively.

Effects of salt stress on plant growth and physiology

Salt stress affected growth, biomass, and some physiological parameters of the rice cultivars. In the hydroponic cultures, the shoot lengths of the four rice cultivars/lines were affected by the salt stress treatment (Fig. 1a). The root lengths of the M-1, the M-3, the Pokkali, and the PTT1 were significantly increased under the salt stress condition by 16.28%, 13.79%, 14.81%, and 1.48%, respectively (Fig. 1b). In the hydroponic culture treatment, the shoot DW of all the four cultivars/lines showed a non-significant decrease under salt stress (Fig. 1c). However, there was a significant decrease in root DW in the PPT1, but not in the others (Fig. 1d). In contrast, in the soil-based pot experiments, almost all cultivars/lines (except Pokkali) had markedly decreased shoot lengths under the salt treatment (Fig. 1e); and only the root length of PTT1 was affected by the salt stress (Fig. 1f). For the soil-based pot experiments, the two mutant lines, M-1 and M-3, showed a non-significant decrease in both shoot and root DWs under the salt stress (Fig. 1(g,h)). These observations suggest that the M-1 and the M-3 were highly salt-tolerant compared with the other two.

Leaf WC was determined to estimate the amount of water lost under stress. As shown in (Fig. S1(a,d)), salt stress resulted in a non-significant decrease in WC in the leaf blades of M-1, M-3, and Pokkali in both screening methods. However, the PTT1 showed decreases in leaf WC under the salt stress treatment of 26.82% and 34.26% in

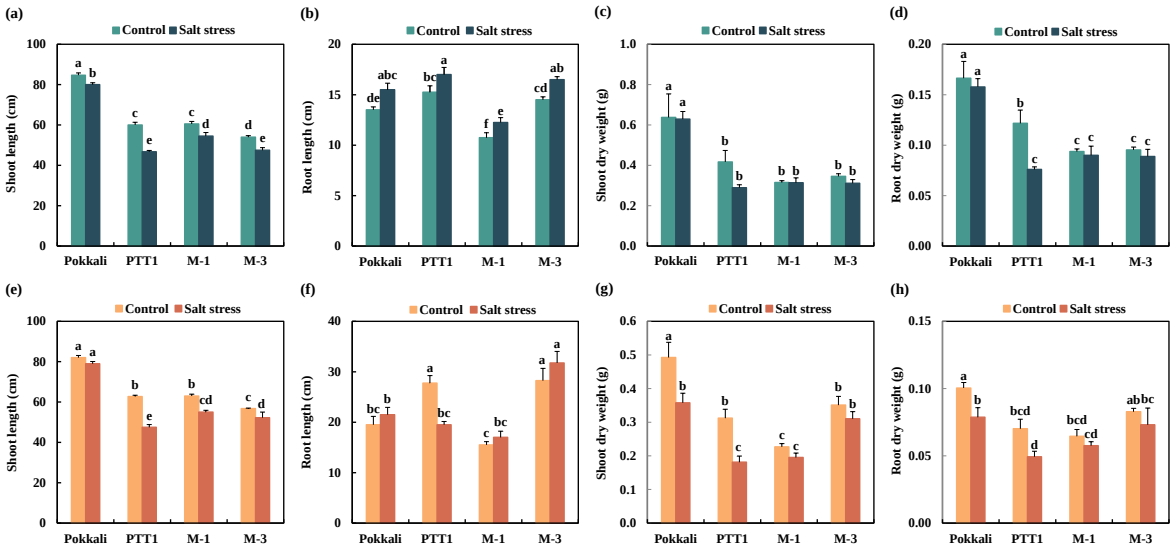

Fig. 1 Growth parameters of the four rice cultivars/lines grown in hydroponic cultures (a–d) and in soil-based pots (e–h) under the control and the salt stress conditions for 12 days: (a,e) shoot length; (b,f) root length; (c,g) shoot dry weight; and (d,f) root dry weight. Value of means followed by different alphabets are statistically significant ($p \leqslant 0.05$) according to DMRT.

the hydroponic culture and in the soil-based pot experiments, respectively.

The ELR value in all four cultivars/lines increased with increasing salt concentration (Fig. S1(b,e)). The two mutant lines, M-1 and M-3, had non-significant increases of ELR, compared with the non-treated group, in both screening methods. However, the ELR value of the tolerant cultivar, Pokkali, was not significantly increased in the soil-based pots and slightly increased (2.4-fold) in the hydroponic condition. Salt stress was found to influence the ELR of the salt-sensitive PTT1 cultivar with 4.6-fold (in hydroponic culture) and 4.4-fold (in soil-based pot) increases in ELR compared with the control seedlings.

Increasing levels of salt stress caused higher proline concentration in the leaf blades. As shown in Fig. S1(c,f), the proline accumulation in the leaves was highly induced in the M-1, the M-3, and the PTT1; but unaffected in the Pokkali, in both screening methods. The highest proline accumulations in the hydroponic culture of 41.56 μg/g FW was found in the M-1 and in the soil-based pot of 61.51 μg/g FW in the PTT1; whereas the Pokkali had the lowest proline contents in both the hydroponic culture (14.39 μg/g FW) and the soil-based pot (16.66 μg/g FW).

The chlorophyll a and b contents of the M-1, the M-3, and the Pokkali were not significantly different in either the hydroponic cultures (Fig. S2(a,b)) or the soil-based pot experiments (Fig. S2(c,d)). In contrast, the sensitive cultivar, PTT1, the chlorophyll a and b contents were greatly decreased under salt stress in both screening methods. However, the chlorophyll a in the hydroponic cultures was not statistically different from that of the control.

Effects of salt stress on the accumulation of Na^+ and K^+ in different tissues

With salt treatment, the concentration of Na^+ significantly increased in the plant tissues of almost all cultivars/lines. The Na^+ concentrations in the leaf sheaths and roots of all the four cultivars/lines were statistically induced in both screening methods. The mutant lines, M-1 and M-3, and the tolerant Pokkali had a lower accumulation of Na^+ in the leaf sheaths and the roots than the sensitive PTT1. However, in the hydroponic culture, Na^+ accumulations in the leaf blades of the M-1 and the M-3 showed a non-significant decrease, but an 18.04 mg/g DW increase in the Pokkali. Interestingly, in the soil-based pot experiments, Na^+ accumulations in the leaf blades of the two mutant lines did not differ from the control Pokkali with the increases in Na^+ concentration of 5.46 and 5.9 mg/g DW in the M-1 and the M-3, respectively. The highest Na^+ accumulations were measured in PTT1: 78.26 mg/g DW in the roots (Fig. 2a), 75.70 mg/g DW in the leaf sheaths (Fig. 2b), and 84.88 mg/g DW in the leaf blades (Fig. 2c) under the salt stress conditions of the

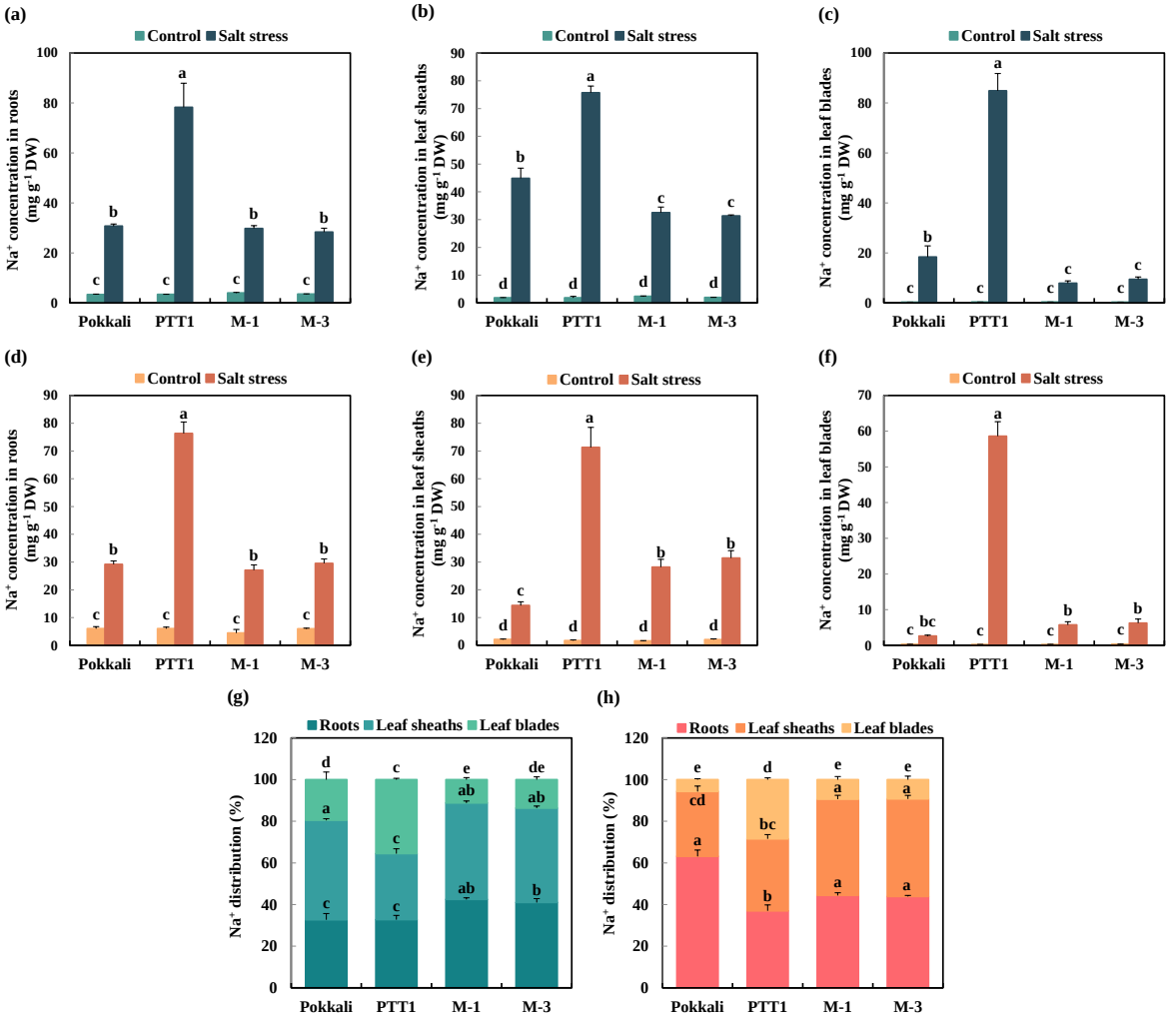

Fig. 2 Na^+ concentrations in the tissues of the four rice cultivars/lines grown in hydroponic cultures (a–c) and in soil-based pots (d–f) under the control and the salt stress conditions for 12 days: (a,d) roots; (b,e) leaf sheaths; and (c,f) leaf blades; and distribution of Na^+ accumulation in the roots, leaf sheaths, and leaf blades of the four rice cultivars/lines grown in hydroponic cultures (g) and in soil-based pots (h). Value of means followed by different alphabets are statistically significant ($p \leqslant 0.05$) according to DMRT.

hydroponic culture; whereas in the soil-based pot experiments, the Na^+ contents were 76.33, 71.30, and 58.62 mg/g DW in the roots (Fig. 2d), the leaf sheaths (Fig. 2e), and the leaf blades (Fig. 2f), respectively.

The Na^+ concentration distribution in plant tissues was estimated by calculating the ratio of Na^+ accumulation in the leaf blades, leaf sheaths, and roots. Na^+ accumulation was found in the leaf sheaths and the roots of the two mutant lines (M-1 and M-3) and the salt-tolerant Pokkali, but only 11.22%, 13.68%, and 19.53% (Fig. 2g) of the absorbed Na^+ were respectively found in their leaf blades in the hydroponic cultures; and 9.40%, 9.29%, and 5.67% (Fig. 2h), respectively, in the soil-based pot experiments. In the contrary, the PTT1 accumulated 35.54% (in hydroponic culture) and 28.42% (in soil-based pot) of the absorbed Na^+ in the leaf blades.

K^+ accumulation in tissues of almost all cultivars/lines was notably decreased. In the hydroponic cultures and the soil-based pot experiments, the K^+ concentration were significantly decreased in the roots (Fig. S3(a,d)), the leaf sheaths (Fig. S3(b,e)), and the leaf blades (Fig. S3(c,f)). However, in the soil-based pot experiments, the K^+ accumulation in the leaf blades of the Pokkali and the PTT1 did not differ from the control.

Differential expression of the genes encoding Na^+ transport proteins in response to salt stress

To clearly understand the mechanisms underlying the differential accumulation of Na^+, the transcript levels of genes encoding the Na^+ transport proteins were analyzed. In response to salt stress at 100 mM NaCl, 7.06-fold induction of *OsHKT1;5* expression was observed in the roots of the salt-tolerant Pokkali under soil-based pot conditions, but its expression was slightly upregulated (1.10-fold) under hydroponic culture conditions (Fig. 3(a,e)). Besides, under hydroponic culture conditions, *OsHKT1;5* expression was markedly upregulated in the roots of the M-1 (3.59-fold) and the M-3 (2.23-fold); and 1.95-fold and 1.42-fold under the soil-based pots for the M-1 and the M-3, respectively. However, *OsHKT1;5* was not detected in the roots of the salt-sensitive PTT1 cultivar under hydroponic conditions, whereas under the soil-based pot experiments, the gene was dramatically repressed (0.24-fold) compared with the others.

OsHKT1;4 is considered an alternative candidate for Na^+ exclusion, which is effective in the leaf sheaths, thus decreasing Na^+ accumulation in the leaf blades. In response to salt stress at 100 mM NaCl, quantitative RT-PCR analyses showed that the *OsHKT1;4* gene expression was slightly upregulated in the leaf sheaths of Pokkali in the soil-based pot experiment by 1.16-fold; however, its expression was 0.37-fold lower in the hydroponic culture. Repression of *OsHKT1;4* expression was observed in the leaf sheaths of the PTT1, the M-1, and the M-3 under both hydroponic culture and soil-based pot screening methods. However, the expression of this gene was significantly lower in the salt-sensitive PTT1 than that in the other rice cultivars (0.18-fold in hydroponics and 0.21-fold in soil-based pots) Fig. 3(b,f)).

After exposure to salt stress at 100 mM NaCl, the expression of *OsNHX1* was markedly induced in the leaf sheaths and the roots of the salt-sensitive PTT1 compared with the others in both the hydroponic and the soil-based pot experiments (Fig. 3(c,d,g,h)). However, the induction magnitude of *OsNHX1* gene expression in the salt-sensitive PTT1 was highly detected in the roots more than in the leaf sheaths.

DISCUSSION

Growth and physiological responses

In the present study, DW partitioning to the shoots and the roots was strongly dissimilar among the four cultivars/lines and persistently linked to salt tolerance. The dissimilarity between the tolerant (M-1 and M-3) lines and the sensitive (PTT1) cultivar was evident from the changes in the shoot and the root DWs. For the M-1 and the M-3 lines, the shoot and the root DWs were slightly increased by salt stress; whereas the other cultivars were retarded. The root length response to salt stress in both screening methods was the opposite (positive growth) to the shoot length response. Salt stress causes stiffening of the cell wall [26] and a reduction in water conductance of the plasma membrane causing a decrease in plant height [26].

The present study found that, under salt stress, cell membrane damages were observed in the salt-sensitive PTT1 cultivar, whereas the membrane permeability of the salt-tolerant M-1 and M-3 lines did not suffer from any damage in the hydroponic and soil-based pot screening methods. This result is in agreement with previous findings of Lutts et al [27], which suggests that the ELR was increased in response to salt stress, and it was higher in the salt-sensitive than in the salt-tolerant rice cultivars.

Under salt stress, the water uptake from the roots to the shoots is often inhibited owing to the higher osmotic pressure generated by the soluble salts in the soil solution than in the root cells. Bell and O'Leary [28] reported that the WCs in both the shoots and the roots of plants grown under non-stressed conditions were significantly higher than those of plants grown under salt stress conditions. Our study revealed that there was no significant difference in the WC of the salt-tolerant Pokkali, the M-1, and the M-3 under the control and the salt stress conditions in both screening methods; however, a higher reduction was found in the salt-sensitive PTT1. These results imply that the growth reduction of the salt-sensitive PPT1 may be related to its low efficiency in maintaining tissue water.

Under salt stress conditions, plants synthesize proline to defend themselves and adjust their physiological status [29]. In this study, a statistically significant difference was found between the proline concentrations of the PTT1 cultivar and the two mutant lines, except the Pokkali that showed a non-significant increase, in both screening methods. The increased proline concentration in the leaves of the two mutant lines might be an indicator of the Na^+ uptake and the identification of salt tolerant cultivars, which effectively exclude Na^+ from their leaf blades by either exclusion or compartmentalization in the leaf sheaths and the roots. In the present study, however, proline accumulation in the leaf blades under salt stress may not be a suitable phys-

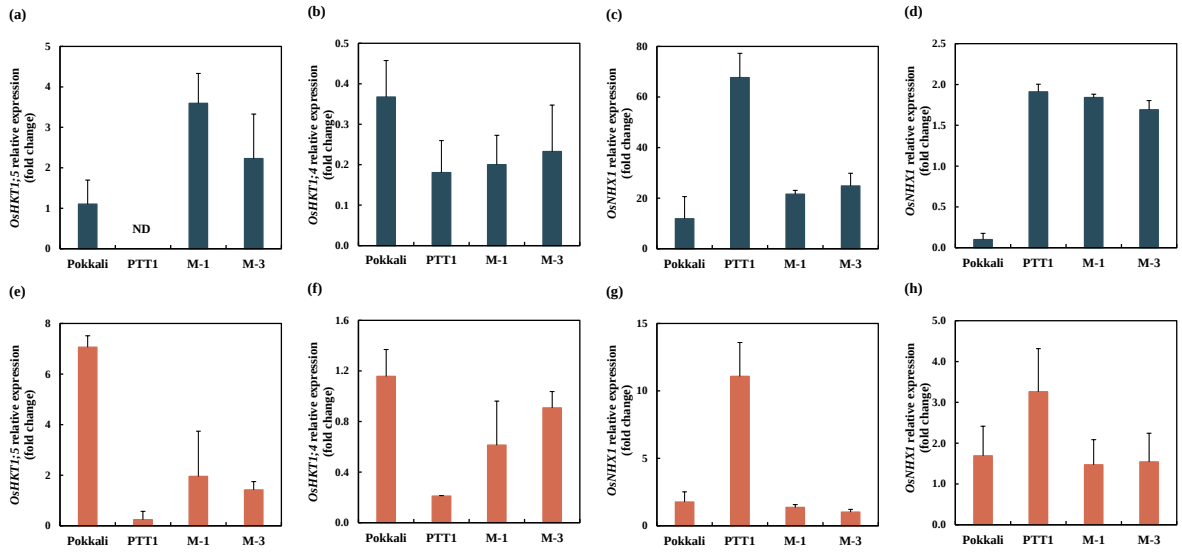

Fig. 3 Relative expression of the genes encoding Na^+ transport proteins of the four rice genotypes grown in hydroponic cultures (a–d) and in soil-based pots (e–h) under the salt stress conditions for 12 days: (a,e) *OsHKT1;5*; (b,f) *OsHKT1;4*; (c,g) *OsNHX1* (roots); and (d,h) *OsNHX1* (leaf sheaths). Values are means of two independent experiments ± standard error.

Table 1 Na^+/K^+ ratio in the leaf blades, leaf sheaths and roots of the four rice cultivars/lines grown in hydroponic cultures and in soil-based pots under the salt stress conditions.

Cultivars/lines	Hydroponic cultures			Soil-based pots		
	leaf blades	leaf sheaths	roots	leaf blades	leaf sheaths	roots
Pokkali	0.76 ± 0.20	1.50 ± 0.20	5.61 ± 0.26	0.10 ± 0.01	0.37 ± 0.02	5.77 ± 0.43
PTT1	3.14 ± 0.21	4.52 ± 0.51	75.43 ± 0.42	1.86 ± 0.15	4.56 ± 0.65	22.72 ± 0.96
M-1	0.32 ± 0.04	1.16 ± 0.07	5.14 ± 0.07	0.26 ± 0.10	1.15 ± 0.09	3.19 ± 0.48
M-3	0.48 ± 0.04	1.36 ± 0.09	5.44 ± 0.11	0.35 ± 0.09	1.32 ± 0.13	3.08 ± 0.53

Values are the mean of four replicates ± standard error.

iological parameter of salt-tolerant cultivars/lines.

A decrease in chlorophyll content may be caused by toxic ion accumulation and functional disorders observed during the closing and the opening of stomas under the salt stress conditions [30, 31]. In the present study, the chlorophyll a and chlorophyll b contents of the M-1, the M-3, and the Pokkali were not statistically significant in either the hydroponic culture or soil-based pot screening methods. However, there was a significant decrease in the contents of both chlorophylls in the leaf blades of the salt-sensitive PTT1 upon exposure to salt stress in both screening methods, except for chlorophyll a in the hydroponic culture, which implied that the high accumulation of Na^+ in the leaf blades of the PTT1 might decrease photosynthesis activity and thus lower the growth rate of the plant.

In our study, Na^+ accumulation in the leaf blades of the M-1 and the M-3 lines in the hydroponic culture showed a non-significant decrease under salt stress conditions compared with the non-treated samples. However, the rankings in the Na^+ exclusion of leaf blades in the hydroponic cultures were unrelated to the rankings found in the soil-based pot experiments. Previous studies have demonstrated the genetic differences in Na^+ exclusion of bread wheat in hydroponic cultures [14] and the dissimilar results for the barley cultivar, suggesting that the discrimination level in Na^+ exclusion was much lower in the hydroponics than in the soil [15].

The M-1 and the M-3 lines exhibited lower root Na^+/K^+ ratios (3.19 and 3.08, respectively) than the PTT1 cultivar (22.72) in the soil-based pot experiment (Table 1). In addition, the root Na^+/K^+ ratios of the M-1 (5.14) and the M-3 (5.44) in hydroponic cultures were higher than those in the soil-based pots. The Na^+/K^+ ratios of the M-1 and

the M-3 leaf blades in the soil-based pots were lower than those in the hydroponic cultures. The mutant lines showed lower Na^+/K^+ ratios, compared with the PTT1, in all tissues under the salt stress conditions in both the hydroponic culture and the soil-based pot experiments (Table 1). Responses to salt stress in hydroponics and soil may be different [15, 32]. An important point is that it can take days to weeks for such variation to develop in the soil, and plants grown in soil will have more time for salt adjustment than those grown in hydroponic culture [33]. Therefore, there must be an adaptation mechanism of specific significance (such as osmotic adjustment) requiring the uptake of ions and the arrangement of consistent solutes happened in the soil; and such activities do not occur in the hydroponic systems [15]. This was shown in the significant Na^+ exclusion and the better maintenance of leaf blade K^+ concentrations under salt stress condition, which are associated with higher tolerance to salt stress. Furthermore, the Na^+/K^+ ratio has been found associated with tolerance to salt stress [5].

Mechanisms of Na^+ retrieval at the tissue level and Na^+ exclusion at the cell level

To further understand the mechanisms underlying the limited Na^+ transport to the leaf blades in the mutant lines, the transcription level of the *OsHKT1;5* gene was examined. Under salt treatment, the *OsHKT1;5* gene expression was greatly upregulated in the roots of the M-1 and the M-3 lines (Fig. S4) but reduced or not detected in the roots of the PTT1 cultivar in both screening methods. In rice plants, the *OsHKT1;5* transporter is localized in the roots, where it mediates Na^+ retrieval from the xylem to the xylem parenchyma cells before entering the transpiration stream to the shoots [7, 9]. An induction in the *OsHKT1;5* activity was observed in the salt tolerant Pokkali, but the activity decreased in the sensitive IR29 [34]. Therefore, it is possible that in this experiment, the improved growth of both mutant lines might be due to the upregulated *OsHKT1;5* gene expression in the roots, which is pivotal to the reduced Na^+ accumulation in the leaf blades. Whereas, the salt sensitivity of the PTT1 might be due to the downregulation of *OsHKT1;5* expression in the roots, consequently leading to disordered Na^+ transport to the leaf blades and growth impairment. Thus, the M-1 and the M-3 mutant lines may have a greater ability to restrict the Na^+ accumulation by *OsHKT1;5* gene in the roots than the PTT1.

An alternative mechanism in the Na^+ transport restriction in the leaf sheaths of the four cultivars/-lines was also assessed by studying the *OsHKT1;4* gene expression. By comparing salt-tolerant and salt-sensitive cultivars and their patterns of Na^+ concentration, Wangsawang et al [10] showed that *OsHKT1;4*, may participate in a Na^+ retrieving mechanism from the rice xylem in the leaf sheaths. Under the salt stress in the hydroponic experiment, the *OsHKT1;4* gene expression was repressed in the leaf sheaths of all rice cultivars/lines, and the magnitude of the downregulation was greater in the salt-sensitive PTT1 than in the salt-tolerant Pokkali and the two mutant lines. In contrast, under the soil-based pot experiment, the expression of the *OsHKT1;4* gene in the Pokkali was slightly upregulated by the salt treatment, leading to a less Na^+ accumulation in the Pokkali leaf blades than in the others. Molecular analysis suggested that under hydroponic conditions, the Na^+ retrieval in the leaf sheaths mediated by *OsHKT1;4* did not contribute to a restriction of Na^+ accumulation in the leaf sheaths of either the tolerant or the sensitive cultivars when salt stress was applied. However, to understand the association between the *OsHKT1;4* gene induction in the salt-tolerant Pokkali and the soil rhizosphere, the root system architecture of the Pokkali should be further investigated.

Accumulating the excess of Na^+ into the vacuoles is considered an effective strategy for plants to tolerate salt stress through the reduction of Na^+ toxicity in the cytosol. *OsNHX1* of rice encodes a vacuolar Na^+ (K^+/H^+ antiporter) and plays an important role in the compartmentalization of excess cytosolic Na^+ and K^+ into the vacuoles [11]. Our study found that under the salt stress of 100 mM NaCl, the expression level of the *OsNHX1* gene was highly upregulated in the leaf sheaths and the roots of the salt-sensitive PTT1 in both hydroponic and soil-based pot experiments, corresponding to a high amount of Na^+ accumulation in two tissues. The increased Na^+ concentration is a consequence of *OsNHX1* activity, as an antiporter, facilitating the Na^+ uptake into the vacuoles in an exchange for H^+ in the cytoplasm. We further showed that, in response to salt stress, *OsNHX1* gene expression in the tolerant genotypes showed a distinct pattern in both screening methods. In the roots, the level of *OsNHX1* induction was much higher in hydroponics culture than in the soil-based pot, whereas its expression remained relatively unchanged in the leaf sheaths of the M-1 and the M-3 lines in both conditions. Therefore, the upregulated expression

of *OsNHX1* was mainly in response to elevated Na^+ levels in both the tolerant (Fig. S4) and the sensitive cultivars/lines; however, the sensitive cultivar may not completely compartmentalize the excess Na^+ into the vacuoles, hence causing accumulation of Na^+ in the other tissues. Conversely, the Na^+ retrieval mechanisms governed by *OsHKT1;4* in the leaf sheaths and *OsHKT1;5* in the roots were not active in the salt-sensitive PTT1; thus, the high concentration of Na^+ absorbed in the roots under salt stress conditions was transported to the aerial parts.

CONCLUSION

The two mutant lines, M-1 and M-3, expressed different adaptation mechanisms under salt stress conditions, with Na^+ concentration highly accumulated in the roots and the leaf sheaths corresponding to the low Na^+ accumulation in the leaf blades. The growth and physiological parameters, shoot DW, root DW, ELR, leaf WC, and chlorophyll (a and b) contents, of the two mutant lines were unaffected by the salt stress compared with the non-treated. However, the increase of proline accumulation in the leaf blades was an indicator for the response to salt stress in all four cultivars/lines. Differences in the mechanisms of salt tolerance in the M-1 and the M-3 lines suggested that Na^+ exclusion mechanisms via *OsHKT1;5* might enable the mutant lines to tolerate salt stress by preventing Na^+ accumulation in the aerial parts, but that might not happen in the salt-sensitive PTT1. In addition, the upregulated expression of *OsNHX1* was mainly in response to elevated Na^+ levels in the tolerant lines. Divergent regulation of Na^+ transporters may be involved in maintaining the low Na^+/K^+ ratios in the mutant lines under salt stress conditions. The present study demonstrated that differences in screening methods of hydroponic cultures and soil-based pots may not be able to differentiate the salt tolerance between cultivars. The results of our study also suggested that assessing salt tolerance at the seedling stage may not predict salt tolerance at the later stages.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2021.106.

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Appendix A. Supplementary data

Table S1 Primers used for quantitative real-time RT-PCR.

Genes	Forward primer (5′–3′)	Reverse primer (5′–3′)
OsNHX1	AATGATCACCAGCACCATCA	AAGGCTCAGAGGTGACAGGA
OsHKT1;4	GTCGAAGTTGTCAGTGCATATGG	TGAGCCTCCCAAAGAACATCAC
OsHKT1;5	TGCATTCATCACTGAGAGGAG	GGTGCAGTTTCTGCAACCTC
OsUBC	GCGGTTTGTTTCACGGATGTT	CTCCCTAACTCTCCGGTTGTA

Table S2 Salt tolerant rating of the four rice cultivars/lines (grown in hydroponic cultures and in soil-based pots) at seedling stage and 12 days of salt stress.

Cultivars/lines	Hydroponic cultures		Soil-based pots	
	SES	Degree of salt tolerance	SES	Degree of salt tolerance
Pokkali	3.5 ± 0.50	Tolerant	3.5 ± 0.50	Tolerant
PTT1	8.5 ± 0.50	Highly sensitive	8.0 ± 0.58	Highly sensitive
M-1	3.5 ± 0.50	Tolerant	2.5 ± 0.50	Tolerant
M-3	4.0 ± 0.58	Tolerant	2.0 ± 0.58	Tolerant

Values are the mean of four replicates ± standard error.

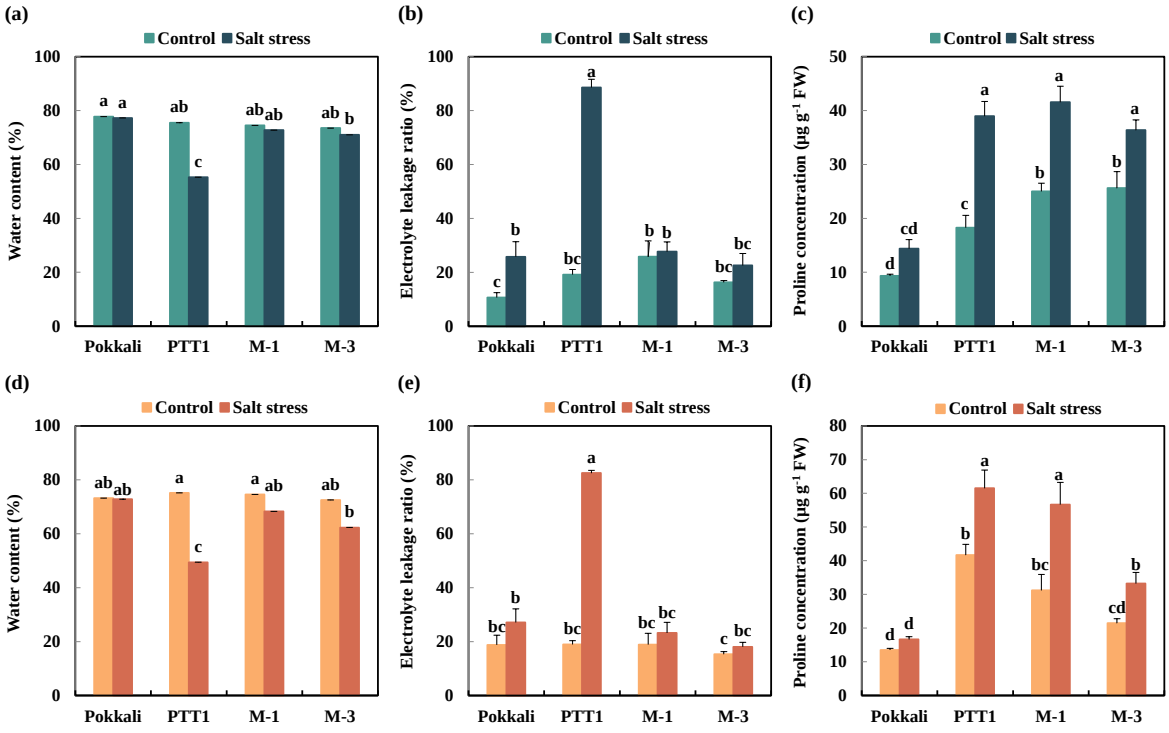

Fig. S1 Water content (a,d), electrolyte leakage ratio (b,e), and proline concentration (c,f) of the four rice cultivars/lines grown in hydroponic cultures (a–c) and in soil-based pots (d–f) under the control and the salt stress conditions for 12 days. Value of means followed by different alphabets are statistically significant ($p \leqslant 0.05$) according to DMRT.

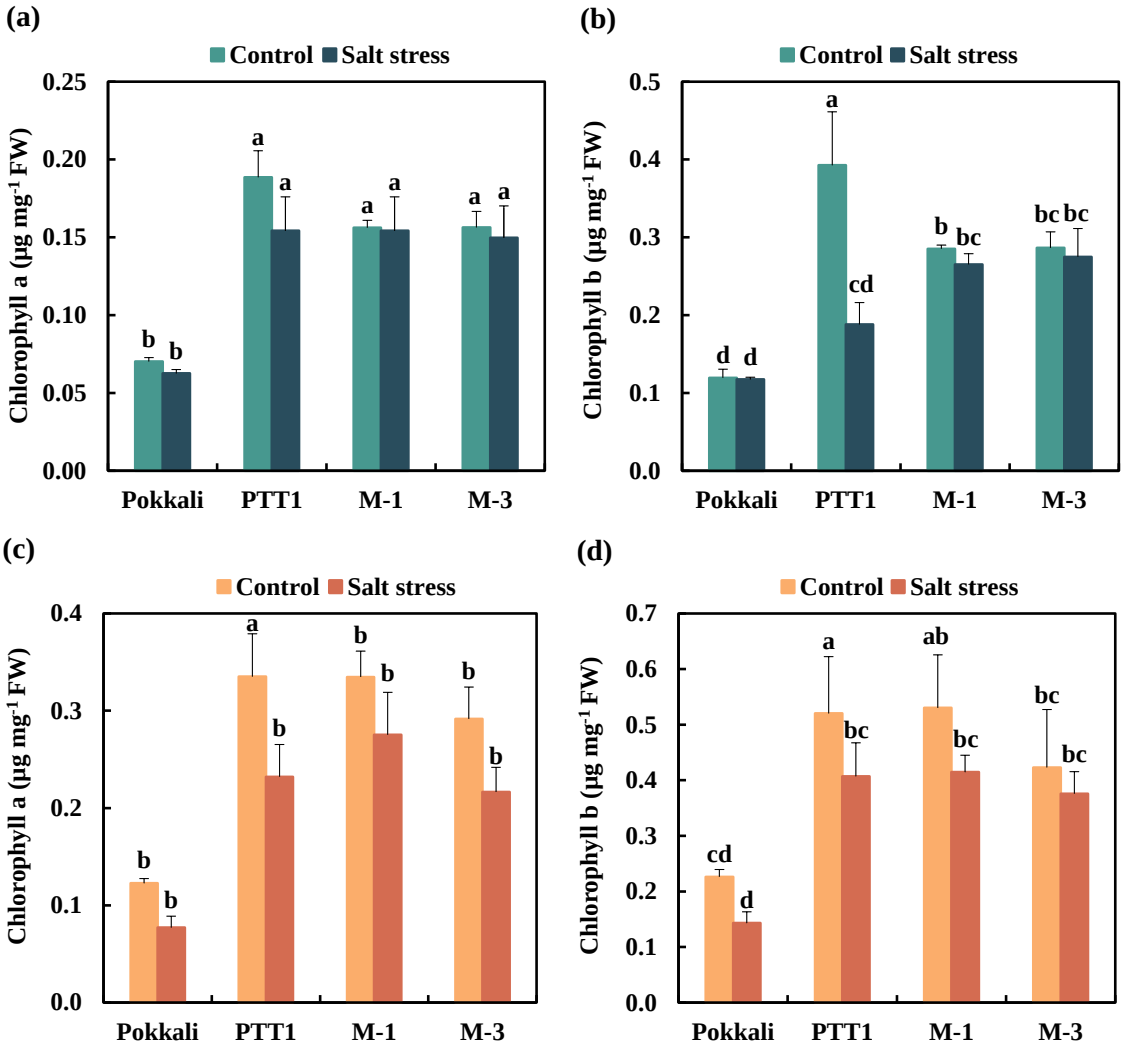

Fig. S2 Chlorophyll a (a,c) and chlorophyll b (b,d) of the four rice cultivars/lines grown in hydroponic cultures (a–b) and in soil-based pots (c–d) under the control and the salt stress conditions for 12 days. Value of means followed by different alphabets are statistically significant ($p \leqslant 0.05$) according to DMRT.

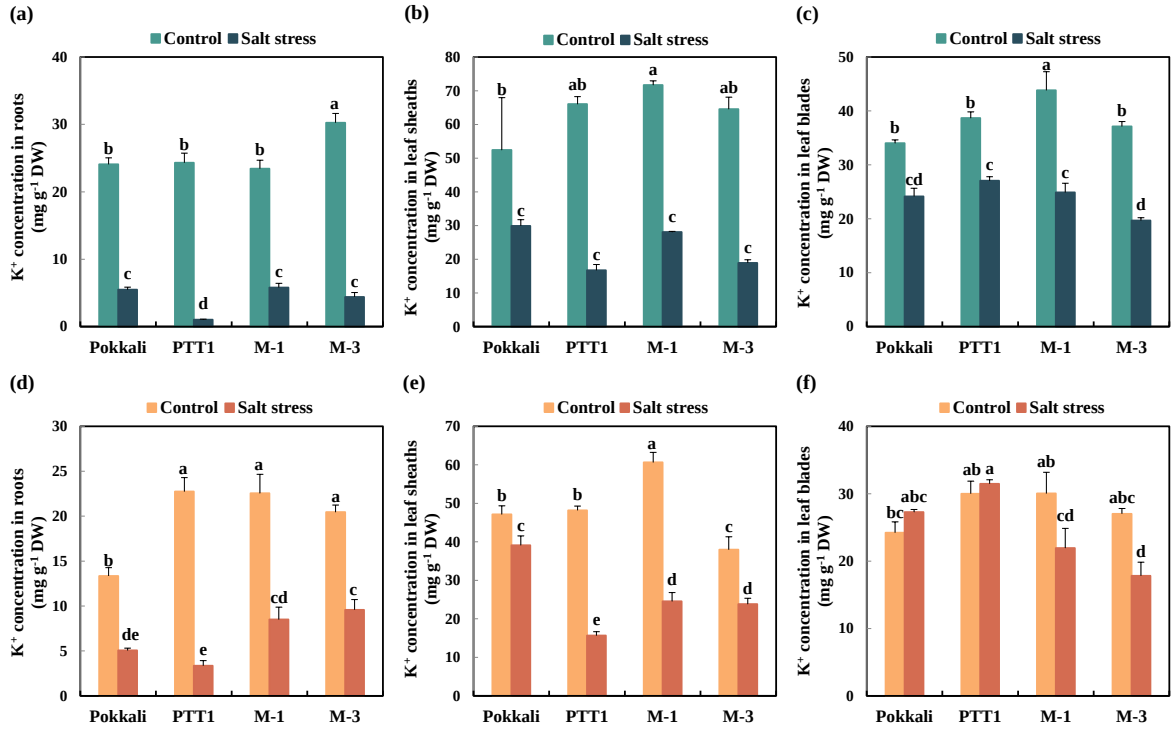

Fig. S3 K^+ concentrations in the tissues of the four rice cultivars/lines grown in hydroponic cultures (a–c) and in soil-based pots (d–f) under the control and the salt stress conditions for 12 days: (a,d) roots; (b,e) leaf sheaths; and (c,f) leaf blades. Value of means followed by different alphabets are statistically significant ($p \leqslant 0.05$) according to DMRT.

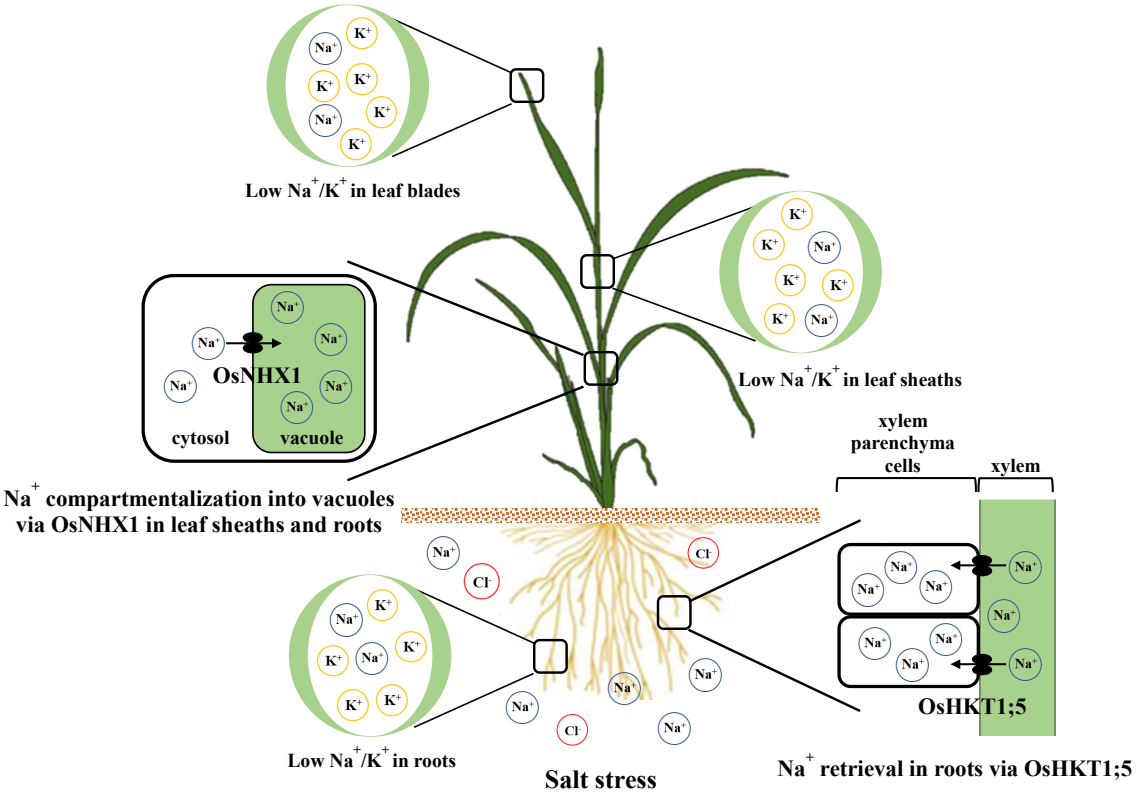

Fig. S4 A generalized schematic representation of salt tolerance mechanism in the mutant lines. The Na^+ retrieval mechanism via *OsHKT1;5* in the roots might up-regulate the salt stress tolerance of the mutant lines by preventing Na^+ accumulation in their aerial parts, whereas the Na^+ exclusion via *OsNHX1* in the leaf sheaths might respond to the elevated Na^+ sequestration in the vacuoles. The mutant lines showed lower Na^+/K^+ ratios in all tissues (leaf blades, leaf sheaths, and roots) under salt stress conditions.