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Unravelling the physiological basis of salinity stress tolerance in cultivated and wild rice species

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ABSTRACT

Wild rice species provide a rich source of genetic diversity for possible introgression of salinity stress tolerance in cultivated rice. We investigated the physiological basis of salinity stress tolerance in *Oryza* species by using six rice genotypes (*Oryza sativa* L.) and four wild rice species. Three weeks of salinity treatment significantly ($P < 0.05$) reduced physiological and growth indices of all cultivated and wild rice lines. However, the impact of salinity-induced growth reduction differed substantially among accessions. Salt tolerant accessions showed better control over gas exchange properties, exhibited higher tissue tolerance, and retained higher potassium ion content despite higher sodium ion accumulation in leaves. Wild rice species showed relatively lower and steadier xylem sap sodium ion content over the period of 3 weeks analysed, suggesting better control over ionic sodium xylem loading and its delivery to shoots with efficient vacuolar sodium ion sequestration. Contrary to this, saline sensitive genotypes managed to avoid initial Na^+ loading but failed to accomplish this in the long term and showed higher sap sodium ion content. Conclusively, our results suggest that wild rice genotypes have more efficient control over xylem sodium ion loading, rely on tissue tolerance mechanisms and allow for a rapid osmotic adjustment by using sodium ions as cheap osmoticum for osmoregulation.

Keywords: chlorophyll content, Na^+ sequestration, osmoregulation, salinity stress tolerance, stomata, tissue tolerance, wild rice, xylem ion loading.

Introduction

Soil salinity is a major environmental limitation for crop production which affects more than 20% of irrigated land worldwide (Shahbaz and Ashraf 2013; Munns and Gilliham 2015; Yang and Guo 2018). The current trends in climate change are leading to limited precipitation, elevated temperatures, and increased evapotranspiration, subsequently exacerbating the issue of soil salinity (Shabala 2013; Suzuki *et al.* 2016). The threats of salinity are more obvious in the arid and semi-arid areas of the world with insufficient rainfall, combined with poor water and soil management practices (Lodeyro and Carrillo 2015; van Zelm *et al.* 2020; Liu *et al.* 2021).

Being a staple food crop, rice (*Oryza sativa* L.) feeds more than half of the world population. But it is also the most salt susceptible amongst cereal crops. Salinity stress adversely affects plant growth primarily by reducing water availability, damaging photosynthetic machinery, initiating oxidative stress, and causing sodium ion (Na^+) toxicity (Munns and Tester 2008; van Zelm *et al.* 2020). The last component is due to Na^+-induced disturbance to potassium ion (K^+) homeostasis (Shabala and Pottosin 2014; Chakraborty *et al.* 2016; Assaha *et al.* 2017) which interferes with the normal functioning of K^+ in growth and metabolism. Given the importance of K^+ essentiality in maintaining the activity of several enzymes, keeping a tight balance between absorption and translocation of Na^+ and K^+ from roots to shoots is crucial in conferring salinity stress tolerance (Bose *et al.* 2014; Shabala *et al.* 2016; Ishikawa and Shabala 2019). Plants have evolved a range of strategies including efficient osmotic adjustment, Na^+

exclusion and/or sequestration, cytosolic K^+ retention, oxidative stress tolerance as well as control of xylem ion loading to minimise salinity-induced damages (Ashraf *et al.* 2008; Adem *et al.* 2014; Bose *et al.* 2014; Chakraborty *et al.* 2016). Overall, control of xylem ion loading, and its regulation is considered as a crucial physiological mechanism to achieve the above-mentioned ion balance. This process is facilitated by the synchronised activity of various ion transporters located at the xylem parenchyma boundary (Ishikawa *et al.* 2018). However, the activities and role of these transporters have not been comprehensively studied in salt stress tolerance of wild rice species.

According to the model proposed by Shabala *et al.* (2003) and Shabala (2013), a certain amount of Na^+ is supposed to be transported to the shoots for achieving osmotic adjustment in the initial stage of salinity stress. Once this is accomplished, reducing Na^+ xylem loading or retrieval of Na^+ from xylem would be a 'wise' option for plants to prevent Na^+ accumulation in the leaf tissue, where the photosynthetic activity could be disrupted by excessive cytosolic Na^+. With advances in applied molecular and biological techniques, numerous ion transporter candidate genes involved in xylem ion loading (e.g. Na^+, K^+ and Cl^-) have been characterised. The most critical of these are plasma membrane Na^+/H^+ antiporter *SOS1* (Shi *et al.* 2000, 2003; Olías *et al.* 2009; Feki *et al.* 2011, 2014, 2017; El Mahi *et al.* 2019), high-affinity Na^+ selective uniporter HKT (Davenport *et al.* 2007; Horie *et al.* 2009), chloride cation co-transporter CCC (Colmenero-Flores *et al.* 2007; Chen *et al.* 2016), and Shaker-like outward channel SKOR (Véry *et al.* 2014; Hu *et al.* 2016). All these transporters have been shown to potentially mediate xylem loading and unloading of K^+ and Na^+ in different plant species. Thus, the question remains: to what extent do different rice genotypes control the kinetics of xylem Na^+ loading?

Generally, plants exclude more than 98% of Na^+ back to the soil rhizosphere for maintaining normal growth, thus Na^+ exclusion is considered as one the most important features mediating the internal salt load of plants (Munns *et al.* 2020). The plasma membrane Na^+/H^+ antiporter (*SOS1*) has been characterised in *Arabidopsis* as a unique Na^+ efflux transporter localised at the root tip epidermis and xylem parenchyma cells (Shi *et al.* 2003). Recently, the *OsSOS1* was reported from rice, which mediates both net root Na^+ uptake and its redistribution to the roots and shoots (El Mahi *et al.* 2019) and is known to partially regulate Na^+ efflux at the xylem–parenchyma boundary (Liu *et al.* 2019). However, it is believed that *SOS1* genes of wild alleles have been lost due to the domestication process making them highly salt sensitive (Prusty *et al.* 2018). At present, current breeding programs for inducing salinity stress tolerance have focused largely on genetic diversity, particularly of those species which are part of the primary gene pool of cultivated rice (*Oryza sativa* and *Oryza glaberrima*), for the purpose of introgressing favorable alleles into the elite rice cultivars (Gur and Zamir 2004; Sharma *et al.* 2013; Prusty *et al.* 2018; Solis *et al.* 2020). Therefore, the rice germplasm contains another 22 wild rice species which are indeed a possible source of new salinity tolerance genes/alleles due to a largely pool of available genetic diversity. These species/accessions might be useful for breeding programs after a better understanding of their phylogenetical relationships and physiological mechanisms of salinity tolerance is obtained.

Thus, this study aims to investigate the unique traits and potentially unidentified strategies that wild rice species adopt to combat salinity stress. The following key questions were asked: (1) do wild rice species possess better control over stomatal aperture? (2) How does salinity stress affect tissue ionic content (Na^+ and K^+) in cultivated and wild rice species? and (3) is differential salinity tolerance related to the difference in plants' ability to control xylem Na^+ loading?

Materials and methods

Plant materials and growth conditions and preparation of treatments

In this study, six rice cultivars (*Oryza sativa* L.) and four wild rice species accessions (*Oryza alta*, *Oryza barthii*, *Oryza australiensis*, and *Oryza punctata*) were used. The experiment was conducted in 2018–2019 (from October to January) in glasshouse conditions in Sandy Bay campus, University of Tasmania, Hobart (Supplementary Fig. S1). Plants were grown in pots using standard potting mixture comprising of 80% composted pine bark, 10% sand and 10% coir peat, plus slow-release fertiliser (N:P:K ratio 8:4:10). Briefly, seeds of different rice genotypes were directly sown in plastic trays which were maintained at 30°C for germination and seedling establishment. Seven-day old rice seedlings were transplanted to 1.5 L pots (5–6 plants per pot) and grown in the glasshouse under controlled temperature conditions (26–28°C) for 2 weeks for seedling establishment. For salinity treatments, three salt concentrations were applied as: (1) control with no salt, (2) 50 mM NaCl: moderate salt stress, and (3) 100 mM NaCl: severe salt stress. To achieve uniformity across the treatments, six pots of different genotypes were placed in a 40-L polyvinyl chloride (PVC) containers and water was filled up to one inch of plant depth for uniform treatment application. For this purpose, the required concentration of salt was mixed with tap water and the water level was maintained with additional tap water. Water was replaced after 4–5 days with freshly prepared salt concentrations. Rice seedlings were treated for 21 days with different salinity treatments before final measurements were recorded.

Chlorophyll content and stomatal conductance

Measurements for chlorophyll content (SPAD value, arbitrary units) and stomatal conductance (Gs, mmol $m^{-2}s^{-1}$) were recorded from the youngest fully expanded leaves at the end of experiment i.e. 21 days after treatment (DAT). Briefly, the relative chlorophyll content in leaves was measured using a SPAD-502 meter (Konica Minolta, Osaka, Japan). All the measurements were carried out from leaves in a region positioned at about one-third from the leaf tip. Eight to ten random recordings were taken and averaged to consider as one replicate, and 10 replicates were measured from each treatment. For stomatal conductance (Gs) measurements, a Decagon Leaf Porometer (Decagon Devices Inc., WA, Australia) was used to measure Gs (mmol $m^{-2}s^{-1}$) under full daylight conditions between 10:00 and 12:00 h. Ten individual replicates were taken from each treatment.

Fresh and dry plant biomass

Plants were harvested after 3 weeks of salt treatment, shoot and root fresh weight (SFW, RFW) per plant were determined immediately by using an electronic balance. Fresh samples were then put into the paper bags, placed in an oven, and dried at 70°C for 72 h for measuring shoot and root dry weight (SDW, RDW).

Tissue Na^+ and K^+ content

To measure the tissue ion content, shoot and root dried tissues were manually ground into powder. Dried plant material was then digested using microwave Reaction System Marc6 (Falakboland *et al.* 2017). Briefly, 0.3 g of dry matter was put into the vessel with an additional 10 mL of nitric acid (HNO_3). The microwave was run at 200°C for 15 min for complete digestion. Acid digested samples were taken out from the microwave after a certain cooling time, these samples were then transferred to plastic tubes and distilled water was added to achieve a final volume of 50 mL. Shoot and root ion contents (Na^+ and K^+) were then determined using flame photometer (PFP7, Jenway; Bibby Scientific Ltd., Stone, UK).

Xylem sap analysis

To understand the kinetics of xylem sap ion contents (Na^+ and K^+), xylem sap was collected by using the Scholander pressure chamber method (Plant Moisture Systems, Santa Barbara, CA, USA). Briefly, the seedling shoot was cut 5–10 mm above the potting medium and immediately inserted (cut surface up) into a pressure chamber (Zhu *et al.* 2017; Ishikawa and Shabala 2019). Compressed air was then filled into the chamber by applying pressure (5–10, 10–20 and 20–30 bars for 0, 50 and 100 mM sodium chloride (NaCl) treatments, respectively) to squeeze the xylem sap. To avoid the dilution effect, the rate of air flow was kept relatively low. After squeezing out sap, it was immediately collected with a micropipette and placed in a 1.5 mL Eppendorf tube. One to three xylem sap specimens from each pot were collected at every day of harvest, and each salinity treatment was replicated three to six times each. All the measurements were conducted at different time points (4, 7 and 21 days after the salinity imposition). Using a 0.1 mg readability balance, the collected samples were weighed to an accuracy of four decimal places. The samples were then diluted typically 20-fold using double distilled water, frozen and kept at −18°C until ion contents (Na^+ and K^+) were recorded using flame photometer (PFP7, Jenway; Bibby Scientific Ltd., Stone, UK).

Statistical analysis

Statistical software IBM SPSS Statistics 24 was used to determine significant differences between treatments using Student's *t*-test at a significance level of $P < 0.05$. Data shown in all the figures and tables are means ± standard error (s.e.).

Results

Biomass accumulation

Plant exposure to 50 mM and 100 mM NaCl stress resulted in significant ($P < 0.05$) reduction in shoot fresh weight (SFW) of all the genotypes (Fig. 1*a*). However, the impact of salinity-induced growth reduction differed substantially among the rice cultivars and wild rice species. Rice cultivar IR29 and wild rice species *O. punctata* were the most susceptible, showing 45–60% and 44–55% reduction in SFW under severe and moderate stress, respectively (Fig. 1*b, c*). Salt tolerant cultivars Pokkali and IR1 and wild rice species *O. barthii* and *O. alta* performed better than the rest of rice genotypes/wild rice species, showing the least reduction in SFW (25–42%) compared to their counterpart sensitive genotypes (Fig. 1*b, c*).

Three weeks of salinity stress significantly ($P < 0.05$) reduced the shoot dry weight (SDW) of all the tested genotypes and wild rice species (Fig. 1*d*). The average SDW was reduced to 47–87% relative to control in moderate salt stress alone, IR29 showed the most reduction in DW while *O. alta* and *O. barthii* showed least reduction, respectively (Fig. 1*d–f*). The reduction in SDW was more pronounced under severe salt stress ranging from 27 to 64% for Pokkali and IR29, respectively (Fig. 1*d–f*). Taken together, we suggest that salinity tolerance occurs in the following order for rice cultivars and wild rice species based on their relative shoot dry weight; Pokkali > *O. alta* > *O. barthii* > IR1 > Nipponbare > H-86 > *O. australiensis* > Pusa Basmati > *O. punctata* > IR29 (Table 1).

Exposing roots to salinity stress significantly decreased the RFW of all the rice cultivars ($P < 0.05$; Fig. 2*a–c*). However, the magnitude of root fresh and dry weight reductions were least pronounced in moderate salt stress. The average RFW

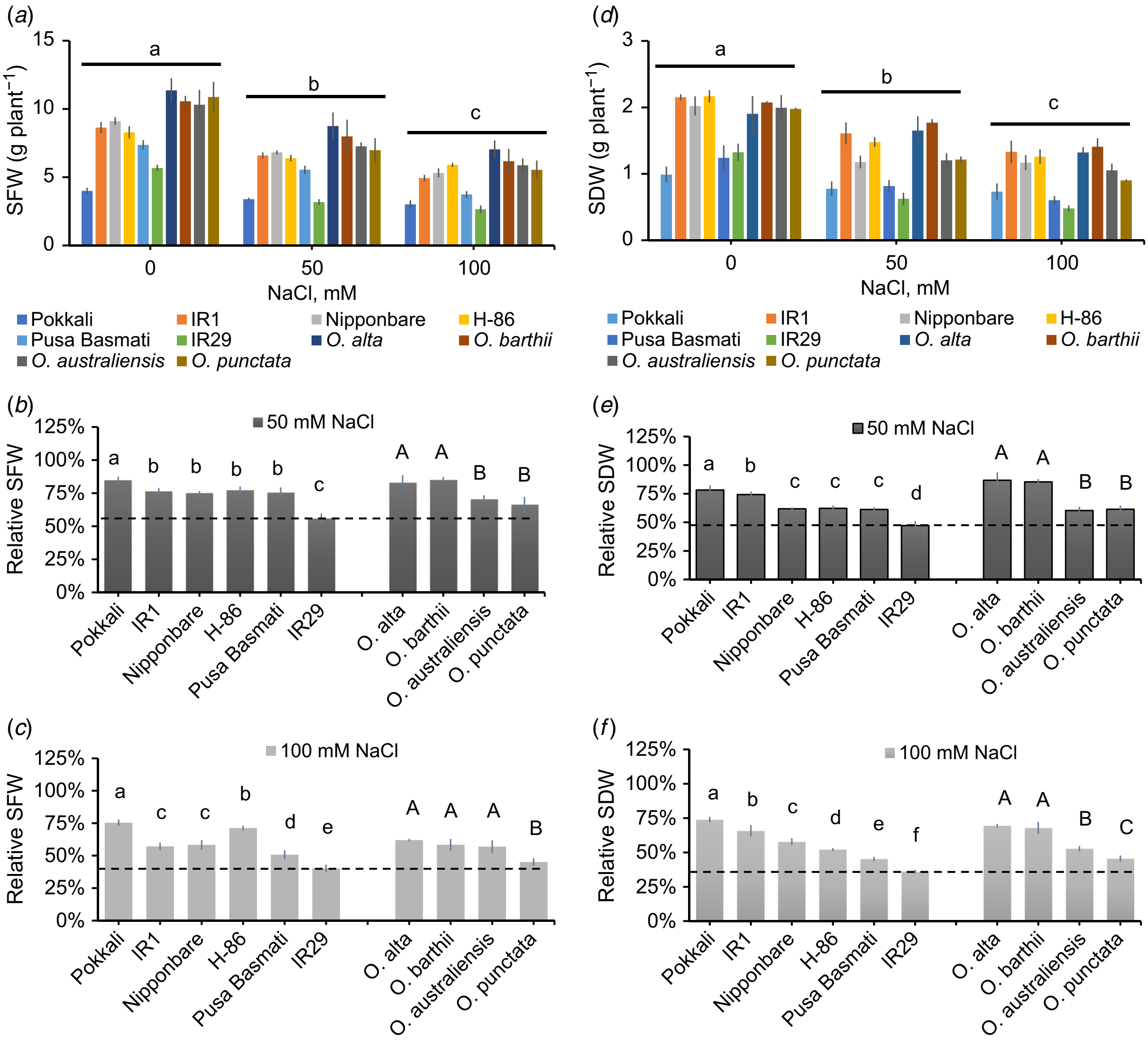

Fig. 1. Shoot fresh and dry weight (SFW, SDW) (*a*, *d*) and relative fresh and dry weights (b, c, e, f) of 10 rice accessions (six cultivars and four wild rice species) grown at 0, 50, and 100 mM NaCl for 2 weeks. The lowercase letters (*a*, *d*) indicate the significant difference among the salinity stress levels while lowercase and uppercase letters (*b*, *c*, e, *f*) indicate the significant difference at ($P < 0.05$) among the cultivated rice genotypes and among wild rice species. The error bars indicate the standard error (s.e.) for all the replicated data for each treatment. Data shown as mean ± s.e. ($n = 6$–8).

reduction was 67–81% relative to control under moderate stress, while 42–71% under severe stress, IR29 and Pokkali had the most and the least decrease in RFW, respectively (Fig. 2*a*–*c*). The average RDW of all the cultivars was reduced to 11–46% and 25–65% in moderate and severe salt stress, respectively (Fig. 2*d*–*f*). Rice cultivars IR29 and Pokkali showed the most and the least reductions under both salinity levels (Fig. 2*d*–*f*).

Chlorophyll content and stomatal conductance

Salinity stress affected plant physiological characteristics, but to different extents (Fig. 3). Three weeks of salinity treatment significantly ($P < 0.05$) reduced the SPAD values of all the rice genotypes and wild rice species (Fig. 3*a*–*c*). Wild rice species showed relatively higher SPAD content compared to the rice cultivars. For IR29 and *O. barthii*, the average SPAD values were 74–93% and 57–81% relative to control under severe and moderate salt stress, respectively, indicating the most and least reduction under both salt levels (Fig. 3*a*–*c*).

Stomatal conductance (Gs, mmol m^{-2} s^{-1}) of all the rice genotypes was significantly ($P < 0.05$) decreased under salinity stress (Fig. 3*d*–*f*), though the magnitude of Gs reduction was more pronounced in the cultivated rice genotypes. The average Gs values under moderate and severe salinity ranged between 53–66% and 20–28% for

Table 1. Relative salt stress tolerance (SDW%) of different rice genotypes exposed to 100 mM NaCl stress for 3 weeks.

	Genotype	Source/accession	Salinity tolerance (%)	Salt tolerance level
Cultivars (*Oryza sativa* L.)	Pokkali	IRGC 108921	73	Tolerant
	IR1	–	65	Tolerant
	Nipponbare	IRGC 117274	57	Moderate tolerant
	H-86	–	52	Moderate tolerant
	Pusa Basmati	Pusa 615-140-10-1	45	Sensitive
	IR29	IRGC 30412	36	Sensitive
Wild rice	*Oryza alta*	Philippines/IRGC 105143	69	Tolerant
	Oryza barthii	Philippines/ IRGC 100936	67	Tolerant
	Oryza australiensis	Philippines/IRGC 100882	52	Moderate tolerant
	Oryza punctata	Philippines/IRGC 105690	45	Sensitive

IR29 and *O. alta* respectively, showing the most and the least reduction in Gs (Fig. 3*d*–*f*). Interestingly, salt tolerant genotypes from both cultivated and wild species groups showed the least reduction in stomatal conductance suggesting that the former possess a better ability to control gas exchange properties as a means of coping with severe

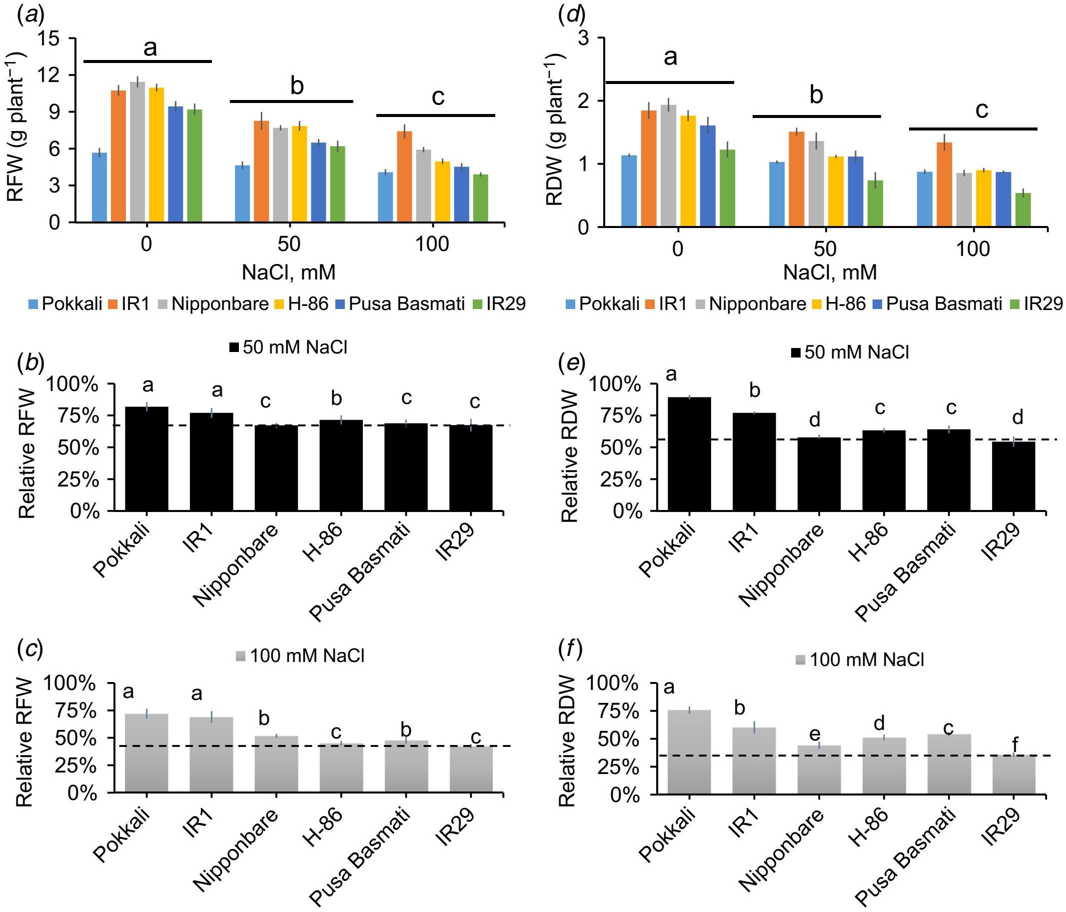

Fig. 2. Root fresh and dry weight (RFW, RDW; *a*, *d*) and relative fresh and dry weights (*b*, *c*, e, f) of six cultivars grown at 0, 50, and 100 mM NaCl for 3 weeks. The lowercase letters (*a*, *d*) indicate the significant difference among the salinity stress levels while lowercase letters (*b*, *c*, e, *f*) indicate the significant difference at ($P < 0.05$) among the cultivated rice genotypes. The error bars indicate the standard error (s.e.) for all the replicated data for each treatment. Data shown as mean ± s.e. ($n = 6$–8).

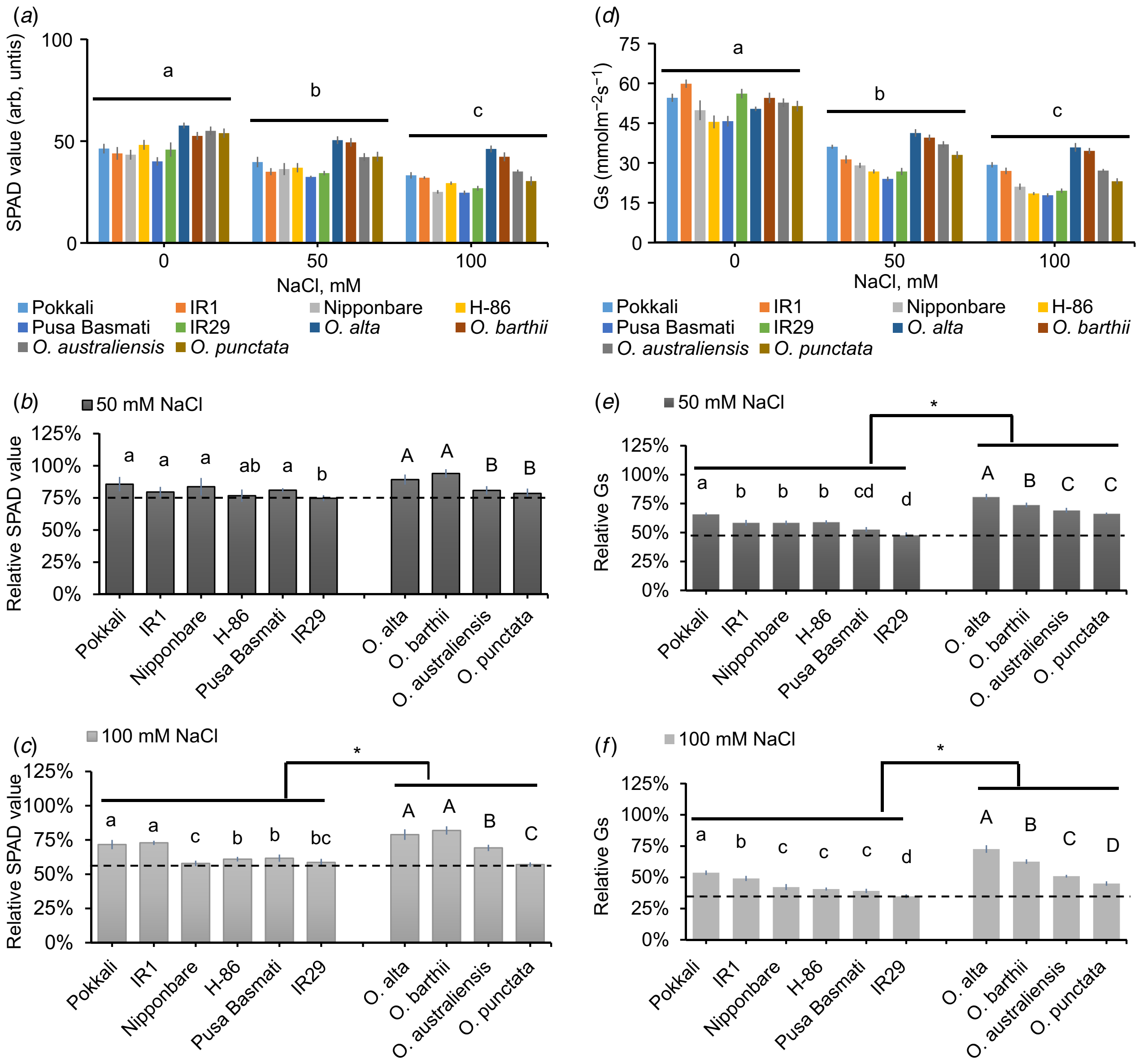

Fig. 3. Chlorophyll content (SPAD value) (*a*), relative SPAD value (*b*, *c*), stomatal conductance (Gs value; *d*) and relative Gs (*e*, *f*) of 10 rice accessions (six cultivars and four wild rice species) grown at 0, 50, and 100 mM NaCl for 3 weeks. The lowercase letters (*a*, *d*) indicate the significant difference among the salinity stress levels while lowercase and uppercase letters (*b*, *c*, *e*, *f*) indicate the significant difference at ($P < 0.05$) among the cultivated rice genotypes and among wild rice species. The error bars indicate the standard error (s.e.) for all the replicated data for each treatment. Data shown as mean ± s.e. ($n = 10$). The data difference is significant at $^{*}P < 0.05$ according to Student's *t*-test.

salt stress, which is common in both cultivated and wild rice species.

Tissue Na$^+$ content

Tissue Na$^+$ contents were significantly increased in shoots and roots of all the rice genotypes and wild rice species regardless of their salt tolerance after exposure to salt stress for 3 weeks. Shoot Na$^+$ contents were significantly higher ($P < 0.05$) in sensitive rice cultivars than tolerant cultivated rice exposed to 50 mM and 100 mM NaCl stress (Fig. 4*a*–*c*). Interestingly, salt tolerant wild rice species *O. alta* showed higher Na$^+$ accumulation (two-fold higher than the salt sensitive wild rice species *O. punctata*) under moderate salinity stress. However, there was no significant difference in Na$^+$ accumulation observed under severe salt stress between the wild rice species *O. alta* and *O. punctata*. The average shoot Na$^+$ content for cultivated rice genotypes were recorded two-fold higher for IR1 and 2.5-fold for *O. alta* when exposed to 50 mM NaCl. Moreover, wild rice

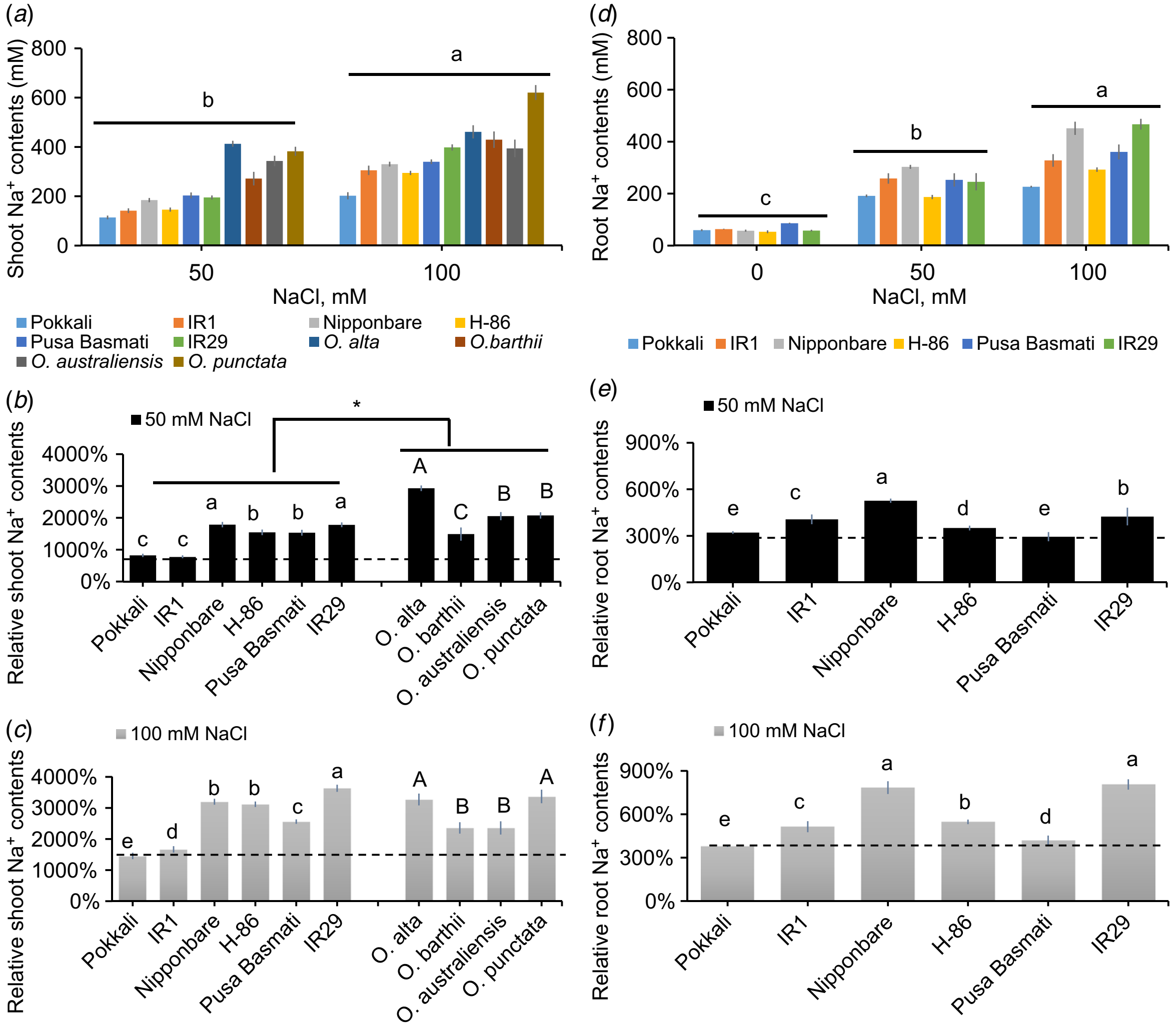

Fig. 4. Shoot and root Na^+ contents (*a*, *d*), relative values of shoot (*b*, *c*) and root Na^+ (e, *f*) at 50 and 100 mM NaCl for 10 rice accessions (six cultivars and four wild rice species) grown at 0, 50, and 100 mM NaCl for 3 weeks. The lowercase letters (*a*, *d*) indicate the significant difference among the salinity stress levels while lowercase and uppercase letters (*b*, *c*, *e*, *f*) indicate the significant difference at ($P < 0.05$) among the cultivated rice genotypes and among wild rice species. The error bars indicate the standard error (s.e.) for all the replicated data for each treatment. Data shown as mean ± s.e. ($n = 6–8$). The data difference is significant at $^*P < 0.05$ according to Student's *t*-test.

species showed relatively higher Na^+ contents compared to the cultivated rice genotypes under 100 mM NaCl (Fig. 4*a*–*c*). Among the wild rice species, *O. alta* and *O. punctata* showed higher shoot Na^+ accumulation compared to *O. australiensis* and *O. barthii*. This suggests there exists great genetic variability among different cultivated and wild rice genotypes/species in their ability to take up and accumulate Na^+ in their shoots. Overall, wild rice genotypes have more tendency to transport Na^+ from roots to shoots regardless of their salt tolerance levels.

To further dissect the impact of salinity stress, six cultivated rice genotypes were assessed for root Na^+ contents. Salinity stress significantly increased the root Na^+ contents in cultivated rice subjected to 50 mM and 100 mM NaCl stress (Fig. 4*d*–*f*). Tolerant rice cultivars showed lower root Na^+ contents compared to the sensitive genotypes. The average root Na^+ contents increased by 294–526%, and 380–806% under 50 mM and 100 mM NaCl stress, respectively. Salt sensitive cultivar Pusa Basmati showed the least Na^+ content while Nipponbare showed the maximum increase in root Na^+ content under moderate salt stress. Like shoots, moderate and sensitive cultivars showed higher Na^+ contents in roots compared to salt tolerant cultivars which showed one–two-fold lower Na^+ content (Fig. 4*d*–*f*).

Tissue K^+ content

Tissue K^+ content was significantly changed in the shoots and roots of all the rice genotypes and wild rice species (Fig. 5*a*–*c*).

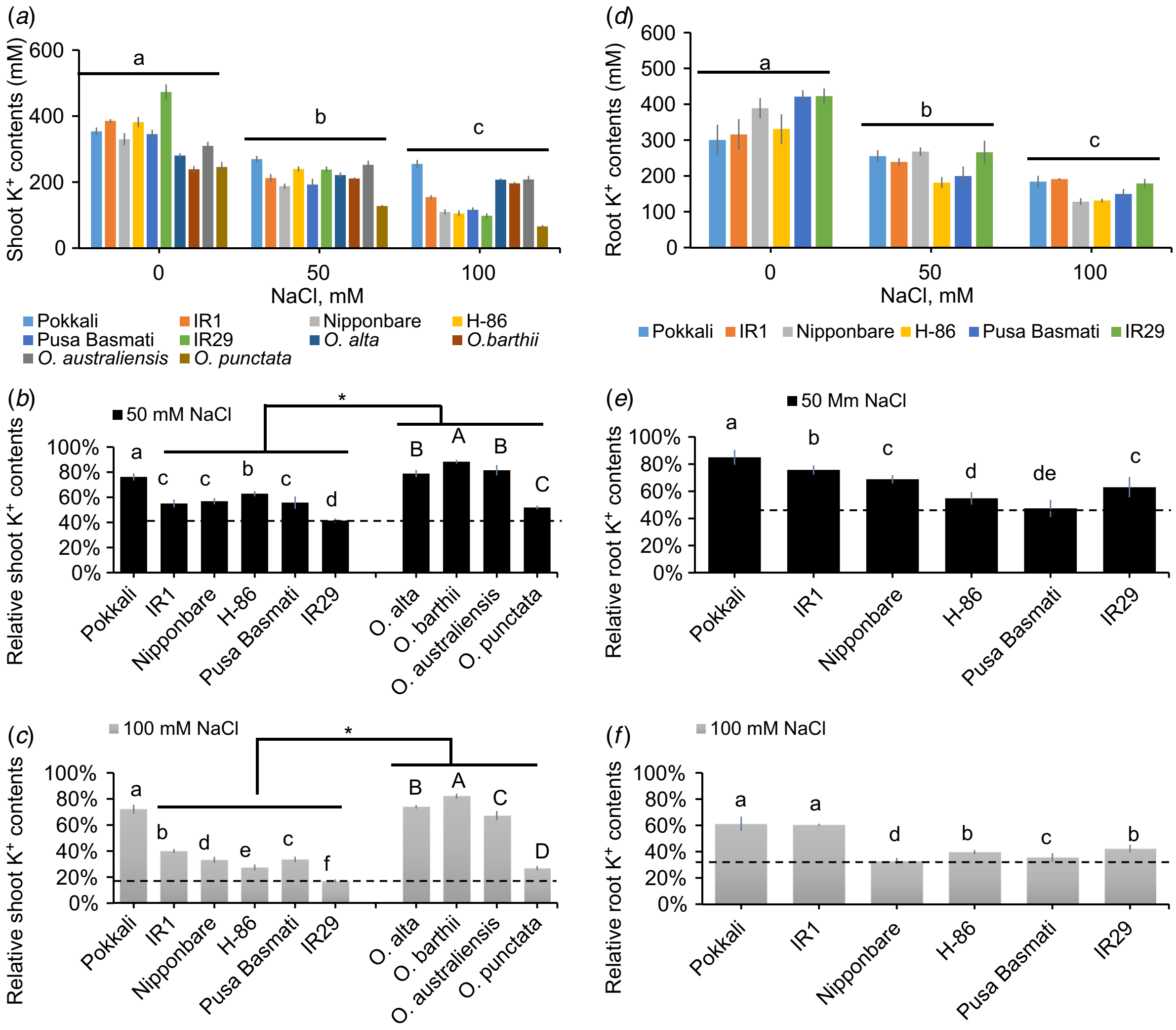

Fig. 5. Shoot and root K^+ content (*a*, *d*), relative values of shoot (*b*, *c*) and root K^+ (e, *f*) at 50 and 100 mM NaCl for 10 rice accessions (six cultivars and four wild rice species) grown at 0, 50, and 100 mM NaCl for 3 weeks. The lowercase letters (*a*, *d*) indicate the significant difference among the salinity stress levels while lowercase and uppercase letters (*b*, *c*, e, *f*) indicate the significant difference at ($P < 0.05$) among the cultivated rice genotypes and among wild rice species. The error bars indicate the standard error (s.e.) for all the replicated data for each treatment. Data shown as mean ± s.e. ($n = 6$–8). The data difference is significant at $^*P < 0.05$ according to Student's *t*-test.

Among the rice cultivars, Pokkali showed higher K^+ contents in both roots and shoots under 50 mM and 100 mM NaCl treatments (Fig. 5). Wild rice species, on the other hand, retained relatively higher K^+ content. Salt sensitive wild rice species *O. punctata* showed the least K^+ retention, a similar pattern to cultivated sensitive genotype IR29 (Fig. 5*a*–*c*). Moderate salt stress resulted in more than 50% loss in K^+ content while maximum reduction was observed under acute salt stress. Based on salt tolerance grouping, the tolerant group showed the least decrease in K^+ content compared to the sensitive group. The shoot K^+ contents decreased from 29–73% to 7–50% in rice cultivars IR29 and Pokkali, respectively, under 50 and 100 mM NaCl treatment (Fig. 5*b*, *c*). Cultivated rice IR29 showed the highest reduction under 50 mM NaCl treatment while wild rice species *O. australiensis* had the least. Similarly, the highest reduction was recorded for IR29 against 100 mM NaCl stress whereas salt tolerant wild rice species *O. barthii* showed the lowest K^+ loss. These results suggest that wild rice species tend to retain more K^+ in shoots compared to cultivated rice genotypes.

Salinity stress significantly ($P < 0.05$) decreased root K^+ content in cultivated rice genotypes after 3 weeks of salt treatment (Fig. 5*d*–*f*). The average root K^+ content for all the six rice cultivars decreased from 34 to 52% relative to control under 50 mM NaCl treatment. Cultivars H-86 and IR29 showed the most and the least decrease in root K^+ content, respectively. Under 100 mM NaCl stress, the relative root

K^+ was 11–26% for Nipponbare and IR29 having the most and the least K^+ loss, respectively (Fig. 5*d–f*). Interestingly, salt-sensitive cultivar IR29 showed higher root K^+ content compared to the other cultivars indicating that sensitive genotypes may have a tendency to compensate K^+ loss by mobilising K^+ from shoots under severe stress.

Xylem sap Na^+ and K+ kinetics

To further investigate the possible mechanisms of salt stress tolerance in cultivated and wild rice species, xylem sap was collected at three time points (4, 7 and 21 days after commencing salt treatments) to measure Na^+ and K^+ contents. Under control conditions, xylem sap Na^+ content differs significantly ($P < 0.05$) across all accessions examined, though values remain rather low during the whole period (Figs 7 and S2). Moderate (50 mM) and severe (100 mM NaCl) stress significantly increased xylem sap Na^+ content in all the six rice cultivars while wild rice species showed relatively low and steady values. Four days after commencing salt treatment, tolerant cultivars Pokkali and IR1 showed significantly higher xylem sap Na^+ content compared to the sensitive genotypes at both salt levels (Figs 7 and S2). However, salt tolerant wild rice species showed relatively lower and steady xylem sap Na^+ content over the period of 3 weeks. Contrary to tolerant accessions, the sensitive rice genotypes managed to avoid initial Na^+ loading but fail to control this in the longer term (after 3 weeks) and hence showed higher sap Na^+ content (Figs 7 and S2).

Xylem sap K^+ content in control plants were higher in sensitive rice genotypes than in tolerant plants over the period of the 3-week treatment (Figs 7 and S3). Under moderate salt stress, xylem sap K^+ content was reduced significantly ($P < 0.05$) in sensitive genotypes but remained unchanged in the wild rice species. Interestingly, salt tolerant rice genotypes and wild rice species showed a gradual decrease in K^+ content over the period of 3 weeks while sensitive genotypes/wild rice species showed a further decrease in xylem sap K^+ content (Figs 7 and S3).

Discussion

Physiological mechanisms underlying salinity tolerance in cultivated and wild rice species

The present study demonstrates that there is a high genetic variability in salt stress tolerance within cultivated rice as well as large genetic diversity among different wild rice relatives. This study also offers a better understanding of salinity-induced effects on rice growth and physiology and proposes mechanistic approaches that may potentially confer salinity tolerance in rice.

Crop performance is severely compromised under saline conditions. The Na^+ ions absorbed by the root cells are transported to the xylem *via* different transporters and channels and then transported to the shoots *via* the transpiration stream (Munns and Tester 2008). In the current study, salinity treatment for 3 weeks significantly increased Na^+ accumulation in both roots and shoots, resulting in ionic imbalance (Na^+/K^+ ratio) and specific ion toxicity, indicating the damaging effect of Na^+ toxicity. Regardless of which salinity treatment was imposed, a significant decrease in plant growth and biomass accumulation was observed in all the rice genotypes and wild rice species (Fig. 6). In this context, there was a significant decrease in the observed SFW and SDW. Interestingly, wild rice species showed higher values for SPAD and Gs (~25%), and higher SFW and SDW (>30%) compared to cultivated rice accessions under 100 mM NaCl stress despite accumulating higher amounts of Na^+. These results indicate that wild rice species perhaps possess superior tissue tolerance. Under saline conditions, plants acquire different strategies to cope with excessive Na^+, by either excluding it back to the xylem parenchyma or by sequestrating in vacuoles. This is achieved at cellular and sub-cellular levels to keep the cytoplasm free from toxic Na^+ concentrations (Chen *et al.* 2007; Cuin *et al.* 2011; Shabala *et al.* 2020), especially in leaf mesophyll cells. The sequestration of Na^+ and K^+ allows plants to maintain a relatively low tissue Na^+/K^+ ratio that is considered a critical factor in determining plant salinity tolerance (Maathuis and Amtmann 1999; Anschütz *et al.* 2014; Shabala and Pottosin 2014; Wu *et al.* 2019). In the current study, rice cultivars Pokkali and IR1 showed superior salinity stress tolerance along with wild rice species *O. alta* and *O. barthii* compared to the salt-sensitive genotypes and wild rice species (Fig. 4). Salt tolerant cultivars Pokkali and IR1 were able to maintain lower Na^+/K^+ ratios with least Na^+ accumulation in the leaf tissue compared to wild rice species *O. alta* and *O. barthii*. These findings of lower Na^+/K^+ ratio of tolerant cultivars are consistent with previous studies in which tolerant rice cultivars selectively transport K^+ over Na^+ to achieve ionic balance under salinity stress (Liu *et al.* 2019; Chakraborty *et al.* 2020; Solis *et al.* 2021).

However, this study also demonstrates that wild rice species i.e. *O. alta* and *O. barthii* can employ different strategies to combat salinity stress (Fig. 6). Wild rice species *O. alta* showed an overall efficient plant performance (SPAD, Gs, SFW and SDW) with the least shoot damage despite significantly higher tissue Na^+ accumulation, suggesting it may have superior tissue tolerance compared to the salt susceptible cultivars. Tissue tolerance has been described as the capacity of plant tissues to endure and sustain their proper function without being injured in the presence of internally toxic Na^+ concentrations (Munns *et al.* 2016). Recently, Prusty *et al.* (2018) and Solis *et al.* (2021) highlighted the importance of tissue tolerance in many wild rice species, including *O. coarctata*, a known halophytic wild rice species (Mondal *et al.* 2018; Mangu *et al.* 2019).

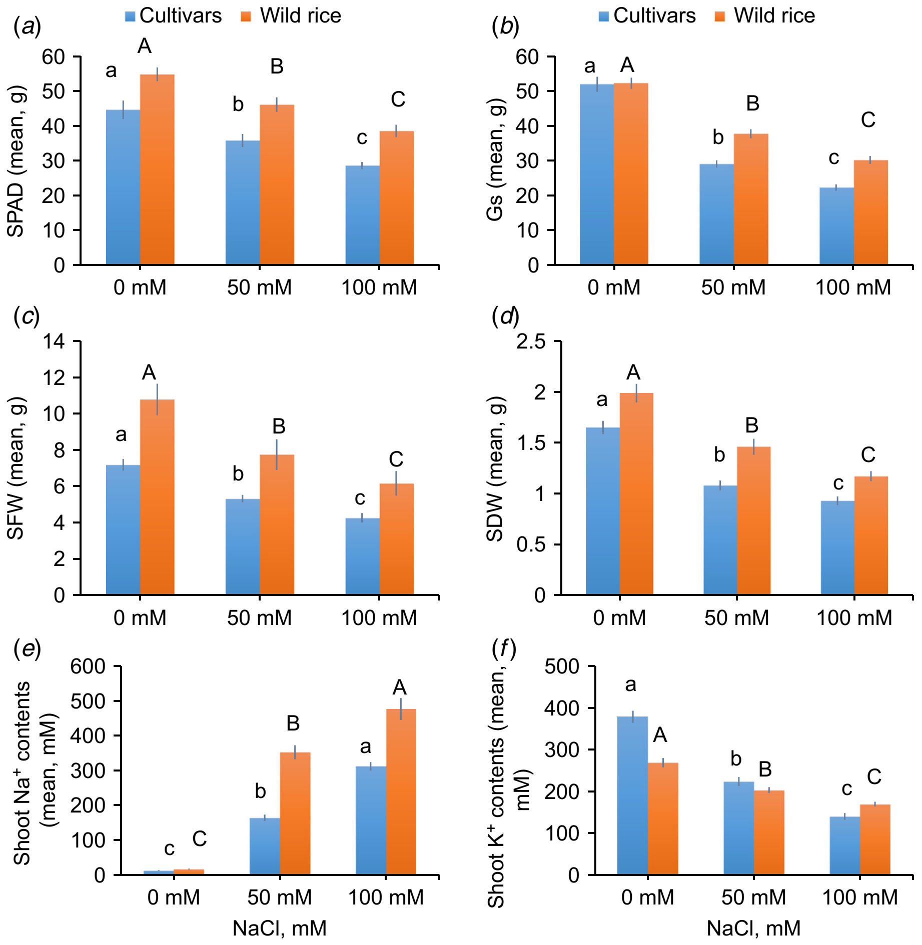

Fig. 6. Different physiological attributes such as SPAD (*a*), Gs (*b*), shoot fresh and dry weight (*c*, *d*), and shoot Na^+ and K^+ (e, *f*) were measured in six cultivated and four wild rice species grown at 50 and 100 mM NaCl stress for 3 weeks. Means of all the cultivated rice genotypes and wild rice species were averaged. Different lowercase letters indicate the significant difference ($P < 0.05$) among NaCl treatments for the cultivated rice genotypes, while different uppercase letter refer to wild rice species. The error bars indicate the average standard error (s.e.) for all the replicated data for each treatment/accession. Data shown as mean ± s.e. ($n = 6$–8).

Vacuolar Na^+/H^+ exchangers (*NHXs*) have been characterised in *O. coarctata* which are important membrane transporters mediating vacuolar compartmentalisation of Na^+/K^+ thereby preventing Na^+ toxicity in the cytosol (Kizhakkedath *et al.* 2015; Jegadeeson *et al.* 2019). Hence, our results suggest that wild rice species can utilise tissue Na^+ tolerance mechanisms to mitigate salt-induced detrimental effects which may have been lost from cultivated rice due to selective breeding for Na^+ exclusion.

Salinity stress reduces photosynthetic efficiency in crop plants, and this may be derived from the negative impacts of salinity on stomatal regulation (Dionisio-Sese and Tobita 2000; Liu *et al.* 2017). Salinity stress lowers stomatal conductance (Gs) by decreasing the leaf water content and water potential which reduces turgor pressure and ultimately decreases net photosynthesis. Therefore, plants tend to increase the production of organic/inorganic osmolytes for the sake of osmotic adjustment that is crucial for maintaining leaf expansion and stomatal conductance under prolonged salinity stress (Roy *et al.* 2014). It is osmotically efficient for plants to use inorganic ions for osmotic adjustment rather than synthesising energetically expensive organic solutes (Shabala *et al.* 2020). It is plausible to suggest that the salt tolerant wild rice species *O. alta* is able to maintain optimum stomatal conductance (Gs) under salinity stress by utilising Na^+ as an osmoticum that allows rapid osmoregulation at a very low energetic cost. In contrast, rice cultivars (*O. sativa*) showed reduced Gs content along with lower biomass accumulation. Thus, low plant biomass accumulation under salinity stress may be explained by lower Gs values in *O. sativa* rice cultivars. It also suggests that wild rice species *O. alta* may not use Na^+ ion exclusion as a salt tolerance

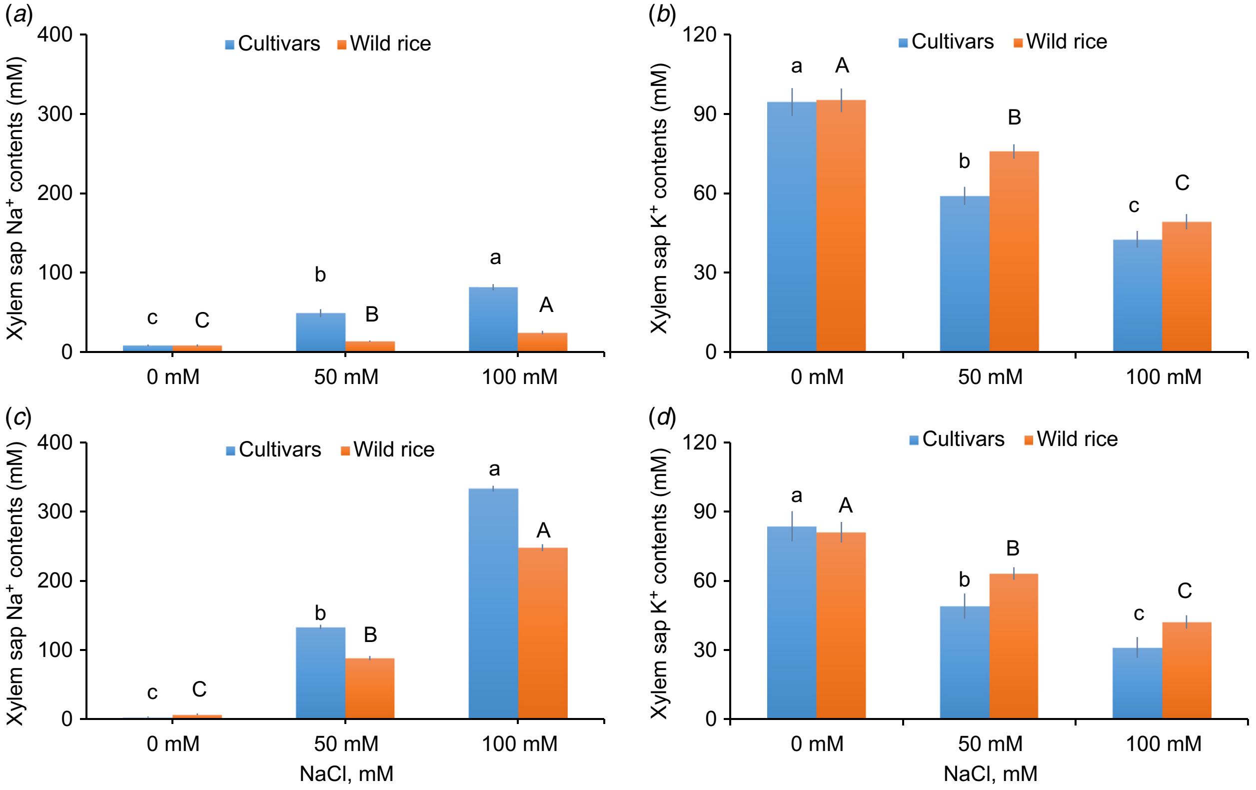

Fig. 7. Kinetics of xylem sap Na^+ and K^+ content in cultivated and wild rice species grown under 50 and 100 mM NaCl measured at 7 days (*a*, *b*) and 21 days (*c*, *d*) after salt treatment. Means of all the cultivated rice genotypes and wild rice species were averaged. Different lowercase letters indicate the significant difference ($P < 0.05$) among NaCl treatments for the cultivated rice genotypes, while different uppercase letters refer to wild rice species. The error bars indicate the standard error (s.e.) for all the replicated data for each treatment. Data shown as mean ± s.e. ($n = 6–8$).

mechanism to a great extent, as shown by significantly higher Na^+ content in the leaves. Instead, it has superior tissue tolerance to alleviate salinity-induced effects and continue operational physiological activities despite the presence of toxic concentrations of Na^+ ions with minor tissue damage.

Wild rice species show superior control of xylem loading of Na^+ and K^+

The ability of plants to maintain shoot Na^+ below non-toxic levels is considered crucial for achieving better plant growth and survival under prolonged saline conditions. Seven days after the commencement of salinity treatment, wild rice species tend to lower xylem Na^+ loading while cultivated rice genotypes failed to prevent Na^+ entry into the xylem (Fig. 7). Thus, cultivated rice genotypes showed symptoms of shoot Na^+ toxicity which were least evident in wild rice species. The plasma membrane Na^+/H^+ antiporter (*SOS1*) has been characterised in *Arabidopsis* and rice as a unique Na^+ transporter localised at the epidermis root tip, which mediates xylem Na^+ loading (Shi *et al.* 2003; Zarei *et al.* 2020). The lowered Na^+ content in xylem sap of wild rice species may be potentially attributed to the downregulation of *SOS1* and/or enhancement of Na^+ re-mobilisation from the xylem sap (Horie *et al.* 2012).

Typically, Na^+ retrieved from the shoot is restored in the root vacuole, and this process is mediated by the recirculation of Na^+ back from the phloem (Munns and Tester 2008; Shabala and Munns 2012; Ishikawa and Shabala 2019). This may explain that a progressive Na^+ accumulation may occur in the roots of salt tolerant rice cultivars due to Na^+ recirculation, after achieving osmotic adjustment in the shoots by means of Na^+ accumulation (Fig. 4). Thus, the two phase model of Na^+ kinetics conferring salt stress tolerance observed in cultivated and wild rice species may be explained as follows: Salt tolerant rice genotypes follow active xylem Na^+ loading from the roots to the shoots for osmotic adjustment; xylem loading and Na^+ accumulation in the root tissues is reduced under extended salinity exposure. Cultivated rice genotypes showed poor control over xylem Na^+ loading by initially preventing Na^+ xylem loading and accumulation in root tissues to avoid Na^+ toxicity in shoots but failed in longer run to keep Na^+ in the roots. This resulted in the massive Na^+ entry into the xylem followed by its transport to the shoot.

Wild rice species on the other hand possess superior salinity stress tolerance that is achieved by employing several mechanisms. Wild rice species (i) show efficient control over xylem Na^+ loading, (ii) rely on superior tissue tolerance mechanisms and (iii) allow for rapid osmotic adjustment by using Na^+ as cheap osmoticum for osmoregulation.

Supplementary material

Supplementary material is available online.

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Data availability. Data generated during this study is available on request.

Conflicts of interest. Sergey Shabala is an Editor of Functional Plant Biology but was blinded from the peer review process for this paper.

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