

RESEARCH PAPER

Root apoplastic barriers block Na⁺ transport to shoots in rice (*Oryza sativa* L.)

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Abstract

Rice is an important crop that is very sensitive to salinity. However, some varieties differ greatly in this feature, making investigations of salinity tolerance mechanisms possible. The cultivar Pokkali is salinity tolerant and is known to have more extensive hydrophobic barriers in its roots than does IR20, a more sensitive cultivar. These barriers located in the root endodermis and exodermis prevent the direct entry of external fluid into the stele. However, it is known that in the case of rice, these barriers are bypassed by most of the Na⁺ that enters the shoot. Exposing plants to a moderate stress of 100 mM NaCl resulted in deposition of additional hydrophobic aliphatic suberin in both cultivars. The present study demonstrated that Pokkali roots have a lower permeability to water (measured using a pressure chamber) than those of IR20. Conditioning plants with 100 mM NaCl effectively reduced Na⁺ accumulation in the shoot and improved survival of the plants when they were subsequently subjected to a lethal stress of 200 mM NaCl. The Na⁺ accumulated during the conditioning period was rapidly released when the plants were returned to the control medium. It has been suggested that the location of the bypass flow is around young lateral roots, the early development of which disrupts the continuity of the endodermal and exodermal Casparian bands. However, in the present study, the observed increase in lateral root densities during stress in both cultivars did not correlate with bypass flow. Overall the data suggest that in rice roots Na⁺ bypass flow is reduced by the deposition of apoplastic barriers, leading to improved plant survival under salt stress.

Key words: Apoplastic barriers, bypass flow, hydraulic conductivity, lateral roots, *Oryza sativa*, PTS, salt stress.

Introduction

Salt stress is one of the most deleterious environmental stress factors affecting crop growth and productivity worldwide. In saline soils, Na⁺ is the principle toxic ion imposing both ionic and osmotic toxicity (Hagemann and Erdmann, 1997). Maintaining low levels of Na⁺ in the cytoplasm is critical for survival under saline stress, and plants have been shown to maintain low cytoplasmic Na⁺ by intracellular (Fukuda *et al.*, 2004; Anil *et al.*, 2007) and extracellular (Anil *et al.*, 2005) compartmentalization. Excessive Na⁺ in the apoplast has been shown to

correlate with poor survival of plants (Oertli, 1968; Flowers *et al.*, 1991; Krishnamurthy *et al.*, 2009). Rice is an important cereal and is very sensitive to salt stress (Munns and Tester, 2008). On the other hand, it also exhibits enormous varietal differences with respect to salt sensitivity (Munns and Tester, 2008), with some of these cultivars growing in very high salt concentrations. This availability of cultivars with widely differing sensitivity to salinity can be exploited to explore mechanisms of salt tolerance.

The mechanisms by which Na⁺ enters the shoots of plants are still ambiguous (Kronzucker and Britto, 2011), but apoplastic transpirational bypass flow of water and solutes has been shown to play a significant role in rice (Yeo *et al.*, 1987; Ochiai and Matoh, 2002). Ca²⁺ reduces bypass flow in rice roots, and hence the accumulation of Na⁺ in shoots, with consequent improvement in survival (Anil *et al.*, 2005). A similar effect has been reported on supplementing the growth medium with silicon (Gong *et al.*, 2006). An earlier study showed a negative correlation between the extent of deposition of apoplastic barriers in the roots and Na⁺ uptake into the shoots. Deposition of hydrophobic material was directly correlated with enhanced survival of rice plants (Krishnamurthy *et al.*, 2009). The study also demonstrated that subjecting rice plants to a sublethal conditioning exposure to 100 mM NaCl for a week resulted in significant deposition of additional hydrophobic substances. Casparian bands (CBs) in the anticlinal walls of root endodermis and exodermis have been reported to block apoplastic flow of ions and fluorescent dyes (Steudle and Peterson, 1998; Lux *et al.*, 2011). The specific role of these barriers in resisting the bypass (apoplastic) flow of Na⁺ in rice needs to be addressed.

Apoplastic barriers in the endo- and exodermis also affect radial water transport in roots (Steudle and Peterson, 1998). The water balance of a plant is generally thought to be maintained by regulation of stomatal transpiration (Kramer and Boyer, 1995). However, there is now increasing evidence to show that roots also have a major role to play (Kramer and Boyer, 1995; Steudle, 2000; Ranathunge *et al.*, 2003). A composite transport model explains water flow through roots (Steudle and Peterson, 1998; Steudle, 2001), with water moving through an apoplastic or cell to cell path. Apoplastic barriers (i.e. CBs) resist the apoplastic water flow (Steudle and Peterson, 1998) while suberin lamellae restrict water flow through aquaporins by impeding access to the membranes (Tyerman and Skerrett, 1999). It is well known that the hydraulic conductivity (water movement) of roots varies with species and environmental conditions (Kramer and Boyer, 1995; Steudle and Peterson, 1998; Zimmermann *et al.*, 2000; Miyamoto *et al.*, 2001). However, not much work has been done to help in understanding the role of apoplastic barriers in modulating the hydraulic conductivity of rice roots under salt stress.

The continuity of apoplastic barriers is temporarily interrupted by the emergence of lateral roots, generating sites through which water and minerals could leak into the main root (Yeo *et al.*, 1987; Ma *et al.*, 2001). In monocots, lateral roots are initiated from the pericycle near the phloem and disrupt the continuity of the endodermal CB. Growth through the cortex is facilitated by development of a digestive pocket in the cortex. Eventually the root breaks through the exodermis and epidermis (Ranathunge *et al.*, 2005). A recent study by Faiyue *et al.* (2010a) found no evidence indicative of Na⁺ entry at these sites in rice plants, but the issue still remains open.

Lateral root development is regulated by hormones and environmental factors (Malamy, 2005; Chhun *et al.*, 2007).

An increase in lateral root density was observed in *Arabidopsis* in response to phosphate and sulphate deficiency (Williamson *et al.*, 2001; Kutz *et al.*, 2002). Lateral root density is affected by salt stress in different ways: a marked reduction in *Arabidopsis* (Brusselsens *et al.*, 2000) and an increase in the case of chickpea were seen (Boominathan *et al.*, 2004). In contrast to earlier results, He *et al.* (2005) demonstrated an increase in the lateral root density with high levels of NaCl in *Arabidopsis*. In the absence of detailed studies in monocots, the influence of salinity on lateral root development takes on added significance in the light of the contribution of bypass flow to Na⁺ uptake in rice. The essential involvement of auxins in the development of lateral roots has been studied extensively (Casimiro *et al.*, 2003; Fukaki *et al.*, 2007). AUX1 is involved in auxin transport and affects the lateral root initiation in *Arabidopsis* (Chhun *et al.*, 2007). It has been recently reported that a redistribution of auxins occurs at the root apex in the case of *Arabidopsis*, when subjected to salinity stress (Wang *et al.*, 2009). *RAU1* (RELATED TO AUX1) and *RAU4* in rice are *AUX1*-like genes which are important for lateral root initiation and development. Arf8 (auxin response factor 8) is a transcription factor that controls auxin-responsive transcription (Guilfoyle and Hagen, 2007) and plays a role in regulating the development of lateral roots (Tian *et al.*, 2004; Yang *et al.*, 2006). However, the involvement of these genes in development of lateral roots, especially in response to salt stress, is still not clear in rice.

In the present study, the role of root apoplastic barriers in rendering resistance to water uptake and bypass flows of PTS (trisodium 3-hydroxy-5,8,10-pyrenetrisulphonate) and Na⁺ in two rice cultivars varying in their sensitivity to salt stress was investigated. In particular, whether the additional barriers induced by conditioning with moderate salinity contribute to a reduction in Na⁺ uptake and an increase in survival was investigated. Further, the role of lateral roots in bypass flow was investigated.

Materials and methods

Plant material and growth conditions

Seeds of *indica* rice (*Oryza sativa* L. cv. Pokkali, and IR20) obtained from Regional Research Station, V.C. Farm (Mandya, Karnataka, India) were germinated in the dark for 3–4 d on moist tissue paper at 27 °C. Seedlings were transferred to half-strength Hoagland's nutrient solution (Epstein, 1972) in 10.0 l containers. The seedlings were allowed to grow for 1 month (days post-germination) with continuous media aeration at 25 °C illuminated at 450 μmol m⁻² s⁻¹ using fluorescent lighting (Philips, Kolkata, India) in a day and night cycle of 12 h each.

Salt stress protocol

One-month-old rice plants grown in aerated hydroponic medium (Hoagland's solution) were stressed with (i) 100 mM NaCl for 1 week (conditioning); (ii) 200 mM NaCl for 48 h (acute stress); (iii) conditioning in 100 mM NaCl for 1 week followed by 200 mM NaCl stress for 48 h; and (iv) conditioning in 100 mM NaCl for 1 week followed by a recovery period of 1 week with a subsequent

stress of 200 mM NaCl for 48 h. At the end of each treatment, plants that retained more than one-third of their leaf area open, without curling up, were counted to estimate survival rates in the rice cultivars.

Measurement of root exudation in the absence (osmotic exudation) and in the presence of hydrostatic pressure gradients

The measurements were carried out following Miyamoto *et al.* (2001). Prior to starting the measurements, 1-month-old control and stressed rice plants were transferred to the nutrient solution in the pressure chamber. Shoots were then cut off using razor blades at distances of 40–70 mm from their base. All tillers except the main stem were closed using clamps. Using a syringe, xylem sap exuding from the cut surface of the main stem was collected in Eppendorf tubes and weighed. In the absence of hydrostatic pressure gradients, differences in osmotic pressure between xylem sap and the medium drives water uptake by the root. The osmotic concentration of the medium and the xylem sap was measured using a freezing point depression osmometer (OSMOMAT 030-D; Genotec, Berlin, Germany). The osmotic pressure of the nutrient solution was 0.0075 MPa.

Plants used for measuring osmotic water flows through the root were also used to measure the hydrostatic water flows of root systems. For these measurements, roots were enclosed in a steel chamber to apply pneumatic pressures to the root medium (Fig. 1 in Miyamoto *et al.*, 2001). Silicone seals with the same diameter as that of the base of the main stem were used to seal the base tightly. Flexible rubber material (Terostat, Germany) was used for further fine adjustment. The pressure in the chamber was raised stepwise from 0 MPa to 0.25 MPa above atmospheric with intervals of 0.05 MPa. The xylem sap exuded was collected as described earlier and weighed to determine the volume (assuming a density of 1). For a given applied gas pressure, the volume exuded from the root system was plotted against time (Fig. 1). The slopes of these relationships were determined and normalized to the surface area. This gave the volume flow J_{vr} in $\text{m}^3 \text{m}^{-2} \text{s}^{-1}$. Root hydraulic conductivity (Lp_r) was determined from the slopes of J_{vr} plotted against applied driving forces in the linear region of the plots (Fig. 1B).

Measurement of PTS and Na^+ bypass flow

For bypass flow measurements, the medium contained the apoplastic tracer PTS at a concentration of 0.01% (w/v) and NaCl at a concentration of 50 mM. The concentration of PTS in the root medium and in the exuded xylem sap was measured using a spectrofluorimeter (excitation wavelength, 405 nm; emission wavelength, 515 nm). A 5-point calibration curve was constructed over the concentration range from 2×10^{-8} M to 2×10^{-6} M PTS. The calibration curve was linear over this range. The Na^+ concentration in the root medium and the exuded xylem sap was estimated using a flame photometer (Systronics Flame Photometer 128, Bangalore, India).

Root anatomy and microscopy

Freehand cross-sections at 10 mm and 20 mm from the root tip were made using the adventitious roots of control and stressed rice plants grown in aerated hydroponics. To check for CBs, sections were stained for 1 h with 0.1% (w/v) berberine hemisulphate and for another hour with 0.5% (w/v) aniline blue (Brundrett *et al.*, 1988). Stained sections were viewed with an epifluorescence microscope using blue light (filters: excitation 450–490 nm, dichroic mirror 510 nm, emission LP 520).

Estimation of total Na^+ and K^+ in shoots and roots

Plants exposed to salt stress were harvested and washed thoroughly 2–3 times over 2 min with distilled water to remove

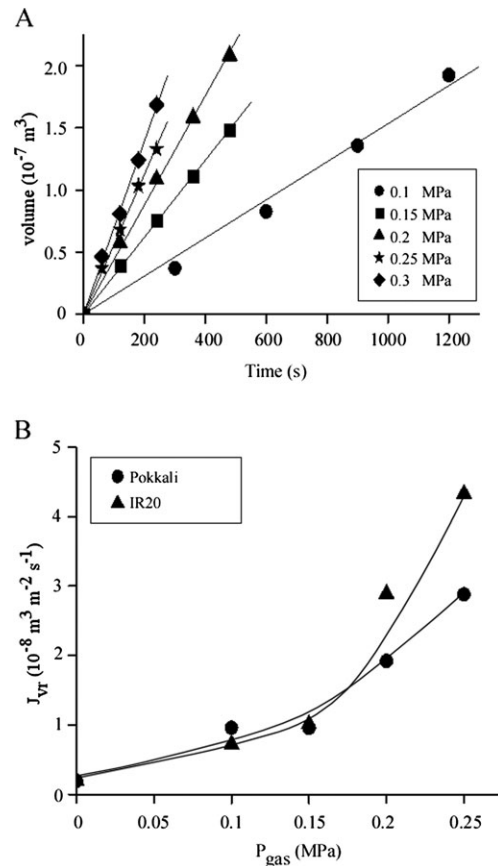


Fig. 1. Results from a typical steady-state experiment for measuring root hydraulic conductivity (Lp_r). One-month-old, hydroponically grown rice plants were stressed with 100 mM NaCl for 1 week and allowed to recover in control nutrient solution for 12 h before starting the measurements. (A) Extruded xylem sap in the presence of hydrostatic pressure gradients (P_{gas}) is plotted against time. (B) Steady-state water flow per unit surface area of the root system (J_{vr}) as a function of applied driving force. Lp_r values were calculated from slopes of the linear ranges of J_{vr} (P) curves. Lines passing through the data points in B are intended to guide the eye. Circles, Pokkali; triangles, IR20.

surface contaminating Na^+ . The shoots were separated from the roots and dried at 50 °C for 3–4 d. The tissue was ground into a powder in liquid nitrogen. This powder was acid digested by suspending it in 5 ml of concentrated nitric acid overnight. A 5 ml aliquot of a 10:4 (v/v) diacid mixture of nitrate and perchlorate was added to the tissue powder, and allowed to digest it completely for 2 h on a sand bath. The digested solution was made up to 25 ml with double-distilled water. The Na^+ and K^+ levels in the acid-digested tissue sample were measured by flame photometry.

Apoplastic fluid was released from shoot segments using the method described by Anil *et al.* (2005) with minor modifications. Freshly harvested shoots were cut transversely into 1 cm segments and gently rocked for 1 h in 50 ml of double-distilled water. The Na^+ content in apoplastic fluid that has equilibrated with water was estimated by flame photometry.

Measurement of lateral root density, their anatomy, and permeability to PTS

To determine lateral root density, adventitious roots of 1-month-old rice plants were cleared with 1% NaOH for 24 h at 60 °C

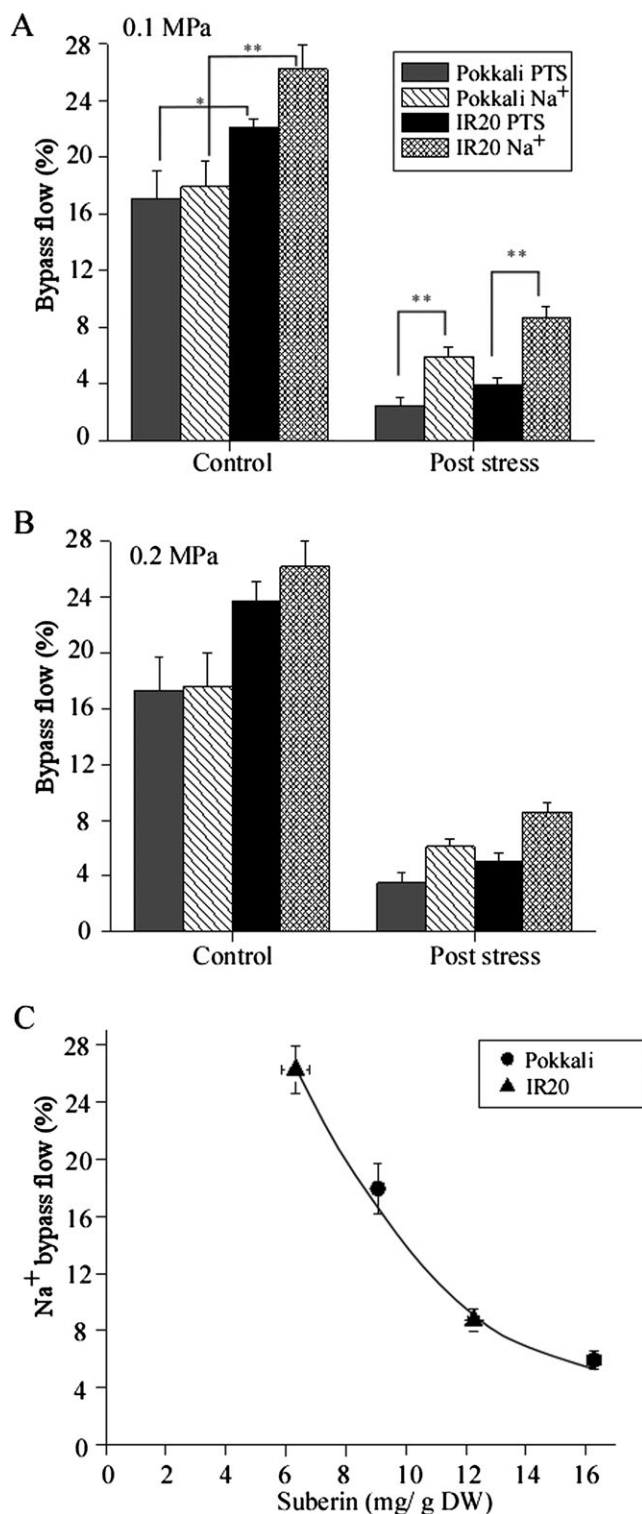


Fig. 2. Bypass flow of Na⁺ and PTS in rice roots. One-month-old, hydroponically grown rice plants were stressed with 100 mM NaCl for 1 week and allowed to recover in control nutrient solution for 12 h before starting the measurements. Apoplastic bypass flow is given as the percentage (w/v) of externally applied PTS/Na⁺. (A) Percentage bypass flow of PTS and Na⁺ from control and post-stressed (stressed with 100 mM NaCl for 1 week) rice roots with the application of an external gas pressure of 0.1 MPa. (B) Percentage bypass flow of PTS and Na⁺ from control and post-stressed rice roots with the application of an external gas pressure

Table 1. Hydraulic conductivity (L_p) of rice roots as determined by hydrostatic and osmotic experiments. Experiments were performed under steady water flow. In order to vary the driving force, pneumatic pressures were applied to the root medium. Values are means \pm SE, n , number of root systems; A_r , the overall surface areas of the root systems; ND, not determined

Treatment	n	A_r (10^{-3} m ²)	L_p (10^{-8} m s ⁻¹ MPa ⁻¹)	
			Hydrostatic	Osmotic
Control				
Pokkali	11	6.4 \pm 0.5	29.8 \pm 7.4	4.95 \pm 0.53
IR20	8	4.8 \pm 0.6	42.5 \pm 9.2 ^a	4.60 \pm 1.15
Stress				
Pokkali	10	5.9 \pm 0.6	19.7 \pm 3.6 ^b	ND
IR20	9	3.7 \pm 0.5	21.8 \pm 3.2 ^c	ND

^a Comparison between control Pokkali and IR20 ($P < 0.05$).

^b Comparison between Pokkali control and stress ($P < 0.05$).

^c Comparison between IR20 control and stress ($P < 0.01$).

(Peterson and Lefcourt, 1990). The lateral roots were counted using a stereomicroscope (Olympus SZX16, Japan). The lateral roots were classified into two types: emerged (lateral roots that had emerged through the epidermis) and unemerged (lateral root primordia that had not yet emerged through the epidermis and were observed like bulbs in the cleared roots). The lateral root density was calculated as the number of lateral roots per unit length (cm) of the primary root. From each condition, 8–10 roots were used for the measurements.

To visualize the CBs in lateral roots, adventitious root segments with laterals were cleared with lactic acid, saturated with chloral hydrate for 1 h at 70 °C. The samples were then stained with berberine hemisulphate for 1 h (Lux et al., 2005). The stained samples were washed with 50% ethanol three times and were mounted on 75% glycerol and viewed with an epifluorescence microscope with a UV filter set (excitation filter BP 365, dichroic mirror FT 395, emission LP 397).

PTS was used as an apoplastic tracer dye to check if the break created by the emergence of lateral roots in the primary root provides a path for bypass flow. One-month-old, intact rice plants were transferred to a container with 0.01% (w/v) PTS in the root medium; the shoots were allowed to transpire for 1 h. Roots were then rinsed under running tap water for 2–3 min to remove excess dye on the surface. Roots were then mounted on a glass slide in 75% glycerol and observed with an epifluorescence microscope with UV light.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from whole leaf, stem and roots (apical, basal) of hydroponically grown, 1-month-old Pokkali plants under control conditions, and from roots stressed for times ranging from 0 h to 24 h with 100 mM NaCl using TRI-reagent (Sigma-Aldrich, MO, USA) according to the instructions of the manufacturer. Reverse transcription was performed with 1 μ g of RNA using 100 U of Moloney murine leukaemia virus reverse transcriptase

of 0.2 MPa. (C) Correlation between Na⁺ bypass flow and amount of suberin in the rice roots. Circles, Pokkali; triangles, IR20. The line passing through the data points in C is intended to guide the eye. Data represent means \pm SE, $n=10$ roots. Asterisks indicate statistically significant differences at * $P \leq 0.05$ and ** $P \leq 0.01$ (t -test); the line bars are introduced to indicate the data/bars used for t -test comparison.

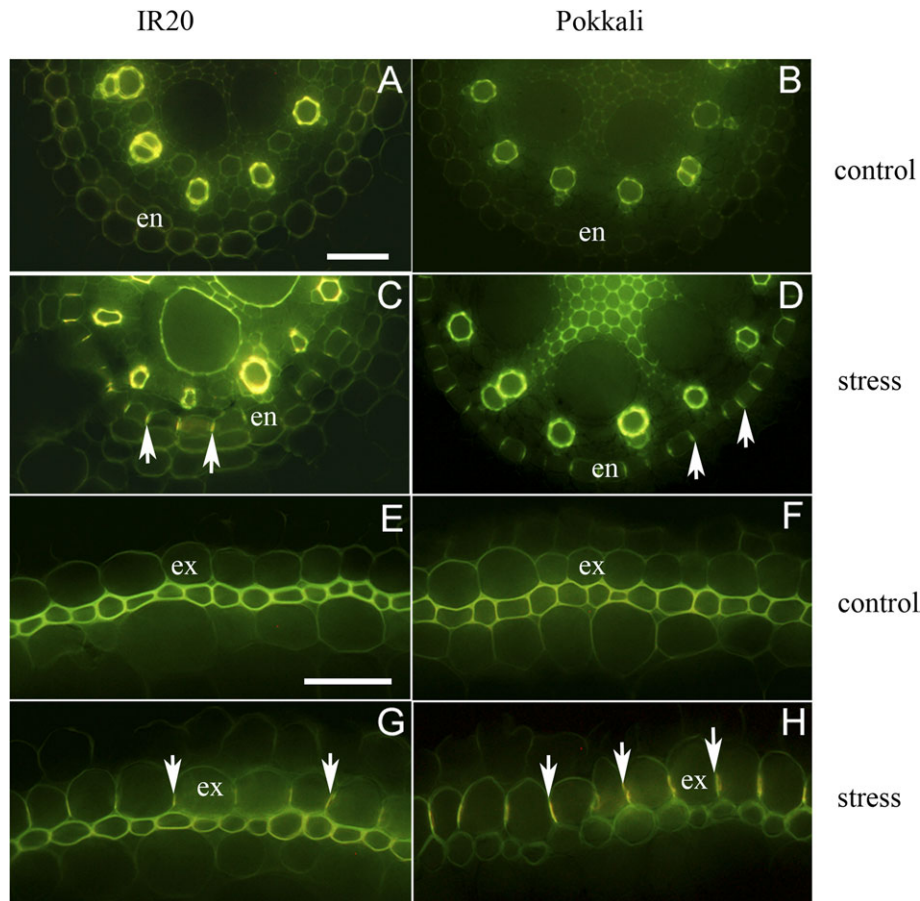


Fig. 3. Development of Casparian bands under salt stress. Free hand cross-sections were made from the adventitious roots of 1-month-old rice plants and the sections were stained with berberine aniline blue and viewed using blue light. Arrows show Casparian bands in the endodermis and exodermis. (A, E) Control IR20. (C, G) Stress IR20. (B, F) Control Pokkali. (D, H) Stress Pokkali. Ex, exodermis; en, endodermis, bar=100 μm

(Invitrogen) following the manufacturer's instructions. The cDNA thus obtained was subjected to a 25-cycle PCR with the following conditions: 3 min at 94 °C followed by 25 cycles of 60 s at 94 °C, 60 s at 55 °C, and 80 s at 70 °C, and finally 5 min at 70 °C. Actin was used as a control. The primer sequences and predicted amplicon sizes were 5'-TG TAGCGAGAAAGCTGAGGA-3' (forward) and 5'-GAGCTGCGAGAAGGAGTACG-3' (reverse) for *RAU1* (300 bp); 5'-AAGAGGAGTCGTCGGAGATG-3' (forward) and 5'-AGTCCCACATTCCTCCAGTG-3' (reverse) for *RAU4* (300 bp); 5'-ATTGGTCGTGATGTGCAAAA-3' (forward) and 5'-ATAACCGGACAATCGGTGAA-3' (reverse) for *Arf8* (926 bp); and 5'-CCTCTTCCAGCCTTCCTTCAT-3' (forward) and 5'-ACGGCGATAACAGCTCC TCTT-3' (reverse) for Actin-1 (Os03g50890) (400 bp).

Statistical analysis

Data presented in the figures are the mean values \pm SE. Significant differences at $P \leq 0.01$ and $P \leq 0.05$ between the two cultivars and various stress conditions were estimated by the Student's *t*-test.

Results

Effect of salt stress on hydraulic conductivity of rice roots

The volume of xylem sap exuded from the shoot under constant pneumatic pressure (P_{gas}) increased linearly with

time (Fig. 1A), indicating a constant rate of fluid exudation. This rate, normalized for root surface area, gives the volume flow of the root (J_{vr}). In both Pokkali and IR20 roots, J_{vr} increased in a non-linear manner with applied hydrostatic pressures, though the curvature was much more prominent for IR20 (Fig. 1B). At higher gas pressures (>0.15 MPa) water flows were appreciably higher and also displayed linear slopes. The hydraulic conductivity (Lp_r) was estimated from the slope of the curve in this regime. The roots of unstressed IR20 (control) had a significantly higher hydrostatic hydraulic conductivity than those of Pokkali (42.5 versus $29.8 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$, $P=0.04$; Table 1). Lp_r decreased significantly in both the cultivars after conditioning the plants with 100 mM NaCl for a week, the decrease being much steeper in the case of IR20 (from 42.5 to $21.8 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$, $P=0.01$). There was no significant difference in root Lp_r between Pokkali and IR20 after stress (19.7 versus $21.8 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$; Table 1).

Active nutrient uptake ensures exudation of xylem fluid even in the absence of applied hydrostatic pressure. Osmotically driven hydraulic conductivity, in which water moves predominantly through the aquaporins in the plasma membrane, was measured using nutrient solution as the external root medium with an osmotic pressure of 0.0075

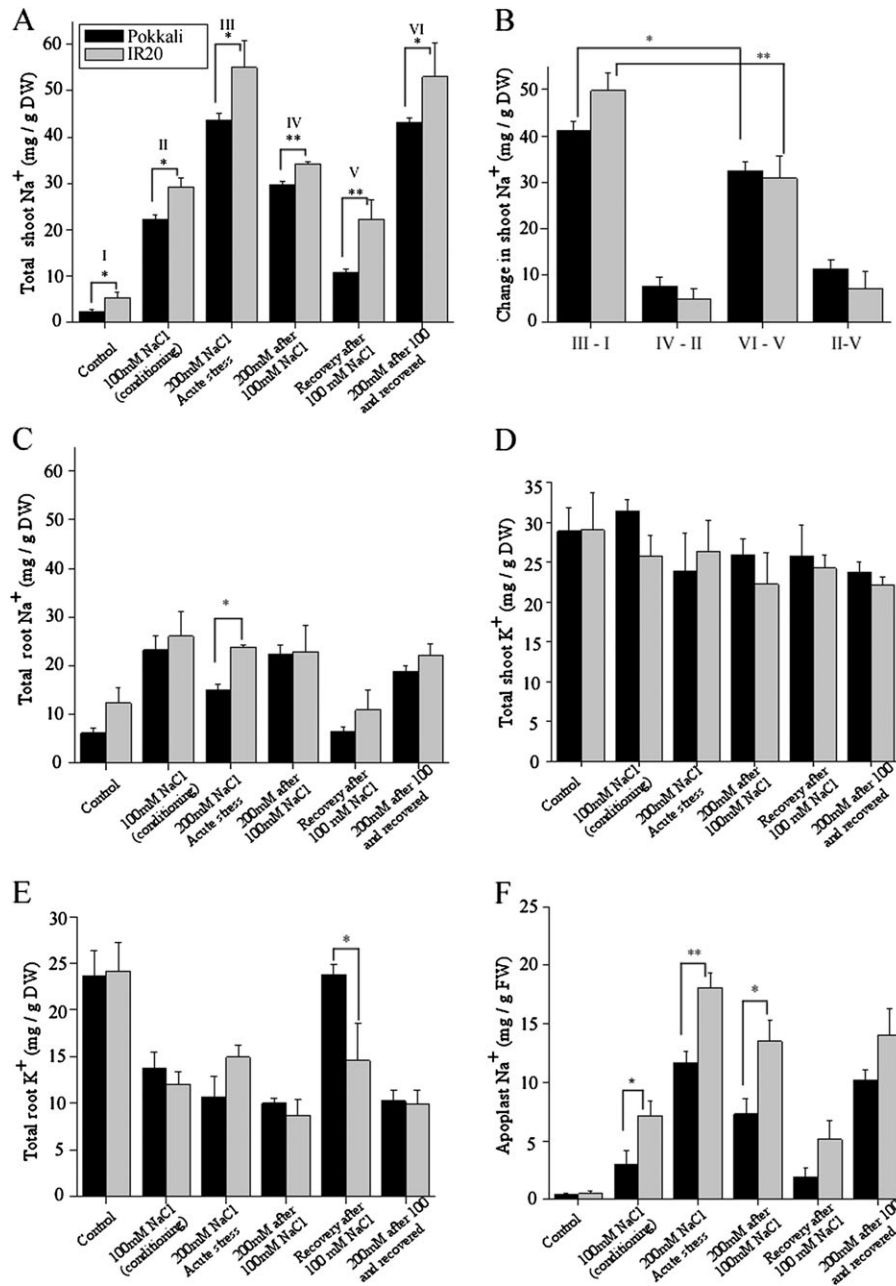


Fig. 4. Variation in Na^+ and K^+ uptake by rice cultivars. One-month-old, hydroponically grown rice plants subjected to various salt stress conditions were washed, dried, and acid-digested to estimate Na^+ levels by flame photometry. (A) Variation in Na^+ uptake by rice shoots under various stress conditions. The bars are labelled with Roman letters I–VI; each letter corresponds to the respective treatment. (B) Change in Na^+ amounts in the shoots of rice cultivars with acute (III–I), moderate (IV–II), acute stress with prior moderate stress (VI–V) and Na^+ release/efflux upon 1 week of recovery after a conditioning stress (II–V) from A is plotted. (C) Variation in Na^+ uptake by rice roots under various stress conditions. (D) Variation in K^+ uptake by rice shoots under various stress conditions. (E) Variation in K^+ uptake by rice roots under various stress conditions. (F) Na^+ amounts in the apoplastic fluid of rice shoots under various stress conditions. Data represent means \pm SE, $n=6$. Asterisks indicate statistically significant differences at $*P \leq 0.05$ and $**P \leq 0.01$ (t -test); the line bars are introduced to indicate the data/bars used for t -test comparison.

MPa. The measured reflection coefficient, σ_{sr} of the nutrient salts was 0.4. The osmotic Lp_r of the two cultivars was indistinguishable, 4.9 and $4.6 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ for Pokkali and IR20, respectively. The rate of xylem exudation declined precipitously after conditioning and no measurable quantity of osmotically driven xylem sap was collected from conditioned plants.

Bypass flow in rice roots: effect of salt stress

PTS is a large anion with three sulphonates that are ionized at physiological pH, rendering it unlikely to permeate cell membranes. It has been used as a tracer for the bulk flow of fluid moving through the root apoplast and into the xylem bypassing hydrophobic barriers, namely CBs which are

located in the anticlinal walls of the endo- and exodermis. Bypass flow was measured with the pressure chamber technique utilized for hydraulic conductivity experiments (Miyamoto *et al.*, 2001). In all the samples tested, volume flow increased linearly with applied hydrostatic pressure (Fig. 1B). The concentrations of PTS and Na⁺ in the xylem exudates did not change with varying flow rate or applied gas pressure (Fig. 2A, B). PTS and Na⁺ concentrations in the xylem sap have been expressed as a percentage of those present in the external medium in Fig. 2. In unstressed Pokkali samples (controls), the xylem fluid was observed to contain similar proportions of PTS and Na⁺ as a fraction of that in the external medium (17.1% versus 17.9% for PTS and Na⁺, respectively). In contrast, bypass of Na⁺ was slightly higher than that of PTS in IR20 (26.2% versus 22.1%). Bypasses of both PTS and Na⁺ were significantly higher in IR20 than in Pokkali ($P=0.03$ and $P=0.003$, respectively; Fig. 2A). On conditioning in 100 mM NaCl, the performance of the hydrophobic barriers improved strikingly, with Na⁺ bypass dropping from 17.9% to 6% in Pokkali while the decline in PTS was still larger: from 17.1% to 2.5%. Even more dramatic changes were seen in IR20 roots (Fig. 2A, B). The difference in bypass flow between cultivars was much smaller after conditioning than in the control samples. These differences in bypass flow closely paralleled the extent of suberin deposition over the course of exposure to moderate salinity. The Na⁺ concentration in the xylem sap of unstressed plants was negatively correlated with suberin deposits (Fig. 2C).

Effect of salt stress on development of Casparian bands

At 20 mm from the root tip, CBs were not detected in either endodermis or exodermis of both IR20 (Fig. 3A, E) and Pokkali (Fig. 3B, F) roots under control conditions. In contrast, very prominent and bright yellow fluorescence indicated well-developed CBs in the radial walls of the endodermis as well as in the exodermis of both IR20 (Fig. 3C, G) and Pokkali (Fig. 3D, H) roots upon a conditioning stress of 100 mM NaCl for 1 week at 20 mm from the root tip.

Sodium and potassium uptake by rice plants

Under almost all conditions tested, IR20 shoots had significantly higher levels of Na⁺ than Pokkali shoots (Fig. 4A). Na⁺ content in shoots of plants subjected to 200 mM NaCl for 2 d was much higher than in control plants (Fig. 4A, III versus I). However, plants that had been conditioned with 100 mM NaCl accumulated much less Na⁺ in their shoots (Fig. 4A, IV). For example, Na⁺ levels dropped from 43.6 mg g⁻¹ dry weight (DW) to 29.7 mg g⁻¹ DW and from 55 mg g⁻¹ DW to 34.1 mg g⁻¹ DW in Pokkali and IR20, respectively (Fig. 4A, III versus IV). Note that the conditioned plants started the 200 mM stress episode with significantly more Na⁺ in the shoot than did plants directly stressed with 200 mM NaCl (Fig. 4A). The amount of Na⁺ taken up into the shoots during the 2

d stress protocol was thus much lower in plants that had been conditioned by exposure to 100 mM NaCl (Fig. 4A, III versus IV; B, III–I versus IV–II). The final Na⁺ level in the shoot was similar in plants subjected to 200 mM NaCl stress without conditioning and plants subjected to the same stress after a conditioning protocol followed by a week of recovery (Fig. 4A, III versus VI). However, these latter plants also entered the acute stress protocol with a significant amount of Na⁺ in their shoot (Fig. 4A, V). Thus, the amount of Na⁺ entering the shoot during the acute stress period was significantly lower in conditioned plants than in plants subjected to 200 mM stress without conditioning (Fig. 4B, III–I versus VI–V).

Having established that exposure to 100 mM NaCl improved barrier performance, it was important to check whether the Na⁺ that accumulated during this conditioning period could be released on subsequent removal of salinity. Na⁺ levels in plants tested after 1 week of recovery in control medium were down to about half those measured at the end of the conditioning period, indicative of a rapid efflux mechanism (Fig. 4A, V versus II).

Na⁺ levels increased in the roots of both cultivars upon salt stress (Fig. 4C), but to a smaller extent than seen in shoots. The Na⁺ levels were slightly higher in IR20 than in Pokkali in almost all stress conditions except the treatment with direct exposure to 200 mM NaCl where IR20 had significantly higher Na⁺ than Pokkali. Different from Na⁺, the amounts of K⁺ in the shoots of rice cultivars under varying stressed conditions remained essentially the same (Fig. 4D). However, the K⁺ levels in the roots declined under varying stress conditions (Fig. 4E). The level of Na⁺ in the shoot apoplast was maintained significantly lower in Pokkali than in IR20 under all stressed conditions (Fig. 4F). The Na⁺ levels in the shoot apoplast were lower in plants subjected to acute stress after conditioning compared with those stressed without conditioning.

Survival of rice plants subjected to salinity stress

Rice plants subjected to an acute stress of 200 mM NaCl for 2 d exhibited poor survival of 60% for Pokkali and only 25% for IR20 (Fig. 5A). Survival was much better on subjecting the plants to a conditioning stress of 100 mM NaCl for a week (~100% and ~80% for Pokkali and IR20, respectively). Interestingly, when conditioned plants were stressed with 200 mM NaCl, their survival increased significantly to 90% and 80% for Pokkali and IR20, respectively. Survival under acute stress was also improved by conditioning in 100 mM NaCl followed by a recovery period of 1 week, the difference in survival being much larger in the case of IR20 (Fig. 5A). It has been previously shown that survival of different rice cultivars subjected to salinity stress is negatively correlated with Na⁺ levels in the apoplast (Anil *et al.*, 2005; Krishnamurthy *et al.*, 2009). Survival is plotted against apoplastic Na⁺ levels for both Pokkali and IR20 plants under all of the conditions described above (Fig. 5B). While all the data points for Pokkali lie above 60% survival, they ranged from 20% to

80% in the case of IR20. The negative correlation between survival and the apoplastic Na^+ levels observed earlier were borne out under all the manipulations described in this work. Conditioned plants of both cultivars looked much healthier, with leaves open, than those stressed without prior conditioning (Fig. 5C).

Lateral root density, anatomy, and PTS leak

There was a significant increase in the number of lateral roots that had emerged from the primary root following a conditioning stress of 100 mM NaCl for a week compared with control plants. The increase was higher in IR20 than in Pokkali (from 9 to 18 versus from 11 to 17 for IR20 and Pokkali, respectively). A small increase was also seen upon a direct acute stress for 2 d, but this was not statistically significant (Fig. 6A). However, a conditioning stress followed by an acute stress of 200 mM NaCl for 2 d resulted in an increase in emerged lateral roots in both Pokkali and IR20 roots that is comparable with that seen after the conditioning stress alone (Fig. 6A; $P < 0.05$ level). On the other hand, a significant increase in the number of unemerged lateral root primordia was seen under various stress conditions in both the cultivars, the increase being most prominent (3–4 times) upon a direct acute stress for 2 d (Fig. 6A).

CBs appeared like a net in the endodermis of unstained lateral roots. No CBs were found in the exodermis (Fig. 6B). Upon staining with berberine hemisulphate, very prominent CBs were found in the endodermis of both Pokkali and IR20 under control as well as stressed conditions (Fig. 6B–D).

The apoplastic tracer PTS was found to leak through the breaks in the primary root that were created by lateral root emergence. However, the PTS was found only in the epidermal and cortical cells of the lateral roots and was not found in the xylem vessels (Fig. 6G, H). PTS fluorescence was also not observed at the base of a mature lateral root due to wound healing (Fig. 6I).

Expression of genes involved in lateral root development

The expression of auxin transport genes, which have been implicated in root development, were monitored. *RAUI*, *RAU4*, and *Arf8* genes were surveyed across both cultivars with 100 mM NaCl stress for different time points. While *RAUI* was expressed abundantly in both root and stem, *RAU4* had a much more restricted pattern of expression (Fig. 7A). It was seen primarily in the apical segments of the root and was minimally expressed in other regions and the stem. On the other hand, *Arf8*, a transcription factor, was predominantly expressed in the above-ground parts. Interestingly, while the more widespread *RAUI* showed little variation in expression level under stress, *RAU4* was down-regulated within 30 min of administering the salinity stress of 100 mM NaCl. There was an up-regulation in the

expression of *Arf8* transcript levels upon salt stress up to 8 h (Fig. 7B).

Discussion

Plants deploy a number of strategies to survive under variable environmental conditions. Stresses such as drought and salinity may have devastating effects on plant survival. The shoot is the first part of the plant to feel drought stress, as it is the location of water loss. Salinity, on the other hand, would affect the roots first, since they are in direct contact with the soil. Consequently, it may be expected that roots would display adaptations to cope with variation in soil salinity. The hydraulic conductivity of roots of both herbaceous and woody species has been extensively investigated (Steudle and Heydt, 1997; Barrowclough *et al.*, 2000; Miyamoto *et al.*, 2001; Lee *et al.*, 2004). These studies indicate that unfavourable environmental conditions reduce hydraulic conductivity (Steudle, 1994; Kramer and Boyer, 1995; Steudle and Peterson, 1998; Zimmermann *et al.*, 2000). Suberization of roots such as formation of CBs and suberin lamellae localized in cell walls, as those of the endo- and exodermis contribute to the observed variability of water uptake in roots, with more extensive barriers effectively reducing conductivity (Cruz *et al.*, 1992; Stasovsky and Peterson, 1993). However, all barrier structures do not contribute equally to the reduction in hydraulic conductivity. The exodermal barrier, for instance, has been shown to present relatively little resistance to water flow in maize and rice (Zimmermann and Steudle, 1998; Ranathunge *et al.*, 2003). In rice, it is the highly suberized endodermal barrier that presents the major resistance to radial water flow (Miyamoto *et al.*, 2001; Ranathunge *et al.*, 2003). Further, the chemical composition of suberin in the apoplastic barriers is reported to affect the hydraulic conductivity of roots (Schreiber *et al.*, 2005).

One of the hydrophobic barriers, namely CBs, serve to prevent transport of the external medium including ions and fluorescent dyes directly into the xylem stream, effectively ensuring that fluid has to pass through at least one cell membrane en route (Steudle and Peterson, 1998). This strategy, in turn, allows for some control of solutes and fluid that are transported to the shoot via the xylem stream. Most of the Na^+ that enters the shoots of rice plants has been reported to do so through the so-called ‘apoplastic bypass’, where Na^+ ions move through the apoplast by solvent drag (Ranathunge *et al.*, 2005), bypassing CBs (Ochiai and Matoh, 2002; Gong *et al.*, 2006). Exposure of rice to moderate saline stress of 100 mM NaCl for 1 week resulted in deposition of additional barrier material (i.e. suberin), strengthening the apoplastic barriers (Krishnamurthy *et al.*, 2009). The present study was designed to test whether or not these induced barriers resist the bypass flow, thereby contributing to a reduction in Na^+ uptake and hence enhance survival of rice plants that remain in a saline environment.

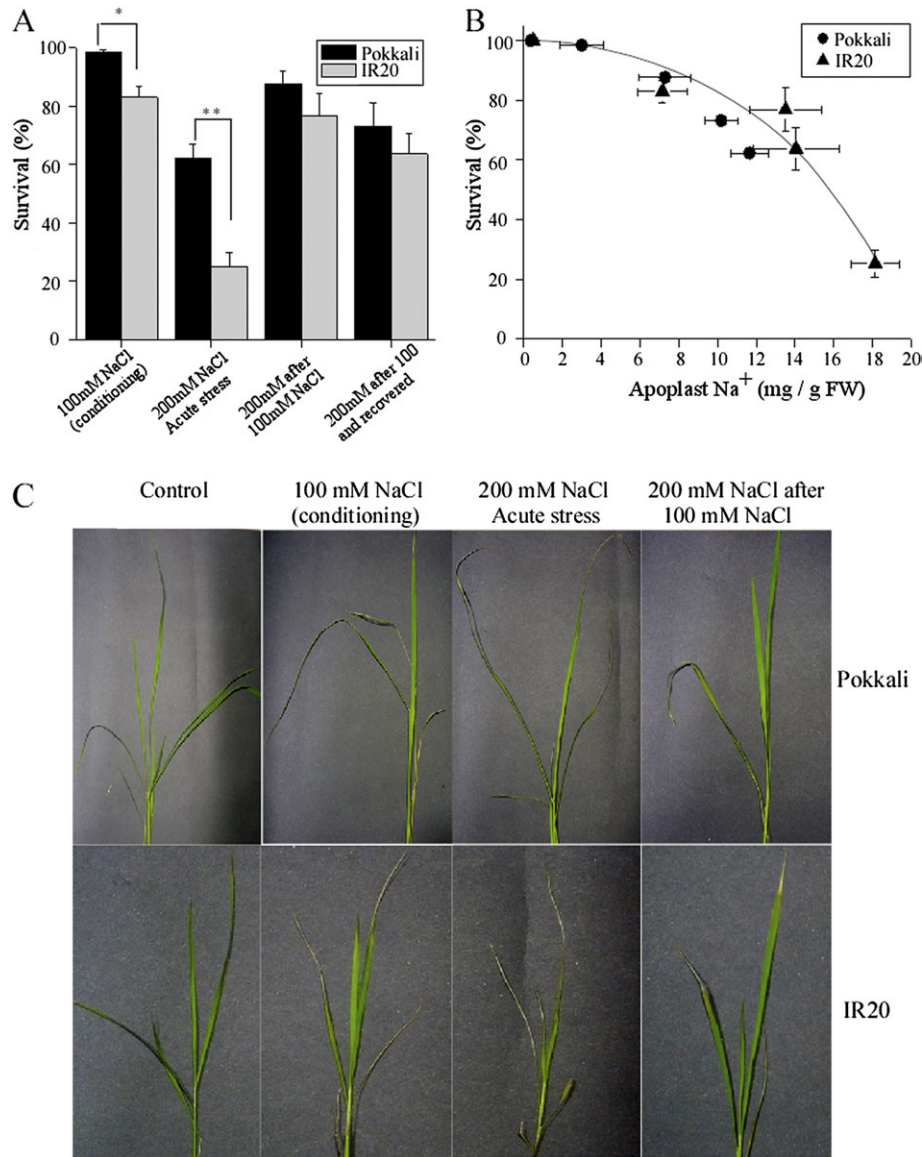


Fig. 5. Survival of rice plants under salt stress. One-month-old, hydroponically grown rice plants were subjected to various salt stress conditions, and plants that retained more than one-third of their leaf area open without curling up were counted to estimate survival rates. (A) Survival of rice plants under various stress conditions. (B) Correlation between survival and Na^+ in the apoplastic fluid in the shoot. (C) Pictures of rice plants under various stress conditions. Data represent means \pm SE, $n=6$ plants. Asterisks indicate statistically significant differences at $*P \leq 0.05$ and $**P \leq 0.01$ (t -test); the line bars are introduced to indicate the data/bars used for t -test comparison.

In the absence of stress, the sensitive cultivar, IR20, exhibited significantly higher hydraulic conductivity than did the tolerant Pokkali (Table 1)—in good agreement with earlier results on the suberin contents of the respective roots (Krishnamurthy *et al.*, 2009). Subjecting the plants to a moderate conditioning stress of 100 mM NaCl resulted in a large reduction in hydraulic conductivity, which is also consistent with data on the deposition of additional suberin during this period and formation of the CBs in the endo- and exodermis close to the root tips (Fig. 3). The extent of barrier deposition (i.e. the fold increase in total root suberin) was significantly greater for IR20 than for Pokkali (Krishnamurthy *et al.*, 2009). Indeed the reduction of

hydraulic conductivity was also more dramatic for IR20 than for Pokkali (Table 1).

The volume flow (J_{vr}) of water through the root increased with applied pneumatic pressure in a non-linear fashion (Fig. 1). In the absence of applied pneumatic pressure, J_{vr} is exclusively due to osmotically driven movement of xylem sap. As the applied pressure is increased, an increasing fraction of the fluid collected in the experiment is due to hydrostatic pressure-driven flow through the apoplastic route (i.e. xylem sap is diluted by the Fiscus Effect; Fiscus, 1975; Miyamoto *et al.*, 2001). At high hydrostatic pressures, J_{vr} is dominated by apoplastic water movement and the relationship is linear. The hydraulic conductivity, L_{Pr} , was

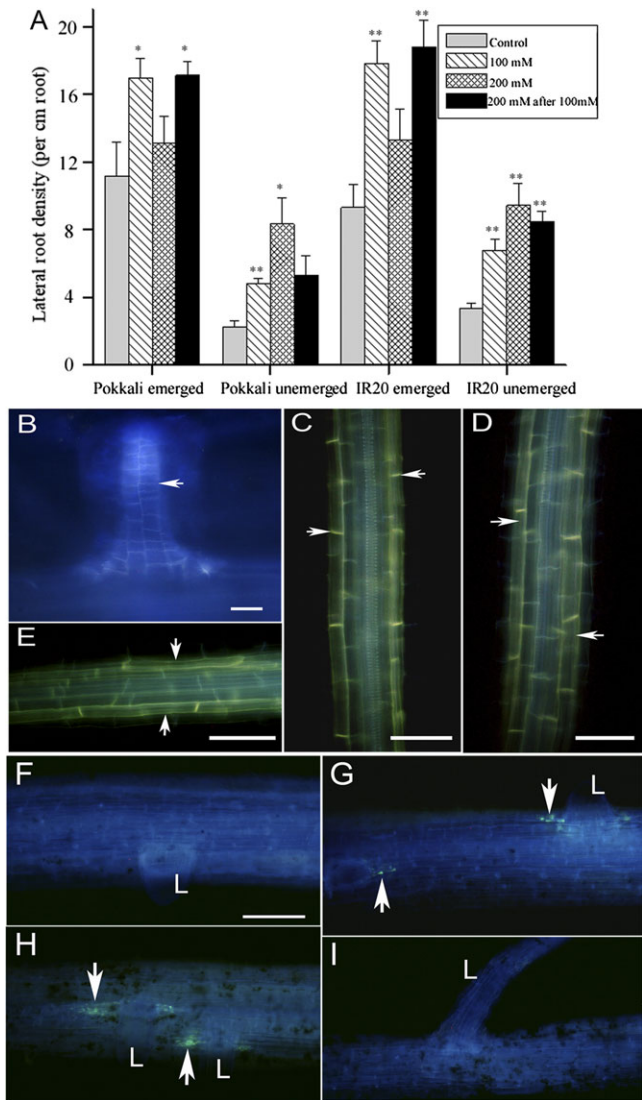


Fig. 6. Lateral root density, anatomy, and PTS leak. Roots from 1-month-old, hydroponically grown rice plants subjected to various salt stress conditions were used to score lateral root density. (A) Lateral root density of rice roots under various stress conditions. Data represent means \pm SE, $n=8$. Emerged, lateral roots that had emerged through the epidermis; unemerged, lateral roots that had not yet emerged through the epidermis. (B–E) Development of Casparian bands in the lateral roots of rice plants. Root segments with lateral roots from 1-month-old rice plants were cleared with lactic acid saturated with chloral hydrate for 1 h at 70 °C and were then stained with berberine hemisulphate for 1 h. The stained samples were washed with 50% ethanol and were mounted in 75% glycerol and viewed under an epifluorescence microscope with a UV filter set. (B) Image of an unstained lateral root segment. (C, D) Images of stained lateral root segments from control Pokkali and IR20 plants. (E) Image of a stained lateral root segment from a stressed Pokkali plant. Arrows indicate the Casparian bands in the endodermis. (F–I) PTS fluorescence from the roots stained with 0.01% (w/v) PTS for 1 h and observed under an epifluorescence microscope with UV excitation. (F) Image of an unstained Pokkali root lacking PTS fluorescence. (G, H) Images of stained IR20 and Pokkali roots; arrows indicate PTS leak at the base of lateral roots as green precipitates. (I) Image of a stained Pokkali root with a mature lateral root, lacking PTS fluorescence. L, lateral root, bar=200 μ m.

estimated from this linear part of the curve and is thus expected to reflect apoplastic water movement. Osmotically driven water flow moves primarily through plasma membranes and requires the activity of aquaporins. Gating of aquaporins is known to be osmotically regulated (Ye *et al.*, 2004; Lee *et al.*, 2005). Hence, salt-stressed plants were transferred to control medium for 12 h prior to starting water permeability measurements to avoid osmotic stress-induced closure of water channels. On the other hand, deposition of suberin lamellae very close to the root tips of these cultivars was observed following a conditioning salt stress of 100 mM in an earlier study (Krishnamurthy *et al.*, 2009). These barriers could have contributed to the inability to observe osmotically driven fluid flow in the stressed plants.

The performance of the barriers laid down during conditioning differed from the pre-existing barriers in terms of the pore size distribution. Bypass of Na^+ and PTS was similar in unstressed roots of both cultivars, indicating that the cell wall pores were too large to distinguish between Na^+ and PTS. On conditioning, bypass reduced significantly for these solutes in both cultivars (Fig. 2A, B). This reduction in bypass flow during conditioning suggests that either the total number of pores or their size is significantly reduced, probably both. Bypass of PTS was more severely curtailed than that of Na^+ , suggesting that the newly deposited suberin clogged the intermicrofibrillar spaces in the cell walls, making tight suberized barriers with reduced pore sizes. A significant fraction of the pores in the pre-existing barriers are much larger than the diameter of PTS, as the drag on PTS and Na^+ is similar. However, the freshly deposited suberized barriers seem to sieve PTS more effectively than Na^+ , indicative of a pore size distribution with relatively few pores exceeding the diameter of PTS.

In rice, it has been suggested that K^+ and Na^+ enter the shoot by distinct mechanisms which are genetically regulated (Garcia *et al.*, 1997). The present data show that the K^+ content of the shoots is invariant across all the conditions imposed in this study, while the Na^+ content in shoots varies widely (Fig. 4A, C). The size of the pores bypassing the hydrophobic barriers is much larger than the diameter of the Na^+ ion both before and after conditioning. The largest pores both before and after conditioning are larger than the diameter of PTS, and so cannot distinguish between Na^+ and K^+ . It follows that the amount of K^+ taken up by the more specific and better regulated mechanism of loading endodermis and xylem via transporters in the plasma membrane greatly exceeds the amount entering through apoplastic bypass flow.

The present estimates of the hydraulic conductivity and bypass flow of unstressed (control) plants are somewhat

higher than those previously reported for rice, but in the same range (Miyamoto *et al.*, 2001; Ranathunge *et al.*, 2003). Care was taken to handle the roots very gently and carefully to ensure that the high values obtained were not caused by artificial injuries or physical damage. In addition, similarly handled roots of stressed plants had significantly lower hydraulic conductivity, which fall well within the range reported. Further, it is known that rice plants exhibit enormous individual variability in bypass flow and Na^+ uptake (Yeo and Flowers, 1983). It is thus likely that the values reported here are higher than those in earlier studies because of varietal differences or growth conditions or the age of the preparations used. Varietal differences are clearly substantial as Lp_r for unstressed IR20 was almost double that seen for Pokkali (Table 1). Growth conditions are also critical inasmuch as Lp_r of IR20 dropped by a factor of ~ 2 after conditioning. On the other hand, not all cultivars are equally sensitive to salinity, as Pokkali Lp_r was reduced by only a third (33%) under the same conditions. Indeed, the final Lp_r for both cultivars after exposure to 100 mM NaCl was essentially indistinguishable.

Suberized, hydrophobic barriers present the major resistance to radial flow of water, and a good correlation was seen between the earlier report on the deposition of these barriers in IR20 and Pokkali (Krishnamurthy *et al.*, 2009) and formation of CBs (Fig. 3) and the hydraulic conductivity measurements conducted in the present study. However, it has been suggested that the locations where lateral roots emerge from primary roots are leaky to water as barriers are interrupted at these points, presenting 'open windows' for water and solute flows (Peterson *et al.*, 1981; Ranathunge *et al.*, 2004). PTS leak into the cortex was seen at the primary root–lateral root junctions where the lateral roots emerge. On the other hand, no such leak was found at the base of mature lateral roots, where the wound created due to lateral root emergence could have healed (Fig. 6F–I). This finding disagrees with that of Faiyue *et al.* (2010a) who have reported PTS entry into the xylem. However, they also suggested that the emerging lateral roots themselves may admit Na^+ as they lack an exodermis (Faiyue *et al.*, 2010b). On the other hand, the exodermis plays a relatively small role in restricting water and solute entry into the root compared with the endodermis (Miyamoto *et al.*, 2001; Ranathunge *et al.*, 2003). Very prominent CBs were seen in the endodermis of lateral roots in control as well as stressed rice plants (Fig. 6B–E), which could be involved in restricting the bypass flow of Na^+ into the root xylem.

The present data indicate a significant increase in the density of lateral roots on exposure to salinity, together with a dramatic reduction in hydraulic conductivity and bypass flow. This would suggest that the contribution of lateral roots, if any, to bypass flow is small compared with the resistance presented by the newly deposited hydrophobic barriers in the primary root. Functionally, the extent of Na^+ uptake correlates very well with the hydrostatic hydraulic conductivity, suggesting that uptake of Na^+ through exposed surfaces of lateral roots and subsequent cell to cell movement is negligible in this context.

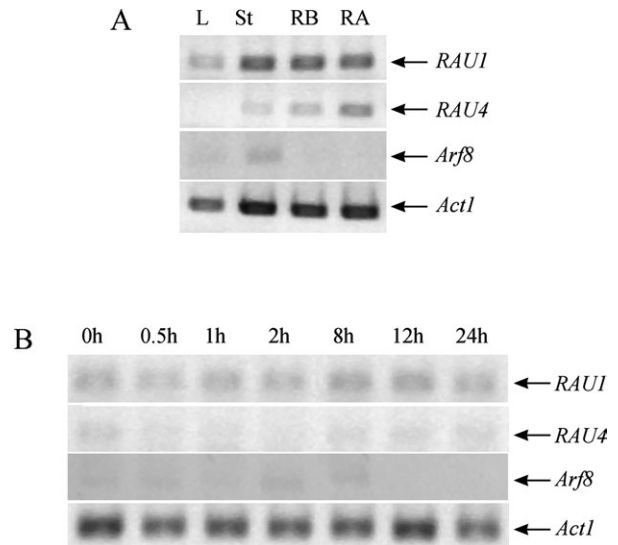


Fig. 7. Tissue-specific and NaCl-induced transcriptional up-regulation of genes involved in lateral root development in hydroponically grown, 1-month-old Pokkali plants. (A) Tissue-specific expression of *RAU1*, *RAU4*, and *Arf8* was monitored by RT-PCR in Pokkali plants. L, leaf; St, stem; RB, root basal; RA, root apical. (B) Temporal changes in the *RAU1*, *RAU4*, and *Arf8* transcript levels were monitored by RT-PCR in Pokkali roots upon stressing with 100 mM NaCl for varying times. *ACTIN* (*Act1*) was used as control. In each reaction, 1 μg of total RNA was used. A 20 μl aliquot of each PCR product was loaded onto a 1% agarose gel from a 25 μl reaction.

While the emergence of lateral roots did not appear to increase the extent of bypass flow, the number of lateral roots emerging on exposure to salinity was significantly greater than in control plants. The total number of lateral roots initiated during stress was significantly higher than in control plants under all stress protocols used. Indeed, a significant enhancement in initiation is seen following the 2 d, acute stress protocol. However, emergence of these roots from the primary root appears to take >2 d inasmuch as the number of emerged lateral roots was much greater after a week of stress compared with a 2 d stress protocol (Fig. 5A). This observation of increased lateral root density following stress is consistent with earlier reports on *Arabidopsis* (Nibau *et al.*, 2008). It is conceivable that this increase is a means of combating the impairment of root hydraulics caused by the extensive suberization.

The deposition of suberized hydrophobic barriers during conditioning is well correlated with a sharp reduction in Na^+ uptake into shoots during subsequent exposure to acute stress. Indeed, the present data indicate that the entry of Na^+ into shoots under these conditions is reduced by almost an order of magnitude compared with the uptake on directly exposing plants to 200 mM NaCl (Fig. 4A, B, III–I versus IV–II). Barrier function was still good 1 week after returning the plants to control medium (Fig. 4B, VI–V versus III–I). It may be expected that this would be reflected in enhanced survival. Survival of plants conditioned with 100 mM NaCl stress was significantly better on subsequent

exposure to toxic 200 mM NaCl compared with unconditioned plants (Fig. 5A, C). In fact, there appears to be little additional mortality on exposure to acute stress after the conditioning (Fig. 5A).

Interestingly, a significant fraction of the Na⁺ taken up into the shoot in the course of the conditioning protocol was released on return to control medium (Fig. 4A, V; B, II–V). The amount of Na⁺ released was larger in the case of Pokkali than for IR20, but it was significant in both cases. The amount of Na⁺ released over a week of recovery was comparable with the amount of Na⁺ taken up into the shoots with an acute stress of 200 mM NaCl for 2 d after conditioning in both the cultivars (Fig. 4B, II–V versus IV–II). Mechanisms for reduction of shoot Na⁺ may include efflux through the phloem back into the root, and exudation from the hydathodes of leaves. Subjecting plants which had gone through a recovery period of a week after the conditioning to an acute stress of 200 mM NaCl resulted in Na⁺ levels in the shoot comparable with those of plants subjected to acute stress without pre-conditioning (Fig. 4A, VI and III). However, the survival of the conditioned plants was better than that of plants that had not been conditioned (Fig. 5A). It was previously shown that survival is best correlated not with total shoot Na⁺ content, but with the Na⁺ content of the apoplast fraction of the shoot (Anil *et al.*, 2005). The apoplastic fraction of shoot Na⁺ for IR20 was lower in plants stressed after recovery from conditioning [14 mg g⁻¹ fresh weight (FW)] than in plants subjected to 200 mM NaCl stress without prior conditioning (~18 mg g⁻¹ FW) (Fig. 4F). The correlation between the survival of plants of both cultivars subjected to the range of treatments studied here with their shoot apoplastic Na⁺ contents is presented in Fig. 5B. Irrespective of stress protocol or cultivar, the negative correlation holds very well. Thus, the conditioning protocol served not only to build up hydrophobic barriers, but also to activate mechanisms for partitioning Na⁺ that enters the shoot in a manner that reduces its presence in the shoot apoplast.

Candidate genes for the regulation of lateral root growth include those for auxin transport, *RAUI* and *RAU4*. These correspond to the *AUX1*-like genes in *Arabidopsis*, which are involved in phloem-based indole acetic acid (IAA) transport (Marchant *et al.*, 2002; Chhun *et al.*, 2007). The present data indicate that neither of these genes is responsible for the initiation of lateral root development under stress in rice as *RAUI* levels were invariant under the stress protocols used, while *RAU4* levels declined under stress (Fig. 7B). It is conceivable that other transporters play a role in auxin transport under these circumstances. However, the transcript levels of *Arf8* were up-regulated upon salt stress, indicating a role for this in lateral root development of rice (Fig. 7B).

In conclusion, the present data indicate a good correlation between apoplastic barrier deposition and resistance to radial flow of water and solutes in the roots of rice. Further, it is demonstrated that the apoplastic barriers deposited under moderate salinity stress (conditioning protocol) resist

the flow of bulk water and dissolved solutes, resulting in reduced uptake of Na⁺ into shoots and consequently better survival under subsequent acute stress. These salinity-induced barriers have a pore size distribution with relatively few pores greatly exceeding the diameter of the apoplastic tracer dye, PTS, whereas the pre-existing barriers have much larger pores. Finally, while salinity stress induces the emergence of lateral roots, these do not appear to play a significant role in enhancing Na⁺ uptake into shoots.

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