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RESEARCH PAPER

Differentially expressed membrane transporters in rice roots may contribute to cultivar dependent salt tolerance

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Abstract

Salinity tolerance in rice, like in other glycophytes, is a function of cellular ion homeostasis. The large divergence in ion homeostasis between the salt-tolerant FL478 and salt-sensitive IR29 rice varieties can be exploited to understand mechanisms of salinity tolerance. Physiological studies indicate that FL478 shows a lower Na^+ influx, a reduced Na^+ translocation to the shoot, and maintains a lower Na^+:K^+ ratio. To understand the basis of these differences, a comparative investigation of transcript regulation in roots of the two cultivars was undertaken. This analysis revealed that genes encoding aquaporins, a silicon transporter, and N transporters are induced in both cultivars. However, transcripts for cation transport proteins including *OsCHX11, OsCNGC1, OsCAX*, and *OsTPC1* showed differential regulation between the cultivars. The encoded proteins are likely to participate in reducing Na^+ influx, lowering the tissue Na^+:K^+ ratio and limiting the apoplastic bypass flow in roots of FL478 and are therefore important new targets to improve salt tolerance in rice.

Key words: Monovalent ion uptake, rice, root membrane transporters, salinity tolerance, silicon accumulation-transciptomics.

Introduction

Soil salinity is one of the major abiotic stresses limiting agricultural production in many areas of the world. In plants, salinity severely decreases growth through osmotic stress, large increases in cellular Na^+ and Cl^- contents and negative effects on K^+, Ca^{2+}, and NO_3^- nutrition (Flowers and Colmer, 2008). These deleterious effects occur at both the cellular and the whole tissue level.

Salt tolerance in glycophytes is predominantly associated with the restriction of toxic ion absorption at the root level, extrusion of ions into the apoplast, and sequestration of excess ions into intracellular compartments and tissues that are less sensitive (Blumwald, 2000; Flowers and Colmer, 2008). Plants face dilemmas in executing these strategies. For example, where the osmotic imbalance is concerned, ion uptake is beneficial to lower the osmotic potential but excess ion uptake, particularly of Na^+, may be toxic. Increased uptake capacity for the less toxic cation K^+ may be problematic due to the physicochemical similarity between Na^+ and K^+ that leads to a lack of discrimination between these cations at the transport sites (Maathuis and Amtmann, 1999). Salt-tolerant species are more capable of achieving this delicate balance and, in glycophytes, this is often exemplified by lower levels of Na^+ accumulation in tolerant species and ecotypes (Munns, 2002).

Amongst the major crops, rice is highly sensitive to salt stress and generally only tolerates salinities ranging between 1.9–3 dS m^{-1} (Grattan *et al.*, 2002), which is comparable to a concentration of around 20–30 mM NaCl. Rice sensitivity to salinity is dependent on growth stage with most stress symptoms being displayed in the seedling and panicle induction stages (Akbar *et al.*, 1972). Although some non-symplastic uptake of ions occurs in most plants, rice is unique in the sense that its apoplastic pathway has considerable conductance for Na^+ (Yeo *et al.*, 1987; Garcia *et al.*, 1997). This so-called 'bypass flow' is increased by an

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inadequate silicon supply (Gong *et al.*, 2006) and possibly depends on Ca^{2+} homeostasis (White, 2001).

Rice salt sensitivity varies considerably across cultivars, a phenomenon that can potentially be exploited to discover genes and proteins that contribute to tolerance. The overall mechanisms of salt tolerance in rice, as in other crops, depend on the control of salt uptake at the root level, regulation of influx into cells, control over long-distance transport, and compartmentation at the cellular and tissue levels. Tolerance also relies on maintaining a high cytosolic K^+:Na^+ ratio and a reduction of cytosolic Na^+ load (Maathuis and Amtmann, 1999). However, the implementation of these strategies may be significantly different in tolerant and sensitive cultivars, both at the cellular and whole tissue levels. For example, short-term measurements using the Na^+ sensitive dye SBFI showed that cells derived from tolerant cultivars show a much smaller Na^+ conductance in their plasma membrane (Kader and Lindberg, 2005; Anil *et al.*, 2007) which could underlie the often observed lower tissue [Na^+] in tolerant varieties.

Transcriptomics approaches have been applied to identify differentially regulated rice genes in response to salt stress for shoots (Chao *et al.*, 2005; Zhou *et al.*, 2007) and roots (Kawasaki *et al.*, 2001) and also comparing rice with other cereals (Ueda *et al.*, 2006). However, such approaches are likely to identify many false positives due to unwanted side-effects of the treatment and/or to the use of different species. To minimize these risks, the use of cultivars with different salt sensitivity for comparative transcriptomics studies is far more preferable.

An extensive transcriptomics study comparing cultivars with varying salt tolerance was carried out on shoot tissues derived from plants at the seedling or panicle initiation stages (Walia *et al.*, 2005, 2007). Genes from many functional classes were found to be differentially regulated in shoots of the tolerant cultivar and included those encoding transcription factors, signal transduction components, cell wall components, and membrane transporters. In the latter group, differential regulation was observed for genes encoding carriers and channels involved in transporting cations (*HKT, HAK, KAT, CNGC, GLR*), anions (*CLC*) and organic substrates (sugar transporters).

Root tissue forms the initial defence barrier against salt stress and many functions in roots, particularly related to ion transport, are specific for this organ. Therefore, the physiological and morphological properties and transcriptional regulation in root tissue of the well-characterized sensitive and tolerant cultivars IR29 and FL478 has been investigated. Since significant differences in tissue [K^+] and [Na^+] is one of the most obvious manifestations of variation between FL and IR, our focus was on the role of membrane proteins that are responsible for Na^+ and K^+ homeostasis. Our analyses yielded a number of root-specific transcripts that are likely to contribute to salt tolerance and a further number of transcripts that were specifically regulated in the tolerant variety.

Materials and methods

Plant growth and salt treatment

Seeds of two rice cultivars, FL478 (FL) and IR29 (IR), were obtained from the International Rice Research Institute (Los Baños, Pillippines). Seeds were germinated and seedlings were transferred to hydroponic medium (1.25 mM KNO_3, 0.5 mM $Ca(NO_3)_2.4H_2O$, 0.5 mM $MgSO_4.7H_2O$, 42.5 μM FeNaEDTA, 0.625 mM KH_2PO_4, and 1.0×10^{-2} μM Cu^{2+}, Zn^{2+}, Mn^{2+}, B^{3+}, Mo^{2+}, and Co^{2+}) (Arteca and Arteca, 2000) 10 d after sowing (DAS) and grown under controlled conditions at 22/19 °C day/night temperatures, 100 μmol m^{-2} s^{-1} of irradiance for 16 h d^{-1}, and 40% relative humidity. Seedlings were exposed to salinity stress by adding 50 mM or 100 mM NaCl to the hydroponic solution at 15 DAS. Hydroponic solution was renewed every 2 d. Plants were harvested at different time intervals for analyses. For monovalent cation analysis tissue was collected at various time points as indicated in the figure legends. Total RNA extraction for transcriptome analysis, and measurement of long-term accumulation of Na^+, K^+, and Ca^{2+} and recording photosynthetic rates were done on the 12th day of the treatment (27 DAS). Seeds of the *lsi1* mutants were received from Professor Jian Feng Ma. They were cultivated and treated as described above except for the addition of 3 mM Si in the form of $Na_2O_7SiO_3$ to the hydroponic medium 3 d prior to the salinity treatment.

Photosynthesis, stomatal conductance, and relative growth rate measurements

Net photosynthesis per unit leaf area and stomatal conductance of the youngest fully expanded leaf were determined at the 12th day of salt treatment using a Li-Cor 6400 infrared gas analyser (Li-Cor Biosciences, Nebraska, USA). Measurements were made at 500 μmol m^{-2} s^{-1} of photosynthetic active radiation, 400 μmol mol^{-1} of chamber CO_2 concentration, 24 °C and 42% relative humidity in the leaf chamber.

To measure the relative growth rates of plants, a minimum of three plants from three independent replicates was randomly selected from three treatments (control, 50 mM NaCl, and 100 mM NaCl) at the beginning and end of the treatment. *RGR* was determined using the equation as described by Poorter and Garnier (1999).

Tissue cation and N analysis

Both long- and short-term Na^+, K^+, and Ca^{2+} content measurements of leaves, culms, and roots were measured using flame photometry. Harvested tissues were washed with cold 20 mM $LaCl_3$ solution for 10 min. Fresh weights of the sample were noted and samples were subsequently dried at 80 °C for 3 d. Dried samples were incubated in 5 ml of 20 mM $LaCl_3$ for 24 h and measurements were recorded using a flame photometer (Sherwood flame photometer-410 Cambridge, UK). For N analysis, dried plant material was wrapped in aluminium foil prior to

loading into a CHNOS elemental analyser 'vario Micro' (Elementar, Hanau, Germany).

RNA isolation and microarray hybridization

Root RNA was isolated from control and salt-treated (50 mM) FL and IR plants using Trizol^{R} reagent. RNA was purified by RNAeasy spin columns (Qiagen, London, UK). RNA was pooled from 3–4 independent sets of 6–8 plants for each experiment. This procedure was repeated three times for each cultivar and treatment, i.e. a total of 12 RNA samples was collected.

The 12 samples were sent to the Arizona microarray facility (http://ag.arizona.edu/microarray) where cDNA synthesis, Cy3 and Cy5 labelling and hybridization was carried out on NSF 45K 70-mer oligo microarrays. The arrays contain around 45 000 elements, representing all known ORFs present in the rice genome. For each cultivar, in one out of three replicate hybridizations, Cy3 and Cy5 dye labelling was swapped between treatment and control.

Microarray data analysis

All raw fluorescence data for both Cy3 and Cy5 labelled probes can be found for each biological replicate in the supplementary data. Data were analysed using SNOMAD software (available at http://pevsnerlab.kennedykrieger.org/snomadinput.html) for lowess signal correction, and spreadsheet software for other manipulations such as background subtraction, global mean normalization, calculation of average signals, and standard deviations as previously described (Maathuis, 2006; Moscatiello *et al.*, 2006). Transcripts were included for further analysis when the following criteria were met for signal (S), background signal (BS), signal ratio (SR), and standard deviation of average SR (SD): (i) S-SB $\geqslant$100 and S/SB $\geqslant$1.5 in at least one channel and this fulfilment would assign a 'present' label; (ii) a 'present' signal in all three replicas; (iii) if criteria (i) and (ii) were met, SR/SD $\geqslant$1.5. Although the background signal intensity can have a large impact on the interpretation of microarray data (Pan *et al.*, 2005), a doubling or halving of the chosen value (100) did not significantly change the general analysis outcome.

Transcripts were annotated as significantly regulated when, in addition to the above criteria, (i) the average of SR values for treated and control transcripts differed more than 2-fold and (ii) a *t* test of significance at $P < 0.05$ was met. The fold-change cut-off criterion (2-fold) was based on the distribution of fold-changes observed in normalized control data. These provide a measurement for inherent variability and thus an estimate for the proportion of false positives that can be expected (Maathuis, 2006). A cut-off criterion of 2-fold should yield a false positives rate of less than 10%.

Prediction of transmembrane domains (TMDs) was carried out using the bulk sequence data retrieval from TIGR at: http://www.tigr.org/tdb/e2k1/osa1/batch_download.shtml and subsequent sequence analysis according to TMHMM at http://www.cbs.dtu.dk/services/TMHMM/.

RT-PCR validation of microarray data

Semi-quantitative RT-PCR was carried out on selected genes to confirm microarray data. A total of 2 μg RNA, isolated from the roots of treated and control samples, was used to synthesize first strand cDNA using the transcriptor high-fidelity cDNA synthesis kit (Roche, Mannheim, Germany). PCR was applied to 1 μl of cDNA with gene-specific primers against, *OsHAK1* (Os04g32920), *OsCAX* (Os02g04630), *OsCHX11* (Os05g31730), *OsTPC1* (Os01g48680), and a high affinity nitrate transporter (Os02g02170). A list of primer sequences used in this analysis is given in the Supplementary data at *JXB* online. The house-keeping gene, tubulin α-1 (Os07g38730) was used as the control. PCR consisted of 35 cycles of 45 s at 56 °C, 1 min 30 s at 72 °C, and 30 s at 95 °C. For *HAK, CHX,* and the high affinity nitrate transporter, PCR was optimized for 35–45 cycles.

Statistical analysis

All data shown were derived from experiments carried out across a minimum of three biological replications. Growth, ion content, and microarray results were subjected to unpaired two-tailed *t* tests to identify significance at the $P < 0.05$ level.

Results and discussion

Physiological responses of FL478 and IR29 to salinity stress

Previous studies (Walia *et al.*, 2005, 2007) and our current results show that FL478 (FL) and IR29 (IR) exhibit discrete differences in many physiological parameters when exposed to salt stress. The most obvious is the significantly greater relative growth rate of FL (Fig. 1) which is particularly apparent in FL roots. Elongation of roots in the presence of salinity as seen in FL may be a stress-avoidance strategy where roots grow away from surface layers that tend to accumulate high levels of salts. Other parameters such as relative growth and photosynthetic rates, although negatively affected in both cultivars, remained higher in FL (Fig. 1) while maintaining a relatively low stomatal conductance compared to IR.

One potentially important tolerance mechanism is the restriction of Na^+ into the plant, particularly into shoot tissues (Moradi *et al.*, 2003), whereas an associated function is that of maintaining adequate nutrition of other minerals such as K^+ and Ca^{2+} (Maathuis and Amtmann, 1999; Davenport and Tester, 2000). Earlier work showed that long-term (7–10 d) Na^+ accumulation is lower in FL whereas that of K^+ is higher (Walia *et al.*, 2005), an observation that was repeated by us over a period of 12 d: after exposure to either 50 or 100 mM NaCl, FL maintained significantly lower [Na^+], especially in leaf tissue, compared

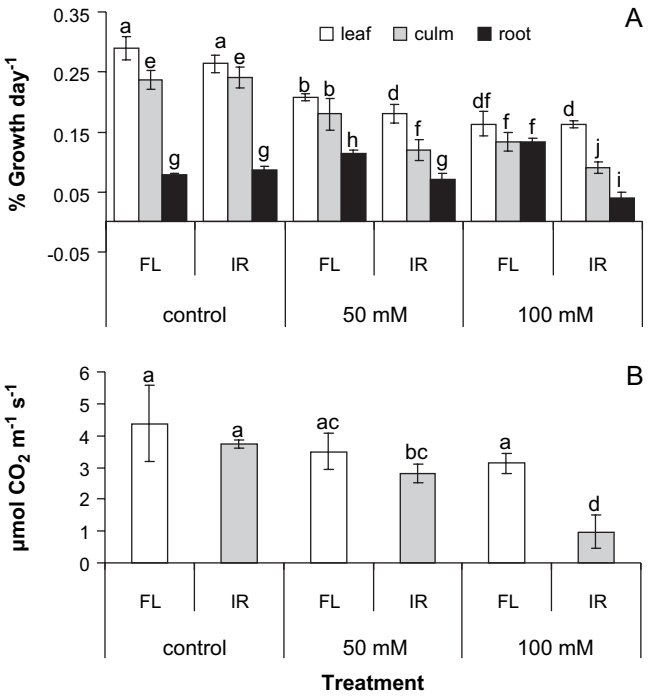

Fig. 1. Relative growth rate (*RGR*) and rate of photosynthesis (*RPS*) in FL478 (FL) and IR29 (IR) after exposure to salt treatment. (A) Fifteen-day-old rice seedlings were exposed to salt treatment of 50 mM and 100 mM NaCl in hydroponic medium. *RGR* was measured after 12 d of salt treatment for the various tissues. (B) *RPS* was measured in the youngest fully expanded leaf after being exposed to 50 mM and 100 mM NaCl treatments for 12 d. Bars in (A) and (B) represent the mean ±SD of three replicates with six readings each for RPS measurements. Different letters indicate significant differences at $P < 0.05$.

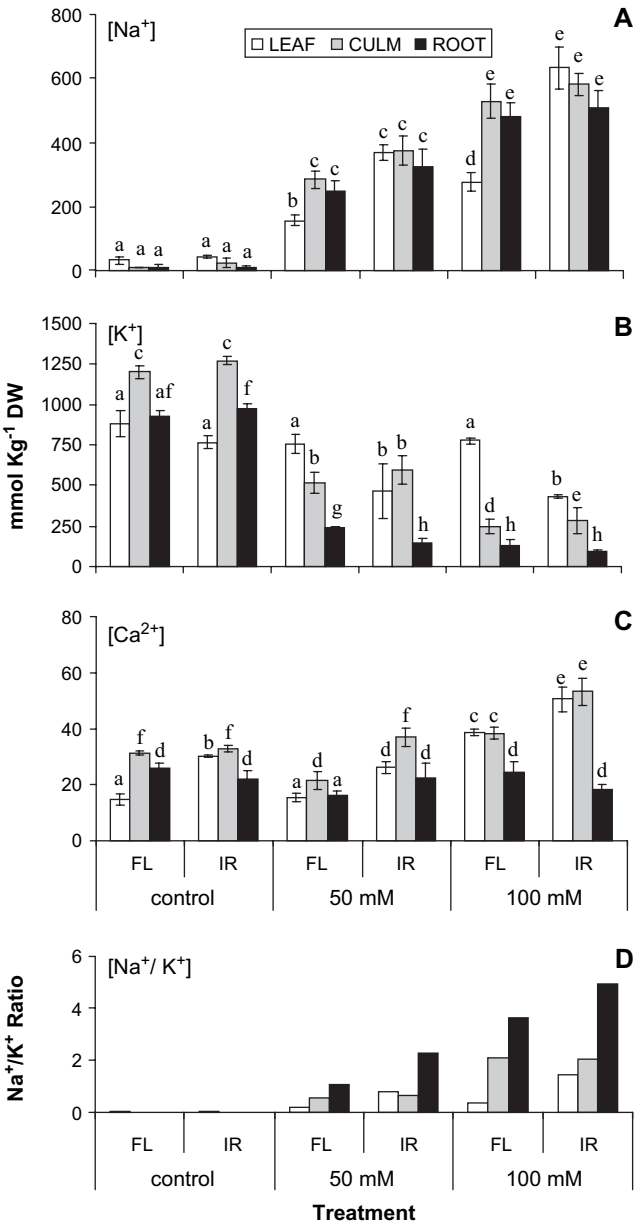

Fig. 2. Long-term accumulation of Na^+, K^+, and Ca^{2+} ions in tissues of FL478 and IR29. At 15 DAS, plants were exposed to salt treatment by adding NaCl to the hydroponic medium to final concentrations of 50 mM and 100 mM NaCl. At the end of 12 d of treatment, Na^+ (A), K^+ (B), and Ca^{2+} (C) concentrations and the Na^+/K^+ ratio (D) in shoot, culm, and root of the tolerant FL478 and sensitive IR29 were determined. Bars show the mean ±SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

to IR (Fig. 2A). By contrast, concentrations of K^+, which are comparable in control conditions, were significantly higher in leaves of the tolerant FL, culminating in a notably lower Na^+:K^+ ratio in FL leaves (Fig. 2C, D). Similarly, Na^+:K^+ ratios in FL roots were two times lower than in IR (Fig. 2D) indicating a larger capacity in this cultivar to maintain adequate K^+ nutrition in both roots and shoots.

Short-term Na^+ uptake experiments (3 h) were carried out to see if the trends discussed above also pertain to shorter periods. Figure 3 shows that a 3 h exposure to 50 mM NaCl resulted in a different pattern of tissue Na^+ in the two cultivars, indicating that FL and IR may also employ varying strategies to maintain Na^+ homeostasis during relatively short periods of exposure to salt. Na^+ accumulation increased in both cultivars but a clear difference was already apparent after 50 min uptake with higher Na^+ concentrations in IR leaf and culm tissues. More importantly, the leaf [Na^+] in FL quickly stabilizes and even reduces slightly after 3 h, whereas that of IR leaves continues to increase. Thus, after 3 h, IR actually contained higher [Na^+] in its leaf tissue than in its roots.

The findings from these short-term uptake experiments agree with those made in protoplasts and suspension culture cells where [Na^+] was measured with the Na^+ reporting dye SBFI (Kader and Lindbergh, 2005; Anil *et al.*, 2007). These studies reported a much smaller Na^+ influx in the tolerant cultivar Pokkali compared to the sensitive BRRI Dhan29 or Jaya variety and the main conclusion was that plasma membranes from Pokkali have a considerably lower Na^+ conductance. Our short-term net Na^+ uptake data appear to confirm this model and show a 2–3-fold larger Na^+ uptake in the sensitive cultivar.

Ca^{2+} has multiple effects on plant salinity and in rice, as in many other species, ameliorates stress symptoms (Pua *et al.*, 2001; Shah *et al.*, 2002). In many plants Ca^{2+} translocation to the shoot is compromised during salinity, due to the combination of reduced transpiration and the symplastic immobility of Ca^{2+} (Lynch and Lauchli, 1985). However, shoot Ca^{2+} deficiency does not appear to occur in rice: in both varieties, shoot Ca^{2+} levels remained unchanged or even increased in the presence of NaCl (Fig. 2C), particularly in the sensitive IR. The substantial apoplastic conductance of rice roots, or 'bypass flow' (Garcia *et al.*, 1997), compared to other plants may explain why Ca^{2+} supply to the leaves is not affected by salinity. Given that substantial amounts of Ca^{2+} are transported via the apoplastic route (White, 2001), the higher accumulation of leaf Ca^{2+} in IR suggests the conductance of this pathway is larger in this cultivar compared to FL.

In combination, the short and long exposure data show that (i) net Na^+ influx is considerably lower in FL than in IR, both during short- and long-term exposure to salt, (ii) Na^+ translocation to the shoot in FL is limited, and (iii) in both cultivars [K^+] decreases but FL maintains a higher concentration in its leaves. This variation could significantly contribute to the higher level of tolerance in FL and must be related to transport functions, particularly of Na^+ and K^+ in roots and their translocation to the shoots. To investigate further which molecular mechanisms could underlie these observed differences, whole genome transcriptomics were used to assess the transcriptional regulation of genes that encode root membrane transporter proteins and those that are potentially involved in the above phenomena were identified. To validate microarray data, RT-PCR was carried out on a selection of transcripts that ranged from around 0.3–14-fold changes and Fig. 4 shows excellent agreement between the two types of analysis.

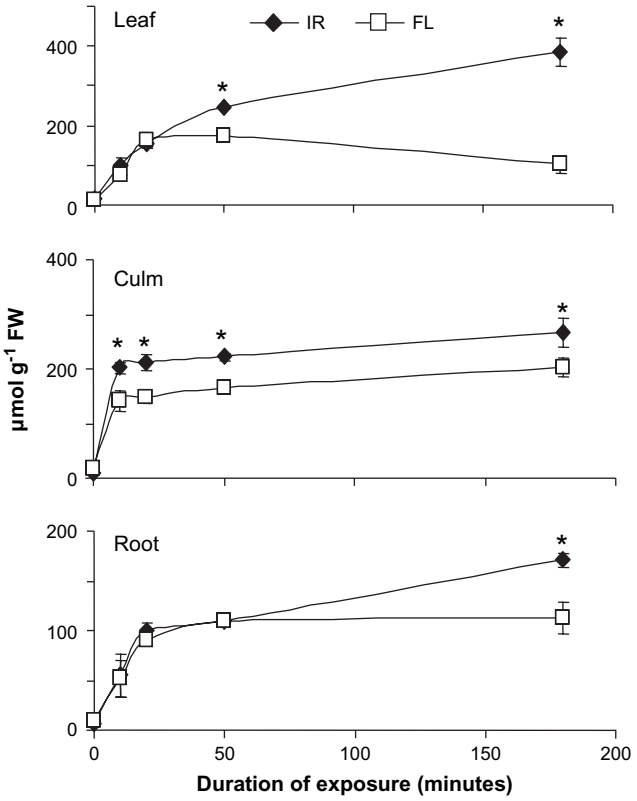

Fig. 3. Short-term Na^+ uptake into different tissues of FL478 and IR29 at different time intervals after exposure to salinity treatment. At 15 DAS, seedlings were exposed to salt (50 mM NaCl). Plants were withdrawn from the growth medium after different time intervals and analysed for Na^+ levels in different tissues. Data show the mean ±SD of three replicates and asterisks indicate significant differences at P <0.05.

Transporter genes that may contribute to salinity tolerance in both cultivars

In both cultivars (Tables 1, 2), genes encoding nitrogen transporters are significantly up-regulated although not necessarily for the same isoform. Many other studies showed that salinity affects transcript levels of nitrogen transporters (Maathuis *et al.*, 2003; Walia *et al.*, 2007) which may point to generic nutrient deficiency during stress. In addition, high Cl^- concentrations can inhibit NO_3^- transport and thus up-regulation of nitrate and ammonium uptake would be beneficial to withstand salt stress (Ehlting *et al.*, 2007). Ambient NaCl will also depolarize root cells and, therefore, reduce ammonium uptake which partly occurs through membrane potential-driven uniport (Ludewig *et al.*, 2002). Analysis of N content in roots and shoots of both cultivars showed that %N is not significantly affected in FL by salt treatment. However, there is a significant (t test, P <0.05) decrease in the N content of NaCl treated

Fig. 4. Semi-quantitative RT-PCR analysis of selected genes to validate microarray data. Genes were selected to represent the different regulatory responses recorded in transcriptomics. Differential expression values are shown and calculated using densitometry for NaCl-treated roots (FL50 and IR50) relative to the control condition (FL0 and IR0) for both the transcriptomics analysis (T) and semi-quantitative RT-PCR analysis (RT). ('–', No detectable expression.)

Table 1. Membrane protein transcripts regulated in roots of the salt-tolerant rice cultivar FL478

Plants were grown in hydroponics for 3 weeks and subsequently treated with 50 mM NaCl for 12 d. Membrane protein transcripts that changed by ⩾2-fold are listed with the corresponding change in the same transcript from the salt-sensitive cultivar IR29. Open space denotes the absence of signal. TMD: number of predicted transmembrane domains.

Locus	Annotation	Degree of regulation			TMD	Ortho/homologues in *Arabidopsis thaliana*
		FL 478	IR 29	FL/IR		
Water transport						
Os03g05290	Aquaporin *TIP1.1*	2.39	2.32	0.97	6	At2g36830 (AtTIP1.1)
Nitrate and ammonium transport						
Os04g43070	Ammonium transporter (*OsAMT1.1*)	2.08	0.98	2.12	11	At3g24290 (AtAMT1.5)
Os02g40730	Ammonium transporter (*OsAMT1.3*)	3.03	1.2	2.53	11	At4g13510 (AtAMT1.1)
Os02g02170	High affinity nitrate transporter	5.14			11	At5g60770 (AtNRT2.4)
Amino acid transport						
Os02g44980	Amino acid transport protein, putative, amino acid/auxin permease	2.20	1.35	1.63	11	At2g42005 Amino acid transporter
Os08g03350	Plasma-membrane localized histidine transporter (*OsHT1*)	2.94	1.56	1.88	10	At5g40780 (AtLHT1)
Os01g40410	Amino acid transporter family protein	0.37	1.39	3.76	10	At3g28960 Amino acid transporter
Mono- and divalent cation transport						
Os05g31730	Putative cation-proton exchanger (*OsCHX11*)	14.9	2.49	5.99	12	At4g23700 (AtCHX17)
Os07g15370	Metal cation transporter Nramp1	2.04	1.06	1.92	12	At1g80830 (AtNRAMP1)
Os01g56420	Ctr copper transporter family protein, expressed	2.28	1.00	2.28	2	At5g59030 (AtCOPT1)
Os04g36720	Ferric-chelate reductase (*OsFRO1*)	0.50	1.14	0.44	10	At5g49730 (AtFRO6)
Os02g04630	CAX-type proton/calcium exchanger protein	0.48	1.31	0.37	8	At1g55730 (AtCAX5)
Os07g47350	Potassium transporter (*OsHAK7*)	0.41	1.36	0.30	13	At3g02050 (AtKUP3/AtKT4)
Cation channels						
Os06g33600	Cyclic nucleotide-gated ion channel 1	0.44	1.38	0.32	5	At5g53130 (AtCNGC1)
Sugar transport						
Os09g249240	Sugar/sugar alcohol proton symporter	0.40	1.57	0.25	11	At4g02050 (AtSTP7)
Os10g42830	Sugar/sugar alcohol proton symporter	0.45	1.23	0.37	12	At5g17010 xylose transporter
Other transporters						
Os02g51110	Silicon influx transporter (*OsLsi1/OsSIT1/OsNIP2.1*)	2.12	1.82	1.16	6	At5g37820 (AtNIP4.2/AtNLM5)
Os06g38950	ABC transporter family protein,	2.76	1.89	1.46	5	At3g47790 (AtATH7)
Os03g09970	Sulphate transporter 1.2	2.03	0.70	2.86	10	At1g78000 (AtSultr1.2)
Os02g21750	ABC transporter family protein,	0.44	1.68	0.26	4	At1g02520 (AtPGP11/AtMDR8)
Os08g43120	ABC transporter, putative, pleiotropic drug resistance ABC transporter (*OsPDR1*)	0.48	1.28	0.37	13	At2g36380 (AtPDR6)
Os06g19110	Cadmium tolerance factor	0.47			20	
Os11g04830	Cadmium tolerance factor	0.44	1.27	0.34	20	
Os01g19290	Nodulin like protein	0.44	1.46	0.30	10	At3g28050 (AtGPTps3)
Os11g04830	Cadmium tolerance factor	0.44	1.27	0.34	20	

IR leaves (Table 3). Collectively, these results suggest that nitrogen nutrition becomes compromised in the sensitive cultivar IR, whereas the tolerant FL is capable of maintaining its N homeostasis, possibly by up-regulating relevant root N transporters.

The root-specific aquaglyceroporin, OsNIP2;1 has been shown to be an important constituent of silicon uptake in rice (Ma *et al.*, 2006). Rice is a typical silicon accumulator and has a large requirement for this element which is believed to play a multitude of roles, including resistance to abiotic and biotic stress (Liang *et al.*, 2003; Zhu *et al.*, 2004). Maintaining the integrity of the casparian strip in root exo- and endodermis is an important role of silicon which restricts apoplastic entry of ions such as Na^+ (Pary and Soni, 1972; Savant *et al.*, 1997). Thus, silicon reduces the bypass flow and limits Na^+ translocation to the shoot, whereas it does not affect K^+ uptake and distribution (Yeo *et al.*, 1999; Gong *et al.*, 2006). Salinity-induced up-regulation of *OsNIP2;1* would improve silicon uptake and help restrict bypass-mediated Na^+ translocation from the root to the shoot.

The relevance of *OsNIP2;1* in salt tolerance was studied further using a loss of function mutant. The *nip2;1* or *lsi1* mutant is considerably more salt-sensitive than the wild type, even in the presence of 3 mM silicon (see Supplementary Fig. S1 at *JXB* online). As expected in a mutant where the bypass pathway is not, or less, reduced by silicon addition, cation tissue analysis in *lsi1* and the wild type shows a considerably higher Na^+ concentration in *lsi1* leaves (see Supplementary Fig. S2A at *JXB* online).

Table 2. Membrane protein transcripts regulated in roots of the salt-sensitive rice cultivar IR29

Plants were grown in hydroponics for 3 weeks and subsequently treated with 50 mM NaCl for 12 d. Membrane protein transcripts that changed by ⩾2-fold are listed with the corresponding change in the same transcript from the salt-tolerant cultivar FL478. Open space denotes the absence of signal. TMD: number of predicted transmembrane domains.

Locus	Annotation/putative function	Degree of regulation			TMD	Ortho/homologues in *Arabidopsis thaliana*
		IR 29	FL 478	FL/IR		
Water transport						
Os03g05290	Aquaporin *TIP1.1*	2.39	2.32	0.97	6	At2g36830 (AtTIP1.1)
Nitrate and ammonium transport						
Os01g50820	Nitrate transporter	2.10	1.87	0.89	10	At1g12940 (AtNRT2.5)
Os12g29950	Nitrate transporter, putative, nodulin family protein	2.58			12	At2g39210 nodulin-type channel
Os11g23890	Low-affinity nitrate transporter	0.46	0.62	1.35	11	At3g16180 proton-dependent oligopeptide or low-affinity nitrate transporter
Amino acid transport						
Os02g09810	Amino acid transporter family protein	2.11	1.21	0.57	10	*At3g30390* Amino acid transporter
Os01g40380	Amino acid transporter	2.13			4	At2g41190 Amino acid transporter
Mono- and divalent cation transport						
Os05g31730	Cation-proton exchanger (*OsCHX11*)	2.49	14.9	5.99	12	At4g23700 (AtCHX17)
Os12g03830	Major facilitator superfamily antiporter	0.44	1.19	2.68	9	At5g13740 (AtZIF1)
Os11g04020	Major facilitator superfamily antiporter	0.39	0.62	1.58	12	At5g13750 (AtZIFL1)
Cation channels						
Os01g48680	Voltage-gated Ca^{2+}-permeable channel (*OsTPC1*)	2.19	0.53	0.24	11	At4g03560 (AtTPC1)
Other transporters						
Os08g44750	Nodulin MtN21 family protein	0.45	0.79	1.76	10	At1g75500

Table 3. Nitrogen content in shoot (S) and root (R) tissues of FL478 and IR29 rice cultivars grown without (control) and with 50 mM NaCl

At 15 DAS, plants were exposed to salt by adding NaCl to the hydroponic medium to a concentration of 50 mM. After 12 d of exposure to salt, percentage N on a dry weight basis in shoot (S) and root (R) of the tolerant FL478 and sensitive IR29 was determined. Results are mean values of three replicates (±SD) comprising three plants per replicate.

Cultivar	Percentage nitrogen content		
	Control	50 mM NaCl	
FL478	S	4.39±0.16	4.18±0.26
	R	2.46±0.25	2.27±0.33
IR29	S	4.30±0.12	3.63±0.35
	R	2.61±0.13	2.16±0.24

However, Ca^{2+} levels were also considerably higher in *lsi1* mutants (see Supplementary Fig. S2B at *JXB* online).

OsTIP1;1 is a tonoplast-expressed aquaporin found in both root and leaf tissue and is up-regulated in both FL and IR (Tables 1, 2). In roots, it is predominantly found in the rhizodermis and exodermis. TIPs are generally contributing to osmotic and turgor homeostasis (Maurel *et al.*, 1993) and several have been recorded to be up-regulated in *Arabidopsis* roots in response to salt stress (Maathuis *et al.*, 2003). No significant regulation of *OsTIP1;1* occurs in shoot tissue (Walia *et al.*, 2005) and no other aquaporins were identified as being regulated more than 2-fold in roots. This may suggest that *OsTIP1;1* contributes to osmotic homeostasis and salt tolerance in rice roots.

Amongst membrane protein transcripts without functional annotation, Os02g37380 is up-regulated in both cultivars by 3.2-fold and 12.3-fold in FL and IR, respectively (see Supplementary data FL Up Down and IR Up Down at *JXB* online). This one transmembrane span protein does not show homology to any annotated genes, is expressed exclusively in roots (e.g. http://mpss.udel.edu/rice/) and the large increase in its transcript number in response to 50 mM NaCl suggests a function in root salt tolerance.

Transporter genes that may contribute to reduced root Na^+ influx in FL

A major proportion of Na^+ that gets into plant roots is likely to be transported through non-selective cation channels (Demidchik and Maathuis, 2007) although more recent work suggests that, at least in halophytes, K^+ channels may also mediate Na^+ uptake (Wang *et al.*, 2007). The molecular identity of channels that mediate Na^+ influx is largely unknown. In *Arabidopsis*, a member of the cyclic nucleotide gated channel (CNGC) family, AtCNGC3, was shown to have a moderate effect on Na^+ uptake (Gobert *et al.*, 2006). Thus, down-regulation of *OsCNGC1* in FL roots (Table 1), which showed more than 2-fold expression compared with IR in control conditions (Table 4), may similarly contribute to restricting Na^+ entry. *OsCNGC1*

Table 4. Differential transcript levels of membrane proteins in roots of the salt-tolerant and salt-sensitive rice cultivars FL478 and IR29 in control conditions

Plants were grown in hydroponics for three weeks plus 12 d in control medium without NaCl. Normalized transcripts of the control treatment were compared between cultivars. Membrane protein transcripts that showed ⩾2-fold differential expression between salt-tolerant FL478 and salt-sensitive IR29 are listed.

Locus	Annotation	FL/IR (control)
Os11g04460	Calcium-transporting ATPase 4, plasma membrane-type (ACA4)	5.02
Os05g39760	VHS domain-containing protein transporter, expressed	4.50
Os02g45380	MATE efflux protein, putative, expressed	3.88
Os09g18159	Protein kinase domain-containing protein, expressed	2.83
Os05g31730	Cation/hydrogen exchanger family protein, expressed (CHX11)	2.72
Os10g42830	Sugar transporter family protein, expressed	2.71
Os01g48680	Voltage-dependent calcium permeable channel protein TPC1	2.63
Os05g30150	Amino acid transporter family protein, putative	2.63
Os01g40410	Transmembrane amino acid transporter protein	2.44
Os06g33600	Cyclic nucleotide-gated ion channel 1 (CNGC1)	2.41
Os05g39540	ZIP zinc/iron transport family protein, expressed	2.39
Os08g05710	ABC transporter family protein, expressed	2.38
Os07g15370	Metal transporter Nramp1, putative, expressed	2.27
Os12g03640	Protein kinase domain-containing protein, expressed	2.21
Os06g36090	ABC transporter family protein, putative, expressed	2.14
Os06g49240	Peptide transporter protein	2.09
Os09g24924	Sugar carrier protein A, putative, expressed	2.08
Os08g44100	Transmembrane 9 superfamily protein member 2 precursor, putative, expressed	2.07
Os05g41480	Phosphate translocator, putative, expressed	2.03
Os03g45370	K-exchanger, putative, expressed	2.00
Os02g50960	Auxin efflux carrier component 4, putative, expressed	0.49
Os03g56160	Lectin receptor kinase 7, putative, expressed	0.49
Os08g03240	Protein kinase domain-containing protein	0.48
Os09g19680	ABC-type transport protein, putative, expressed	0.33
Os08g07760	Brassinosteroid insensitive 1-associated receptor kinase 1 precursor, putative, expressed	0.33

transcript level in shoots is not significantly affected by salinity in either cultivar (Walia *et al.*, 2005).

Although all HAK high affinity K^+ transporters are competitively inhibited by Na^+, some may also transport this ion, as was shown for barley HvHAK1 (Santa-Maria *et al.*, 1997). If similar properties pertain to OsHAK7, its down-regulation in FL roots may prevent Na^+ leak into the root symplast and, consequently, the overall Na^+ load.

Transporter genes that may contribute to reduced Na^+ translocation to FL shoots

As mentioned above, Ca^{2+} translocation to the shoot is often compromised during salinity, due to a combination of reduced transpiration and the symplastic immobility of Ca^{2+}. Ca^{2+} nutrition in the root may also impact on salinity via limiting the bypass flow (Anil *et al.*, 2005). OsACA4 is a P-type 2B Ca^{2+} ATPase located at the plasma membrane and involved in Ca^{2+} extrusion into the apoplast (Geisler *et al.*, 2000). In control conditions (Table 4), expression of OsACA4 was around 5-fold higher in FL compared to IR which may point to a greater release of Ca^{2+} into the FL root apoplast. This mechanism may be further augmented during salinity stress by the reduced vacuolar Ca^{2+} deposition in FL roots resulting from the down-regulation of the CAX-type antiporter Os02g04630 whose transcript level was not affected in IR by salinity. Augmented levels of apoplastic Ca^{2+} have been proposed to limit the bypass flow by reducing the 'leakiness' of endodermal junctions (Anil *et al.*, 2005) and thus would restrict Na^+ translocation to the shoot.

In *Arabidopsis* roots, CAX transcript levels also responded to salinity stress (Maathuis, 2006) and several CAX isoforms were shown to be important in salt-related Ca^{2+} translocation and signalling (Zhao *et al.*, 2008). The Ca^{2+} permeable channel OsTPC1 has also been shown to impact on rice Ca^{2+} nutrition and sensitivity to environmental stress where overexpression resulted in reduced growth in plants (Kurusu *et al.*, 2004). Its down-regulation in FL and up-regulation in IR as shown in both microarray and RT- PCR data (Table 1; Fig. 4) may point to differential Ca^{2+} homeostasis that impacts on salt tolerance.

Transporter genes that may contribute to a reduced Na^+:K^+ ratio in FL

K^+ nutrition and homeostasis can be negatively affected during salt stress and salinity-induced transcriptional regulation of K^+ transporters has been observed before in rice (Bañuelos *et al.*, 2002; Walia *et al.*, 2005) and *Arabidopsis* (Maathuis *et al.*, 2003). On the basis of microarray data, the Os05g31730 transcript level is considerably higher after salinity treatment, in both FL and IR (Table 1).

Os05g31730 encodes the putative monovalent cation exchanger CHX11. The closest homologue to OsCHX11 in *Arabidopsis* is AtCHX17. *AtCHX17* is primarily expressed in the epidermal and cortical root tissues (Cellier *et al.*, 2004). In *Arabidopsis*, *CHX17* expression also rapidly increased in response to salinity. In addition, it increased after ABA treatment or K^+ deprivation. A loss of function mutant accumulated less K^+ in roots in response to salt stress or K^+ shortage (Cellier *et al.*, 2004). These findings indicate that *AtCHX17* helps maintain K^+ homeostasis by providing extra K^+ acquisition capacity, for example, to compensate for the loss of K^+ uptake through HAK/KUP-type systems which are sensitive to Na^+ inhibition (Santa-Maria *et al.*, 1997). It is tempting to envisage a similar role for *OsCHX11* in rice and it would be interesting to see if ABA and K^+ deficiency induce *OsCHX11* transcription. Its far greater up-regulation in FL could explain this cultivar's ability to maintain a significantly higher K^+ concentration in its tissues compared with IR.

In FL roots, *OsHAK7* showed significantly decreased transcription. The primary high affinity K^+ uptake mechanism in rice root is believed to be *OsHAK1* (Grabov, 2008), but no functional data are available for *OsHAK7*. Its closest *Arabidopsis* homologue is *AtKUP4* which has been shown to be involved in root hair growth (Rigas *et al.*, 2001) and is probably located in the vacuole (Whiteman *et al.*, 2008). If *OsHAK7* is similarly expressed at the tonoplast, as was also shown for other members of the HAK family (Bañuelos *et al.*, 2002), its down-regulation by approximately 2.4-fold in FL might signify retention of vacuole sequestered root K^+.

Concluding remarks

Improvement of rice salt tolerance is urgently required, but necessitates a detailed understanding of the processes, genes, and proteins involved. Comparing physiological and transcriptional parameters in cultivars with divergent levels of sensitivity could provide an excellent basis to increase our knowledge in this respect. For rice cultivars FL478 and IR29, such a study was carried out for root tissue and membrane transporters were identified that may contribute to the difference in tolerance between FL and IR such as OsCHX11 and OsCNGC1. Our data show that transcripts of specific membrane proteins in roots, for example, *OsCHX11*, *OsTIP1;1*, *Lsi1,* and Os02g37380, may form important targets for tolerance that were not identified in shoot tissue. Vice versa, putative target membrane proteins previously detected in shoot tissue (Walia *et al.*, 2005, 2007) show little overlap with those found in roots, emphasizing the existence of different mechanisms in shoots and roots and the necessity to carry out analyses in multiple tissues.

Supplementary data

Supplementary data are available at *JXB* online and comprise the following figures and data files.

Supplementary Fig. S1. Effect of increasing salinity on relative growth rates for the rice mutant *lsi1* and wild-type cultivar *Nipponbare*.

Supplementary Fig. S2. Na^+ and Ca^{2+} ion concentrations in different tissues of rice mutant *lsi1* and wild-type cultivar *Nipponbare* after exposure to salinity stress in the presence of Si.

Supplementary data. Gene expression data of the transcriptome assay of the roots of FL478 and IR29 in response to salinity stress and sequences of the primers used in validating microarray data analysis. Files contain: a 'Legend' describing various data sets; 'Raw data' consisting of signal and background fluorescence intensities of each wavelength for treatment and control for FL and IR; 'Normalized data' for each element; Probes significantly up or down in 'IR'; Probes significantly up- or down-regulated in 'FL'; Differentially regulated probes for 'FL' and 'IR'; Differentially expressed probes for FL and IR in 'control' conditions; 'Primers' used for control RT-PCR analyses.

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