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Comparative proteomic and physiological characterisation of two closely related rice genotypes with contrasting responses to salt stress

Seyed Abdollah Hosseini[A,*], *Javad Gharechahi*[B,*], *Manzar Heidari*[A], *Parisa Koobaz*[A], *Shapour Abdollahi*[A], *Mehdi Mirzaei*[C], *Babak Nakhoda*[A,E] *and Ghasem Hosseini Salekdeh*[D,E]

[A]Department of Molecular Physiology, Agricultural Biotechnology Research Institute of Iran, PO Box 31535-1897, Karaj 3135933151, Iran.
[B]Chemical Injuries Research Center, Baqiyatallah University of Medical Sciences, PO Box 19395-5478, Tehran 1435916471, Iran.
[C]Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW 2109, Australia.
[D]Department of Systems Biology, Agricultural Biotechnology Research Institute of Iran, PO Box 31535-1897, Karaj 3135933151, Iran.
[E]Corresponding authors. Emails: h_salekdeh@abrii.ac.ir; b.nakhoda@abrii.ac.ir

Abstract. Salinity is a limiting factor affecting crop growth. We evaluated the responses of a salt-tolerant recombinant inbred rice (*Oryza sativa* L.) line, FL478, and the salt-sensitive IR29. Seedlings were exposed to salt stress and the growth rate was monitored to decipher the effect of long-term stress. At Day 16, IR29 produced lower shoot biomass than FL478. Significant differences for Na^+ and K^+ concentrations and Na^+ : K^+ ratios in roots and shoots were observed between genotypes. Changes in the proteomes of control and salt-stressed plants were analysed, identifying 59 and 39 salt-responsive proteins in roots and leaves, respectively. Proteomic analysis showed greater downregulation of proteins in IR29. In IR29, proteins related to pathways involved in salt tolerance (e.g. oxidative stress response, amino acid biosynthesis, polyamine biosynthesis, the actin cytoskeleton and ion compartmentalisation) changed to combat salinity. We found significant downregulation of proteins related to photosynthetic electron transport in IR29, indicating that photosynthesis was influenced, probably increasing the risk of reactive oxygen species formation. The sensitivity of IR29 might be related to its inability to exclude salt from its transpiration stream, to compartmentalise excess ions and to maintain a healthy photosynthetic apparatus during salt stress, or might be because of the leakiness of its roots, allowing excess salt to enter apoplastically. In FL478, superoxide dismutase, ferredoxin thioredoxin reductase, fibre protein and inorganic pyrophosphatase, which may participate in salt tolerance, increased in abundance. Our analyses provide novel insights into the mechanisms behind salt tolerance and sensitivity in genotypes with close genetic backgrounds.

Additional keywords: 2D gel electrophoresis, mass spectrometry, *Oryza sativa*, salinity, sensitivity, tolerance.

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Introduction

Salinity is a major environmental limiting factor that affects growth and productivity as well as the geographical distribution of many plant species. High salinity, predominantly in the form of NaCl, affects plant growth in three ways: osmotic effects, ion toxicity and nutrient imbalance. A high salt concentration in the rhizosphere impairs water uptake (osmotic effects) and nutrient absorption (nutrient imbalance) in plant roots. However, excess accumulation of salt within the plant is highly toxic and results in oxidative stresses and enzyme inhibition (ion toxicity) (Munns and Tester 2008). Plants have evolved sophisticated molecular and biochemical mechanisms to minimise the adverse effects of salt stress, especially by actively excluding Na^+ and Cl^- during water absorption and by limiting the transport of salt within the plant and its excess accumulation in the cytoplasm. However, halophytes are able to maintain this exclusion more efficiently at even higher salt concentrations than glycophytes (Munns and Tester 2008). In addition, both glycophytes and halophytes have gained the ability to sequester the excess salt into the vacuoles or to compartmentalise harmful ions in different tissues (Türkan and Demiral 2009).

The response of plants to salinity is usually assessed by measuring the amount of biomass produced under saline and

[*]These authors contributed equally to this work.

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control conditions during long-term salt stress treatment (Munns 2002). Shortly after exposure to salt stress, plants experience a growth reduction, which is largely caused by the salt outside the roots and is known as an osmotic effect (Munns 2002; Munns and Tester 2008). This response is identical to water stress caused by drought. Within days or weeks of salt exposure, excess amounts of salt enter the plant, leading to salt toxicity and premature senescence, especially in older transpiring leaves (a salt-specific or ionic effect) (Munns 2002; Roy *et al*. 2014). This will largely affect the synthesis of assimilates and further limits biomass production. This is the second phase of growth reduction and is the phase that clearly separates genotypes with differing susceptibility to salt stress.

Rice (*Oryza sativa* L.) is a salt sensitive crop especially at the early seedling and reproductive stages of growth (Flowers and Yeo 1981). It is the primary food source consumed by almost half of the world's population (Dowling *et al*. 1998). It has been estimated that a 60% increase in rice production would be required to supply the food demand of Asia's increasing population by the year 2020 (Dowling *et al*. 1998). Considerable effort is underway to increase rice productivity to meet this goal.

Understanding the molecular mechanisms of salt tolerance will pave the way for molecular breeding of plants for salinity tolerance. At the transcriptome level, microarray data have provided a wealth of information regarding the genes and pathways that are modulated during salt stress treatment in rice. In previous studies, changes in the root and leaf gene expression of two *indica* rice genotypes, IR29 and FL478, were monitored during salt tress treatment using Affymetrix microarrays (Walia *et al*. 2005; Cotsaftis *et al*. 2011). Analysis of leaf transcripts showed an induction of the genes involved in the biosynthesis of flavonoids in the salt-sensitive genotype IR29 and cell wall biosynthesis in both genotypes, suggesting cell wall reconstruction as a general adaptive mechanism for salt stress tolerance. Analysis of root transcripts discovered a greater number of salt-responsive genes in FL478 than in IR29, suggesting the induction of a broad spectrum of saline-responsive adaptive pathways in roots during salt stress treatment.

Proteomic analysis have proven to be a powerful tool for discovering candidate genes and pathways that are crucial for stress responsiveness and tolerance (Salekdeh and Komatsu 2007). In recent years, this approach has increased in sensitivity and power as a result of improvements in 2D gel electrophoresis (2DE), protein detection and quantification technologies, protein identification using MS, genomics and bioinformatics. Knowledge from comparative analysis of the responses of plants to stresses at the proteome level in combination with physiological measurements will play a significant role in the development of new strategies in plant breeding (Salekdeh *et al*. 2009).

Proteomics has been applied to discover salt-responsive proteins in rice roots (Yan *et al*. 2005; Cheng *et al*. 2009; Liu *et al*. 2012; Nam *et al*. 2012), leaves (Abbasi and Komatsu 2004; Kim *et al*. 2005; Parker *et al*. 2006; Nohzadeh Malakshah *et al*. 2007; Song *et al*. 2011; Ghaffari *et al*. 2014), young panicles (Dooki *et al*. 2006) and anthers (Sarhadi *et al*. 2012). These studies resulted in the identification of several salt-responsive proteins that may play vital roles in plant adaptation to salt stress. However, proteomics can be more powerful if it is applied to contrasting genotypes. Hence, in this study, a 2DE-based proteomics approach, coupled with tandem MS (MS–MS) for protein identification, was applied to discover the changes in the proteome profiles of the roots and leaves of two contrasting rice genotypes (salt-sensitive IR29 and salt-tolerant FL478) under long-term salt treatment, when the two tested genotypes showed differential growth response to salt stress. Owing to a comparative analysis of tolerant and sensitive plants, several proteins belonging to different molecular pathways emerged as key participants in plant adaptation to salt stress.

Materials and methods

Plant culture and salt stress treatment

To distinguish the key mechanisms contributing to salinity sensitivity or tolerance in rice (*Oryza sativa* L.), two contrasting rice genotypes were phenotypically analysed, including IR29, a modern variety developed at the International Rice Research Institute that is very sensitive to salt stress, and FL478, a highly tolerant recombinant inbred line developed from the cross of IR29 and Pokkali. Comparative phenotypic analyses, including morphological, physiological, and proteomic analyses, were carried out under controlled greenhouse conditions at the Agricultural Biotechnology Research Institute of Iran in Karaj, Iran.

This experiment was conducted in hydroponics under controlled conditions using an Agricultural Biotechnology Research Institute of Iran phytotron glasshouse set at 29°C : 21°C day : night temperatures and 70–75% relative humidity as described previously (Ghaffari *et al*. 2014). Plant seedlings were exposed to salt stress 13 days after sowing by adding NaCl to the culture medium. The electrical conductivity of the nutrient solution was gradually increased in three steps as shown in Fig. 1 and finally maintained at 12 dS m^{-1} (~120 mM NaCl at 25°C). The nutrient solution used for control plants had an electrical conductivity of 0.94–1.1 dS m^{-1}.

Measuring biomass production under saline and control conditions

To show when the sensitive and tolerant genotypes exhibited differential growth responses to salt stress, the total shoot biomass production under saline and control conditions was measured. Four plants per replicate were harvested daily for a period of 16 days. Roots were excised at the base of the stem and shoot FWs were recorded. Shoot were the oven-dried at 70−75°C for 72 h and the DWs were measured.

Morphological analyses

To quantify and compare the responses of the contrasting genotypes under control and saline conditions, morphological parameters such as plant height, root length, root and shoot DWs, and green leaf area were measured after final scoring. After harvesting the samples, roots were blotted dry, and root and shoot FWs were recorded using a portable tabletop digital balance (Sartorius AG, Göttingen, Germany). All entries were monitored and scored based on the visual symptoms as described by Gregorio (1997) using the modified standard evaluation

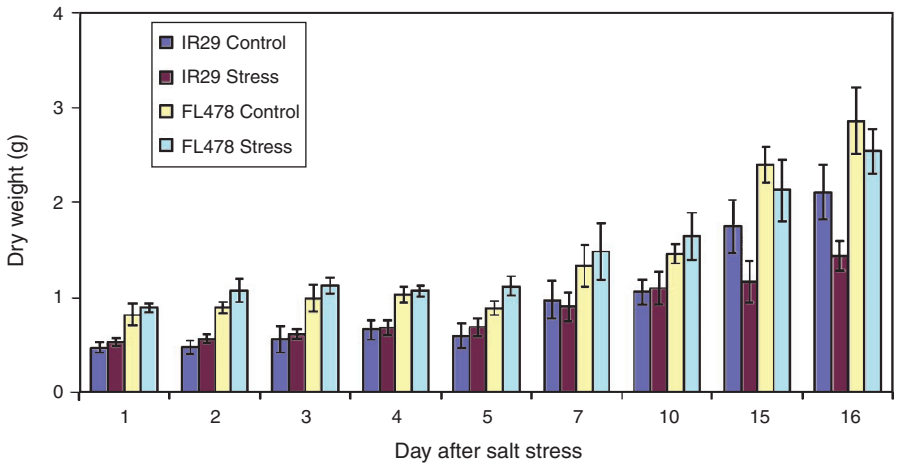

Fig. 1. Effect of 120 mM NaCl on shoot biomass production (shoot DW) in the sensitive (IR29) and tolerant (FL478) rice genotypes. Total shoot DW was measured using four replications with four plants per replication. Error bars represent s.d.

system for rice (SES) at 10 and 16 days after salinisation, as the initial and second scores, respectively.

Leaf area, plant height, root length and dry weight measurement

All the leaves of the five seedlings were excised, with senescing portions removed. The green leaf areas were measured using a leaf area meter (LI-COR, Lincoln, NE, USA). The average leaf area per plant was calculated based on cm^2 per plant. The plant height (in cm) was measured on the same samples from the base of the stem to the tip of the topmost or youngest fully expanded leaf of the plants using a measuring stick. Root lengths were measured similarly. Root and shoot samples were oven-dried at 70−75°C for 72 h and their DWs were determined.

Determination of Na, K and total ion content in plant tissues

To determine the total ion content of plants, Na and K concentrations and $Na^+ : K^+$ ratio in roots and shoots were measured. For each genotype in each replication, five plants were uprooted and washed thoroughly to remove salts from the surface of the tissues and then the total Na^+, K^+ and $Na^+ : K^+$ were determined for each tissue as described previously (Ghaffari *et al*. 2014).

Sampling for proteomic analysis

Leaf and root samples of the two genotypes were collected for protein extraction 16 days after reaching the final electrical conductivity of 12 dS m^{-1}, when IR29 showed differential growth response to salt stress compared with FL478. A total of five plants were harvested per genotype to constitute a single biological replicate. Seedlings were separated into roots and shoots. Root and leaf samples were pooled separately, wrapped in aluminium foil, snap-frozen in liquid N and stored at −80°C until analysis.

Protein extraction

Root and leaf proteins from three independent biological replicates were extracted using the phenol extraction (Hurkman and Tanaka 1986) and trichloroacetic acid and acetone precipitation methods (Damerval *et al*. 1986), respectively. Briefly, 1 g of root or leaf sample was pulverised to a fine powder with liquid N in a mortar and pestle. Root powder was suspended directly in 2.5 mL of Tris-buffered phenol (pH 8.8) and an equal volume of extraction buffer containing 0.1 M Tris–HCl (pH 8.8), 10 mM EDTA, 0.4% 2-mercaptoethanol and 0.9 M sucrose. The homogenate was mixed for 30 min at 4°C and centrifuged afterwards for 15 min at 5000*g* and 4°C. The phenol phase was recovered and proteins were precipitated with 5 volumes of ice-cold 0.1 M ammonium acetate in 100% methanol at −20°C for 16 h. Subsequently, the homogenate was centrifuged for 10 min as described above and the protein pellet was thoroughly washed twice in 20 mL of 0.1 M ammonium acetate in 100% methanol, followed by two washes in ice-cold 80% acetone and 10 mM DTT and then pellets were dried at room temperature.

Leaf powder was suspended in 10% (w/v) trichloroacetic acid in acetone with 0.07% (w/v) DTT at −20°C for 1 h, followed by centrifugation for 15 min at 35 000*g*. The resulting pellets were washed with ice-cold acetone containing 0.07% DTT, incubated at −20°C for 1 h and centrifuged again at 4°C. This step was repeated three times and then pellets were dried at room temperature. After a brief air drying, the leaf or root powder was then solubilised in lysis buffer (9 M urea, 2% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio] propanesulfonate (CHAPS), 0.8% (w/v) Pharmalyte (pH 3–10; (GE Healthcare, Waukesha, WI, USA)) and 1% (w/v) DTT). The protein concentrations were measured by the Bradford assay (Bio-Rad, Hercules, CA, USA) with BSA as a standard.

2D gel electrophoresis and image analysis

2DE was performed as described previously (Gharechahi *et al*. 2013). Silver-stained gels were scanned using a GS-800 densitometer (Bio-Rad) at a resolution of 600 dots per square

inch over multiple wavelengths (400–750 nm). Spot detection, protein quantification and spot pairing were carried out according to the Melanie ver. 6 software user manual (GeneBio, Geneva, Switzerland). Gel image analysis was carried out as previously described. Spots were numbered and coded as originating from the leaves (L) or roots (R).

Three replicate gels were run per tissue and percent volume (%vol) of each spot, as a normalised quantitative measure of that spot, was estimated and subjected to statistical analysis. Considering the two genotypes (IR29 and FL478) and two treatments (control and salt stress), four genotype × treatment combinations were analysed by one-way ANOVA for each tissue (root and leaf) separately ($P \leq 0.01$). All statistical analyses were performed by SAS software ver. 9 (SAS Institute, Cary, NC, USA).

Protein identification and database search

Proteins that showed a statistically significant change in abundance ($P \leq 0.01$) under salt stress conditions were recovered from the coomassie brilliant blue (CBB)-stained gels and analysed using a Bruker Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltronic GmbH, Germany) as described previously (Gharechahi *et al*. 2013). Brukerflex Analysis software was used for the spectral processing and generation of the peak lists for the MS and MS–MS spectra. Combined MS and MS–MS spectral data were subjected to database searching using a copy of MASCOT ver. 2.1 (Matrix Science, London, UK) that was run locally through the BioTools interface, ver. 3.1 (Bruker). Search criteria included: enzyme, trypsin, variable modifications, oxidation (M), peptide tolerance, 100 ppm (parts per million), carbamidomethyl (C) as a fixed modification, MS–MS tolerance, 0.8 Da, instrument and MALDI-TOF/TOF. The database search was run against the National Center for Biotechnology Information nonredundant protein database NCBInr 20090222 (7 894 593 sequences; 2 721 452 874 residues; www.ncbi.nlm.nih.gov/protein/).

Total mRNA extraction and quantitative real time-PCR analysis

Total mRNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two micrograms of RNA were treated with a DNaseI RNase Free Kit (Fermentas, St. Leon-Rot, Germany) to remove any potential genomic DNA contamination. Complementary DNA was synthesised in a reverse transcription reaction with iScript cDNA Synthesis kit (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Quantitative PCR reactions were performed in triplicate using the iCycler iQ, the Multicolor Real-Time PCR Detection System (BioRad) and the iQ SYBR Green Supermix kit (BioRad). Reaction conditions (25-µL volumes) were optimised by changing the annealing temperature to minimise primer–dimer formation and to increase PCR efficiency. The following PCR profile was used: 4 min at 95°C; 1 min at 95°C, 30 s at 60°C and 30 s at 72°C for 30 cycles; and 5 min at 72°C, followed by recording of a melting curve. The presence of primer–dimer or nonspecific product accumulation was checked by melt curve analysis. Each run included standard dilutions and negative reaction controls. The relative expression levels of 12 mRNA transcripts were normalised against the ribosomal 18S rRNA as a housekeeping gene using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Table S1 (available as Supplementary Material to this paper) presents the sequences of primers used for quantitative real-time PCR (qPCR) analysis of selected genes.

Results

Physiological and morphological responses of genotypes to salt stress

Growth response to salt stress

The sensitive and tolerant genotypes (IR29 and FL478, respectively) exhibited the same growth rate for the first 10 days under 120 mM NaCl treatment, even though they had different growth rates under control or saline conditions (Fig. 1). At Day 16, the salt-sensitive genotype, IR29, experienced a significant reduction in total shoot biomass production. At this point, the differences between genotypes in DW production were also significant and showed distinct growth responses to salt stress. This differential growth response was also visible in seedlings in culture trays under control and salt stress conditions (Fig. 2).

Visual effects based on rice SES

To evaluate the visual effects of salt stress on seedlings of the salt-tolerant and sensitive genotypes, we used a modified rice SES scoring system (Table 1). Under control conditions, plant seedlings had normal growth and showed no symptoms of salt injury (average SES score = 1), whereas plants under salt stress were significantly affected and clearly exhibited symptoms of salt injury such as leaf burning, chlorosis and stunted growth (average SES score = 5.5; Fig. 2). IR29 showed the highest salt injury and the highest SES score of 7.0, whereas FL478 was less affected by salt stress and had the lowest average SES score of 4.0. Moreover, the interaction between salinity and genotype in SES scores was also statistically significant.

Effect of salinity on root length, plant height and leaf area

Under salt stress conditions, IR29 had the shortest roots (15 cm), whereas FL478 had the longest roots (23.2 cm). Root length in FL478 was not affected by salt stress; however, the sensitive genotype, IR29, experienced a 40% reduction in root length when exposed to salt (Table 1). In addition, the interactions between genotypes and treatments for root length were highly significant. Plants were significantly shorter under salt stress with an overall reduction in height of ~35% (Table 1). Significant differences were observed between genotypes under salt stress, where FL478 was the tallest (48 cm) and IR29 was the shortest (27 cm). However, the interaction between genotypes and treatment was not significant. Leaf area per plant was dramatically affected by salt stress, with IR29 having the lowest leaf area of only ~3 cm^2 per plant; FL478 had the highest leaf area of ~33 cm^2 (Table 1). The interaction between salinity and genotype was highly significant, with IR29 showing an 89% reduction in leaf area compared to only 49% for FL478.

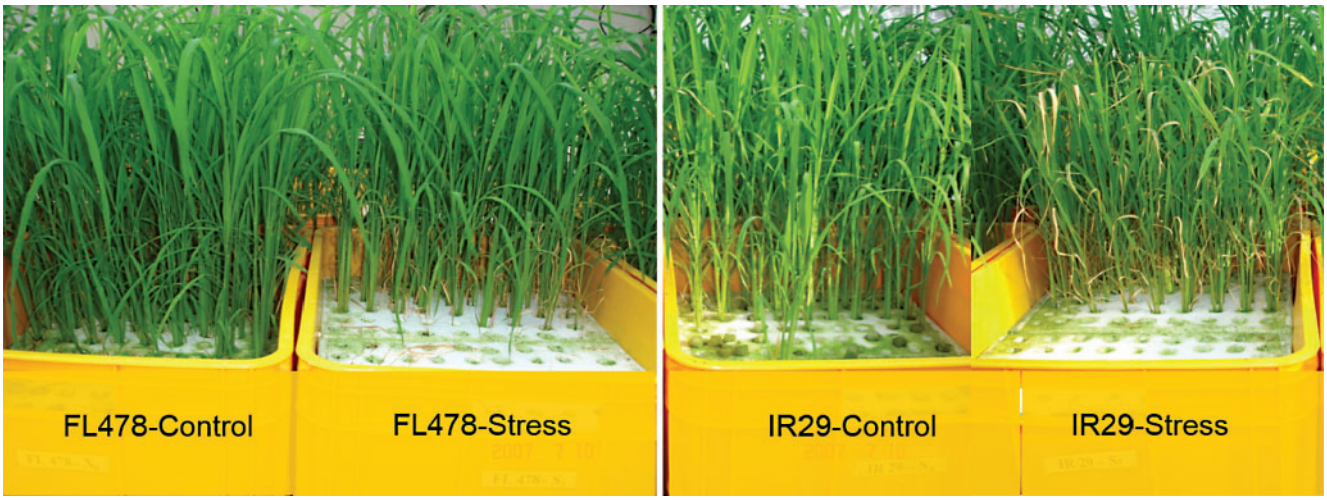

Fig. 2. The stress response of two rice genotypes (IR29 and FL478) with different susceptibility to salt stress. Thirteen-day-old seedlings were exposed to salt stress (120 mM) for 16 days. The salt-sensitive genotype IR29 experienced significant salt injury after 16 days of salt exposure and exhibited a significant reduction in growth compared with salt-tolerant FL478.

Table 1. Effects of salinity on growth and morphological parameters of contrasting rice genotypes under greenhouse conditions

IR29 is the salt-sensitive genotype; FL478 is the salt-tolerant genotype. Data are the means of four replications with five subsamples per replication. Growth parameters are presented on a per-plant basis. *, significant at $P=0.05$; **, significant at $P=0.01$; ***, significant at $P=0.001$; ns, nonsignificant; SES, standard evaluation system; S, salinity treatment; G, genotype

Genotype and treatment	SES scores	Root length (cm)	Plant height (cm)	Leaf area (cm^2)
Saline				
IR29	7.00	15.0	27.0	3.2
FL478	4.00	23.2	48.3	33.6
Mean	5.5	19.1	37.65	18.4
Control				
IR29	1.00	24.8	41.1	16.9
FL478	1.00	22.9	59.2	66.9
Mean	1.00	23.85	50.15	41.9
Significance				
S	***	***	***	***
G	***	***	***	***
S × G	***	***	ns	**

Effect of salinity on growth and root and shoot biomass

In salt-treated IR29, root FW was reduced by ~37%, whereas root DW was reduced even to a greater extent, by ~56% (Table 2). Differences between genotypes in root biomass production were significant, with FL478 showing the highest root FW and DW under both saline and control conditions. The interaction between salinity and genotype was not significant for root FW but was highly significant for both root and shoot DW. At the time of sampling, IR29 showed dramatic reductions in shoot biomass production under salt stress, amounting to ~49% and 50% for FW and DW, respectively. However, shoot biomass production in FL478 was less affected by salt stress (Fig. 1 and Table 2).

Total ion concentration and ion content

To study ion concentration and distribution under salt stress conditions, Na^+ and K^+ concentrations and Na^+ : K^+ ratio were measured in roots and shoots of salt treated and control plants. Root Na^+ concentration was significantly increased (more than fourfold) in IR29 as well as salt-tolerant FL478 (3.7-fold) under stress conditions (Table 3). Root K^+ concentration did not change significantly in both genotypes during the salt stress treatment (Table 3). The large change in root Na^+ concentration under saline conditions resulted in a high Na^+ : K+ ratio in the root. Na^+ concentration in shoots of IR29 behaved similarly and increased significantly up to ninefold (Table 3). The extent of Na^+ accumulation in shoots of IR29 was double that of its roots. Similarly, in FL478, a significant increase (up to fivefold) in shoot Na^+ concentration was also detected. On average, shoot Na^+ concentrations in the sensitive genotype, IR29, was about three times higher than those in the FL478 under salinity. In contrast to roots, shoot K^+ concentration changed significantly in IR29 under salt stress conditions. Salt stress had significant effect on Na^+ : K^+ ratios in the shoots of both genotypes, which was mainly due to higher concentrations of Na^+ in the shoots under saline conditions.

To determine the extent to which roots of the contrasting genotypes exclude salt, the total ion content in roots and shoots was calculated based on the concentration of ions and the corresponding DW. IR29 showed higher concentrations of Na^+ in shoots but a much lower level of total Na content in comparison with FL478 (Table 3). This was mainly due to the dramatic reduction in biomass of this genotype under salt stress and the higher biomass of the tolerant genotype under the same conditions.

Comparative proteomic analysis to identify root and leaf salt-responsive proteins

We further applied 2DE-based proteomic analysis to compare root and leaf proteins of the control and salt-stressed plants

Table 2. The effects of salinity on root and shoot FW and DW (in mg per plant) in two rice genotypes, salt-sentisive IR29 and salt-tolerant FL478
Data are the means of four replications with five subsamples per replication. Growth parameters are presented on a per-plant basis. R : S, root to shoot ratio; *, significant at $P = 0.05$; **, significant at $P = 0.01$; ***, significant at $P = 0.001$; ns, nonsignificant; S, salinity treatment; G, genotype

Genotype and treatment	Root FW	Shoot FW	Total FW	Root DW	Shoot DW	Total DW	R ; S
Saline							
IR29	221.1	494.0	715.2	16.5	105.1	121.6	0.16
FL478	1206.0	2429.1	3635.1	91.6	495.2	586.8	0.18
Mean	713.55	1461.55	2175.15	54.05	300.15	354.2	0.17
Control							
IR29	355.0	983.3	1305.5	37.9	211.0	248.9	0.18
FL478	1474.3	3947.4	5351.5	191.9	871.3	1063.3	0.22
Mean	914.65	2465.35	3328.5	114.9	541.15	656.1	0.2
Significance							
S	**	***	***	***	***	***	**
G	***	***	***	***	***	***	**
S × G	ns	***	**	***	***	***	ns

Table 3. Total ion concentration and content of the two contrasting rice genotypes (salt-sensitive IR29 and salt-tolernant FL478) under greenhouse conditions
Data are the means of four replications with five subsamples per replication. Growth parameters are presented on a per-plant basis. *, significant at $P = 0.05$; **, significant at $P = 0.01$; ***, significant at $P = 0.001$; ns, nonsignificant; S, salinity treatment; G, genotype

Genotype and treatment	Ion concentration (mmol g^{-1} DW)						Total ion content (mg per plant)			
	Root Na^+	Root K+	Root Na^+ : K+	Shoot Na^+	Shoot K+	Shoot Na^+ : K+	Root Na^+	Root K+	Shoot Na^+	Shoot K+
Saline										
IR29	1.14	0.30	3.82	0.99	0.57	1.75	0.43	0.19	2.38	2.36
FL478	1.10	0.33	3.36	0.34	0.67	0.50	2.32	1.18	3.85	12.99
Mean	1.12	0.315	3.59	0.665	0.62	1.125	1.375	0.685	3.115	7.675
Control										
IR29	0.28	0.31	0.90	0.11	0.72	0.15	0.24	0.46	0.52	5.93
FL478	0.29	0.29	1.03	0.06	0.65	0.09	1.30	2.18	1.17	22.08
Mean	0.285	0.3	0.965	0.085	0.685	0.12	0.77	1.32	0.845	14.005
Significance										
S	***	ns	***	***	*	***	***	***	***	***
G	ns	ns	ns	***	ns	***	***	***	***	***
S × G	ns	ns	*	***	**	***	***	***	*	***

after 16 days of salt exposure, when the two contrasting genotypes showed differential growth responses to salt stress and accumulated excess salt in their shoots and roots. We aimed to explore salt-specific responses and to identify salt toxicity-related proteins in genotypes with differing abilities to tolerate salt stress. Proteomic analysis generated a list of candidate salt-responsive protein (SRP) spots (58 in leaves and 120 in roots) that showed significant changes in abundance (up to 1.5-fold, $P \leq 0.01$) in at least one of the genotypes in response to salt stress. Among the candidate leaf SRP spots, 24 increased in abundance and 15 decreased in IR29; in FL478, 14 spots increased in abundance and five decreased. Among the candidate SRP spots in roots, 51 spots increased in abundance and 40 spots decreased in IR29. However, in FL478, 14 spots increased in abundance and 15 spots decreased in response to salt stress. The details of the number of reproducibly detected spots in each treatment group, the number of spots with statistically significant differences and those that were successfully identified after MS analysis are presented in Table S2.

Identification of the candidate salinity-related proteins

The candidate SRP spots that could be excised from preparative CBB-stained gels were subjected to MALDI-TOF/TOF MS analysis (58 leaf spots and 115 root spots). Protein identity was determined based on a combined peptide mass fingerprinting and MS–MS analysis that led to the reliable identification of 39 leaf and 59 root proteins (Table 4 and Tables S3 and S4). Fig. 3 shows the gel positions of the identified leaf and root SRPs on 2DE gel images of IR29 under salt stress conditions. In addition, changes in the spot

Table 4. The candidate leaf (L) and root (R) salt-responsive proteins (SRPs) that showed a decreased or increased abundance in the sensitive (IR29) and tolerant (FL478) genotypes in response to salt stress

Only those proteins that showed statistically significant differences ($P \leq 0.01$) and displayed at least a 1.5-fold change in abundance were accepted as candidate SRPs. The proteins were divided into functional categories based on biological function retrieved from the Uni-prot database (http://www.uniprot.org, accessed 11 February 2015)

Protein identity	Spot ID	
	Decreased	Increased
Antioxidant, oxidoreductase activity and stress response		
Superoxide dismutase (Cu–Zn)	–	8L, 27R (FL478)
GST	–	187R, 287R, 802R (IR29)
Thioredoxin M5, chloroplastic	104L (IR29)	–
Thioredoxin H1	3R (IR29)	4R, 90L (IR29)
Lactoylglutathione lyase	–	452L (IR29, FL478)
Dehydroascorbate reductase	–	117R (IR29)
Peroxiredoxin-2C	670L (IR29)	–
Peroxidase	671R (IR29); 574R (IR29, FL478)	818R (IR29)
Germin-like protein 8	–	801R (IR29)
Putative uncharacterised protein	159R (IR29)	159R (FL478)
CBS domain-containing protein	–	47R (IR29)
Ferritin	–	810L (IR29, FL478); 284L (IR29)
Salt stress root protein RS1	–	621L (IR29)
Fibre protein Fb19	–	73L (FL478)
Photosynthesis		
Rubisco large chain	–	877L (IR29, FL478)
Rubisco small chain	–	260L (IR29, FL478)
Rubisco activase	193L (IR29, FL478)	–
Ferredoxin-thioredoxin reductase	72L (IR29)	72L (FL478)
Oxygen-evolving enhancer protein 3	–	11L (FL478)
Chloroplast 23-kDa polypeptide of PSII	63L (IR29)	189R (IR29)
Putative 33-kDa oxygen evolving protein of PSII	–	426R (IR29, FL478)
ATP synthase ε chain, chloroplastic	629L (IR29)	–
ATP synthase β chain, chloroplast	–	761L (IR29)
Plastocyanin, chloroplastic	499L (IR29)	–
Hypothetical protein	671L (IR29)	–
Carbohydrate and energy metabolism		
Putative tyrosine phosphatase	–	456L (IR29)
Phosphoglycerate kinase	–	486L (IR29)
Fructose-bisphosphate aldolase, chloroplastic	527L (IR29)	–
ADP glucose pyrophosphatase	722L (IR29, FL478)	720L (FL478)
Pyruvate dehydrogenase E1 component subunit β-1, mitochondrial	–	356R (IR29)
Fructokinase 2	363R (IR29)	–
Nucleoside diphosphate kinase 1	384R (IR29)	384R (FL478)
NAD-dependent isocitrate dehydrogenase *c*	600R (IR29, FL478)	–
Putative NADPH-dependent mannose 6-phosphate reductase	–	652R (IR29)
Enolase	699R (IR29, FL478)	–
Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	865R (IR29)	–
Inorganic pyrophosphatase	813R (IR29)	813R (FL478)
Os01g0665400 protein	320R (IR29)	–
Protein biosynthesis and processing		
60S acidic ribosomal protein P3	244L (IR29)	–
Serine carboxypeptidase 1	316L (IR29)	–
Endosperm lumenal-binding protein	–	376L (IR29)
Clp *N*-terminal domain containing protein	–	18L (FL478)
Pi starvation-induced protein	–	102L (IR29)
Chaperonin	727L (IR29)	248R (IR29)
GrpE protein homologue	838L (IR29)	–
Oryzacystatin-I	–	110L (IR29)
Oryzain β chain	–	332R (IR29)
Eukaryotic translation initiation factor 5A-2	–	223R, 224R, 178R (IR29)

(*continued next page*)

Table 4. (*continued*)

Protein identity	Spot ID	
	Decreased	Increased
Protein disulfide isomerase-like 1	–	521R (IR29)
Amino acid biosynthesis		
Cysteine synthase	–	372R, 391R (IR29)
Aspartate aminotransferase	–	563R (IR29, FL478)
Glutamine synthetase	508L (IR29, FL478)	578R (IR29)
Polyamine biosynthesis		
Spermidine synthase 1	–	397R (IR29)
S-adenosylmethionine synthase 1	–	840R (IR29)
Actin cytoskeleton		
Actin-like protein	–	469R (IR29)
Actin	–	482L (IR29)
Actin-depolymerising factor 3		241L (IR29)
Cell wall biogenesis and degradation		
Chitinase	639R (IR29, FL478)	157R, 644R (IR29); 162R (IR29, FL478)
Xyloglucan endotransglycosylase/hydrolase protein 8	268R (IR29)	–
Caffeoyl-CoA 3-O-methyltransferase	–	259R (IR29)
Putative uncharacterised protein	61R (IR29, FL478)	–
Gene transcription and mRNA processing		
NAM, ATAF, and CUC (NAC) transcription factor	683R (IR29, FL478)	–
Hypothetical protein	866R (IR29, FL478)	–
Histone acetyltransferase GCN5	315R (IR29)	–
Putative glycine-rich RNA-binding protein 2	69R (IR29, FL478)	–
Putative 41-kDa chloroplast nucleoid DNA binding protein	326R (IR29)	–
Putative mRNA binding protein	519L (IR29)	–
Signal transduction		
Probable calcium-binding protein CML7	5R (IR29)	–
Receptor like-protein kinase	143R (IR29)	777R (IR29)
Putative remorin 1 protein	–	624R (IR29)
Ion compartmentalisation		
Vacuolar ATPase β subunit	–	583R (IR29)
Putative vacuolar proton ATPase	–	531R (IR29)
Unclassified proteins		
FHA domain-containing protein	–	256L (IR29); 257L (IR29, FL478)
Uncharacterised protein	–	208L (IR29)
3-oxoacyl-(acyl-carrier-protein) synthase	–	705R (IR29)
HSR203J	864R (FL478)	864R (IR29)
Putative PRMC3	–	631R (IR29)

densities of some of the candidate SRPs are shown in the magnified gel images (Fig. 3).

Some of the SRPs were represented in more than one spot and were therefore identified as protein species including GST (three root spots), peroxidase (three root spots), thioredoxin H1 (two root spots), ferritin (two leaf spots), eukaryotic translation initiation factor 5A (eIF5A, three root spots), cysteine synthase (two root spots), chitinase (four root spots) and the forkhead-associated (FHA) domain-containing protein (two leaf spots). In most cases, the same proteins were identified in spots that displayed small changes in pI and showed a horizontal shift in 2DE gels. These positional variations might be due to post-translational modifications, such as phosphorylation, acetylation, methylation and reduction, or other modifications during translocation processes that affect the net charge of the protein.

The candidate leaf and root SRPs were categorised into separate functional groups based on the functional data retrieved from the Uni-prot database (http://www.uniprot.org (accessed 11 February 2015);Table 4). Proteins related to the antioxidative and stress response pathways (13 root and 9 leaf proteins) were highly represented, with significantly higher abundance under salt stress (18 out of 23), indicating that controlling oxidative stresses constitutes an important part of adaptation to salt stress. Fourteen proteins (nine root and five leaf proteins) were categorised as carbohydrate and energy metabolism proteins, and comprised the second most abundant group. Photosynthesis-related proteins were also affected in both genotypes, with significantly decreased abundance in IR29, which suggests that growth reduction in this genotype is related to impaired photosynthesis under salt stress. Proteins involved in protein synthesis and processing, and amino acid biosynthesis were significantly increased in IR29 under salt stress. Proteins related to gene transcription and mRNA processing were also decreased in roots. Proteins involved in ion compartmentalisation (in roots) and the actin cytoskeleton were increased in IR29 in response to salt stress.

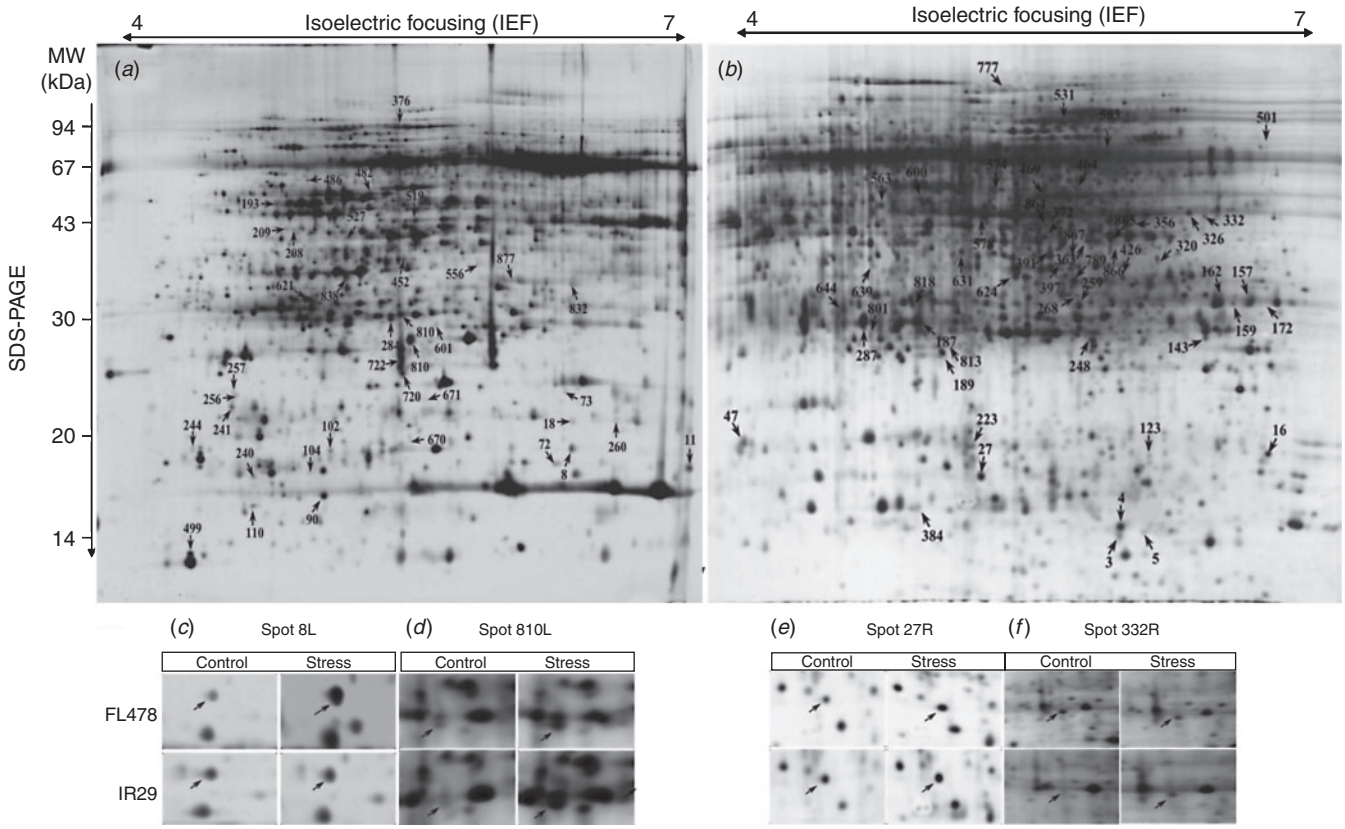

Fig. 3. Representative silver-stained 2D gel electrophoresis images of (*a*) total leaf and (*b*) root proteins separated over a pH range of 4–7 and a molecular weight of 10–100 kDa. The gel position of each of the candidate leaf and root salt-responsive proteins is shown using an arrow a number which refers to spot ID as reported in Table 4 and supplementary Tables 3 and 4. Magnified gel images show the changes in spot intensities of some of the candidate salt-responsive proteins in salt-sensitive IR29 and salt-tolerant FL478: (*c*) Spot 8L; (*d*) Spot 810L; (*e*) Spot 27R; (*f*) Spot 332R.

Quantitative PCR confirmation of the transcript level of some of the candidate SRPs

We used qPCR to further evaluate the mRNA expression signatures of 12 SRPs (five root and seven leaf proteins). Overall, the mRNA expression in the sensitive genotype, IR29, was poorly correlated with the protein abundances estimated from spot densities (Fig. 4). In this genotype, only two mRNAs, corresponding to Spots 452L and 527L, were expressed in the same direction; the remaining were expressed in the opposite direction compared with the protein level. This low level of correlation might be due to the damage imposed by excess salt within the plant, which also significantly affected biomass production in this genotype. In the tolerant genotype, FL478, the correlation was more positive. Nine mRNA transcripts were expressed in the same direction as their corresponding proteins, although their relative levels of expression were highly variable. In contrast, the expression of transcripts corresponding to Spots 621L, 583R and 3R was in the opposite direction. The lack of correspondence between mRNA and protein levels might be due to differences in the relative half-lives of the proteins and the corresponding mRNAs in *in vivo*, post-transcriptional and translational regulatory checkpoints, and the existence of significant error and noise in measuring protein and mRNA levels (Greenbaum *et al*. 2003).

Discussion

When plants are exposed to salt stress, they first encounter with problems with water absorption, which is largely due to osmotic stress imposed by the salt outside the roots. This osmotic stress is usually accompanied by a temporary and recoverable reduction in growth (Munns 2002, 2010). However, the salt-specific effect takes time to develop and happens when excess salt enter the plant and causes salt toxicity (Munns 2002). This salt-specific response is accompanied by a significant growth reduction and differential growth responses in species or genotypes with differing abilities to tolerate salt. Here, we showed that this salt-specific growth reduction appeared 16 days after exposure to 120 mM NaCl in IR29. At this point, salt stress significantly affected most morphological and physiological characteristics of the plants (Tables 1, 2 and 3). These detrimental effects and salt injuries were more severe in IR29 compared with the tolerant FL478. The excess salt injury in IR29 was largely due to higher salt accumulation in roots and shoots, which exceeded the capacity to compartmentalise it into the vacuoles and resulted in salt build-up in the cytoplasm. These findings suggest that FL478 was probably more efficient in excluding Na^+ from the transpiration stream and partitioning it into different organs, or that its roots leaked less salt than those of IR29.

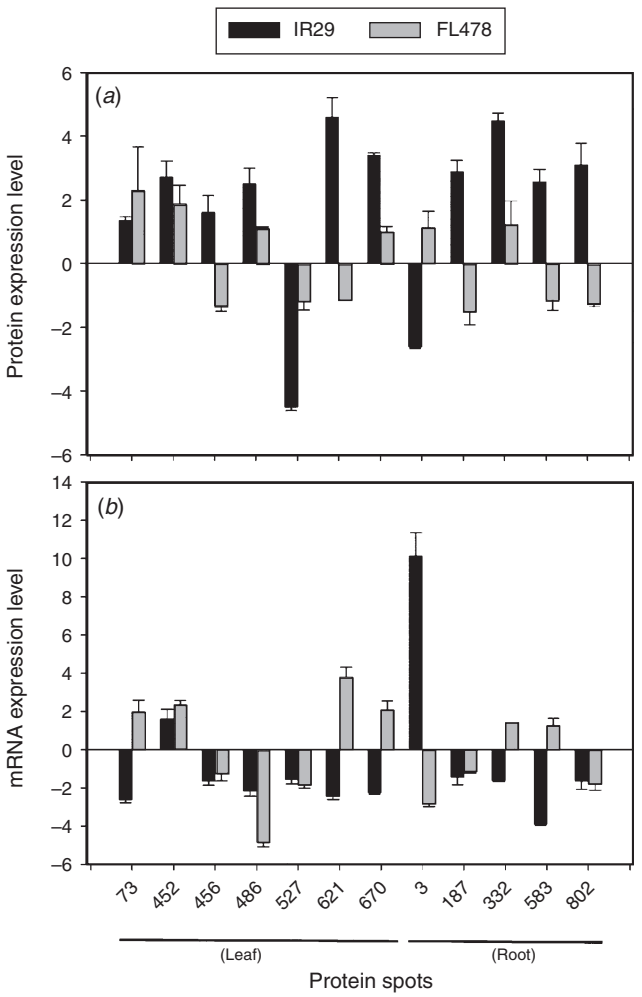

Fig. 4. Quantitative real-time PCR analysis of the mRNA expression of some of the candidate leaf and root salt-responsive proteins (SRPs). (*a*) The abundance of the selected SRPs at the protein level as estimated from spot intensities. (*b*) The mRNA expression levels as measured using qRT-PCR.

To identify the proteomic changes that support salt tolerance in FL478 and those that are associated with salt sensitivity in IR29 when salt accumulates to toxic level in their tissues, a comparative 2DE-based proteomics approach was applied to explore the root and leaf proteomes. Salt stress significantly affected the leaf and root proteomes of the salt-sensitive genotype IR29, in accordance with previous microarray analyses of gene expression (Walia *et al*. 2005; Cotsaftis *et al*. 2011). Sixteen out of 39 candidate leaf SRPs and 22 out of 59 candidate root SRPs were decreased in IR29 (Table 4). In addition, the abundances of 19 leaf and 35 root SRPs were increased under salt stress in this genotype. These results were consistent with the physiological measurements, which showed greater salt injuries and suggested that a broad spectrum of metabolic and regulatory pathways were affected in IR29 by salinity.

Proteins related to antioxidative and stress response pathways mostly increased under salt stress

The abundance of proteins with a protective function against reactive oxygen species (ROS) was changed in both genotypes but to a greater extent in IR29 in response to salt. Of these proteins, the abundance of superoxide dismutase (SOD) increased significantly in both roots (Spot 27R) and leaves (Spot 8L) of FL478, but remained unchanged in IR29. SOD is a metalloenzyme that catalyses the dismutation of superoxide to oxygen and H_2O_2. An increased abundance of SOD has also been reported in rice leaves in response to salt stress (Abbasi and Komatsu 2004; Parker *et al*. 2006) and in rice mitochondria during salt stress-induced programmed cell death (Chen *et al*. 2009). Studies have shown that salt-tolerant genotypes of rice exhibit increased SOD activity and limited lipid peroxidation under salt stress compared with salt-sensitive ones (Dionisio-Sese and Tobita 1998). In addition, overexpressing SOD increased salt stress tolerance in rice (Tanaka *et al*. 1999; Prashanth *et al*. 2008). These results clearly indicate that the accumulation of SOD in FL478 may contribute to its salt tolerance phenotype.

The abundance of GST (Spots 187R, 287R and 802R) increased in IR29 roots but did not show any changes in FL478. GST is implicated in diverse molecular processes, including detoxification of xenobiotics and excess auxin, and protection against oxidative stresses, heavy metals and pathogen attack (Marrs 1996). An increase in GST protein has been reported in rice roots (Nam *et al*. 2012) and shoots (Ruan *et al*. 2011) in response to salt stress. Transgenic rice plants co-expressing GST and catalase from *Suaeda salsa* showed increased SOD and catalase activities, but not GST activity, and enhanced tolerance to salt stress (Zhao and Zhang 2006). In agreement, in *Arabidopsis thaliana* (L.) Heynh. plants overexpressing GST, lipid peroxidation was reduced under salt stress but this reduction was not enough to cope with root growth reduction imposed by salt stress (Katsuhara *et al*. 2005). Here, the increased abundance of GST in IR29 may help to alleviate the effect of salt toxicity but is not likely to be sufficient to minimise salt injuries.

Three root proteins were identified as peroxidase (Spots 671R and 574R decreased, and Spot 818R increased under salinity). Peroxidases are normal apoplastic constituents, which are involved in several processes including lignin biosynthesis, suberisation, auxin catabolism, wound healing and defence against pathogen infection (Hiraga *et al*. 2001). H_2O_2 detoxification by peroxidases is the beneficial consequence of the reactions in which they participate. Changes in the abundance of peroxidases were also reported in rice upon salt stress treatment (Walia *et al*. 2005; Song *et al*. 2011). Changes in the abundance of peroxidases are likely to contribute to salt sensitivity or tolerance, since apoplastic peroxides polymerise proteins and lignin precursors into the cell wall and create physical barriers that limit apoplastic salt uptake. Four proteins with significant downregulation in IR29 were identified as thioredoxin (three H-types and one M-type). Thioredoxin H is involved in the regulation of the redox environment of cells by reducing disulfide bridges (Gelhaye *et al*. 2004). A change in the abundance of H-type thioredoxin was reported in rice panicles in response to salt stress (Dooki *et al*. 2006).

Ferritin (Spots 284L and 810L) was found to be significantly increased in response to salt stress in both genotypes but the extent of this increase was much greater in IR29 than FL478. Similarly, an increased abundance of ferritin has also been reported in rice leaves during salt stress (Kim *et al*. 2005; Parker *et al*. 2006; Liu *et al*. 2012), suggesting it as a candidate SRP. Plant ferritins are plastidic proteins that are involved in iron storage and regulation of iron homeostasis (Lescure *et al*. 1991). Ferritin may be involved in protection against ROS because excess iron can catalyse the formation of hydroxyl radicals via Fenton chemistry (Orino *et al*. 2001). In line with this, it has been shown that ferritin-deficient mutants of *Campylobacter jejuni* are more sensitive to H_2O_2 (Wai *et al*. 1996), suggesting an important role for ferritin in protecting cells against oxidative stresses.

Since there was no clear trend in the level of anioxidative enzymes in IR20 and FL478 that could be linked to salt sensitivity or tolerance, changes in the abundance of antioxidative enzymes might be due to genotypic differences that modulate ROS production during salt stress.

Cell wall biogenesis and degradation-related proteins were mostly affected in roots

Proteins related to cell wall modification such as chitinase (Spots 162R, 157R, 639R and 644R), xyloglucan endotransglycosylase/hydrolase (Spot 268R) and caffeoyl-CoA 3-O-methyltransferase (CCOMT; Spot 259R) changed in abundance in the roots in response to salt stress. A change in the abundance of a similar set of proteins has already been reported in rice shoots in response to salt stress, suggesting a critical role for cell wall reconstruction as an adaptive strategy to combat salt stress (Walia *et al*. 2005; Song *et al*. 2011). Chitinases are enzymes that degrade cell walls and participate in the defence against pathogens as well as environmental stresses (de las Mercedes Dana *et al*. 2006). CCOMT (Spot 259R), an enzyme involved in the lignin biosynthesis pathway, was significantly enriched in the roots of IR29 with no detectable change in FL478. CCOMT catalyses the methylation of caffeol-CoA, which serves as a precursor in the biosynthesis of guaiacyl and syringyl lignin units (Ye *et al*. 2001). Similarly, an increased abundance of CCOMT was reported in rice in response to salt stress (Salekdeh *et al*. 2002). It is thought that in some species such as rice, Na^+ may be taken up apoplastically due to the leaky nature of their roots (Munns and Tester 2008). Increased CCOMT may enhance the cell wall lignification process and impact cell–cell interactions, which may result in greater selectivity and reduced salt uptake, and limited bulk flow of water and solutes along the apoplastic pathway.

We also found an increased abundance of proteins related to the actin cytoskeleton such as actin (Spot 482L), actin-like protein (Spot 469R) and actin depolymerising factor 3 (Spot 241L) in IR29 in response to salt stress. These proteins did not show any changes in FL478, indicating that the actin cytoskeleton was not affected by salinity in this genotype. The actin depolymerising factor is critical for actin dynamics through binding to G- and F-actins and severing actin filaments (Feng *et al*. 2006). Recently an *A. thaliana* mutant that was defective in a xyloglucan galactosyltransferase involving in actin microfilament organisation and cell wall biogenesis has been identified; this mutant shows hypersensitivity to salt stress (Li *et al*. 2013). In this mutant, actin microfilaments cannot assemble and aggregate in the cytoplasm; however, addition of phalloidin, which prevents actin depolymerisation, can rescue salt hypersensitivity in this mutant. These results suggest that actin cytoskeleton rearrangement serves as an adaptive mechanism for salt tolerance.

Carbohydrate and energy metabolism-related proteins showed decreased abundance in IR29

Several differentially accumulated proteins are known to be involved in carbohydrate metabolism (Table 4). Within this category, proteins such as tyrosine phosphatase, phosphoglycerate kinase, pyruvate dehydrogenase, enolase and NADPH-dependent mannose-6-phosphate reductase increased, whereas fructose-bisphosphate aldolase, fructokinase-2 and glyceraldehyde-3-phosphate dehydrogenase 2 decreased in IR29 under salt stress. Interestingly, fructokinase-2 (spot 363R) showed threefold decreased abundance in IR29 but remained unchanged in FL478 under salt stress. Fructokinase-2 has been shown to contribute to stem and root growth, since suppression of this gene in tomato (*Solanum lycopersicum* L.) resulted in much shorter plants (Odanaka *et al*. 2002). Decreased abundance of the same protein was reported in the leaves of a sensitive genotype of rice upon salt stress (Ghaffari *et al*. 2014). Growth reduction under salt stress in IR29 might be related to the decreased abundance of this protein.

In addition to being important for energy production, some glycolytic proteins such as enolase (increased in IR29) and fructose bisphosphate aldolase (decreased in IR29) have been shown to interact with subunits of vacuolar ATPase and modulate its H^+ pump activity, and therefore play a key role in salt stress tolerance (Barkla *et al*. 2009). Interestingly, fructose bisphosphate aldolase stimulates the ATP-binding activity of vacuolar ATPase and thus upregulates the import of salt into the vacuole and promotes salt stress tolerance. A decreased abundance of this protein upon salt stress in the leaves of IR29 might be related to the salt sensitivity phenotype of this genotype.

Another protein in this category was nucleoside diphosphate kinase 1 (NDPK, Spot 384R), which showed opposite changes in IR29 (decreased) and FL478 (increased) upon salt stress. An increased abundance of NDPK was reported in rice panicles (Dooki *et al*. 2006) and roots (Kawasaki *et al*. 2001) in response to salt stress. NDPK is known as a housekeeping enzyme that maintains the intracellular balance between ATP and other nucleoside triphosphates. It also plays a regulatory role in signalling pathways leading to the oxidative stress response (Otero 2000). Stress-inducible overexpression of NDPK2 in potato (*Solanum tuberosum* L.) plants provided evidence that NDPK protects plants against environmental stresses (Tang *et al*. 2008). Its increased abundance in salt-tolerant FL478 and decreased abundance in salt-sensitive IR29 strongly suggests it as a candidate salt tolerance-related protein.

Proteins related to polyamine biosynthesis were increased in the roots of IR29

Salt stress modulates the metabolic pathways leading to the biosynthesis of many secondary metabolites. Spermidine

synthase, a key enzyme involved in the biosynthesis of polyamines, increased (Spot 397R) in IR29's roots but unchanged in those of FL478. Plant polyamines are aliphatic amines with a low molecular weight that play key roles in growth, development, reproduction and response to various environmental stresses (Gill and Tuteja 2010). An increase in spermine content was reported in rice challenged with salt stress (Maiale *et al*. 2004). Studies have also shown that exogenous application of some polyamines enhances salt stress tolerance in rice (Chattopadhayay *et al*. 2002; Ndayiragije and Lutts 2006). Interestingly, tomato plants overexpressing apple (*Malus domestica*) spermidine synthase 1 showed increased salt stress tolerance (Neily *et al*. 2011). However, antisense inhibition of a spermidine synthase gene in European pear (*Pyrus communis* L.) led to increased sensitivity to salinity and cadmium (Wen *et al*. 2011). These studies provide direct evidence that spermidine synthase plays a key role in the establishment of tolerance against salt stress.

S-adenosylmethionine synthetase (SAMS, Spot 840R) behaved similarly to spermidine synthase and increased in IR29 roots but did not show any change in FL478. SAMS catalyses the formation of *S*-adenosylmethionine (AdoMet) from L-methionine and ATP. AdoMet serves as an important methyl donor in most transmethylation reactions, and as a precursor for the biosynthesis of ethylene and polyamines (Gill and Tuteja 2010). SAMS has been shown to be involved in the biosynthesis of lignin and the polyamine glycine betaine during salt stress (Sánchez-Aguayo *et al*. 2004; Tabuchi *et al*. 2005). It is hypothesised that the induction of SAMS by salt stress might be necessary to supply AdoMet, which is required for the lignification process. Increased cell wall lignification may further hinder the apoplastic flow of salt.

Amino acid biosynthesis-related proteins increased in the roots of IR29

Amino acid biosynthesis enzymes were found to be increased in roots under salt stress. Glutamine synthetase (GS) was significantly increased in roots (Spot 578R, cytosolic) of IR29 but decreased in the leaves of both genotypes (Spot 508L, chloroplastic), which is in line with our previous report (Ghaffari *et al*. 2014). An increased abundance of GS has been reported in rice roots in response to salt stress (Jiang *et al*. 2007; Nam *et al*. 2012). GS is an essential enzyme that is responsible for ammonium assimilation and catalyses the condensation of inorganic ammonium with glutamate to generate glutamine in an ATP-dependent reaction. GS is also involved in the biosynthesis of the precursors of proline, an osmolyte involved in adaptation to salt stress (Yan *et al*. 2005). An increased abundance of GS in roots may compensate for increased amino acid degradation under salt stress. However, decreased chloroplastic GS in leaves is likely to be the result of a reduction in net photosynthesis under salinity, since it is well documented that the expression of genes related to nitrate reduction in chloroplasts are under the control of cytosolic sugar concentration, which is largely determined by photosynthetic activity.

Aspartate aminotransferase (Spot 563R) increased significantly in the roots of both genotypes during salt stress. Similar to our results, an increased abundance of aspartate aminotransferase has been reported in rice roots (Nam *et al*. 2012) and shoots (Li *et al*. 2010) during salt stress. Aspartate aminotransferase catalyses the reversible reaction of the transfer of the amino group from aspartate to α-ketoglutarate to generate oxaloacetate and glutamate. It plays a critical role in the regulation of C and N metabolism. An enhanced abundance of root aspartate aminotransferase may indicate that salt stress is inducing significant asparagine transport from roots to the shoot.

Cysteine synthase (Spots 372R and 391R) increased significantly in the roots of salt-sensitive IR29. In plants, cysteine synthase catalyses the final step of the cysteine biosynthesis pathway, which is a rate-limiting step in the biosynthesis of glutathione, a thiol-containing compound that is involved in the response to many biotic and abiotic stresses (May *et al*. 1998). Indeed, cysteine synthase removes sulphide, which eventually might reach toxic concentrations in roots. However, the amino acid cysteine still has some toxic potential. Therefore reduced (organic) sulfur has to be transported via the phloem in form of *S*-methylmethionine. *S*-methylmethionine is synthesised through the enzymatic action of *S*-AdoMet-methionine methyltransferase using AdoMet as a precursor (Wirtz and Droux 2005). As noted above, AdoMet is synthesised by SAMS, which also increased in the roots of IR29.

Protein biosynthesis and processing proteins frequently increased in the roots and decreased in the leaves of IR29

It is well documented that the synthesis of new proteins and the removal of old or unnecessary proteins is an essential prerequisite for plant cells to gain a stress tolerance phenotype. Interestingly, we found a decreased abundance of proteins such as 60S acidic ribosomal protein P3 (Spot 244L), serine carboxypeptidase 1 (Spot 316L), chaperonin (Spot 727L) and GrpE protein homologue (Spot 838L) in the leaves of IR29, suggesting that protein synthesis and folding were significantly affected in this genotype under salt stress. In addition, an increased abundance of proteins such as eukaryotic translation initiation factors 5A (Spots 178R, 223R and 224R), protein disulfide isomerase (Spot 521R), oryzain β (Spot 332R), oryzacystatin-1 (Spot 110L), endosperm luminal binding protein (spot 376 L) and proteinase inhibitor (Spot 102L) was detected in IR29. Of these, oryzacystatin-1 increased significantly in the leaves of IR29 but remained unchanged in FL478. Oryzacystatin -1 is a cysteine proteinase inhibitor that binds and inhibits the papain-like proteases such as oryzain β tightly and reversibly (Abe *et al*. 1991). In addition to being used as transgenes to engineer plants against pests (Schluter *et al*. 2010), cystatin overexpression has been shown to increase tolerance to salt, drought, cold and oxidative stresses, at least in *A. thaliana* (Zhang *et al*. 2008). All of this evidence clearly suggests a critical role for oryzacystatin in response to salt stress.

The protein synthesis process is mainly controlled at translation initiation by fine-tuning the synthesis and activities of translation initiation factors. EIF5A (Spots 223R, 224R and 178R) increased in IR29 but remained unchanged in FL478. EIF5A is a small structurally conserved protein in the eukaryotes and archaea that is the only known protein containing the post-translationally modified amino acid hypusine (Gordon *et al*.

1987). Recent evidence suggests that it may not be required for protein synthesis, but it stimulates (Henderson and Hershey 2011) or facilitates the process by selective stabilisation and transport of a subset of mRNAs from the nucleus to the cytoplasm (Zuk and Jacobson 1998; Feng *et al*. 2007). We recently showed that transgenic tobacco (*Nicotiana tabacum* L.) plants expressing cyanobacterial flavodoxin, which displayed increased tolerance to drought, salt and UV, expressed elevated eIF5A under drought stress (Gharechahi *et al*. 2014), suggesting eIF5A as candidate osmotic stress-related protein.

Proteins with a direct role in ion compartmentalisation increased in IR29 roots

The ability of plant cells to compartmentalise excess Na^+ into the vacuoles is an important strategy for maintaining ion homeostasis within the cytoplasm, since excess accumulation of salt in this compartment will result in enzyme inhibition and has detrimental consequences for plant cells. In line with this, two protein spots (Spots 531R and 583R), which increased significantly up to 2.5-fold in the roots of IR29 were identified as subunits of vacuolar ATPase. However, in FL478, these two proteins displayed no change under salt stress. Vacuolar ATPase is a multienzyme complex and an ATP-dependent proton pump that couples ATP hydrolysis with proton (H^+) transport across the tonoplast (Padmanaban *et al*. 2004). This H^+ electrochemical gradient is subsequently used by Na^+–H^+ antiporters to deposit excess Na^+ into vacuoles and thus aids in adaptation to salt stress. It is believed that an increase in vacuolar ATPase activity is the main strategy that the halophyte plant *Suaeda salsa* exploits to adapt to high salt concentrations. In agreement with this, transgenic rice plants overexpressing a subunit of vacuolar ATPase from the halophyte grass *Spartina alterniflora* Loisel. showed enhanced salt stress tolerance (Baisakh *et al*. 2012). This result suggest that IR29 overproduced vacuolar ATPase to deposit excess ions into the vacuoles; however, the rate of salt accumulation in this genotype exceeded the capacity of cells to compartmentalise it and therefore resulted in salt injury, growth inhibition and salt sensitivity.

Photosynthesis-related proteins mainly decreased in IR29

Accumulation of a toxic level of harmful ions in the cytoplasm will result in enzyme inhibition, loss of photosynthesis, impairment of the energy production processes and finally cell death. Therefore, adjustment of photosynthesis and energy metabolism is of great importance in adapting to high salinity. Interestingly, we found a decreased abundance of proteins related to the photosynthetic electron transport chain in the salt-sensitive genotype IR29, including ferredoxin-thioredoxin reductase (Spot 72L), plastocyanin (Spot 499L), a 23-kDa polypeptide of PSII (Spots 63 L) and ATP synthase Cf1 ε subunit (Spot 629L), which may lead to impaired photosynthesis and further result in increased PSII-dependent ROS production in the light. This result suggests that IR29 is at high risk of ROS production but has a low detoxification potential; the opposite may be assumed for FL478, since the level of the ROS-scavenging enzyme SOD increased in this genotype but did not show any change in IR29. We also found an increased abundance of proteins such as the 33-kDa oxygen-evolving protein of PSII (Spot 426R), the oxygen-evolving enhancer protein 3 (Spot 11L), Rubisco small (Spot 260L) and large (Spot 877L) subunits, and a decreased abundance of Rubisco activase (Spot 193L) under salt stress. Interestingly, Rubisco activase decreased significantly in both genotypes in response to salt stress, which is in accordance with the results of our previous proteomic analysis in rice (Ghaffari *et al*. 2014). Rubisco activase is a chaperone protein that mediates the release of inhibitory sugar phosphates that bind to the active site of Rubisco when the intracellular level of CO_2 diminishes due to stomata closure (Jordan and Chollet 1983). A decreased abundance of this protein may result in increased Rubisco inactivation and reduction in net photosynthesis and growth.

Conclusion

The two tested genotypes, IR29 (salt-sensitive) and FL478 (salt-tolerant), had the same growth rate for the first 10 days of salt treatment (120 mM NaCl). However, at Day 16 they clearly separated and showed differential growth responses to salt stress. At this point, IR29 accumulated greater amounts of Na^+ in the roots and shoots than FL478, and exhibited greater salt injury. Our physiological measurements suggested that FL478 achieves salt tolerance by either actively excluding Na^+ from the transpiration stream, by partitioning excess Na^+ into vacuoles or by being able to dilute the absorbed Na^+ in its tissues through its higher growth rate. Proteomic analysis also showed significant downregulation of proteins in IR29 under salt stress, which might be due to greater salt injury. Interestingly, salt-sensitive IR29 activated molecular mechanisms that may lead to salt stress tolerance by upregulating proteins related to the oxidative stress response, amino acid biosynthesis, polyamine biosynthesis, the actin cytoskeleton and ion compartmentalisation, but could not overcome salt toxicity. These results suggested that the rate of salt accumulation in this genotype exceeded the capacity to tolerate it and therefore ion toxicity happened. In FL478, we observed a significant accumulation of proteins with possible roles in salt tolerance such as SOD and NDPK. Overall, our integrated physiology and proteome analyses provided a novel insight into the mechanisms that contribute to salt stress tolerance and sensitivity.

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