An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina

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Abstract

Proteomic analysis of any biological system by twodimensional gel electrophoresis (2-DE) requires high resolution and high reproducibility. The results presented here demonstrate the reproducible and accurate separation of rice (Oryza sativa L.) proteins using improved procedures for high resolution 2-DE, which were adapted for the separation of rice lamina proteins. Validation of this system was achieved by measuring the effects of sample preparation and biological variation on the coefficient of variation (CV) for replicate spots. The majority of experimental variation was shown to be introduced by the 2-DE technique (CV 0.26). Analysis of biological variation indicated that approximately 93–95% of spots were within a CV of 0.7. This provided a threshold value from which valid differences in expression between experimental groups could be screened. This system was then utilized for the proteomic analysis of short- and long-term salt-stress-responsive proteins in the rice leaf lamina. Analysis resulted in the separation of approximately 2500 protein species of which 32 were observed to be significantly regulated by salinity; so far 11 of these proteins have been identified by tandem mass spectrometry. An increase in eight proteins, including RuBisCO activase and ferritin, occurred by 24 h of exposure to sodium chloride (50 mM) and continued to increase during the following 6 d. Only one protein, a putative phosphoglycerate kinase, was found to increase in expression within 24 h and did not increase over a longer period of exposure to salt. There were also proteins that showed no change 24 h after exposure to salt, but had increased (superoxide dismutase) or decreased (S-adenosyl-L-methionine synthetase) after 7 d salt treatment.

Key words: Experimental error, Oryza sativa, proteomics, rice, salinity, salt stress.

Introduction

Amongst crop plants, rice is particularly sensitive to salinity and while genotypes of rice have been developed that are relatively tolerant to sodic soils (Singh et al., 2002), little progress has been made in enhancing tolerance to soils where sodium chloride is the dominant salt. Although changes in sodium (and chloride) concentrations in the shoot determine leaf longevity and hence overall sensitivity to salt (Yeo et al., 1991; Munns, 1993; Munns et al., 2002), the molecular response of the rice leaf lamina to salt is poorly characterized. Analysis of changes in gene expression in rice plants exposed to salt stress should enable the relative importance of processes hypothesized to be involved in tolerance to be evaluated; processes such as ion compartmentation in cell vacuoles as opposed to those that limit cellular damage. This knowledge should enable the design of effective strategies for the genetic engineering of enhanced salt tolerance in rice.

Rice plants respond to salinity in two distinguishable phases (Yeo et al., 1991). The initial effects of a rise in external sodium chloride concentration are short-term and result from a drop in external water potential. The secondary effects of continued exposure to salt are long-term and result from excess ion accumulation in the shoot: in the leaf blade, excess salt results in photoinhibition and promotes cell death (Yeo et al., 1991). Adaptation to salt stress requires alterations in the cellular machinery that result directly from modifying gene expression, which has been investigated by analysis of transcript abundance after exposure to salt. Analysis of salt-stressed rice shows changes in the expression of large numbers of genes (up
to a few hundred) in roots in response to 150 mM NaCl (Kawasaki et al., 2001; Sahi et al., 2003; Wu et al., 2005) and whole shoots (Shiozaki et al., 2005) or leaves and roots (Rabbani et al., 2003) of plants shocked with 250 mM NaCl. A similar high salt concentration induced changes in genes involved in the metabolism of antioxidants in leaves (Menezes-Benavente et al., 2004). It is, however, difficult to evaluate the importance of particular genes or groups of genes, when such a large number change in their expression and the stress is relatively severe.

Whilst transcriptomic approaches are an important resource, functional gene expression profiles can only be achieved by proteome analysis. Furthermore, proteins undergo significant levels of post-translational modification of their primary sequences and are readily subjected to targeted proteolysis. Thus, quantitative analysis of gene expression at the protein level is essential to dissecting responses to salt stress. Expression profiling at the protein level represents the core of proteomic analysis done today. The most common tool used for revealing the expression of intact proteins is two-dimensional gel electrophoresis (2-DE). Currently, the range of protein concentrations that can be separated, the resolution of separation, and the heterogeneous physicochemical nature of protein mixtures limit analysis. Consequently, in order to generate accurate and reliable data on the relative quantity of proteins present in different samples, significant optimization is required. Once sample preparation is optimal, experimental design and quantification are key to the identification of biologically relevant markers.

Several studies have attempted to analyse alterations in protein expression in response to a multitude of biotic and abiotic stimuli (Rakwal and Agrawal, 2003; Komatsu and Tanaka, 2005) and differential proteomics has been used to analyse the proteome of rice, a useful model organism for the study of salt stress in monocotyledonous plants (Agrawal and Rakwal, 2006). The majority of 2-DE data published shows the consistent regulation of approximately 50 protein species during salt stress. Amongst these, the tolerance to oxidative stress has been highlighted by the up-regulation of superoxide dismutase and ascorbate peroxidase (Abbasi and Komatsu, 2004; Salekdeh et al., 2004). The regulation of enzymes involved in photosynthesis, and carbon metabolism has also been detected in these and other studies (Yan et al., 2005).

Whilst these already published data represent an important contribution to rice proteomics there is a need for the implementation of improved procedures for the proteomic analysis of salt stress in rice. Also, whilst authors provide various statistical tests to validate differences, no meaningful data are provided to examine the overall variation present within the experiments performed. Choe and Lee (2003) suggested that a simple set of experiments using same-sample replicates can be performed to measure the variability present within any 2-DE system. In order to analyse and validate the expression data produced for rice-lamina proteins by 2-DE, a similar experimental procedure was adopted. Before expression analysis was performed, the experimental system was tested for its robustness for quantitative reproducibility. Analysis of this indicated that spot-to-spot variation was mainly due to error introduced by the 2-DE instrumentation and therefore allowed the use of biological replicates for comparative expression profiling. It also showed that a 1.7-fold threshold provides a 93–95% confidence interval and was therefore suitable for determining real biological differences. Utilizing this methodology, it has been investigated whether there are specific differences in the proteins expressed during the first and second phases (Munns, 2002) of response to salt. Comprehensive analysis of short- (24 h) and long-term (7 d) salt stress revealed 32 differentially regulated protein spots, only one of which was unique to changes that occurred within 24 h; 11 of these spots have so far been identified.

**Materials and methods**

**Plant material**

An elite breeding line of rice (*Oryza sativa* L.) from the International Rice Research Institute (IRRI), IR4630-22-2-5-1-3 was used in these experiments. Approximately 200 caryopses were soaked in aerated water for 24 h. Seeds were germinated on Perspex grids floating on Yoshida nutrient solution (Yoshida et al., 1972) modified by replacing sodium salts with potassium and a reduced phosphate concentration of 0.5 mM (Yeo et al., 1991). Plants were grown in a growth chamber (Conviron model E15, Winnipeg) under an irradiance of 450–500 μmol m⁻² s⁻¹ (PAR) and a relative humidity of 30–90% with a 12 h photoperiod and day/night temperatures of 29/27 °C. After 7 d, seedlings were transplanted and grown for another 7 d and 13 d before being treated with 50 mM NaCl for 7 d and 1 d, respectively. At 21 d, all treated plants, as well as untreated controls, were harvested. The proximal third (avoiding necrotic tissue at the leaf tip) of the blade of leaf 4 from 50 plants of each treatment was immediately frozen in liquid nitrogen and stored at −70 °C for ion analysis and protein extraction.

**Ion analysis**

Sodium content was determined in the proximal third of the blade of leaf 4 from approximately 25 plants taken from each box. The leaves were ground twice in liquid nitrogen to a powder, which was oven-dried for 2 d, weighed, and Na was extracted in 100 mM acetic acid for 2 h at 80 °C. The resulting solution was filtered to remove leaf particulates and sodium content was determined by atomic absorption spectrometry (Unicam 919, Unicam, Cambridge, UK). The concentrations of sodium ions in the leaf were expressed per unit of dry mass.

**Protein extraction and 2-DE analysis**

The method for preparation of rice proteins for 2D PAGE was a modified protocol of that used by (Damerval et al., 1986). Rice leaf tissue was ground four times in liquid nitrogen to a fine powder. This powder (400–500 mg fresh weight) was precipitated in 10 vols of 10% (w/v) trichloroacetic acid (TCA) in acetone with 0.5% (w/v) dithiothreitol (DTT) at −20 °C overnight followed by centrifugation for 30 min at 16 000 g at 4 °C. The pellets were then washed with an excess (7.5 ml) of ice-cold acetone and then centrifuged for 30 min at 16 000 g at 4 °C. This wash was repeated a total of three times and the pellets lyophilized to remove any remaining acetone. The sample
powder (25 μL mg⁻¹) was then solubilized in the lysis buffer (9 M urea, (w/v), 4% (w/v) CHAPS and 2% (v/v) ampholytes pH 3–10. To aid solubilization, this solution was incubated at 25 °C for 1.5 h with gentle mixing and then clarified by centrifugation at 16 000 g for 30 min. The supernatant, containing predominantly solubile proteins, was aspirated and reduced by adding 5 mM tributyl phosphate (TBP). Reduction was continued for 1 h at room temperature. Samples were then alkylated by treatment with 15 mM iodoacetamide (IAA) for 1.5 h at room temperature. This reaction was quenched by the addition of 15 mM DTT. Samples were then immediately frozen in liquid N₂ and then stored at −70 °C in aliquots. Total protein quantitation was achieved using the BCA protein assay kit (Pierce) as described in the manufacturer's instructions.

2-DE

The protein extract was diluted into final a volume of 500 μL of rehydration solution (2% (w/v) CHAPS, 2 M thiourea, 6 M urea, 0.5% IPG buffer, 4 mM TBP, and a trace of bromophenol blue) well mixed, and centrifuged at 16 000 g for 15 min. IPG strips 4–7 (24 cm) were actively rehydrated with 450 μL of rehydration solution containing 600 μg of protein for 12 h at 30 V. Isoelectric focusing was carried out on ICPphor (GE Healthcare) with a current limit of 50 μA/IPG strip, in three steps: 500 V for 1 h, 1000 V for 1 h, and 8000 V for 8 h 20 min achieving approximately 68 000 Vh. The IPG strips were then soaked in 10 mL of equilibration buffer (50 mM TRIS-Cl, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS) containing 100 mg of DTT for 15 min, then the soaking repeated but DTT was replaced by 250 mg of iodoacetamide. Second dimension electrophoresis was performed with Laemml gel (7=12.5%) in the Ettan DALT twelve system (GE Healthcare). Electrophoresis was carried out at 2.5 W gel⁻¹ for 15 min and then for 17 W gel⁻¹ 25 °C until the dye front was approximately 1 mm from the bottom of the gel. All gels were stained with colloidal Coomassie Brilliant blue G-250.

Image acquisition and data analysis

Image acquisition was achieved using a flattened transmissive white light scanner (ImageScanner, GE Healthcare). The scanner control software used was Labscan 3.01 (GE Healthcare). Pixel depth was 16 bit, resolution was 300 dpi; brightness and contrast were set to default. Intensity calibration was performed prior to acquiring all gel images using the Kodak photographic step tablet no. 2. Cropped gel images were exported as TIFF files from the scanner control software. Progenesis Workstation 2001.04 (Nonlinear Dynamics, Newcastle upon Tyne, UK) was employed using fully automatic spot detection and matching capabilities. Briefly, after spot detection, all spots from each replicate for a particular treatment were matched to create an average gel for each treatment. Average gels of each treatment were then matched to a reference gel that links all matched spots present in each replicate for the same sample was divided into three equal parts and run on the same gel for 1 d or 7 d in order to separate short- and long-term responses to a salt concentration previously shown to distinguish sensitive and resistant genotypes of rice (Flowers and Yeo, 1981). Analysis of sodium uptake in the leaf blade (Fig. 1) showed a doubling of the sodium concentration between 1 d and 7 d of exposure to salt. To try and identify the molecular components resulting from short- and long-term salt stress, rice leaf proteins were analysed by 2-DE.

Results and discussion

Growth and sodium accumulation in response to salt

Three-week-old rice seedlings were treated with 50 mM salt for 1 d or 7 d in order to separate short- and long-term responses to a salt concentration previously shown to distinguish sensitive and resistant genotypes of rice (Flowers and Yeo, 1981). Analysis of sodium uptake in the leaf blade (Fig. 1) showed a doubling of the sodium concentration between 1 d and 7 d of exposure to salt. To try and identify the molecular components resulting from short- and long-term salt stress, rice leaf proteins were analysed by 2-DE.

Quantification of experimental error in the system

Proteomic analysis is subject to variation introduced by the technology, by sample preparation, and by biological variation. To evaluate the variation present within this experimental system for rice, three experiments were carried out (see supplementary figure at JXB online). In the first, the same sample was divided into three equal parts and run on the same gel for 1 d or 7 d in order to separate short- and long-term responses to a salt concentration previously shown to distinguish sensitive and resistant genotypes of rice (Flowers and Yeo, 1981). Analysis of sodium uptake in the leaf blade (Fig. 1) showed a doubling of the sodium concentration between 1 d and 7 d of exposure to salt. To try and identify the molecular components resulting from short- and long-term salt stress, rice leaf proteins were analysed by 2-DE.
on three different gels; in the second, three preparations were made, independently using the same starting material and each run on separate gels. Finally, three biologically independent replicates were grown, prepared and run in parallel. The same area was selected for 2-DE analysis from each of the three replicate gels for each experiment. This area was chosen to avoid known problem areas of 2-DE maps where spot matching is problematic, thus allowing for a more automated assessment of reproducibility. Normalized volume data was collected for each spot that was matched in at least two out of three gels in each experiment and used for the calculation of coefficients of variation (CV). These settings reflect the minimum requirement for the generation of CV values; in most cases spots were present in all three replicates. This approach is in accord with spot analysis software available from all manufacturers and allows for the rare event where a spot is lost from one gel within an experimental group. By screening samples in this fashion, gel analysis can be performed rapidly and in an automated manner.

The average CV for matched spots increased from 0.26 for the single sample preparation run on separate gels, to 0.29 where three separate preparations were made, to 0.31 where three independent biological samples were processed separately (data not shown). The differences in variance can also be seen in the percentage of spots falling below certain cut-off values for the CV. Within the rice 2-DE system established using biological replicates, a 1.7-fold threshold was determined as a quantitative change in expression is reasonable, as it results in a 93–95% CI (data not shown). Candidate spots altering by 1.7-fold or greater were selected and the probability of changes in expression levels being statistically significant further assessed by a Student’s t test. This stringent approach was adopted to reduce the likelihood of identifying ‘false positive’ changes in protein expression.

2-DE analysis of leaf blade proteins in salt-stressed rice

After image analysis, more than 2500 protein spots were detected and matched between all gels. In order to compare protein expression, a difference map was generated which indicated proteins that differed by 1.7-fold in average normalized volume between control and salt-treated groups (Fig. 2). A total of 32 spots were shown to differ in normalized volume between treatments. The probability of the differences being statistically significant was calculated using the Student’s t test; changes in expression were considered significant if the calculated P values were ≤0.05. Not all of the 1.7-fold changes recorded gave P values of ≤0.05 but were still considered to be relevant as this difference was above the 93–95% threshold value.

The number of spots differentially regulated was substantially more after long-term than after short-term salt stress (Fig. 3). In response to short-term salt stress, the abundance of seven spots increased and two decreased; only one of these spots (1663) was specific to short-term stress, the others all showed similar or enhanced changes in expression by 7 d. After long-term salt stress, a total of 21 spots were found to be up- and 12 down-regulated; of these, 23 were specific to long-term salt stress. The temporal regulation of proteins in response to different salt treatments will be important in understanding their roles in plant adaptation to short- or long-term salt stress.

Identification of proteins responsive to salt stress

Among the 32 spots altering in abundance, 11 have been identified by a combination of matrix-assisted laser-desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS), peptide mass finger printing (PMF), and tandem MS/MS (Table 1). Spot montages for all identified spots are shown in Fig. 4 indicating successful matching between experimental groups. All identifications showed a good correlation of theoretical and experimental pI and Mr. For the majority of identified proteins initial top hits from PMF database searching were confirmed by additional MS/MS analysis of peptides. Each type of data resulted in significant MASCOT scores, which were analyzed statistically and shown to be equal to or greater than the 95% (usually 99–100%) confidence interval cut off for either PMF and/or MS/MS. Some lower intensity samples did not result in enough peptides for PMF analysis; these samples were subjected to MS/MS analysis only. In Table 2, the difference in expression is shown and proteins are grouped into a temporal profile indicating their response to short-, short- and long-, or long-term salt stress. The molecular function, role, and predicted subcellular location for each protein is given in Table 3. The majority of the identified proteins were located to the chloroplast: other proteins identified have roles in mediating iron homeostasis, 1-carbon metabolism, and cell cycle progression.

Identification of proteins responsive to short-term salt stress only

The early effects of salinity on growth are transient so that 24 h after salinization with 50 mM NaCl, leaf growth rates have recovered to their presalinization values and there is no noticeable effect on photosynthesis (Yeo et al., 1991). Nevertheless, a significant change in the level of a chloroplast phosphoglycerate kinase was observed by 24 h. Phosphoglycerate kinase (EC 2.7.2.3) utilizes ATP to phosphorylate 3-phosphoglycerate to form 1,3 bisphosphoglycerate. This reaction represents the first reaction in the reduction step of the Calvin cycle. Increased expression of this enzyme during the initial phase of salt stress could indicate an increase in photosynthetic carbon assimilation following short-term salt stress, perhaps a consequence of a transient increase in the internal concentration of CO₂, following exposure to salt (Yeo et al., 1991).
Identification of proteins responsive to short- and long-term salt stress

Although only a single protein was found whose change in concentration was restricted to the period up to 24 h after exposure to salt, a total of eight proteins changed during both short- and long-term salt stress; of these three have so far been identified (Table 2).

**RuBisCO activase (RCA):** RuBisCO activase was up-regulated 1.7- and 2.5-fold in response to short- and long-term salt stress, respectively. RuBisCO activase is a...
member of the AAA+ family of proteins that have diverse chaperone-like functions. The main role of the activase is the maintenance of the catalytic activity of RuBisCO by removal of inhibitory sugars from the active site of uncarbamylated and carbamylated RuBisCO (Portis, 2003). Increased activase activity may be required to tolerate long-term salt stress due to a direct reduction in stomatal conductance and subsequent low CO2 levels. Low stomatal CO2 will result in increased rates of RuBisCO inactivation through the binding of inhibitory sugars prior to carboxylation. Increases in stromal levels of the large isoform of the activase may directly allow carboxylation to occur at low CO2 levels.

**Ferritin:** Ferritin was found to accumulate by 3.5- and 5.2-fold after short- and long-term salt stress. Plant ferritins are iron-storage proteins able to accommodate 4500 iron atoms in a central cavity (Arosio and Levi, 2002) and are utilized to regulate the iron concentration in chloroplasts in order to prevent iron toxicity. During salt stress, increased oxidative stress could result in the formation of hydroxyl radicals from a reaction between ferrous iron and H2O2 (Fenton reaction). The sequestering of ferrous iron through increased expression of ferritin could act to reduce the production of hydroxyl radicals, the most dangerous reactive oxygen species produced in living cells, during salt stress.

**ATP synthase:** Spot 1900, identified as the ATP synthase β subunit (small isoform), was up-regulated 2.6- and 5-fold at 24 h and 7 d, respectively. The ATP synthase, a large 400 kDa protein complex, consists of an integral membrane CF0 portion and an extrinsic CF1 portion. The CF0 portion forms a transmembrane ion channel for the translocation of protons. The β subunits are found within the extrinsic CF1 portion and are involved in the catalysis of the formation of ATP from ADP and inorganic phosphate (Senior et al., 2002). The increase in ATP synthase could be associated with transiently increased photosynthetic rates and the up-regulation of the Calvin cycle enzyme phosphoglycerate kinase. However, the reason for enhanced expression of ATP synthase after long-term stress is unclear. Photosynthetic rates in rice reduce as the Na+ in the lamina increases (Yeo et al., 1991) and in response to long-term salt stress (Tiwari et al., 1997). Enhanced ATP synthesis in salt-stressed rice may reflect the requirements of processes such as secondary transport mechanisms. ATP may be transported from the stroma to support the increased activity of H+-ATPases required for increased antiporter activity at the plasma and tonoplast membranes during salt stress (NHX1 and SOS1).

**Identification of proteins responsive to long-term salt stress**

A number of proteins showed no change in their expression after 24 h of exposure to salt, but had altered concentrations after 7 d of stress. Seven days of exposure to salt causes significant cellular damage to rice leaves and proteins involved in the repair of cellular damage might be expected to be up-regulated by this time. Other changes may reflect damage to dying cells.

**Superoxide dismutase:** The chloroplast [Cu-Zn] superoxide dismutase was shown to increase in expression after long-term salinity. Superoxide dismutase increased by 1.7-fold after 7 d; no significant change in expression occurred by 24 h. The chloroplast superoxide dismutase forms part of an enzymatic detoxification system for the scavenging of reactive oxygen species (Asada, 1999). Enhanced activity of the chloroplast [Cu-Zn] superoxide dismutase may maintain electron flux in the thylakoids when CO2 levels are decreased in the stroma.

**Porphobilinogen deaminase (PBG deaminase):** Putative porphobilinogen deaminase (PBG deaminase) which catalyses the deamination of porphobilinogen to hydroxymethylbilane (EC 2.5.1.61) (Cornah et al., 2003) and is involved in the synthesis of tetrapyrroles was down-regulated 2.0-fold by long-term salt stress. In photosynthetic cells, the main tetrapyrrole synthesized is chlorophyll, suggesting that down-regulation of PBG deaminase is switching of the biosynthesis of chlorophyll. This could be a pathological consequence of increased Na+ toxicity and the onset of premature senescence.

### Table 1. Table of proteins identified by MALDI-TOF/TOF-MS

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein ID</th>
<th>MALDI-TOF MS</th>
<th>GI</th>
<th>MASCOT score</th>
<th>Protein Cl (%)</th>
<th>Peptide count</th>
<th>M_r</th>
<th>pI</th>
</tr>
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<tbody>
<tr>
<td>1070</td>
<td>Rubisco activase large isoform precursor</td>
<td>8918359</td>
<td>500 (PMF)</td>
<td>100</td>
<td>17</td>
<td>51.8</td>
<td>5.4</td>
<td></td>
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<tr>
<td>1086</td>
<td>S-adenosylmethionine synthetase 1</td>
<td>17529621</td>
<td>489 (PMF)</td>
<td>100</td>
<td>16</td>
<td>43.6</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>1101</td>
<td>GSINBa0011P19.5 (S-adenosylmethionine synthetase)</td>
<td>34809718</td>
<td>391 (PMF)</td>
<td>100</td>
<td>13</td>
<td>43.3</td>
<td>5.7</td>
<td></td>
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<tr>
<td>1663</td>
<td>Putative chloroplast phosphoglycerate kinase</td>
<td>46981258</td>
<td>480 (PMF)</td>
<td>100</td>
<td>10</td>
<td>32.5</td>
<td>9.9</td>
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<tr>
<td>1677</td>
<td>Putative porphobilinogen deaminase</td>
<td>49388602</td>
<td>530 (PMF)</td>
<td>100</td>
<td>17</td>
<td>38.9</td>
<td>7.1</td>
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<tr>
<td>1900</td>
<td>ATP synthase β subunit</td>
<td>6815115</td>
<td>462 (PMF)</td>
<td>100</td>
<td>13</td>
<td>54.0</td>
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<td>2144</td>
<td>Putative 14-3-3 protein</td>
<td>13702816</td>
<td>416 (PMF)</td>
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<td>2400</td>
<td>Ferritin</td>
<td>21686526</td>
<td>116 (MS/MS)</td>
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<td>Ferritin</td>
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<td>123 (PMF)</td>
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<td>2885</td>
<td>Translation initiation factor 5A</td>
<td>3789948</td>
<td>67 (MS/MS)</td>
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<td>2</td>
<td>17.4</td>
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<tr>
<td>2925</td>
<td>Putative superoxide dismutase</td>
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<td>7</td>
<td>20.5</td>
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Fig. 4. Montage of identified spots at control, 24 h and 7 d post-treatment with 50 mM NaCl. Montages were generated automatically after image analysis using Progenesis workstation.
Table 2. Identified leaf lamina proteins altering in expression after short- and long-term salt stress

Changes greater than 1.7-fold are highlighted in bold.

<table>
<thead>
<tr>
<th>Spot</th>
<th>24 h</th>
<th>7 d</th>
<th>Protein ID</th>
<th>Expression</th>
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<td>Fold change</td>
<td>t test (P)</td>
<td>Fold change</td>
<td>t test (P)</td>
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<td>–2.0</td>
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<td>2400</td>
<td>1.3</td>
<td>0.099</td>
<td>2.3</td>
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<td>2885</td>
<td>–1.4</td>
<td>0.021</td>
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Table 3. Protein location, role and molecular function

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein ID</th>
<th>Location</th>
<th>Role</th>
<th>Molecular function</th>
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<td>1663</td>
<td>Putative chloroplast phosphoglycerate kinase</td>
<td>Plastid</td>
<td>Calvin cycle</td>
<td>Phosphoglycerate kinase activity</td>
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<td>1900</td>
<td>ATP synthase β subunit</td>
<td>Plastid</td>
<td>ATP biosynthesis</td>
<td>Nucleotide binding, ATP binding</td>
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<td>1070</td>
<td>Rubisco active large isoform precursor</td>
<td>Plastid</td>
<td>Calvin cycle</td>
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<td>Ferritin</td>
<td>Plastid</td>
<td>Iron homeostasis</td>
<td>Iron sequestration</td>
</tr>
<tr>
<td>1086</td>
<td>S-adenosylmethionine synthetase 1</td>
<td>Unknown</td>
<td>One carbon metabolism</td>
<td>Methione adenosyltransferase</td>
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<td>1101</td>
<td>S-adenosylmethionine synthetase 1, OSJNBa0011P19.5</td>
<td>Unknown</td>
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<td>Methione adenosyltransferase</td>
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<td>1677</td>
<td>Putative porphobilinogen deaminase</td>
<td>Plastid</td>
<td>Porphyrin biosynthesis</td>
<td>Hydroxymethylbilane synthase activity</td>
</tr>
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<td>2959</td>
<td>Putative superoxide dismutase [Cu-Zn], chloroplast</td>
<td>Plastid</td>
<td>Superoxide metabolism</td>
<td>Copper, zinc superoxide dismutase activity</td>
</tr>
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<td>2451</td>
<td>Ferritin</td>
<td>Plastid</td>
<td>Iron homeostasis</td>
<td>Iron sequestration</td>
</tr>
<tr>
<td>2885</td>
<td>Translation initiation factor 5A [Oryza sativa]</td>
<td>Nuclear, Cytoplasmic</td>
<td>Cell cycle</td>
<td>mRNA shuttling</td>
</tr>
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</table>

Translation initiation factor 5A (EIF-5A): Spot 2885, identified as translation initiation factor 5A (eIF-5A), was significantly down-regulated, 1.9-fold, after long-term salt stress. eIF-5A is not required for global translation to occur; its role appears to be in the shuttling of specific mRNAs into the cytoplasm. Transmethylation reactions with AdoMet are vital for the synthesis of lipids, nucleic acids, proteins, and other products of secondary metabolism. AdoMet is also

Ferritin: A second isoform of ferritin was shown to be differentially regulated by long-term salt stress only. This second isoform was resolved by a slight variation in molecular weight, although it was identified as the same protein by MS. The distinct isoforms may be present due to multiple copies of the gene in the rice genome, or due to post-transcriptional, translational modification of the ferritin gene or protein. Unfortunately, the modification cannot be characterized by the experimentation done here. The importance of two distinct isoforms of ferritin in salt stress is evident from their temporal differences in expression. The light isoform is responsive to both short- and long-term salt stress and up-regulated 3.5- and 5.2-fold, respectively. The heavy isoform is shown only to respond significantly (2.3-fold) after long-term salinity. The independent regulation of ferritin isoforms by short- and long-term salinity indicates that different cues control the expression of this protein. Evidence for the induction of different ferritin genes has been observed in maize: induction of two separate ferritin genes (ZmFer1 and ZmFer2) in response to iron appears to include ABA-dependent and -independent components (Fobisloisy et al., 1995; Lobreaux et al., 1995).

S-adenosyl-L-methionine synthetase: Two proteins encoding different isozymes of S-adenosyl-L-methionine synthetase were significantly down-regulated, 4.3- and 2.0-fold, respectively, by long-term salt stress. Expression of several transcripts encoding SAMS have been shown to be down-regulated in rice (Kawasaki et al., 2001). S-adenosyl-L-methionine synthetase (SAMS) (EC 2.5.1.6) catalyses the formation of S-adenosyl-L-methionine (AdoMet) from L-methionine and ATP. AdoMet is an important methyl group donor utilized in most transmethylation reactions. Transmethylation reactions with AdoMet are vital for the synthesis of lipids, nucleic acids, proteins, and other products of secondary metabolism.
utilized as a common precursor for biosynthesis of the phytohormone ethylene, polyamines and is required for the production of phenylpropanoid, a constituent of the cell wall. Reduced SAMS might result in reduced production of the plant hormone ethylene, although in rice, salinity stimulates ethylene biosynthesis in tolerant cultivars, but has little effect in more sensitive lines (Lutts et al., 1996). In *A. thaliana*, reduced expression of the ethylene receptors has been reported after exposure to salt stress (Zhoa and Schaller, 2004). SAMS have also been shown to be involved in the biosynthesis of lignin and the polyamine glycine betaine during salt stress (Sanchez-Aguayo et al., 2004; Tabuchi et al., 2004). In these studies an increase in SAMS abundance or activity has been shown in association with increased lignification and glycine betaine synthesis. The down-regulation of SAMS observed in rice suggests that these isoforms are not involved in the biosynthesis of osmolytes (rice does not synthesize glycine betaine; Hall et al., 1978) or lignin. Without further analysis the roles of the SAMS identified can only be speculative.

**Conclusion**

The quantity of published protein expression data being generated by 2-D gel electrophoresis is rapidly growing. As more laboratories embark on proteomic studies, interpretation of these data will require strict control of data quality and experimental conditions. Procedures that result in accurate expression analysis by 2-DE are required for every biological system under investigation.

The analysis of salt-responsive proteins has indicated that changes in time-dependent expression of specific proteins occurs following salinization. Of the proteins identified, expression analysis identified only one protein, phosphoglycerate kinase, that altered specifically within 24 h. Other proteins, RuBisCO activase and ATP synthase, were shown to respond by 24 h post-salt treatment and continue to be differentially expressed as exposure to salt continued. By contrast, two SAMS protein isoforms showed no change in 24 h, but were down-regulated after long-term exposure to salt. The response of superoxide dismutase (SOD) enzyme identified in this analysis can be interpreted as a pathological response to severe salt stress, correlated with its role in the tolerance of oxidative stress in the chloroplast. The temporal regulation of two isozymes of ferritin indicated a specific role for these isoforms in response to both short- and long-term salinity. The function of ferritin highlights the importance of controlling intra-plastid iron concentrations during both early and late responses to salinity. Two proteins, PBG deaminase and EIF-5A, were down-regulated after long-term salt stress. The function of these proteins in chlorophyll biosynthesis and cell cycle control denotes possible mechanisms for the initiation of premature senescence during long-term salt stress.

The work described here tested the performance, robustness, and reproducibility of a platform developed for comparative proteomics: the aim was an experimental design that produced biologically meaningful data. From the analysis of already published proteomics data on salt stress in rice it was clear that there was considerable room for improvements in data quality. Initial work was focused on testing the robustness and quantitative reproducibility of the rice lamina 2-DE system developed in our laboratory. This work demonstrated the applicability of biological replicates and indicated the threshold value to use for assigning differences in expression. These data also exemplified the overall quality of data produced and thus provides a sound basis for expression study’s and inter-laboratory comparisons. The applicability of this approach to the identification of real biological difference was demonstrated by the analysis of short- and long-term salt stress in the leaf lamina of rice. Analysis resulted in the separation of approximately 2500 protein species of which 32 were observed to be significantly regulated by salinity; 11 of these have so far been identified by mass spectrometry and their molecular function ascertained. Linking the function to a role in salt stress responses remains more elusive and for some proteins will ultimately require complementary approaches. The results illustrate both the strength and weakness of current proteomics studies. By only providing a snapshot of biochemical processes the role of some proteins will be elusive without complementary approaches. The strength of proteomics is to generate new areas of research by revealing novel mechanisms involved in the regulation of biochemical processes.

**Supplementary data**

Supplementary data are available at JXB online.

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**References**


