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# Proteomic analysis of seed germination under salt stress in soybeans*

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**Abstract:** Soybean (*Glycine max* (L.) Merrill) is a salt-sensitive crop, and its production is severely affected by saline soils. Therefore, the response of soybean seeds to salt stress during germination was investigated at both physiological and proteomic levels. The salt-tolerant cultivar Lee68 and salt-sensitive cultivar N2899 were exposed to 100 mmol/L NaCl until radicle protrusion from the seed coat. In both cultivars, the final germination percentage was not affected by salt, but the mean germination times of Lee68 and N2899 were delayed by 0.3 and 1.0 d, respectively, compared with controls. In response to salt stress, the abscisic acid content increased, and gibberellic acid ($GA_{1+3}$) and isopentenyladenosine decreased. Indole-3-acetic acid increased in Lee68, but remained unchanged in N2899. The proteins extracted from germinated seeds were separated using two-dimensional gel electrophoresis (2-DE), followed by Coomassie brilliant blue G-250 staining. About 350 protein spots from 2-DE gels of pH range 3 to 10 and 650 spots from gels of pH range 4 to 7 were reproducibly resolved, of which 18 protein spots showed changes in abundance as a result of salt stress in both cultivars. After matrix-assisted laser desorption ionization-time of flight-mass spectroscopy (MALDI-TOF-MS) analysis of the differentially expressed proteins, the peptide mass fingerprint was searched against the soybean UniGene database and nine proteins were successfully identified. Ferritin and 20S proteasome subunit β-6 were up-regulated in both cultivars. Glyceraldehyde 3-phosphate dehydrogenase, glutathione *S*-transferase (GST) 9, GST 10, and seed maturation protein PM36 were down-regulated in Lee68 by salt, but still remained at a certain level. However, these proteins were present in lower levels in control N2899 and were up-regulated under salt stress. The results indicate that these proteins might have important roles in defense mechanisms against salt stress during soybean seed germination.

**Key words:** Proteomics, Salt stress, Seed germination, Soybean
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## 1 Introduction

Agricultural productivity and the eco-environment are severely affected by soil salinity. It is estimated that about 20% of irrigated land, which yields one-third of the world's food, is affected by salinity. Moreover, a significant proportion of recently cultivated agricultural land has become saline because of land clearing or irrigation (Munns, 2005). Salinity is one of the major constraints limiting plant growth in some of the most productive agricultural regions of the world (Boyer, 1982). Therefore, the need to develop salt-tolerant cultivars is unavoidable.

However, soybean (*Glycine max* (L.) Merrill), an important crop that provides fatty acids and proteins for humans and animals, is a salt-sensitive crop (Luo *et al*., 2005), and its production is severely

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affected by saline soils. Saline soils significantly alter plant metabolic processes (Levitt, 1980). A high concentration of salt causes ion imbalance, hyperosmotic stress, and oxidative damage (Zhu, 2002). In response to these, plants resort to various pro-survival strategies, most of which are preceded by specific changes in expression levels of proteins whose biological functions are related to salt stress tolerance. The proteomic approach, based on reproducible two-dimensional gel electrophoresis (2-DE) and powerful mass spectrometry (MS) analyses, offers the possibility of identifying those proteins (Gygi and Aebersold, 2000). In fact, the proteomics of soybean in response to abiotic stress have been studied (Zhen *et al.*, 2007; Aghaei *et al.*, 2008; Toorchi *et al.*, 2009; Cheng *et al.*, 2010; Nouri and Komatsu, 2010; Sobhanian *et al.*, 2010). Although the majority of defense proteins are expressed in optimal growth conditions, they have been found to be either overexpressed or underexpressed during stress. These investigations showed that a proteomic approach is useful for analyzing the physiological changes and the functions of stress-induced proteins.

Proteomic analyses also have been performed to analyze soybean seed proteins in mature seeds (Mooney and Thelen, 2004), seed filling (Hajduch *et al.*, 2005; Agrawal *et al.*, 2008), and seed germination (Xu *et al.*, 2006). However, seed germination is one of the most crucial and decisive phases in the growth cycle of plants, because it determines plant establishment and the final yield of the crops. Seeds and young seedlings are frequently confronted with much higher salinities than vigorously growing plants, because germination usually occurs in surface soils, which accumulate soluble salts as a result of evaporation and the capillary rise of water (Almansouri *et al.*, 2001). Limited information is available about salt-response genes in soybean, and the study of protein expression in response to salinity may therefore help identify the related genes and provide a detailed network of salt adaptation mechanisms in this important crop (Aghaei *et al.*, 2008). In this study, proteomic analysis was carried out to identify salt stress-responsive proteins in soybean seed germination in two cultivars, Lee68 (salt-tolerant) and N2899 (salt-sensitive). The application of abiotic stress can result in an altered level of plant growth hormones (Morgan, 1990); therefore, we also investigated the levels of the endogenous hormones indole-3-acetic acid (IAA), gibberellic acid (GA), abscisic acid (ABA), and isopentenyladenosine (iPAs) in seeds germinated under salinity.

## 2 Materials and methods

### 2.1 Plant materials and growth condition

Soybean seeds (*Glycine max* cv. Lee68 and cv. N2899) were obtained from the Chinese National Center for Soybean Improvement. Lee68 is known for its high salt tolerance, while N2899 is a salt-sensitive cultivar identified in our laboratory. For each cultivar, hand-selected seeds of uniform size were sterilized in 1 mg/ml $HgCl_2$ (Genebase) for 2 min and thoroughly washed in distilled water. Seeds were germinated in Petri plates containing Whatman No. 1 filter paper moistened with distilled water or with 100 mmol/L NaCl (Genebase) solution and kept in the dark at 25 °C. A seed was scored as germinated if the primary root reached 2–3 mm length. Seeds were germinated up to 12 d in the dark. Every 12 h, the percentage of germinated seeds (containing abnormal growth) was calculated from three replicates of 300 seeds each. Seeds that had germinated such that the radicle protruded by 2–3 mm from the seed coat were harvested and stored at −80 °C until use (Fig. 1).

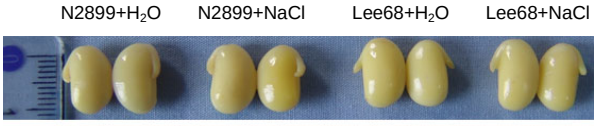


**Fig. 1 Germinated soybean seeds of Lee68 and N2899 used in the experiment**
N2899+$H_2O$: control N2899; N2899+NaCl: NaCl-treated N2899; Lee68+$H_2O$: control Lee68; Lee68+NaCl: NaCl-treated Lee68

### 2.2 Extraction and determination of plant hormones

Extraction and immunoassay of IAA were carried out according to Chen *et al.* (1998b). Extraction and immunoassay of gibberellin $A_1$ & $A_3$ ($GA_{1+3}$) were carried out as described by Chen *et al.* (1998a). Extraction and immunoassay of iPAs and ABA were performed as described by Chen *et al.* (1997). Each assay was replicated three times. The data were

analyzed by analysis of variance (ANOVA) and Student's *t*-test. All data presented are the mean values.

### 2.3 Preparation of the protein extract

Total proteins from germinated seeds were extracted according to a modified procedure based upon that of Watson *et al.* (2003). In brief, a frozen sample was ground in a mortar with liquid nitrogen and incubated with 0.1 g/ml trichloroacetic acid (TCA) (Genebase) and 20 mmol/L dithiothreitol (DTT; Bio-Rad) in acetone (Genebase) at −20 °C for 1 h. The precipitated proteins were pelleted and washed repeatedly with ice-cold acetone containing 20 mmol/L DTT to remove pigments and lipids until the supernatant was colorless. The protein pellet was dried under a vacuum and resuspended in buffer containing 7 mol/L urea (Bio-Rad), 2 mol/L thiourea (Bio-Rad), 0.04 g/ml 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS; Bio-Rad), 0.2% (v/v) carrier ampholyte (pH 3–10 or pH 4–7; Bio-Rad), and a cocktail of protease inhibitors (Sigma). Samples were mixed on a vortex mixer for 30 s and ultrasonicated using a VCX600 for 3 min. The insoluble tissue was removed by centrifugation at 15000×*g* for 15 min (Beckman). The supernatant was stored at −80 °C. The protein concentration was determined according to Bradford (1976), with bovine serum albumin (BSA; Amresco) as a standard.

### 2.4 Two-dimensional polyacrylamide gel electrophoresis and image analysis

Immobilized pH gradient (IPG) strips (17 cm, pH 3–10, linear gradient; or 17 cm, pH 4–7, linear gradient; Bio-Rad) were rehydrated at 50 V for 12 h at 20 °C with 350 μl rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 0.04 g/ml CHAPS, 0.2% (v/v) carrier ampholyte, 0.01 g/ml DTT) containing about 1.0 mg (pH 3–10) or 1.5 mg (pH 4–7) of proteins. Focusing was carried out in a Bio-Rad Protean isoelectric focusing (IEF) cell. The voltage setting was 200 V for 30 min, 500 V for 30 min, 1000 V for 1 h, 2000V for 1 h, and 8000 V for 5 h, to a total about 50000 V·h. After, IPG strips were equilibrated for 2×10 min in 6 mol/L urea, 30% (v/v) glycerol (Bio-Rad), 0.05 g/ml sodium dodecyl sulfate (SDS; Bio-Rad) in 0.05 mol/L Tris-HCl (pH 6.8; Genebase) containing 0.01 g/ml DTT for the first equilibration step and 0.025 g/ml iodoacetamide (Bio-Rad) for the second equilibration step. The samples were then transferred onto a 0.12 g/ml polyacrylamide gel. Electrophoresis was performed in Tris/glycine/SDS buffer on a Multiphor system (Amersham Pharmacia Biotech) according to the manufacturer's recommendations. For calibration, low-molecular weight marker proteins (Amersham Biosciences) were applied on the gel via a small piece of filter paper. Gels were stained overnight with Coomassie brilliant blue G-250 (Genebase) according to Neuhoff *et al.* (1988) and scanned using VersaDoc image system (Bio-Rad). 2-DE gels were processed using PDQuest software V.7.3 (Bio-Rad). Spot quantity normalization occurred through the whole match set, which included all 12 2-DE gels. The gel used as reference in the PDQuest match set corresponded to a co-migration of protein extracts from both Lee68 and N2899 cultivars. Only those with significant (quantitative changes more than two-fold in abundance) and reproducible changes in three replicates were used for further analysis.

### 2.5 Protein in-gel digestion and matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS)

Protein spots of interest were excised from the stained gels and in-gel digestion was performed as follows. Gel pieces were washed three times with Milli-Q water, and 50% (v/v) acetonitrile (Sigma) containing 100 mmol/L ammonium bicarbonate (Genebase) was used to remove the dye. Proteins in the gels were reduced in 10 mmol/L DTT dissolved in 50 mmol/L $NH_4HCO_3$ (Genebase) solution for at least 1 h at 56 °C, and then incubated with 50 mmol/L iodoacetamide in 50 mmol/L $NH_4HCO_3$ at room temperature for 40 min. Gels were then dried by vacuum centrifugation, and incubated for 14 h at 37 °C with 10 μl of 12.5 μg/ml trypsin (modified porcine trypsin, sequencing grade, Promega) in 50 mmol/L $NH_4HCO_3$. The resulting tryptic fragments were eluted by diffusion into 50% (v/v) acetonitrile and 0.5% (v/v) TCA, and dried in a speed vacuum. The dried samples were resuspended in 2 μl 0.5% (v/v) trifluoroacetic acid (Genebase). Each sample was mixed with the supernatant of 60% (v/v) acetonitrile saturated with α-cyano-4-hydroxycinnamic acid (Genebase) (1:1, v/v), and then air-dried on the flat surface of a sample plate. The samples were then analyzed with MALDI-TOF-MS (Reflex III, Bruker, Germany) in

positive ion reflector mode at an accelerating voltage of 20 kV. Spectra were calibrated using trypsin autolysis products (*m*/*z* 842.51 and 2211.10) as internal standards, and a mixture of standard peptides as external standards.

### 2.6 Database queries and protein identification

Protein identification was performed by querying peptide mass fingerprinting (PMF) data in the soybean UniGene database (ftp://ftp.ncbi.nih.gov/repository/UniGene/Glycine_max/) using MS-Fit program of Protein Prospector (http://prospector.ucsf.edu). The following parameters were used for database searches with MALDI-TOF peptide mass data: mono-isotopic peak; mass tolerance, 0.2 Da; missed cleavages, 1; and allowed modifications, carbamidomethylation of Cys and oxidation of Met. A positive identification of the protein followed the procedure of Hajduch *et al.* (2005).

## 3 Results

### 3.1 Effects of salinity on seed germination

Under optimal conditions, over 98% of Lee68 and N2899 seeds germinated after a mean of 1.5 and 2.1 d, respectively. When exposed to 100 mmol/L NaCl, the final germination percentage in both cultivars was not affected. However, the mean germination times of Lee68 and N2899 were delayed by 0.3 and 1.0 d, respectively, compared to the controls. In addition, control Lee68 and N2899 seeds required 1.2 and 1.5 d, respectively, to reach 50% germination, while seeds germinated in the presence of 100 mmol/L NaCl required 1.5 and 2.5 d, respectively. From imbibition till maximum germination, Lee68 and N2899 took 3.5 and 4.5 d, respectively. Under salt stress this period was 3.5 and 6.5 d, respectively (Fig. 2). We also observed that there were many more abnormally germinated N2899 seeds compared to Lee68. Moreover, germination of N2899 seeds was completely inhibited by exposure to over 150 mmol/L NaCl (data not shown). These observations suggest that 100 mmol/L NaCl affected soybean seed germination, especially in N2899. However, moderate salt stress intensity only delayed germination time, and did not have a severe impact on the final germination percentage.

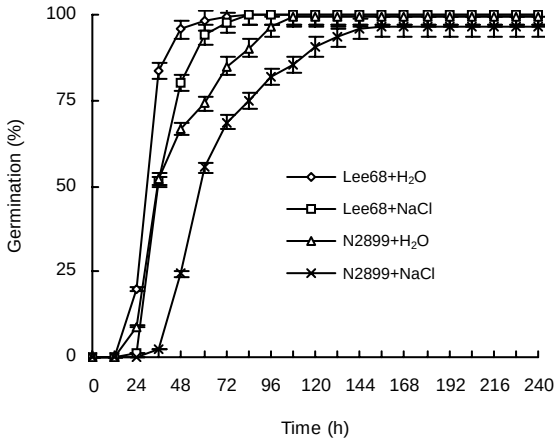


**Fig. 2 Effects of 100 mmol/L salt concentration on the germination percentages of Lee68 and N2899 seeds**
Lee68+$H_2O$: control Lee68; Lee68+NaCl: NaCl-treated Lee68; N2899+$H_2O$: control N2899; N2899+NaCl: NaCl-treated N2899

### 3.2 Effects of salinity on IAA, $GA_{1+3}$, ABA, and iPAs levels

Hormone levels are related to seed germination and stress. Therefore, we detected the endogenous ABA, IAA, $GA_{1+3}$, and iPAs levels in Lee68 and N2899 seeds germinated in moderate salinity conditions (Fig. 3). The IAA content of salt-treated germinated Lee68 seeds was significantly higher than that of the control ($P$<0.01). There was no difference in IAA levels among control Lee68, salt-treated N2899, and control N2899 seeds ($P$>0.05). Salinity had a stronger effect on the $GA_{1+3}$ level. The $GA_{1+3}$ content significantly decreased in both cultivars under salt stress ($P$<0.001). The $GA_{1+3}$ content in control Lee68 was higher than that in control N2899 ($P$<0.05). ABA content significantly increased in response to salinity ($P$<0.001), while the ABA content in salt-treated N2899 was lower than that in control Lee68 ($P$<0.001). The iPAs content in these four samples was low compared with the other hormones and was decreased by salt in both cultivars ($P$<0.01). However, the iPAs content in salt-treated N2899 was higher than that in control Lee68 ($P$<0.01).

### 3.3 2-DE maps of Lee68 and N2899

Soybean Lee68 and N2899 displayed different seed vigors and germination times. Therefore, we investigated the proteins expressed in the germinated

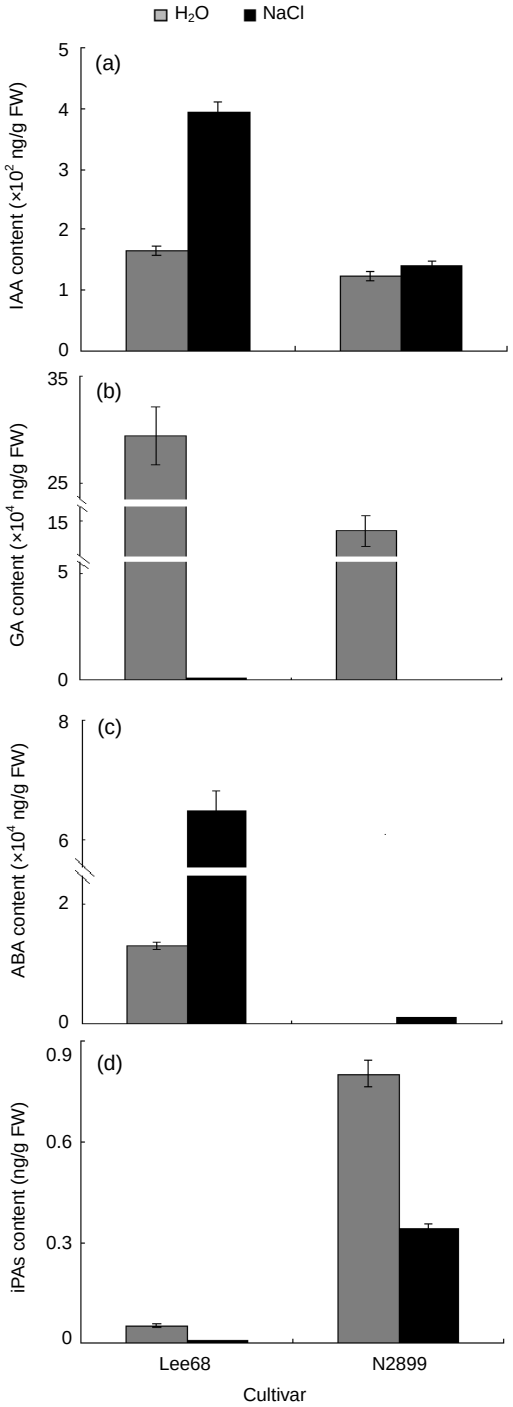


**Fig. 3 Effects of salinity on IAA (a), GA (b), ABA (c), and iPAs (d) in Lee68 and N2899 germinated seeds**

seeds. Total proteins from Lee68 and N2899 germinated seeds were extracted and separated by 2-DE gels. Initial separations were performed with pH 3–10 IPG strips. The gel maps showed that about 70% of the protein spots were in the region of pH 4–7, as analyzed by PDQuest software (Fig. 4). Therefore, additional analyses with pH 4–7 IPG strips were performed to improve spot resolution. The 2-DE maps of the control Lee68 and N2899 germinated seeds in the pH 3–10 and pH 4–7 ranges are shown in Fig. 4. About 350 protein spots could be resolved in pH 3–10 2-DE gels and 650 protein spots in pH 4–7 2-DE gels stained by Coomassie brilliant blue G-250. In all, there were more than 90 differentially expressed proteins between the 2-DE maps of Lee68 and N2899, although the majority of protein spots were similar. These differentially expressed proteins showed varietal differences and might be associated with resistances, agronomic traits, and qualities between cultivars.

### 3.4 Salt-responsive proteins

To investigate the response of soybean seed germination to non-lethal concentration salinity levels, Lee68 and N2899 seeds were exposed to 100 mmol/L NaCl until the radicle protruded from the seed coat. Whole proteins from germinated seeds were analyzed by high-resolution 2-DE. Eighteen protein spots showed more than two-fold reproducible differences in abundance as a result of salt stress in both cultivars. The positions of these proteins are indicated by number in Fig. 4. Ten proteins were in the pH 4–7 2-DE gels and eight in the pH 3–10 2-DE gels. The relative abundances of these proteins are shown in Fig. 5. Compared with their controls, 10 proteins were differentially displayed in Lee68 2-DE gels and 12 in N2899 2-DE gels under salt stress. Fig. 6 shows the enlarged maps of the 18 protein spots. These changes were relatively small when compared with the soybean cultivars, but they directly responded to salt stress.

These differentially expressed proteins were excised from the 2-DE gels and subjected to MALDI-TOF-MS. All proteins obtained PMF. By querying the PMF in the soybean UniGene database, nine proteins were successfully identified (Table 1). The data in Table 1 include assigned protein spot numbers, experimental molecular mass and pI, theoretical molecular mass and pI, number of peptides matched, sequence coverage, number of UniGene accessions, and homologous proteins. The possible functions of these proteins are discussed below.

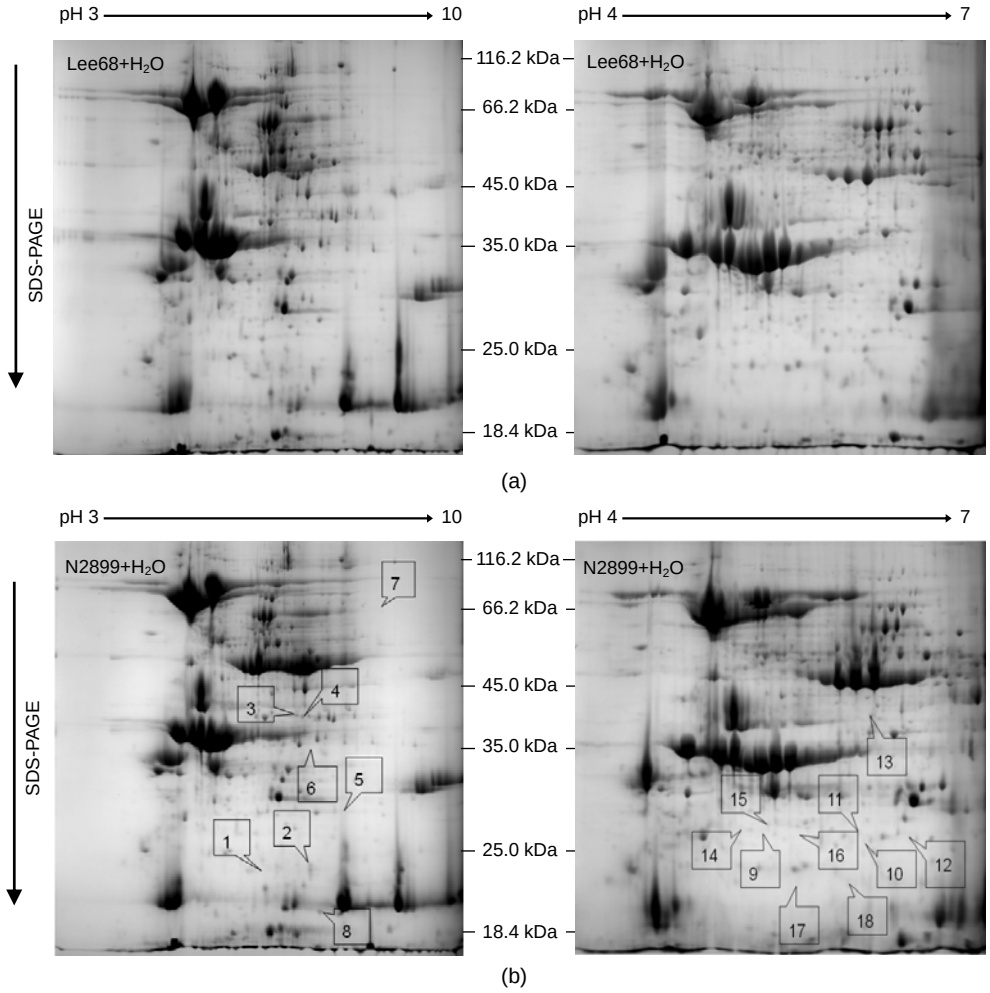


**Fig. 4 2-DE maps of the control Lee68 and N2899 seeds germinated at pH 3–10 and pH 4–7**
(a) Lee68+$H_2O$: control Lee68; (b) N2899+$H_2O$: control N2899

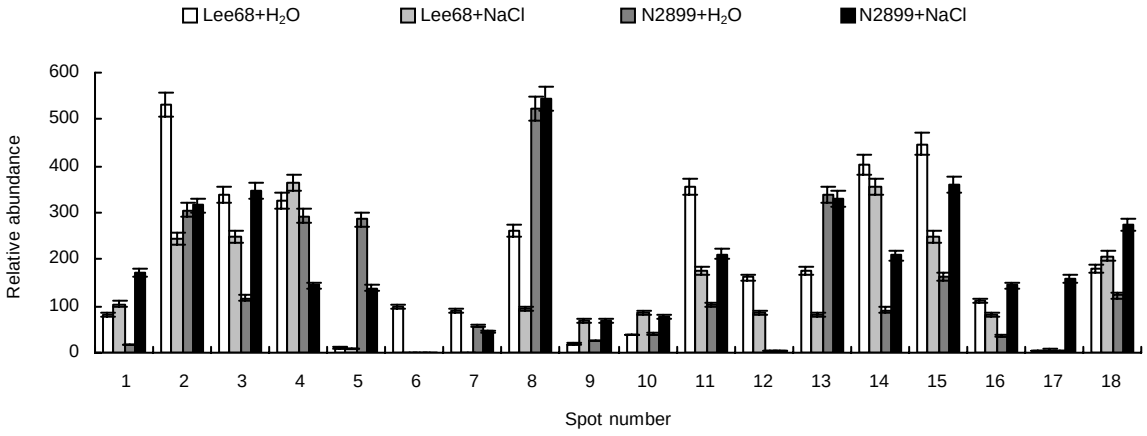


**Fig. 5 Histograms showing the volume changes of 18 differentially expressed spots in Lee68 and N2899 seeds germinated under salt stress**
Lee68+$H_2O$: control Lee68; Lee68+NaCl: NaCl-treated Lee68; N2899+$H_2O$: control N2899; N2899+NaCl: NaCl-treated N2899

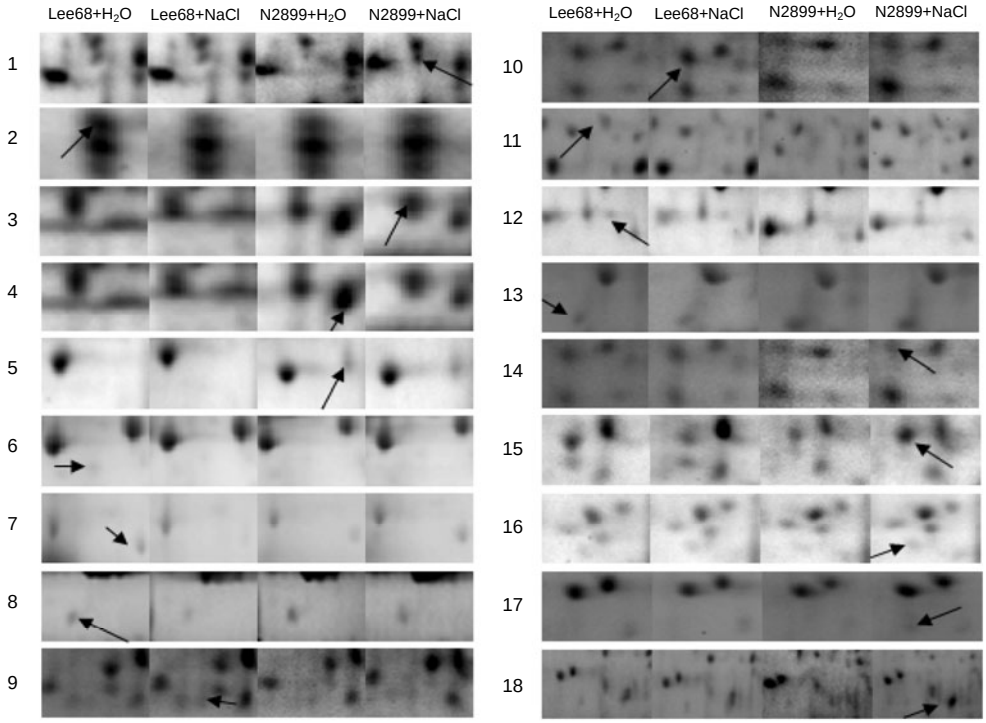


**Fig. 6 Enlarged maps of the 18 differentially expressed protein spots in Lee68 and N2899 seeds germinated under salt stress**

Lee68+$H_2O$: control Lee68; Lee68+NaCl: NaCl-treated Lee68; N2899+$H_2O$: control N2899; N2899+NaCl: NaCl-treated N2899

**Table 1 Differentially expressed proteins identified by PMF query**

| Spot No. | Theoretical $M_r$/pI | Experimental $M_r$/pI | No. of peptides matched | Sequence coverage (%) | Accession No. | Protein name |
|---|---|---|---|---|---|---|
| 1 | 28.7/6.3 | 22.7/6.7 | 8 | 31 | Gma.15591 | Unknown protein |
| 3 | 36.9/6.4 | 38.7/7.2 | 7 | 61 | Gma.17053 | Glyceraldehyde 3-phosphate dehydrogenase |
| 7 | 63.8/8.2 | 62.9/8.3 | 19 | 25 | Gma.8130 | Malate synthase |
| 9 | 28.1/5.7 | 26.7/5.3 | 6 | 23 | Gma.18200 | Ferritin |
| 10 | 24.9/6.3 | 26.2/6.1 | 5 | 26 | Gma.448 | 20S proteasome β subunit |
| 11 | 25.4/5.7 | 27.0/6.0 | 7 | 30 | Gma.1917 | Glutathione *S*-transferase (GST) 10 |
| 14 | 26.1/5.8 | 26.2/6.1 | 5 | 27 | Gma.7612 | Hypothetical 26.0-kDa protein |
| 15 | 27.4/5.9 | 27.0/6.0 | 5 | 19 | Gma.8540 | Seed maturation protein PM36 |
| 16 | 23.6/5.7 | 26.8/5.6 | 4 | 25 | Gma.8517 | Glutathione *S*-transferase (GST) 9 |

## 4 Discussion

### 4.1 Phytohormones

We investigated the effects of salinity on endogenous IAA, $GA_{1+3}$, ABA, and iPAs in germinated seeds of Lee68 and N2899. The ABA content increased, and $GA_{1+3}$ and iPAs decreased in response to salinity, while the change of IAA content depended on the cultivar. IAA has a major role in regulating plant growth. Bianco and Defez (2009) reported that the IAA-overproducing RD64 strain showed an increased tolerance to 0.5 mol/L NaCl in *Medicagotruncatula*. IAA can strengthen the capacity of resistance of the soybean to saline environment (Wei and Chen, 2000). The level of IAA in salt-treated Lee68 was more than two-fold higher than that in the control, but was the same as that in N2899. The increased IAA in salt-tolerant Lee68 might help seed

germination under salt stress. The decreased endogenous GA and increased ABA contents have been observed in salt-stressed soybean. $GA_3$ ameliorates the adverse effects of salt stress and restores normal growth and development of soybean (Hamayun *et al.*, 2010b). Germination of stressed seeds was partially restored by the addition of exogenous cytokinin (CTK) (Gidrol *et al.*, 1994). However, Kaur *et al.* (1998) found that $GA_3$ was more effective than CTK in enhancing the reduced germination and seedling growth of chickpea seeds under salt stress. GA synthesis affects not only alterations in protein expression, but also increases in germination rate, promotes root and shoot length during seed germination, and promotes early seedling development (Gallardo *et al.*, 2002; Kim *et al.*, 2008). In the present study, GA content was significantly reduced and seed germination time was delayed by salt. ABA is involved in responses to environmental stress such as salinity (Jia *et al.*, 2002), and is required by the plant for stress tolerance (Hamayun *et al.*, 2010a). Umezawa *et al.* (2001) found that the leaf ABA content in Lee (salt-tolerant) increased significantly under salt stress, while that in Enrei (salt-sensitive) showed only a slight increase. It is thus possible that ABA enhances salt tolerance in soybean. Our results also showed that salinity increased the ABA contents of both cultivars. Moreover, the ABA level in Lee68 was much higher than that in N2899, confirming that it might be related to the salt stress tolerance of Lee68. The contents and change ranges of hormones, except iPAs, in Lee68 were higher than those in N2899. This might be one of the reasons why N2899 is more sensitive to salt than Lee68.

### 4.2 Differentially expressed proteins

In the plant life cycle, seed germination and seedling stages are key developmental stages conditioning the final yield of crops. Both are very sensitive to salt stress. Consequently, researchers have submitted crop seeds to salt treatments and sampled them after varying stress time. However, germination and seedling growth are delayed by salt. Therefore, we studied all soybean seeds that completed the germination process under salt stress in two cultivars with different salt tolerances. We then sampled the germinated seeds whose radicles protruded from the seed coat by 2–3 mm. Eighteen protein spots showed changes in abundance in response to 100 mmol/L NaCl stress in the two cultivars, of which nine proteins were identified.

Spots 9 and 10 were up-regulated by salt in both cultivars. Spot 9 was identified as ferritin. Ferritin, a class of iron-storage proteins, is composed of at least two different subunits and its level decreases gradually during soybean germination (Masuda *et al.*, 2001). However, the abundance of ferritin increased in response to salt stress in both cultivars. A high concentration of salt leads to oxidative damage. Ferritin could be involved in defense mechanisms against iron-mediated oxidative stress (Briat *et al.*, 1999). Proteomic investigations on soybean root also revealed the accumulation ferritin under drought stress (Alam *et al.*, 2010). Therefore, it might have an important role in soybean seed germination and seedlings under stress. Induction of ferritin by ABA has been documented at both transcript and protein levels (Ravet *et al.*, 2009). In maize plantlets, iron overload led to a five-fold increase in ABA concentration in roots and leaves, and ferritin mRNA accumulated in response to exogenous ABA treatment (Lobréaux *et al.*, 1993). In this study, the endogenous ABA and ferritin levels were up-regulated by salt. The result supports the possible involvement of ABA in ferritin gene regulation.

Spot 10 was identified as 20S proteasome subunit β-6. The 20S proteasome is the catalytic core of the 26S proteasome, a proteolytic complex involved in recognizing and catabolizing ubiquitin-protein to remove abnormal proteins (Sassa *et al.*, 2000; Smalle and Vierstra, 2004). The proteasome also operates in the stress response by removing abnormal proteins (Imin *et al.*, 2006). 20S proteasome α subunit A was up-regulated in soybean under osmotic stress (Toorchi *et al.*, 2009). The up-regulation of the 20S proteasome in both cultivars after stress could be associated to the degradation of oxidatively-damaged proteins caused by salt stress. Recent evidence has suggested a role for the ubiquitin-proteasome pathway in CTK, GA, and ABA signalings (Itoh *et al.*, 2003). 26S ubiquitin-proteasome regulatory subunit 4 homolog levels were increased during rice seed germination by GA and ABA (Kim *et al.*, 2008).

Spots 1, 3, 14, and 16 were up-regulated by salt in N2899 and were unaffected in Lee68. Although Spots 1 and 14 were matched with UniGenes, providing evidence for the existence of the proteins, they

were annotated as unknown proteins. Glyceraldehyde 3-phosphate dehydrogenase (GPD) (Spot 3) is a ubiquitous enzyme involved in glycolysis and gluconeogenesis (Duée *et al.*, 1996). Basic metabolism change is a general response to stress. GPD is one of target genes regulating the response of cells to salt stress and may aid in the development of new salt-tolerant cultivars in soybean (Nouri *et al.*, 2011). Jeong *et al.* (2001) transferred *GPD* gene to potato to improve salt tolerance in transgenic potato plants. Spot 16 was identified as glutathione *S*-transferase (GST) 9. GSTs are encoded by a large and diverse gene family in plants and there are 25 identified accurate full-length sequence clones in soybean. It is clear that GST activity levels frequently increase in response to stimuli that cause oxidative damage, but the mechanisms involved in protection are unclear (McGonigle *et al.*, 2000). Overexpression of GSTs and glutathione peroxidases enhanced the growth of transgenic tobacco seedlings during chilling and salt stress (Roxas *et al.*, 1997). GSTs have an important role in the response of plants to changing environmental conditions. Therefore, gene engineering strategies involving GSTs could improve soybean salt tolerance. Furthermore, they might have a role in hormonal regulation (Marrs, 1996). Kim *et al.* (2008) reported that GST was modulated by GA and ABA.

Spots 11 and 15 were down-regulated in Lee68, but up-regulated in N2899 under salt stress. Interestingly, Spot 11 was identified as GST 10. This result indicates that one protein changes its expression differently between cultivars and that different members of a family are differentially regulated in the same cultivar under salt stress. Spot 15 was identified as seed maturation protein PM36. It is synthesized at late embryogenesis and is degraded rapidly at the early stage of seed germination (Blackman *et al.*, 1991; Hsing *et al.*, 1998). In contrast to most seed maturation proteins, protein PM36 contains a TENA_THI-4 conservative domain. It was predicted that PM36, similarly to seed maturation protein PM4, acted as a storage form of biotin to support seedling growth during germination (Hsing *et al.*, 1998). In the present study, PM36 was up-regulated by salt in N2899. We infer that PM36 might be similar to other late embryogenesis abundant (LEA) proteins and has a protective role in the plant cell under stress conditions (Skriver and Mundy, 1990).

Spot 7 was down-regulated by salt in Lee68, but was unaffected in N2899. Malate synthase (Spot 7) is a characteristic enzyme of the glyoxylate cycle. In germinating oilseeds, the glyoxylate cycle has a key role in converting acetyl-coenzyme A produced by fatty acid β-oxidation into oxaloacetate, and subsequently into sugar (Smith, 2002). It was confirmed that malate synthase was activated in the prolonged anaerobic environment (Ying *et al.*, 2005). The significance of the diminished levels of malate synthase in Lee68 requires further research.

In summary, the proteins we identified are related to salt stress. GPD, GST 9, GST 10, and seed maturation protein PM36 have protective roles in stress tolerance. In control Lee68, these proteins were maintained a high level and, although they were down-regulated by salt stress, they still remained at a certain level. However, in control N2899, their levels were the lowest and were up-regulated by salt stress. It can be hypothesized that these proteins help seed germination under salt stress and Lee68 itself has a stronger resistance mechanism. NaCl at 100 mmol/L might represent low salinity to Lee68, but relatively high salinity to N2899. We could also infer this interpretation from the delayed mean germination time and increased duration of germination. Therefore, N2899 was injured more than Lee68 under a non-lethal concentration of NaCl (100 mmol/L). To achieve the process of germination, these defensive proteins were up-regulated.

The expression and regulation of genes under salt stress is a complicated process, and is affected by experimental materials, salt concentration, treatment methods, and stress time. Our research contributes to the understanding of the mechanisms of seed germination in response to salt stress. Future studies will be directed towards the function of the identified proteins and the regulatory network of hormones in the salt response.

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