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ORIGINAL ARTICLE

Proteome analysis of soybean hypocotyl and root under salt stress

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Abstract To evaluate the response of soybean to salt stress, the related changes in protein expression were investigated using the proteomic approach. Soybean plants were exposed to 0, 50, 100, and 200 mM NaCl. Especially at 200 mM, the length and fresh weight of the hypocotyl and root reduced under salt stress, while the proline content increased. Proteins from the hypocotyl and root treated with 100 mM NaCl were extracted and separated by two-dimensional polyacrylamide gel electrophoresis; 321 protein spots were detected. In response to salt stress, seven proteins were reproducibly found to be up- or down-regulated by two to sevenfold: late embryogenesis-abundant protein, β-conglycinin, elicitor peptide three precursor, and basic/helix-loop-helix protein were up-regulated, while protease inhibitor, lectin, and stem 31-kDa glycoprotein precursor were down-regulated. These results indicate that salinity can change the expression level of some special proteins in the hypocotyl and root of soybean that may in turn play a role in the adaptation to saline conditions.

Keywords Proteome · Soybean · Salt stress · Proline

Abbreviation

2D-PAGE Two-dimensional polyacrylamide gel electrophoresis

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Introduction

Throughout the world, soybean is one of the main sources of edible vegetable oil and high-protein livestock feed, and it is the most important dicot crop due to the high content of oil and protein in its seeds (Luo et al. 2005; Umezava et al. 2000; Banzai et al. 2002). However, its production is low in arid and semi-arid regions in which salinity is the main problem. Soybean has been considered as a salt-sensitive to moderately salt-tolerant crop (Luo et al. 2005; Umezava et al. 2000). In order to produce salt-tolerant cultivars of soybean, it is imperative to identify genes whose products confer improved salt tolerance. A homologue of oxysterol-binding protein has been characterized in soybean; this protein is encoded by a novel salt-inducible gene, and it may be involved in some physiological reactions of stress response in soybean (Li et al. 2007). Another salt-inducible gene encoding an acidic isoform of pathogenesis-related protein group 5 (PR-5) has been characterized in soybean. The PR-5 protein plays an important role in the extracellular space of soybean roots in response to high salt stress and dehydration (Onishi et al. 2006). 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 genes responsible for the response to salt stress and provide a detailed network of salt adaptation mechanisms in this important crop.

Salt stress severely limits plant growth (Banzai et al. 2002) and production (Amitai et al. 1995). Salinity is one of the most widespread agricultural problems in arid and semi-arid regions, which renders fields unproductive (Sharifia et al. 2007). Plants suffer from composite stresses caused by salinity, including water deficit (Sha Vali Khan et al. 2007), ion imbalance, ion toxicity (Banzai et al. 2002;

Amini et al. 2007; Wang et al. 2007), susceptibility to injury, and oxidative stress (Lu et al. 2007; Benevides et al. 2000; Mottiva et al. 2004). Adaptation to salt stress requires alterations in the cellular machinery that directly result from the modification of gene expression (Parker et al. 2006). Such salt stress-induced modifications may lead to the accumulation of certain metabolites and alterations in the level (increase or decrease) or the presence (appearance or disappearance) of some cellular proteins (Kong-Negren et al. 2005). Identification of salt stress-responsive genes requires molecular tools; key molecular tools include genome-wide genetic and physical maps of chromosomes in plants for mapping, tools for isolating and sequencing important genes, microarray analysis, and proteomics. Determination of gene function requires the identification of encoded proteins by proteomics (Abbasi and Komatsu 2004).

The proteomic approach has been used to analyze salt-responsive proteins in rice leaf sheaths, and 8 proteins were found to be up-regulated by salt stress (Abbasi and Komatsu 2004). The protein expression level changed in grapevines under salt stress, and 48 proteins showed differential expression patterns after salt stress—32 were up-regulated; 9 were down-regulated; and 7 new protein spots were observed (Jelloulia et al. 2007). While transcriptomic approaches are important, functional gene expression profiles can only be obtained by proteomic analysis. Thus, the quantitative analysis of gene expression at the protein level is essential for determining the responses to salt stress. Expression profiling at the protein level represents the core of current proteomic approaches (Parker et al. 2006). Several techniques are available for the differential analysis of protein expression, many of which involve the use of protein arrays. Multidimensional separation techniques are based on differential isotopic labeling methods. Despite these new developments, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) remains the most widely used method for the separation of proteins, and it is the method of choice for obtaining quantitative data (Jelloulia et al. 2007). The analysis of proteins by high-resolution 2D-PAGE has become increasingly popular, and its most important application is the resolution of a large number of proteins (Jelloulia et al. 2007). 2D-PAGE has been successfully applied for the examination of induced gene products in several plant species subjected to a wide range of abiotic treatments, including treatment with salt (Srivastava et al. 2004), drought (Vincent et al. 2005), high and low temperatures (Sule et al. 2004), ultraviolet radiation (Rakwal et al. 1999), heavy metals (Hajduch et al. 2001), and herbicides (Castro et al. 2005).

The current popularity of the proteomic approach for the analysis of responses to salt and drought is due to technical developments by which proteins extraction is easier and gel running and analysis is easier and more reproducible; further, mass spectrometry (MS) is highly sensitive for fingerprinting and partial sequencing of proteins (Abbasi and Komatsu 2004). In this study, proteome analysis was used to determine the effects of salt stress on the hypocotyl and root of soybean at the protein expression level, and major differentially expressed proteins were identified by Edman sequencing.

Materials and methods

Plant materials and treatment

Seeds of soybean (*Glycin max* [L]. Merr. Cv. Enrei) were planted in trays containing sand and treated with 0, 50, 100 and 200 mM NaCl and grown in growth chamber under white fluorescent light (600 μmol m^{-2} s^{-1}; 16 h light/8 h dark) at 25°C and 70% relative humidity. Length and fresh weight of hypocotyls and roots fully grown seedlings were measured after 1, 2, 3 and 4 days post germination. Finally, for proteome analysis, hypocotyls and roots from untreated (control) and 100 mM salt treated plants were used from 3 days old seedlings.

Free proline measurement

Proline content was estimated using ninhydrin reaction method (Bates et al. 1973). A portion (0.5 g) of hypocotyl and root were homogenized with 10 mL of 3% (*w*/*v*) sulphosalicylic acid and passed through whatman No. 2 filter paper. Then, 2 mL of the filtered extract were taken for the analysis to which 2 mL of ninhydrin reagent and 2 mL of glacial acetic acid were added. The mixture was incubated in 100°C water bath for 1 h and the reaction was finished in an ice bath. The reaction mixture was extracted with 4 mL toluene and absorption of chromophore was read at 520 nm against toluene as blank using spectrophotometer (Beckman, Fullerton, CA, USA). Proline content was calculated using L-proline corresponding on the standard curve.

Protein extraction

A portion (200) mg of hypocotyl and root was homogenized in phosphate saline buffer (pH 7.6) containing 65 mM K_2HPO_4, 2.6 mM KH_2PO_4, 400 mM NaCl and 3 mM NaN_3 at 4°C using a glass mortar and pestle on ice. The homogenate was centrifuged at 15,000×*g* for 10 min, and trichloro acetic acid (TCA) was added to the supernatant to a final concentration of 10%. The solution was

kept on ice for 30 min and then centrifuged for 10 min at 15,000×*g* at 4°C. The resultant precipitate was homogenized with 200 µL of lysis buffer containing: 4.8 g urea, 0.2 mL NP-40, 0.2 mL ampholine (pH 3.5–8), 0.5 mL 2-mercaptoethanol and polyvinyl pyrrolidone (PVP) (O'Farrell 1975) in a glass mortar and pestle on ice. The homogenate was centrifuged at 15,000×*g* at 4°C for 10 min. The supernatant was subjected to electrophoresis.

Two-dimensional polyacrylamide gel electrophoresis

Proteins were separated by 2D-PAGE in the first dimension by IEF tube gels (Daiichi pure chemicals, Tokyo, Japan) and the second dimension by SDS-PAGE. An IEF tube gel of 11 cm length and 3 mm diameter was prepared. IEF gel solution consisted of 8 M urea, 3.5% acrylamide, 2% Nonidet p-40, 2% Ampholines (pH 3.5–10.0 and pH 5.0–8.0), ammonium peroxodisulfate and tetra methyl ethylene diamine (TEMED). Electrophoresis was carried out at 200 V for 30 min, followed by 400 V for 16 h and 600 V for 1 h. After IEF SDS-PAGE in the second dimension was performed using 15% polyacrylamide gels with 5% stacking gels. The gels were stained with Coomassie brilliant blue (CBB) and image analysis was performed. 2D-PAGE images were visualized and the positions of individual proteins on gels were evaluated automatically with PDQuest soft ware (Bio-Rad, Richmond, CA, USA). The pI and Mr of each protein was determined using 2D-PAGE markers (Bio-Rad)

Image acquisition and data analysis

Spot detection, spot measurement, background subtraction and spot matching were performed specifically after CBB staining of the gels using PDQuest software. Following automatic spot detection, gel images were carefully edited. Before spot matching, one of the gel images was selected as the reference gel. The amount of a protein spot was expressed as the volume of that spot which was defined as the sum of the intensities of all the pixels that make up the spot. In order to correct the variability due to CBB-staining and to reflect the quantitative variations in intensity of protein spots, the spot volumes were normalized as a percentage of the total volume in all of the spot present in the gel. The resulting data from image analysis were transferred to PDQuest software for querying protein spots, which show quantitative or qualitative variations. Statistical analysis of the data was carried out using Microsoft Excel 2000 then the standard error (SE) was calculated from three spots in different gels and used as error bars.

Cleaveland peptide mapping

Following separation by 2D-PAGE, gel pieces containing protein spots were removed and proteins were electroeluted from the gel pieces using an electrophoretic concentrator (Nippon Eido, Tokyo, Japan) at 2 W constant powers for 2 h. after electroelution, protein solutions were dialyzed against deionized water for 2 days and then lyophilized. Proteins were dissolved in 20 µL of SDS sample buffer (pH 6.8) and applied to sample wells in an SDS-PAGE gel. Sample solutions were overlaid with 20 µL of a solution containing *Staphylococcus aureus* V8 protease (0.1 µg/µL; Pierce, Rockford, IL, USA). Electrophoresis was performed until the stacking of the sample and protease in the stacking gel, and then it was interrupted for 30 min to digest the protein and then continued until the dye line came to end of gel and then subjected to western blotting (Cleveland et al. 1977).

N-terminal and internal amino acid sequence analysis and homology searches

Following separation by 2D-PAGE or Cleaveland method (Cleveland et al. 1977), proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Pall, Port Washington, NY, USA) using a semidry transfer blotter (Nippon Eido) and detected by CBB-staining. The stained protein spots were excised from PVDF membrane and applied to the upper glass block of the reaction chamber in a gas-phase protein sequencer, procise 494 (Applied Biosystems, Foster City, CA, USA). Edman degradation was done according to the standard program supplied by Applied Biosystems. The amino acid sequences were obtained and compared with those of known proteins in the Swiss-Prot, PIR, GenPept and PDP databases with the web accessible search program FastA (http://www.dna.affre.go.jpl).

ESI-Q/TOF-MS/MS analysis

CBB-stained protein spots were excised from gels, washed with 15 mM potassium ferricyanide and 50 mM sodium thiosulfate and destained with 50 mM NH_4HCO_3 in 50% methanol at 40°C for 15 min. Proteins were reduced with 10 mM dithiothreitol in 100 mM NH_4HCO_3 at 50°C for 1 h and incubated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min the gel pieces were minced and allowed to dry and then rehydrated in 10 mM Tris-HCl (pH 8.5) with 1 pM trypsin at 37°C for 10 h. The digested peptides were extracted from gel slices with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile/water

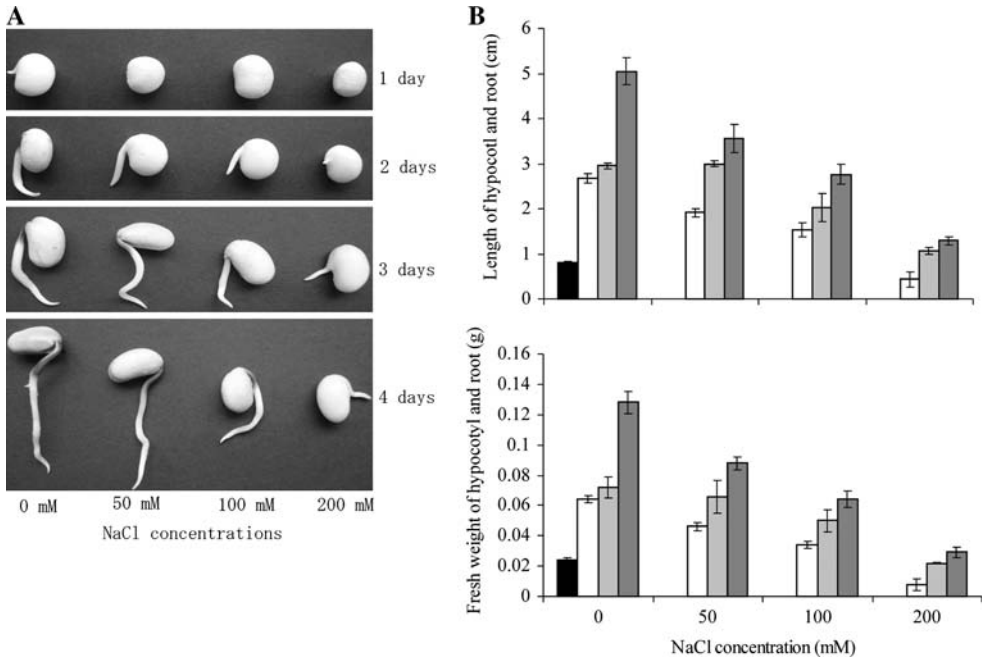

Fig. 1 Effect of NaCl concentrations on the length and fresh weight of soybean hypocotyl and root. Seeds were sown and treated with or without 50, 100, and 200 mM NaCl (**a**), and the length and fresh weight of the hypocotyl and root were measured at 1 (*black*), 2 (*white*), 3 (*light grey*) and 4 days (*dark grey*) after treatment (**b**). Values are the mean ± SE from three experiments

for 3 times. The peptide solutions were analyzed by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q/TOF-MS/MS) (Q-TOF micro; Micromass, Manchester, UK). MS/MS data were processed with a maximum entropy data enhancement program MaxEnt 3 (Micromass). The resultant spectra were interpreted with SeqMS, a software aid for denovo sequencing by MS/MS. The obtained sequence tags were also used for the homology search in the database with Mascot software (Matrix Science Ltd, London, UK).

Results

Effect of salt stress on length and fresh weight of soybean hypocotyl and root

To evaluate the effects of salt stress on the morphological characteristics of the treated soy plants, the length and fresh weight of the hypocotyl and root of seedlings were measured after 1, 2, 3, and 4 days of treatment with 0, 50, 100 and 200 mM NaCl. At all treatment concentrations, salt stress prevented root emergence in 1-day-old seedlings as compared to the control (Fig. 1a). Salinity severely affected the length and fresh weight (Fig. 1b) of the hypocotyl and root of soybean. The extent of length and fresh weight lowering was similar under all treatment conditions. The effect of treatment at 50 mM NaCl on seedling growth was not severe even after 4 days (Fig. 1a, b), but that of treatment at 200 mM NaCl was lethal. In order to investigate the effects of salt stress at the early stage of plant growth, the hypocotyls and roots of untreated and 100 mM NaCl-treated seedlings were excised, the protein expression levels were determined, and proteome analysis was conducted.

Free L-proline content increased in the hypocotyl and root of soybean under salt stress

The proline content was measured to determine whether this amino acid accumulates in response to salinity. As the NaCl concentration increased, the proline content of the hypocotyl and root increased, and it was particularly high at 100 and 200 mM NaCl (Fig. 2). The proline content at 200 mM NaCl was approximately fivefold that of the control. The difference in proline content was not

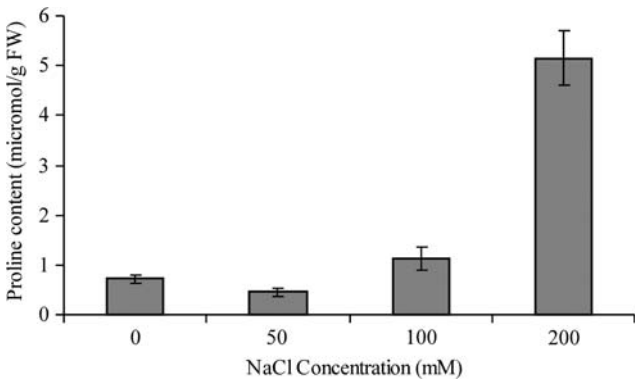

Fig. 2 Effect of salt stress on proline content in the hypocotyl and root of soybean. Seeds were sown and treated with or without 50, 100, and 200 mM NaCl, and the proline content was measured 3 days after treatment. Values are the mean ± SE from three experiments

considerable between the control plants and those treated with 50 mM NaCl.

Expression level of some proteins changed under salt stress

To investigate the response of soybean to salt stress, 3-day-old untreated or 100 mM NaCl-treated seedlings were used for proteome analysis. Proteins were extracted from the hypocotyls and roots, separated by 2D-PAGE, and stained with Coomassie brilliant blue (CBB). Digital image analysis revealed 321 protein spots (Fig. 3a). Of these, 20 proteins were responsive to salinity in that they were either up- or down-regulated. Seven proteins consistently showed significant and reproducible changes in their levels (two-fold to sevenfold) under salt stress (Fig. 3b) in each replicate experiment.

Identification of amino acid sequences

These seven proteins were selected for amino acid sequencing. They were designated SOY 1, SOY 2, SOY 3, SOY 4, SOY 5, SOY 6, and SOY 7. In order to elucidate the structure of these salt-inducible proteins, the accumulated protein spots were excised from the SDS-PAGE gels, and sequence analysis was performed. Homology searches were carried out using the FastA program (Table 1) for protein identification. Among these seven proteins, six

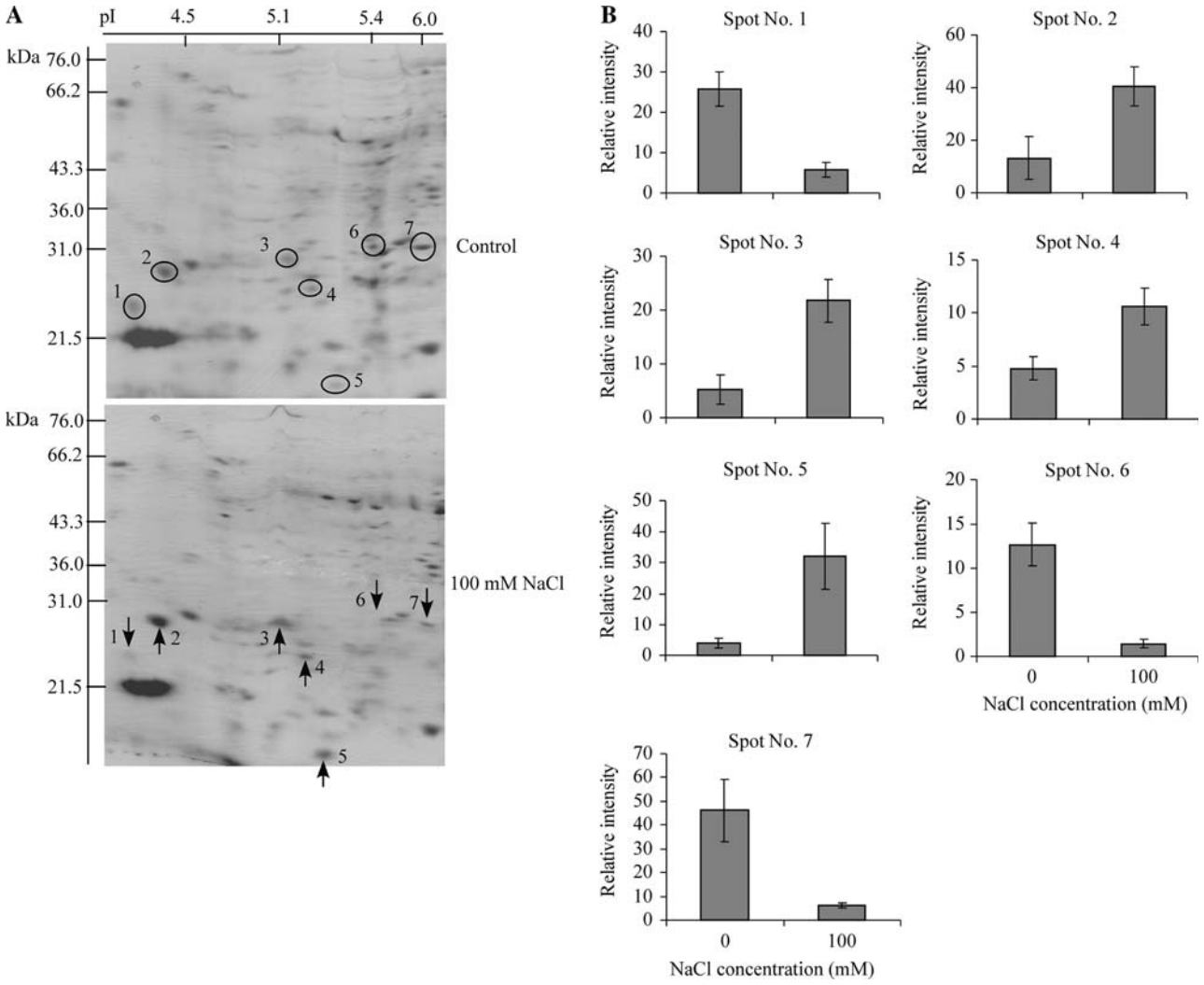

Fig. 3 Protein expression pattern in the hypocotyl and root of soybean induced by salt stress. Seeds were sown and treated with or without 50, 100, and 200 mM NaCl. After 3 days, proteins were extracted from the hypocotyl and root, separated by 2D-PAGE, and assessed by CBB staining. In the first dimension of the 2D-PAGE, IEF was used. *Arrows* indicate protein changes induced by NaCl treatment, and *circles* mark the position of the same proteins from the control (**a**). A portion (70 μg) of the protein was applied in each experiment, and the experiment was repeated three times. Relative levels of protein expression were analyzed (**b**). Values are the mean ± SE from three experiments

Table 1 Identified proteins, which differentially accumulated in hypocotyl and root of soybean after salt stress

Spot number	Mr	pI	Sequences	Homologous protein (percentage of homology)	Accession number
SOY1	19.56	4.1	N-AEPEPVVDKQ	Protease inhibitor (pi1 gene) (100%)	AJ441323
SOY2	27.59	4.29	N-ISSEDKPFNL I-LSQDIFVIP	β-Conglycinin, alpha chain precursor (100%)	P13916
SOY3	32.42	4.97	N-KEEEEEEEED	Elicitor peptide 3 precursor (100%)	Q8LAX3
SOY4	25.19	5.07	N-KLPNNS	Basic/helix-loop-helix (100%)	AC142095
SOY5	5.89	5.16	*n*-blocked (ESI-Q/TOF-MS/MS)	Late embryogenesis-abundant (24%)*	GI1762955
SOY6	35.63	5.42	N-TDTVSFTFNK	Lectin (100%)	DQ235094
SOY7	34.83	5.93	N-ERSSEVKXAS I-YDEXVNKG	Stem 31 kDa glycoprotein precursor (90%)	P10743

N: N-terminal amino acid sequence, I: internal amino acid sequence

* Coverage

were characterized using the protein sequencer as a protein inhibitor (SOY 1), β-conglycinin alpha chain (SOY 2), an elicitor peptide three precursor (SOY 3), a basic/helix-loop-helix (bHLH) protein (SOY 4), lectin (SOY 6), and a stem 31-kDa glycoprotein precursor (SOY 7); the last protein was identified using ESI-Q/TOF-MS/MS as a late embryogenesis-abundant protein (SOY 5).

Discussion

Salt stress severely decreased the growth of the hypocotyls and roots of the salt-treated soybean seedlings. This deleterious effect was higher at 200 mM NaCl. The reduction in soybean biomass due to salt stress has been reported previously (Kao et al. 2006). However, the present results confirm that soybean is a relatively salt-sensitive crop (Kao et al. 2006; Katerji et al. 2000). The harmful effects of salinity are mainly due to the resultant down-regulation of most genes and their corresponding proteins (Kao et al. 2006).

Analysis of the free proline content showed that at high NaCl concentrations, soybean increases the content of proline as an osmoprotectant in order to cope with the resulting high osmotic potential. The accumulation of free proline is a common response to a wide range of biotic and abiotic stresses, including those related to salt (Martinez et al. 1996), drought, and freezing (Van Heerden et al. 2002), and it is often the most striking consequence of lower leaf and root water potential (Van Heerden et al. 2002). A positive correlation between proline content and salt stress tolerance was previously observed in a wide range of plant species (Martinez et al. 1996). The precise role of proline accumulation as well as its physiological significance remains unclear. Many studies suggest that proline may be involved in intracellular osmotic adjustments between the cytoplasm and vacuole (Sharp et al. 1990). Another hypothesis suggests that proline is a protective agent for enzymes and intracellular structure and that it functions as a free radical scavenger or as a storage compound for carbon and nitrogen to allow lipid recovery from stress (Ehsanpour et al. 2003).

In order to investigate the early effects of salt stress on soybean at the protein expression level, i.e., before its severe effects are exhibited, the hypocotyls and roots were subjected to proteome analysis. A total of 321 protein spots were detected; more than 20 proteins were differentially expressed by salt stress, and 7 of these were structurally analyzed. The relative intensity of four proteins increased by twofold to sevenfold and that of three proteins decreased by fourfold to sixfold in salt-stressed plants as compared to the untreated control. β-Conglycinin (SOY 2), elicitor peptide 3 precursor (SOY 3), bHLH protein (SOY 4), and late embryogenesis-abundant protein (SOY 5) were up-regulated, while protease inhibitor (SOY 1), lectin (SOY 6), and stem 31 kDa glycoprotein precursor (SOY 7) were down-regulated.

One of the proteins that were up-regulated in our experiment is a late embryogenesis-abundant (LEA) protein. This protein may be suggested to play a pivotal role in the response of soybean to salt stress, and it may confer relative salt tolerance to soybean. LEA proteins accumulate at high concentrations in plant embryos during the late stages of seed development before desiccation (Baker et al. 1988). These proteins also accumulate in vegetative tissues exposed to exogenous abscisic acid as well as dehydration, osmotic stress, and low temperature (Chandler et al. 1994). Induction of LEA proteins has also been observed in cereals under cold stress (Ndong et al. 2002), in rice exposed to salt stress and inadequate water (Xu et al. 1996), and in soybean under salt stress (Soulages et al. 2002). Thus, our results confirm that the up-regulation of LEA proteins in soybean can be considered to be a part of the response to salt stress.

β-Conglycinin is a major storage protein in soybean, which differentially accumulates during seed development (Krishman 2002). During seed germination and the early stages of growth, β-conglycinin is degraded in order to nourish the rapidly growing embryo and seedling. In our experiment, the β-conglycinin expression level in the stressed plants was higher than that in the controls; based on this, we suggest that salinity negatively affected the growth of the seedlings such that they were unable to use β-conglycinin for growth, and they therefore showed a higher storage protein content as compared to the controls.

Elicitor peptides have been identified in plants as defense-related proteins in response to pathogens. In this experiment, one elicitor peptide protein was induced under salt stress. Treatment of cells with various elicitors induced ion fluxes and the expression of genes encoding defense-related proteins (Honee et al. 1998). Ion injury caused by NaCl treatment is suggested to induce elicitor peptides that can play a role in the defense mechanisms underlying injury repair.

The bHLH proteins comprise a superfamily of transcription factors. These proteins are important regulatory components in transcriptional networks and control diverse processes from cell proliferation to lineage establishment (Toledo-Oritz et al. 2003). The up-regulation of one of these proteins, which is a transcriptional factor, shows that under salt stress, some salt-responsive genes were induced in soybean to enable it to cope with the stress.

The protease inhibitor gene was found to be down-regulated in our study. To fight against pathogens, an important line of defense exhibited by plants is the expression of protease inhibitors that neutralize the proteases produced by the pathogens (Habib et al. 2007). After plants are subjected to salt stress, some proteases appear to be released from injured vacuoles into the cytosol, which may lead to the expression of some protease inhibitors in tolerant plants whereby these vacuolar proteases can be neutralized to prevent their deleterious effects on cell proteins. Lectins in plants belong to the defense system, and most lectins play a role in the defense against different kinds of organisms (Peumans et al. 1995). The down-regulation of protease inhibitors and lectins in addition to that of glycoprotein precursors that can be used to repair injured membranes following salt stress suggests that this cultivar of soybean (Enrei) cannot appropriately respond to salinity.

In conclusion, the soybean plants in our study responded to salt stress by changing their protein expression pattern. Some proteins, especially LEA proteins, were up-regulated, and this process may be involved in the process of adaptation to salt stress; meanwhile, other proteins were down-regulated. Most of the down-regulated proteins such as lectins belong to the plant defense system and are necessary for the repair of salt-induced injuries; consequently, soybean plants exhibited a severe decrease in growth under saline conditions and could not appropriately withstand salt stress. However, the up-regulation of some proteins observed in this study provides new insights for future studies on the improvement of salt tolerance in soybean.

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