Identification and Characterization of Osmotic Stress Responsive Proteins in Soybean using Quantitative Proteomics Approach

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Abbreviations

ABA	Abscisic acid
BiP	Binding immunoglobulin protein
CBB	Coomassie brilliant blue
CHAPS	3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
GRAVY	Grand average of hydropathy
HSP	Heat shock protein
IEF	Isoelectric focusing
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MGF	Mascot generic file
MMT	Million metric tons
MOPS	3-(N-morpholino) propanesulfonic acid
NCBI	National center for biotechnology information
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
PVP	polyvinylpyrrolidone
ROS	Reactive oxygen species

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
2-DE	Two dimensional polyacrylamide gel electrophoresis

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INTRODUCTION

Global climate changes have become a major concern of humanity in this century. Several environmental factors including weather, soil and accessible water have been affected by these alterations. Limitation of water resources caused more intense and longer drought since the 1970s particularly in the tropics and subtropics. Increasing the temperature along with the decrease of precipitation is contributing to cause drought (IPCC, 2007). Such adverse environmental conditions are highly affecting factors in plant growth and production. It is well known that abiotic stress which is a general term for unfavorable environmental conditions is one of the main causes of reduction of crop yield (Wang et al., 2003b). It was reported that for most crops, abiotic stresses are leading to reduction of the average yield by more than 50% (Bray et al., 2000). A number of abiotic stresses such as extremes in temperature, drought, salinity, heavy metals and radiation have detrimental effects on plant growth and yield.

Among abiotic stresses, drought is one of the major stresses on plants and it occurs in the nature when the available water in the soil is reduced because of lowering rainfall and continuing of transpiration or evaporation. One-third of the world's population resides in water-stressed regions and because of climate changes it could become more frequent and severe in the future (Manavalan et al., 2009). Salinity is another major constraint that adversely affects crop productivity and quality. Saline soil is characterized by toxic levels of chlorides and sulfates of sodium with the electrical conductivity of more than 4 dS/m (Marschner, 1995). It was reported that about 20% of arable land and nearly half of the irrigated lands in the world are affected by salinity (Yeo, 1998; Zhu, 2001). Drought and salinity are becoming widespread in many regions

and increased salinization of arable land is expected for 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang et al., 2003b). Cold or chilling stress which is defined as exposure of plant to a non-freezing low temperature is another stresses threaten the plant growth (Solanke and Sharma, 2008).

Plants due to their sessile nature are constantly exposed to a variety of abiotic stresses. This has led to evolution of adaptive mechanisms to sense the environmental changes and activate a responsive mechanism to cope with the stress (Heino and Tapio Palva, 2004). There are several determining factors affecting the stress response mechanism and in fact a wide range of stress responses exist in different plants. A diagram represented in Figure 1 has classified the abiotic stresses according to the cellular mechanism responses against the stress. Several reports have confirmed a cross-talk among abiotic stresses. In fact, adverse abiotic factors are tending to occur together and they almost never present as individual entities in the nature. Drought, salinity and cold are the stresses with several common cellular responses. It is well known that these stresses form osmotic stress in plants (Beck et al., 2007; Jonak et al., 1996; Nuccio et al., 1999). Reduction of osmotic potential and relative water contents of the cell, inhibition of photosynthesis and deleterious effects on carbohydrate metabolism and reduction in cell wall biosynthesis are happens by these stresses (Bartels and Souer, 2004; Desikan et al., 2004; Solanke and Sharma, 2008). All these stresses cause oxidative stress with production of reactive oxygen species (ROS) and disruption of ion homeostasis which finally leads to program cell death in plant (Figure 1). Therefore, to study of abiotic stresses, osmotic stress as a common phenomenon to the major abiotic stresses can be used aiming to identify a common pathway in plants exposed to varieties of abiotic stresses.

In order to study of osmotic stress in plants, several compounds can be used to adjust osmotic potential. Some of these compounds are usually exist in the cell as osmolytes to adjust the osmotic potential of the cell. For instance, mannitol, a widely distributed sugar alcohol in nature, has been reported in more than 100 species of plants. It was reported that mannitol producing plants exhibit a high degree of salt tolerance as a result of acting as a compatible solute (Stoop et al., 1996). Polyethylene glycol (PEG) is another compound that has been widely used as inert and nonionic solutes in the water related studies of plants (Money, 1989). It is more widely used than other compounds such as mannitol, sucrose, or glucose in inducing osmotic stress in plants because, as a result of its high relatively molecular mass, it mimics the effects of soil drying by causing cytorrhysis rather than plasmolysis (Oertli, 1985). In addition, PEG is not taken up by the plant and it has no toxic effects in roots and total plant (van der Weele et al., 2000).

Soybean (*Glycine max* L.) is one of the crops in which the abiotic stresses can severely affect the growth and productivity (Gutierrez-Gonzalez et al., 2010; Meyer and Boyer, 1972; Tran and Mochida, 2010). Osmotic stress which happens by drought, salinity and cold significantly affects the cellular mechanisms in soybean. Drought reduces soybean yield by about 40%, affecting all stages of plant development from germination to flowering and reducing the quality of seeds (Manavalan et al., 2009). Soil salinity is another limiting factor of soybean growth involving several deleterious effects such as osmotic stress, ion toxicity, and mineral deficiencies. Soybean is a relatively salt-sensitive crop and it was reported that salinity can highly affect the growth and development of soybean (Sobhanian et al., 2010). Low temperature and cold stress is also highly affects soybean productivity, quality, and post-harvest life. It was

reported that cold stress impart maximum damage during imbibitions of soybean seeds, reducing seedling emergence and ultimately cause severe loss in yield (Cheng et al., 2010). In plants under osmotic stress, the pressure associated with turgor decreases as a result of osmotic dehydration.

Soybean belongs to the family of Leguminosae or Fabaceae which is a large family of plants with approximately 20,000 species. This family, commonly known as legume, is the second important family in agriculture based on area harvested and total production and is the third largest family of higher plants (Gepts et al., 2005). The global production of soybean in the world in 2007 was approximately 219.8 million metric tons (MMT). The US was the largest producer with 70.4 MMT, followed by 2 other major producer countries, Brazil and Argentina by production of 61 and 47 MMT, respectively (Manavalan et al., 2009). Soybean is native to East Asia and it has long been important source of human nutrition in this region. Among Asian countries, China which is ranked as the fourth world producer of soybean is also the main importing country from US. Japan is the second largest importing country in Asia. It was reported that 22% of soybean in Japan, is used for human consumption and 75% is being used for oil and 3% for feedstuffs (Yamaura and Xia, 2010).

Soybean is one of the most important sources of protein for human and animal nutrition, as well as a major source of vegetable oil. It contains approximately 40-42% protein and 20-22% oil, on a dry-matter basis (Panizzi and Mandarino, 1994). In addition, soybean contains several secondary metabolites such as isoflavonoids (Sakai and Kogiso, 2008). Consumption of soybean-based products is increasing worldwide because of the beneficial effects of soybean seed on human health (Friedman and Brandon, 2001). Furthermore, the ability to fix atmospheric nitrogen (Burris and

Roberts, 1993) and recent reports of biodiesel production from soybean (Hill et al., 2006; Pimentel and Patzek, 2005) make this crop a wealth of resources for utilization.

In the cellular level, upon exposure to osmotic stress plants adopt a wide range of responses to cope with the stress. The mechanism of sensing the stress signal and transduction into the cell is well known as an initial reaction of plant cells subjected to the stress (Desikan et al., 2004). Stress signals perception is in outer parts of the cell and plasma membrane plays a key role in this process. A simplified process of cellular mechanisms for osmotic stress response in plant cells is presented in Figure 2. Osmotic stress causes expression or suppression of a wide range of genes which ultimately regulates proteins expression. In the other hand, proteins can be subjected to modifications to be functionally active or translocation or targeting to the specified region of the cell. Such kinds of mechanisms help the plant to improve the tolerance or resistance against stress and keep the plant survive.

Six major protein groups are involving in osmotic stress response mechanisms (Figure 2). One of the protein groups is those which regulate the accumulation of osmoprotectants such as sugar, proline, betaine, manitol and amino acids. These compounds increase osmotic pressure in the cell preventing further water loss and maintaining turgor (Galaud et al., 1997; Johansson et al., 1998). ROS scavenger proteins are another protein groups involving stress defense mechanism. Changes in flux of ions such as H^+, K^+, Cl^- and Ca^{2+} across the plasma membrane, alters cytosolic pH and transmembrane electric potential difference. It causes transient production of various ROS such as hydrogen peroxide, hydroxyl radicals and superoxide anions which should be removed by the scavengers. Enzymatic ROS scavengers in plants are consist of superoxide dismutase, ascorbate peroxidase, glutathione peroxidase and

catalase (Apel and Hirt, 2004; Blokhina et al., 2003). Molecular chaperons are other groups of proteins that their functions in quality control of proteins and protein folding are already known. Heat shock proteins, calnexin, calreticulin and Binding immunoglobulin protein (BiP) are some of the molecular chaperone proteins regulated by osmotic stress (Zang and Komatsu, 2007; Nouri and Komatsu, 2010; Wang et al., 2004). Osmotic stress not only activates enzymes in biosynthesis pathways but also regulates those in proteolysis. The mechanism of protein breakdown and recycling are an essential part of the plant response to environmental stress (Hieng et al., 2004). Ubiquitin-mediated and protease pathways are 2 types of protein breakdown mechanisms regulated by osmotic stress (Galaud et al., 1997).

Regulation of ion and water channel proteins is other cellular mechanisms in response to osmotic stress which happens in membranes especially in plasma membrane. Since intracellular ion homeostasis is important for the activity of many cellular enzymes, proper regulation of ion efflux is necessary (Beffagna et al., 2005; Ingram and Bartels, 1996; Waditee et al., 2006). Several proteins such as Na^+/H^+ antiporters and plasma membrane H^+-ATPase are involved in ion sequestration and ion balance under osmotic stress (Bohnert et al., 1995; Nouri and Komatsu, 2010). Water channel proteins such as aquaporins are the membrane proteins that control the water content of the cell. (Brown et al., 1998; Vera-Estrella et al., 2004). Ubiquitous proteins of aquaporins facilitate the transport of water across cell membranes. It was reported that 35 homologs of aquaporins are known in Arabidopsis and plasma membrane intrinsic protein 2 is one of the abundantly expressed aquaporin isoforms in Arabidopsis root plasma membranes (Javot et al., 2003).

The role of plasma membrane in response to osmotic stress is highlighted by

explanation of various defense mechanisms of the cell. Plasma membrane has a dynamic state to maintain cell components and structure and to adjust the permeability capabilities. It consists of the phospholipid bilayer containing hydrophobic tails and hydrophilic heads. There are mainly 2 types of proteins association with plasma membrane, embedded in the membrane lipid core or peripheral proteins. Later proteins are associated to plasma membrane by reversible interactions with either lipids or other membrane proteins (Marmagne et al., 2004). It is known that plasma membrane not only interact with the outer parts of the cell, it has a close relationship with the inner cellular organelles. Therefore, plasma membrane has a pivotal role in the cell especially under stress condition. Among several techniques for studies of plasma membrane functions, proteomics can give valuable information. However, finding suitable approaches for extraction and identification of the entire set of mostly hydrophobic plasma membrane proteins is still challenging (Komatsu et al., 2007).

Efficiency of any proteomics approaches is directly related to the availability of genome and proteome information. In soybean, sequencing of the 1100 Mbp of total soybean genome (1115 Mbp) predicts the presence of 46,430 protein-encoding genes, 70% more than in *Arabidopsis* (Schmutz et al., 2010). Soybean genome database containing 75,778 sequences and 25,431,846 residues has been constructed on the basis of Soybean Genome Project in the United states Department of Energy Joint Genome Institute (http://www.phytozome.net). Although the genome sequence information is almost completed, no high-quality genome assembly is available because the results from the computational gene-modeling algorithm are imperfect. Apart from genome information, proteome database for soybean is available (http://proteome.dc.affrc.go.jp/Soybean). Soybean proteome database provides valuable information from various

omics including proteome maps and functional analysis of soybean proteins (Sakata et al., 2009).

Proteomics is a powerful tool for investigating the molecular mechanisms of the responses of plants to stresses, and it provides a path toward increasing the efficiency of indirect selection for inherited traits. Proteome analyses of a specific tissue, organ or cell organelles are directly related to the quality and quantity of the extracted proteins. A protein extract, even from a purified fraction, consists of a huge number of individual proteins and several other components. The aim when analyzing such fraction is to obtain as much qualitative and quantitative information on the proteome as possible. In classical proteome analyses, known as 'gel-based proteomics', proteins are initially separated by a two dimensional polyacrylamide gel electrophoresis (2-DE) technique (O'Farrell, 1975) with isoelectric focusing (IEF) as first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as second dimension. The separated proteins can be subsequently identified by sequencing or by mass spectrometry. By introducing mass spectrometry into protein chemistry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) have become the methods of choice for high-throughput identification of proteins (Gevaert and Vandekerckhove, 2000).

An alternative technique, known variously as 'gel-free proteomics', 'shotgun proteomics', or 'LC MS/MS-based proteomics' can also be used in high-throughput protein analysis. This approach is based on LC separation of complex peptide mixtures coupled with tandem mass spectrometric analysis (Swanson and Washburn, 2005). The LC MS/MS-based technique has the advantage of being capable of identifying

low-abundance proteins, proteins with extreme molecular weights or pI values, and hydrophobic proteins that cannot be identified by using gel-based technique. Several studies confirmed the efficiency of this technique for identifying integral membrane proteins. For instance, application of multidimensional protein identification technology in shotgun proteomics resulted that the 72% out of 670 proteins from enriched membrane fraction were integral membrane proteins (Blackler et al., 2008). Hahne et al. (2008) compared 3 approaches of LC MS/MS-based quantification techniques with efficient separation of integral membrane proteins.

Taken together, abiotic stress is considered as one of the main reasons of yield reduction. In the other hand, soybean is one of the main sources of human and animal nutrition. It contains high level of vegetable oil and protein, both in the quantity and quality, related to other crops. It was reported that consumption of soybean-based products was increasing worldwide (Manavalan et al., 2009). Furthermore, the abilities of nitrogen fixation and biodiesel production (Burris and Roberts, 1993; Hill et al., 2006; Pimentel and Patzek, 2005) are other reasons for increasing demands for soybean. Continuous reduction of arable lands and increasing demands for soybean production are major challenges in current and future agriculture. Since commercial cultivars of soybean with enough tolerance to abiotic stresses are not available, in order to improve the tolerance of soybean varieties to abiotic stresses, study of plant response against the stresses is very important. Elucidation of molecular mechanisms of plant responses to abiotic stresses is an informative approach. Proteomics is the method of choice for studies of a dynamic state processes in the cell such as stress response. Performing a comprehensive soybean proteome analysis under abiotic stress and providing an appropriate proteomics approach is necessary to better understand the cell mechanisms

upon exposure to the stress. Genes which are responsible for stress-related proteins expression are a great source to improve crop productivity through transformation or marker-assisted breeding programs.

In this study, plasma membrane as a cells interface and a major part for controlling water content and ion homeostasis under osmotic stress has been considered for proteomics study. Purified plasma membrane proteins were obtained from soybean seedlings under osmotic stress condition and analyzed using quantification proteomics techniques. The efficiency and applicability of comparative proteomics for analysis of membrane proteins were evaluated. Functional analysis and classification of stress responsive proteins would help to interpret the cellular mechanisms involving plant tolerance and survival. Characterization of a candidate protein which is responsive to osmotic stress was performed by immunoblot analysis in various conditions and cellular fractions.

CHAPTER 1

COMPARATIVE ANALYSIS OF SOYBEAN PLASMA MEMBRANE PROTEINS UNDER OSMOTIC STRESS USING PROTEOMICS APPROACH

1.1 Introduction

Osmotic stress, which occurs in several abiotic stresses such as drought, cold and high salinity, can severely affect plant growth. Upon exposure to osmotic stress, plants exhibit a variety of responses (Xiong and Zhu, 2002). At the whole plant level, different mechanisms such as stomatal closure and increase in root/shoot ratio are involved in response to the stress (Gadallah, 2000). Imposing any kind of osmotic stress on the cell causes changes in cell volume, shape and loss of turgor which finally leads to the cellular plasmolysis. Plant cell under dehydration condition adopt a wide range of responses to cope with the stress. It alters numerous biochemical mechanisms in the cell, especially in the plasma membrane (Garcia-Gomez et al., 2000; Santoni et al., 2000). At the cellular level, plasma membrane is probably the most diverse membrane of the cell with a protein composition that varies with cell type, developmental stage, and environment (Alexandersson et al., 2004). Plasma membrane has a critical role under stress condition for perception of stress signals and transduction into the cell. It is an organized system as communicative interface between the intracellular and extracellular environments. Changes in expression of plasma membrane proteins are the main responses of the cell to perception of a signal and transduction into intrecellular parts.

Reliability and validity of any organellar proteomics is directly depends on the degree of purification. Therefore, in plasma membrane proteomics obtaining highly enriched plasma membrane proteins is an initial step for proteome study. Although several techniques have been developed to isolate plant plasma membrane proteins, purification of plasma membrane proteins is difficult, possibly because of the presence of the rigid cell wall (Komatsu, 2008). Two-phase partitioning method has been widely

used for isolation and purification of plasma membrane proteins. In this technique, aqueous polymers of dextran and PEG can effectively separate plasma membrane vesicles from other subcellular fractions (Larsson et al., 1987). The purity of plasma membrane fraction can be verified by assaying the activity of the plasma membrane-localized ATPase in the presence of specific inhibitor (Palmgren et al., 1990). This technique has been successfully applied to isolate a wide range of plasma membrane proteins in plant cells.

Extraction of proteins and preparation of samples are the earliest and most challenging steps in any proteome studies. In plant proteomics, the type of the plant species, tissues, organs, cell organelles, and the nature of desired proteins affect the techniques for protein extraction. Furthermore, the presence of vacuoles, rigid cell walls, and membrane plastids makes the extraction process difficult in plants (Komatsu, 2008; Lee and Cooper, 2006). There are some other interfering substances, such as phenolic compounds, proteolytic and oxidative enzymes, terpenes, organic acids, and carbohydrates in soybean affecting the protein extraction (Komatsu and Ahsan, 2009). Therefore, minimizing the effects of interfering substances has a high priority when proteins were extracting from soybean. An ideal extraction method would involve reproducibly capturing and solubilizing the full complement of proteins from a given sample, while minimizing post-extraction artifact formation, proteolytic degradation, and nonproteinaceous contaminants (Cho et al., 2006; Rose et al., 2004).

In order to identify proteins, 2-DE coupled to mass spectrometry is a commonly used method (Komatsu et al., 2003). Using this integrated technology, proteins can be separated, detected, and quantified. However, it has been shown that 2-DE does not represent a truly global technique because of limitations in identifying specific classes

of proteins. These include basic proteins, excessively large or small proteins, and low abundance and hydrophobic proteins (Zhang et al., 2007). Although membrane-associated proteins are detectable in a 2-DE pattern, integral membrane proteins have some distinct features that make them difficult to identify. They contain transmembrane domains and have a hydrophobic nature which cannot be solubilized in buffer for 2-DE (Ephritikhine et al., 2004). LC MS/MS-based proteomics is an alternative method in which a wide range of proteins including very basic or acidic, low abundance or hydrophobic proteins can be identified. Tandem mass spectrometry with its high sensitivity, accuracy, and throughput in protein identification has been the most powerful tool for proteomics (Morel et al., 2006; Sun et al., 2007). In this method, plasma membrane proteins digested with protease can be identified directly using mass spectrometry. Vast efforts have been made to identify membrane proteins either using gel-based or LC MS/MS-based proteomics, but to our knowledge these 2 techniques have not come together for comparative analysis.

The mechanisms of reaction and response of the cell to the stress are determining factor for cell survival. Several groups of proteins are believed to have a critical function in soybean under stress condition (Manavalan et al., 2009). It is known that not only regulation of all responsive proteins are depends on signal perception in plasma membrane but also a large varieties of the proteins expresses in this membrane. Therefore, proteome study of soybean plasma membrane using appropriate proteomics approaches is an informative technique for identification of stress responsive proteins. In this chapter, to investigate the effect of osmotic stress on the early growth stage of soybean, the plasma membrane was purified using a two-phase partitioning method. The gel-based and LC MS/MS-based proteomics approaches were applied for

identification of osmotic-stress-related proteins. The efficiency and applicability of comparative proteome studies in both proteomics techniques were investigated. Evaluation of enzyme activity and analysis of gene expression was performed for candidate proteins which identified as osmotic stress responsive proteins in soybean.

1.2 Experimental procedures

1.2.1 Plant materials

Soybean (*Glycine max* L. cv. Enrei) seeds were surface sterilized in 1.25% NaClO for 2 min followed by rinsing several times with water. The seeds were grown in plastic pots containing 400 mL of washed sand under white fluorescent light (600 μmol/m^2 s and 16 h light/8 h dark photoperiod) at 25°C and 75% relative humidity in a growth chamber. To control irrigation, each pot was placed in a container with a fixed water level during the growth period. For morphological analysis of soybean seedlings under osmotic stress, 2-day-old seedlings were treated with or without 10% PEG (M_r 6000, Wako, Osaka, Japan) for 1, 2, 3 and 4 days. PEG was removed by washing the sand after 1, 2 and 3 days. The length of hypocotyl and main root, fresh and dry weight, and numbers of lateral roots were measured every day. Mean comparisons were performed using Tukey's range test at $P<0.05$. Based on the morphological analysis, 2-day-old seedlings were treated for 2 days and then used for plasma membrane isolation and proteome analysis. For quantitative real-time polymerase chain reaction (PCR), 2-day old seedlings were treated with or without 10% PEG for 0, 6, 12, 24 and 48 h in 3 individual replicates.

1.2.2 Plasma membrane purification

All purification procedures were carried out on ice or at 4°C. A portion (20 g) of seedlings including fresh roots and hypocotyls was chopped and ground in 210 mL of extraction buffer containing 0.4 M sucrose, 75 mM MOPS/KOH (pH 7.6), 5 mM EDTA/KOH (pH 7.5), 5 mM EGTA/KOH (pH 8.2), 10 mM KF, 1 mM dithiothreitol (DTT), and 2% polyvinylpyrrolidone (PVP)-40 using a mortar and pestle. The homogenates were filtered through 4 layers of Miracloth (Calbiochem, San Diego, CA, USA) and centrifuged at 10,000 × g for 15 min. Membrane vesicles were pelleted from the resulting supernatant by centrifugation at 200,000 × g for 30 min and resuspended using a tissue homogenizer in 3mL microsome suspension buffer of 0.33 M sucrose, 5 mM KH_2PO_4 /K_2HPO_4 buffer (pH 7.8), 3 mM KCl followed by dilution with 6 mL extraction buffer.

A plasma membrane-enriched fraction was obtained from two-phase partitioning in a mixture composed of 6.2% (w/w) PEG 3350, 6.2% (w/w) dextran (M_r ~ 500,000, Sigma-Aldrich, Stockholm, Sweden) in 27 mL microsome-suspension buffer (Larsson et al., 1994) (Figure 3). The mixture was centrifuged at 2,450 × g for 4 min and the upper phase of the solution was collected. The PEG upper phase is enriched in plasma membrane vesicles and the dextran lower phase, the endomembrane fraction, contains all other membranes (Marmagne et al., 2004). To increase the purity of the plasma membrane, the initial upper phase with fresh lower phase was repartitionated for 3 times (Larsson et al., 1987). The upper phase of the final phase partitioning, which contained the plasma membrane vesicles, was diluted 4 times with 20 mM Tris-HCl (pH 7.5) and pelleted by 30 min centrifugation at 200,000 × g. Finally, the pellet was resuspended in

2 mL membrane buffer containing 0.25 M sucrose and 20 mM Tris-HCl (pH 7.5) and centrifuged at 50,000 × g for 1 h. Resulted pellet was dissolved in 100 µL membrane buffer and kept at -30°C until use.

1.2.3 Protein quantification and ATPase activity measurement

Quantification of purified plasma membrane fraction was performed according to the Bradford method (Bradford, 1976). Activity of ATPase was measured colorimetrically based on the production of free Pi in 10 individual plasma membrane fractions of control and PEG treatment. In order to evaluate the purity of isolated plasma membrane, ATPase activity was assayed in the presence of inhibitors as marker enzymes. The inhibitors for plasma membrane, tonoplast and mitochondrial membrane were Na_3VO_4, KNO_3 and NaN_3, respectively. Vanadate-inhibited P-ATPase is a classic marker for plasma membrane. Effectiveness of vanadate for evaluation of plasma membrane purity was checked by measuring the ATPase activity in crude extracts of control and PEG treatment before purification. ATPase activity was assayed in a reaction solution containing 30 mM MES-Tris (pH 6.5), 50 mM KCl, 3 mM $MgSO_4$ and 3 mM ATP with or without ATPase inhibitors. Plasma membrane-enriched fraction (1 µg) was added to the reaction solution and incubated at 30°C for 15 min. The reactions were stopped by the addition of 0.5% ammonium molybdate, 1% SDS and 0.8 N H_2SO_4, to which ascorbate was added to a final concentration of 0.3%. The reaction mixtures were incubated at room temperature and the absorbance was measured at 750 nm after 30 min. According to a standard curve generated by K_2HPO_4, various ATPase activities were calculated.

1.2.4 Solubilization of protein pellets

Plasma membrane proteins (100 µg) were precipitated by trichloroacetic acid (TCA) and the resulting pellet was washed 2 times with cold ethanol. The lysis buffer was prepared following O'Farrell (1975) containing 8 M urea, 2% Nonidet P-40, 2% Ampholine (pH 3.5-10.0, GE Healthcare, Piscataway, NJ, USA), 5% 2-mercaptoethanol, and 5% PVP-40. Since results of preliminary experiments showed that this lysis buffer could not efficiently dissolve membrane proteins, the combination of lysis buffer has been changed. The new lysis buffer which modified to solubilize more hydrophobic proteins was containing 7 M urea, 0.2 M thiourea, 0.2 mM tributylphosphine, 5% PVP-40, 0.4% 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) and 0.2% Bio-Lyte ampholytes (pH 3.0-10.0, Bio-Rad, Hercules, CA, USA). Protein pellets were homogenized in lysis buffer using pestle and mortar and applied to 2-DE.

1.2.5 Two-dimensional polyacrylamide gel electrophoresis

The solubilized plasma membrane proteins were separated by 2-DE (O'Farrell, 1975). For the first dimension, isoelectric focusing (IEF) tube gels were prepared in an 11 cm long, 3 mm diameter glass tube containing 8 M urea, 3.5% acrylamide, 2% Nonidet P-40 and 2% Bio-Lyte ampholytes (pH 3.5-10 and 5-8; 1:1 mixture). Electrophoresis was performed at 200 V for 30 min, followed by 400 V for 16 h and 600 V for 1 h. IEF tube gels were equilibrated by SDS-sample buffer containing 10% glycerol, 60 mM Tris-HCl (pH 6.8), 0.1 M SDS and 0.5 M 2-mercaptoethanol for 15 min, twice before subjecting to second dimension. SDS-PAGE was carried out in the

second dimension using 15% polyacrylamide gels with 5% stacking gels. The protein spots were detected by silver staining (Sil-Best stain, Nacalai, Kyoto, Japan) or Coomassie brilliant blue (CBB) staining.

1.2.6 Image analysis

Three individual biological replicates for analyses of 2-DE patterns were scanned with a GS-800 calibrated densitometer (Bio-Rad) and analyzed with PDQuest software (version 8.0.1, Bio-Rad). Image analysis included image filtration, spot detection and measurement, background subtraction and spot matching. All gels were compared with one of the selected gels as a reference gel and spots were automatically matched followed by adding unmatched spots to the gels, manually. 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 that spot. To accurately compare spot quantities between gels, the spot volumes were normalized as a percentage of the total volume in all of the spots in the gel. Significantly changed spots were identified according to the F-test through analysis of variance ($P<0.05$). Coefficient of variation was considered to confirm precision of the analysis.

1.2.7 Sample preparation for mass spectrometry

For protein identification, protein spots stained with CBB were digested using a DigestPro 96 automated protein digestion system (Intavis AG, Koeln, Germany). According to the procedure, spots placed in 96-well plates were incubated in 50%

acetonitrile, followed by washing in 50 mM NH_4HCO_3 for 15 min each. Proteins were reduced with 10 mM DTT in 50 mM NH_4HCO_3 for 20 min and alkylated with 40 mM iodoacetamide in 50 mM NH_4HCO_3 for 15 min, then digested at 37°C with 1 pM trypsin (TPCK treated Type XIII, Sigma-Aldrich). The resulting peptides were concentrated and desalted using a NuTip C-18 pipet tips (Glygen, Columbia, MD, USA) and analyzed by nanoLC MS/MS for protein identification.

To prepare LC MS/MS-based proteomics samples, a ReadyPrep 2D cleanup kit (Bio-Rad) was used to further purify the plasma membrane protein pellet. For digestion, the pellet was incubated in denaturing solution containing 8 M urea, 0.1 M NH_4HCO_3 and 0.1 M EDTA (pH 8.5) and in reduction solution containing 30 mM DTT at 37°C for 90 min, and then alkylated in 50 mM iodoacetamide for 30 min. Sample proteins were digested with 1 µL of 10 pM trypsin at 37°C for 16 h followed by concentration and desalting with a NuTip C-18.

Tryptic digested spots from the gel-based or LC MS/MS-based protein samples were injected using an auto sampler into an Ultimate 3000 nanoLC (Dionex, Germering, Germany) coupled to a nanospray LTQ XL Orbitrap MS (Thermo Fisher, San Jose, CA, USA). Peptides (4 µL) were loaded in 0.1% formic acid onto a 300 µm ID × 5 mm C_{18} PepMap trap column at a 25 µL/min flow rate. The peptides were eluted and separated from the trap column using 0.1% formic acid in acetonitrile on a 75 µm ID × 15 cm C_{18} column at a flow rate of 200 nL/ min. To spray sample in the MS, a PicoTip emitter (20 µm ID, 10 µm Tip ID, Woburn, MA, USA) was used with a spray voltage of 1.8 kV. The MS operated in positive ion mode using Xcalibur software (version 1.4, Thermo Fisher) and data acquisition was set to cover a scan range of *m/z* 100-2000 followed by 3 MS/MS scans in exclusion dynamic mode. The running time of the program for spot

detection and for LC MS/MS-based samples was set to 60 and 120 min, respectively.

1.2.8 Data acquisition and comparative analysis of LC MS/MS-based data

Tandem mass spectrum files were converted to mascot generic file (MGF) using Bioworks software (version 3.3.1, Thermo Fisher) and were used to search for matches in the National Center for Biotechnology Information database (NCBI nr 2009.08.03, 9363125 sequences) using Mascot search engine (version 2.2.04, Matrix Science Ltd., London, UK). A homologous protein search was performed using the soybean genome sequence database (version 4; 75788 sequences) (http://www.phytozome.net) followed by a BLAST search of the NCBI database (www.ncbi.nlm.nih.gov/blast) for those spots which were not identified in the NCBI database. To set the parameters for database search, for both gel-based and LC MS/MS-based data, accuracy of peptide mass was 10 ppm and MS/MS fragment ion mass value was 0.5 Da. Only 1 missed trypsin cleavage was allowed and carbamidomethylation of cysteines and oxidation of methionine were considered as fixed and variable modifications, respectively. Positive precursor peptide charge states of 1, 2 and 3 were specified. The proteins with at least 5 matched peptides in the Mascot search results with more than 10% sequence coverage were considered. The proteins with ion scores greater than 41 and 20 were significant for NCBI and soybean genome sequence databases, respectively ($P<0.05$).

Differential expression analysis for LC MS/MS-based proteomics was carried out using SIEVE software (version 1.1.0, Thermo Fisher). Using raw spectral data files of control and treatment generated by MS, the analysis was performed. False discovery rate for peptide matches above identity threshold for the datasets was less than 5%

according to the mascot search. To validate the P-value in SIEVE, duplicates of 3 individual biological samples of controls and treatments were used. For frame creation, *m/z* ranges were set to 500~1500 and retention time to 10~115 min. Frame *m/z* width was 0.02 amu and frame time width was 2.5 min. The threshold parameter indicates the lowest signal intensity at which a frame is generated; in this study, it was set to 500,000. Soybean database searching was performed using SEQUEST search engine in SIEVE software with a maximum of 10,000 frames to be searched. Based on the results of comparative analysis, proteins with significant changes in peak intensities were identified.

1.2.9 GRAVY and transmembrane helix prediction

To determine the hydrophobicity of identified proteins, the grand average of hydropathy (GRAVY) index was calculated using Sosui software version 1.10 (http://bp.nuap.nagoya-u.ac.jp/sosui), which takes into account the size and charge of the whole protein. A hydrophobicity value ranges classically from -2 to +2, and positive values refer to hydrophobic proteins. Using the same software, the numbers of transmembrane helices were predicted.

1.2.10 Protein function, localization and signal peptide prediction

Subcellular localization is an important step to understand the function of specific protein. Using N-terminal sequence information, localization of the proteins was predicted by TargetP server (www.cbs.dtu.dk/services/TargetP) (Emanuelsson et al.,

2000). Under stress condition some proteins might be secreted (signal peptide protein) or translocated to the plasma membrane. In order to elucidate whether identified proteins can transport through the secretory pathway, the presence of signal peptide were predicted using SignalP server (www.cbs.dtu.dk/services/SignalP) (Bendtsen et al., 2004). Furthermore, according to the review of the literatures, presence of the proteins in plasma membrane and function of the proteins were indicated.

1.2.11 Quantitative real-time PCR analysis

Real-time PCR was used to examine the expression analyses of calnexin (accession number, Q39817) and plasma membrane H^+-ATPase (accession number, BAC77531) genes in different time courses. Soybean seedlings quick-frozen in liquid nitrogen were ground to powder using a mortar and pestle. Total RNA was isolated by the RNeasy plant mini kit (Qiagen, Germantown, MD, USA) based on the manufacturer's instructions. First-strand cDNA was synthesized from 40 ng of total RNA using iScript cDNA synthesis kit (Bio-Rad). The cDNA template equivalent to 2.5 ng of RNA was used for real-time PCR. Gene-specific primers for calnexin were F-5'ACCAAGGTAGTGGAGAGAAT3' and R-5'ACTGAAAACAGAGTGCACCT3' and for plasma membrane H^+-ATPase were F-5'AGAAGTTGCAAGAGAGGAAG3' and R-5'TCCTTGTCTCTCCGTTCATT3'. Real-time PCR reactions were performed on a MyiQ Real-time PCR detection system using iQ SYBR Green Supermix (Bio-Rad). 18s ribosomal RNA with primer sets of F-5'TGATTAACAGGGACAGTCGG3' and R-5'ACGGTATCTGATCGTCTTCG3' was used as internal control. According to the

transcript level of the control, relative transcript abundance of the samples were calculated and normalized.

1.3 Results

1.3.1 Osmotic stress affects on soybean root and hypocotyl length

Two-day-old seedlings were treated with or without 10% PEG for 1-4 days. Although seedlings length was changed after 2 days of treatment with 10% PEG, the number of lateral roots was not changed significantly (Figure 4A). Analysis of morphological characteristics showed that only main root and hypocotyl length were significantly changed. The length of root and hypocotyl were 128% longer and 39% shorter than control, respectively, after 2 days treatment. Prolonging the treatment for 4 days further increased the root length and decreased the hypocotyl length by 158% and 41%, respectively (Figure 4B). Removing the stress allowed partial recovery of growth by improving hypocotyl growth and slowing down root growth.

Reduction in plant growth is a common visible response of dehydrated cells during osmotic stress. However, it has been proven that under water deficit, plants increase root depth as a major mechanism for improving water uptake (Turner, 1986). Surowy and Boyer (1991) evaluated soybean seedlings in response to water deficit and concluded that an increase in root area improves the water status. In their experiments, root growth was inhibited only when the normal water potential gradient between roots and stems was reversed. According to the results, morphological changes were started from the second day of osmotic stress. Therefore, total vegetative parts of 4-day-old seedlings with or without 2 days of PEG treatment were used for proteome analysis.

1.3.2 The plasma membrane is purified using two-phase partitioning method

Plasma membrane was purified by a two-phase partitioning method using buffer containing PEG and dextran. The purity of fractions was evaluated by assaying ATPase activity in the presence of specific inhibitors. Na_3VO_4, KNO_3 and NaN_3 were used as inhibitors of plasma membrane, tonoplast and mitochondrial membrane enzyme activity, respectively. The purity was calculated based on the differences in activity of ATPase determined by absorbance measurement in the presence or absence of the inhibitors. The average purity of plasma membrane, tonoplast and mitochondrial membrane was 92.5%, 9.7% and 6.3%, respectively, based on 10 individual plasma membrane-enriched fractions (Table 1). Plasma membrane purity in crude protein extracts was less than 50%, confirming the efficiency of selected inhibitor and purification steps. Therefore, enriched plasma membrane protein fractions using two-phase partitioning method can be used for plasma membrane protein identification.

1.3.3 Expressions of 12 protein spots are changed under osmotic stress as identified by gel-based proteomics technique

Two-day-old soybean seedlings were treated with 10% PEG for 2 days and roots and hypocotyls of 4-day old seedlings were used for plasma membrane purification. Proteins purified from the plasma membrane preparation (100 µg) were pelleted and solubilized using lysis buffer and applied to 2-DE. The silver-stained 2-DE patterns of control and treatment were analyzed by PDQuest software. Image analysis of 3 individual experiments could reveal 202 protein spots with high reproducibility (Figure

5). According to the results of analysis of variance, 12 differentially changed spots were identified; of which 4 and 8 spots were up- and down-regulated, respectively (Figure 6). To identify the selected proteins, 1 mg of purified plasma membrane proteins were applied to 2-DE followed by CBB staining. Loading lower quantity of proteins could not visualize low abundance proteins. Spots from CBB-stained gels were analyzed using nanoLC MS/MS. Generated data files from MS data sets were searched against NCBI and soybean genome sequence databases. Identified proteins were varied from single to multiple proteins within a spot, ranked by ion score (Table 2). However, in this stage it may not be concluded that all proteins in one spot were changed by PEG treatment.

Up-regulated spots were spot number 2 with 5 proteins including calnexin homolog, BiP isoform B, heat shock protein 90-1, unnamed protein product and phosphoinositide-specific phospholipase C P13, and spot number 6 with 2 proteins including elongation factor 1-beta and 14-3-3-like protein. Two other up-regulated proteins were identified as putative quinone oxidoreductase (spot number 9) and nucleoside diphosphate kinase 1 (spot number 11) (Table 2). Down-regulated spots were spot number 3 including unknown protein, cysteine proteinase precursor and fasciclin-like arabinogalactan protein 13 precursor. Spot number 4 was identified as plasma membrane intrinsic polypeptide, unnamed and unknown proteins; spot number 5 was as vacuolar ATPase subunit E, glyceraldehyde-3-dehydrogenase C subunit and delta 24-sterol-C-methyltransferase, and spot number 7 as 14-3-3-like protein D and band 7 family protein. Other down-regulated proteins were patellin 1 (spot number 1), 14-3-3-like protein D (spot number 8), temperature-induced lipocalin (spot number 10) and profilin (spot number 12).

Patellin 1 is a carrier protein that is involved in membrane trafficking (Supplemental Table 1). It has already been reported that this protein binds to some hydrophobic molecules such as phosphoinositides and promotes their transfer between different cellular sites (Zhang et al., 2008). Two isoforms of 14-3-3 like protein were identified in spot numbers 6, 7 and 8. It has been shown that most of higher plants have more than one isoform for this signaling protein (Xu and Shi, 2006). In this study, 14-3-3-like protein D (spot numbers 7 and 8) was down-regulated under 10% PEG treatment. This protein has been reported to have a regulatory role for plasma membrane H^+-ATPase (Shanko et al., 2003).

In the gel-based method, spot number 2, identified as endoplasmic reticulum (ER) chaperone proteins was up-regulated. Clapham (2007) explained the role of calcium-sensitive chaperones and their relationship with plasma membrane in ion transfer. Nucleoside diphosphate kinase 1 (spot number 11) is one of the up-regulated proteins under osmotic stress. This protein is associated with H_2O_2-mediated mitogen-activated protein kinase signaling in plants. In *Arabidopsis*, under oxidative stress condition the expression of nucleoside diphosphate kinase gene was strongly induced and its overexpression reduced the accumulation of ROS (Moon et al., 2003).

1.3.4 Eighty six proteins are changed under osmotic stress as identified by LC MS/MS-based proteomics technique

Tryptic digested peptides of control and PEG treatment were eluted and separated in a nanoLC trap column and sprayed in MS. Individual searches of converted data files of control and treatment against NCBI database resulted about 480 protein hits with

negligible variations among samples. In this study, SIEVE software was applied to demonstrate automated analysis of differential proteomic expression profiling in control and treatment. Three biological replicates were applied twice in MS and generated raw data files were used for label-free differential analysis. All chromatograms of controls and treatments aligned to the one of the controls as reference control and total of 40858 peaks were detected. The chromatographic alignment of triplicate of control and treatment data over a 20-100 min retention time shows the reproducibility of the experiment (Figure 7). Log_{10} of peak intensities for reference control were plotted against average of those of controls (Figure 8A). Distribution of peak intensities in the scatterplot approves the peak quality for further analysis (America and Cordewener, 2008). Scatterplot of the average Log_{10} of controls versus average Log_{10} of 10% PEG treatment performed broader distribution due to the differences between control and treatment (Figure 8B).

According to the SEQUEST result and visual inspection of the frame quality of the ion chromatograms, 86 significantly changed proteins were identified, of which 11 proteins were up-regulated and 75 proteins were down-regulated. Using SIEVE software the ratio of up- and down-regulated proteins were calculated in which the values >1 indicates up-regulated proteins (Table 3). Up-regulated proteins were calnexin homolog, H^+-transporting ATPase, 2 homologues of plasma membrane H^+-ATPase, phototropin, protease inhibitor, protein phosphatase 2C, universal stress protein and 2 hypothetical proteins as well as a predicted protein. Classification of the identified proteins based on the protein function revealed that all up-regulated proteins were categorized as transporter, signal transduction and defense- related proteins. Interestingly, the proteins which classified in cell structure, metabolism, protein folding

and protein synthesis were down-regulated under osmotic stress (Supplemental Table 2).

Three out of 11 up-regulated proteins were related to proton transport proteins. These proteins containing 10 transmembrane helices play an important role in abiotic stress condition. Plasma membrane H^+-ATPase, for instance, provides an electrochemical H^+ gradient across the membrane to prepare the energy needed for secondary transport and for the regulation of cell turgor and intracellular pH (Michelet and Boutry, 1995). Activation of this protein is modulated by signaling molecules such as 14-3-3 protein and protein kinases (Xu and Shi, 2006). Low abundance protein kinases have a great family and it was reported that more than 1000 genes in *Arabidopsis* encode protein kinases (Wang et al., 2003a). It was shown that protein kinase inhibits the activity of plasma membrane H^+-ATPase by phosphorylation. By contrast, removing the phosphate using phosphatase can highly activate this protein (Lino et al., 1998). Using LC MS/MS-based proteomics, 3 kinase proteins were identified which down-regulated under osmotic stress and protein phosphatase 2C was highly up-regulated (Table 3).

Phototropin, a photoreceptor protein, is another modulator of plasma membrane H^+-ATPase, which is up-regulated under osmotic stress. This plasma membrane-associated protein undergoes autophosphorylation upon exposure to blue light. It was reported that phototropin can activate the plasma membrane H^+-ATPase in stomatal opening (Sakamoto and Briggs, 2002). In *Arabidopsis,* while this protein negatively affects on hypocotyl growth, it promotes drought tolerance by enhancing the efficiency of root growth (Galen et al., 2007; Folta and Spalding, 2001). Based on the previous reports in *Arabidopsis* and present result in soybean, phototropin may play role in morphological changes under stress condition.

1.3.5 Two proteomics techniques are complementary in comparative analysis

Seven mutual proteins were identified by comparison of 2 proteomics techniques. Calnexin was the only common up-regulated protein in both techniques. Down-regulated proteins were fasciclin-like arabinogalactan protein, plasma membrane intrinsic polypeptide, vacuolar ATPase subunit E, glyceraldehyde-3-dehydrogenase C subunit, temperature-induced lipocalin and isoforms of 14-3-3-like protein. This low overlap implies necessity of multiple strategies to obtain high coverage of the proteome (Zhao et al., 2008). Furthermore, not all of differentially changed proteins in gel-based proteomics could be identified in LC MS/MS-based techniques, indicating that later method cannot be completely replaced for gel-based proteomics (Lambert et al., 2005). Therefore, the 2 proteomics techniques play a complementary role for analysis of soybean plasma membrane proteins under osmotic stress.

The distribution of molecular weight versus pI of the stress responsive proteins using gel-based and LC MS/MS-based techniques were plotted (Figure 9A). It has been showed that the LC MS/MS-based method could identify the proteins with a wider range of molecular weight and pI. Furthermore, the identified proteins using LC MS/MS-based technique had the higher number of transmembrane helices and broader range of GRAVY (Figure 9B). In this study, 3 ion transporter proteins with 10 transmembrane helices, including 2 homologues of plasma membrane H^+-ATPase and H^+-transporting ATPase protein (Supplemental Table 2) were identified. Kawamura and Uemura (2003) reported that proteins in a solubilized fraction separated on 2-DE were peripherally or non-covalently bound to the plasma membrane and the majority of the identified proteins had no transmembrane domains. Comparison of hydrophobic feature

of identified proteins indicated that only one protein with positive GRAVY index was identified by gel-based proteomics. In LC MS/MS-based technique, however, 15 proteins were predicted to have positive GRAVY (Supplemental Table1 and 2). According to the aforesaid comparisons, some limitations of 2-DE method for identification of plasma membrane proteins were indicated. Low abundance or hydrophobic features of plasma membrane proteins are the main reasons for incompatibility of the 2-DE method for plasma membrane proteome studies (Hood et al., 2004; Komatsu, 2008; Zhang et al., 2007). Aebersold and Goodlett (2001) described low abundance proteins such as transcription factors, protein kinases, and other proteins of regulatory function not being detectable by the approach of standard 2-DE followed by MS.

To classify the identified proteins functions, plasma membrane localized proteins were separated from putative novel candidates. Out of 86 identified proteins in LC MS/MS-based technique, the numbers of transporter proteins were 14 comparing to 2 transporter proteins out of 24 in gel-based proteomics. Subsequently, in gel-based technique the numbers of proteins involve in cell structure and signal transduction were 2 and 4, respectively, comparing to 11 cell structure related and 7 signal transduction related proteins in LC MS/MS-based technique (Figure 10). Prediction of protein localization revealed that in gel-based proteomics, only 4 out of 24 proteins were localized in plasma membrane comparing to 18 out of 86 proteins in LC MS/MS-based method. The presence of proteins in plasma membrane was searched in literatures, especially under stress conditions. In gel-based and LC MS/MS-based proteomics, 10 and 28 non-plasma membrane proteins were reported to be localized in plasma membrane (Supplemental Tables1 and 2). Using SignalP server, the numbers of proteins

with predicted secretory signal peptide were 5 and 18 proteins in gel-based and LC MS/MS-based techniques, respectively. Emanuelsson et al. (2007) explained translocation of the secretory signal proteins across the ER, transport through the Golgi apparatus and export by secretory vesicles. Although all proteins with signal peptides are not necessarily secreted or integrated to the plasma membrane, it is reliable for current study of purified plasma membrane proteins.

1.3.6 Calnexin and plasma membrane H^+-ATPase are 2 highlighted proteins under osmotic stress

The numbers of down-regulated proteins were much higher than that of up-regulated proteins. It may refer to slowing down protein synthesis during osmotic stress (Irsigler et al., 2007). An extensive down-regulation of the proteins related to metabolism was observed, indicating that the metabolic pathways in soybean seedlings are highly affected by osmotic stress. For instance, fructose-bisphosphate aldolase which is a key enzymes involved in the metabolism of glycolysis was down-regulated. Hewezi et al. (2008) reported down-regulation of fructose-bisphosphate aldolase and S-adenosyl-L-methionine synthetase gene expression under high light stress. They concluded that high light stress repressively affect on glycolysis pathway. Furthermore, under osmotic stress all identified proteins involve in cell structure were down-regulated. Actin depolymerizing factor-like protein and 6 homologues of tubulin as well as profilin which is an actin-binding protein related to the restructuring of the actin cytoskeleton were down-regulated under osmotic stress.

In this study, molecular chaperones performed both up- and down-regulation.

While calnexin was up-regulated in both proteomics approaches, calreticulin-1, BiP, heat shock 70 kDa protein and heat shock cognate 70 kDa protein were down-regulated as identified by LC MS/MS-based technique. Zang and Komatsu (2007) identified osmotic stress related proteins in rice and concluded that down-regulation of molecular chaperones after 48 h manitol treatment may refer to action of these molecules within the first 24 h. Calnexin which predicted to contain secretory signal peptide was the only mutually up-regulated protein. This multi-functional ER protein was up-regulated more than 2 times under osmotic stress. Furthermore, among all transporter proteins identified by 2 techniques, only 3 homologues of plasma membrane H^+-ATPase were up-regulated under osmotic stress. These homologue proteins with high number of transmembrane domains play pivotal role during osmotic stress interacting with wide range of proteins.

1.3.7 Gene expression of plasma membrane H^+-ATPase is increased within 12 h after 10% PEG treatment

Calnexin and plasma membrane H^+-ATPase were evaluated for gene expression analysis using real-time PCR. The transcripts were monitored in 5 time courses from stress initiation up to the second day of the treatment. Relative transcript abundance of calnexin was not changed within time courses comparing to the control (Figure 11A). In plasma membrane H^+-ATPase, however, expression of the gene was increased 2 times in 12 h after 10% PEG treatment (Figure 11B). The expression level was decreased later on and backed to the control level. Although plasma membrane H^+-ATPase was modulated by interaction with other proteins under stress condition, the gene and subsequently mRNA expression was enhanced in 12 h after stress exposure. Niu et al.

(1993) reported that mRNA level of plasma membrane H^+-ATPase in different organs of *Atriplex nummularia* was highly up-regulated under NaCl stress. Using different time course experiment, they showed that the increase could be detected within 8 h after treatment. In soybean root, it was reported that ATPase transcript levels was markedly increased at low water potential (Surowy and Boyer, 1991).

1.3.8 Total activity of ATPase is enhanced in plasma membrane under 10% PEG treatment

In order to verify the accumulation of H^+-ATPase in plasma membrane, total activity of ATPase was measured in plasma membrane-enriched fractions. The absorbance was measured in 10 individual samples of control and 10% PEG treatment and ATPase activity was calculated using standard curve generated by K_2HPO_4. The activity of ATPase in plasma membrane fraction under 10% PEG treatment was approximately 30% higher than that of control (Figure 12). Osmotic stress increased the total activity of ATPase in soybean plasma membrane which can be inferred as enhancement of ion efflux in plasma membrane. Liu et al. (2005) reported that activity of plasma membrane H^+-ATPase was highly increased in wheat roots under PEG treatment and concluded that tolerance of wheat seedlings to osmotic stress is associated with the activity of H^+-ATPase.

1.4 Discussion

In this study, two-phase partitioning could efficiently purify plasma membrane

proteins. An approximate purity of 95% was reported in previous studies by applying two-phase partitioning method, indicating the efficiency of the technique for plasma membrane purification (Alexandersson et al., 2004; Palmgren et al., 1990). The impurity of plasma membrane fraction by other membranes such as tonoplast was evaluated using specific inhibitor of the enzyme. In this experiment, the contamination by vacuolar ATPase was 9.7% in the purified plasma membrane fraction. Robinson et al. (1996) reported that vacuolar type H^+-ATPase exists not only in the tonoplast, but also in highly purified plasma membrane fractions of maturing pea cotyledons. This enzyme is widely recognized as being associated with various endomembranes as well as the plasma membrane (Herman et al., 1994; Sze and Palmgren, 1999).

Results of 2 proteomics techniques for the numbers of transmembrane helices, positive GRAVY and protein localization imply the higher efficiency of LC MS/MS-based proteomics for identification of integral plasma membrane proteins. Furthermore, gel-based method was comparatively limited to identify transporter proteins. According to the result, mutually identified proteins were not integral plasma membrane proteins. Plasma membrane intrinsic polypeptide was reported to be a membrane-associated protein (Lei et al., 2005). Fasciclin-like arabinogalactan protein is a subclass of arabinogalactan proteins which are abundant in cell wall and plasma membrane (Johnson et al., 2003). This protein is mainly involved in the hydrolysis of cell wall polysaccharides, leading to the modification of cell wall structures. Calnexin is the only mutually up-regulated protein with more than 2 times increase in protein expression in both proteomics techniques. As the transcript level of calnexin was not changed under PEG treatment, the idea of targeting of this signaling protein to plasma membrane under osmotic stress condition may explain the higher expression of this

protein.

Under osmotic stress condition, numerous changes in protein expression were observed, both in integral and peripheral plasma membrane proteins. The peripheral plasma membrane proteins associate with the plasma membrane proteins and cooperate in cell defense mechanism. Plasma membrane H^+-ATPase is one of the transporter protein which interacts with activator or suppressor proteins for regulation. This protein plays an outstanding role during abiotic stresses in cell turgor and intracellular pH (Beffagna et al., 2005). Protein phosphatase and a photoreceptor protein, phototropin which activate plasma membrane H^+-ATPase (Folta and Spalding, 2001), were highly up-regulated in this study. Moreover, 3 homologues of protein kinase were down-regulated in this study. These proteins may negatively affect the activity of plasma membrane H^+-ATPase by phosphorylation (Lino et al., 1998). In current study, activation of plasma membrane H^+-ATPase under osmotic stress has been shown in proteome and gene expression analyses and confirmed by measuring ATPase activity. These results imply acceleration of ion efflux under hyperosmotic conditions by up-regulation of proteins responsible for ion transport. It is concluded that the plant uses complicated tolerance mechanism against osmotic stress by combining enhancement of gene expression as well as up- and down-regulation of activator and suppressor proteins, respectively.

1.5 Summary

To study the soybean plasma membrane proteome under osmotic stress, 2 methods were used: a gel-based and an LC MS/MS-based proteomics method.

Two-day-old seedlings were subjected to 10% PEG for 2 days. Plasma membranes were purified from seedlings using a two-phase partitioning method and their purity was verified by measuring ATPase activity. Using the gel-based proteomics, 4 and 8 protein spots were identified as up- and down-regulated, respectively, whereas in the LC MS/MS approach, 11 proteins were up-regulated and 75 proteins were down-regulated under PEG treatment. Out of osmotic stress responsive proteins, most of the transporter proteins and all proteins with high number of transmembrane helices as well as low abundance proteins were identified by the LC MS/MS-based method. Three homologues of plasma membrane H^+-ATPase which are transporter proteins involved in ion efflux, were up-regulated under osmotic stress. Gene expression of this protein was increased after 12 h of stress exposure. Among the identified proteins 7 proteins were mutual in 2 proteomics techniques, in which calnexin was the highly up-regulated protein. These results suggest that under hyperosmotic conditions calnexin accumulates in the plasma membrane and ion efflux accelerates by up-regulation of plasma membrane H^+-ATPase protein.

CHAPTER 2

CHARACTERIZATION OF CALNEXIN, AN UP-REGULATED PROTEIN IN SOYBEAN UNDER OSMOTIC STRESS

2.1 Introduction

The ER is one of the most complex organelles in the cell performing large varieties of functions because, not only this organelle involves in synthesis, folding and degradation of proteins, but also has functions in lipid synthesis, lipid transfer and calcium signaling and calcium storage. The rough ER with membrane bound ribosomes is mostly responsible for the synthesis, folding and processing and trafficking of a large variety of proteins (Chen et al., 2010). This organelle provides an oxidizing environment that favors formation of disulfide bonds and folding and subunit assembly of nascent proteins (Bergeron et al., 1994). Adverse environmental conditions cause ER-stress in the cell leading to increase in the accumulation of unfolded or misfolded proteins. It is known that a coordinated adaptive program will be activated in response to imbalance between the unfolded proteins and folding capacity. This program alleviates stress by inhibition of protein synthesis, upregulation of ER chaperones and activation of ER associated degradation pathways (Kim et al., 2006; Schröder and Kaufman, 2005). In plants, several abiotic stresses such as drought and osmotic stress can activate ER-stress sensor proteins. Calnexin expression was used as a marker for ER-stress activation in a time course experiment (Irsigler et al., 2007). Because of the presence of calcium-binding protein such as BiP, glucose-regulated protein of 94 kDa, calreticulin and calnexin, ER plays a major role in calcium homeostasis and signaling and it was reported that upon depletion of calcium in ER, a sensor protein of ER signals to the plasma membrane to increase calcium influx (Cahalan, 2009; Chen et al., 2010).

Calnexin is a membrane-bound molecular chaperone protein of the ER. The structure of calnexin contains distinct regions of transmembrane domains, P-domain

including calcium-binding site, and lectin site. This protein interacts with many nascent membrane and soluble proteins of the secretory pathway and participates in the folding and quality control of newly synthesized glycoproteins (Bergeron et al., 1994; Brockmeier and Williams, 2006). Glycoproteins, the proteins with carbohydrate chains, are consisting most of the plasma membrane and secretory proteins and a database survey showed that over half of all proteins are glycoproteins (Agrawal et al., 2010; Apweiler et al., 1999). Calnexin binds to monoglucosylated glycoprotein through lectin site with specificity for Glc1Man9GlcNAc2 oligosaccharides. This leads to the transient association with almost all of the glycoproteins that are synthesized in the ER (Ellgaard and Helenius, 2003). Binding of calnexin to non-glycosylated substrates is also reported which enables to suppress the aggregation of these types of proteins (Brockmeier et al., 2009).

Several studies have been performed for analysis of calnexin in living cells (Bergeron et al., 1994; Danilczyk et al., 2000; Schrag et al., 2001). Sarwat and Tuteja (2007) reviewed this protein for function, structure in several organisms and in plants. One of the earliest studies on calnexin in plants was performed in *Arabidopsis thaliana* (Huang et al., 1993). Comparison of calnexin in plants and human shows that calnexin in plants has less amino acids (about 46-60 amino acids) than human in their N-terminal domain. However, there are several conserved regions in the middle parts of amino acid sequences of calnexin in different organisms. It was reported that the first 100 and the last 150 residues of calnexin are highly diverged (Huang et al., 1993). The central domains of calnexin and calreticulin in several organisms are highly conserved. They contain 2 types of repeats including DP (E/D) (A/D) XKPEDWD (D/E) and GXWXXPXIDNP and it was reported that this part is involved in calcium binding and

regulation of calcium homeostasis in the ER (Huang et al., 1993; Sarwat and Tuteja, 2007; Wada et al., 1991).

In chapter 1, calnexin has been identified as a highly up-regulated protein in plasma membrane fraction of soybean under osmotic stress. This protein was the only common up-regulated protein in gel-based and LC MS/MS-based techniques. The presence of this ER-resident protein in plasma membrane has already been reported and the mechanisms of calnexin migration have been explained. Myhill et al. (2008) have explained the mechanism by which calnexin targets to the plasma membrane. Interaction of calnexin with a cytosolic sorting protein PACS-2 reportedly distributes this protein in the plasma membrane (Myhill et al., 2008). The amount of calnexin in plasma membrane varies depend on the cell type or cellular homeostasis and can affect cell surface properties (Gagnon et al., 2002). Okazaki et al. (2000) were reported that a small fraction of calnexin is continuously expressed in plasma membrane of several types of mammalian cells. They concluded that although it can dynamically be turned over by endocytosis, the amount of this protein on the plasma membrane results from the balance of the rates of exocytosis and endocytosis. Although several attempts have been made to unravel the behavior of calnexin, there is no comprehensive characterization of calnexin in plants under stress conditions.

In this chapter, firstly using provided antibody from overexpressed calnexin antigen, expression of calnexin under various conditions such as osmotic stress severity, time course, organ specific and stress specific were evaluated by immunoblot analysis. In the second step using rice, a model plant of cereals, calnexin expression was evaluated and the behavior of calnexin expression was compared with those of in soybean.

2.2 Experimental procedures

2.2.1 Plant material preparation

In order to evaluate the expression of calnexin in soybean seedlings under various conditions, several individual experiments have been performed. In all experiments, soybean seeds were planted in the sand and grown in the growth chamber with the same conditions as described in chapter 1. To evaluate the effects of osmotic stress severity, 2-day-old soybean seedlings were subjected to 0, 5, 10 and 20% PEG for 2 days and roots and hypocotyls of 4-day-old seedlings with or without treatments were used for protein extraction. To evaluate the expression of calnexin in different organs, 2-day-old soybean seedlings were treated without or with 10% PEG for 2 days. Six parts of the 4-day-old seedlings including 3 main parts, cotyledon, hypocotyl and root as well as 3 sections of the root containing lateral part, elongation section and root tip were collected and used for protein extraction. To check the expression of calnexin under various abiotic stresses, 2-day-old soybean seedlings were subjected to 5 types of the stresses for 2 days. Seedlings were subjected to 10% PEG for osmotic stress, withholding water for drought stress, 100 mM NaCl for salt stress, 50 µM ABA (Wako) for ABA stress and 5 ^{o}C for cold stress. Roots and hypocotyls of 4-day-old seedlings were used for protein extraction.

Four individual time course experiments were performed to evaluate the expression of calnexin. In the first time course experiment, roots and hypocotyls of 2- to 6-day-old soybean seedlings grown in control condition were used for protein expression. In another experiment, 2-day-old seedlings were treated with 10% PEG for 1 to 4 days and seedlings with various stress durations were used for protein extraction.

To check the effect of recovery from stress on calnexin expression, PEG was removed from the seedbed by washing the sand with tap water and seedlings were allowed to continue the growth in normal condition. Samples with various durations of stress and recovery were used for analysis. Soybean seedlings were kept in the growth chamber to continue the growth up to 2 weeks and seedling roots and hypocotyls at the ages of 4, 9 and 14-day-old treated without or with 10% PEG for 2 days were collected. In this part of the experiment, morphological characteristics such as length and fresh weight of roots and shoots were measured in 3 individual replications and 15 seedlings were measured in each replicate.

Rice (*Oryza sativa* cv. Nipponbare) seeds have been sterilized and grown in plastic pots containing 400 mL of washed sand along with soybean in the growth chamber with the same conditions as described in chapter 1. Soybean and rice seedlings at the age of 12-day-old were subjected to various PEG concentrations including 0, 5, 10% PEG for 2 days. Because of differences in root expansion of soybean and rice, an experiment was conducted to evaluate the effects of PEG on morphology of plants. Soybean and rice seedlings were treated with PEG solution in seedbed or the seedlings were removed from the sand and transferred to PEG solution for 2 days. Morphological characteristics such as lengths and fresh weights of roots and shoots in both soybean and rice were measured in 3 independent experiments. Roots of 14-day-old soybean and rice seedlings were collected for protein extraction. To study of stress specificity, various aforementioned abiotic stresses were subjected to 12-day-old seedlings of soybean and rice for 2 days and roots were collected for protein extraction. Morphological traits of soybean and rice were measured in 3 replicates.

2.2.2 Subcellular fractionation

Various fractions of the cell were prepared from roots and hypocotyls of 4-day-old soybean seedlings with or without 10% PEG treatment for 2 days. All isolation procedures were carried out on ice or at 4°C. Plasma membrane fraction was purified according to the procedure explained in chapter 1. Microsomal and mitochondrial fractions were obtained using Qproteome mitochondria isolation kit (Qiagen**,** Hilden**,** Germany). Soybean roots and hypocotyls were excised and homogenized in the lysis buffer containing 0.4 M manitol, 4 mM L-cysteine, 1 mM EGTA, 25 mM MOPS and 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 1000 × g for 10 min. The pellets were resuspended using provided disruption buffer and centrifuged at 6000 × g for 10 min. The supernatant was considered as microsomal fraction containing ER and other membranes. The pellet was resuspended in provided storage buffer and considered as mitochondrial fraction according to the manufacturer's protocol. Crude protein extracts were obtained from homogenization of roots and hypocotyls in SDS-sample buffer containing 10% glycerol, 60 mM Tris-HCl (pH 6.8), 0.1 M SDS and 0.5 M 2-mercaptoethanol followed by 2 times centrifugation at 15,000 × g for 10 min by saving supernatant.

Cytosolic and membrane fractions obtained by ultracentrifugation are known as S100 and P100 fractions, respectively. Roots and hypocotyls were homogenized in the buffer containing 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 1m M DTT and 1mM PMSF followed by centrifugation at 260,000 × g for 15 min and the supernatant was considered as S100 fraction. For P100, remaining pellet washed with homogenizing buffer for 2 times and solubilized in the buffer containing 1% Triton

X-100, 20 mM Tris-HCl (pH 7.5), 50 mM 2-mercaptoethanol and 1 mM EDTA. After incubation of proteins solution on ice for 8 min, the supernatant from centrifugation at 260,000 × g for 15 min was considered as P100 fraction. A preliminary immunoblot analysis was performed using all fractions to check the cross-reaction of calnexin antibody. According to the result of this experiment, P100 fraction has been selected and used for all immunoblot analyses.

2.2.3 Preparation of calnexin antigen for making antibody

According to the proteomics results from chapter 1, soybean calnexin homolog (accession number in NCBI database, Q39817) has been selected as candidate protein. Amino acid sequence of this protein was used for BLAST search in soybean genome sequence database. In order to prepare antibody against calnexin, full-length cDNA of this protein (clone: GMFL01-40-N08) was provided from national bioresource project of Japan (Umezawa et al., 2008). The pEU-E01 vector containing 6 residues of Histidin coding codons (Cellfree Sciences, Yokohama, Japan) was cut using *SmaI* restriction enzyme and PCR-cloned cDNA of calnexin was phosphorylated and inserted to the dephosphorylated vector (Figure 13). The vector was transformed into the *E.coli* strain JM109 using heat-shock method. DNA sequencing of the transformed cDNA was performed using specific primers of the vector and target cDNA in DNA sequencer (ABI PRISM 3100 genetic analyzer, Applied Biosystems, Foster, CA, USA).

The recombinant His-calnexin protein was produced by automatic protein synthesizer using wheat germ cell and purified by affinity purification column (Protemist DT, Cellfree Sciences). To check the in vitro synthesized protein, protein

solutions before and after purification along with the flow through solution were loaded on SDS-PAGE followed by CBB staining or transferred onto polyvinylidene fluoride (PVDF) membrane to check the amino acid sequence of overexpressed protein by a gas-phase protein sequencer (Procise cLC, Applied Biosystems). Antibody was prepared in rabbit using overexpressed protein of calnexin (Scrum, Tokyo, Japan).

2.2.4 Immunoblot analysis using calnexin antibody

Crude protein extracts of control and 10% PEG treatment were used for 2-DE. Proteins precipitated by TCA were washed with cold ethanol for 2 times. The pellets were solubilized by lysis buffer containing 8.5 M urea, 2.5 M thiourea, 5% CHAPS, 2% Triton X-100, 100 mM DTT and 1% ampholine and solubilized protein was quantified according to the Bradford method (Bradford, 1976). A portion (300 µg) of protein was applied to IEF tube gel for first dimension followed by SDS-PAGE for second dimension (See chapter 1). In order to evaluate calnexin expression in various experimental conditions, P100 protein fractions were separated using one dimensional SDS-PAGE. An equal amount (10 µg) of protein samples were separated in 15% SDS-PAGE in mini slab gel with 25 mA current for about 65 min and used for immunoblot analyses or stained by CBB.

In order to perform immunoblot analysis, proteins were electroblotted onto PVDF membrane using semidry transfer blotter (Nippon-Eido, Tokyo, Japan) in a current of 1 mA/cm^2 for 90 min. The membrane was blocked in TBS-T solution containing 20 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.1% (v/v) Tween 20 with 5% (w/v) nonfat dry milk at 4°C for overnight. The blot was incubated with first antibody in blocking

solution at 1:5000 dilution for 1 h at room temperature. After 6 washes in TBS-T, the membrane was probed with HRP- conjugated secondary antibody at 1:10,000 dilution in blocking solution for 1 h followed by washing in TBS-T. The membrane was developed with Amersham ECL plus western blotting detection reagents (GE Healthcare) following the manufacturer's protocol and visualized by a LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

2.3 Results

2.3.1 Four predicted genes are exist for calnexin

Amino acid sequence of calnexin has been used for BLAST search in soybean genome sequence database to identify the number of predicted encoded genes. Four encoded genes including Glyma04g38000.1, Glyma05g33330.1, Glyma06g17060.1 and Glyma08g00920.1were available for soybean calnexin. Among these genes, Glyma06g17060.1 has performed the highest homology (92%) with Q39817.1. Alignment of amino acid sequences of these genes has been performed (Figure 14). Glyma06g17060.1 and Glyma04g38000.1 have shown 85% homology and 2 others have presented 94% homology. Homologies between other pairs were about 75 % indicating diversity in amino acid sequences for calnexin in soybean. Most of the differences among amino acid sequences were from both ends of the sequences. About 40 amino acids from N-terminal and 80 amino acids from C-terminal domain of calnexin have shown more diversity and the middle parts of the sequence were conserved region. Huang et al. (1993) have compared *Arabidopsis* and dog calnexin and concluded that while the N-terminal and C-terminal parts were highly diverged, the

middle part of calnexin was highly conserved.

2.3.2 *In vitro* synthesized calnexin is purified using His-binding column

Full length cDNA of calnexin was inserted to the expression vector containing Histidin coding codons and recombinant His-calnexin protein was produced using protein synthesizer. Calnexin was purified using His-binding affinity column and collected for preparation of antibody. The purified calnexin was loaded onto SDS-PAGE and stained by CBB (Figure 15). Calnexin has shown a clear single band with the molecular weight of 80 kDa. Electroblotted calnexin band was excised and applied to protein sequencer to analyze the N-terminal amino acid sequence. The N-terminal amino acid sequence was 'HHHHHHLEDPMGERK' and it has been confirmed the correct sequences of calnexin.

In order to preliminary evaluate cross-reaction of calnexin antibody, crude protein extracts of control and 10% PEG treatment were separated by 2-DE and subjected to immunoblot analysis. Two main cross-reacted spots were identified which were up-regulated under treatment (Figure 16). Spots from CBB-stained gels were cut and digested and applied to mass spectrometry for protein identification. Spot number 1 was identified as calnexin and heat shock protein 90-2 and spot number 2 was identified as heat shock protein 90-2 and beta-tubulin. Heat shock protein 90 was previously identified along with calnexin in gel-based proteomics approach (Table 1). Cross-reaction in second spot might be because of crude protein extract which was used for protein separation in 2-DE.

2.3.3 Calnexin accumulates in plasma membrane under 10% PEG stress

The expression of calnexin has been evaluated in various cell fractions from 4-day-old soybean roots and hypocotyls with or without 10% PEG for 2 days. In the first step, an immunoblot analysis was performed using purified plasma membrane protein and microsomal protein fractions (Figure 17). It was shown that calnexin is significantly accumulated in plasma membrane fraction upon exposure to osmotic stress. However, expression of this protein was not changed in microsomal fraction which is supposed to contain more ER proteins. Three other cellular fractions such as mitochondrial fraction, S100 and P100 fractions have been isolated from soybean seedlings with or without 10% PEG to evaluate calnexin expression. It has been shown that the expression of calnexin did not change in the fractions under osmotic stress (Figure 18). P100 fraction contains various membranes and the result shows that calnexin was accumulated only in purified plasma membrane fraction under 10% PEG stress. Expression of calnexin in plasma membrane fraction of animal cells was already reported (Myhill et al., 2008; Okazaki et al., 2000). While an equal amount of 10 µg has been applied to SDS-PAGE, P100 fraction presented a strong band for calnexin, indicating that calnexin accumulation in P100 is more than other cell fractions. Therefore, P100 fraction has been considered for further immunoblot analyses of calnexin expression.

2.3.4 Calnexin constantly expresses under stresses in the first week of growth

The membranes enriched fraction of the cell (P100) has been prepared for

immunoblot analyses of calnexin. In order to evaluate the effect of osmotic stress severity on expression of calnexin, two-day-old seedlings were subjected to 5, 10, 20% PEG for 2 days and compared with control (Figure 19). It was shown that increasing PEG concentration did not affect the expression of calnexin in 4-day-old soybean seedlings. In order to check the expression pattern in various sections of soybean, 6 sections were isolated from seedling. Cotyledon, hypocotyl, total root, lateral root part, elongation section and root tip were isolated from seedlings with or without 10% PEG treatment and P100 fractions were obtained. Immunoblot analysis has indicated that the expression of calnexin was reduced in cotyledon under osmotic stress and in other parts of seedlings it remained unchanged (Figure 20).

Effects of various abiotic stresses were evaluated for expression of calnexin in early growth stage of soybean seedlings. Two-day-old seedlings were treated with osmotic, drought, salinity, ABA and cold stresses for 2 days and compared to the control. Morphological traits such as length and fresh weight of roots and hypocotyls were measured to confirm the efficiency of stresses on seedlings (Figure 21). It has been shown that the length and fresh weight of hypocotyls were significantly reduced under abiotic stresses. The length of root was highly reduced in cold stress and the fresh weight of root was reduced under PEG, salinity and cold. Immunoblot analysis for calnexin expression in 4-day-old soybean seedlings under various abiotic stresses were performed (Figure 22). The expression of calnexin was constant in P100 fraction under various stress conditions. It can be concluded that soybean seedlings do not show variation for calnexin expression in the early growth stage under osmotic stress severity, different organs and various abiotic stresses.

Calnexin expression was evaluated in several conditions of time course

experiments. In the first experiment, calnexin expression behavior was investigated in the first week of growth. Roots and hypocotyls from 2 to 6-day-old soybean seedlings were collected and P100 fractions were extracted. Result of immunoblot analysis was showed that the expression of calnexin did not change in the first week of growth (Figure 23). In order to evaluate the effects of osmotic stress with various duration of exposure time on calnexin expression within the first week of growth, seedlings have been treated by 10% PEG for 1 to 4 days. A diagram shows the pattern of stress exposure for this experiment (Figure 24A). Roots and hypocotyls from 3 to 6-day-old seedlings without or with 1 to 4 days stress have been collected for protein extraction. Immunoblot analyses were performed in samples without or with 10% PEG treatment (Figure 24B). It was shown that calnexin expression was not affected by stress duration in the first week of seedling growth. Using the same stress pattern presented in Figure 24A, treated seedlings were recovered by washing the sand and the growth was continued in normal condition. Calnexin expression was presented in Figure 25 in panels A, B and C for 4, 5 and 6-day-old seedlings, respectively. Each panel performs calnexin expression behavior with indicated days for stress exposure or recovery. It was shown that there are no significant differences in calnexin expression of recovered seedlings. Taken together of the experiments in the first week of soybean growth, it can be concluded that the expression of calnexin in the early growth stage is not affect by abiotic stresses.

2.3.5 Abiotic stresses reduce calnexin expression in the second week of soybean growth

In order to compare the expression of calnexin from first week to the second week of seedling growth, soybean seedlings were grown for 2 weeks. The seedlings at the ages of 4, 9 and 14-day-old with or without 10% PEG for 2 days have been selected for analysis (Figure 26A). Morphological analysis was performed to evaluate the effect of stress on growth of seedlings (Figure 26B). While shoot lengths in 4 and 14-day-old seedlings were significantly reduced in plants subjected to 10% PEG stress, it was not changed in 9-day-old seedlings. The responses of root length to the stress were different and upon exposure to 10% PEG stress, roots length were increased and decreased in 4 and 14-day-old seedlings, respectively. The fresh weight of roots and shoots were reduced in 14-day-old seedlings. Seedlings in the second week have extended roots and leaves and it is shown that seedlings in this stage are more sensitive to stress condition.

Roots and hypocotyls were excised from 4, 9 and 14-day-old seedlings with or without 10% PEG and P100 protein fractions were extracted. The result of immunoblot analysis indicated that the expression of calnexin was significantly reduced in the roots of 14-day-old seedlings upon exposure to 2 days stress (Figure 27). However, calnexin expression was not changed in hypocotyls of 14-day-old seedlings. Furthermore, calnexin expression was not changed in 4 and 9-day-old seedlings treated by 10% PEG for 2 days. The reduction of calnexin expression in soybean roots indicates that soybean seedlings are more sensitive to osmotic stress in the second week of the growth.

2.3.6 Soybean is more sensitive to abiotic stresses than rice

To evaluate the expression of calnexin in rice, possibility of cross-reaction of soybean calnexin antibody in rice, was checked by comparison of amino acid sequences.

The homology between soybean and rice calnexin amino acid sequences was 72% (Figure 28). Alignment of amino acid sequences of these 2 crops indicated that N-terminal and C-terminal domains have more diversity and the middle section has several conserved regions. The calcium binding sites of KPEDWD and GEWEAPKI were similar in both crops.

In order to evaluate the expression of calnexin in soybean and rice, 2-week-old soybean and rice were compared under various stress severity. Twelve-day-old soybean and rice seedlings were subjected to 0, 5, 10% PEG for 2 days. Because of differences in root expansion of 12-day-old soybean and rice, a preliminary experiment was conducted to evaluate the effects of PEG on both plants. The seedlings were irrigated with PEG solution in seedbed or removed from the seedbed and transferred to PEG solution for 2 days (Figure 29). Results of morphological evaluation were indicated that the length of roots and shoots of soybean seedlings were significantly reduced by 5 and 10% PEG stress. However, fresh weights were only affected under 10% PEG. In rice, while the length of roots and shoots were not affected by stress, fresh weight was reduced under stress condition. Immunoblot analyses of soybean and rice under various PEG concentrations were performed (Figure 30). It was shown that 10% PEG significantly reduced the expression of calnexin in soybean roots. A mild stress by subjecting 5% PEG did not affect the expression of calnexin in soybean. In rice, however, calnexin expression was not changed under stress conditions. It seems that soybean at 2-week-old is more sensitive that rice in response to osmotic stress.

Effects of various abiotic stresses on 2-week-old soybean and rice were evaluated. Morphological analysis in soybean indicated that lengths and fresh weights of soybean shoots and roots were significantly affected by most of stresses (Figure 31). The effect

of abiotic stresses on rice morphology was less than that of soybean (Figure 32). For instance, fresh weight of rice roots was not changed under various stresses. While drought stress caused significant increase in rice root length, PEG stress reduced the root length and other stresses did not present any significant changes. Comparison of immunoblot analysis in soybean and rice under various abiotic stresses was performed (Figure 33). It was shown that all abiotic stresses significantly reduced the expression of calnexin in soybean roots. However, in rice, the expression of calnexin under abiotic stresses was not changed. Taken together, it can be concluded that 2-week-old soybean is more sensitive to abiotic stress condition than rice. It might be because of the differences in developmental stages of 2 crops in the second week of the growth. While soybean has an extended root and shoots in the second week of the growth, rice is in the 2 leaves stage.

2.4 Discussion

Calnexin is an ER-resident molecular chaperon protein and according to the results of proteomics, it was highly up-regulated in soybean plasma membrane fraction under osmotic stress. The presence of this protein in purified plasma membrane fraction was confirmed by immunoblot analysis. Gene expression of this protein was not changed within time courses and the expression of this protein in ER-enriched membrane fraction was not changed. Therefore, it can be concluded that calnexin targets to the plasma membrane under osmotic stress. The presence of this protein in plasma membrane was already reported in animal cells and effect of changes in cellular conditions on accumulation of this protein in plasma membrane was explained. Gagnon

et al. (2002) reported that the cell types or cellular homeostasis are indicating factors for calnexin expression in plasma membrane. Another mechanism for the presence of calnexin in plasma membrane is that this protein escapes from the ER through a specific binding to glycoproteins (Okazaki et al., 2000). Association of this protein with plasma membrane localized transporter proteins such as plasma membrane H^+-ATPase was reported in oat seedlings (Li et al., 1998). It can be concluded that osmotic stress conditions change ion homeostasis of plant cells and activates the pathways for increasing the accumulation of calnexin in plasma membrane.

Four predicted genes were available for calnexin according to the soybean genome database. It was reported that duplications in the genome of soybean result in nearly 75% of the genes being present as multiple copies (Schmutz et al., 2010). Presence of multiple copies of specific proteins in soybean makes this crop more complicated for functional analyses. Considering to the amino acid sequence alignments of soybean predicted genes and rice in this study and based on the reviewed literatures in other organisms, it can be concluded that calnexin has conserved regions in the middle parts of the sequence. P-domain of calnexin especially calcium-binding domain is highly conserved in calnexin sequence.

In the first week of soybean growth, calnexin expression in several stress conditions as well as in normal growth condition was evaluated. The results indicate that not only expression of this protein is constant in normal condition; exposure of various abiotic stresses does not change calnexin expression in P100. Soybean in the second week of growth was highly responded to osmotic stress, both in morphology and calnexin expression. The differences in calnexin expression behavior in the early growth stage at the first week of growth comparing to the second week, may imply the

existence of different cellular mechanism. In this hypothesis, behavior of molecular chaperon of calnexin in the cell is different in the first and second week of growth. In the other hand, the expression of this protein was not changed in rice under various stress conditions. Since, rice growth is much slower than soybean in the early growth stages, it might be concluded that the expression of molecular chaperon of calnexin is only affected in morphologically extended crop such as soybean.

2.5 Summary

Calnexin was identified as an up-regulated protein in plasma membrane fraction of soybean seedlings under osmotic stress. This protein is an ER-resident molecular chaperone protein involves in protein folding and quality control of proteins. There are several conserved regions in amino acid sequences of calnexin, especially in calcium-binding domains between soybean and rice. An overexpressed calnexin antigen was synthesized using full length cDNA of soybean to prepare antibody for immunoblot analyses. To evaluate the expression of calnexin in soybean seedlings under abiotic stresses especially 10% PEG, proteins were extracted from seedlings within the first week and in the second week of growth and immunoblot analysis was performed. A comparison between soybean and rice has been performed for calnexin expression in 2-week-old seedlings under abiotic stress. Results of immunoblot analysis indicated that calnexin was accumulated in plasma membrane fraction of 4-day-old seedlings treated by 10% PEG stress for 2 days. Despite of significant morphological changes, this protein was constantly expressed within the first week of growth under various abiotic stresses, time courses and in different organs. Results of immunoblot analyses in

2-week-old seedlings of soybean and rice indicated that the expression of calnexin in soybean is highly decreased under various abiotic stresses. Expression of calnexin in rice was not affected by abiotic stresses indicating difference between soybean and rice in response to abiotic stresses. These results suggest that calnexin expression in soybean under stress conditions is highly depends on the seedling age and soybean is more sensitive than rice at the same age.

CONCLUSION AND PROSPECTS

Cultivation of economically important crops such as soybean and rice would have the risk of yield loss because of various stresses which threaten plants growth and productivity. It is known that abiotic stress is one of the main causes of reduction of crops yield leading to an average yield loss of more than 50% (Bray et al., 2000; Wang et al., 2003b). Among the stresses, those which cause cellular dehydration, changes in osmotic potential and disturbance of ion homeostasis are more considerable. Drought and salinity are two main constraints in agriculture and along with cold cause osmotic stress in plant and are known as major abiotic stresses in crop. Soybean is one of the main sources of human and animal nutrition containing high level of vegetable oil and protein, both in the quantity and quality. It was reported that consumption of soybean-based products is increasing worldwide (Manavalan et al., 2009). In order to improve crop productivity under adverse environmental conditions, elucidation of the molecular mechanisms of stress is highly important in soybean.

A comprehensive proteome study has been performed on purified plasma membrane fraction of soybean using gel-based and LC MS/MS-based proteomics approaches. A wide range of proteins have been identified by both approaches as osmotic stress responsive proteins in which 7 proteins were mutual. This result clearly showed the efficiency of each technique for plasma membrane proteome analysis in that LC MS/MS-based technique could identify more plasma membrane proteins. The efficiency of LC MS/MS-based quantification techniques for identification of integral membrane proteins was already reported (Blackler et al., 2008; Hahne et al., 2008). The comparative proteome analysis which performed in this study is one of the first reports

in this sphere. It could reveal the capacities and limitations of each proteome studies and confirmed the complementary role of gel-based and LC MS/MS-based techniques for protein identification. It has also been pointed out that a wide range of proteins are involved as osmotic stress responsive proteins.

Identified proteins have been classified according to their function, GRAVY index, signal peptide, number of transmembrane domains and their localization in the cell. LC MS/MS-based proteomics technique could identify more proteins with transmembrane domains, positive GRAVY index and plasma membrane-localized proteins. These results clearly show the efficiency of LC MS/MS-based technique for identification of integral membrane proteins related to gel-based proteomics. Gel-based method was comparatively limited to identify transporter proteins. For instance, plasma membrane H^+-ATPase protein, is a critical ion transporter protein under stress condition involving in cell turgor and intracellular pH (Beffagna et al., 2005). Various homologues of this protein with 10 transmembrane domains have been identified as up-regulated proteins under osmotic stress using LC MS/MS-based technique. In this study identification of several regulators of this protein such as up-regulation of protein phosphatase and phototropin as activator and down-regulation of protein kinases as suppressor has confirmed the significant role of this protein. Activation of plasma membrane H^+-ATPase gene expression after 12 h of stress exposure and increase in activity of the enzyme confirmed the proteomics results. These results imply acceleration of ion efflux under hyperosmotic conditions by up-regulation of ion transport proteins.

Calnexin has been identified as only mutually up-regulated protein with more than 2 times increase in protein expression under osmotic stress in both proteomics

techniques. This protein which is an ER-resident molecular chaperon was identified in enriched plasma membrane fraction. The possibility of ER contamination in plasma membrane fraction and actual accumulation of this protein in plasma membrane under osmotic stress was investigated. Calnexin was identified as stress responsive protein in proteomics approaches, individually, using different methods of protein identification. Furthermore, plasma membrane fraction was prepared in several replicates for control and treatment and calnexin was up-regulated in the treatment replicates. Additional experiments on immunoblot analysis using several cell fractions have confirmed that accumulation of calnexin in plasma membrane is because of osmotic stress exposure. Calnexin expression was up-regulated under osmotic stress only in purified plasma membrane fraction and there was no change in the expression in other cellular fractions especially in membrane and microsomal fractions, known as total membrane and ER-enriched fractions, respectively. Taken together, since gene expression of calnexin is not changed under stress, it can be concluded that calnexin has been targeted to plasma membrane under osmotic stress condition in soybean seedlings. While several studies have reported targeting of this protein to plasma membrane (Gagnon et al., 2002; Myhill et al., 2008; Okazaki et al., 2000; Wiest et al., 1995), to the best of my knowledge this is the first report of calnexin expression in plant plasma membrane. Furthermore, none of previous reports were explained the stress condition as a factor for up-regulation of this protein in plasma membrane.

Evaluation of calnexin expression in various conditions of seedling ages, organs, time courses of soybean declared that calnexin expressed constantly in the first week of seedlings growth. Furthermore, expression of this protein was only affected in 2-week-old seedlings by various stresses and osmotic stress severity. These results

indicated that expression of molecular chaperone of calnexin reduces by increasing seedling age and this reduction is significantly more under osmotic stress or other abiotic stress conditions. It may imply the existence of different cellular mechanism between the first days of emergence and after 2 weeks of growth for quality control of proteins. Evaluation of rice roots for calnexin accumulation under various conditions of stress did not show any significant changes in calnexin expression. Since, emergence and seedling growth of rice is much slower than soybean; it seems that 2-week-old rice present the same behavior of 4-day-old soybean seedlings for calnexin expression. Further evaluation might be necessary to show the expression pattern of calnexin in different plants.

A schematic representation of cell behavior under osmotic stress is proposed in Figure 34. Stress signal percepts in outer part of the cell and transduces into the cell (Shinozaki and Yamaguchi-Shinozaki, 1997). Osmotic stress causes ER-stress in the cell and regulates large varieties of proteins including molecular chaperones. In the other hand, osmotic stress reduces the expression of several cell wall biosynthesis and many other proteins which known as cell wall related proteins. It affects on cell turgor and adhesion and disturbs ion homeostasis of the cell. Several homologues of cell adhesion related proteins are affected by the stress. These processes might activate a mechanism for acceleration of translocation of molecular chaperone protein of calnexin to plasma membrane via various pathways such as secretory pathway. Furthermore, considering to the calcium-binding properties of calnexin, the role of this protein in ion homeostasis of the cell along with other ion transporter proteins such as plasma membrane H^+-ATPase might be important.

Environmental stresses are known as complex phenomena in the nature and there

are various determining factors for degree of their deleterious effects to the plant cells. Understanding the mechanisms of stress response would be beneficial for increasing crop productivity (Bartels and Souer, 2004). This study could elucidate parts of the cell mechanisms against osmotic stress highlighting the critical role of several proteins especially molecular chaperone of calnexin. Although proteins and enzymes have main function in cellular processes, there are several other components such as cellular ions contents, metabolites and secondary products which have indicating role especially under stress conditions. Moreover, transcriptional expression and post translational modification of proteins as well as protein interactions are other important factors. Generation of a comprehensive network for intracellular interactions under stress condition using cellular information would be the main goal in molecular studies. Selection of a bio-marker according to the network to develop crop cultivars would be one of the best ways to improve crop productivity.

SUMMARY

Abiotic stress has deleterious effects on developmental stages and productivity of crop. Osmotic stress happens by drought, salinity and cold is a major abiotic stress and highly reduces the production of economical crop. Soybean is one of the important plants containing high level of vegetable oil and protein with increasing consumption worldwide. Study of molecular behavior of the cell and understanding plant response against osmotic stress would help to improve plant production. Proteome analysis is one of the main options for cell studies because of the capacity for elucidation of molecular pathways through identification of proteins and enzymes involve in stress response. In this study, comparative analyses of gel-based and mass spectrometry-based proteomics were performed for identification of stress responsive proteins from plasma membrane fraction. A selected candidate protein was characterized and further evaluated for its functions under stress condition.

Plasma membrane has a pivotal role in the cell under stress condition, interacting with the outer parts of the cell and inner cellular organelles. Plasma membrane fraction was purified from 4-day-old soybean seedlings subjected to 10% polyethylene glycol as osmotic stress for 2 days. A two-phase partitioning method has been used for purification of plasma membrane fraction and the purity was verified by measuring ATPase activity. Using the gel-based proteomics, 4 and 8 protein spots were identified as up- and down-regulated, respectively. In the mass spectrometry-based approach, however, 11 proteins were up-regulated and 75 proteins were down-regulated under osmotic stress. Gel-based and mass spectrometry-based proteomics approaches were complementary for identification of osmotic stress responsive proteins in soybean plasma membrane. Out of osmotic stress responsive proteins, most of the transporter proteins and all proteins with high number of transmembrane helices as well as low

abundance proteins were identified by mass spectrometry-based approach. Plasma membrane transporter proteins such as plasma membrane H^+-ATPase were up-regulated and the expression of gene in this protein was increased under osmotic stress. These results suggest that under hyperosmotic conditions, ion efflux accelerates by up-regulation of plasma membrane H^+-ATPase protein.

Among the identified proteins, 7 proteins were mutual in 2 proteomics techniques, in which the molecular chaperone of calnexin was the highly up-regulated protein. Calnexin did not change in gene expression level within time courses. Expression of calnexin was evaluated in several cell fractions using immunoblot analysis and it was significantly increased in plasma membrane fraction under osmotic stress. Immunoblot analysis confirmed the results of proteome study for accumulation of calnexin in plasma membrane. Evaluation of calnexin expression in various conditions of seedling ages, organs and time courses of soybean declared that calnexin expressed constantly in the first week of seedlings growth. Furthermore, expression of this protein was reduced in 2-week-old seedlings by various stresses and osmotic stress severity. Evaluation of rice roots for calnexin accumulation under various conditions of stress did not show any significant changes in calnexin expression. These results suggest that calnexin expression in membrane fraction of soybean reduces by increasing seedling age and this reduction is significantly more under osmotic stress.

These results suggest that osmotic stress causes endoplasmic reticulum stress in the cell and regulates large varieties of proteins in plasma membrane. The mechanism of calnexin translocation from endoplasmic reticulum to plasma membrane can explain the accumulation of this protein in plasma membrane of soybean seedlings.

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Table 1. ATPase activity of plasma membrane proteins with or without marker enzymes.

	μmol Pi μg protein^{-1} h^{-1}	Purity (%)
Total		
Control	0.094 ± 0.014	
Specific (marker enzyme)[a]		
Na_3VO_4 (0.1 mM)	0.007 ± 0.002	92.5
KNO_3 (50 mM)	0.085 ± 0.013	9.7
NaN_3 (10 mM)	0.088 ± 0.014	6.3

[a] The sensitivity of ATPase activity to vanadate, nitrate and azide was used to distinguish between plasma membrane, vacuolar and mitochondrial membrane enzymes, respectively.

Numbers shown are average ± S.E. of 10 independent plasma membrane-enriched fraction.

Table 2. Differentially expressed proteins in soybean plasma membrane under PEG stress identified by gel-based proteomics.

Spot no.[a]	Accession no.[b]	Description	Score[c]	Cov.[d] (%)	M.P.[e]	Id.[f] (%)	Theo.[g] Mr(Da)/pI		Exp.[h] Mr(kDa)/pI		F.C.[i]	P value[j]
1	ABB77236	patellin1	257	19	18	87	65127	4.8	86	4.4	-2.54	0.042
2	Q39817	calnexin homolog	744	39	24	100	62080	4.8	70	5.7	2.1	0.041
	AAA81954	BiP isoform B	403	19	8	100	73674	5.1				
	ACI31552	heat shock protein 90-1	392	20	10	100	80700	4.9				
	CAO21687	unnamed protein product	329	22	7	100	71696	5.1				
	AAB03258	phosphoinositide-specific phospholipase CP13	314	20	10	100	69053	5.7				
3	ABK95079	unknown protein	82	14	7	88	43303	4.9	45	4.3	-6.1	0.049
	CAB17076	cysteine proteinase precursor	80	13	6	92	53279	5.5				
	NP_199226	fasciclin-like arabinogalactan protein 13	54	18	6	75	26443	9.2				
4	CAB61742	plasma membrane intrinsic polypeptide	1269	51	12	81	24255	5.0	41	4.8	-1.85	0.011
	CAO70926	unnamed protein	478	36	13	92	30508	6.2				
	ABK92956	unknown protein	247	16	5	96	30188	6.7				
5	AAO69667	vacuolar ATPase subunit E	653	40	17	100	26029	6.5	43	6.7	-2.74	0.039
	ABA07956	glyceraldehyde-3-dehydrogenase C subunit	352	26	6	100	36815	6.7				
	AAB04057	delta 24-sterol-C- methyltransferase	132	20	5	100	42005	7.5				
6	AAT40505	elongation factor 1-beta	102	32	7	87	24300	4.5	35	4.3	1.6	0.022
	AAF64040	14-3-3-like protein	60	23	5	88	19180	4.9				
7	Q96453	14-3-3-like protein D	621	37	13	100	29614	4.8	33	4.5	-2.03	0.042
	NP_177142	band 7 family protein	250	18	5	95	31652	5.1				

8	Q96453	14-3-3-like protein D	226	28	8	100	29614	4.8	32	4.8	-1.9	0.025
9	CAD31838	putative quinone oxidoreductase	224	25	5	100	21708	6.5	26.8	6.4	2.15	0.045
10	ABB02384	temperature-induced lipocalin	273	41	8	100	21281	7.8	25	6.5	-1.83	0.037
11	Q39839	nucleoside diphosphate kinase 1	591	47	11	100	16489	5.9	22	5.9	1.84	0.043
12	ABU97472	profilin	376	56	6	100	11167	4.3	21	4.2	-2.42	0.033

[a] Spot no., The spot numbers as given in figure 5.

[b] Accession no., accession number according to the NCBI database.

[c] Score, Ions score of identified protein using NCBI and soybean genome sequence databases (http://www.phytozome.net).

[d] Cov., sequence coverage, the proteins with less than 10% sequence coverage were excluded from the result.

[e] M.P., number of query matched peptides, the proteins with > 5 matched peptides were considered.

[f] Id., Identity of protein identified by NCBI database.

[g] Theo., theoretical ; Mr, molecular weight; pI, isoelectric point.

[h] Exp., experimental.

[i] F.C., fold change.

[j] P value, indicates the significance of up- or down-regulation of spots according to the F-test through analysis of variance ($P<0.05$).

Table 3. Plasma membrane proteins with significant changes in expression level under PEG treatment identified by LC MS/MS-based proteomics using SIEVE (P<0.05).

No.	Accession No.[a]	Description	Score[b]	Theo.[c] Mr(Da)/pI		Ratio[d]
1	ABC49719	actin depolymerizing factor-like protein	254	16090	6.1	0.29
2	EEF41529	alpha-soluble NSF attachment protein	425	32535	5.1	0.22
3	XP_002316384	amino acid transporter	461	53241	5	0.27
4	AAB01221	ascorbate peroxidase 2	441	27140	5.6	0.34
5	EEF42393	ATP binding protein	828	95276	4.8	0.26
6	NP_001151807	ATP synthase beta chain	872	58979	5.9	0.2
7	ACM78033	beta-tubulin	850	49880	4.8	0.19
8	BAD95470	BiP	1159	73594	5.1	0.38
9	P17928	calmodulin	297	16861	4.1	0.3
10	Q39817	calnexin homolog	858	62080	4.8	2.3
11	BAF36056	calreticulin-1	683	48174	4.4	0.37
12	XP_002298432	cellulose synthase	1825	120484	6.7	0.3
13	AAY60847	cellulose synthase 5	986	121881	6.4	0.24
14	2IUJ_A	chain A, crystal structure of soybean lipoxygenase-B	1588	96658	5.6	0.42
15	EEF34069	clathrin heavy chain	2971	193214	5.3	0.34
16	EEF48730	conserved hypothetical protein	751	51924	9.7	0.33
17	CAE47488	copper amino oxidase	1284	75615	6.9	0.37
18	AAX94775	cyclophilin	310	18124	8.7	0.39
19	AAF85975	cytosolic phosphoglycerate kinase	733	42287	5.7	0.14

20	XP_002316776	fasciclin-like arabinogalactan protein	428	34946	5.9	0.2
21	ABV27483	fasciclin-like arabinogalactan protein12	398	42927	5.3	0.27
22	ABV27484	fasciclin-like arabinogalactan protein 13	395	43911	5.4	0.29
23	BAE71278	fasciclin-like arabinogalactan protein FLA2	488	44822	6.1	0.32
24	O65735	fructose-bisphosphate aldolase	634	38452	6.2	0.31
25	ABC75834	glyceraldehyde-3-phosphate dehydrogenase	637	36764	6.7	0.28
26	EEF33338	glycerophosphoryl diester phosphodiesterase	969	83323	5.6	0.17
27	AAD48471	glycine-rich RNA-binding protein	166	15847	6.6	0.47
28	CAA59799	H^+-transporting ATPase	1580	104443	6.8	1.7
29	P26413	heat shock 70 kDa protein	1141	70879	5.4	0.32
30	ABA95501	heat shock cognate 70 kDa protein	1066	67320	4.9	0.22
31	BAE71297	hypothetical protein	339	39140	7.7	0.37
32	XP_002266279	hypothetical protein	271	24062	4.7	0.23
33	XP_002277247	hypothetical protein	112	8380	9.5	1.55
34	XP_002281459	hypothetical protein	301	24133	5.3	0.56
35	XP_002285741	hypothetical protein	674	44564	10.4	0.24
36	ABD32399	hypothetical protein	245	21419	5.4	2.03
37	XP_002279106	hypothetical protein isoform 2	88	11408	4.3	0.68
38	ABX60408	lipoxygenase L-3	1484	96768	6.1	0.3
39	ABQ41114	monodehydroascorbate reductase	720	47279	5.9	0.38
40	NP_192400	NSF (N-ethylmaleimide sensitive factor)	1090	81487	5.7	0.24
41	NP_001056941	Os06g0172600	105	6931	12.1	0.12
42	BAD89967	phototropin	1555	110725	6.5	3

43	CAB69824	plasma membrane H^+ ATPase	1584	105346	5.9	1.57
44	BAC77531	plasma membrane H^+-ATPase	1581	104865	6.4	1.43
45	CAB61742	plasma membrane intrinsic polypeptide	182	23346	4.9	0.22
46	XP_002300761	predicted protein	442	26925	5.8	0.2
47	XP_002301813	predicted protein	142	27058	8.6	2.4
48	XP_002316240	predicted protein	228	12811	10.7	0.26
49	XP_002320784	predicted protein	150	21491	6.7	0.2
50	XP_002321856	predicted protein	199	21443	7.6	0.27
51	AAC97524	protease inhibitor	106	8772	9.1	3.6
52	BAG16714	protein disulfide isomerase	900	58591	5.1	0.32
53	AAO92595	protein kinase Pti1	691	40386	8.1	0.44
54	EEF31736	protein phosphatase 2c	1255	97094.5	6.6	2.2
55	CAG14984	putative lipid transfer protein GPI-anchored	181	19576	5.8	0.35
56	AAU10526	putative receptor-like protein kinase 2	1236	110103	5.6	0.32
57	AAL06644	quinone oxidoreductase	432	36195	6.6	0.32
58	EEF50502	remorin	173	20743	8.7	0.25
59	ABN08656	ribosomal protein S10	232	13711	9.5	0.16
60	BAH03477	secretory carrier-associated membrane protein 2	445	30529	7.7	0.21
61	ABO61376	serine hydroxymethyltransferase	868	45302	8.6	0.27
62	P10743	stem 31 kDa glycoprotein	500	29280	6.7	0.16
63	Q7M1R5	superoxide dismutase [Cu-Zn]	284	15194	5.3	0.39
64	ABB02390	temperature-induced lipocalin	336	21465	6.2	0.3
65	AAR15081	translational elongation factor 1 subunit B	246	25307	4.4	0.25

66	ABA86966	triosephosphate isomerase	446	27204	5.9	0.22
67	AAX86047	tubulin A	815	49692	5	0.68
68	Q9ZRR5	tubulin alpha-3 chain	828	49729	4. 9	0.37
69	EEF51188	tubulin beta chain	836	50027	4.8	0.3
70	EEF52727	tubulin beta chain	819	50261	4.7	0.29
71	P12460	tubulin beta-2 chain	844	50628	4.8	0.33
72	ABV89642	universal stress protein	203	17588	7.8	2.1
73	ABK93473.1	unknown	173	21450	5.4	0.42
74	ACJ83843	unknown	234	16209	10.1	0.14
75	ACJ83940	unknown	225	20785	4.8	0.3
76	ACJ83959	unknown	354	21930	9. 9	0.34
77	ACJ85277	unknown	224	12368	9.6	0.53
78	ACJ85422	unknown	313	32596	5.1	0.31
79	ACJ86044	unknown	174	24261	4.6	0.46
80	ACJ86147	unknown	349	21782	9.3	0.25
81	AAO69667	vacuolar ATPase subunit E	392	25816	6.4	0.18
82	ABO61030	vacuolar H^+-ATPase B subunit	944	54075	4.9	0.22
83	P13548	V-type proton ATPase catalytic subunit A	1192	68681	5.3	0.17
84	Q40079	V-type proton ATPase subunit B 2	460	53726	5.1	0.33
85	Q96450	14-3-3-like protein A	459	29049	4.7	0.23
86	AAM93434	40S ribosomal S4 protein	4491	29961	10.3	0.37

[a] Accession no., accession number according to the BLAST search in NCBI database.

[b] Score, score of the BLAST search in NCBI database.

[c]Theo., theoretical ; Mr, molecular weight; pI, isoelectric point.

[d] Ratio, protein abundance changes in osmotic stress compared to the control. Proteins with the ratio greater than 1 are up-regulated.

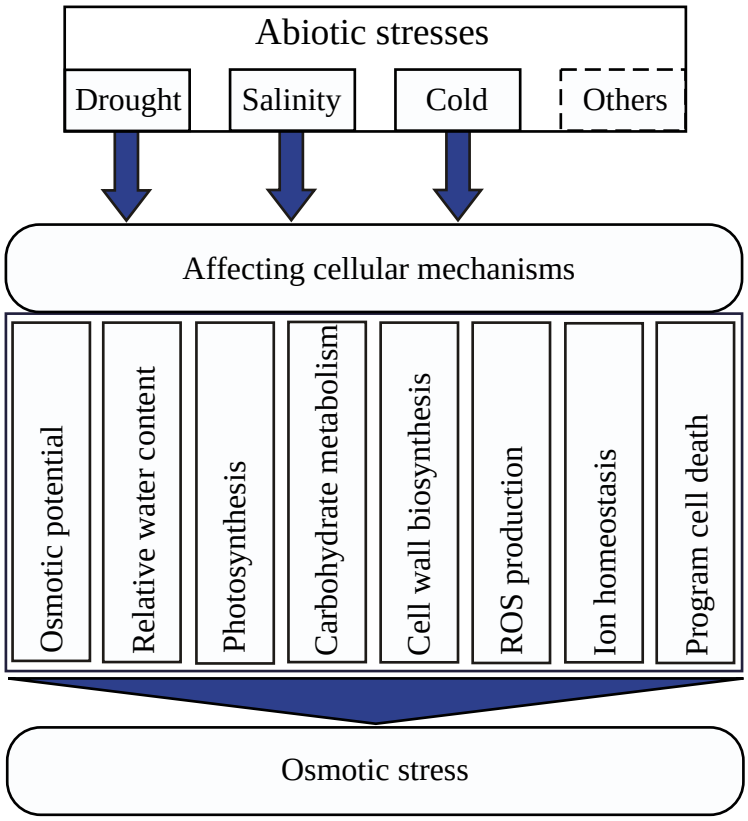


Figure 1. Representation of some common cell responses in abiotic stresses. Drought, salinity or cold perform several common responses with osmotic stress in plant cells.

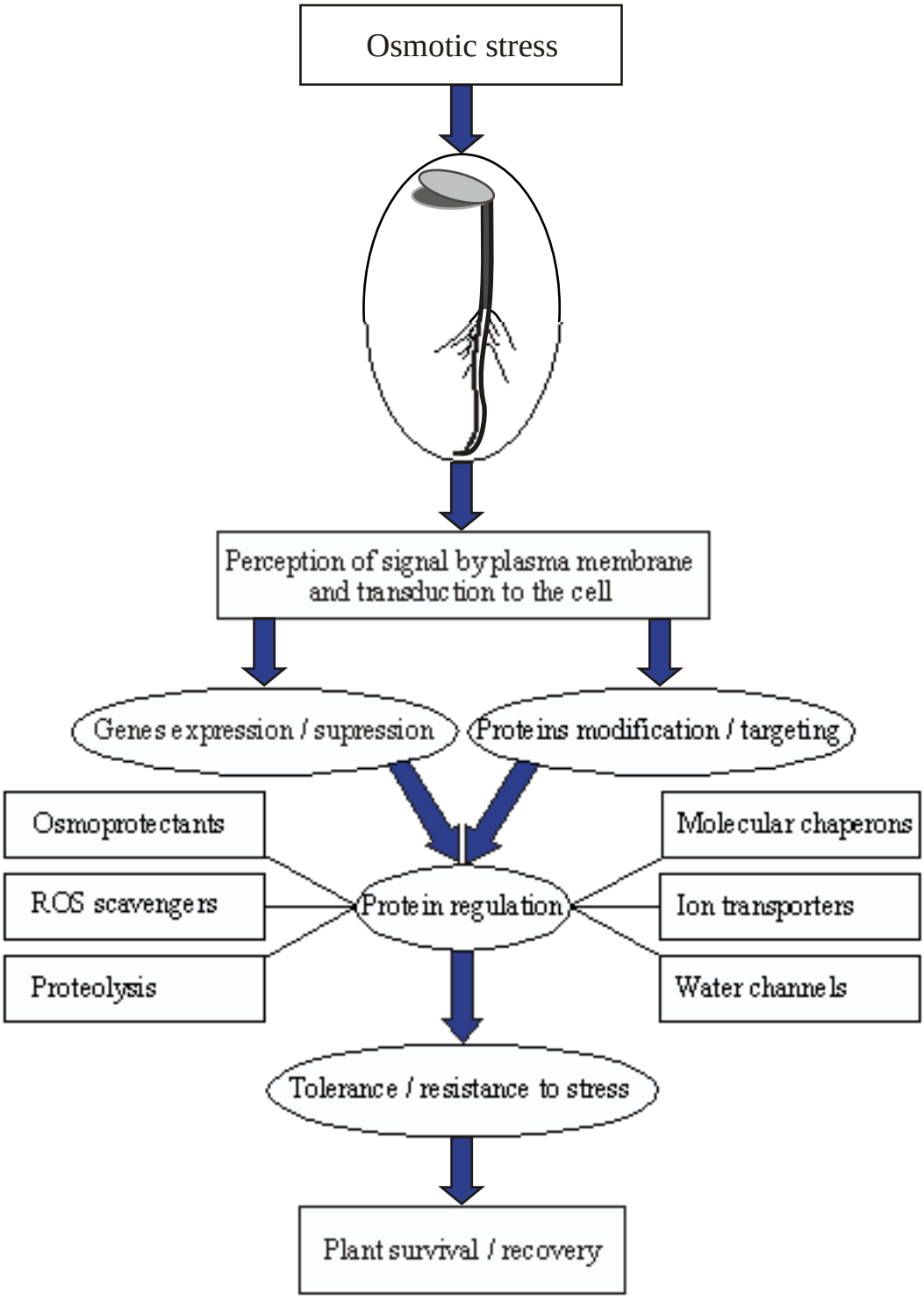


Figure 2. Schematic representation of molecular mechanisms associated with osmotic stress. Stress signals cause changes in various molecular mechanisms in the cell leading to regulation of several groups of proteins to cope with the stress.

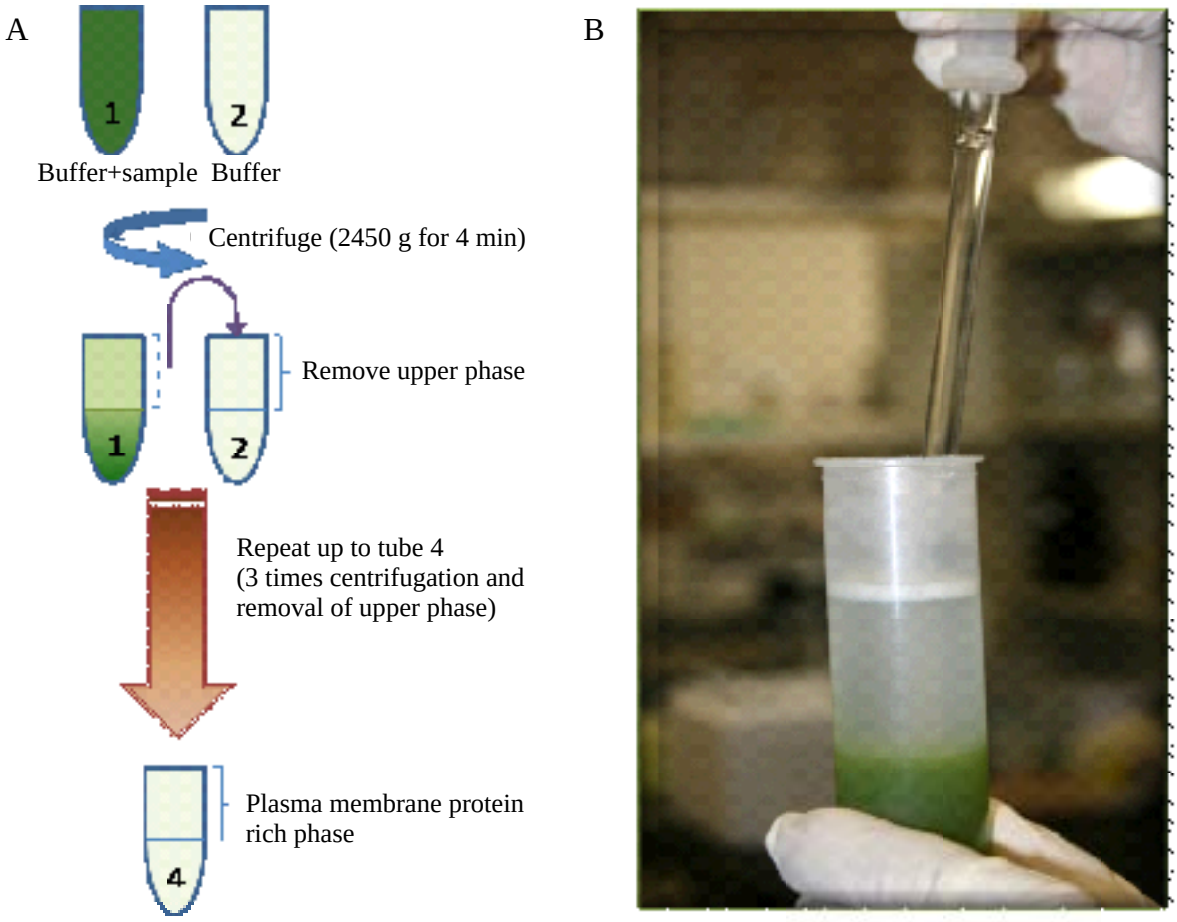


Figure 3. Plasma membrane purification using two-phase partitioning technique. Extracted proteins were added to the buffer containing dextran and PEG. The upper phase was added to a fresh lower phase for 4 times after each centrifugation (A). Photo shows the collection of upper phase after the first round of centrifugation (B).

A

B

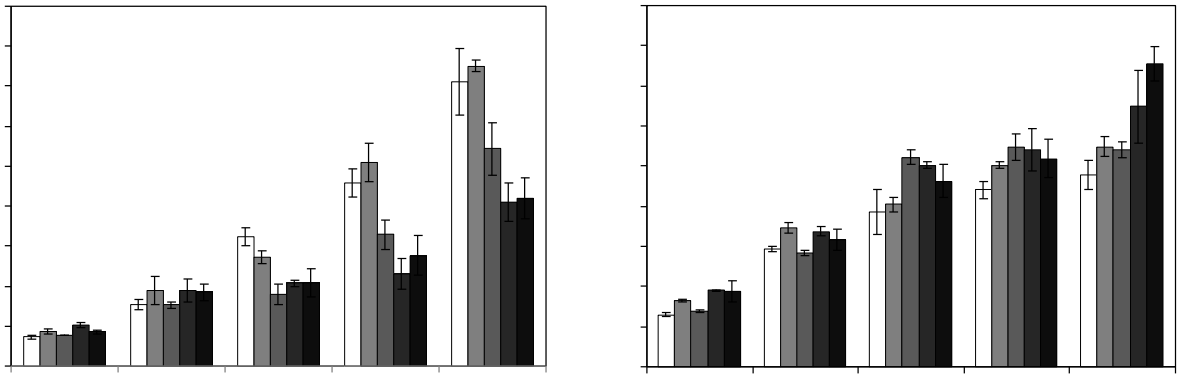


Figure 4. Effect of 10% PEG on the morphological characteristics of soybean seedlings. Two-day-old seedlings were treated with or without 10% PEG for 1, 2, 3 or 4 days. After 1, 2 and 3 days of the treatment, the PEG was removed by washing the sand. Photograph shows the morphological differences of 6-day-old soybean seedlings (A). Root and hypocotyl lengths were measured in control (white columns), 1 day (light-gray columns), 2 days (gray columns), 3 days (dark-gray columns) and 4 days (black columns) treatments (B). The values are the average ± S.E. from 3 independent experiments. Means with at least 1 similar letter are not significantly different at the 0.05 level according to the Tukey`s range test.

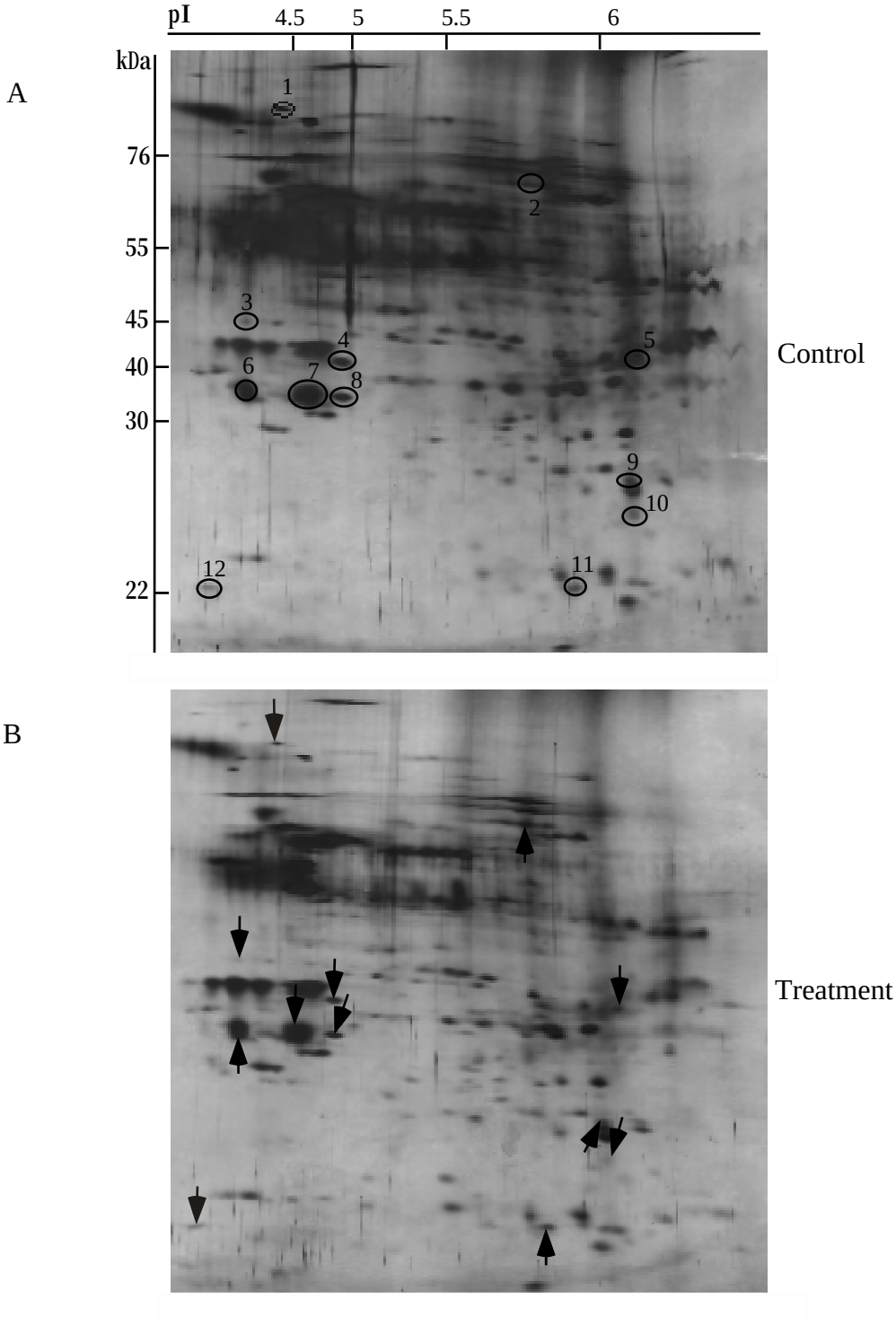


Figure 5. 2-DE patterns of plasma membrane proteins from roots and hypocotyls of soybean under 10% PEG treatment. Two-day-old soybean seedlings were treated without (A) or with 10% PEG (B) for 2 days. Purified plasma membrane proteins were separated by 2-DE and visualized by silver staining. The pI and Mr of selected proteins were determined using a 2-DE marker (Bio-Rad). Circles mark the position of the proteins on the control. Arrows indicate protein changes caused by 10% PEG; upward arrows indicate up-regulation and downward arrows indicate down-regulation.

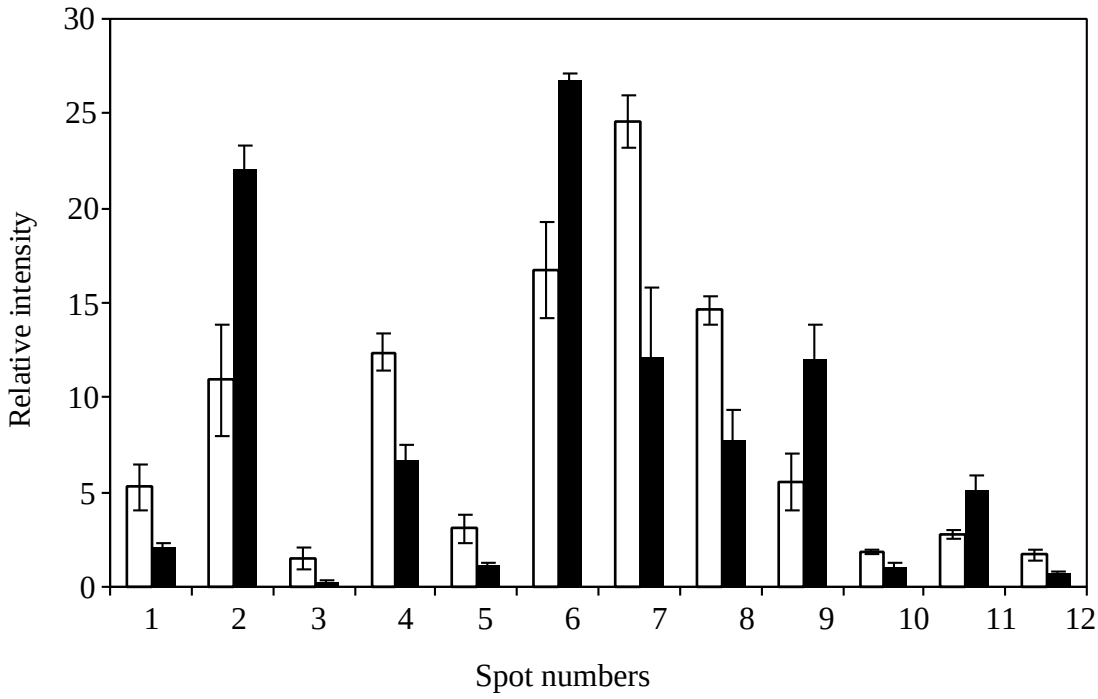


Figure 6. Relative intensity of protein spots regulated by osmotic stress. Changes in protein expression under 10% PEG treatment were calculated by PDQuest software using 3 replications. The values are relative intensities of spots for control (white bar) and treatment (black bar) ± S.E. ($P<0.05$).

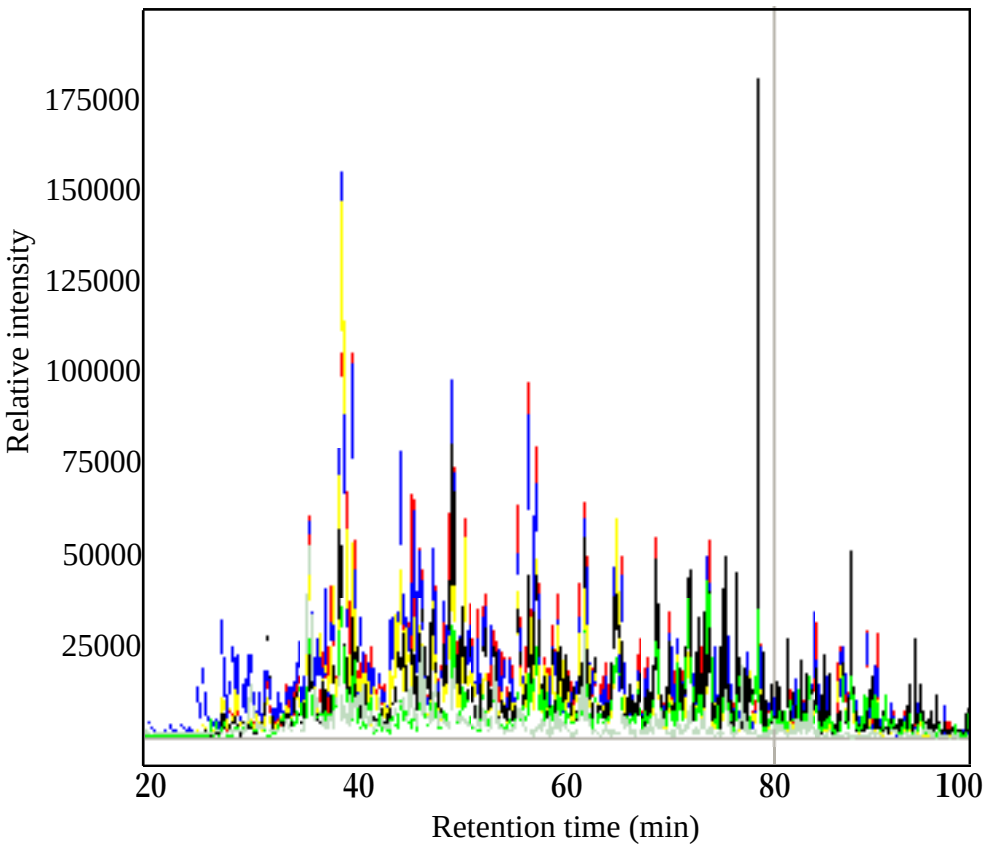


Figure 7. Chromatographic alignment of 6 data files of control and treatment in gel-free quantification technique using SIEVE. Three biological replicates of plasma membrane proteins from soybean seedlings without or with osmotic stress were applied to LC- MS/MS and generated data files were used for quantification. Overlap of the chromatographic surfaces were maximized using a fast fourier transform algorithm and using full MS scan, all components of the samples were fully aligned according to the maximization of the sum of correlation co-efficient of peaks for a specified retention time. Each color stands for an individual data file.

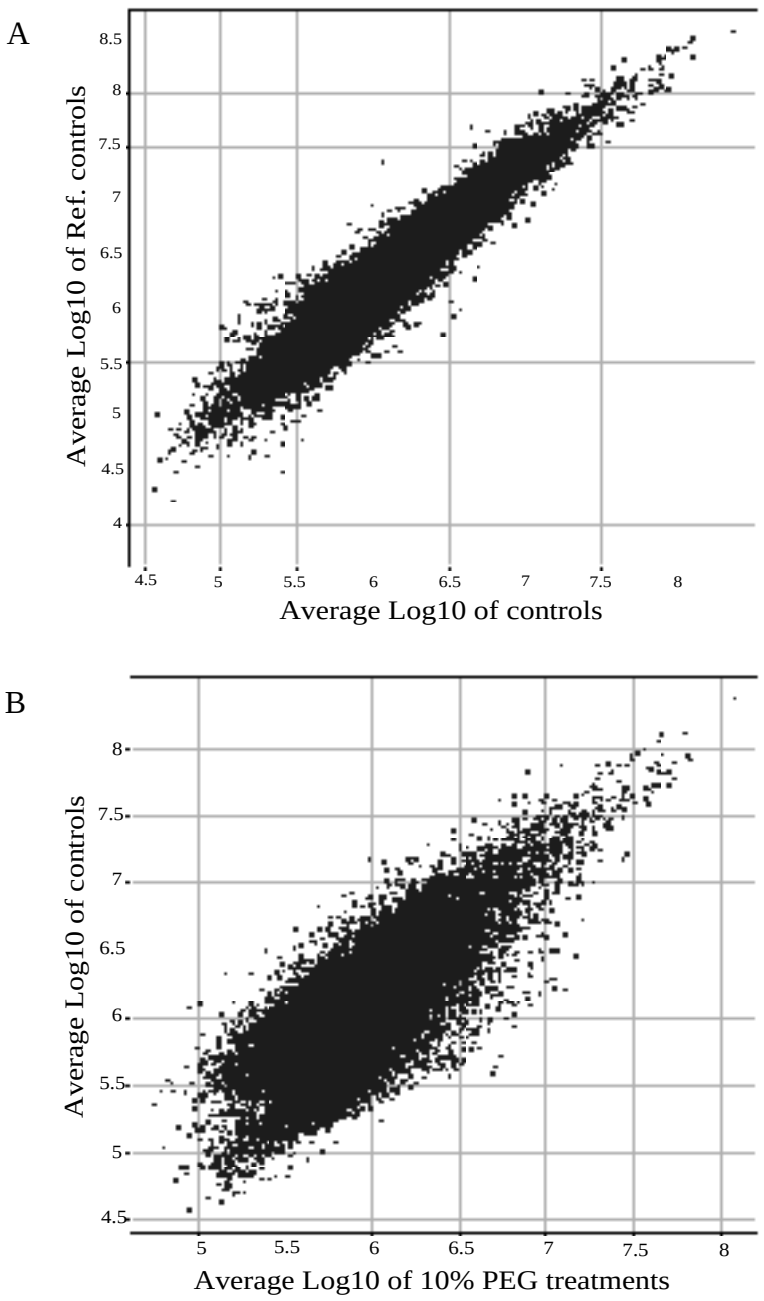


Figure 8. Distribution plots of peak intensities of LC MS/MS-based proteomics data sets. Two-day-old soybean seedlings were treated without or with 10% PEG for 2 days. Plasma membrane proteins were purified and digested. Three biological replicates of control and treatment were subjected to nano LC MS/MS, twice. Raw spectral data files of control and PEG treatment were analyzed by SIEVE software. Peak intensities of data sets were detected and quantified and transformed to log10 scale. Log10 of peak intensity of reference control was plotted versus average log10 peak intensities of all controls (A). Average log10 of peak intensities of control plotted versus Average log10 of peak intensities of treatment (B).

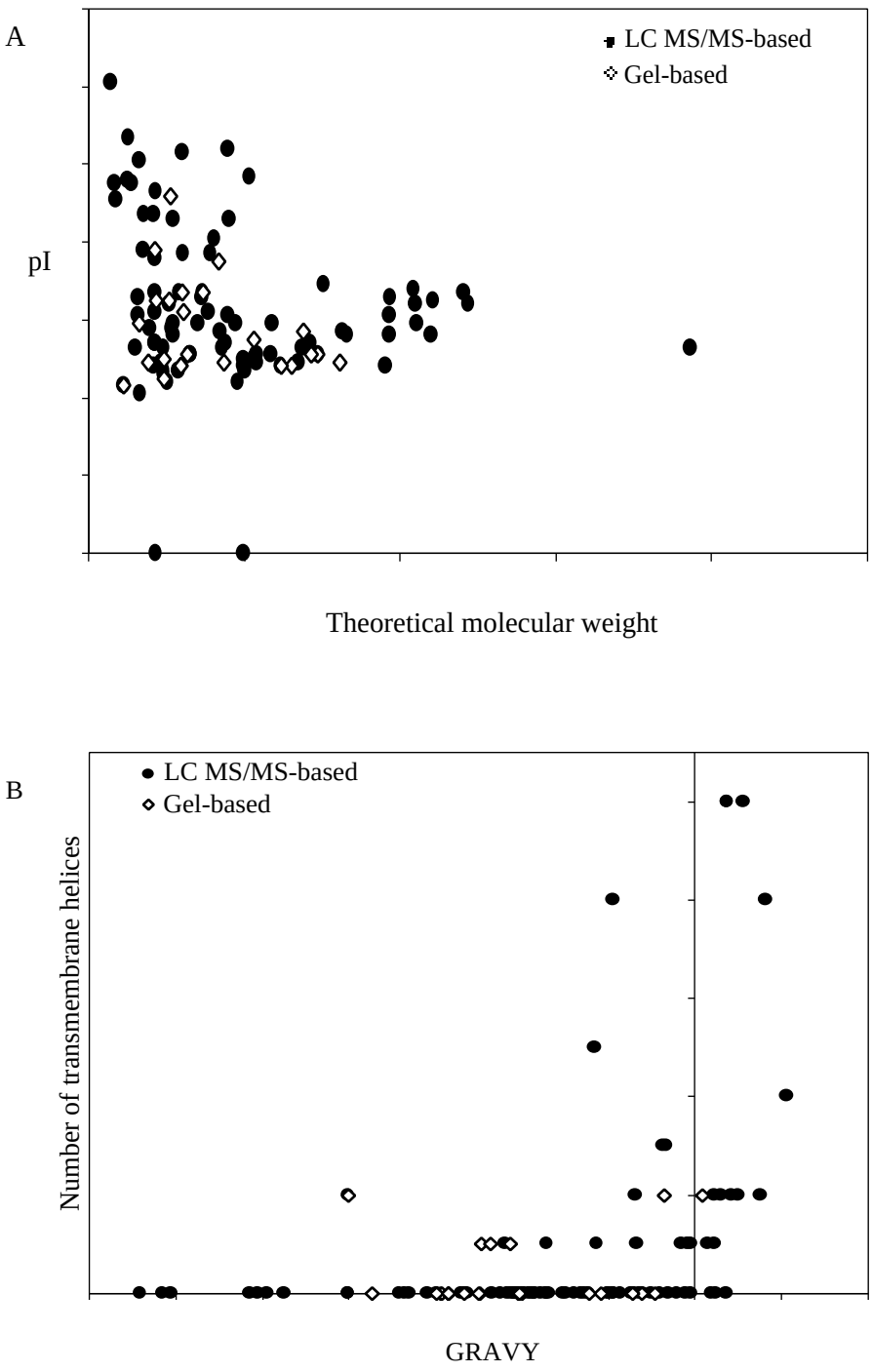


Figure 9. Scatterplot of stress responsive proteins identified by gel-based and LC MS/MS-based approaches. Distribution of 10% PEG stress responsive proteins were plotted for molecular weight and pI (A) or GRAVY and number of transmembrane helices (B).

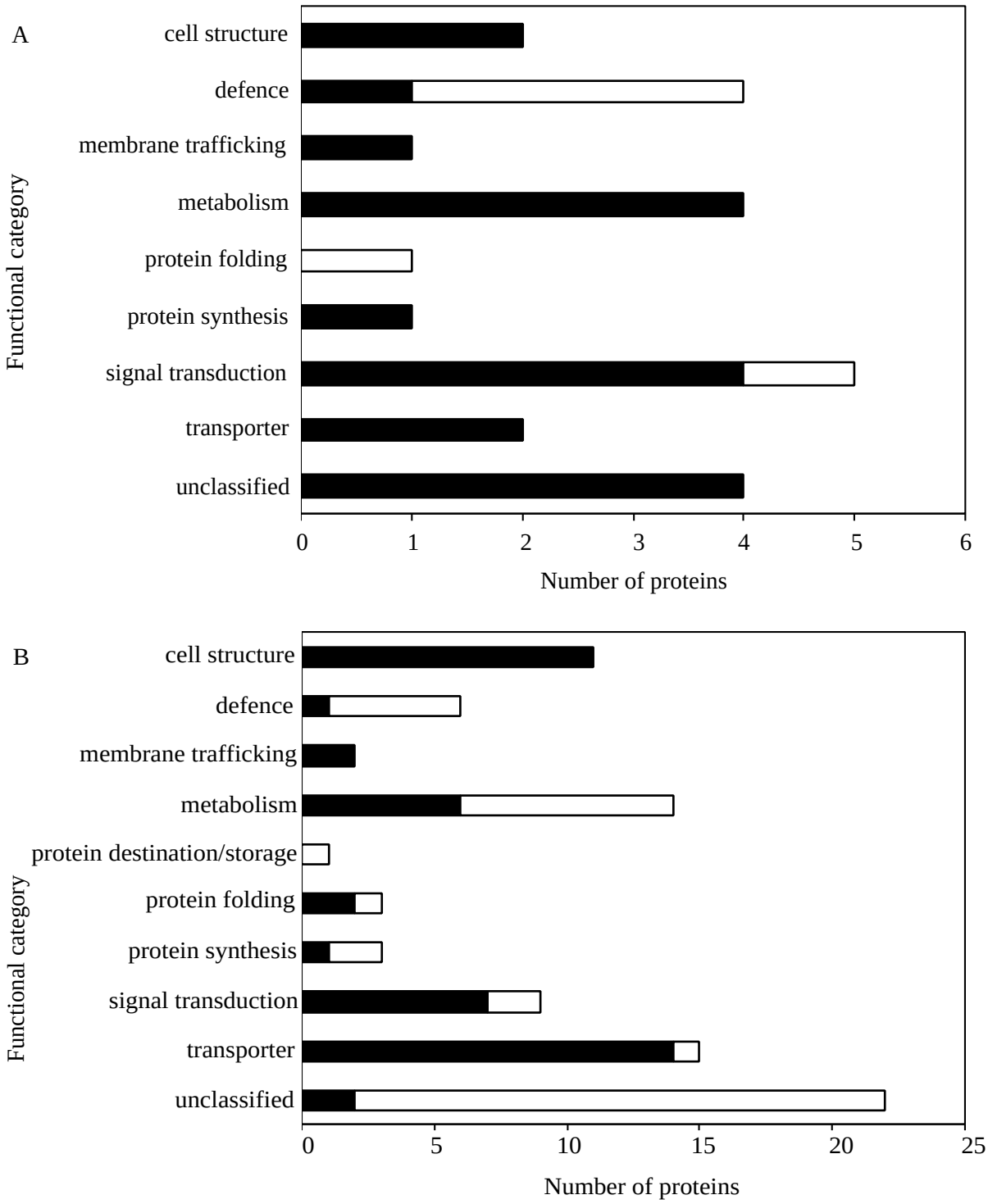


Figure 10. Functional classification of differentially changed proteins under 10% PEG stress. Proteins were assigned using classifications described by Bevan et al. (1998) (Supplemental Tables 1, 2). The number of proteins in each functional category is shown for plasma membrane proteins from gel-based (A) or LC MS/MS-based proteomics techniques (B). The number of proteins were separated according to the predicted/ reported plasma membrane localized (black bar) or putative candidate (white bar) proteins.

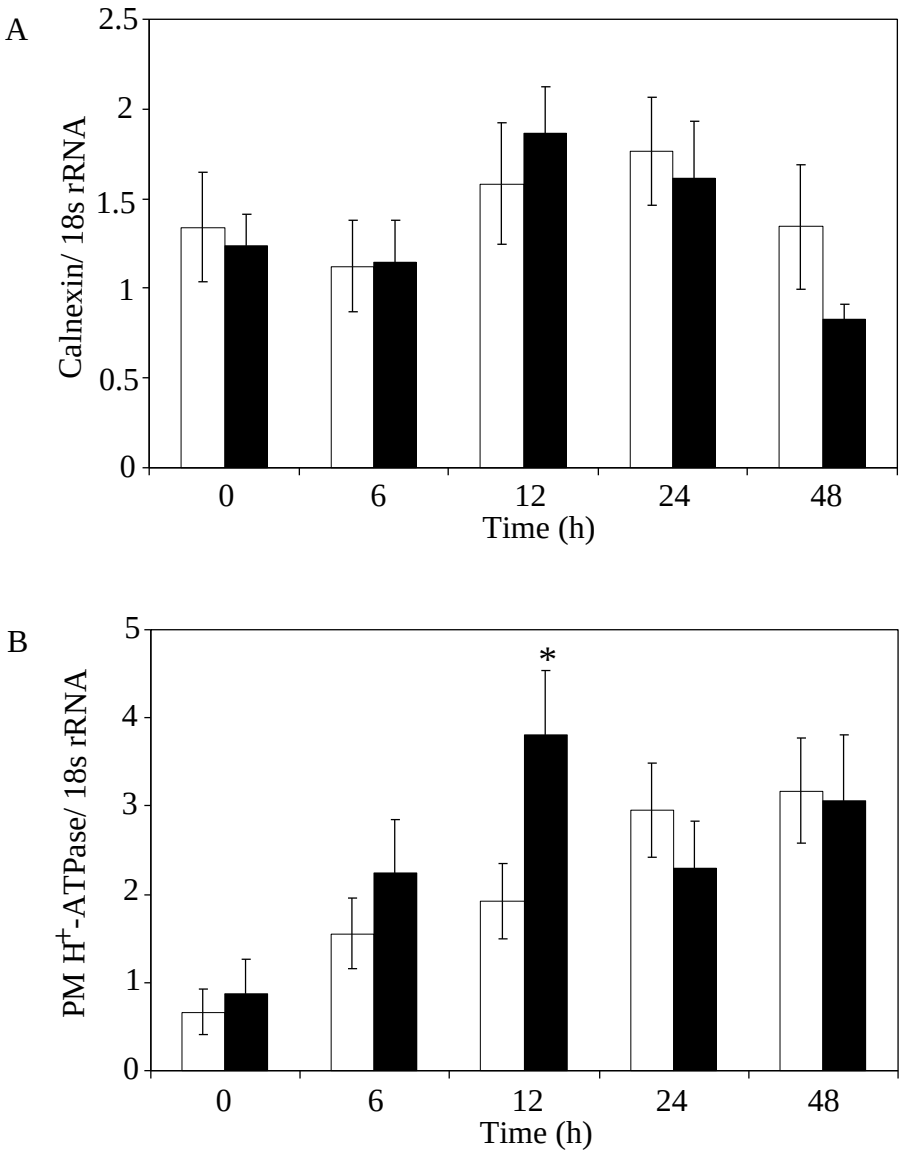


Figure 11. Gene expression analysis of highlighted proteins in response to 10% PEG treatment. Total RNA was isolated from soybean seedlings after 0, 6, 12, 24 and 48 h of 10% PEG treatment. cDNA was prepared by reverse transcription and subjected to quantitative real-time PCR. Relative transcript abundance was calculated and normalized with respect to the 18s rRNA transcript level for calnexin (A) and plasma membrane H^+-ATPase (B). The values are average ± S.E. from 3 independent reactions. Asterisk indicates significant change in gene expression at $P<0.05$.

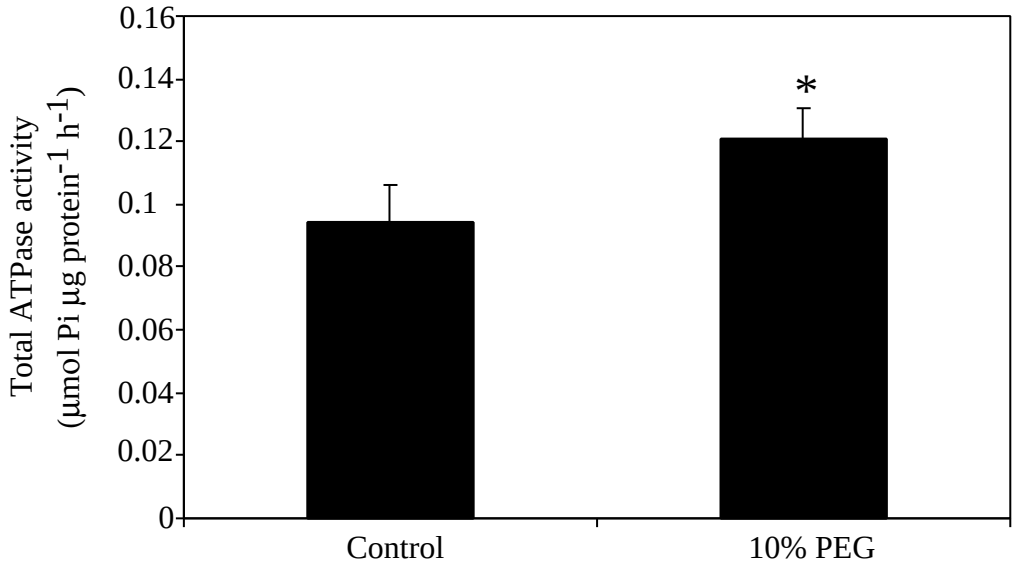
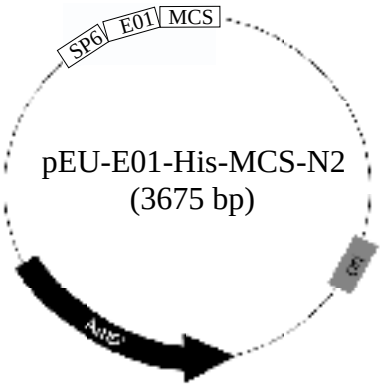


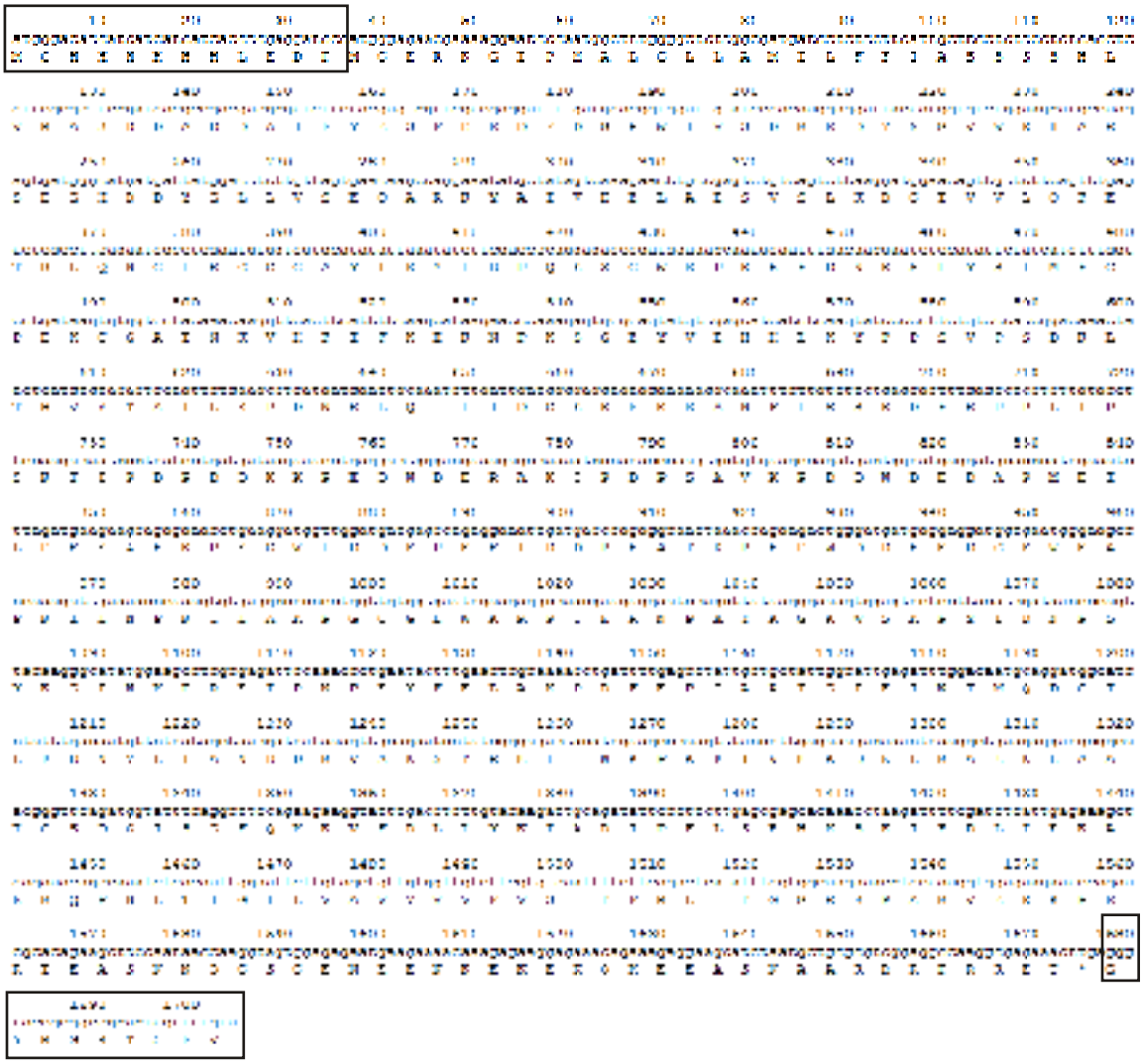
Figure 12. Total activity of ATPase in soybean plasma membrane in control and 10% PEG treatment. Two-day-old soybean seedlings were treated without or with 10% PEG for 2 days. ATPase activity was measured in purified plasma membrane proteins and standard curve was generated using K_2HPO_4. The values are average ± S.E. of 10 individual plasma membrane-enriched fractions. Asterisk indicates significant change at $P<0.05$.

A



B

His tag+ Multiple cloning site



Multiple cloning site

Figure 13. Vector sequence information for calnexin overexpression. The pEU-E01 vector containing 6 residues of Histidin (His) coding codons has been used for insertion of full length sequence of calnexin (A). Calnexin has been inserted to the multiple cloning site (MCS) of vector after cutting by SmaI restriction enzyme (B).

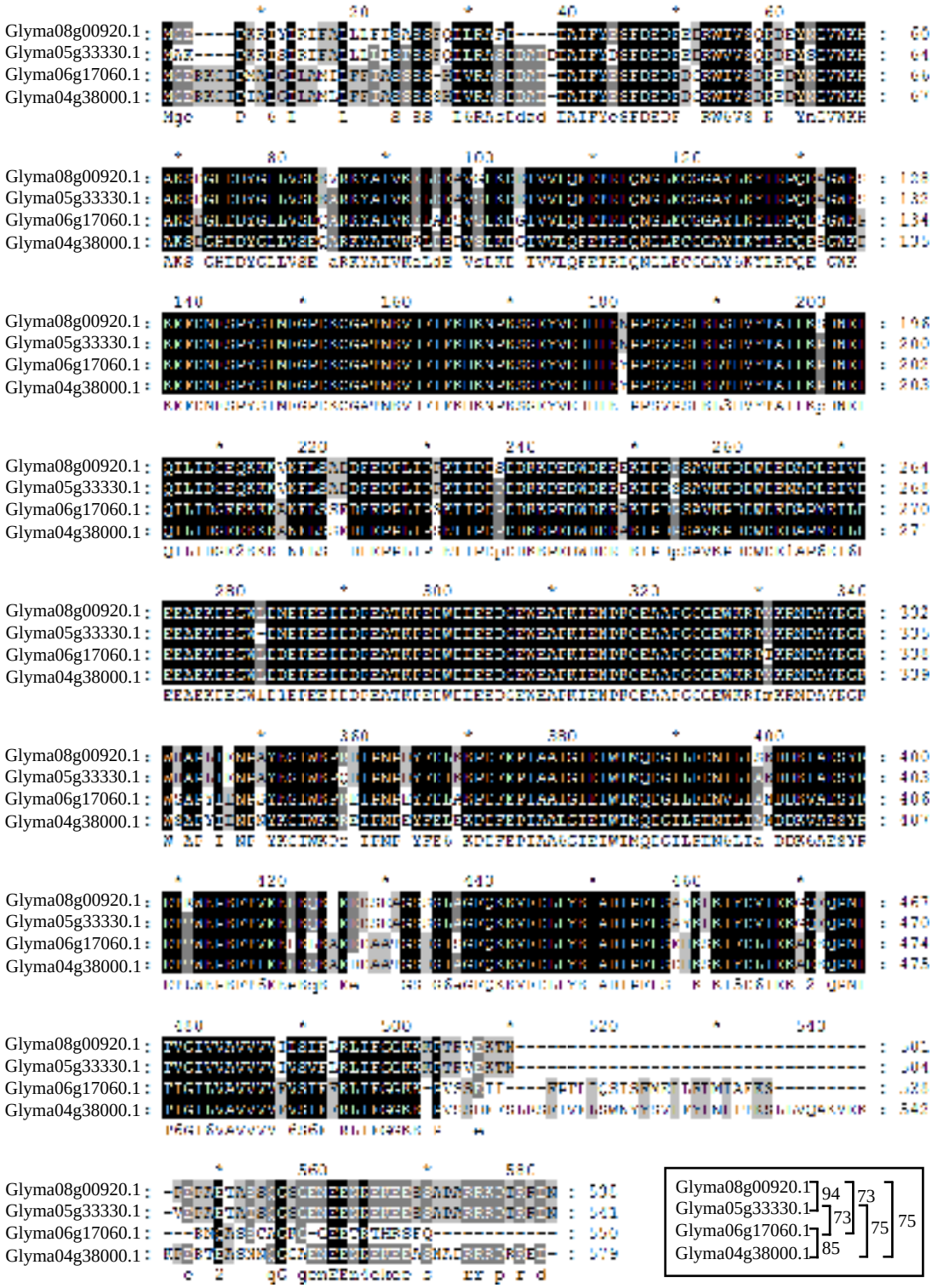


Figure 14. Alignment of amino acid sequences in encoded genes for calnexin in soybean. Homology of amino acid sequences of 4 predicted genes from Phytozome database were represented according to the percentage.

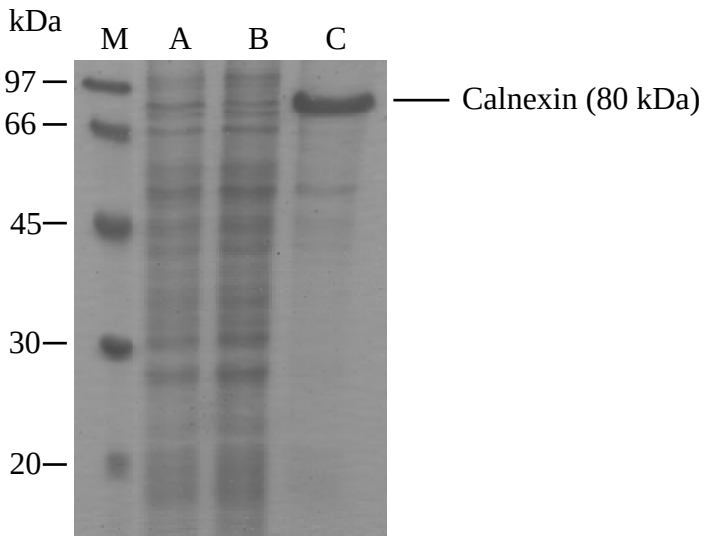


Figure 15. SDS-PAGE pattern of overexpressed calnexin. The recombinant His-calnexin protein was produced by automatic protein synthesizer using wheat germ cell and purified by affinity purification column. A purified in vitro synthesized calnexin (C) along with flow through solution (A) and unpurified protein (B) were loaded on SDS-PAGE followed by CBB staining.

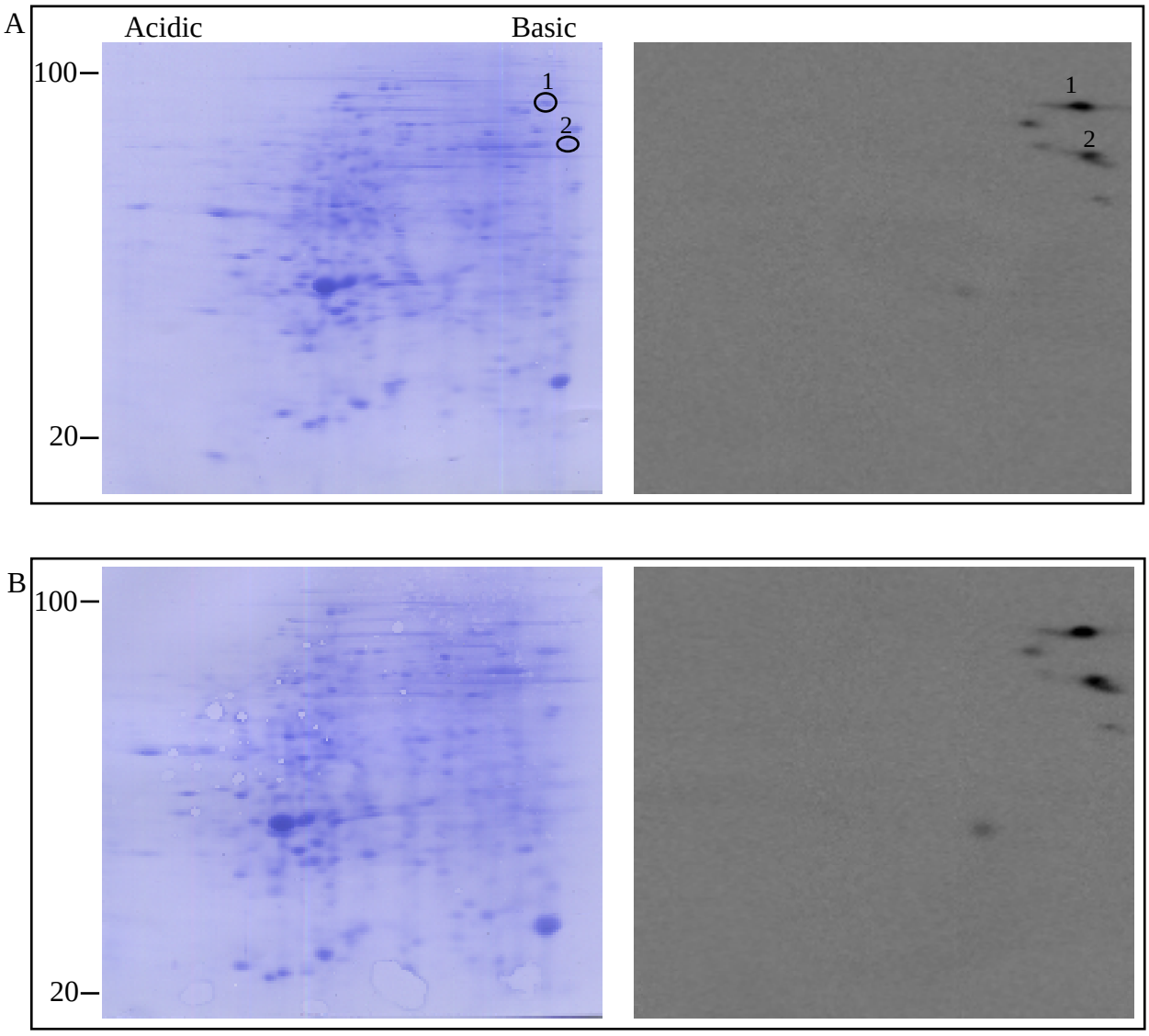


Figure 16. 2-DE patterns of crude fraction in soybean seedlings with specified cross-reacted spots. Two-day old soybean seedlings were treated without (A) or with (B) 10% PEG for 2 days. Crude protein fractions obtained from roots and hypocotyls of 4-day-old seedlings were separated by 2-DE. Immunoblot analysis was performed using calnexin antibody in control (A, right) and 10% PEG treatment (B, right).

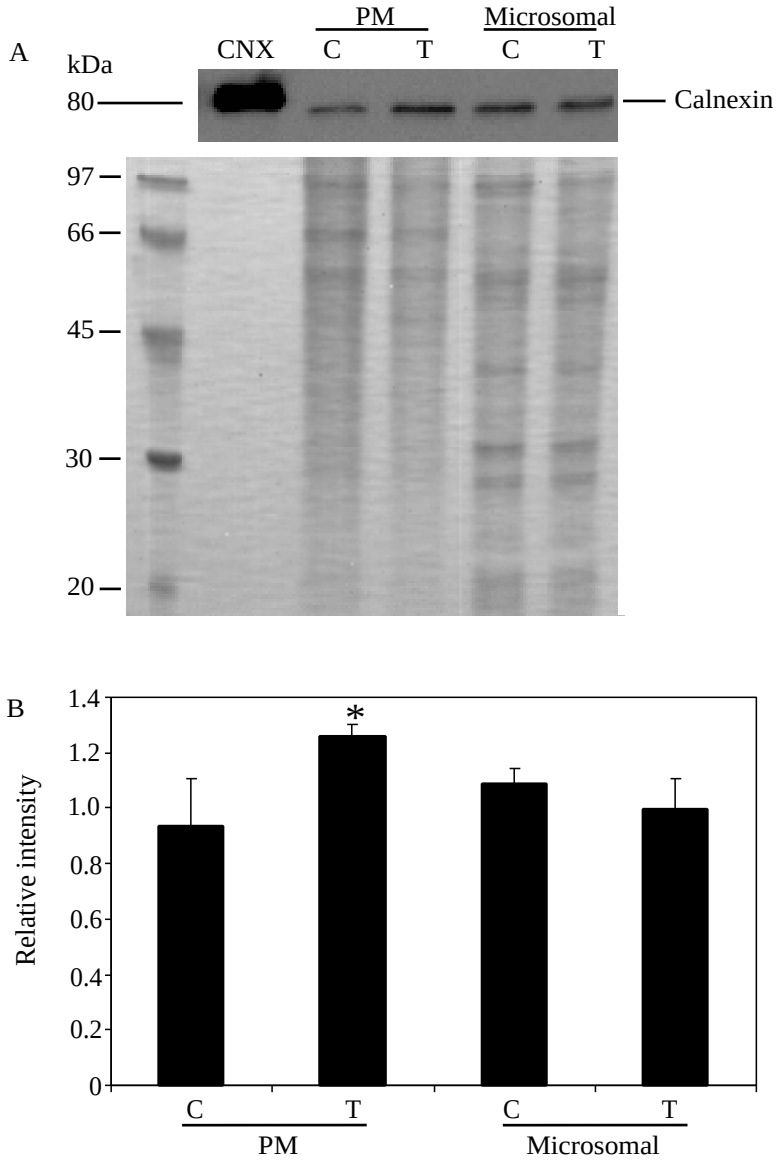


Figure 17. Immunoblot analysis of calnexin in plasma membrane and microsomal fractions of soybean seedlings with or without 10% PEG treatment. Synthesized calnexin (CNX) and a total of 10 μg of purified plasma membrane (PM) and microsomal fractions, without (C) or with (T) 10% PEG treatment were separated by SDS-PAGE and used for immunoblot analysis (A, top panel). CBB stained SDS-PAGE indicates the quality and loading quantity of protein samples (A, lower panel). The values are average ± S.E. from 3 independent immunoblots. Asterisk indicates significant change at $P<0.05$ (B).

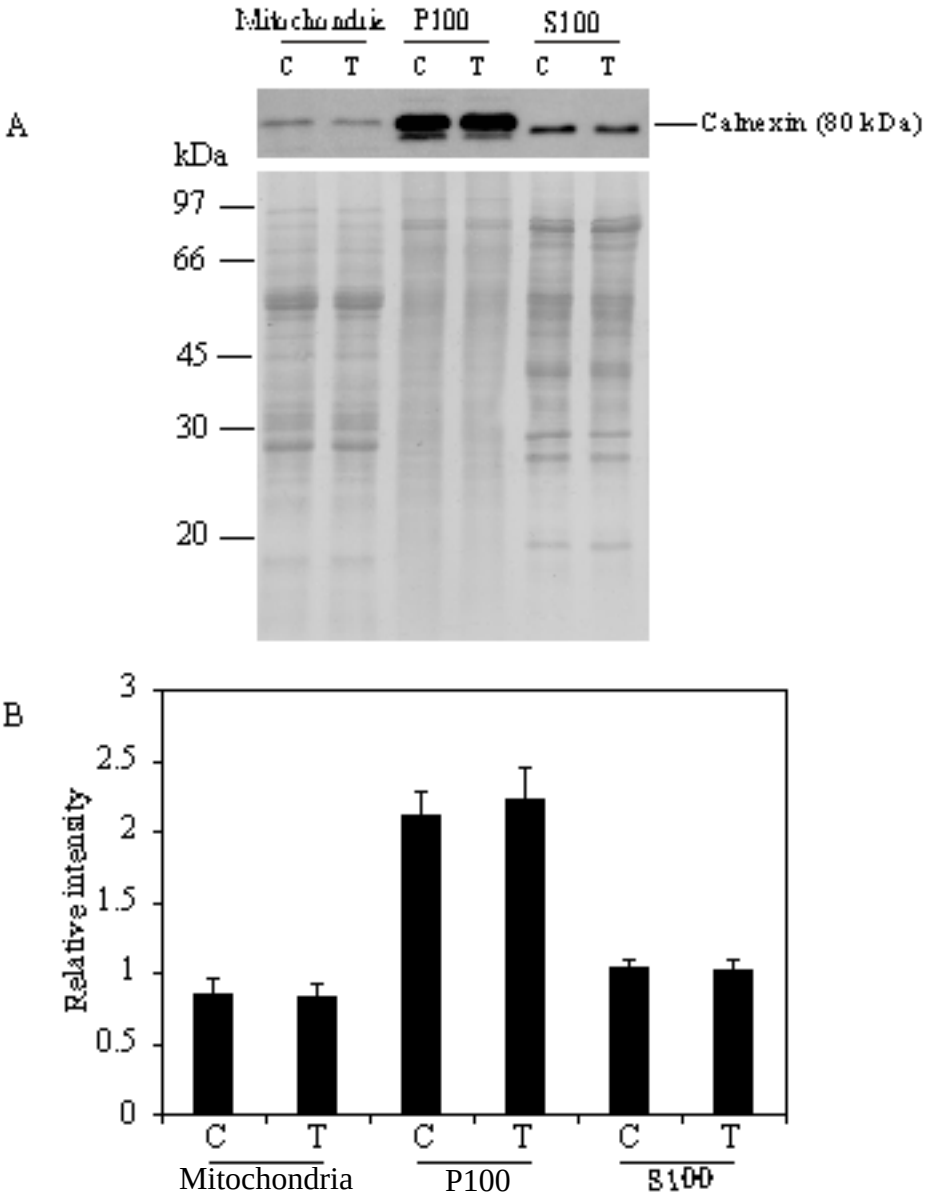


Figure 18. Evaluation of calnexin expression in various cell fractions. Four-day-old soybean seedlings without (C) or with (T) 10% PEG treatment for 2 days were used. Cell fractions consist of mitochondrial fraction, total membrane fraction (P100) and cytosolic fraction (S100) were prepared from roots and hypocotyls.

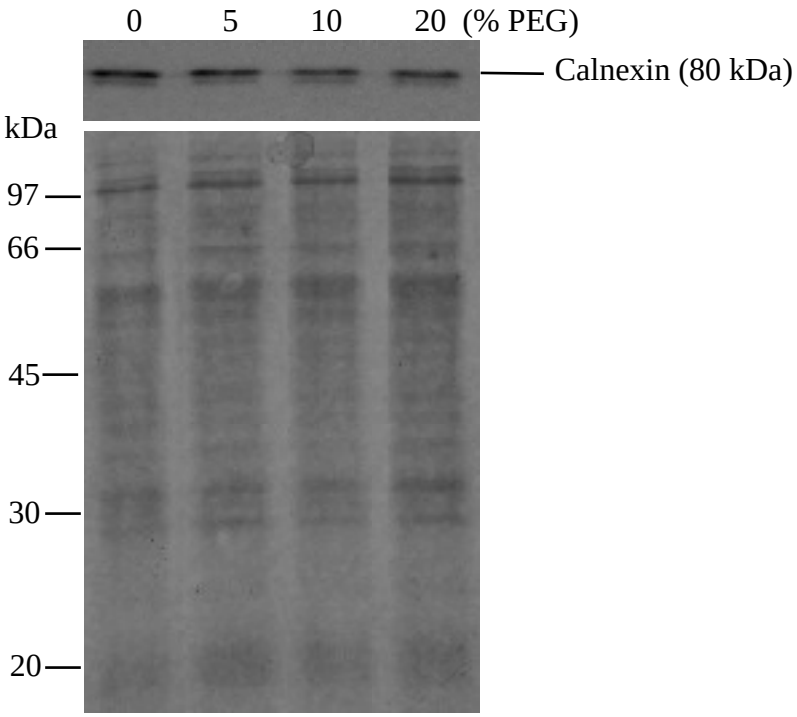


Figure 19. Expression of calnexin under various severities of osmotic stress. Two-day-old soybean seedlings were subjected to 0, 5, 10 and 20 % PEG treatment for 2 days. Total membrane fraction (P100) was extracted from roots and hypocotyls and immunoblot analysis was performed using calnexin antibody.

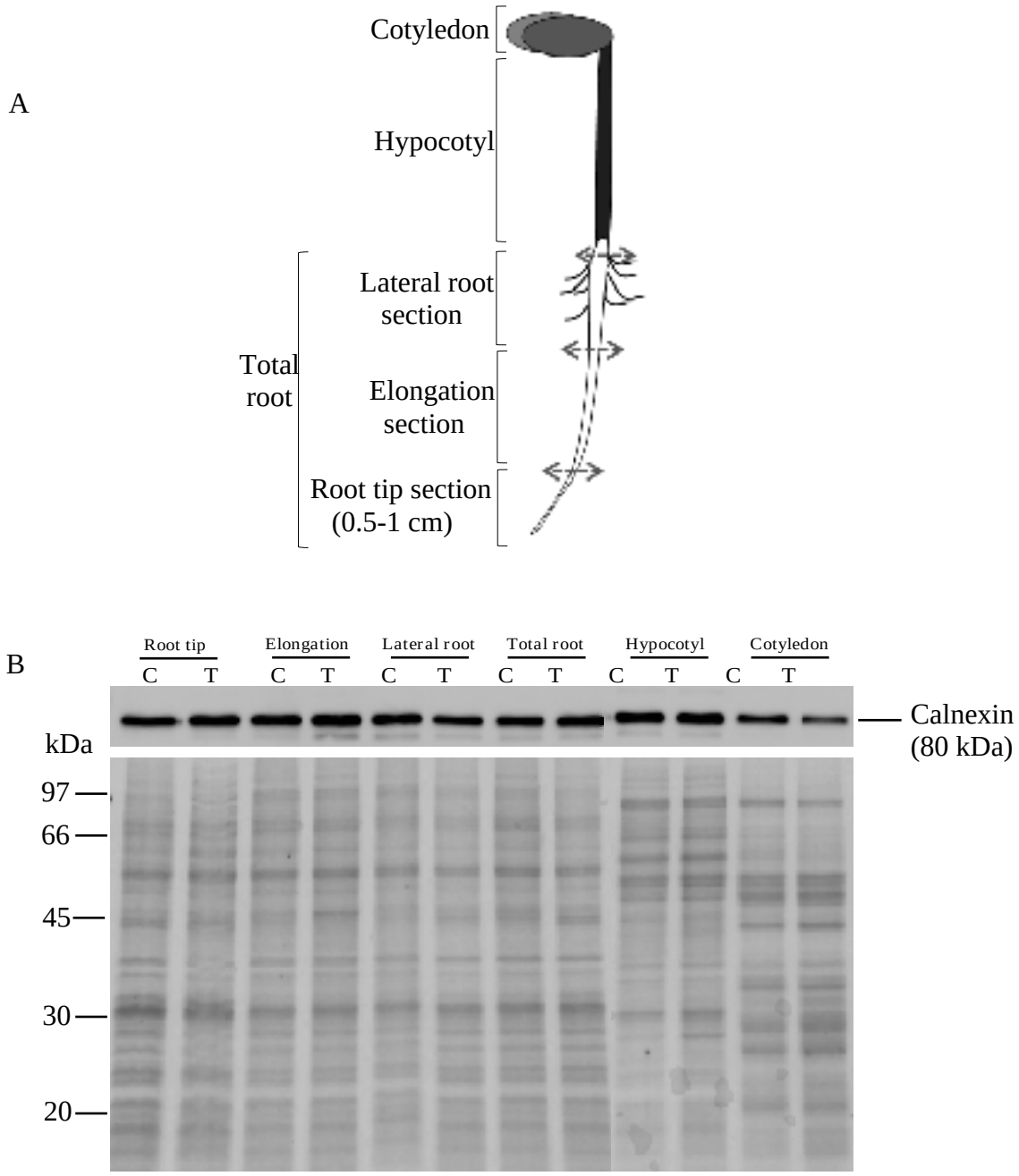
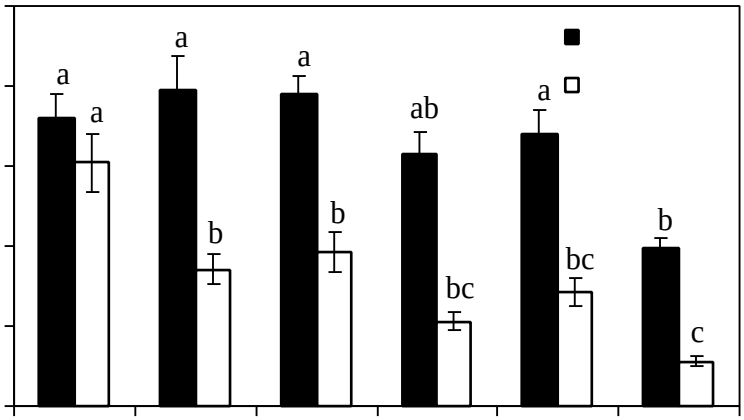


Figure 20. Expression of calnexin in various organs and sections of soybean seedling. Four-day-old soybean seedlings were treated with or without 10% PEG for 2 days. Total membrane fraction (P100) was extracted from cotyledon, hypocotyl, total root and 3 sections of root including root tip, elongation and lateral root sections (A) and immunoblot analysis was performed using calnexin antibody (B).

A

B



C

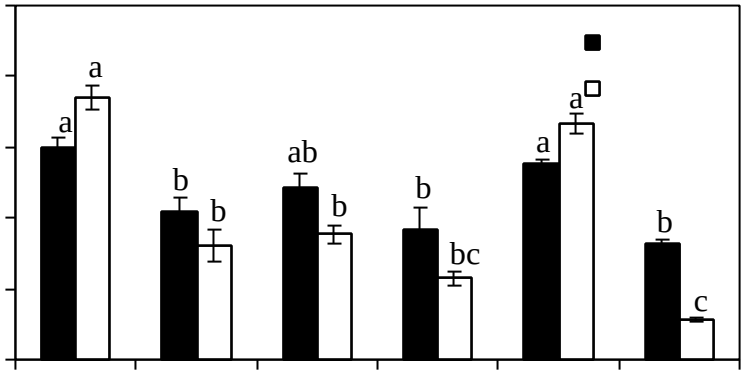


Figure 21. Effects of various abiotic stresses on morphology of soybean seedlings. Two-day-old seedlings were normally irrigated as control (T1) or treated with 10% PEG (T2), withholding water for drought (T3), 100 mM NaCl (T4), 50 μM ABA (T5) and cold (5 degree) (T6) for 2 days (A). Roots and hypocotyls length (B) and fresh weight/ seedling (C) were measured under various stresses. The values are average ± S.E. from 3 independent experiments. Means with at least 1 similar letter are not significantly different according to the Tukey's range test ($P<0.05$).

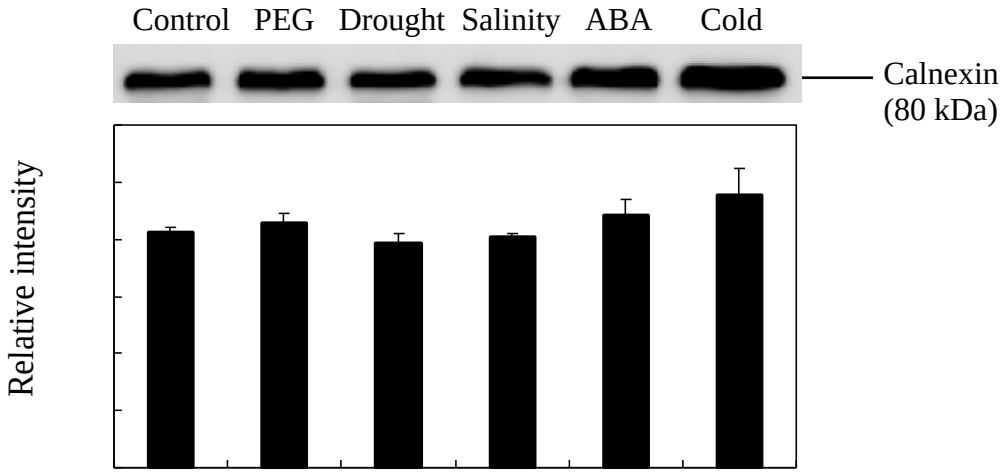


Figure 22. Expression of calnexin in 4-day-old soybean seedlings under various abiotic stresses. Total membrane fraction (P100) was extracted from roots and hypocotyls of 4-day-old seedlings subjected to PEG, drought, salinity, ABA and cold stresses for 2 days. Immunoblot analysis was performed using calnexin antibody. The values are average relative intensities of calnexin expression ± S.E. from 3 independent experiments.

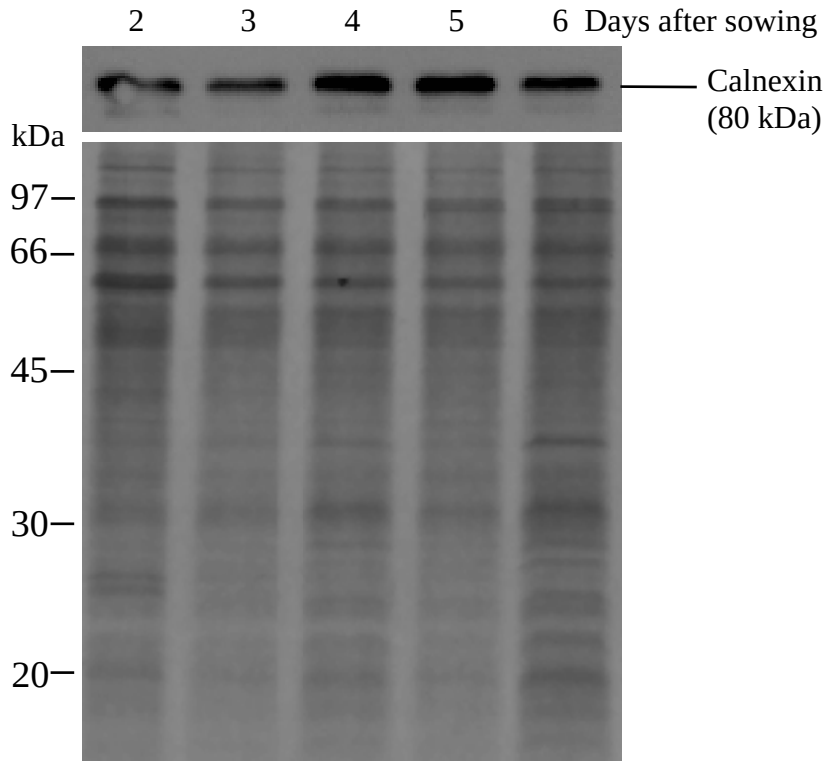


Figure 23. Expression of calnexin in the first week of soybean growth. Total membrane fractions (P100) were extracted from roots and hypocotyls of seedlings at 2, 3, 4, 5 and 6 days after sowing. Immunoblot analysis was performed using calnexin antibody.

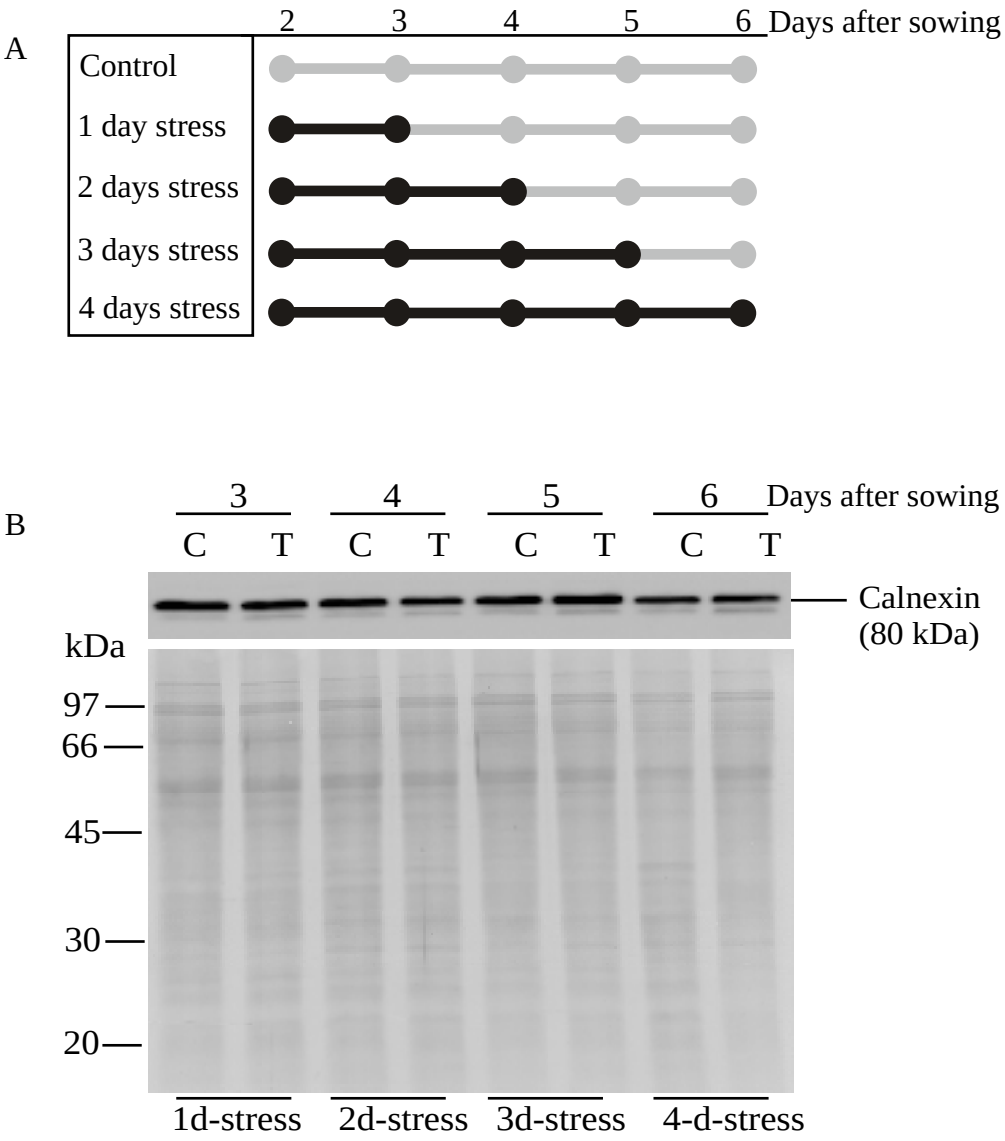


Figure 24. Effects of prolonging of PEG stress on calnexin expression in the first week of soybean growth. Two-day-old seedlings were treated with 10% PEG for 1, 2, 3 and 4 days. Schematic representation of the days after sowing without (gray) or with 10% PEG (black) shows the stress and recovery situation (A). Total membrane fractions (P100) were extracted from roots and hypocotyls of control and treated seedlings at 3, 4, 5 and 6 days after sowing. Immunoblot analysis was performed using calnexin antibody (B).

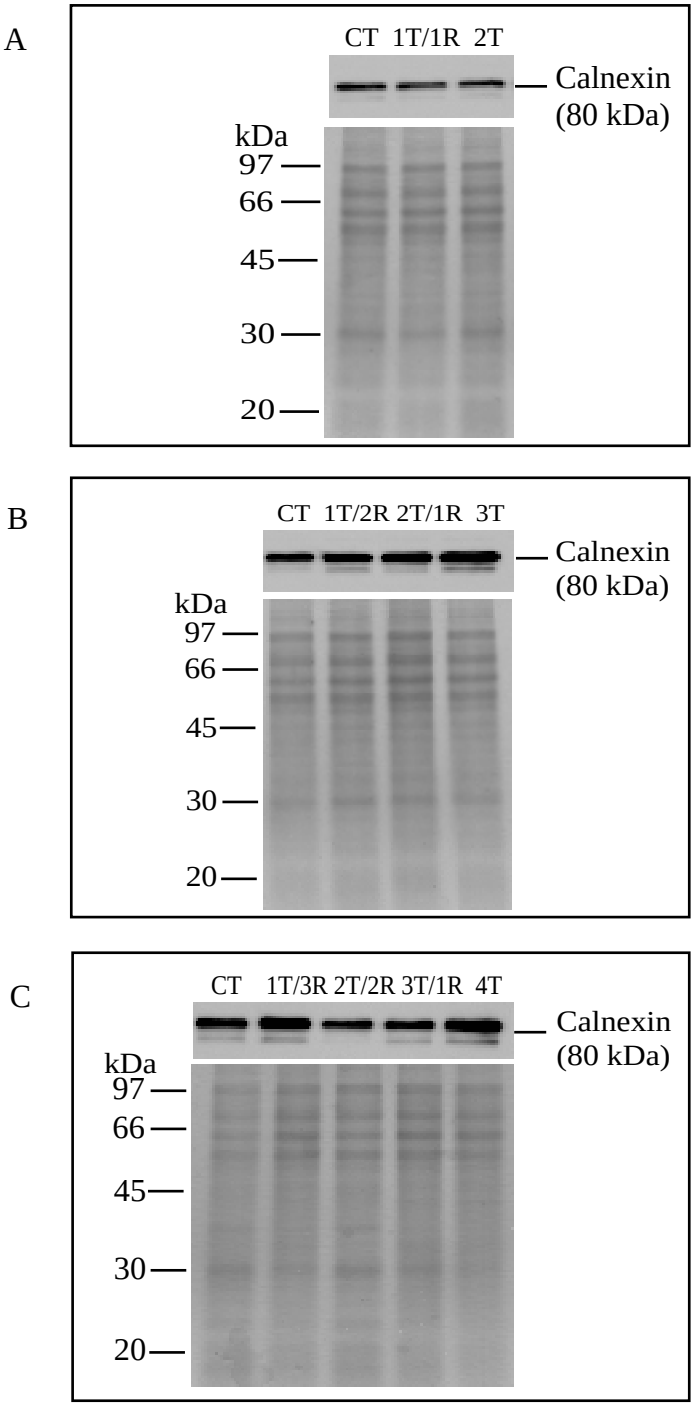
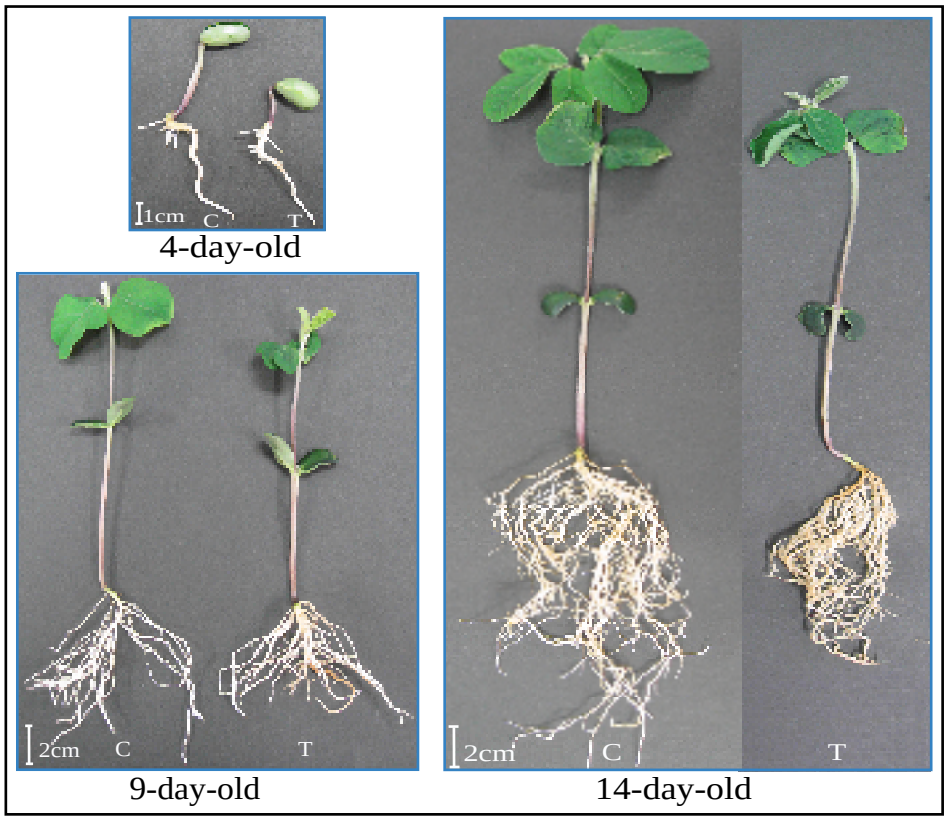


Figure 25. Effects of prolonging of stress and recovery on calnexin expression. Two-day-old seedlings were treated with 10% PEG for 2 days (2T) or 1 day treatment followed by 1 day recovery (1T/1R) or without treatment (CT) (A). Expression of calnexin in 5-day-old seedlings with 3 days treatment (3T), 1 or 2 days treatment with recovery (1T/2R, 2T/1R) (B). Expression of calnexin in 6-day-old seedlings with 4 days treatment (4T), 1, 2 or 3 days treatment with recovery (1T/3R, 2T/2R & 3T/1R) (C).

A



B

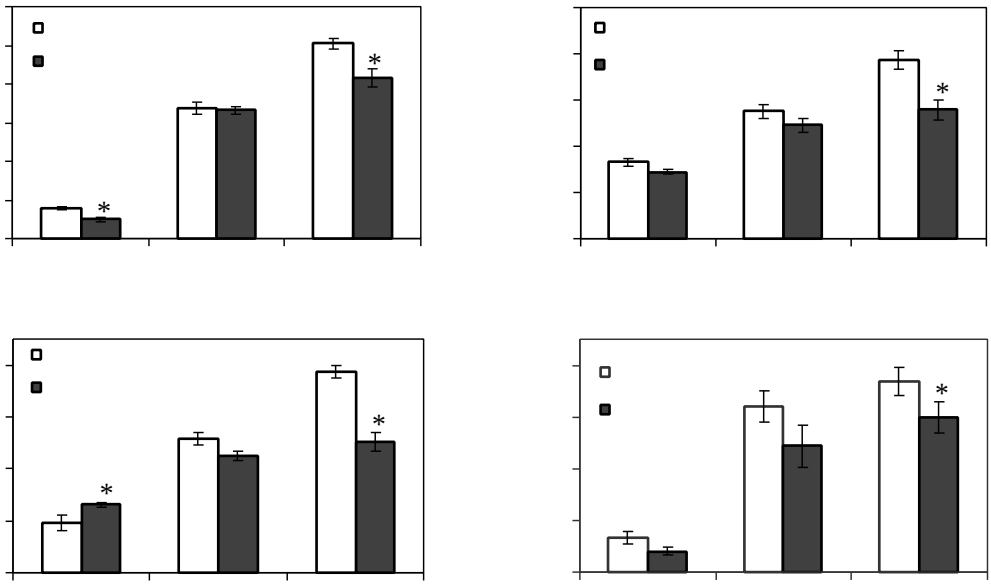


Figure 26. Effect of 10% PEG on the morphological characteristics of soybean seedlings. Photograph shows the morphological differences of 4, 9 and 14-day-old soybean seedlings without (C) or with (T) 10% PEG treatment for 2 days (A). The values are average ± S.E. of length and fresh weight of shoots and roots from 3 independent experiments. Asterisk indicates significant changes in treatment related to relevant control at $P<0.05$ (B).

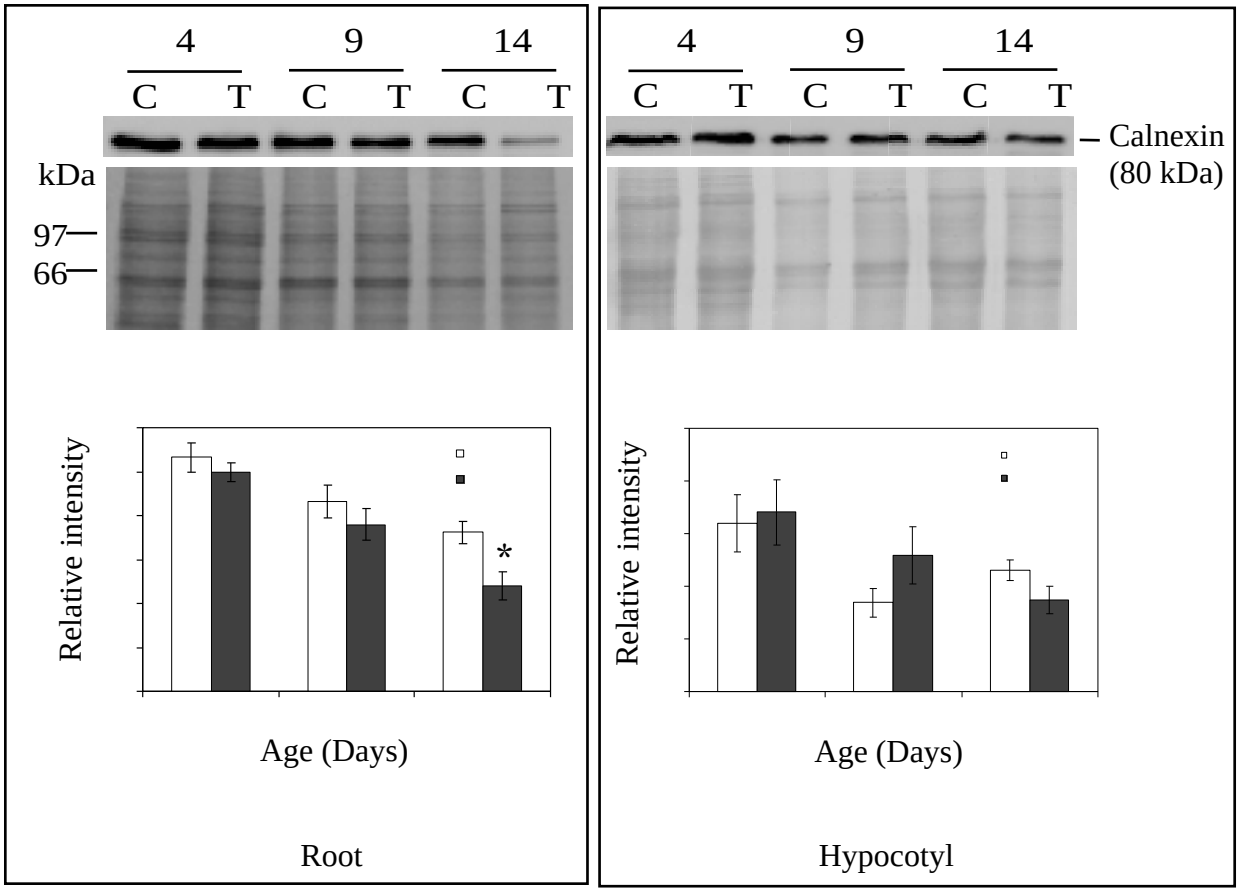


Figure 27. Expression of calnexin in soybean roots and hypocotyls within first two weeks of growth without (C) or with (T) 10% PEG treatment for 2 days. Immunoblot analyses were performed in total membrane fractions (P100) of soybean roots (Left panel) and hypocotyls (Right panel) within time courses. The values are average relative intensities of calnexin expression in control and treatment ± S.E. from 3 independent experiments. Asterisk indicates significant changes in treatment related to relevant control at $P<0.05$.

Figure 28. Alignment of calnexin amino acid sequences in soybean and rice. Homology of amino acid sequences of calnexin in soybean (Q89317.1) and rice (ACS13098.1) was 72%.

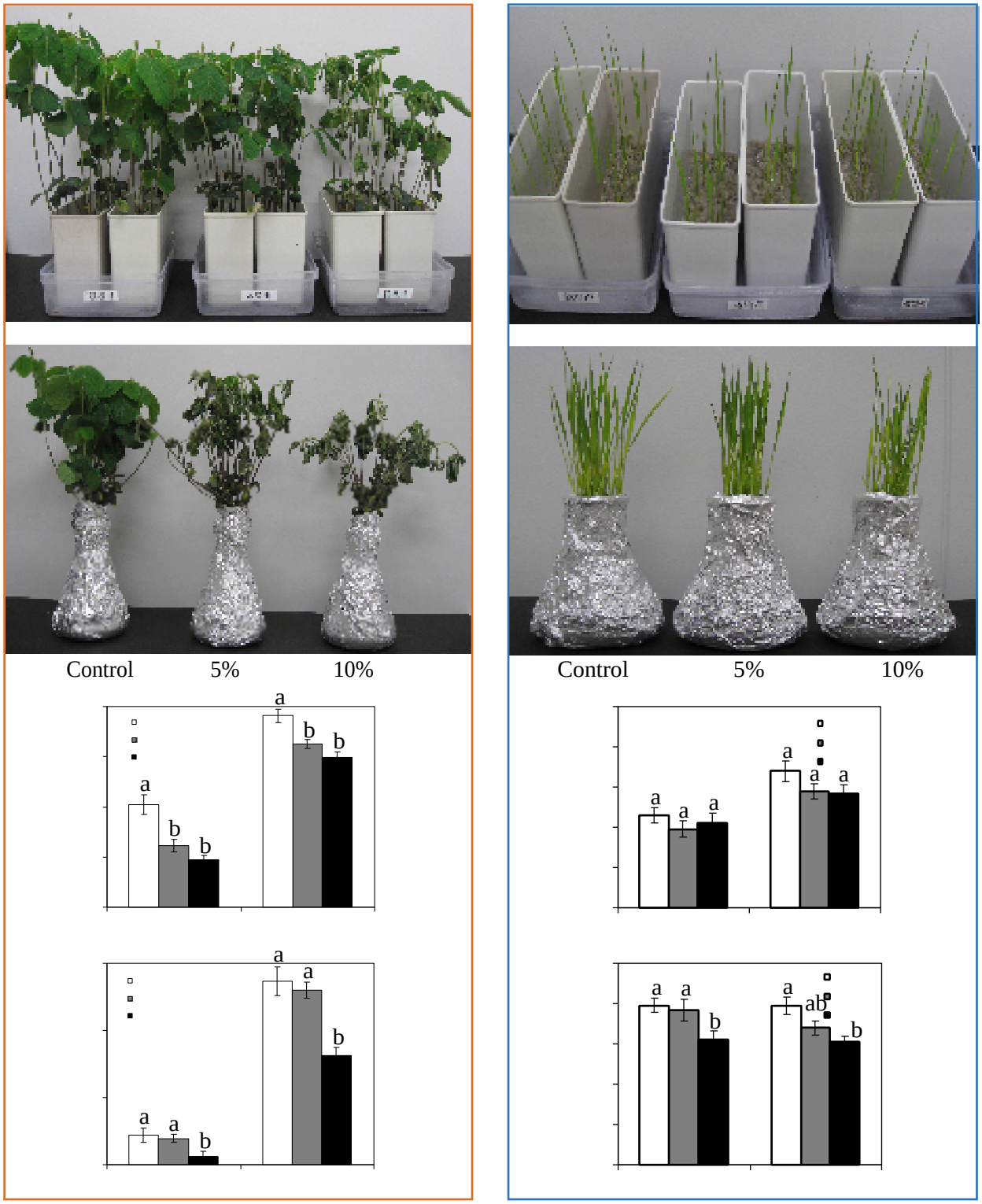


Figure 29. Effects of PEG concentrations on morphology of soybean and rice seedlings. Soybean and rice were grown in the sand for 12 days and subjected to 0, 5 and 10% PEG for 2 days (upper panel photos) or transferred to PEG solutions for 2 days (lower panel photos). The values are average ± S.E. of length and fresh weight of shoots and roots of soybean and rice from 3 independent experiments. Means with at least 1 similar letter are not significantly different according to the Tukey's range test ($P<0.05$) (B).

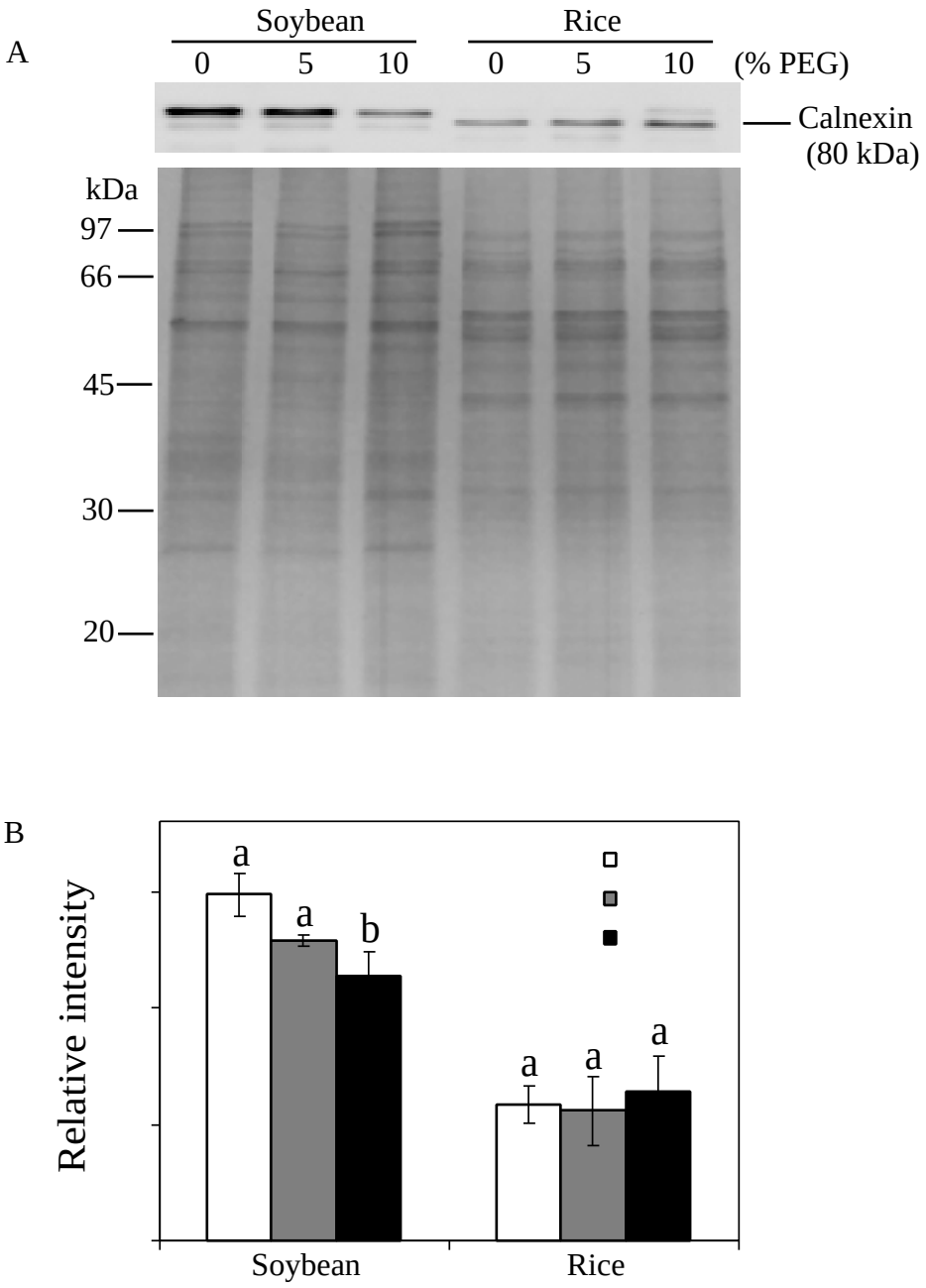


Figure 30. Expression of calnexin in soybean and rice roots under different PEG concentrations. Twelve-day-old soybean and rice seedlings were subjected to 0, 5 and 10 % PEG for 2 days. Total membrane fractions (P100) were extracted from roots and immunoblot analysis was performed using calnexin antibody (A). The values are average ± S.E. of relative intensities of calnexin expression from 3 independent experiments. Means with similar letter are not significantly different according to the Tukey's range test ($P<0.05$) (B).

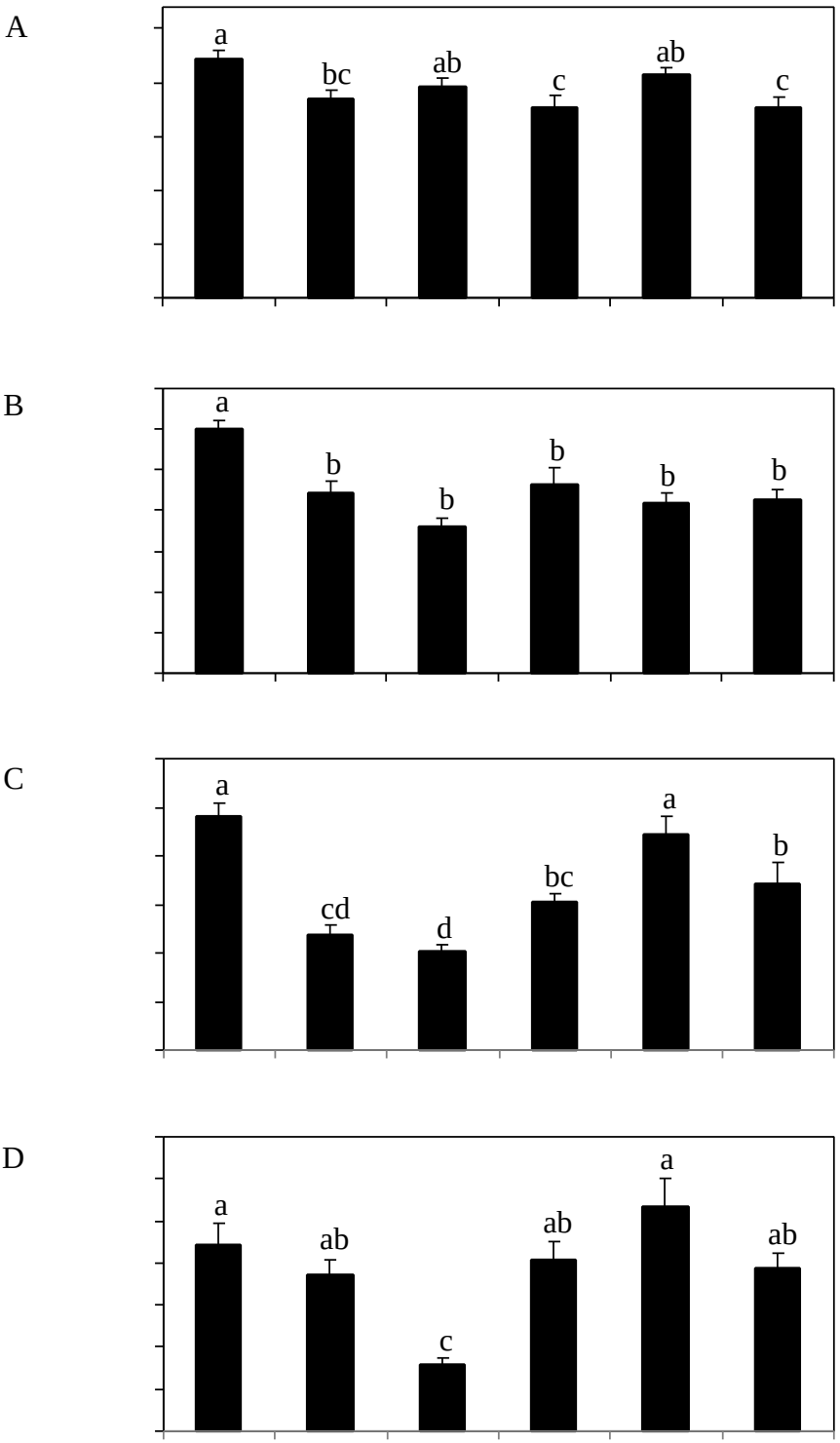


Figure 31. Evaluation of morphological characteristics of soybean seedlings treated by various stresses. Twelve-day-old soybean seedlings were subjected to 10% PEG, withholding water for drought, 100 mM NaCl for salinity, 50 μM ABA and cold (5 degree) and compared to the control. Shoots length (A), roots length (B), shoot fresh weight/seedling (C) and root fresh weight/ seedling (D) were measured under various stresses. The values are average ± S.E. from 3 independent experiments. Means with at least 1 similar letter are not significantly different according to the Tukey's range test ($P<0.05$).

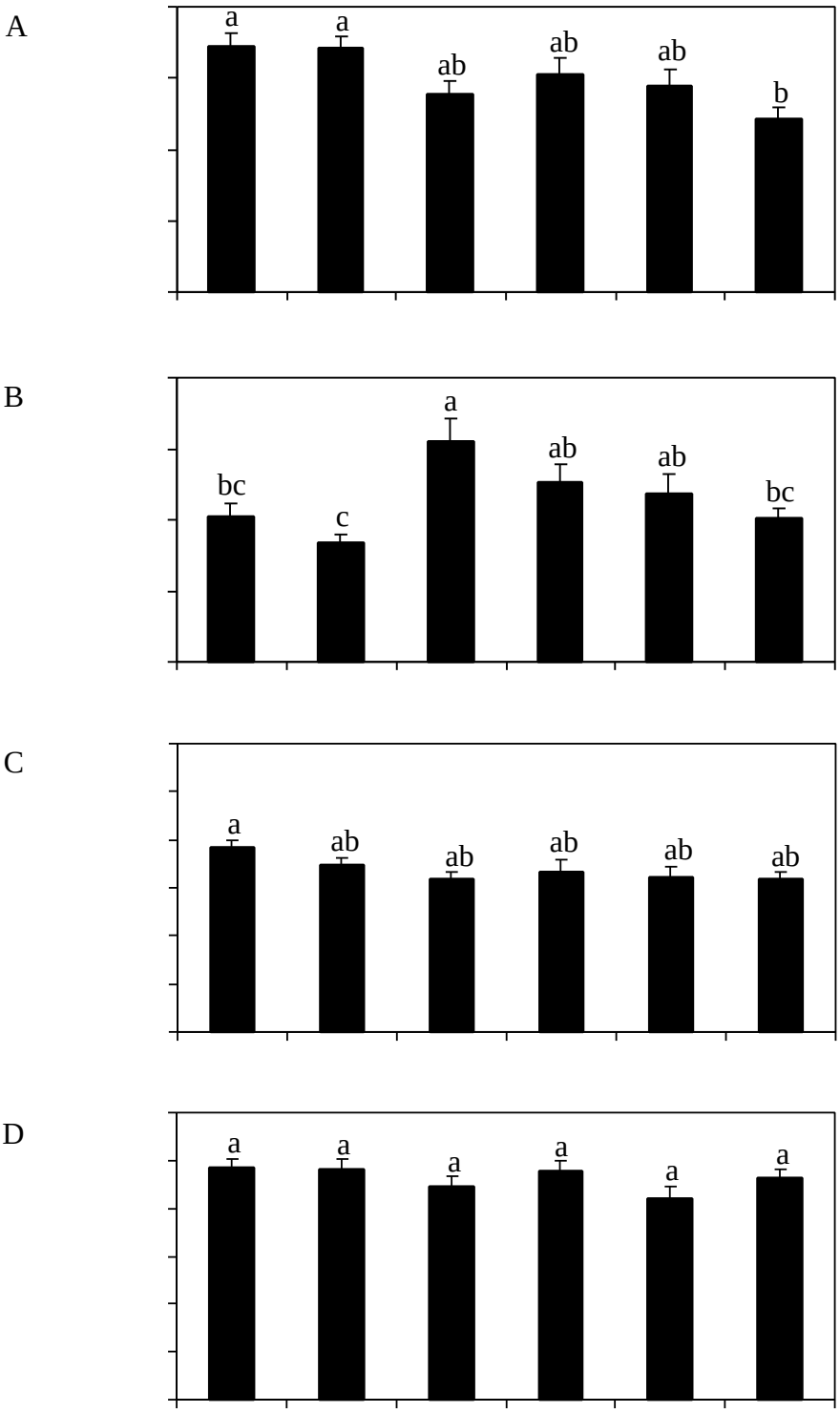


Figure 32. Evaluation of morphological characteristics of rice seedlings treated by various stresses. Twelve-day-old rice seedlings were subjected to 10% PEG, withholding water for drought, 100 mM NaCl for salinity, 50 μM ABA and cold (5 degree) and compared to the control. Shoots length (A), roots length (B), , shoot fresh weight/seedling (C) and root fresh weight/ seedling (D) were measured under various stresses. The values are average ± S.E. from 3 independent experiments. Means with at least 1 similar letter are not significantly different according to the Tukey's range test ($P<0.05$).

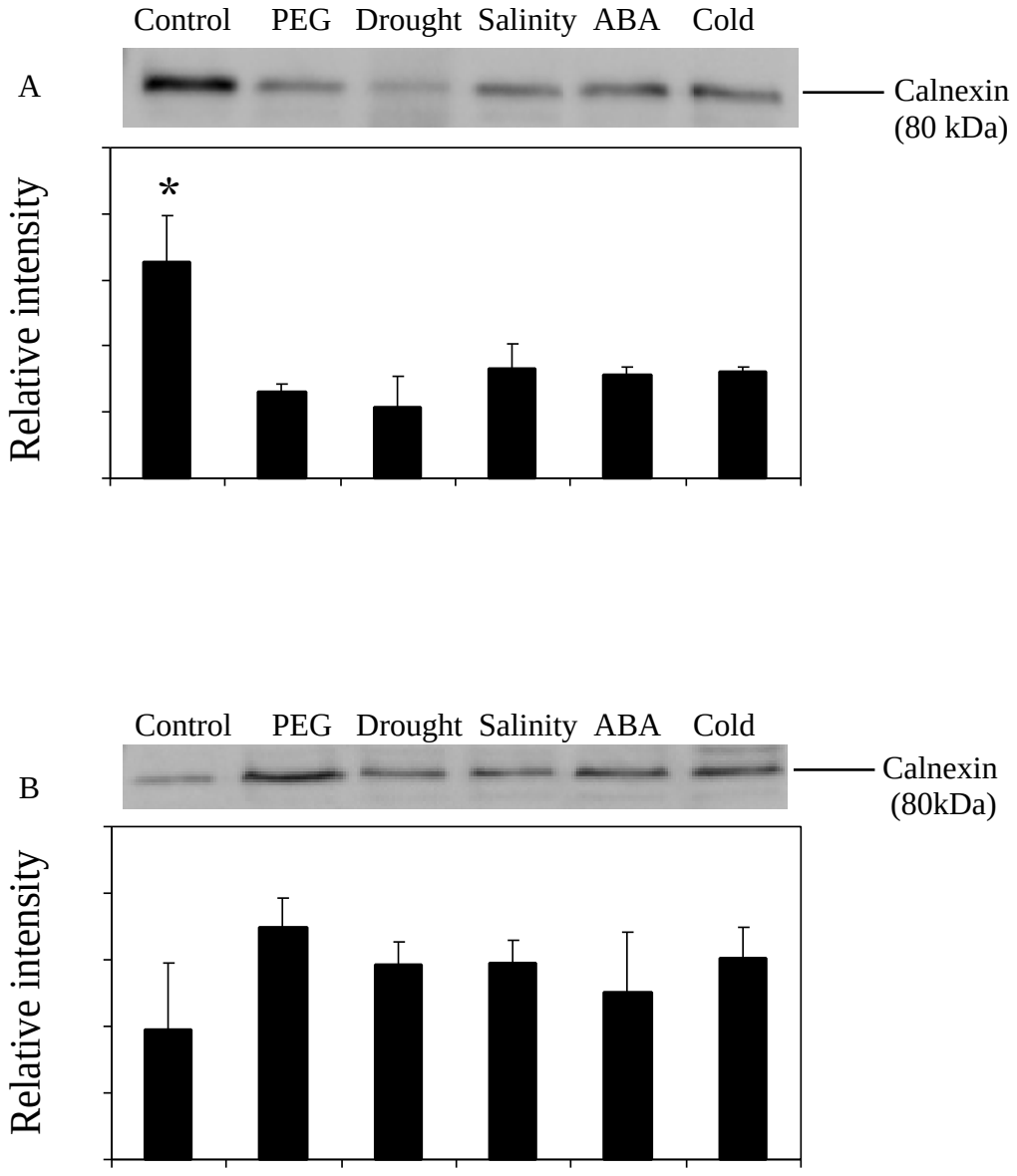


Figure 33. Expression of calnexin in soybean and rice roots under various abiotic stresses. Treatments were 10% PEG, withholding water for drought,100 mM NaCl for salinity, 50 μM ABA or cold (5 degree) for 2 days comparing to control. Total membrane fractions (P100) were extracted from roots of 14-day-old soybean (A) and rice (B) and immunoblot analyses were performed using calnexin antibody. Asterisk indicates significant change in calnexin expression ($P<0.05$).

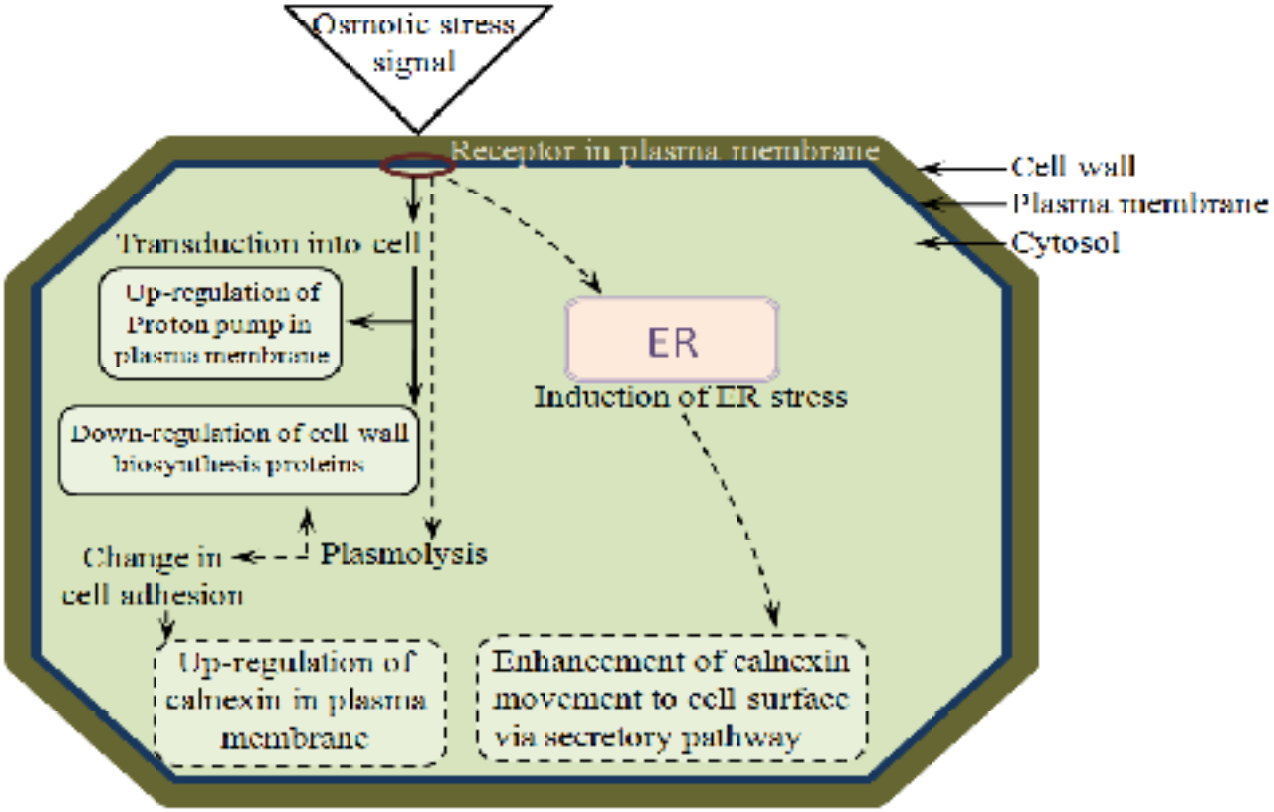


Figure 34. Schematic representation of effects of osmotic stress on plant cell. Osmotic stress reduced the expression of cell wall biosynthesis proteins and increased the expression of plasma membrane proton pump. Two pathways (dotted lines) have been proposed for accumulation of calnexin in plasma membrane.