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Proteomics Approach for Identifying Abiotic Stress Responsive Proteins in Soybean

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1. Introduction

Soybean (*Glycine max* L.) is an important source of protein for human and animal nutrition, as well as a major source of vegetable oil. The seeds consist of approximately 40–42% protein and 20–22% oil, on a dry-matter basis (Panizzi & Mandarino, 1994). Although soybean is adapted to grow in a range of climatic conditions, several adverse environmental factors, known as abiotic stresses, affect the growth, development, and global production of soybean. For instance, drought reduces the yield of soybean by about 40%, affecting all stages of plant development from germination to flowering and reducing the quality of the seeds (Manavalan et al., 2009). Salinity and cold are other inhibition factors in soybean growth and production and together with drought cause osmotic stress in plant (Beck et al., 2007; Nuccio et al., 1999). Several other abiotic stresses, such as flooding, high temperature, irradiation, or the presence of pollutants in the air and soil have detrimental effects on the growth and productivity of soybean.

Along with morphological and physiological studies on the responses of plants to stress conditions, several molecular mechanisms from gene transcription to translation as well as metabolites were investigated. Recent advances in genomic research, particularly in the field of proteomics, have created an opportunity for dissecting quantitative traits in a more meaningful way. 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. In soybean a comprehensive functional genomics is yet to be performed; therefore, proteomics approaches form a powerful tool for analyzing the functions of the plant's genes and proteins (Komatsu & Ahsan, 2009).

In this chapter, recent methodologies for the extraction of proteins from soybean are explained first, and then protein identification techniques are discussed briefly. Analyses of the proteome of soybean subjected to various types of abiotic stresses are explained in two main categories: 1) stresses that induce a negative osmotic pressure in plants, such as drought, salt, cold, or osmotic stress, and 2) other abiotic stresses, such as flooding, high temperature, ultraviolet radiation, toxicity of ozone and the two heavy metals cadmium and aluminum. Studies on the expression of proteins in soybean cultivars subjected to conditions

of stress, and the functional analysis of stress-responsive proteins provide a clear insight into the complex mechanisms involved in the response of plants to stress.

2. Methodologies for extraction and identification of soybean proteins

2.1 Extraction of proteins

The extraction of proteins and the preparation of samples is one of the most challenging steps in any proteomics study. In plant proteomics, the type of the plant species, tissues, organs, cell organelles, and the nature of desired proteins affect the techniques that can be used for protein extraction. Furthermore, the presence of vacuoles, rigid cell walls, and membrane plastids makes the extraction process more difficult (Komatsu, 2008; Lee & Cooper, 2006). 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). Whereas the proteome of model plants such as *Arabidopsis* and rice have widely been studied, less attention has been paid to the analysis of valuable crops such as soybean. Soybean generally contains high levels of interfering substances, such as phenolic compounds, proteolytic and oxidative enzymes, terpenes, organic acids, and carbohydrates, which make the protein extraction process more difficult (Komatsu & Ahsan, 2009). Therefore, the minimization of the effects of interfering substances needs to be a priority when extracting proteins from soybean.

A proteome analysis of various tissues of soybean and rice by using three extraction methods and two lysis buffers found that the proteome map of soybean contained a relatively small number of total protein spots. This suggested that the method used to homogenize the protein pellet and the contents of the lysis buffer have marked effects on protein solubilization and separation in the classical proteomic analysis of soybean (Toorchi et al., 2008). Soybean contains large quantities of secondary metabolites. These consist mainly of flavone glycosides, such as kaempferol and quercetin glycosides (Buttery & Buzzell, 1973), and phenolic compounds (Cosio & McClure, 1984). The presence of several secondary metabolites and lipids, as well as large amounts of carbohydrates, not only hampers high-quality protein extraction, but also impedes high-resolution protein separation in two-dimensional polyacrylamide gel electrophoresis (2-DE), resulting in streaking and a reduction in the number of resolved protein spots (Komatsu & Ahsan, 2009). In protein extraction and solubilization procedures, the combinations and concentrations of detergents, reducing agents, and chaotropic agents that are present in the buffer all have marked effects on the quality of the extracted proteins.

Although all of the reported proteomics studies on soybean subjected to abiotic stresses have been performed in recent years, there are several differences in the compositions and concentrations of the extraction buffers that were used (Tables 1 & 2). Although some of these differences arose from the personal preferences of the investigators or from the nature of the tissue or organ that was sampled, their existence implies a lack of consensus regarding the choice of appropriate buffer compositions for the extraction of soybean samples. There are two main groups of extraction buffers: trichloroacetic acid (TCA)/acetone-based buffers and phenol-based buffers. A comprehensive proteomic study was performed on nine organs from soybean plants in various developmental stages by using three different methods for protein extraction and solubilization (Ahsan & Komatsu, 2009). The results showed that whereas the use of an alkaline phosphatase buffer followed

by TCA/acetone precipitation caused horizontal streaking in 2-DE, the use of a Mg/NP-40 buffer followed by extraction with alkaline phenol and methanol/ammonium acetate produced high-quality proteome maps with well-separated spots, high spot intensities, and high numbers of separate protein spots in 2-DE gels (Ahsan & Komatsu, 2009). Sarma et al. (2008) also reported a similar preference for the use of alkaline phenol and methanol/ammonium acetate rather than TCA/acetone for the extraction of soybean tissues. Natarajan et al. (2005) reported that, in the case of soybean seeds, thiourea/urea and TCA methods produced a higher protein resolution and greater spot intensity for all proteins than did the phenol extraction method. They also showed that several less abundant and high molecular weight proteins were clearly resolved and strongly detected by using thiourea/urea and TCA method (Natarajan et al., 2005).

The phenol extraction procedure might be more suitable for tissues such as leaves that contain interfering compounds, whereas both the phenol and the TCA/acetone methods might be useful for other tissues (Tables 1 & 2). The buffer contents for solubilizing the pellets obtained by both methods are another determining factor that should not be neglected. It has been shown that the numbers of proteins identified in gels and the separation and resolution of these proteins are highly dependent on the composition of the protein solubilization buffer (Ahsan & Komatsu, 2009). The extraction methodologies explained here are generally used in separation of total proteins by classical proteomics approaches such as 2-DE. In the case of organelle proteomics particularly that of membrane proteomics, a different extraction procedure is required that involves modifications to dissolve hydrophobic proteins and additional purification steps (Komatsu et al., 2009a; Nouri & Komatsu, 2010). Furthermore, when studying protein-protein interactions, it is necessary to extract protein complexes by using buffers with less or no detergent to get the proteins in their native states.

2.2 Protein identification

A protein extract, even from a purified fraction, consists of a huge number of individual proteins as well as several other components, and the aim when analyzing such a fraction is to obtain as much qualitative and quantitative information on the proteome as possible. In classical proteome analyses, proteins are initially separated by a 2-DE technique (O'Farrell, 1975) with isoelectric focusing (IEF) as the first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as the second dimension. A greater resolution in protein separation has been achieved by introducing immobilized pH gradients (IPGs) for the first dimension (Bjellqvist et al., 1982). Tables 1 and 2 show that either IEF or IPG strip gels coupled with SDS-PAGE which generally termed 'gel-based proteomics' has been used for comparative proteome analysis of soybean subjected to abiotic stresses. A combination of an IEF tube gel (for the low *pI* range 3.5 to 8.0) and an IPG gel (for the high *pI* range, 6.0 to 10.0) has been used for protein identification over a broad range of *pI* values to produce a reference map (Hashiguchi et al., 2009) and to identify flooding-responsive proteins in soybean (Komatsu et al., 2009b). Methodological advances in 2-DE have led to the introduction of two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) (Ünlü et al., 1997), which has been used for the comparative analysis of the proteome of early-stage soybean seedlings subjected to flooding stress (Table 2) (Nanjo et al., 2010).

The Separated proteins can be subsequently identified by sequencing or by mass spectrometry. By introduction of 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 & Vandekerckhove, 2000; Natarajan et al., 2006). 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 & Washburn, 2005). A multidimensional protein identification technology (MudPIT) that usually incorporates separation on a strong cation exchange, reverse-phase column and MS/MS analysis (Wolters et al., 2001) helps the efficient separation of complex peptide mixtures. The gel-free 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. A combination of gel-based and gel-free proteomics has been used for identification of soybean plasma membrane proteins under flooding (Komatsu et al., 2009a; Nanjo et al., 2010) or osmotic stress (Nouri & Komatsu, 2010), suggesting that these two methods are complementary to one another for protein identification. Methods for protein identification are not usually organism specific, and they can be applied to a wide range of living organisms in addition to soybean.

Identification of proteins normally performs by using a database search engine such as MASCOT or SEQUEST. Soybean has an estimated genome size of 1115 Mbp, which is significantly larger than those of other crops, such as rice (490 Mbp) or sorghum (818 Mbp). Sequencing of the 1100 Mbp of total soybean genome 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, DOE Joint Genome Institute; this database is available at <http://www.phytozome.net> (Schmutz et al., 2010). 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. In addition, duplications in the genome of soybean result in nearly 75% of the genes being present as multiple copies (Schmutz et al., 2010), which further complicates the analysis. Soybean proteome database which is available at <http://proteome.dc.affrc.go.jp/Soybean> is also provides valuable information of various omics including 2-DE maps and functional analysis of soybean proteins (Sakata et al., 2009). However, the presence of a considerable number of proteins with unknown functions highlights the limitations of bioinformatic prediction tools and the need for further functional analyses.

3. Proteomics of soybean subjected to osmotic stresses

3.1 Drought

Drought has been recognized as a primary constraint in limiting the grain yield of crops, including soybean. Among the factors that contribute to enhance drought resistance, the characteristics of the root are believed to be vital in the mechanisms of delaying dehydration, as these contribute by regulating plant growth and by extracting water and nutrients from deeper unexplored soil layers (Norouzi et al., 2008). Proteome analysis of soybean roots subjected to severe but recoverable drought stress at the seedling stage demonstrated significant variations in 45 protein spots as detected on Coomassie Brilliant Blue (CBB)-stained 2-DE gels. Of these spots, the expression of five proteins was up-regulated and that of 21 proteins was down-regulated, while two new proteins were detected only under drought

conditions. When the stress was terminated by watering the plants for four days, the protein levels generally tended toward the control levels (Larrainzar et al., 2007).

The major reason for loss of crop yields under drought stress is a decrease in carbon gain through photosynthesis. Drought stress has been shown to inhibit photosynthesis in soybean leaves within a few days of limiting the water supply, thereby causing the CO₂ assimilation rate to drop to almost zero (Ribas-Carbo et al., 2005). Carbohydrate metabolism is likely to be most affected by drought stress after photosynthesis. Proteome analysis of soybean root under drought condition showed that two key enzymes involved in carbohydrate metabolism, UDP-glucose pyrophosphorylase and 2,3-bisphosphoglycerate independent phosphoglycerate mutase, were down-regulated upon exposure to drought (Alam et al., 2010b). The levels of expression of both enzymes tended to revert to that of the control plants when watering was restored. Because the shift in carbon partitioning under drought stress is an adaptive response, a decrease in the expression of glycolytic enzymes in response to drought stress may be a consequence of reduced growth as well as a mechanism for accumulating sugars as an energy source for recovery and rapid growth once water is available again.

Oxidative stress and programmed cell death caused by cysteine proteases have been shown to accompany many environmental stresses (Prasad et al., 1994). In the case of drought stress, up-regulation of reactive oxygen species (ROS) scavengers such as superoxide dismutase (SOD) was reported in soybean seedlings (Toorchi et al., 2009) and in rice (Ali & Komatsu, 2006). Proteomic investigations on soybean root revealed the accumulation of dehydrin and ferritin under drought stress (Alam et al., 2010b). Dehydrins, which belong to the group of late-embryogenesis-abundant (LEA) proteins, have been reported to function in abiotic stress tolerance by minimizing the negative effects of ROS (Mowla et al., 2006). Ferritin sequesters highly reactive intracellular iron and reduces the formation of toxic hydroxyl radicals. Control of free iron is important because Fe³⁺ can be reduced to Fe²⁺ by O₂⁻ radicals. Induction of ferritins by excess iron, water stress and abscisic acid has been documented at both transcript and protein level (Ravet et al., 2009).

The proteome analysis of two-day-old soybean seedlings subjected to drought stress by withholding of water for two days revealed a variety of responsive proteins involved in metabolism, disease/defense and energy including protease inhibitors (Toorchi et al., 2009). The up-regulation of protease inhibitors in soybean seedling induced by drought stress indicate their defense response to drought stress; this up-regulation also occurs in response to other abiotic stresses, such as cold or salinity (Pernas et al., 2000). It appears that some proteases are released from injured vacuoles into the cytosol in plants exposed to drought stress; this may lead to the expression of protease inhibitors to neutralize the vacuolar proteases, thereby suppressing their deleterious effects on cell proteins. The S-adenosylmethionine synthetase gene is expressed in all living cells, and its product, S-adenosyl-L-methionine, is the major methyl donor in all cells. It has been shown that the expression of S-adenosylmethionine synthetase in soybean root decreases upon exposure to drought stress (Alam et al., 2010b). Down-regulation of this enzyme under drought is consistent with the inhibition of photosynthetic activity as a general feature of abiotic stresses. A proteomic study in soybean revealed that levels of caffeoyl-CoA O-methyltransferase are significantly decreased at the seedling stage when water is withheld for two days (Toorchi et al., 2009). Down-regulation of this protein correlates with the possibility of root elongation under drought stress, and helps the plant to continue root growth and to delay root lignification.

Taken together, these results show that drought stress results in an increase in the accumulation of ROS and subsequent lipid peroxidation. The proteins identified by proteomic studies of soybean under drought stress are involved in a variety of cellular functions, including carbohydrate and nitrogen metabolism, cell wall modification, signal transduction, cell defense, and programmed cell death, and they contribute to the molecular mechanism of drought tolerance in soybean plants. Further analyses of protein expression patterns has revealed that proteins associated with osmotic adjustment, defense signaling, and programmed cell death play important roles in the adaption of soybean plants to drought. The identification of proteins such as UDP-glucose pyrophosphorylase, 2,3-bisphosphoglycerate, ferritin, and S-adenosylmethionine synthetase has provided new insights that may lead to a better understanding of the molecular basis of responses to drought stress in soybean.

3.2 Salinity

Throughout the world, agricultural productivity is being severely affected by increasing levels of soil salinity, mainly as a result of inappropriate agricultural activities and changes in climate. The effects of salinity on plant growth are complex and involve osmotic stress, ion toxicity, and mineral deficiencies (Yeo, 1998). The detrimental effects of salt on plants are consequences of both a water deficit, resulting from the relatively high solute concentrations in the soil, and Na⁺-specific stresses, resulting from altered K⁺/Na⁺ balances and Na⁺ ion concentrations that are inimical to plants (Bandehhagh et al., 2008). In soybean subjected to 40 mM NaCl, the Na content of the leaves, hypocotyls, and roots increased, whereas the K content remained unchanged (Sobhanian et al., 2010b). Soybean is a relatively salt-sensitive crop, and salinity suppresses the expression of most genes and their corresponding proteins (Kao et al., 2006).

The proteome of soybean subjected to salinity has been analyzed using roots and hypocotyls of young seedlings (Aghaei et al., 2009) and using different tissues (Sobhanian et al., 2010b) (Table 1). It has been reported that 50S ribosomal protein, which contributes to soybean protein biosynthesis and presumably leads to the consequent reduction in plant growth was down-regulated in leaves. Furthermore, photosynthesis-related proteins in leaves are mainly down-regulated, suggests that NaCl affects photosynthesis and leads to energy reduction inside the plant with a consequent reduction in plant growth. The chaperone protein of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activase is down-regulated by NaCl in soybean (Sobhanian et al., 2010b), and this led to the main inhibitory effect of NaCl on soybean photosynthesis (Parker et al., 2006). Chaperones act by repairing potential damage caused by misfolding of proteins. Up-regulation of the 20-kDa chaperonin (Sobhanian et al., 2010b) suggests that protection of proteins by chaperonins in soybean is very important in preventing misfolding of proteins under salt stress.

Metabolic-related proteins in the leaves, hypocotyls, and roots of soybean seedlings are mainly down-regulated under salt stress. Adenosine triphosphate (ATP) is vital for many biosynthetic pathways in plant cells, and energy requirements may increase considerably during periods of external stress. Adenosine kinase and ATP synthase are up-regulated by salinity in wheat (Wang et al., 2008), rice (Kim et al., 2005), potato (Aghaei et al., 2008), and the C4 plant *Aeluropus lagopoides* (Poaceae) (Sobhanian et al., 2010a). This shows that the maintenance of ATP-dependent salt tolerance by increasing the formation of ATP is one strategy that plants adopt to cope with salt stress. Caffeoyl-CoA O-methyltransferase, which can catalyze the conversion of caffeoyl-CoA to sinapoyl-CoA (Grimmig & Matern, 1997), is

down-regulated in the presence of salinity (Sobhanian et al., 2010b). The products of this enzyme are intermediates in the lignification of the cell wall, and down-regulation of the enzyme suggests a reduction in cell wall lignification and a consequent decrease in growth of soybean seedlings under salt stress.

An examination of the secreted proteins in the root, hypocotyl, or leaf may help to unravel the regulation of salt-stress progression. Knowledge regarding the coordination between signaling molecules and factors that regulate extracellular matrix formation may aid in the development of new salt-tolerant varieties. Studies on the proteins present in roots or other tissues may facilitate the identification of salt-responsive proteins and their corresponding genes. Overall, the analysis of membrane proteins, intracellular components of signaling pathways, and factors that interact in regulating the response of cells to salt stress may eventually provide target genes, such as glyceraldehydes-3-phosphate dehydrogenase, for genetic engineering. Unfortunately, at present, the nature, signal molecules, and even the function of such proteins are poorly classified and understood; however, by applying the techniques of proteomics, our knowledge of these factors should become greatly improved.

3.3 Cold

Cold is one of the major environmental stresses that limit crop productivity, quality, and post-harvest life. Nonfreezing temperatures from 0 to 15 °C correspond to chilling or cold stress for plants (Renaut et al., 2004). Most temperate plants acquire tolerance to chilling and freezing through prior exposure to cold stress, a process called cold acclimation, although many agriculturally important crops, such as soybean, are incapable of cold acclimation. Soybean is very sensitive to cold stress (Cheesbrough, 1990) and to heat shock, both of which affect protein synthesis and cell metabolism (Roberts & Key, 1991). Low temperatures affect the uptake of water and nutrients, the fluidity of membranes, and the conformations of proteins and nucleic acids; they also have a marked influence on cellular metabolism, either directly through a reduction in the rates of biochemical reactions or indirectly through reprogramming of gene expression (Chinnusamy et al., 2007). The generation of ROS is a common feature of many plants when they are exposed to cold temperatures. The resulting ROS interact with several cellular components including proteins, and plants have been shown to develop complementary protective responses to cope with the cold stress (Yamaguchi-Shinozaki & Shinozaki, 2006).

Limited information is available regarding the proteome response of soybean subjected to cold stress. A differential proteome analysis of soybean seeds exposed to a low temperature (4 °C) during imbibition revealed a total of 40 affected protein spots of which 25 were up-regulated and 15 were down-regulated (Cheng et al., 2010) (Table 1). These proteins are involved in many metabolic pathways, including those related to cell defense, energy, protein synthesis, cell growth/division, storage, transcription, and transport. Cheng et al. (2010) found that alcohol dehydrogenase I and RAB21 may contribute in decreasing the effect of the anoxia resulting from water uptake during imbibition, whereas, stress-related proteins, such as LEA and GST24, probably play a pivotal role in reacting to low temperature stress. In addition, enhancements in levels of malate dehydrogenase and phosphoenolpyruvate carboxylase, which are involved in the tricarboxylic acid cycle, are associated with cold tolerance of seeds during germination. Accumulation of chloroplastic LEA proteins has been correlated with the capacity of various wheat and rye cultivars to develop freezing tolerance. Furthermore, *Arabidopsis* plants transformed with the *Wcs19* gene, which encodes chloroplastic proteins related to LEA, show a significant increase in

their freezing tolerance (Ndong et al., 2002). It appears that the functional role of LEA protein across all species is related to the response to stress conditions from desiccation, osmotic stress, or cold. Induction of LEA proteins has also been observed in soybean under salt stress (Aghaei et al., 2009; Soulages et al., 2002).

Short-term exposure of two-day-old soybean seedlings to cold stress resulted in up-regulation of pathogenesis-related protein 1 (PR1), but down-regulation of PR10 and caffeoyl-CoA 3-O-methyltransferase (Toorchi et al., 2009). It is well documented that the *PR* genes are regulated by pathogenic infections and by damage caused by insect attacks and wounding. It is noteworthy that several *PR* genes are also regulated by environmental factors such as cold, osmotic stress, or light (Zeier et al., 2004). In addition, one group of *PR* proteins, including PR1-type proteins, chitinases, and thaumatin-like proteins, are synthesized in overwintering monocots such as barley, wheat, and grasses under cold stress; these proteins exhibit antifreeze activities (Griffith & Yaish, 2004) suggesting that they may also be involved in resistance to extreme temperatures. These reports have shown that *PR* proteins have a significant role in protecting the components of the plant cell from the deleterious effects of abiotic stresses. It has also been proposed that plant responses to biotic and abiotic stresses share certain aspects of signaling molecules and pathways (O'Donnell et al., 2003). However, the effects of a range of abiotic stresses on the expression of *PR* genes have not been extensively explored.

Cold stress signaling is an important aspect with regard to increasing the productivity of plants. Similarly, variations in the resistance of plants to chilling may require a series of metabolic reorganizations that could involve a range of mechanisms for reducing the vulnerability of enzyme structures and functions in cold conditions. The mechanisms by which chill-resistant plants resist such changes are poorly understood, and much more research is required before we can understand how membranes and proteins can be modified to stabilize them at low temperature. A deeper understanding of the transcription factors regulating the relevant genes, the products of the major stress-responsive genes, and crosstalk between divergent signaling components is likely to remain an area of intense research activity in the future.

3.4 Osmotic stress

Osmotic stress is a general feature of most abiotic stresses, including drought, salinity, and low temperatures (Beck et al., 2007; Nuccio et al., 1999), in which the pressure associated with turgor decreases, and water is lost as a result of osmotic dehydration. It has been reported that salinity, low water availability, and osmotic potential are indirectly related to one another, and part of the reduction in osmotic potential is caused by a decrease in the relative water content of plant (Chaparzadeh et al., 2003). Polyethylene glycol (PEG) is more widely used than other compounds such as mannitol, sucrose, or glucose in inducing osmotic stresses 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). To cope with osmotic-related stresses, plants have developed various responses such as the production of osmolites for osmotic adjustment and the synthesis of Na^+/H^+ antiporters for ion sequestration (Bohnert et al., 1995). A hybrid-type histidine kinase has been reported to function as osmosensor and to transmit a stress signal to a downstream mitogen-activated protein kinase cascade (Urao et al., 1999). In soybean, an induction of an osmotic potential of -0.3 MPa for two days modulated by PEG 6000 caused reductions in the root length and thickness (Toorchi et al., 2009).

Proteome analysis of soybean root subjected to osmotic stress was analyzed with CBB staining, and 37 responsive proteins were identified, of which 19 were up-regulated and 18 were down-regulated. Functional classification of the responsive proteins revealed that 28% contributed directly to disease/defense, suggesting that the root is actively engaged in these processes. On the other hand, 16% of the proteins contributed to metabolism, indicating that the root is damaged by PEG treatment. SOD and thioredoxine, which are categorized as being involved in defense, were up-regulated by PEG treatment (Toorchi et al., 2009). Plant stress tolerance requires the activation of complex metabolic pathways within the cells, including antioxidative pathways (especially ROS-scavenging systems) that can contribute to continue growth under water stress (Esfandiari et al., 2007). Within a cell, SODs form the first line of defense against ROS, whereas thioredoxin is an important antioxidant for eliminating ROS. The increased abundance of SOD and thioredoxin in soybean plants correlates with enhanced tolerance of the plants to osmotic stress.

Nouri & Komatsu (2010) investigated the soybean plasma membrane proteome under osmotic stress by using gel-based and gel-free proteomics approaches. They adapted a two-phase partitioning method to purify plasma membranes from soybean seedlings that had been treated for two days with 10% PEG. The numbers of proteins identified as being up-regulated (11) and down-regulated (75) by means of the gel-free proteomics approach were clearly greater than those identified by the gel-based approach (4 and 8, respectively). Three homologues of plasma membrane H⁺-ATPase and calnexin (a molecular chaperone protein) were highlighted as being up-regulated under osmotic stress. Plasma membrane H⁺-ATPase 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. The chaperone protein calnexin accumulates in the plasma membrane, and ion efflux is accelerated by up-regulation of plasma membrane H⁺-ATPase protein. Down-regulation of other chaperone proteins, such as the calreticulin precursor and HSP, have been reported in a study of rice leaf sheath treated with mannitol (Zang & Komatsu, 2007). Calreticulin is an important calcium-binding protein with chaperone functions, and it plays a pivotal role in regulating calcium homeostasis and protein folding in the endoplasmic reticulum of plants (Wang et al., 2004). Taken together, calcium signaling and ion transporting are the pathways that are involved in the defense mechanism of cells subjected to osmotic stress. Although considerable information has been accumulated on responses to osmotic stress, and some signaling elements have been identified, we are still far from having a clear picture of the actions of osmotic stress on plants, particularly soybean.

4. Soybean proteomics under other abiotic stresses

4.1 Flooding

The term 'flooding' refers to submersion in water or soil waterlogging, and it entails an excess of available water for the plant. Higher plants have a wide range of tolerances to flooding, and soybean is classified as an intolerant crop. Soybean is particularly susceptible to flooding stress during its germination and early vegetative stages, and the grain yield is markedly affected by flooding (Githiri et al., 2006). Flooding stress is usually accompanied by hypoxia stress resulting from an inadequate supply of oxygen to submerged tissues (Armstrong, 1980), and cellular depletion of oxygen results in a rapid signal to enhance transient survival or long-term tolerance (Bailey-Serres & Chang, 2005). Sensing of the stress starts at the cell wall and a signal is transmitted to the cell through the plasma membrane by

altering the expression of several metabolites and proteins. Proteomics analysis provides a novel approach for identifying the proteins and pathways that play critical roles in responses to flooding stress. Most studies of the proteome of soybean under flooding stress have focused on the early growth stage, since even a short period of stress at this stage can cause a considerable amount of damage.

Komatsu et al. (2009b) studied total proteins from roots and hypocotyls of 2-day-old soybean seedlings subjected to 12 hours of flooding stress (Table 2) and they identified inducible genes and proteins. Within 12 hours of stress, genes associated with alcohol fermentation, ethylene biosynthesis, pathogen defense, and cell wall loosening were significantly up-regulated. Furthermore, the expression of hemoglobin, acid phosphatase, and Kunitz trypsin protease inhibitor were altered at both the transcriptional and the proteome level. In a separate experiment, in a separate experiment, 2D-DIGE and gel-free and gel-free techniques were used to identify stress-responsive proteins (Nanjo et al., 2010); proteins related to glycolysis, fermentation enzymes, and inducers of heat shock proteins were identified as being key elements in the early responses to flooding stress. An analysis of the carbohydrate content and measurements of enzyme activities in flooded soybean confirmed that activation of glycolysis and down-regulation of sucrose-degrading enzymes caused acceleration in glucose degradation and accumulation of sucrose in flooded seedlings. Another early response to flooding is that of dephosphorylation of proteins involved in protein folding and synthesis. This modification may affect metabolic pathways under flooding stress.

Effects of one day flooding stress on the proteome of soybean have been studied for total protein extracts (Hashiguchi et al., 2009) and for plasma membrane proteins (Komatsu et al., 2009a). Proteins classified as defense- and disease-related proteins accounted for most of the differentially expressed proteins in the total protein extract (Hashiguchi et al., 2009). Gel-based and gel-free techniques showed the presence of up-regulation of cell wall proteins, SOD, and heat shock cognate 70 kDa protein in the plasma membrane fraction, and it has been suggested that signaling proteins such as 14-3-3, serine/threonine protein kinase and band 7 family protein may operate cooperatively in regulating plasma membrane H⁺-ATPase and in maintaining ion homeostasis (Komatsu et al., 2009a). Cell wall proteins were identified as being up-regulated in the purified plasma membrane fraction, suggesting that plasma membranes contribute to the construction of the cell wall. To understand the mechanism of the responses of the soybean cell wall to flooding stress, cell wall proteins from roots and hypocotyls of four-day-old soybean subjected to flooding stress for two days were purified, separated by 2-DE, and stained with CBB (Komatsu et al., 2010a). A comparison of the 2-DE gel patterns showed that, under stress, a copper amine oxidase protein shifted from the basic zone to the acidic zone. Komatsu et al. (2010a) also reported that lignification is suppressed in the roots of soybean following flooding stress. Several studies have identified ROS scavengers as common flooding-responsive proteins in total protein and cell wall fractions of soybean, and various ROS scavengers have been reported to be down-regulated under flooding stress in soybean seedlings (Alam et al., 2010a; Hashiguchi et al., 2009; Komatsu et al., 2009b; Komatsu et al., 2010a, Komatsu et al., 2010b; Shi et al., 2008).

Flooded soybean seedlings are also subject to hypoxia stress as a result of low oxygen levels in the submerged tissues. The seedlings inevitably show anaerobic pathways in which they generate ATP through glycolysis and they regenerate NAD⁺ through ethanol fermentation. Several studies confirmed that the expression of proteins involved in glycolysis and

fermentation pathways, such as UDP-glucose pyrophosphorylase, fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, and alcohol dehydrogenase are up-regulated in response to flooding stress (Alam et al., 2010a; Hashiguchi et al., 2009; Komatsu et al., 2010b; Nanjo et al., 2010). These results confirm that flooding stress also involves stress from oxygen starvation. Analyses of the proteome of soybean subjected to flooding stress could throw some light on the physiology of soybean subjected to flooding; however, several cellular mechanisms for plant survival, such as the pathways involved in energy metabolism and transpiration, are not yet well understood.

4.2 High temperature

Soybean is a crop that is sensitive to high temperatures during both its vegetative and reproductive stages (Khan et al., 2007; Salem et al., 2007). Exposure of soybean plant to a temperature of 35 °C for 10 hours per day resulted in about a 27% reduction in yield (Gibson & Mullen, 1996). High temperatures during seed development changed the seed components, in which concentrations of palmitic, stearic, and oleic acids increased, whereas those of linoleic and linolenic acids decreased; there was also a marked decrease in seed vigor (Ren et al., 2009). One of the main deleterious effects of high temperatures during soybean growth is the disruption of photosynthesis, and it has been reported that, among the various components of the photosynthetic apparatus, photosystem II (PS II) is particularly sensitive to high temperatures (Thompson et al., 1989). Nishiyama et al. (2006) showed that in a suspension culture of soybean cells, a moderately high temperature induces the synthesis of proteins responsible for the thermal stability of PS II. Depending on the severity of this stress, changes occurred in the expression of carbohydrate biosynthesis enzymes, metabolic pathway enzymes and proteins for survival at high temperatures.

Only few proteomics studies have been performed on soybean subjected to high-temperature stress. In one of the experiments, total proteins have been extracted from mature seeds of soybean plants grown under normal and high temperatures in growth chambers (Ren et al., 2009), and 20 heat stress-responsive proteins were identified by using the 2D-DIGE technique. The accumulation of heat shock protein 22 (HSP 22) was shown to increase in seeds that developed at a high temperature. It was concluded that an increase in the levels of HSPs protects the seeds from damage by high temperatures (Ren et al., 2009). Recently, a proteome analysis has been performed on soybean seedlings subjected to a high temperature, and differentially expressed proteins were identified (Ahsan et al., 2010a). Total proteins extracted from leaves, stems, and roots of two-week-old soybean seedlings exposed to a temperature of 40 °C for 6, 12, or 24 hours were compared with those of control plants. Of 150 proteins, 10 were common among the three tissues, whereas 21, 10, and 34 were unique in leaves, stems, and roots, respectively, when these were subjected to a high temperature. Ahsan et al., (2010a) reported that nearly half of the differentially expressed proteins belong to the HSP family and were up-regulated under the stress; some of these proteins, such as heat shock cognate 70, HSP 70, HSP 22, HSP 18.5, and HSP 17.5, were common to all three tissues.

In terms of functional classification, most of the proteins involved in antioxidant defense were up-regulated. The expression of two antioxidant enzymes, SOD [Cu-Zn] and cytosolic APX1, were significantly increased in soybean leaves exposed to high temperature; these enzymes act as a defense against heat stress-induced ROS. Proteins associated with photosynthesis, secondary metabolism, and protein biosynthesis were down-regulated in response to heat stress, as reported previously (De Ronde et al., 2004; Thompson et al., 1989).

Furthermore, the expression of proteins related to primary carbon assimilation, Calvin cycles, PS I/II, and electron transport were down-regulated following exposure to high-temperature stress. Except for a few isolated studies, little attention has been paid to the soybean proteome analysis subjected to a high temperature and, with the growing threat of global warming, we need to increase our knowledge of the high-temperature defense or tolerance mechanisms of plants.

4.3 Ultraviolet radiation

The depletion of the stratospheric ozone layer has increased the amount of ultraviolet-B (UV-B; 280–315 nm) as a proportion of the Sun's radiation reaching the Earth's surface (Blumthaler & Ambach, 1990; McKenzie et al., 1999). Modeling by the Goddard Institute for Space Science estimated that the annual increase in the dose of UV radiation that will reach ground levels in the Northern and Southern Hemisphere will increase by 14% and 40%, respectively, for the period 2010–2020 compared with conditions during 1979–1992 (Taalas et al., 2000). Enhanced UV-B radiation initiates diverse responses in plants, including effects on plant development, metabolism, and viability. Soybean genotypes exhibit a wide range of sensitivities to UV-B radiation, and it has been reported that differences in the flavonoid contents of cultivars is one factor that affects their response to UV (Xu et al., 2008). Several studies have been performed to evaluate morphological and physiological aspects of damage in UV-irradiated soybean plant. For instance, UV radiation causes a decrease in the accumulation of dry matter in soybean roots, stems, and leaves (Peng & Zhou, 2010); it also inhibits growth (Peng & Zhou, 2010), reduces the chlorophyll content and photosynthesis (Wang et al., 2009), and results in smaller flowers and pollen grains with lower pollen germination (Koti et al., 2004). Furthermore, UV-B radiation causes oxidative stress in plants and leads to the generation of ROS. It has been shown that levels of ROS scavengers, such as SOD, peroxidase, and catalase, are considerably increased in soybean plants affected by UV-B radiation (Wang et al., 2009). UV radiation also increases the expression of heme oxygenase, at both the mRNA and protein levels, as a mechanism for protecting the cell against oxidative damage (Yannarelli et al., 2006).

Xu et al. (2008) analyzed the proteome of soybean leaves subjected to natural levels of UV-B radiation in the field for two isolines with moderate and reduced flavonol glycoside contents, respectively. A total of 67 responsive proteins were identified, among which the proteins related to photosynthetic photosystems were up-regulated and enzymes involved in primary carbon and nitrogen metabolism were down-regulated. The role of flavonoides as screening compounds in protecting plants from UV-B radiation was confirmed. Although physiological and metabolic aspects of soybean plant subjected to UV radiation have been extensively studied, there is an obvious lack of a comprehensive proteome analysis of soybean plants subjected to harmful solar irradiation. Furthermore, depending on the geographical region, side effects of irradiation on the plant probably occur in conjunction with several other abiotic stresses, such as high temperature or drought, and these need to be considered in future studies.

4.4 Ozone

Tropospheric ozone is a photochemically generated air pollutant that negatively affects plant growth and development. Depending on the concentration of ozone, the length of exposure, the age of the plant tissue, and the genetic susceptibility of the plant, ozone can

damage plant through inhibition of photosynthesis, premature senescence, visible tissue necrosis, and reduction of yield (Darrall, 1989; Krupa & Manning, 1988). Ozone enters the intercellular space of leaves through stomata, and it can be converted into ROS and hydrogen peroxide, initially in the cell wall and plasma membrane (Pellinen et al., 1999), causing oxidative stress in the plant. Several physiological studies on plants have shown that ozone stress causes a decrease in the CO₂ assimilation rate (Soldatini et al., 1998), an inhibition of PS II (Guidi et al., 2001), and a reduction in RuBisCO activity in leaves (Feng et al., 2008; Pelloux et al., 2001), which ultimately affect the plant's normal growth and productivity.

A comparative proteome analysis has been performed for soybean seedling subjected to stress by 120 ppb of ozone for three days. Total soluble proteins and chloroplast proteins were extracted and subjected to analysis (Table 2) (Ahsan et al., 2010b). Although the ozone treatment did not produce any visible symptoms of ozone-induced necrosis, it significantly increased the accumulation of hydrogen peroxide and thiobarbituric acid-reactive substance in leaves. At the protein level, 45% of responsive proteins were involved in photosynthesis, indicating that the photosynthetic pathways are the most affected by ozone stress. Measurements of the starch and soluble sugar contents of soybean leaves in the presence and absence of ozone stress showed that the starch content was significantly decreased whereas the sucrose content increased. Ahsan et al. (2010b) concluded that short-term acute ozone exposure feeds the tricarboxylic acid cycle, and that the availability of sucrose plays a pivotal role in oxidative stress signaling and the regulation pathways of the antioxidative processes. As climate changes are likely to cause increases in ozone levels in the field, further studies on the proteome of soybean are necessary to reveal the complex defense mechanisms by which the plant copes with this oxidative stress.

4.5 Heavy metals

4.5.1 Cadmium

Cadmium (Cd) is an environmental pollutant of soil that arises from both natural and anthropogenic sources, such as atmospheric emissions, general urban and industrial emissions, as a by-product of phosphate fertilizers and sewage sludge (Alloway & Steinnes, 1999). The presence of this heavy metal in the atmosphere, soil, and water, even in trace amounts, can endanger all organisms, and the accumulation of Cd in the food chain can cause serious problems. In addition to being a worldwide concern with regard to human health (Waalkes, 2003), Cd also has deleterious effects on plants. Plants show differences in their uptakes of Cd that depend on the concentration of Cd in the soil and its bioavailability. The latter is modulated by the presence of organic matter, the pH, the redox potential, the temperature, and the concentrations of other elements present in the soil (Toppi & Gabbrielli, 1999).

The proteome of suspension-cultured cells of soybean subjected to various concentrations and time courses of Cd exposure has been analyzed (Sobkowiak & Deckert, 2006). Stress-induced protein SAM22, which is classified as a PR10 protein, was identified in an SDS-PAGE band that was enhanced by Cd treatment. Antioxidant enzymes, such as SOD [Cu-Zn], were also up-regulated, providing a clue regarding the defense reaction of soybean to metal toxicity. A group of glutathione S-transferases were also up-regulated on treatment with Cd; these enzymes have known functions in detoxification of a wide range of xenobiotic substances, including heavy metals (Frova, 2003). The expressed proteins in soybean treated with Cd, like those in other plants, generally belong to the group of proteins that are active against oxidative stress and in detoxification. Soybean, as a legume, has

symbiosis with rhizobia in the root system, which could affect the degree of sensitivity or tolerance of plant to Cd, and this aspect needs more investigation in future studies.

4.5.2 Aluminum

The toxicity of aluminum (Al) is a major factor in limiting the growth and development of plants in acidic soils. An increase in the solubility of Al as a result of a decrease in soil pH causes a significant inhibition of plant growth in acidic soils (Foy et al., 1978). The toxicity of Al in acidic soils and its detrimental effects on plants has been extensively documented. For instance, Al inhibits both cell division and elongation of root tips (Ryan et al., 1993); it also increases susceptibility to drought (Foy et al., 1978), inhibits calcium uptake (Huang et al., 1992), produces an alteration in the cytoskeleton, causes a depolarization of the plasma membrane, and induces the formation of callose (Sivaguru et al., 1999). The presence of toxic Al in the soil leads to strong binding of Al to the cell walls of the root apex and induces callose formation by binding to negatively charged sites on the plasma membrane. In response to Al stress, many plants secrete organic acids, such as citric, malic, and oxalic acids. This kind of exudation is known to be an important mechanism for resistance to Al toxicity in plants (Shen et al., 2005). Soybean secretes citrate in response to Al toxicity, and it has been reported that under Al stress an Al-tolerant soybean cultivar, Suzunari, may specifically secrete more citrate than does an Al-sensitive cultivar (Yang et al., 2000).

In a study on the expression of plasma membrane H⁺-ATPase at the transcriptional and translational levels in soybean roots under Al stress, it was found that the effects of Al stress on citrate secretion were mediated through modulation of the activity of plasma membrane H⁺-ATPase (Shen et al., 2005). The proteome of soybean subjected to Al toxicity was analyzed by Zhen et al. (2007) (Table 2). One-week-old soybean seedlings were exposed to 50 μ M AlCl₃ for various time courses, and Al-responsive proteins were identified from total protein extracts. Activation of sulfur metabolism was detected in the soybean seedlings in consistent with that of observed in rice. *O*-Acetylserine (thiol) lyase, one of the up-regulated proteins, is a key enzyme in the sulfur metabolism of plants, catalyzing the biosynthesis of cysteine from inorganic sulfide and *O*-acetylserine. The proteome studies therefore confirmed the existence of an antioxidative mechanism for responding to Al toxicity in soybean roots. Although several physiological studies have examined Al-tolerance mechanisms in soybean, there is an obvious lack of a comprehensive proteomics analysis. Because acid soils occupy up to 40% of the arable lands in the world (Kochian, 1995), the toxicity of Al needs be considered in more detail in future research.

5. Challenges for soybean proteomics

5.1 Organelle proteomics

The analysis of proteome of organelles and subcellular fractions is one of the most informative approaches to the functional analysis of living cells. Because each organelle of a plant cell generally has its own specific functions in addition to communicating with other parts of the cell, a study of an enriched fraction of a desired organelle offers many advantages in proteome research. The study of cell organelles is usually subject to two major constraints: the availability of an appropriate purification technique, and the verification of the purity of extract. A review has been published on the isolation of plant nuclei, mitochondria, and chloroplasts in pure forms and the preparation of other organelles (Lilley

& Dupree, 2007). The evaluation of the purity or the degree of contamination of organelle extracts is necessary step, otherwise any novel proteins identified by proteomics analyses cannot be definitely assigned to a particular organelle (Komatsu & Ahsan, 2009). In the case of hydrophobic proteins, such as membrane proteins, in subcellular fractions of soybean seedlings, or in the case of low-copy-number proteins, the identification of the individual proteins remains a challenge (Huber et al., 2003).

Analyses of the proteome of soybean organelles have been performed for mitochondrial fractions from roots and nodules (Hoa et al., 2004), peribacteroid membranes (Panter et al., 2000), and etiolated cotyledon peroxisomes (Arai et al., 2008). In relation to abiotic stress-responsive proteins in soybean organelles and subcellular fractions, the effects of osmotic stress on the plasma membrane, of flooding on the cell wall and plasma membrane, and of ozone on chloroplasts have been examined (Tables 1 & 2). In all such experiments, the purification of protein extracts and the verification of their purity determine the validity of the results of the proteomics analysis. Plasma membrane extracts were purified by using a two-phase partitioning method and their purity was verified by measurement of the activity of P-type ATPase (Komatsu et al., 2009a; Nouri & Komatsu, 2010). A cell wall fraction was obtained by using calcium chloride, and its purity was confirmed by assaying the activity of glucose-6-phosphate dehydrogenase (Komatsu et al., 2010a). Chloroplasts from soybean leaves were purified by using a Percoll gradient, and their purity was assayed by immunoblot analysis using specific antibodies (Ahsan et al., 2010b).

Although techniques for organelle proteomics continue to be improved, achieving to a pure fraction free of contaminants from other parts of the cell remains a challenging problem. This aspect is particularly important under stress conditions where several proteins, such those related to quality control, defense, or metabolism, can migrate through secretory pathways in the cell to cope with the imposed stress. In such cases, protein localization may be capable of verifying the existence of a given protein in a specific fraction (Lilley & Dupree, 2007). It is expected that the completion of annotation of the soybean genome and the corresponding database information should result in further improvements the analysis of the proteome of organelles of soybean.

5.2 Protein–Protein interactions

Proteins in the cell are usually found as complexes, and biological processes within the cell are controlled by interactions between various proteins (Alberts et al., 2002). For instance, the molecular mechanisms involved in the synthesis and use of ATP and in the replication and translation of genes are reportedly controlled by interacting proteins in metabolic and signaling pathways (Marcotte et al., 1999). Several procedures exist for identifying potential protein partners and studying protein–protein interactions. Among these, yeast two-hybrid analysis has been reported to be a robust method for the detection of pairwise protein–protein interactions (Parrish et al., 2006). Along with these improvements in experimental techniques, a number of improvements in computational methods are available to assist in the prediction of protein interactions (Salwinski & Eisenberg, 2003).

Studies on protein–protein interaction are generally performed in model organisms and in some model plants. Although the resulting database of protein–protein interactions is a valuable resource for understanding signaling networks in various organisms (Ding et al., 2009), the expansion of this study to other crops remains a challenging problem. Moreover,

the identification of interacting proteins in their natural environments, especially under stress conditions, can give a better insight in relation to comprehensive plant proteome research. A mathematical gene interaction network has been used in studies on protein-protein interactions in soybean seedlings under conditions of flooding stress (Hashiguchi et al., 2009), and it was shown that flooding stress affects the interactions of proteins in soybean seedlings. The development of these types of computational technique or improvements in experimental approaches for protein interaction studies depend directly on the reliability of databases, a factor that requires further consideration.

6. Conclusion

Abiotic stresses caused by adverse environmental conditions alter the metabolism of soybean cells and ultimately affect the growth, development, and potential productivity of the plant. Plant cells defend against stresses by altering their expression of genes and, consequently, their proteome. Gene expression varies according to the type and severity of the stress and the developmental stage of the plant. Analysis of the proteome is a powerful tool for linking gene expression to cell metabolism. It also provides the possibility of studying individual organelles within the cell. Proteomics, with its growing collection of technologies for extraction and identification of proteins and for studies of their interactions, permits the elucidation of the mechanisms that are involved in the responses of cells to abiotic stresses. Soybean contains many secondary metabolites, phenolic compounds, lipids, and carbohydrates that hamper high-quality protein extraction and separation. A range of methods has been developed for extracting and identifying the proteins from various tissues and organelles. These methods permit the analysis of the proteome of soybean subject to a given abiotic stress, thereby providing an insight into causes of morphological and physiological changes induced by stress. Studies on molecular mechanisms within the cell, such as studies on the proteome, help in achieving a better interpretation and explanation of the morphological behaviors of plants subjected to stress. Depending on the type, severity, and duration of the stress, soybean presents both common and specific responses at the proteome level. For instance, in almost all abiotic stresses, the production of ROS and the expression of ROS scavengers occur, indicating the involvement of oxidative stress in the plant. Therefore, common pathways are expected to be found in cells that permit them to cope with a range of abiotic stresses.

Although several proteomic studies have been performed on the responses of soybean to various abiotic stresses, knowledge about the stresses leading to improving plant tolerance is inadequate. In studies on stress, it should be noted that under natural conditions adverse environmental factors are almost never present as individual entities; on the contrary, they tend to occur together, a factor that should be considered when designing comprehensive studies. The involvement of several signaling molecules and the activation of signaling pathways in the cell under stress conditions have been partly studied, but an understanding of the signal transduction pathways that mediate responses to stresses remains a challenge. The application of high-throughput proteomics approaches is expected to accelerate progress in our understanding of these signaling elements. Elucidation of the signaling networks through proteomics should pave the way for more rational engineering of stress-tolerant soybean plants.

| Cultivar | Protein fraction/ Organ, Tissue/ Age | Protein extraction buffer | Stress duration | Protein separation & identification | Responsive protein spots (up/down) | Key identified proteins | Ref. |
|-----------------|--|---|-----------------|--|--|--|-------------------------------|
| Drought | | | | | | | |
| Taeg-wang | Total protein/ root/ 3- weeks-old | 8 M urea, 1% CHAPS, 0.5% IPG buffer, 20 mM DTT, bromophenol blue | 5 days | IPG/SDS- PAGE MALDI-TOF MS | 28 (7/21) | glycerol kinase, arogenate/ prephenate dehydratase, phloem serpin | Alam et al., 2010b |
| Salinity | | | | | | | |
| Enrei | Total protein/ root, hypocotyl, leaf/ 7-day- old | 8.5 M urea, 2.5 M thiourea, 5% chaps, 100 mM DTT, 0.5% Bio-Lyte (pH 3-10 and 5-8), | 7 days | IEF/SDS- PAGE MALDI-TOF MS, protein sequencer | leaf: 19 (11/8) hypocotyl: 22 (12/10) root: 14 (7/7) | glyceraldehyde -3-phosphate dehydrogenase ,fructokinase 2, stem 31 kDa glycoprotein | Sobhania n et al., 2010 |
| Enrei | Total protein/ root, hypocotyl/ 3- day-old | 65 mM K ₂ HPO ₄ , 2.6 mM KH ₂ PO ₄ , 400 mM NaCl, 3 mM NaN ₃ | 3 days | IEF/SDS- PAGE ESI-Q/TOF- MS/MS, protein sequencer | 7 (4/3) | LEA, defense related proteins | Aghaei et al., 2009 |
| Cold | | | | | | | |
| Genotype Z22 | Embryonic axes/ seed | 7 M urea, 2 M thiourea, 4% CHAPS, 0.4% IPG buffer (pH 4-7), 1% DTT | 1 day | IPG/SDS- PAGE MALDI-TOF MS | 40 (25/15) | LEA , GST24, malate dehydrogenase , phosphoenol pyruvate carboxylase | Cheng et al., 2010 |
| Osmotic | | | | | | | |
| Enrei | Plasma membrane/ root, hypocotyl/ 4- day-old | 0.4 M sucrose, 75 mM MOPS, 5 mM EDTA, 5 mM EGTA, 10 mM KF, 1 mM DTT, 2 % PVP-40 | 2 days | IEF/SDS- PAGE LC- MS/MS | Gel-based:12 (4/8) Gel-free: 86 (11/75) | calnexin, plasma membrane H ⁺ - ATPase | Nouri & Komatsu, 2010 |
| Enrei | Total protein/ root/ 2-day- old | 8 M urea, 2% NP-40, 0.8% ampholine (pH 3.5-10), 5% 2-ME, 5% PVP-40 | 2 days | IEF/SDS- PAGE MALDI-TOF MS, protein sequencer | 37 (19/18) | caffeoyl-CoA O- methyltransfer ase, 20S proteasome alpha subunit A | Toorchi et al., 2009 |

Table 1. A summary of proteome analyses of soybean treated by osmotic stresses.

| Cultivar | Protein fraction/ Organ, Tissue/ Age | Protein extraction buffer | Stress duration | Protein separation & identification | Responsive protein spots (up/down) | Key identified proteins | Ref. |
|----------|--|---|-----------------|---|---------------------------------------|--|-------------------------|
| Flooding | | | | | | | |
| Enrei | Total protein/ root, hypocotyl/ 2.5-day-old | TCA/acetone precipitation followed by resuspension using 8 M urea, 2 M thiourea, 5% CHAPS, 2 mM tributylphosphine | 12 h | 2D-DIGE LC-MS/MS | 17 (8/9) | glycolysis and fermentation enzymes, inducers of HSPs | Nanjo et al., 2010 |
| Enrei | Total protein/ root, hypocotyl/ 2.5-day-old | 8 M urea, 2% Nonidet P-40, 0.8% Ampholine (pI 3.5-10), 5% 2-ME, 5% PVP-40 | 12 h | IEF& IPG/SDS-PAGE MALDI-TOF MS, LC-MS/MS, protein sequencer | 34 (14/20) | hemoglobin, acid phosphatase, kunitz trypsin protease inhibitor | Komatsu et al., 2009b |
| Enrei | Total protein/ root, hypocotyl/ 3-day-old | 8 M urea, 2% Nonidet P-40, 0.8% Ampholine (pI 3.5-10), 5% 2-ME, 5% PVP-40 | 1 day | IEF& IPG/SDS-PAGE LC-MS/MS, protein sequencer | 51 (35/16) | glycolytic enzymes, ROS scavengers | Hashiguchi et al., 2009 |
| Enrei | Plasma membrane/ root, hypocotyl/ 3-day-old | 400 mM sucrose, 75 mM MOPS, 5 mM EDTA, 5 mM EGTA, 10 mM KF, 1 mM DTT, 2% PVP-40 | 1 day | IEF/SDS-PAGE MALDI-TOF MS, LC-MS/MS, protein sequencer | 22 (20/2) | cell wall-related proteins, antioxidative proteins, heat shock cognate | Komatsu et al., 2009a |
| Enrei | Cell wall/ root, hypocotyl/ 4-day-old | 5 mM acetate buffer containing 0.4 M sucrose, 1 mM PMSF, 2% PVP-40 | 2 days | IEF/SDS-PAGE MALDI-TOF MS, LC-MS/MS, protein sequencer | 16 (4/12) | lipoxygenases, germin-like protein precursors, stem 28/31 kDa glycoprotein precursors, SOD [Cu-Zn], copper amine oxidase | Komatsu et al., 2010a |
| Enrei | Total protein/ root, hypocotyl/ 4-day-old | 65 mM K ₂ HPO ₄ , 2.6 mM KH ₂ PO ₄ , 400 mM NaCl, 3 mM NaN ₃ | 2 days | IEF& IPG/SDS-PAGE MALDI-TOF MS, protein sequencer | 28 (21/7) | alcohol dehydrogenase | Komatsu et al., 2010b |
| Enrei | Cytosolic and membrane/ root/ 5-day-old | Cytosolic fraction: 20 mM Tris-HCl, 10 mM EGTA, 1 mM DTT, 1 mM | 3 days | IEF/SDS-PAGE ESI-Q-TOF MS | 10 | cytosolic ascorbate peroxidase 2 | Shi et al., 2008 |

| Cultivar | Protein fraction/ Organ, Tissue/ Age | Protein extraction buffer | Stress duration | Protein separation & identification | Responsive protein spots (up/down) | Key identified proteins | Ref. |
|------------------|---|---|-----------------|---|---------------------------------------|---|---------------------|
| | | PMSF and for membrane fraction additionally used 1% Triton X-100, 20 mM Tris-HCl, EDTA, 50 mM 2-ME | | | | | |
| Asoagari | Total protein/ root/ 3-week-old | 0.5 M Tris-HCl, 2% NP-40, 20 mM MgCl ₂ , 2% 2-ME, 1mM PMSF, 0.7 M sucrose, Tris-HCl saturated phenol | 3, 7 days | IPG/SDS-PAGE MALDI-TOF, ESI-MS/MS | 24 (19/5) | glycolysis and fermentation pathway enzymes | Alam et al., 2010a |
| High temperature | | | | | | | |
| Enrei | Total protein/root, stem, leaf / 2-week-old | 0.5 M Tris-HCl, 2% NP-40, 20 mM MgCl ₂ , 2% 2-ME, 1 mM PMSF, 0.7 M sucrose,Tris-HCl saturated phenol | 6, 12, 24 h | IPG/SDS-PAGE MALDI-TOF MS, LC-MS/MS, protein sequencer | 54, 35, 61 | low molecular weight HSPs and HSP70 | Ahsan et al., 2010a |
| line N98-4445A | Total protein/ seed | 50% phenol, 0.45 M sucrose, 5 mM EDTA, 0.2% 2-ME, 50 mM Tris-HCl | R5 ~ R8 | 2D-DIGE MALDI TOF/TOF MS | 20 (13/7) | sucrose-binding protein, acidic endochitinase, HSP22, late embryo abundant protein, Bowman-Birk proteinase inhibitor, formate dehydrogenase | Ren et al., 2009 |
| Ultraviolet | | | | | | | |
| Clark | Total protein/ leaf/ 12-day-old | TCA/acetone precipitation followed by 9 M urea, 1% CHAPS, 1% DTT, 1% pharmalyte | 9 days | IPG/SDS-PAGE MALDI-TOF MS, LC-MS/MS | 67(31/36) | photosynthetic photosystem proteins, primary carbon and nitrogen-metabolism proteins | Xu et al., 2008 |
| Ozone | | | | | | | |
| Enrei | Total protein/ leaf/ 13-day-old | 0.5 M Tris-HCl , 2% NP-40, 20 mM MgCl ₂ , 2% 2-ME, 1 mM PMSF, 0.7 M sucrose, Tris-HCl saturated phenol | 3 days | IEF/SDS-PAGE MALDI-TOF MS, protein sequencer | 20 (6/14) | photosystem I/II and carbon assimilation- and metabolism-related proteins, antioxidant defense proteins | Ahsan et al., 2010b |

| Cultivar | Protein fraction/ Organ, Tissue/ Age | Protein extraction buffer | Stress duration | Protein separation & identification | Responsive protein spots (up/down) | Key identified proteins | Ref. |
|-----------------|---|--|-----------------|--|---------------------------------------|---|---------------------------|
| Enrei | Chloroplast/ leaf / 13-day-old | 0.3M sorbitol, 5mM MgCl ₂ , 5mM EGTA, 5mM EDTA | 3 days | IEF/SDS-PAGE MALDI-TOF MS, protein sequencer | 32 (10/22) | glutamine synthetase precursor, Fructose-bisphosphate aldolase, Photosystem I subunit | Ahsan et al., 2010b |
| Cadmium | | | | | | | |
| Naviko | Total protein/ suspension cultured cells/ 4-day-old | 100 mM Tris-HCl, 15 mM MgCl ₂ , 15 mM EDTA, 75 mM NaCl, 1 mM DTT, 0.5 mM PMSF, 1 mM NaF | 24, 48, 72 h | SDS-PAGE Q-TOF MS | 12 | superoxide dismutase, histone H2B, chalcone synthase , glutathione transferase | Sobkowiak & Deckert, 2006 |
| Aluminum | | | | | | | |
| Baxi 10 | Total protein/root / 7-day-old | TCA/acetone precipitaton followed by 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 2% pharmalyte | 24, 48, 72 h | IPG/SDS-PAGE MALDI-TOF MS | 30 (26/4) | HSP, glutathione transferase, chalcone synthetase, GTP-binding protein, ATP-binding protein | Zhen et al., 2007 |

Table 2. A summary of proteome analyses of soybean treated by non-osmotic stresses.

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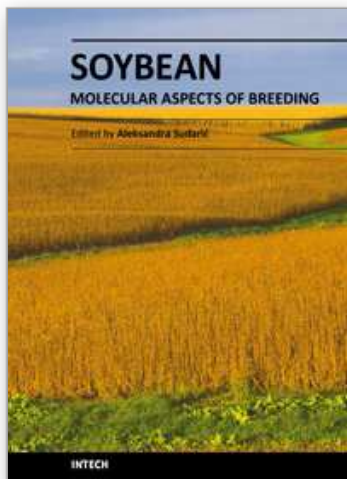
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Soybean - Molecular Aspects of Breeding

Edited by Dr. Aleksandra Sudaric

ISBN 978-953-307-240-1

Hard cover, 514 pages

Publisher InTech

Published online 11, April, 2011

Published in print edition April, 2011

The book Soybean: Molecular Aspects of Breeding focuses on recent progress in our understanding of the genetics and molecular biology of soybean and provides a broad review of the subject, from genome diversity to transformation and integration of desired genes using current technologies. This book is divided into four parts (Molecular Biology and Biotechnology, Breeding for Abiotic Stress, Breeding for Biotic Stress, Recent Technology) and contains 22 chapters.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Mohammad-Zaman Nouri, Mahmoud Toorchi and Setsuko Komatsu (2011). Proteomics Approach for Identifying Abiotic Stress Responsive Proteins in Soybean, Soybean - Molecular Aspects of Breeding, Dr. Aleksandra Sudaric (Ed.), ISBN: 978-953-307-240-1, InTech, Available from:
<http://www.intechopen.com/books/soybean-molecular-aspects-of-breeding/proteomics-approach-for-identifying-abiotic-stress-responsive-proteins-in-soybean>

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