# Proteomic Dissection of Abiotic Stress Response in Crop Plants

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## Abstract

Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50%. In addition, future agricultural production and management will encounter multifaceted challenges from global climate change. Therefore, it is necessary to study the molecular response of crop plants to the stresses in order to develop appropriate strategies to sustain food production under adverse environmental conditions. We carried out a large scale proteomic analysis of soybean plants in response to various abiotic stresses, including drought, salinity, waterlogging and their interactions. Proteins were analyzed by two dimensional polyacrylamide gel electrophoresis followed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. The identified proteins are involved in a wide range of cellular functions. In addition to the well known stress-associated proteins, we identified several novel proteins, which were not reported before. In many cases our proteomic data bridges the gap between mRNA and metabolite data. Our studie provides new insights into identification of abiotic stress responsive proteins in soybean, and demonstrates the advantages of proteomic analysis in dissecting metabolic and regulatory networks.

***Keywords :*** *Abiotic stress, drought, proteome, salinity, soybean, waterlogging*

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## Introduction

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and result in the deterioration of the environment. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for major crop plants by more than 50% (Wang *et al*., 2003). Elucidating the various mechanisms of plant response to stress and their roles in acquired stress tolerance is thus of great practical and basic importance. Various genomic and functional genomics tools have helped to advance our understanding of stress signal perception and transduction and the associated molecular regulatory network. These studies

discovered some of the major tolerance mechanisms, including ion transporters, osmoprotectants, free-radical scavengers, late embryogenesis abundant proteins and factors involved in signaling cascades and transcriptional control (Wang *et al*., 2003).

The availability of vast DNA sequence data has opened new opportunities for studying biological systems, and as a consequence tremendous advances have been seen in genomic technologies. DNA-based microarray technology (oligonucleotide- and cDNA-based microarray systems;(Schena *et al*., 1995) and serial analysis of gene expression (SAGE; (Velculescu *et al*., 1995) have become increasingly popular to profile genome-wide expression of genes at the mRNA level. However, genomics does not provide a biological snapshot of an organism at a particular time point. Because, genome itself is static, and functional proteins allow an organism to interact and to adapt to their environment. In this way proteins, not genes, are directly responsible for the functions and phenotypes of cells such as physical features (as in xerophytes) or by encountering stressors directly (antioxidant enzymes and chaperons) or indirectly (enzyme in osmolyte synthesis). In addition, the powerful genomic tools do not provide any direct information on protein levels and their state of modification (Anderson and Anderson 1998) mainly due to post-translational regulation, which results in a lack of correlation between mRNA and protein abundance (Fiehn *et al*., 2000; Gygi *et al*., 1999). Hence, the study of proteins is not only necessary, it has become essential, and is destined to play an indispensable role in the functional genomics era (Geisow 1998). Proteomics, the comprehensive and quantitative analysis of proteins that are expressed in a given organ, tissue or cell line, provides unique insights into biological systems that cannot be acquired from genomic or transcriptomic approaches. Proteomics bridges the gap between knowing the sequences of genomes and understanding how particular mechanisms are governed at the molecular level. Indeed, collective information from both genomics and proteomics is necessary to answer how biological systems work and interact in the environment, and to improve stress tolerance.

Soybean (*Glycine max*) is one of the most important crop in terms of its wide usage as edible vegetable oil and as a high-protein feed supplement for livestock. Soybean seed products are widely used in industrial and pharmaceutical applications. Recently, soybean biodiesel was recognized as one of the alternatives to fossil fuels for the future (Hill *et al*., 2006). Soybeans grow in field which is exposed to a large variety of abiotic and biotic stresses that affect their growth and development. In particular, tolerance against waterlogging, drought and temperature stresses has been targeted as priority works using functional genomics tools (Stacey *et al*., 2006). It is predicted that water deficit and in many cases excess moisture will continue to be a major abiotic factor affecting global crop yields. One-third of the world's population resides in water-stressed regions, and with elevated $CO_2$ levels in the atmosphere and climatic changes predicted in the future, drought could become more frequent and severe. The resilience of legume crops against present-day weather extremes, such as

drought, excess water, heat, cool weather during grain filling, and early frost, is considered to predict their adaptation to future climate change. On the basis of impact on soybean growth and productivity, and implication our project was intended to study three major environmental stresses: waterlogging, drought and salinity which affect plant and soil relationship. To develop soybean plants with enhanced tolerance to stress, a basic understanding of the physiological, biochemical and gene regulatory networks is essential. Although the emphasis of this work is on soybean, the basic concepts would be applicable for improvement of other crops as well.

Being sessile organisms, plants rely on proteomic plasticity to remodel themselves during periods of developmental change, to endure varying environmental conditions, and to respond to biotic and abiotic stresses. Different families of proteins are known to be associated with a plant's response to stresses by being newly synthesized, accumulating or decreasing. Among other things, these proteins are involved in signaling, translation, host- defense mechanisms, carbohydrate metabolism and amino acid metabolism. Compared to other approaches, research on changes at the protein level, which occur before observed functional changes, is still in sufficient. Therefore, the objective of this work was to carried out proteomic analysis of soybean plants under abiotic stresses to identify possible regulatory networks and useful candidates for genetic engineering.

## Materials and methods

### *Plant growth and treatments*

Seeds of two soybean (*Glycine max*) cultivars (Taegwang, waterlogging susceptible) and Asoagari (waterlogging tolerant) were planted in plastic pots containing commercial potting mix (Horticulture Nursery Medium®, Biomedia, Korea) and grown in a growth chamber maintained at a temperature of 25°C and 55-65% humidity. Two-week-old seedlings were subjected to drought (Alam *et al*., 2010b) or waterlogging (Alam *et al*., 2010a) as described previously. To test salinity and flooding interactions, germinating seedlings were irrigated or the completely flooded with a 100 mM NaCl solution. Roots, hypocotyls or leaves were collected from control and treated plants and immediately frozen in liquid nitrogen, and used for proteomics analysis.

### *Protein extraction and 2-D PAGE*

Proteins were extracted from the hypocotyl or root samples using a phenol extraction method described previously (Alam *et al*., 2010b). PEG fractionation method was used to extract proteins from leaves due to its high Rubisco content. The protein samples were then quantified using the Lowry method and subjected to two-dimensional gel electrophoresis (2-DE) using a standard procedure. The gels were stained with colloidal Coomassie Brilliant Blue (CBB) and the images were acquired using GS-800 Calibrated Imaging Densitometer; Bio-Rad, Hercules, CA, USA).

Spots were detected, quantified and then matched using the Bio-Rad PDQuest software (Version 7.2; Bio-Rad).

### *In-gel digestion, MALDI-TOF MS and bioinformatics analysis*

Differentially expressed protein spots were excised manually from the CBB-stained gels and subjected to trypsin digestion according to Alam *et al* (2011). The samples were analyzed using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Parent ion masses were measured in the reflectron/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 76.000%, a guide wire voltage of 0.01%, and a delay time of 150 ns. The peptide mass fingerprintings (PMFs) obtained from each digested protein were compared with PMFs in the non-redundant National Center for Biotechnology Information database using the ProFound program (http://prowl.rockefeller.edu/ prowl-cgi/profound.exe). The search was performed within all green plants (Viridiplantae) using the following parameters : the maximum number of missed cleavages was set at one, the complete carbamidomethylation of cysteines and variable oxidation of methionines was assumed, monoisotopic masses were used and a mass tolerance of 50 ppm was allowed. Protein matches were therefore considered significant (a nonrandom protein hit) when the expectation values were 5e-2 (confidence 95%).

## Results and discussion

### *Proteomic alteration of soybean seedlings in response to waterlogging stress*

To investigate differentially expressed proteins in soybean plants, we compared the proteomic alterations between a waterlogging sensitive and a waterlogging tolerant cultivar. In roots, a number of proteins were increased or decreased in expression. Some proteins were also newly induced in response to waterlogging. These proteins include numbers of glycolysis and fermentation pathway enzymes such as, UDP-glucose pyrophosphorylase, enolase, pfkb-type carbohydarate kinase family protein, cytosolic phosphoglycerate kinase and alcohol dehydrogenase. These proteins are highly upregulated in both the cultivars. Several spots such as enolase and alcohol dehydrogenase appeared as multiple spots. However, their distribution was observed as cultivar specific. Level of coproporphyrinogen oxidase was increased, which may play a crucial role in linking heme synthesis to the oxygen/heme-dependent control of gene expression during waterlogging. A number of identified proteins are involved in regulating programmed cell death (PCD) such as eukaryotic translation initiation factor 5A-1, apyrase 2, apoptosis antagonizing transcription factor. It has been reported that xylem formation requires PCD in tracheary element (Ye, 2002). Overexpression of eIF5A1 showed enhanced xylem formation while it is formed in fewer amounts when the gene is suppressed (Liu *et al*., 2008). Thus, there might be a possible link between our PCD-related protein

expression and cell lysis which is essential for aerenchyma formation during waterlogging stress. From a proteomic comparison, it could be concluded that several factors are involved in improved hypoxic tolerance, all of which are associated with the fermentation pathway. These include better energy status due to higher rates of glycolysis and ethanol fermentation, limitations of lactate accumulation, and better regulation of cytoplasmic pH. In addition, differential accumulation of several programmed cell death-related proteins indicated that regulation of PCD is an interesting aspect of waterlogging tolerance. Thus, protein associated with aerenchyma and adventitious roots development may favor waterlogging tolerance. Oxidative stress also seems lower in tolerant genotype.

The responses observed in leaves are different; the effects observed are mostly secondary effects. Fractionation of leaf proteins with 15% PEG provided high resolution 2DE map (Fig 1). The differentially expressed identified proteins are involved in several processes, i.e. photosynthesis and energy metabolism, protein turn over, antioxidant metabolism, stress defense, signaling and PCD. In addition to the previously known proteins, we also identified several novel proteins including In2-1 protein, senescence-related gene 3 and a ethylene response factor. This study provides a new insight in response to waterlogging stress as well as in the finding of genes of interest for transgenic research in healing premature leaf senescence of legume plants.

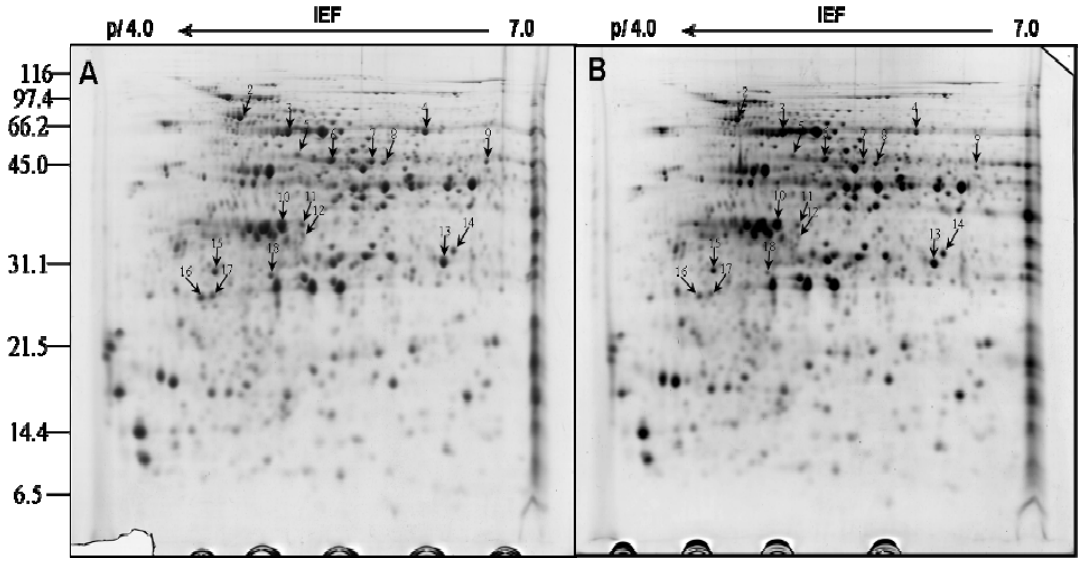


**Fig. 1.** 2-D PAGE analysis of soybean leaf proteins subjected to waterlogging stress. A total of 500 μg of proteins were extracted and separated by 2-D PAGE and visualized with CBB stain. A and B shows the 2-D gel patterns of the PEG-fractionated supernatant samples prepared from the control and the 3days waterlogging treated plants, respectively.

### *Proteomic alteration of soybean seedlings in response to drought stress*

Drought is one of the serious threat to global agriculture affecting food security. However, as a primary sensing organ, the plant root response to drought has not been well-documented at the proteomic level. we have investigated the molecular responses to drought stress in the soybean root proteome and identified the probable drought responsive proteins that contribute to a variety of cellular functions. These proteins include: carbohydrate metabolism proteins such as phosphoglycerate mutase, UDP-glucose pyrophosphorylase; nitrogen metabolism proteins such as glutamine synthetase, SAM synthetase isoforms, defense-related proteins such as HSP70, chitinase, NBS/LRR resistance protein. Cell signaling and PCD-related proteins such as eIF 5A, MADS-domain transcription factor, PPR repeat-containing protein, phloem serpin and a number of secondary metabolism protein such as flavanone 3-hydroxylase, isoflavone reductase isoforms, COMT were also altered. Several members of cellular protection from drought also identified including glycerol kinase, arogenate dehydratase, ferritin isoforms and dehydrin isoforms.

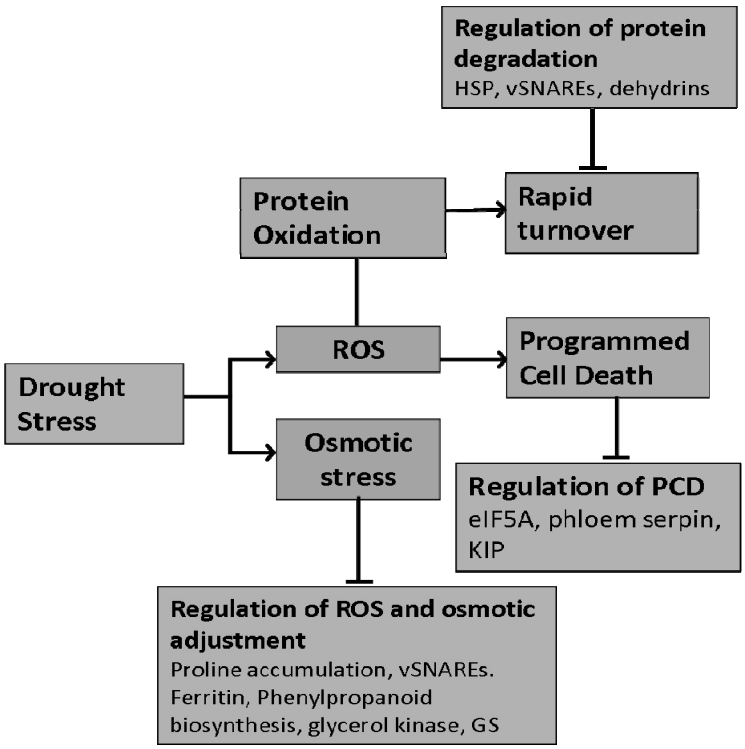


**Fig. 2.** Schematic representation of the potential roles of drought stress-regulated proteins in soybean seedlings as depicted from the proteome profile.

Nevertheless, there are many overlapping mechanisms that participate in the drought response. For example, the accumulation of compatible solutes, such as proline and glycerol, plays an

important role in dehydration adaptation by helping to maintain the relative water content. The accumulation of proline may play a role in dehydration tolerance by protecting protein and membrane structure, regulating redox status, and acting as a scavenger of ROS. Drought signaling components aid transferring signals while the accumulation of compatible solutes protect cellular components. Different kinds of transcription and translation factors are modulated to alter protein synthesis, turnover and PCD. Probable osmotic adjustment was seen as a result of modulation in proteins leading to compatible solute accumulation. Plants undergo cell wall modifications to minimize water loss and avoid dehydration. Proteins related to the cell defense pathways are modulated and may aid the survival of plants under severe dehydration.

Apart from these, drought stressed leaves showed different response. Here, most of the differentially expressed were involved in Celvin cycle including phosphoribulose kinase, 1-deoxy-D-xylulose 5-phosphate reductoisomerase, phosphoglycerate kinase, fructose-bisphosphate aldolase, 2-deoxyglucose-6-phosphate phosphatase and sedoheptulose-1,7-bisphosphatase. These proteins appeared as multiple spots on the 2DE gel. A number of osmotic-stress-related proteins such as dehydrin, ferritin and phosphatidylinositol kinase, myo-inositol-1-phosphate synthase and energy related proteins such as ATP synthase CF1 alpha subunit and ATPase subunit I were also modulated during drought stress. The identification of would provide new insight into the drought response of soybean as well as upland crops. Future works should focus on comparative proteomic approaches and the dynamics associated with the differentially expressed proteins involved in the dehydration response, as well as determining their functions in order to generate effective engineering strategies to improve the outcome of drought stress.

### *Salinity-flooding interaction results in unique proteome profile*

Waterlogging can significantly reduce crop yield or kill plants on soils containing only small amounts of salt (Akhtar *et al*. 1994). Therefore, it is necessary to understand the molecular and physiological mechanism of the combined stress in order to develop appropriate strategies in a changing climate. We studied the global effects of saline flooding stress on multiple metabolic pathways. Our proteomic analysis showed that the saline flooding combined affect the cellular quantity of a complex series of proteins which are both known and unknown for their single effect (salt or flooding alone). Most notably, redistribution of seed storage protrins such as glycinin and β-conglycinin and accumulation of diverge phenylpropanoid components such as isoflavone reductase, dihydroflavonol 4-reductase, halcone synthase, UDP-glycosyltransferases, flavonol 4'-sulfotransferase and CCOMT. Proteins involved in photosynthesis, including the Rubisco large chains, the Rubisco large subunit-binding protein subunit beta and oxygen-evolving enhancer protein, were significantly upregulated by the saline-flooding combined stress, whereas, single

stress alone did not alter their expression levels. As saline-flood inhibit aerenchyma formation, higher level of alcohol dehydrogenase expression was observed compared to flooding stress alone. An important feature of this study was the ability to compare the combined salinity-flooding stress with the two individual stresses to more precisely identify changes at the proteomic level that are either 'specific' or part of a 'common' or 'shared' response. These results could provide new insight into the cellular homeostasis involved in the combination of saline and flooding stresses in glycophyte crop plants.

## Acknowledgment

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2007-211-F0006). I. Alam is supported by a post doctoral grant; K-H Kim and S.A. Sharmin are supported by the scholarship from BK21 program at Gyeongsang National University, Republic of Korea.

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