Functional Analysis of Post-Flooding Recovery in Soybean using Gel-Free Proteomic Technique

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Functional Analysis of Post-Flooding Recovery in Soybean using Gel-Free Proteomic Technique

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Abbreviations

ROS	Reactive oxygen species
APX	Ascorbate peroxidase
GA	Giberrellic acid
ABA	Abscisic acid
MS	Mass spectrometry
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction

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INTRODUCTION

Meeting the food needs of growing population has become more challenging in the twenty-first century due to climate changes around the globe. The effects of climatic changes include extreme weather conditions such as increasing temperature of the earth, severe droughts, and frequent flooding events around the globe (Reichstein et al., 2013). It is expected that the frequency of drought and flooding events is increasing (Mittler and Blumwald, 2010). Extremes in precipitation have limited food and forest production world-wide (Easterling et al., 2007). The change in rainfall and temperature variability has influence on nutritional quality and yield of crops (Porter and Semonov, 2005). The global carbon cycle is influenced by climate changes leading to increase in terrestrial carbon uptake (Reichstein et al., 2013). Climatic changes cause abiotic stresses to decrease agricultural productivity and are an issue of major concern around the globe (Lobell and Gourdji, 2012). Therefore, climate change is a potential threat to plants and efforts are needed to cope with agricultural losses.

Numerous stresses caused by unfavorable environmental conditions are grouped as abiotic stresses which are UV, heavy metals, high/low temperature extremes, freezing, drought, salinity, hypoxia, and flooding (Versulues et al., 2006). Plants are often subjected to abiotic stresses that play a major role in determining productivity of crop yields (Boyer et al., 1982) and also the differential distribution of the plant species across different types of environment (Chaves et al., 2003). Abiotic stress is a key limiting factor that impairs growth and yield of agricultural crops around the world (Hossain et al., 2013). Under abiotic stress, hormones and amino acids are synthesized in root and minerals and nutrients are taken by root and transported to the shoot to enable normal leaf functioning (Ghanem et al., 2011). Changes in temperature, amount of carbon dioxide, and the frequency and intensity of extreme weather have significant impacts on crop yields.

A remarkable feature of plant adaptation to abiotic stresses is the activation of multiple responses involving complex gene interactions and crosstalk with many molecular pathways (Basu et al., 2012; Umezawa et al., 2006). Abiotic stresses elicit

complex cellular responses that have been elucidated by progresses made in exploring and understanding plant abiotic responses at the whole-plant, physiological, biochemical, cellular, and molecular levels (Grover et al., 2001). Stress sensing is a complicated phenomenon and there is not a single sensing mechanism common to all stresses. Stresses like drought and flooding directly affect the underground parts of plant bodies, whereas other stresses affect directly the aboveground structures of plant bodies (Duque et al., 2013). Following sensing, one or more signal transduction cascades are activated, preparing for restitution counter reactions which will lead to the phase of tolerance to stress (Kazuko and Shinozaki, 2006). Functional declines are observed, including the photosynthetic performance (Lawlor, 2002), transport or accumulation of metabolites and uptake and translocation of ions (Cook et al., 2004; Sanchez et al., 2012; Sicher and Barnaby, 2012). Elevated production of active oxygen species in plants under stresses caused increased damage (de Carvalho et al., 2012). These reports suggested that stresses perceived by plants invoke complicated responses.

Severe climatic changes have caused increased flooding events over the past six decades (Bailey-Serres et al., 2012). Flooding frequency is expected to increase in this century in Asia, USA and Africa (Hirabayashi et al., 2013). Flooding due to heavy rainfall in ill-drained areas has been depicted as abiotic stressor on many crops (Jackson and Colmer, 2005). Flooding reduces the active gaseous exchange between the plant tissues and atmosphere (Armstrong and Drew, 2002), and decrease availability of light (Vervuren et al., 2003). Prolonged exposure of plant to flooding stress results in root injuries, which in turn restrict photosynthetic capacity by inducing certain alteration in biochemical reactions of photosynthesis (Ashraf, 2012). Flooding shifts the metabolism to anaerobic condition (Gibbs and Greenway, 2003) and increases the production of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Jackson and Colmer, 2005). Flooding also causes hormonal imbalance leading to stomatal closure (Jackson et al., 1996). Flooding increased the uptake of toxic metals by plant (Michalcova et al., 2011). Flooding-induced damages in crops are major concern for the ever-growing food needs in the modern era.

The physical strength of the root and shoot is weakend by flooding. Flooding-tolerant rice has developed the strategies of low-oxygen escape and low-oxygen

quiescence (Voesenek et al., 2006; Bailey-Serres and Voesenek, 2008). In escape strategy, rate of gas exchange between the plant and environment is increased in plant parts above the water level, leaves bend upward, shoot elongation is enhanced (Fukao et al., 2006). The less energy-consuming quiescence strategy restricts the growth and brings metabolic changes (Fukao et al., 2006). High energy consuming processes such as DNA replication and protein synthesis are either suppressed or selectively carried out under flooding stress (Branco-Price et al., 2008). In flooding-intolerant crops such as soybean, the mechanism of response to flooding is different from rice. Aerenchyma formation takes place in roots, shoots, and secondary tissues, thus enabling plant to sustain oxidative phosphorylation (Shimamura et al., 2010). Adventitious roots formation and leaf gas films formation are also synchronized developments (Sauter, 2013). Plant responses to flooding differ from plant to plant depending on the capacity of plant to deal with the stress as well as the severity and duration of flooding.

Some plants manage to recover following stress removal, although complete recovery may not be possible. Recovery from a stress depends upon many factors such as type of plant, type of stress, and its duration. Upadhyaya et al. (2011) reported that $CaCl_2$ improved post-drought recovery in *Camellia sinensis* by increasing activities of antioxidative enzymes such as superoxide dismutase, catalase, peroxidase, and glutathione reductase. The recovery after chilling treatment in *Phaseolus vulgaris* influenced the accumulation of the proteins implicated in calcium-dependent signal transduction pathways, secondary metabolism, and those promoting cell division and expansion (Badowiec and Weidner, 2014). Seeds of *Vitis californica* were studied for polyethyleneglycol-induced osmotic stress and recovery (Weidner et al., 2011), resulting that total concentration of catechin increased under stress conditions and declined during post-stress recovery. The seeds which underwent post-stress recovery, the total level of phenolic compounds was increased than in the stress conditions. Analyzing post-stress recovery responses in various plants may provide valuable knowledge in the improvement of tolerance of plant species to stresses.

Flooding is one of the lethal stresses for the plants when felt for long durations of the time; however, some plants are still able to recover from a specifc period of flooding/submergence stress. In two flooding-tolerant riparian species *Alternanthera*

philoxeroides and *Hemarthria altissima,* growth and photosynthesis recovered after the end of the submergence treatment, with recovery of photosynthesis preceding growth recovery (Luo et al., 2009). The effective quantum yield of photosystem II and non-photochemical quenching was diminished during submergence and rapidly increased upon de-submergence. In perennial ryegrass, carbohydrate production, plant height, and growth rate were recovered following desubmergence (Yu et al., 2012). Investigations about nitrogen fixation activity in soybeans following flash flooding concluded that nitrogen fixation ability was rapidly recovered without any lasting damage (Justino et al., 2013). These studies suggested that some plant species were able to recover from short-term flooding after removal of the stress. The ability for photosynthetic acclimation may be essential for adaptation to wetland habitats in which water levels fluctuate. These reports suggested that post-flooding recovery studies can provide information about the changes at physiological level which can be utilized as basis for further analysis.

Soybean is a protein and oil rich legume crop grown in many parts of the world (Panizzi and Mandarino, 1994). The whole-genome of soybean cultivar Williams 82 consists of 950 Mbp DNA (Arumuganathan et al., 1991), and that of cultivar Enrei consists of about 947 Mbp DNA (Shimomura et al., 2015). The sequenced genome information of soybean has added to the efforts of analyzing the interactions of plant with environment. Top producers of soybean are USA, Argentina, Brazil, and China (Figure 1). The production by Argentina and China has increased over the time in twenty-first century. Soybean is adapted to grow in a wide range of climatic conditions; however, growth and yield of soybean are greatly affected by several abiotic stressors such as flooding, drought, salinity, and heavy metals (Hossain et al., 2013). In addition to morphological and physiological responses of soybean, several molecular studies elucidating stress-induced changes in gene expressions and metabolites have been documented (Komatsu et al., 2012a; Mohammadi et al., 2012; Sobhanian et al., 2010; Hossain et al., 2012).

Soybean is sensitive to flooding stress which reduces its growth and yield (Githiri et al., 2006). Flooding initially damges root growth than subsequently shoot (Sauter, 2013). Flooding caused a decrease in nutrient uptake (Sallam and Scott, 1987), and

reduced nitrogen fixation capacity of soybean (Sung, 1993). Total number of root, the length of the main, lateral, and adventitious roots, and the fresh weight of the root were significantly suppressed in soybean seedling under flooding stress (Shi et al., 2008). Aerenchyma development was primarily evident in new adventitious roots, whereas, primary roots of flooded plants exhibited tightly packed cortical cells (Shimamura et al., 2010). Flooding reduced the hypocotyl pigmentation, decreased the fresh weight and lengths of the root and hypocotyl in soybean (Hashiguchi et al., 2009). van Toai et al. (2012) examined the effect of flooding on seed composition of soybean and found that the linoleic/linolenic acids, daidzein, genistein, and glycitein contents were significantly decreased under flooding stress. Flooding stress substantially resulted in reduction of biomass, tap-root length, and pod number, inhibition of carbon/nitrogen content in root/nodule, decrease of nodule dry weight and number, and reduction of grain yield in soybean (Miao et al., 2012). These reports suggest that flooding is one of the major constraints on growth of soybean.

Proteomic techniques have been applied to study physiological and molecular responses of soybean to flooding stress (Komatsu et al., 2015). These studies are summarized in Table 1. The proteomic studies on flooded soybean seedlings in early stage revealed increase in protein abundance of glycolysis, fermentation, and cell wall-related proteins; whereas, ROS scavenging enzymes, proteins related to cell organization and amino acid metabolism were decreased (Hashiguchi et al., 2009; Nanjo et al., 2012; Nanjo et al., 2010). The role of various phytohormones has been investigated in soybean under flooding stress. Protein phosphorylation is linked with flooding response mechanisms in root tips of soybean via ethylene signaling pathway (Yin et al., 2014). Flooding stress decreased the calcium oxalate crystals in the soybean cotyledon indicating that calcium ion level was raised under flooding stress (Komatsu et al., 2013a). Abscisic acid (ABA) enhanced flooding tolerance of soybean through regulation of energy conservation via glycolytic system (Komatsu et al., 2013b). Gibberellic acid (GA) treatment of flooding-stressed soybean restored the abundance of secondary metabolism, cell, protein synthesis/degradation related proteins (Oh et al., 2014a). Jasmonic acid and salicylic acid play important roles in regulation of developmental processes and signaling networks in plants under abiotic stresses (Yoon

et al., 2009; Gemes et al., 2011). However, their role in flooding response mechanism in soybean yet needs to be investigated. Proteomic studies on flooding response mechanism and role of phytohormones in soybean can provide valuable knowledge that may be used as basis for developing flooding-tolerant soybean varieties.

The post-flooding recovery has been relatively less studied in plants. Elucidating the mechanisms involved in post-flooding recovery may provide valuable insight. In soybean, only one proteomic study (Salavati et al., 2012) reported that the cell structure was modified through alteration of cell wall metabolism and reorganization of the cytoskeleton in soybean during recovery from flooding. This study used gel-based proteomic technique and could not indicate the abundance changes in low abundance proteins. The proteomic analysis of plants during post-flooding recovery can identify proteins that undergo changes in abundance during the transition from stress to post-stress periods (Salavati et al., 2012). Elucidating the mechanisms involved in post-flooding recovery can better guide towards the goal of getting flooding-tolerant soybean. In this study, to unravel the post-flooding recovery mechanism in hypocotyl and root of soybean, gel-free proteomics was utilized. Additionally, the role of jasmonate and salicylate in post-flooding recovery with organ-specifc and time-dependent manner was analyzed.

Table 1. A summary of proteomic analyses of soybean under flooding stress.

Stress duration [a](additional stress)	Functional category of proteins	Protein abundance	Reference
3 h	cell wall, protein metabolism	Decreased	Yin et al., 2014
3 h	transport, RNA regulation	Increased	Yin and Komatsu, 2015
12 h	glycolysis, fermentation	Increased	Nanjo et al., 2010
1 day	glycolysis, fermentation, cell wall	Increased	Nanjo et al., 2012
	redox, cell structure, amino acid metabolism	Decreased	
1 to 3 days (Al nanoparticles)	glycolysis, fermentation, tricarboxylic acid, amino acid metabolism, nucleotide metabolism	Increased	Mustafa et al., 2015b
1 to 4 days	metal handling	Decreased	Kamal et al., 2015
	stress, protein synthesis/degradation	Increased	
2 days	hormone metabolism, stress, redox	Decreased	Komatsu et al., 2010a
	tricarboxylic acid, electron transport chain	Increased	Komatsu et al., 2011
	redox, protein folding/degradation	Increased	Komatsu et al., 2009b
	glycolysis, redox, defense	Increased	Hashiguchi et al., 2009
	secondary metabolism	Increased	Komatsu et al., 2012b
	stress, hormone metabolism, DNA repair	Decreased	
	Protein folding/translocation	Increased	Komatsu et al., 2013a
	Stress	Decreased	Mustafa and Komatsu, 2014
(GA)	secondary metabolism, cell cycle, protein degradation/synthesis	Decreased	Oh et al., 2014a
(Calcium)	cell wall, hormone metabolism, protein metabolism, DNA synthesis	Decreased	Oh et al., 2014b
2 and 4 days	cell wall, tricarboxylic acid, secondary metabolism	Increased	Komatsu et al., 2013b
(ABA)	Stress	Decreased	
(Ag nanoparticles)	fermentation	Increased	Mustafa et al., 2015a
	defense	Decreased	
	energy	Increased	Kamal and Komatsu, 2015
3 days	cell wall	Decreased	Nanjo et al., 2013

[a]additional stress, applied in addition to flooding

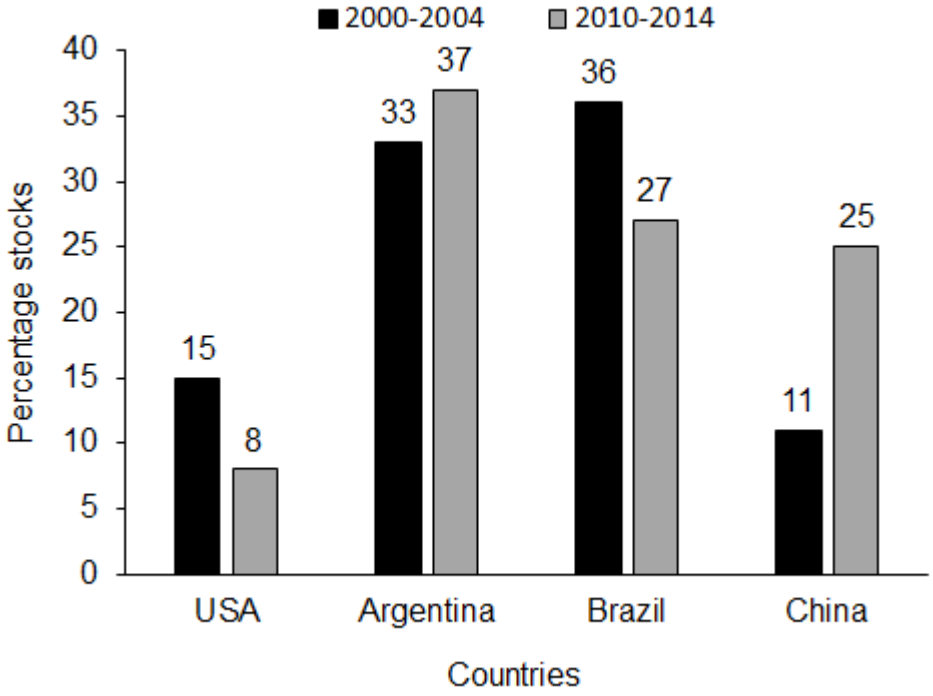


Figure 1. World top producers of soybean from year 2000 to 2004 vs 2010 to 2014. The percentage shows the stocks held by these countries. The figure is prepared using data from United States Department of Agriculture Office of the Chief Economist. "World Agricultural Supply and Demand Estimates. http://www.usda.gov/oce/index.htm.

CHAPTER 1
MORPHOLOGICAL ANALYSIS OF SOYBEAN DURING POST-FLOODING RECOVERY

1.1. Introduction

Soybean is flooding intolerant crop and exhibits drastically reduced growth and yield under flooding conditions (Githiri et al., 2006). The responses to flooding vary in flooding tolerant and intolerant crop species. Rice is considered as less susceptible to flooding/submergence stress but mechanism of response to submergence varies in different rice cultivars (Fukao et al., 2011). Flash flooding can cover the entire plant for prolonged periods, and most rice cultivars die within 7 days of complete submergence (BaileySerres et al., 2010). Deepwater rice responds to submergence by promoting internode elongation to outgrow floodwaters. This escape response is regulated by a polygenic locus that encodes two DNA binding proteins, SNORKEL1 and SNORKEL2 (Hattori et al., 2009). By contrast, submergence-tolerant lowland rice restrains elongation growth, economizing carbohydrate reserves to enable development of new leaves upon desubmergence. This quiescence response is regulated by *SUB1A* gene (Xu et al., 2006). The highly submergence-inducible *SUB1A* gene that confers this tolerance is absent in all japonica and most indica accessions. Flooding response mechanism is diverse in plants that involves morphological as well as molecular factors.

Flooding affects the morphology and physiology of the flooding-intolerant soybean (Githiri et al., 2006). Flooding affects both root and shoot of soybean, although roots are primarily affected in the initial stages of flooding stress (Sauter, 2013). Morphological changes in soybean under flooding stress include rerstriction of root and hypocotyl elongation, decrease in root and hypocotyl weight, discoloration of hypocotyl and cotyledons, and fragility of the plant organs. Studies on the morphology changes in response to flooding stress provided a direction for molecular level analysis. Morphological changes of soybean under flooding stress have been documented in previous studies (Khatoon et al., 2012a; Nanjo et al., 2013; Yin et al., 2014; Oh et al., 2015). The results depicted suppression of growth under flooding stress. Root and hypocotyl lengths were restrained and weights could not increase under flooding stress.

Morphological changes in soybean during recovery after flooding are less studied. Soybean appears to attempt to reduce flooding injury through extensive adventitious root development, which is reported to enhance oxygen transport from the stem to the roots (Shimamura et al., 2003; Lee et al., 2003). Aerenchyma development

primarily occurs in new adventitious roots, whereas the primary roots of flooded plants exhibited tightly packed cortical cells (Bacanamwo and Purcell, 1999; Shimamura et al., 2010). Slavati et al. (2012) reported morphological and proteomic changes in soybean during post-flooding recovery stage. The study revealed that root and hypocotyl elongation and development of first leaf was significantly delayed compared to control seedlings. In order to recover, seedlings try to repair the damge caused to their morphology by flooding. In this experiment, to reveal the morphological responses during recovery after flooding, root and hypocotyl were analyzed.

1.2. Material and Methods

1.2.1 Materials and treatments

Soybean (*Glycine max* L. cv Enrei) seeds were sterilized with 2% sodium hypochlorite solution and then thoroughly rinsed in water. The sterilized seeds were sown 4 cm inside 450 mL of quartz sand in seedling cases (180 X 140 X 45 mm^3) wetted with 150 mL water and grown at 25°C in a growth chamber (Sanyo, Tokyo, Japan) under white fluorescent light (160 μmol m^{-2} s^{-1}, 16 h light period/day). Ten seeds were grown in each seedling case for each treatment, and the average grown were used for analysis. Two-day-old soybeans were flooded for 2, 4, and 6 days and morphological changes such as lengths and weights of root and hypocotyl were measured at 2, 4, and 6 days of flooding and then every second day during the 6-day recovery period after water removal (Figure 2). Three independent biological replicates were performed for each experiment**.**

1.2.2 Statistical analysis

The mean values of length and weight measurements of root and hypocotyl of soybean from 3 independent replications were analyzed for statistical significance using One-way ANOVA and Duncan's Multiple Range test.

1.3. Results

Two-day-old soybeans were flooded for 2, 4, and 6 days and morphological changes were periodically measured after the removal of water. Recovery from flooding

was correlated with flooding duration and was highest in soybeans flooded for 2 days, whereas soybeans flooded for 6 days failed to recover. The appearance of plants clearly depicts the differences under flooding and during recovery stage (Figure 3). Hypocotyl length of 2-day-flooded soybeans was 10% and 68% greater than 4 and 6 days flooded soybeans, respectively (Figure 4A). Hypocotyl weight of 2-day-flooded soybeans was 20% and 58% greater than 4- and 6-day-flooded soybeans, respectively (Figure 4A). Root length, when compared at the end of recovery period, was 12% longer in 2-day flooded soybeans than that of 4-day-flooded soybeans and 48% longer than that of 6-days flooded soybeans (Figure 4B). The mean of root length of 4-day-flooded soybeans was 42% greater than 6-day-flooded soybeans at the end of the recovery period (Figure 4B). Consistent with the findings of root length, the root weight of 2-day-flooded soybeans was 13% and 58% greater than 4- and 6-day-flooded soybeans, respectively (Figure 4B).

1.4. Discussion

Flooding stress causes injury in soybean due to excessive intake of water that causes physical disruption of tissues (Komatsu et al., 2012a). Morphological analysis provide a base for indicating changes in response to abiotic stresses. Results of the morphological analysis can be used for design of further experiment planning. Morphological analyses of soybean under flooding stress have been documented. Khatoon et al. (2012a) carried out organ-specific morphological analysis and revealed that flooding suppressed the growth in root, hypocotyl, and leaf. The length and weights were decreased under flooding stress. A suppression in growth was reported in many studies on soybean under flooding stress (Khatoon et al., 2012a, 2012b; Nanjo et al., 2013; Yin et al., 2014; Oh et al., 2015). Morphological analysis in other crops also depict growth suppression under flooding/waterlogging stress. In wheat, growth was reduced as root length and dry mass were decreased (Haque et al., 2011). Water-logging in tomato leads to reduced photosynthesis due to stem closure, decreased chlorophyll content, increased hydrogen peroxide levels, leaf chlorosis and senescence, reduced stem elongation, and adventitious root formation (Jackson et al., 1996; Ahsan et al., 2007). All these reports indicated that flooding causes growth suppression in soybean

and other plants.

Recovery after flooding stress in soybean is scarcely studied area with only very few studies appeared so far. Salavati et al. (2012) studied post-flooding recovery in soybean, and reported that growth was suppressed with increasing stress duration; however, plants were able to recover. In the current morphological analysis, it was noted that flooding stress suppressed the growth of soybean and caused injury. As the flooding duration was increased, injury level was also raised in the stressed seedlings. However, when the stress was removed, plants tried to recover following removal of 2 and 4 days of flooding stress. Soybeen seedlings flooded for 6 days were not able to recocver as this duration proved lethal in young seedlings. The status of recovery was evident from the phenotypes (Figure 3). The seedlings which lost their hypocotyl and cotyledon pigmentation under flooding stress, were getting back their normal pigmentation during the post-flooding recovery stage.

Morphological measurements revealed that flooding suppressed the growth of soybean plants at all treatment durations (Figure 3).The evident differences were noted in morphological parameters in seedlings under flooding stress and during the post-flooding recoverty stage. The changes observed shown a clear tendency towards recovery following removal of 2 and 4 days of flooding stress. The hypocotyl pigmentation was increased during the recovery in a direct proportion with stress duration. Hashiguchi et al. (2009) also reported decrease in hypocotyl pigmentation in soybean hypocotyl under flooding stress. The morphological measurements of hypocotyl length and weight were increased during the recovery following removal of 2 and 4 days of flooding stress (Figure 4). However, 6 days flooding proved lethal for the plants and they could not recover. The length of main root and its weight increased markedly after removal of flooding stress, however, the rate of length and weight increase was slowed as stress duration increased from 2 to 4 days (Figure 5). These results suggest that flooding causesd growth suppression in soybean at all treatment durations, but flooding of more than 4 days at seedling stage is lethal and soybean could not recover.

1.5. Conclusion

To unravel the changes in morphology of soybean during post-flooding recovery stage, morphological analysis was carried out. Based on this result, experimental design for chapters 2 and 3 was constructed. Root has primary importance in plant life as it manages the drawl of minerals and water from the soil for the growth and development of the plant (Russel, 1977). Under flooding stress, morphology of root is severely affected. In this experiment, it was demonstrated that root can both effectively recover from 2 and 4 days flooding and efficiently resumes the growth. However, longer flooding durations at seedling stage also prove lethal and root becomes much weak and could not recover following flooding removal. Hypocotyl is an important organ in the secondary growth of the plant as well as it manages the transportation of nutrients, minerals and water through phloem and xylem tissues (Reid and Howell, 1995). In soybean, hypocotyl pigmentation was reduced and morphology was affected by flooding, but recovered during the period of recovery following 2 and 4 days flooding. However, longer duration of flooding proved lethal for hypocotyl as its tensile strength was weakened. Results of this morphological analysis incite the need to search the post-flooding recovery mechanism used by hypocotyl and root. For this purpose, both hypocotyl and root were analyzed using gel-free proteomic technique.

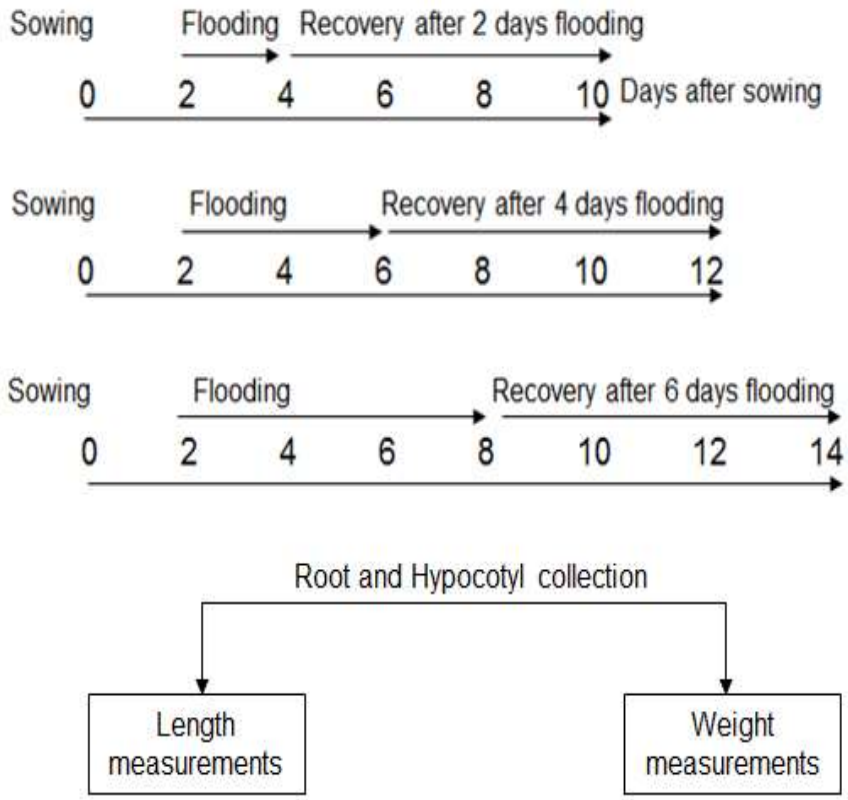


Figure 2. Experimental design used in this study. Two-day-old soybeans were flooded for 2, 4, and 6 days and then allowed to recover after water removal. Hypocotyl and root lengths and weights were measured under flooding and after flooding stages. Three independent biological replicates were performed for each treatment.

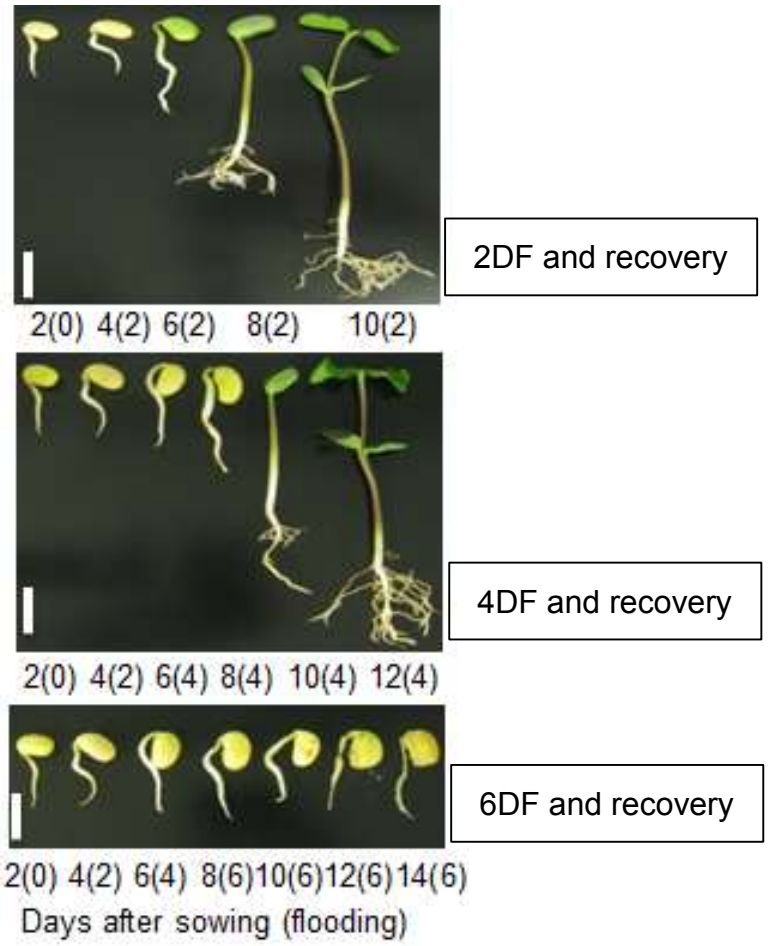


Figure 3. Phenotypic diffrences depicted in soybean under flooding and during recovery after flooding. Two-day-old soybeans were exposed to flooding for 2 (2DF), 4 (4DF), and 6 days (6DF), and allowed to recover after water removal. Photographs show phenotypic differences among treatments and during recovery stage. Bar indicates 10 mm.

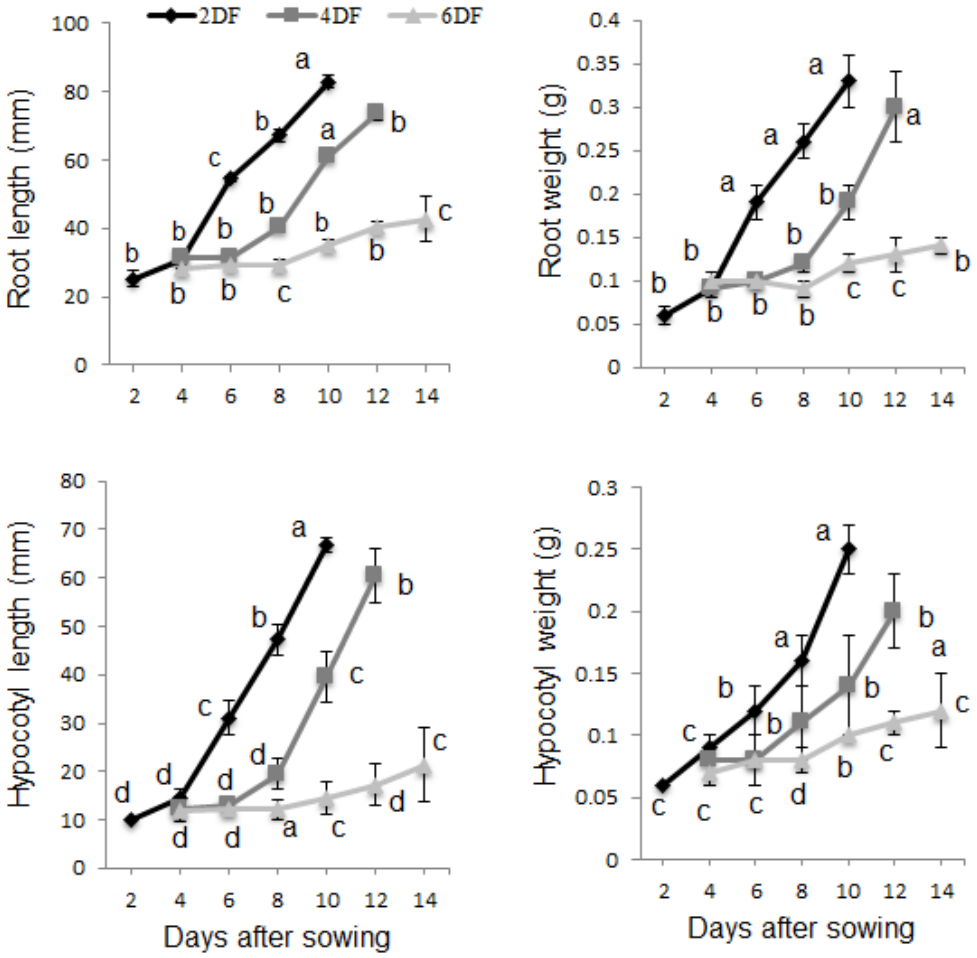


Figure 4. Morphological measurements of soybean hypocotyl (A) and root (B) under flooding and during recovery after flooding stress. Two-day-old soybeans were exposed to flooding for 2, 4, and 6 days, and morphological changes were measured at 2, 4, and 6 days of flooding and then every second day during the 6-day recovery period. The lengths and weights of hypocotyl and root were measured on the indicated days after sowing. Black, grey, and light grey lines indicate 2-day flooded (2DF), 4-day flooded (4DF), and 6-day flooded (6DF) soybeans during recovery stage, respectively. Plotted values in graphs are mean ± SE from three independent biological replications (n=3). The data was subjected to One-way ANOVA and Duncan's Multiple Range test. The different letters indicate significant difference.

CHAPTER 2
QUANTITATIVE PROTEOMICS OF HYPOCOTYL IN SOYBEAN DURING POST-FLOODING RECOVERY

2.1. Introduction

The hypocotyl plays an important role in both the primary and secondary growth of plants. The *Arabidopsis* hypocotyl, which is widely used as a model for studying secondary growth in plants (Ragni et al., 2014), grows exclusively by cell expansion and does not undergo cell division (Derbyshire et al., 2007). Hypocotyl cell elongation in *Arabidopsis* is controlled by a central growth-regulation circuit that integrates hormonal, environmental, and developmental signals (Oh et al., 2014). Phytohormones such as auxins, gibberellins, and brassinosteroids promote hypocotyl growth (Clouse et al., 1996), whereas cytokinins, ethylene, and ABA inhibit its growth (Reid et al., 1995). In addition to its importance to plant growth and development, the hypocotyl is involved in plant tolerance to abiotic stresses, including salinity, drought, and flooding. In soybean, which is an important economic crop worldwide, calcineurin B-like protein enhances hypocotyl elongation during salt and drought stresses (Li et al., 2012), and polyamine catabolism involving copper-containing amine oxidase promotes hypocotyl growth under salt stress (Campestre et al., 2011). The hypocotyl responses to flooding have not yet been explored.

Flooding is a major constraint on the growth and productivity of crops (Jackson et al., 2005). Plant responses to flooding vary depending on the species and extent of damage. At the metabolic level, most flooding-stressed plants change from aerobic respiration to glycolysis and fermentation for energy production (Bailey-Serres et al., 2012). The hypocotyl surface of soybean at late growth stage contains hypertrophic lenticels that enable the entry of atmospheric oxygen into the secondary aerenchyma under flooding stress (Shimamura et al., 2010). The metabolic and morphological changes that occur in flooding-stressed soybean are likely an attempt to cope with the anaerobic conditions caused by flooding. The changes in hypocotyl in response to flooding at early growth stages needs to be unveiled.

Proteomic investigations of flooding stress-responsive mechanisms in soybean root have demonstrated that numerous proteins and boilogical processes are involved. Undeer flooding, soybean seedlings show differential regulation of proteins involved in hormonal signaling, transcriptional control, glucose degradation/ sucrose accumulation, alcohol fermentation, gamma amino butyric acid shuns, mitochondrial impairment, cell

wall loosening, and suppression of reactive oxygen scavenging (Komatsu et al., 2015). Nanjo et al. (2013) identified several cell wall-related proteins that serve as indicators for assessing the severity of flooding stress in soybean root including hypocotyl. In addition, proteomic profiling for determining flooding tolerance of different soybean varieties revealed that the levels of RNA binding/processing-related proteins were positively correlated in untreated soybeans, whereas flooding stress indicator proteins were negatively correlated in flooded soybeans (Nanjo et al., 2014). Proteomic studies analyzed root including hypocotyl in soybean under flooding stress (Hashiguchi et al., 2009; Komatsu et al., 2010b; Nanjo et al., 2010; Komatsu et al., 2011); however, studies on soybean hypocotyl separately are lacking.

Plant responses to flooding are being widely researched; however, considerably less attention has been paid towards understanding the mechanisms linked to post-flooding recovery. Salavati et al. (2012) analyzed the protein profiles in soybean roots during recovery after flooding and found that cell wall metabolism and cytoskeleton reorganization were predominantly affected. Despite these studies in roots, the changes that occur in soybean hypocotyls during post-flooding recovery have not been examined in detail. Proteomics of soybean hypocotyl during recovery after flooding provides useful knowledge about proteomic changes. In chapter 1 results, it was demonstrated that flooding affected the length, weight, and pigmentation in hypocotyl, which was recovered after the removal of stress. These results invoke the need of proteomic analysis to reveal changes at protein level. To determine the mechanisms involved in post-flooding recovery in soybeans, the temporal profiles of hypocotyl proteins were analyzed using gel-free proteomic technique.

2.2. Materials and methods

2.2.1. Plant material and treatments

For growth conditions, please see chapter 1, section 1.2.1. For proteomic analysis, 2-day-old soybeans were flooded for 2 days and hypocotyl samples were collected at the day of removal of flooding and then at 2 more points during the 4-day-recovery period. For mRNA expression and enzyme activity assays, the hypocotyl of soybean recovering after 2- and 4-day-flooding was analyzed. Three independent

biological replicates were performed for each experiment.

2.2.2. Protein extraction

A portion (0.5 g) of collected hypocotyl was ground to powder in liquid nitrogen using a mortar and pestle. The powder was transferred to an acetone solution containing 10% trichloroacetic acid and 0.07% 2-mercaptoethanol, and the resulting mixture was vortexed and then sonicated for 10 min. The suspension was incubated for 1 h at -20°C with vortexing every 15 min and then centrifuged at 9,000 × g at 4°C for 20 min. The supernatant was discarded and pellet was washed twice with 0.07% 2-mercaptoethanol in acetone. The pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hickville, NY, USA) and then resuspended in lysis buffer, consisting of 7 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine, by vortexing for 1 h at 25°C. The suspension was centrifuged at 20,000 × g for 20 min at 25°C and the supernatant was collected as protein extract. Protein concentrations were determined using the Bradford assay (Bradford et al., 1976) with bovine serum albumin as the standard.

2.2.3. Protein purification and digestion for mass spectrometry analysis

Protein extracts (100 μg) were purified with methanol and chloroform to remove any detergent from the sample solutions (Nanjo et al., 2012). Briefly, 400 μL methanol was added to 100 μL sample. After mixing, 100 μL chloroform and 300 μL water were added to the samples, which were mixed and centrifuged at 20,000 × g for 10 min to achieve phase separation. The upper aqueous phase was discarded, and 300 μL methanol was added slowly to lower phase. The samples were centrifuged at 20,000 × g for 10 min, the supernatants were discarded, and the pellets obtained were dried. The dried samples were reduced with 0.24 M dithiothreitol for 30 min at 56°C, followed by alkylation with 0.28 M iodoacetamide for 30 min at 37°C in the dark. Alkylated proteins were digested with trypsin and lysyl endopeptidase at 1:100 enzyme/protein concentrations at 37°C for 16 h. The resulting tryptic peptides were acidified with formic acid, desalted with a C-18 pipet tip (SPE C-TIP, Nikkyo Technos, Tokyo, Japan), and then analyzed by nano-liquid chromatography (LC) mass spectrometry (MS).

2.2.4. Nanoliquid chromatography-tandem mass spectrometry analysis

A nanospray LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated in data-dependent acquisition mode with the installed XCalibur software (version 2.0.7, Thermo Fisher Scientific). Peptides in 0.1% formic acid (2 μL out of 10 μL) were loaded onto a C18 PepMap trap column (300 μm ID × 5mm; Dionex, Germering, Germany) of an Ultimate 3000 NanoLC system. The peptides were eluted from the trap column with a linear acetonitrile gradient (8%-30% over 120 min) in 0.1% formic acid at a flow rate of 200 nL/min. The peptides eluted from the trap column were separated and sprayed on a C18 capillary tip column (75 μm ID × 120 mm, Nikkyo Technos) at a spray voltage of 1.5 kV. Full-scan mass spectra were acquired in the LTQ Orbitrap mass spectrometer over 400-1500 m/z with a resolution of 30,000. A lock mass function was used for high mass accuracy (Olsen et al., 2005). The six most intense precursor ions were selected for collision-induced fragmentation in the linear ion trap at a normalized collision energy of 35%. Dynamic exclusion was employed within 90 sec to prevent the repetitive selection of peptides (Zhang et al., 2009).

2.2.5. Protein identification by Mascot search

Proteins were identified from a soybean peptide database (73,320 sequences) constructed from the soybean genome database (Phytozome version 9.1, http://www.phytozome.net/soybean) (Schmutz et al., 2010) using the Mascot search engine (version 2.4.0.2, Matrix Science, London, UK). The data files were processed using Proteome Discoverer software (version 2.3.2, Thermo Fisher Scientific). The parameters used in Mascot searches were as follows: carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Peptide mass tolerance was set at 10 ppm, fragment mass tolerance was set at 0.8 Da, and peptide charge was set at +2, +3, and +4. An automatic decoy database search was performed as part of the search. Mascot results were filtered with the Mascot Percolator package to improve the accuracy and sensitivity of peptide identification. False discovery rates for peptide identification of all searches were less

than 1.0%. The Mascot results were exported for SIEVE software analysis (version 2.1; Thermo Fisher Scientific).

2.2.6. Differential analysis of acquired mass spectrometry data

A commercial label-free quantification software package SIEVE was used for comparing the relative abundances of peptides and proteins between control and experimental groups. The chromatographic peaks detected by MS were aligned and the peptide peaks were detected as frames using the following settings: frame time width, 5 min; and frame m/z width, 10. Frames were generated for all parent ions scanned by MS/MS and were matched to exported Mascot results to identify peptides. In the differential analyses of protein profiles, total ion current was used as a normalization factor. For all differential analyses, data from three biological replicates were analyzed and only proteins with at least two peptide matches across the data from all sample groups and replicates were defined as identified proteins.

The mass spectrometry proteomics data have been deposited with the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaino et al., 2013) with the data set identifier PXD001567.

2.2.7. Cluster and *in silico* protein-protein interaction analyses

Protein ratio data from the differential analysis performed with SIEVE software was subjected to cluster analysis using Genesis software (version. 1.7.6; http://genome.tugraz.at) (Sturn et al., 2002). Cluster analysis was performed using hierarchical clustering with a Euclidean distance metric and a centroid linkage clustering method. Clustered proteins were analyzed for *in silico* protein-protein interactions that were estimated by temporal expression profiling utilizing *S*-system differential equation (Voit, 2000) as a mathematical model. In this analysis, we interpolated expression levels at day 4 by fitting a quadratic function to logarithmic values of expression levels at days 4, 6, and 8. The interpolated expression level was used to estimate the interactions with the expression levels at day 4, 6 and 8. Each interaction between proteins was tested based on a goodness-of-fit which indicates how

well the *S*-system differential equation simulates expression of the corresponding target protein. The interactions showing an r^2 value (coefficient of determination) > 0.9 were considered as candidate interactions. In the model protein interaction diagram, a red arrow indicates an inductive interaction and corresponds to $gij > 0$ in the S-system differential equation, and a blue T-bar indicates a suppressive interaction and corresponds to $gij < 0$ in the *S*-system differential equation (Tanaka et al., 2005).

2.2.8. Functional categorization

The identified proteins were classified on the basis of functional category through MapMan bin codes using MapMan software (http://mapman.gabipd.org/) (Usadel et al., 2005).

2.2.9. RNA extraction and quantitative reverse transcription-polymerase chain reaction

A portion (100 mg) of hypocotyl was ground to powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) with DNase treatment. Total RNA (800 ng) from each sample was reverse-transcribed to cDNA in a 20 μL reaction volume using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using an amount of cDNA product corresponding to 20 ng total RNA in a 20 μL reaction volume using SsoAdvanced SYBR Green Supermix (Bio-Rad) on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). The PCR conditions were as follows: 95°C for 30 sec, followed by 45 cycles of 95°C for 10 sec and 60°C for 30 sec. The quantity of each transcript was calculated using the delta-Ct method. To normalize gene expression, 18S rRNA (X02623) was used as an internal control. All primers used in the qRT-PCR analysis (Supplemental Table 1) were designed using the Primer3Plus web interface (http://primer3plus.com) (Untergasser et al., 2007). In the qRT-PCR experiments, three biological replicates with two experimental replicates were analyzed.

2.2.10. Analyses of enzyme activities

Pyruvate kinase: A portion (125 mg) of hypocotyl was homogenized with 1 mL

extraction buffer consisted of 50 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 2% polyvinylpyrrolidone, 0.1% triton X-100, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 15,000 × g at 4°C for 20 min, and the supernatant obtained was again centrifuged at the same settings. The resulting filtrate was used as enzyme extract. A reaction mixture for determining pyruvate kinase activity consisted of 50 mM HEPES-KOH (pH 6.9), 25 mM KCl, 10 mM $MgCl_2$, 2 mM PEP, 1 mM ADP, 1 mM DTT, 5% polyethylene glycol 8,000, 0.15 mM NADH, and 4 U/mL lactate dehydrogenase. The reaction was started by the addition of ADP and PEP, and was incubated for 5 min at room temperature. The absorbance of the reaction solution was then measured at 340 nm (Tang et al., 2003; Turner et al., 2005).

Nucleotidyl transferase: A portion (125 mg) of hypocotyl was homogenized at 4°C in 1 mL of 100 mM buffer, consisting of potassium phosphate (pH 7.4), 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM phenylmethyl sulfonylflouride. The homogenate was incubated at 56°C for 10 min and then transferred to 4°C for 5 min. The suspension was centrifuged at 13,000 × g at 4°C for 20 min, and 1 M cold acetic acid was added dropwise to the supernatant until the pH reached 5.0 and further centrifuged at 20,000 × g for 20 min. The supernatant obtained was used as enzyme extract. For the measurement of enzyme activity, a reaction mixture consisted of 100 mM HEPES (pH 7.4), 40 mM $MgCl_2$, 12.5 mM ATP, 50 mM nicotinamide mononucleotide, 0.50 mg/mL ADH, and 390 µL ethanol reagent in a 1.5 mL tube was incubated at 37°C. The reaction was initiated by the addition of nicotinamide mononucleotide, and absorbance was recorded at 340 nm (Balducci et al., 1995).

Beta-ketoacyl reductase: A portion (125 mg) of hypocotyl was homogenized at 4°C in 1 mL of 50 mM phosphate buffer (pH 7.0). The resulting homogenate was sonicated for 10 min and then centrifuged at 15,000 × g at 4°C for 20 min. The supernatant was collected and saturated with 45% ammonium sulphate. The resulting precipitate was pelleted by centrifugation at 15,000 × g for 20 min and was then discarded. The supernatant was collected, adjusted to pH 7.0 with 1 N potassium hydroxide, and was used as enzyme extract. The reaction mixture for the assay contained 0.1 M sodium phosphate (pH 7.0), 1 mM 2-mercaptoethanol, 239 µM

NADPH, 58.7 μM acetoacetyl-CoA, and 40 μL enzyme extract. The reaction was started by the addition of enzyme extract. The amount of NADPH oxidation prior to the addition of acetoacetyl-CoA was used as blank. The enzyme activity was measured by monitoring the absorbance at 340 nm (Toomey et al., 1966; Lai et al., 2004).

2.2.11. Statistical analysis

Significant changes in protein abundance were analyzed by two-way ANOVA using Prism 6 software (GraphPad Software, La Jolla, CA, USA). mRNA expression and enzyme activity changes were analyzed for significance using the Student's *t*-test by comparing age-matched control and treated seedlings. A value of $p < 0.05$ was considered statistically significant.

2.3. Results

2.3.1. Changes in protein abundance in soybean hypocotyl during post-flooding recovery

To identify differentially changed proteins in soybean hypocotyl during post-flooding recovery, a gel-free proteomic technique was used to analyze the protein profiles of soybeans that had been grown for 2 days, flooded for 2 days and recovered for 2 and 4 days following stress removal (Figure 5). The hypocotyls collected from untreated soybeans at the same time points were used as controls. To analyze the changes occurring during recovery from flooding, hypocotyl proteins of control and 2-day flooding-stressed soybeans were compared with proteins from 2-day-old plants. A total of 4,471 and 4,640 proteins with matched peptides 2 or more were identified in control and flooding-stressed soybean hypocotyls, respectively. Out of the total identified proteins, 498 proteins with 2 or more matched peptides and $p < 0.05$ were significantly changed at all time points in control hypocotyls and 70 proteins with more than 2 matched peptides and $p < 0.05$ were significantly changed at all time points in the flooding-stressed soybean hypocotyls. Between the control and post-flooding recovering hypocotyls, a total of 20 proteins that significantly changed in abundance were commonly identified (Table 2).

Proteins identified in the control and post-flooding recovering hypocotyls were

grouped by clustering analysis based on their abundance ratios. The 20 common proteins identified in each condition were grouped into 3 clusters (I, II, and III). In control hypocotyls (Figure 6A), Cluster I proteins increased during the growth period from 2 to 8 days after sowing and belonged to photosystem (1 protein) and unassigned (2 proteins) categories. Cluster II proteins did not markedly change during growth and included 2 proteins related to secondary metabolism. Cluster III proteins decreased during the growth period and included proteins categorized as development (4 proteins), protein (6 proteins), not assigned (2 proteins), stress (1 protein), glycolysis (1 protein), and secondary metabolism (1 protein). In post-flooding recovering hypocotyls (Figure 6B), Cluster I grouped proteins were highly increased under flooding and during the initial recovery period, consisted of secondary metabolism (2 proteins) and unassigned (2 proteins) categories. Proteins grouped in Cluster II were either slightly decreased or remained unchanged during the recovery period. The main categories of Cluster II were protein (2 proteins), glycolysis (1 protein), and photosystem (1 protein). Proteins grouped in Cluster III decreased under flooding and during the initial recovery period, and belonged to protein (4 proteins), development (4 proteins), secondary metabolism (1 protein), and unassigned (3 proteins) categories.

2.3.2. *In silico* protein-protein interactions during post-flooding recovery

Protein-protein interactions were estimated by the expression time course through utilizing S-system differential equation as a mathematical model of the protein interaction in control (Figure 7A) and post-flooding recovery (Figure 7B) in soybean hypocotyl. Protein interactions in control showed two subnetworks which were linked to each other by 2Fe-2S ferredoxin-like superfamily protein (protein no. 13) on one side and pyruvate kinase family protein (protein no. 8) on the other side. The 2Fe-2S ferredoxin-like superfamily protein had maximum number of suppressive interactions. Main interacting proteins in control with inductive interactions were cupin family protein (protein nos. 10, 19 and 20), Rmlc-like cupins superfamily protein (protein nos. 1 and 11), and MLP-like protein 423 (protein no. 7). In post-flooding recovery, the interaction network was much closer with inductive interactions dominating the network although suppressive interactions were also present. The main interacting proteins with

dominating inductive interactions were cupin family protein (protein nos. 9 and 20), granulin repeat cysteine protease family protein (protein nos. 16, 15 and 6), and Rmlc-like cupins superfamily protein (protein nos. 1 and 11). Pyruvate kinase family protein (protein no. 8), nucleotidyl transferase superfamily protein (protein nos. 3 and 14), and beta-ketoacyl reductase 1 (protein nos. 12 and 17) were showing only inductive interactions during the post-flooding recovery. The main proteins with suppressive interactions in post-flooding recovery were calcium-dependent lipid-binding family protein (protein no. 2) and tautomerase/MIF superfamily protein (protein no. 18).

2.3.3. Protein abundance changes among the selected proteins

The temporal abundance patterns of the 20 significantly changed proteins in recovery phase following 2 days of flooding stress were compared with those of control hypocotyls. Among the 20 proteins, pyruvate kinase family protein (Glyma10g34490.1), nucleotidyl transferase superfamily protein (Glyma03g25740.1), and beta-ketoacyl reductase 1 (Glyma11g37560.1) were highly increased in abundance under flooding conditions and during the initial recovery period as compared to control plants (Figure 8). Pyruvate kinase and nucleotidyl transferase increased in abundance in response to flooding stress, but displayed a decreasing trend during the 4-day recovery period, finally reaching the levels found in control plants. The protein abundance ratio of beta-ketoacyl reductase 1 was 6-fold higher after 2 days of flooding stress than that in control plants. However, during the 4-day post-flooding recovery period, the abundance ratio of beta-ketoacyl reductase 1 sharply decreased to the level of control.

2.3.4. mRNA expression level changes during post-flooding recovery

The expression of the genes encoding three significantly changed proteins in the hypocotyls was analyzed at the mRNA level. Total RNA was extracted from hypocotyls under flooding stress and during post-flooding recovery and was compared with control hypocotyls (Figure 9). Primer specificities were confirmed by agarose gel electrophoresis and melt-curve analysis of the obtained PCR products. The analysis revealed that expression of the pyruvate kinase gene was significantly up-regulated in hypocotyls on days 3 and 4 during flooding stress, but was down-regulated to the level

of the control on days 5 and 6 during the recovery period. In the case of soybeans exposed to 4 days of flooding, a similar trend was observed, in which gene expression of pyruvate kinase was up-regulated under flooding and down-regulated to the level of control plants during the recovery period. The gene encoding nucleotidyl transferase superfamily protein was down-regulated on days 3 and 4 of flooding stress, but the expression increased to more than 2 fold the level of the control on days 5 and 6 during the recovery period. A similar trend in expression was observed for the 4-day flooded soybean, in which the gene was down-regulated on days 5 and 6 during flooding stress but was up-regulated on day 7 during the recovery stage compared to the control. The beta-ketoacyl reductase 1 gene was up-regulated on days 3 and 4 during the flooding stress, but was down-regulated to the level of the control during the recovery stage on days 5 and 6. A similar trend of beta-ketoacyl reductase 1 gene expression was observed in hypocotyls exposed to 4 days of flooding.

2.3.5. Pyruvate kinase, nucleotidyl transferase, and beta-ketoacyl reductase enzyme activities during post-flooding recovery

The enzyme activities of pyruvate kinase, nucleotidyl transferase, and beta-ketoacyl reductase in soybean hypocotyls were measured after exposing 2-day-old soybeans to flooding for 2 or 4 days, and during the post-flooding recovery period (Figure 10). Pyruvate kinase activity increased significantly in hypocotyls under flooding conditions, but decreased to control levels during the post-flooding recovery period. In contrast, the activities of nucleotidyl transferase and beta-ketoacyl reductase decreased 2 fold during the initial post-flooding recovery period as compared to control but gradually increased during the latter stage of recovery.

2.4. Discussion

2.4.1. Protein synthesis, degradation, and development-related storage proteins decrease during recovery after flooding in soybean hypocotyl

To elucidate the mechanisms involved in post-flooding recovery in soybeans, the temporal profiles of hypocotyl proteins were analyzed using gel-free proteomics. Flooding suppressed the growth and reduced the degree of pigmentation of soybean

hypocotyls, as was reported previously by Hashiguchi et al. (2009). Proteins related to protein and development categories were decreased during post-flooding recovery (Figure 7) and included ribosomal protein L19e family protein, which is a structural component of eukaryotic 60S ribosomal subunits. This finding is consistent with studies by Branco-Price et al. (2008), who suggested that mRNA translation is tightly regulated in *Arabidopsis* under oxygen deprivation conditions to conserve energy.

Another family of proteins that decreased in soybean hypocotyls during post-flooding recovery was the granulin-repeat cysteine proteases. Cysteine proteases have been linked to protein remobilization during seed germination, development, senescence, and programmed cell death, as well as biotic and abiotic stress responses (Grudkowska and Zagdanska, 2004). In *Arabidopsis*, *AtCYSa* and *AtCYSb* inhibit cysteine proteases and thereby increase plant resistance to salt, drought, cold, and oxidative stresses (Zhang et al., 2008). Quain et al. (2014) reported that the inhibition of cysteine proteases by phytocystatin ectopic OCI is important for controlling lifespan and drought stress tolerance in soybean and *Arabidopsis*. Here, the observed reduction in granulin repeat cysteine proteases in hypocotyls after exposure to flooding indicates that decreased protein degradation might have facilitated post-flooding recovery.

RmlC-like cupin superfamily proteins and cupin family, which are storage proteins belonging to the development category, were not markedly affected under flooding with respect to abundance, but were decreased during the post-flooding recovery stage to the levels found in control plants. Development-related storage proteins were increased in soybean roots exposed to flooding due to delayed degradation (Salavati et al., 2012; Komatsu et al., 2010b). Nishizawa and Komatsu (2011) also reported that storage protein degradation was reduced under flooding stress, as was found here. Cupins with nutrient reservoir activity were also increased in abundance in hypocotyls under flooding conditions compared to controls and were decreased during the post-flooding recovery period. Taken together, these results suggest that cupin family proteins were used by soybeans to recover from flooding injury.

2.4.2. Pyruvate kinase, nucleotidyl transferase, and beta-ketoacyl reductase are key

enzymes involved in recovery after flooding in soybean hypocotyl

Three selected enzymes, pyruvate kinase, nucleotidyl transferase, and beta ketoacyl reductase, which changed significantly in abundance in soybean hypocotyls, were also analyzed at the mRNA and enzyme activity levels. The observed changes in the mRNA expression and enzyme activity levels of these three enzymes between the control and flooding-stressed plants indicate that they have important roles in post-flooding recovery processes (Figures 8, 9, and 10).

Pyruvate kinase increased in soybean hypocotyls in response to flooding and during the initial post-flooding recovery period, but then decreased to the levels of controls with respect to protein abundance, mRNA expression, and enzyme activity. Pyruvate kinase, which is a key regulatory enzyme of the glycolytic pathway, generates ATP while converting phosphoenolpyruvate to pyruvate (Plaxton, 1988). Nanjo et al. (2012) reported that the protein, mRNA, and activity levels of pyruvate kinase are increased in the root tips of flooded soybean. However, as the previous study was focused on identifying flooding-responsive proteins, and did not examine the expression profile of pyruvate kinase during post-flooding recovery, here, pyruvate kinase activity in soybean hypocotyls was analyzed under flooding and during post-flooding recovery. The level of pyruvate kinase activity detected in the hypocotyl of soybean is consistent with that reported in roots under flooding stress. In plants, the oxidative stress caused by flooding shifts metabolism to anaerobic pathways. Thus, during the recovery from flooding, it appears that soybean attempts to promote aerobic metabolism by decreasing pyruvate kinase activity to the level of control.

Nucleotidyl transferase increased in protein abundance under flooding and during the initial post-flooding recovery period, but then decreased to control levels. An opposite trend was observed with respect to mRNA expression and enzyme activity, which decreased under flooding and gradually increased during latter stage of post-flooding recovery. The decrease in mRNA expression under flooding was much evident than decrease in enzyme activity. Nicotinamide mononucleotide adenylyltransferase catalyses NAD biosynthesis from nicotinamide mononucleotide and ATP. Ishikawa et al. (2009) suggested that the pyrophosphohydrolase AtNUDX7 maintains NAD^+ levels in *Arabidopsis* under oxidative stress by recycling nucleotides from free ADP-ribosyl

molecules and hence regulates defense mechanisms against oxidative DNA damage. Hashida et al. (2007, 2010) reported that nicotinamide mononucleotide adenyltransferase via the NAD pathway is required for normal pollen growth and seed production in *Arabidopsis*, and also protects guard cells from reactive oxygen species in ABA-mediated stomatal movement, thus maintaining NAD homeostasis. These findings, taken together with the present results, indicate that the increase in Nucleotidyl transferase abundance and gradual increase in its activity during the recovery period might be an effort to maintain plant growth and recover from stress.

In this study, the protein abundances and mRNA expression levels of two beta-ketoacyl reductases, which are related to secondary metabolism, were increased under flooding and during the initial post-flooding recovery period. The enzyme activity slightly decreased under flooding but gradually increased during the post-flooding recovery. In control soybeans, the protein abundance of these enzymes remained nearly constant at each analyzed time point. In *Arabidopsis*, beta-ketoacyl reductase was involved in microsomal fatty acid elongation, with the suppression of beta-ketoacyl reductase activity resulting in reduced cuticular wax load and impaired very long-chain fatty acids content of sphingolipids and triacylglycerols (Beaudoin et al., 2009). Lipids containing very long-chain fatty acids are deposited on primary plant surfaces to act as a barrier for pathogens (Jenks et al., 1994). As waxes serve as a protective barrier for plant cells, an increase in beta-ketoacyl reductase during post-flooding recovery may help plants tolerate and recover from flooding stress by strengthening the exterior surface of cells.

2.5. Conclusions

The present study is the first to investigate the proteins and mechanisms involved in post-flooding recovery in soybean hypocotyls. It was previously reported that cell wall metabolism-related proteins and cytoskeletal reorganization proteins play important roles in post-flooding recovery in soybean root (Salavati et al., 2012). In the present study, analyses of protein abundance, mRNA expression, and enzyme activity levels indicated that glycolysis-related pyruvate kinase, protein/nucleotide metabolism-related nucleotidyl transferase, and secondary metabolism-related beta-ketoacyl

reductase were appreciably changed under flooding and during post-flooding recovery. The identified enzymes are involved in energy generation, nucleotide metabolism, and complex fatty acids synthesis and may coordinate cellular responses to protect against flooding-induced damage and promote growth during post-flooding recovery. These results suggest that pyruvate kinase, nucleotidyl transferase, and beta-ketoacyl reductase are induced in soybean under flooding conditions and induce metabolic changes that promote survival and recovery from flooding-induced damage.

Table 2. Identified proteins that changed significantly during recovery after 2 days of flooding stress in hypocotyl.

S.No.	Protein ID[a]	Description	MP[b]	Ratios (Control)				Ratios (Post-flooding recovery)				Functional category[c]
				4(0)/ 2(0)	6(0)/ 2(0)	8(0)/ 2(0)	*P*[d] value	4(2)/ 2(0)	6(2)/ 2(0)	8(2)/ 2(0)	*P* value	
1	Glyma01g38340.2	RmlC-like cupins superfamily protein	2	0.03	0.02	0.03	0.00	0.17	0.26	0.02	0.00	not assigned
2	Glyma03g01750.1	Calcium-dependent lipid-binding family protein	5	1.94	1.81	4.18	0.01	1.71	2.31	3.39	0.04	not assigned
3	Glyma03g25740.1	Nucleotidyl transferase superfamily protein	3	0.43	0.45	0.16	0.01	1.21	1.04	0.17	0.00	protein
4	Glyma03g36920.1	Ribosomal protein L19e family protein	3	0.04	0.05	0.03	0.00	0.08	0.10	0.03	0.01	protein
5	Glyma04g40030.1	Chalcone-flavanone isomerase family protein	6	0.49	0.29	0.58	0.00	0.73	0.47	0.47	0.00	secondary metabolism
6	Glyma05g20930.1	Granulin repeat cysteine protease family protein	2	0.26	0.06	0.07	0.00	0.42	0.29	0.17	0.00	protein
7	Glyma07g37240.1	MLP-like protein 423	4	0.11	0.01	0.02	0.00	0.81	0.68	0.11	0.00	stress
8	Glyma10g34490.1	Pyruvate kinase family protein	9	0.52	0.20	0.06	0.00	1.67	1.50	0.40	0.00	glycolysis
9	Glyma10g39150.1	Cupin family protein	14	0.02	0.00	0.01	0.00	0.25	0.11	0.01	0.00	development
10	Glyma10g39170.1	Cupin family protein	4	0.01	0.00	0.01	0.00	0.27	0.47	0.01	0.00	development
11	Glyma11g07020.1	RmlC-like cupins superfamily protein	2	0.03	0.02	0.03	0.00	0.17	0.26	0.02	0.00	not assigned
12	Glyma11g37560.1	beta-ketoacyl reductase 1	3	0.85	0.87	1.30	0.04	6.24	2.86	0.70	0.00	secondary metabolism
13	Glyma12g29100.1	2Fe-2S ferredoxin-like superfamily protein	2	6.95	8.26	13.02	0.02	1.66	0.95	0.13	0.02	photo system
14	Glyma13g11390.1	Nucleotidyl transferase superfamily protein	2	0.45	0.45	0.20	0.02	1.23	1.10	0.22	0.00	protein
15	Glyma16g16290.1	Granulin repeat cysteine protease family protein	3	0.50	0.06	0.07	0.00	0.44	0.36	0.17	0.00	protein
16	Glyma17g18440.1	Granulin repeat cysteine protease family protein	2	0.26	0.06	0.07	0.00	0.42	0.29	0.17	0.00	protein
17	Glyma18g01510.1	beta-ketoacyl reductase 1	3	0.85	0.87	1.30	0.04	5.62	2.81	0.83	0.00	secondary metabolism
18	Glyma18g43830.1	Tautomerase/MIF superfamily protein	3	0.71	1.39	4.93	0.00	1.10	2.18	3.63	0.04	not assigned
19	Glyma20g28650.2	cupin family protein	9	0.10	0.00	0.01	0.00	0.27	0.09	0.01	0.00	development
20	Glyma20g28660.1	cupin family protein	10	0.10	0.00	0.01	0.00	0.27	0.09	0.01	0.00	development

[a] Protein ID is according to the Phytozome database; [b] MP means matched peptides; [c] Functional category is obtained from MapMan bin codes; [d] *p* value is calculated by one-way ANOVA using Prism 6 GraphPad software

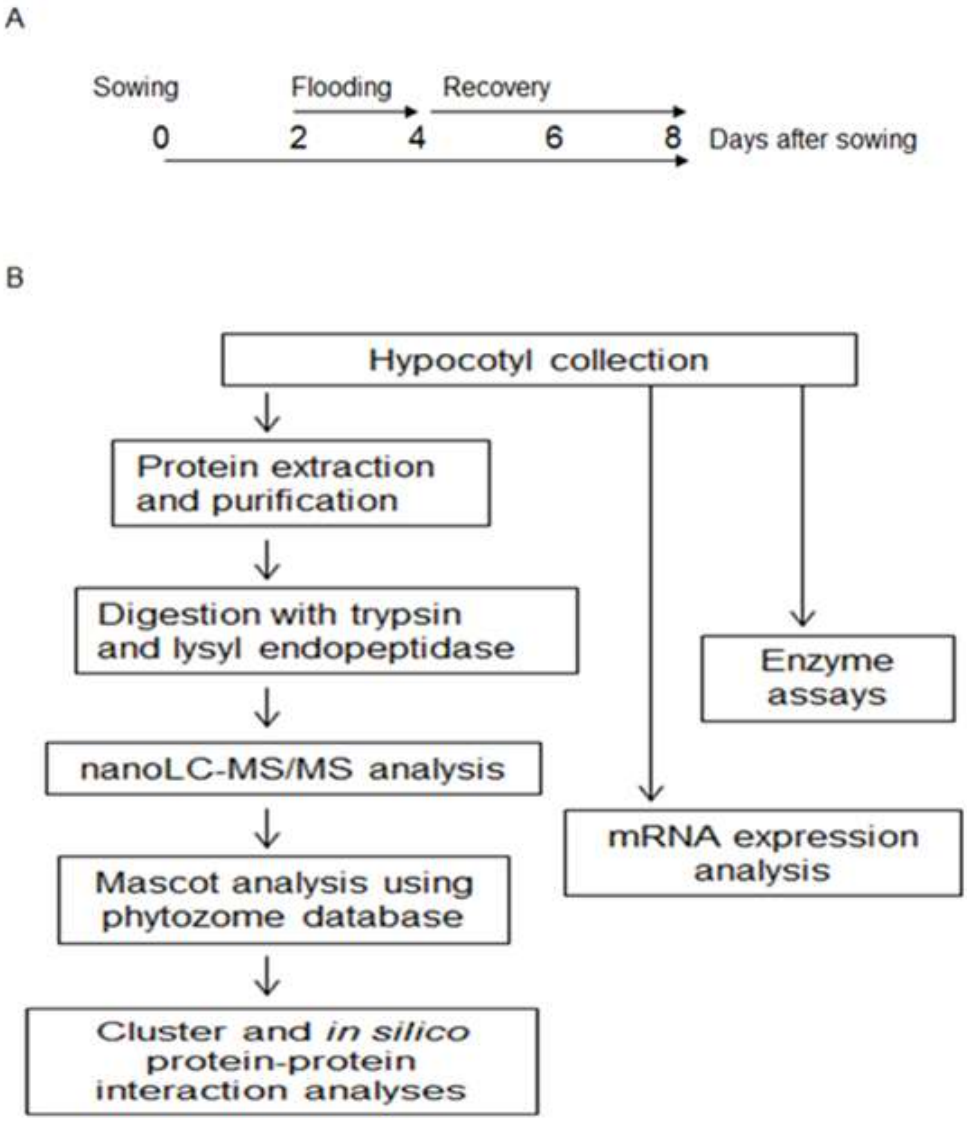


Figure 5. Experimental design used in the analysis of soybean hypocotyl. Two-day-old soybeans were flooded for 2 days and then allowed to recover for 4 days. Hypocotyl was collected at 2, 4, 6, and 8 days after sowing (A). The collected hypocotyls from control and flooded soybeans in the recovery phase were examined using proteomic, mRNA expression, and enzyme activity analyses (B). Three independent biological replicates were performed for each experiment.

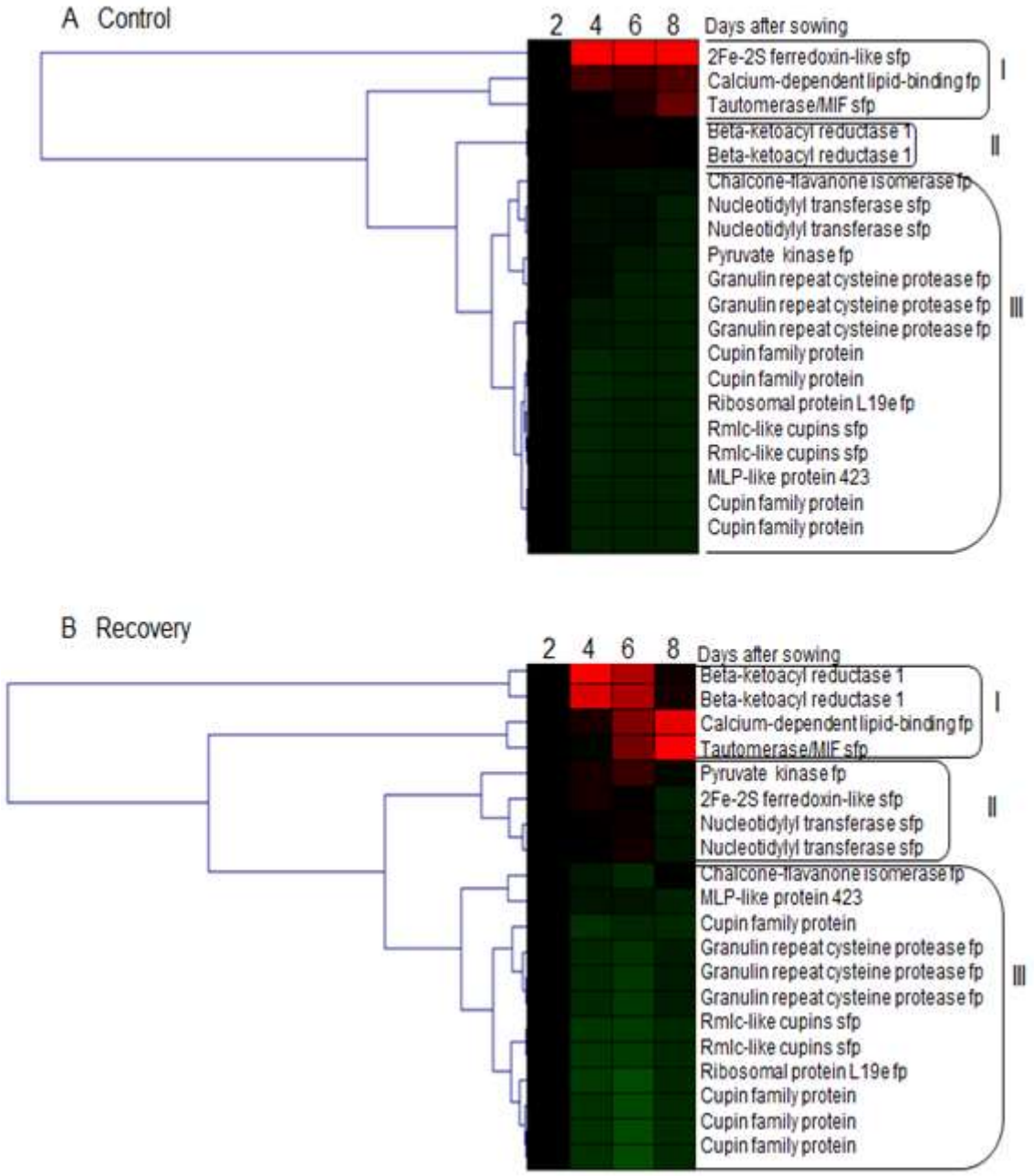


Figure 6. Cluster analysis of recovery-responsive proteins in soybean hypocotyl. Twenty proteins that changed in abundance during recovery after 2 days of flooding were analyzed based on their accumulation levels. Clustering results of the proteins in control (A) and recovery stage (B) were estimated based on time course data of the ratios when compared with 2-day-old soybeans. I, II, and III indicate the cluster number.

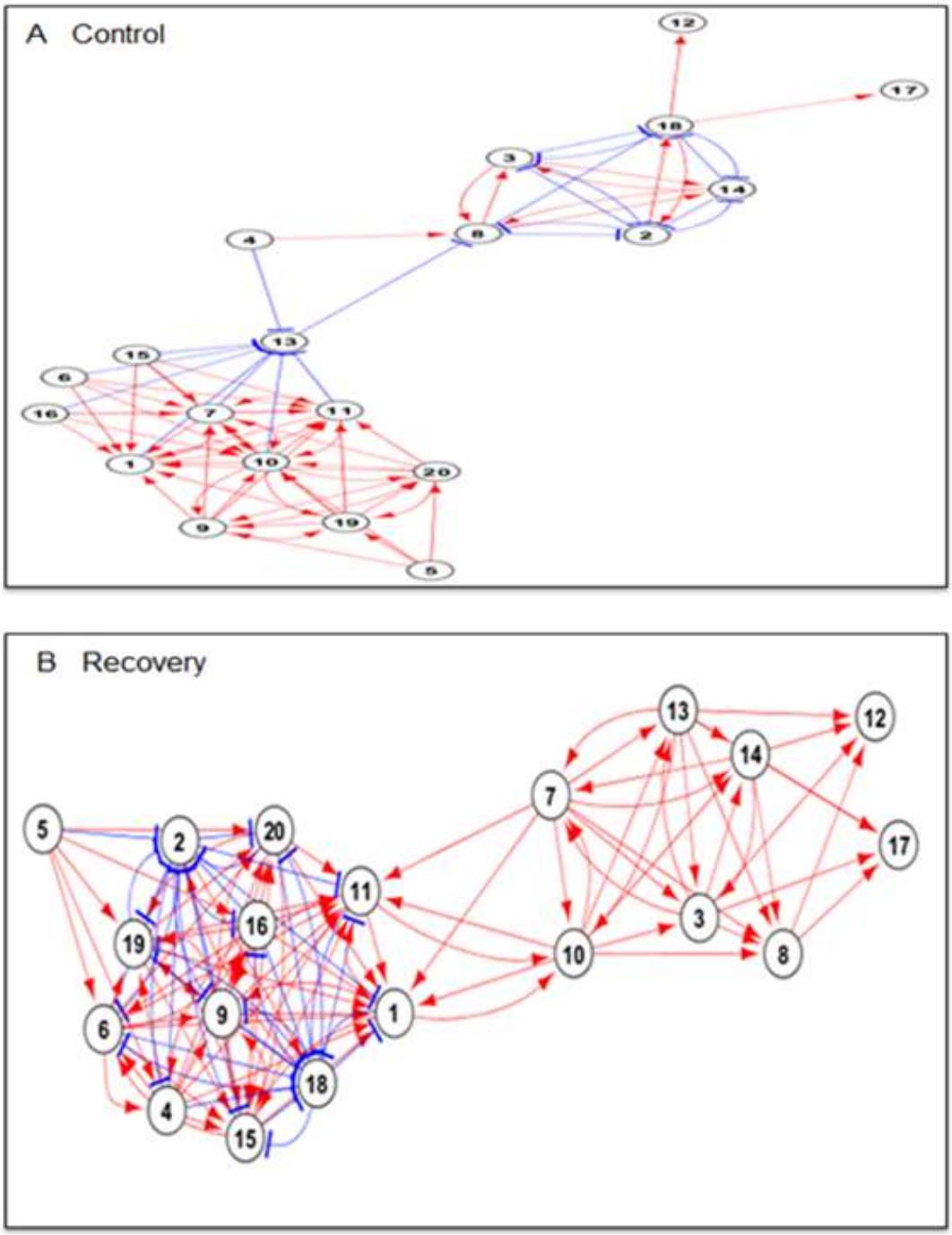


Figure 7. *In silico* protein-protein interaction analysis of recovery-responsive proteins in soybean hypocotyl. Protein interactions of the control (A) and post-flooding recovery (B) estimated based on time course data of the ratios when compared with 4-day-old control soybeans and 2-days flooded 4-day-old soybeans. A red arrow shows an inductive interaction, and a blue T-bar shows a suppressive interaction. Protein numbers are same as in Table 2.

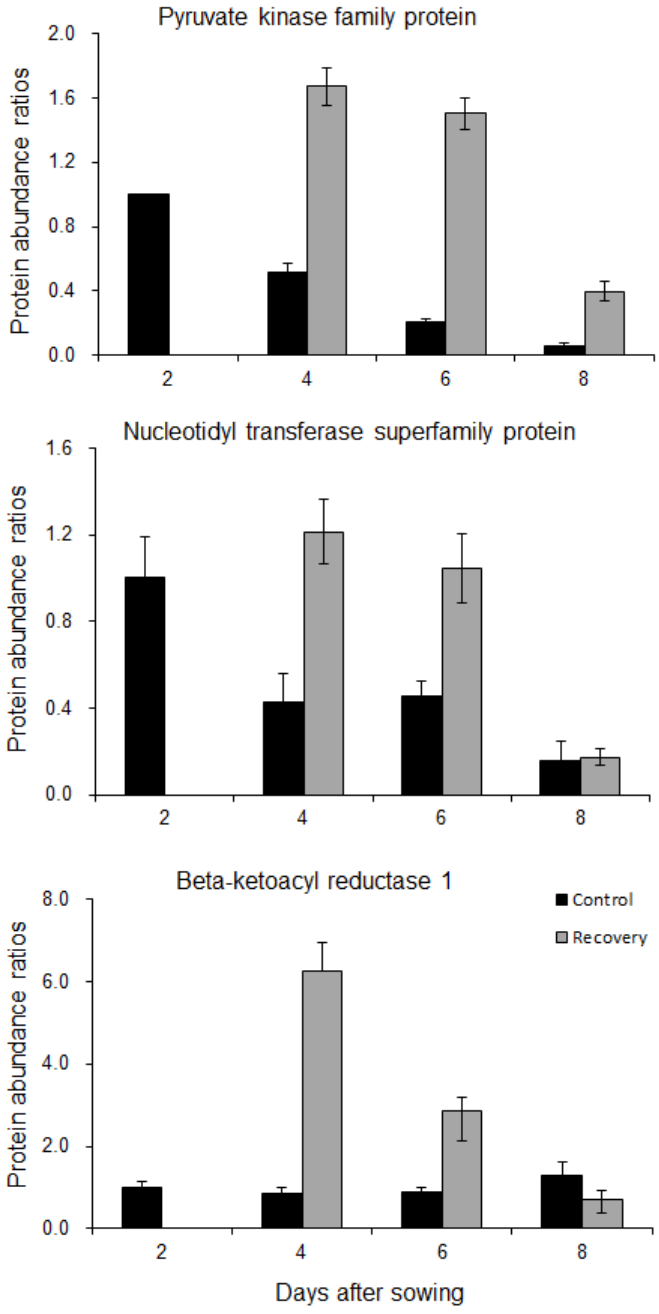


Figure 8. Changes in protein abundance in soybean hypocotyl during recovery after 2 days flooding. Changes in the abundance ratios of pyruvate kinase, Nucleotidyl transferse, and beta-ketoacyl reductase 1 during post-flooding recovery were calculated. Columns graph shows protein abundance in control (black) and recovering hypocotyl following 2-days of flooding (grey). Protein abundance ratios from SIEVE analysis were used. Data are means ± SE from three independent biological replicates (n=3).

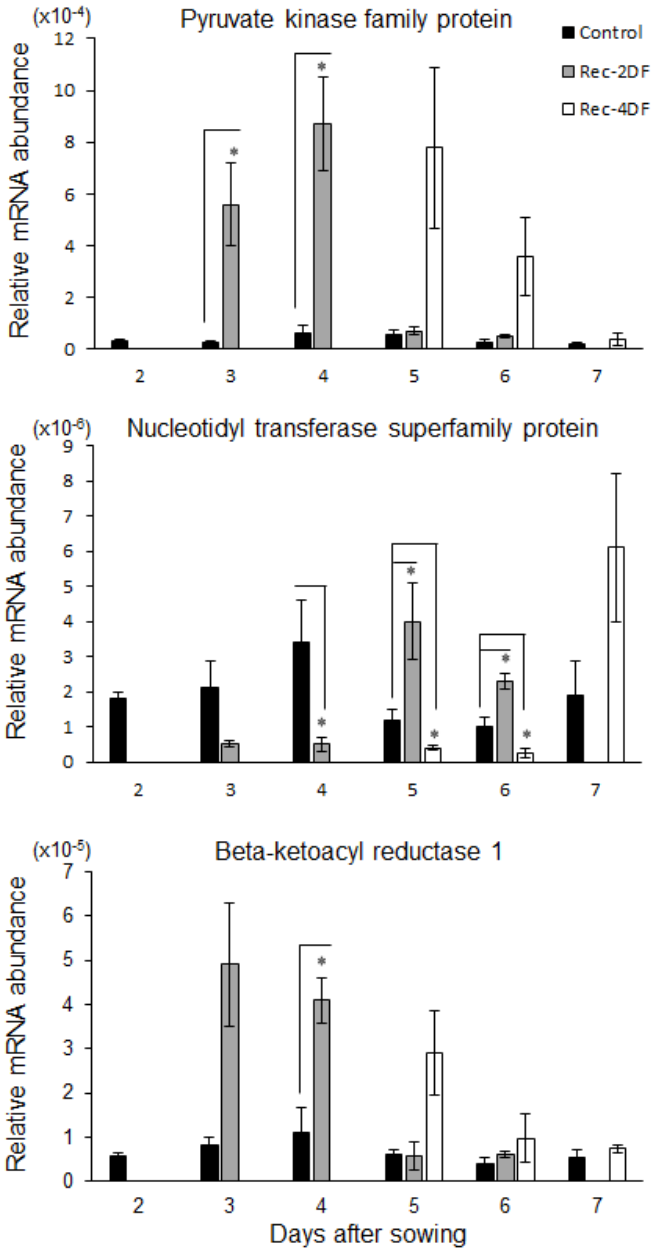


Figure 9. mRNA expression changes of three target genes during recovery after flooding in soybean hypocotyl. Two-day-old soybeans were flooded for 2 and 4 days, and the relative abundance of Nucleotidyl transferase, pyruvate kinase, and beta-ketoacyl reductase mRNA was analyzed during flooding and post-flooding recovery after 2 days flooding (Rec-2DF) and 4 days flooding (Rec-4DF) by using qRT-PCR. The expression data were normalized using 18S rRNA. The column graphs show changes in mRNA abundance of control hypocotyls (black), hypocotyls recovering after 2 days of flooding (grey), and hypocotyls recovering after 4 days of flooding (white). Data are means ± SE from three independent biological replicates (n=3). Asterisks (*) indicate significant changes between the age-matched control and treated seedlings as determined by the Student's *t*-test ($p < 0.05$).

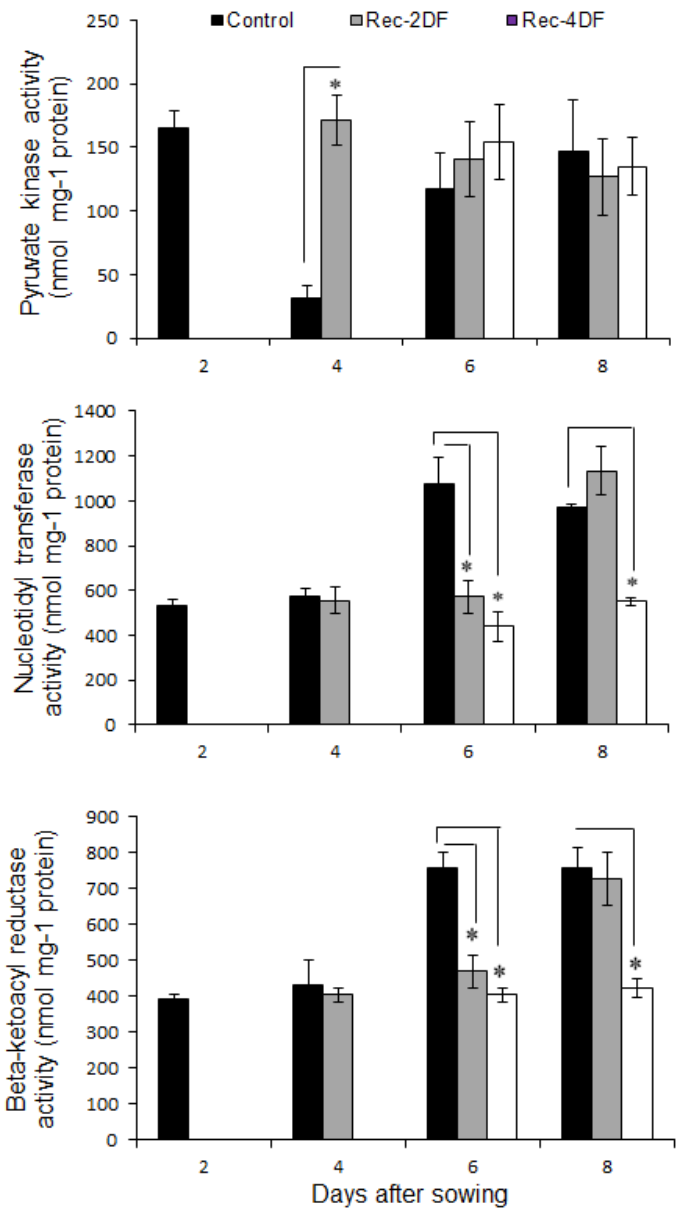


Figure 10. Enzyme activities of pyruvate kinase, Nucleotidyl transferse, and beta-ketoacyl reductase during recovery after flooding in soybean hypocotyl. Two-day-old soybeans were flooded for 2 and 4 days, and then allowed to recover for 4 and 2 days, respectively. Hypocotyl was collected during the flooding and post-flooding recovery after 2 days (Rec-2DF) and 4 days flooding (Rec-4DF), to analyze pyruvate kinase, Nucleotidyl transferase, and beta-ketoacyl reductase activities. The column graphs show enzyme activities in control hypocotyls (black), hypocotyls recovering after 2 days of flooding (grey), and hypocotyls recovering after 4 days of flooding (white). Data are means ± SE from three independent biological replicates. Asterisks (*) indicate significant changes between the age-matched control and treated seedlings as determined the by Student's *t*-test ($p < 0.05$).

CHAPTER 3
QUANTITATIVE PROTEOMICS OF ROOT IN SOYBEAN DURING POST-FLOODING RECOVERY

3.1. Introduction

Root is an important organ and first to recognize the flooding stress. Under flooding stress, roots undergo several structural and functional modifications at the molecular, cellular, and phenotypic level (Atkinson and Urwin, 2012). Flooding weakens the hydraulic conductivity of roots, leading to reduced root permeability (Clarkson et al., 2000). Plant initially responds to flooding by reducing root permeability, water absorption, and mineral uptake, followed by decreasing photosynthesis, altering hormonal balance, and developing aerenchyma and adventitious roots (Vartapetian and Jackson, 1997). Carbohydrate reserves in the root are considered a hallmark of flooding resistance. During hypoxic or anoxic conditions the starch reserves in the root are rapidly used up and maintenance of root growth and function becomes difficult or impossible (Sauter, 2013). These findings indicate that root is an important organ for understanding the mechanisms by which soybean regulates flooding stress response.

Plant recovery responses to flooding vary depending on the type of damage, which may become even more severe after water recedes. Rice tissue injuries caused by flooding were exacerbated when shoots were re-exposed to the atmosphere (Srakar et al., 2006). Quiescence and escape strategies that are activated during flooding not only help plants cope with submerged condition, but also promote the recovery of photosynthesis following the removal of water (Luo et al., 2011). In perennial ryegrass, carbohydrate production, plant height, and growth rate were recovered following de-submergence (Yu et al., 2012). Investigations about nitrogen fixation activity in soybean following flash flooding concluded that nitrogen fixation ability was recovered rapidly without any lasting damage (Justino and Sodek, 2013). These studies suggested that some plant species were able to recover from short-term flooding after removal of the stress. However, the underlying mechanisms and specific proteins involved in these recovery responses remained largely unknown.

Proteomic techniques have been extensively applied for investigating the effects of flooding on soybeans to identify flooding stress-responsive proteins. Proteins involved in the detoxification of reactive oxygen species, anaerobic catabolism, storage, and disease resistance, were commonly affected by flooding (Nanjo et al., 2010). A cell

wall proteomic study of flooded soybean revealed down-regulation of lipoxygenases and superoxide dismutase, awhile the lignification was suppressed (Komatsu et al., 2010a). Proteomics and metabolomics analyses of mitochondria in flooding-stressed soybean showed direct negative impacts on electron transport chain as ATP production decreased despite increasing NADH concentration (Komatsu et al., 2011). Proteomic analyses have revealed that proteins involved in storage, transport, energy metabolism, disease defense, and cell signaling were induced by flooding in soybean roots (Komatsu et al., 2012a). Energy-related proteins were increased whereas those involved in protein folding and cell structure organization were decreased in flooded soybean root tips (Nanjo et al., 2012). Flooding stress increased the levels of heat shock protein-70 and decreased calcium oxalate crystals in the cotyledon of soybean, resulting in increased levels of calcium ions (Komatsu et al., 2013a). In addition, polygalacturonase inhibitor-like and expansin-like B1-like proteins were identified as indicator proteins for assessing the severity of flooding stress in soybean (Nanjo et al., 2013).

The plant responses to flooding stress are well characterized; however, a very little attention has been paid to unwind the mechanisms associated with plant recovery after flooding. In chapter 1, it was demonstrated that flooding affected the morphology of soybean roots and growth was recovered after the removal of the stress. However, this result indicated the need of further analysis to get a clear insight into the recovery mechanism. In an only proteomic study on soybean root during post-flooding recovery, Salavati et al. (2012) reported that the cell structure of soybean was modified through alteration of cell wall metabolism and reorganization of the cytoskeleton. The causes of recovery mechanisms behind the survival are unclear. In this study, to unravel the mechanism involved in post-flooding recovery in soybeans, the temporal profiles of root proteins were analyzed using gel-free proteomic technique.

3.2. Materials and methods

3.2.1. Plant material and treatments

Plant growth conditions are same as described in section 2.2.1. Soybeans were flooded for 2 and 4 days followed by recovery after removal of the stress. (Figure 11). Root was collected for proteomic, mRNA expression, and enzyme activity analyses.

Sodium azide was used to inhibit activity of peroxidases. Briefly, 3 mM solution of sodium azide was used to flood the 2-day-old seedlings for 2 and 4 days in glass tubes. After removal of treatment, seedlings were grown in sand. Morphological changes and peroxidase activity was measured at the day of removal of flooding and then at two more time points during the recovery period following slightly modified method of Tewari et al. (2002).

3.2.2. Protein extraction

Same as described in section 2.2.2.

3.2.3. Protein purification and digestion for mass Sspectrometry analysis

Same as described in section 2.2.3.

3.2.4. Nanoliquid chromatography-tandem mass spectrometry analysis

Same as described in section 2.2.4.

3.2.5. Protein identification

Same as described in section 2.2.5.

3.2.6. Differential analysis

Same as described in section 2.2.6.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaino et al., 2013) with the dataset identifier PXD000986.

3.2.7. Cluster and *in silico* protein-protein interaction analyses

Protein ratio data obtained from differential analysis using SIEVE software, were subjected to cluster analysis using the MultiExperiment Viewer application (version 4.8.1) (Saeed et al., 2006). For cluster analysis, hierarchical clustering with a Euclidean distance metric and a centroid linkage clustering method was used. For *in silico* protein-protein interaction analysis, method was same as described in section 2.2.7.

3.2.8. Functional categorization

Same as described in section 2.2.8.

3.2.9. RNA extraction and quantitative real-time polymerase chain reaction

Same as described in section 2.2.9 (Supplemental Table 1).

3.2.10. Analysis of enzyme activities

Peroxidase: Peroxidase assay was performed by slightly modified method of Tewari et al. (2002). Briefly, a portion (125 mg) of frozen samples were homogenized at 4°C in 1 mL of extraction buffer consisting of 25 mM phosphate buffer (pH 7.8), 0.4 mM EDTA, 1 mM ascorbic acid and 2% polyvinylpyrrolidone with a mortar and pestle. The homogenate was centrifuged at 15,000 × g at 4°C for 20 min. Supernatant collected and centrifuged again. The filtrate was used as enzyme extract. Protein concentrations were determined using Bradford assay. A reaction mixture for determining activity was prepared using 100 mM phosphate buffer (pH 7.8), 4% *p*-phenylenediamine, 2% hydrogen peroxide, and 5 N H_2SO_4. Enzyme extract (100 µL) was mixed with 900 µL reaction mixture, vortexed and kept for 5 min. The mixture was then centrifuged at 15,000 × g at 4°C for 5 min. The absorbance of the resulting supernatant was measured at 485 nm using UV/Vis Spectrophotometer (Beckman Coulter, Brea, CA, USA).

Lipoxygenase: Lipoxygenase assay was performed by slightly modified method of Axelrod et al. (Axelrod et al., 1981). Briefly, a portion (100 mg) of frozen samples was homogenized in 1 mL of deionized water with a mortar and pestle. The homogenate was centrifuged at 3000 × g at 4°C for 15 min. The supernatant was used as enzyme extract. Protein concentrations were determined using Bradford assay. For determining lipoxygenase1 activity, substrate was prepared by suspending linoleic acid in 2.24 mM borate buffer (pH 9.0) and the suspension was neutralized by drop-wise addition of 5 mM NaOH solution and shaken by adding two drops of Tween-20 to it. For assaying activity, 150 µL of enzyme extract was added to 1 mL substrate suspension. The mixture was shaken and incubated at 30°C for 3 min. The reaction was terminated by adding 2.5 mL of ethanol to the mixture and then 2.5 mL of deionized water was added to the

mixture. The reference blank sample was prepared that contained all reaction components except lipoxygenase. Absorption was measured at 234 nm using UV/Vis Spectrophotometer.

3.2.11. Statistical analysis

Same as described in section 2.2.11.

3.3. Results

3.3.1. Identification of proteins during post-flooding recovery

To study the protein changes during post-flooding recovery in soybean root, gel-free proteomics was used. Soybeans grown for 2 days were flooded for 2 and 4 days and then allowed to recover for 6 days following stress removal. Proteomic analysis of soybean roots was performed before flooding, at the day of removal of water, and at three more points during 6 days recovery period with sampling at alternate days. Soybeans grown at same time points without treatment were used as control. To study the changes occurring during recovery after flooding, the root proteins of 2 days flooding-stressed and control plants were compared. A total of 1,662 proteins with 2 or more matched peptides were identified in control; while 1,645 proteins were identified in soybean roots recovering after 2 days flooding. Between control and recovery, 73 proteins were commonly identified in both with significant changes (Table 3). A total of 1,826 proteins with 2 or more matched peptides were identified in control; while 1,707 proteins were identified in roots recovering after 4 days flooding. Between control and recovery, 21 proteins were commonly identified in both with significant changes (Table 4). These 73 and 21 commonly changed proteins were used for further analyses.

3.3.2. Changes in protein abundance in soybean root during post-flooding recovery

Proteins identified in post-flooding recovery of 2 days flooding were subjected to clustering analysis using their abundance ratios. The clustering analysis grouped the 73 proteins into 3 clusters (I, II, and III) in the control (Figure 12A) and recovery stage (Figure 12B). Proteins grouped in Cluster I were highly increased during the recovery stage and belonged to stress (31%), lipid metabolism (8%), secondary metabolism (8%),

and xenobiotic biodegradation (7%) categories. Proteins grouped in Cluster II were either slightly increased or unchanged during the recovery stage. The main categories of Cluster II proteins included secondary metabolism (17%), stress (13%), and protein (8%). Proteins grouped in Cluster III decreased during the recovery stage and belonged to protein (38%), hormone metabolism (11%), stress (11%), amino acid metabolism (8%), cell (8%), RNA (5%), and other categories.

Proteins identified in post-flooding recovery of 4 days flooding were subjected to clustering analysis using their abundance ratios. Clustering analysis led to the grouping of 21 proteins into 3 clusters (I, II, and III) in the control (Figure 12C) and recovery stage (Figure 12D). Proteins of Cluster I were highly increased during the recovery stage and belonged to stress (29%), cell wall (14%), and protein (14%) categories. Proteins within Cluster II were either slightly increased or unchanged and predominantly belonged to secondary metabolism (13%) and hormone metabolism (12%) categories. Proteins grouped in Cluster III were decreased during the recovery phase and mainly belonged to protein (33%), hormone metabolism (33%), and development (17%) categories.

3.3.3. *In silico* protein-protein interactions during post-flooding recovery

Protein-protein interactions were estimated by the expression time course through utilizing S-system differential equation as a mathematical model of the protein interaction in control and treated soybeans. Protein interactions in control (Figure 13A) showed two sub-networks which were linked to each other by ATP-citrate lyase A-1 (protein no. 50) as most interacting protein with both inductive and repressive types of interactions. Other main interactive proteins in control were isoflavone reductase (protein no. 28), ribosomal L5P protein (protein no. 24), P450 reductase 2 (protein no. 54) and aldolase type TIM barrel family protein (protein no. 4). In case of recovery (Figure 13 B), a uniform network of protein interactions was structured in the recovery process from flooding stress as compared to control. Aldolase type TIM barrel family protein (protein no. 65), *S*-adenosyl-L-methionine dependent methyltransferase (protein no. 57), ribosomal protein S19e family protein (protein no. 67), sterol methyl trasferase 2 (protein no. 8) and ribosomal protein L14p (protein no.35) were more interacting

proteins.

In post-flooding recovery of 4 days flooding stress, a well coordinated uniform network among interacting proteins was detected (Figure 13D). Lipoxygenase 1 (protein no. 38 and 42), MLP-like protein 43 (protein no. 103) and peroxidase superfamily proteins (protein no. 110 and 44) were more interactive proteins with both inductive and repressive interactions. In contrast, control soybeans at the same time points showed a segregated network of interacting proteins, with different types of interactions predicted among proteins.

3.3.4. Protein abundance changes among the six selected common proteins

The temporal abundance patterns of the six significantly changed proteins that were common in recovery phase following 2 and 4 days flooding were calculated (Figure 14). The abundance pattern of lipoxygenase 1 showed a marked decrease under flooding stress conditions, but increased more than the control during the recovery phase, most notably after 4 days of post-flooding. Peroxidase superfamily protein increased continuously during the recovery period after significantly decreasing in response to flooding and reached or exceeded the level of control; however, this protein showed a decreasing tendency at the later stages of recovery. Root hair specific 19, a type of peroxidase, also increased during the early stage of recovery for both 2 and 4 days flooded soybeans, but the degree of increase was less than that found in control plants. HAD-superfamily acid phosphatase slightly increased during the recovery phase in flooded soybeans. Isoflavone reductase significantly increased in the post-flooding recovery period following 2 and 4 days flooding and exceeded the levels found in control plants. An unknown protein (Glyma16g07750.1) also significantly increased during the recovery stage following 4 days of flooding stress and exceeded the amount found in control plants.

3.3.5. mRNA expression level changes during post-flooding recovery

RNA was extracted from soybean roots during the post-flooding recovery period and compared to age-matched control soybeans. The genes of 6 significantly changed proteins that were commonly identified in the roots of 2 and 4 days flooded soybeans

during the recovery phase were analyzed at the mRNA level to examine changes in expression (Figure 15). Primer specificities were assessed by agarose gel electrophoresis and melt-curve analysis of PCR products. The qRT-PCR analysis revealed that the gene encoding lipoxygenase 1 was significantly up-regulated until 3 days after sowing in control and 2-day flooded soybean plants; however, the gene expression was significantly and steadily down-regulated during the recovery stage. A similar trend was observed during the recovery period for 4 days flooded plants, but the expression of the lipoxygenase 1 gene was significantly up-regulated by day 7 compared to the control. In contrast, the gene encoding peroxidase superfamily protein was down-regulated in response to flooding, as can be seen for days 3 and 4 after sowing, but was significantly up-regulated by days 5 and 6 after sowing during the recovery stage following 2 days flooding. Similarly, the peroxidase gene was up-regulated compared to the controls on days 7 and 8 after sowing following 4 days flooding. Root hair specific 19 displayed a similar trend to peroxidase superfamily protein as the corresponding gene was significantly up-regulated during the recovery phase for both flooding durations. HAD-superfamily acid phosphatase gene expression was completely down-regulated under flooding stress and only slightly increased during the recovery period. The isoflavone reductase gene showed significant up-regulation under 2 days flooding condition from days 3 to 4 after sowing, but then significantly down-regulated on days 5 and 6 after sowing during the recovery period. This gene was also significantly up-regulated on days 5 and day 6 in soybeans treated with 4 days flooding, but was then down-regulated on days 7 and 8 after sowing during the recovery period. Finally, the gene encoding unknown protein (Glyma16g07750.1) was up-regulated in the recovery stage as compared to 2 days flooding conditions, although the expression level was less than that in the control plants; however, in recovery following 4 days flooding, this gene was up-regulated more than control.

3.3.6. Peroxidase and lipoxygenase enzyme activities during post-flooding recovery

Peroxidase superfamily protein, being changed significantly during post-flooding recovery was analyzed at enzyme activity level (Figure 16A). The peroxidase activity was decreased under flooding stress when measured at 4 days after sowing as

compared to control plants, but was almost doubled during the recovery stage when measured at day 6 and 8 after sowing in the soybean roots following 2 days flooding. This increase in activity was also statistically significant. Peroxidase activity had increased significantly than control by day 8 after sowing in the roots of soybeans exposed to 4 days flooding.

Lipoxygenase activity was also measured under flooding conditions and during the post-flooding recovery stage (Figure 16B). The enzyme activity was slightly decreased under flooding stress when compared to control plants. A continuous decreasing trend of activity was observed during the recovery period, reaching the level of significance on day 6 after sowing for 2 days flooding soybeans. Lipoxygenase activity was slightly increased compared to controls in plants treated with 4 days flooding when assayed during the recovery phase on days 6 and 8 after sowing; however, this change was not significant.

3.3.7. Peroxidase activity inhibition by sodium azide treatment

Sodium azide was used to reveal whether peroxidases are key players in the post-flooding recovery or not. Root length and weight were measured. Root in flooded seedlings continued to elongate while root elongation was evidently less in seedlings treated with sodium azide solution (Figure 17A, B). Similar trend was noted in root weight. Coincident with morphological changes, peroxidase activity was significantly less in sodium azide treated flooded seedlings as compared to those flooded without sodium azide (Figure 17C).

3.4. Discussion

3.4.1. Stress-responsive proteins increase and protein metabolism-related proteins decrease during recovery after flooding in soybean root

Flooding stress causes injury in soybean (Komatsu et al., 2012a). In chapter 2, hypocotyl proteomic analysis during post-flooding recovery demonstrated that proteins involved in energy and secondary metabolism were induced. In the present study, several stress-related proteins such as disease resistance-responsive protein, germin-like protein 5, isoforms of peroxidase, and MLP-like protein 43 were increased in soybean

roots during recovery after flooding (Figure 12B and D). Disease resistance-responsive (dirigent-like) proteins have been shown to be involved in plant defense response and secondary metabolism in Spruce *(Picea spp.)* (Ralph et al., 2007), and also played a regulatory role in lignin formation in *Forsythia intermedia* (Burlat et al., 2001). In current study, disease resistance-responsive protein decreased under flooding stress, but subsequently increased during recovery. Germin-like protein 5 was stimulated within ROS-dependent pathways and was bound to the cell wall, indicating its role in plant defense (Soares et al., 209). Germin-like proteins, which are expressed at specific stages of plant development, are decreased in soybean under flooding (Komatsu et al., 2010a). In the present study, it was confirmed that the levels of disease resistance-responsive proteins and germin-like protein decreased in soybean roots in response to flooding stress, but were increased during post-flooding recovery. This increase during the recovery period suggests that these proteins have important trade-off roles in soybean during post-flooding recovery.

Isoforms of peroxidase, which are well-characterized stress-related proteins, also decreased under flooding and subsequently increased during the post-flooding recovery stage. Peroxidases act as free radical-scavenging enzymes and play roles in lignification as well as intracellular signaling (Kausar et al., 2012; Herrero et al., 2013) and are therefore thought to help plants recover from oxidative damage caused by flooding. The present results are consistent with the findings of previous studies, confirming beneficial functions of peroxidase with respect to scavenging ROS, thus helping plants to recover efficiently from flooding-induced damages. MLP-like protein 43 belongs to the Kunitz-type protease inhibitor superfamily, whose members are involved in plant defense against biotic and abiotic stresses through inhibiting the activity of proteases such as trypsin (Tsukuda et al., 2006). The increase of this protein in soybean roots during post-flooding recovery indicate that the activity of proteases was decreased, which would have helped to maintain the integrity of cellular proteins and thus facilitated recovery.

The gel-free proteomic analysis also revealed that many proteins belonging to the protein metabolism category are decreased in soybeans during post-flooding recovery. The identified proteins, which were involved in protein synthesis and post-translational modifications, included various ribosomal proteins, initiation and elongation factors,

and ribonucleo-protein. Branco-Price et al. (2008) also suggested that mRNA translation was regulated in *Arabidopsis* to conserve energy under oxygen deprivation. The results of the present study suggest that protein synthesis does not take place during the early recovery stages from flooding because it is an energy-intensive process. However, once the stress is removed and plants can completely resume ATP production, protein synthesis is expected to take place in the later stages of recovery.

3.4.2. Cell wall metabolism-related proteins increase lignification in soybean root during recovery after flooding

Cell wall-related proteins such as *O*-methyltransferase 1, *S*-adenosyl-L-methionine -dependent methyl transferase superfamily protein, and plant invertase/pectin methylesterase inhibitor were increased in soybean roots during post-flooding recovery stage. *O*-methyltransferase reportedly catalyzed reactions leading to lignin G-unit and S-unit formation and is involved in controlling cell wall thickness (Do et al., 2007; Day et al., 2009). *S*-adenosyl-L-methionine-dependent methyl transferases play a variety of roles in the biosynthesis of important plant products, such as lignin and flavonoid (Joshi and Chiang, 1998). Plant invertase/pectin methylesterase inhibitor was increased in soybean roots during the early stages of recovery following 4 days of flooding stress. Notably, this enzyme was not detectable during recovery after 2 days flooding that might suggest a relatively less degree of damage to the cell wall under 2 days flooding. Pectin methylesterase affects the properties of pectin and cell wall rigidity by de-methylesterification of homogalacturonans (Micheli, 2001). Pectin methylesterase inhibitor is associated with plant defense and stress reactions, such as injury (An et al., 2008). Previous reports about soybean under flooding stress have revealed that majority of proteins associated with flooding stress were linked to cell wall synthesis and modification (Komatsu et al., 2010a; Nanjo et al., 2013). The present results suggest that increased abundance of cell wall-related proteins during post-flooding recovery enhance lignification and thereby triggered reactions that led to re-arrangements of the cell wall structure and hence facilitated recovery.

3.4.3. Peroxidases, lipoxygenase, and isoflavone reductase are key enzymes during

recovery after flooding in soybean root

Various isoforms of peroxidase were decreased in soybean roots under flooding and significantly increased during the post-flooding recovery stage at both protein abundance and mRNA expression levels. Plant peroxidases belong to a large multigenic family, which includes 138 members in rice that play role in the lignification and imparting rigidity to the cell wall (Herrero et al., 2013; Passardi et al., 2004). ROS such as superoxide radicals, hydroxyl radicals, and hydrogen peroxide are produced as toxic products of normal cell metabolism and also act as regulatory molecules in stress and signal transduction pathways (Navrot et al., 2006; Mittler et al., 2011). ROS accumulate in soybean cells under flooding, as fewer ROS scavenging enzymes such as peroxidases are available to detoxify these harmful radicals (Kausar et al., 2012). In the present study, peroxidases increased continuously during the flooding recovery stage, suggesting that peroxidases help soybeans to recover from flooding by scavenging harmful radicals as well as by enhancing cell wall lignification through signaling.

Proteomic analysis showed that lipoxygenase was decreased in soybean roots under flooding stress, as also reported previously (Komatsu et al., 2010a). The levels of lipoxygenase increased slightly during recovery following 2 days flooding, but were markedly increased compared to control soybeans during the recovery of plants exposed to 4 days flooding. At the mRNA level, lipoxygenase was up-regulated under flooding, but was steadily down-regulated during recovery. The slight increase in lipoxygenase at the protein level during recovery was considerably less than pre-flooding levels, confirming the role of this enzyme in growth and development of roots during germination, as reported earlier (Junghans et al., 2004). The observed differences between protein abundance and mRNA expression suggest that post-translational modification affects the function of lipoxygenase. The slight increase in lipoxygenase suggests plant's attempt to resume growth suppressed by flooding.

Isoflavone reductase protein was increased during post-flooding recovery at higher levels in 2 days flooded than compared to 4 days flooded soybeans. This protein was previously shown to be decreased in soybean under flooding stress (Khatoon et al., 2012a). Isoflavone reductase is involved in the biosynthesis of secondary metabolites, including lignins and isoflavonoids (Dixon, 2001), and also has a role in defense against

oxidative damage (Bors et al., 1990). Isoflavone reductase-like genes were involved in growth and ROS tolerance in rice under oxidative stress (Kim et al., 2010). Taken together, these findings indicate that isoflavone reductase-like proteins are increased during flooding stress recovery in soybeans as a mechanism to facilitate secondary metabolism and thereby promote post-flooding recovery.

3.4.4. Peroxidases inhibition assay confirmed the key role of peroxidases in the post-flooding recovery

Sodium peroxide has been used as an effective inhibitor of peroxidases (Tuisel et al., 1991; Sukalovic et al., 2015). Azide forms azidyl radicle with the heme prosthetic group and inhibits enzyme activity. Peroxidase inhibition assay was performed for confirming the role of peroxidases during post-flooding recovery in soybean. The morphological as well as activity level analyses showed that recovery was evidently suppressed when seedlings were flooded with sodium azide solution as compared to those flooded only with water without sodium azide. Peroxidase inhibition assay confirmed that peroxidases reduce the oxidative damage caused by flooding and hence facilitate post-flooding recovery in soybean roots. The peroxidase gene exhibited up-regulated mRNA expression in soybean roots during post-flooding recovery. An enzyme activity assay revealed that the activity of peroxidase was decreased under flooding stress, but was significantly increased during the post-flooding recovery stage. The temporal profiles of peroxidase activity are consistent with the measurements of protein abundance and mRNA expression. Ascorbate peroxidase was reduced in protein abundance in soybean under flooding stress (Kausar et al., 2012). Peroxidase activity was reported to be reduced in rice under flooding stress (Ismail et al., 2009). As peroxidases are involved in the scavenging of toxic peroxides and cell wall lignification (Herrero et al., 2013). Peroxidases might comprise a cascade to generate a coordinated response following flooding that facilitated soybean recovery.

3.5. Conclusions

A previous study examining soybean recovery after flooding (Salavati et al., 2012) reported that root growth during recovery was regulated through cell wall

modification and cytoskeletal reorganization. In the present study, several cell wall-related proteins including methyltranferase and pectin methylesterase inhibitor were identified in soybean roots during post-flooding recovery. Furthermore, proteomic analysis indicated that a broad range of proteins involved in stress responses, primary and secondary metabolism are induced following water removal. Changes in these proteins may regulate metabolic pathways that facilitate the post-flooding recovery of soybean. In particular, proteins involved in ROS scavenging and cell wall metabolism might be key players in the morphological, metabolic, and molecular pathways involved in strengthening the cell wall as first line of defense against subsequent abiotic stress. It is concluded that scavenging of toxic radicals and associated lignification by peroxidases play vital role in post-flooding recovery in soybean root.

Table 3. Identified proteins in soybean roots that changed significantlty during recovery after 2 days of flooding stress.

Protein No.	Protein ID	Protein description	MP	P value	Ratios (Control)				Ratios (Post-flooding recovery)				Functional category
					4(0)/2(0)	6(0)/2(0)	8(0)/2(0)	10(0)/2(0)	4(2)/2(0)	6(2)/2(0)	8(2)/2(0)	10(2)/2(0)	
1	Glyma02g00340.1	HXXXD-type acyl-transferase family protein	5	0.00	2.43	23.67	1.25	1.72	1.05	2.03	1.29	1.97	stress
2	Glyma02g40310.1	Eukaryotic translation initiation factor 2 beta subunit	4	0.00	0.08	0.12	0.04	0.03	0.23	0.56	0.17	0.25	protein
3	Glyma03g05481.1	Disease resistance-responsive (dirigent-like) family protein	5	0.00	6.88	11.01	11.45	14.62	1.72	4.43	5.72	6.22	stress
4	Glyma03g16940.1	AAldolase-type TIM barrel family protein	4	0.01	2.74	2.06	0.53	0.97	1.89	2.64	1.86	2.18	OPP
5	Glyma03g26100.1	Hyaluronan / mRNA binding family	3	0.00	0.07	0.04	0.03	0.02	0.10	0.26	0.25	0.23	RNA
6	Glyma03g40490.1	O-acetylserine (thiol) lyase (OAS-TL) isoform A1	5	0.01	2.19	2.35	1.47	2.10	0.40	1.96	1.21	1.99	amino acid metabolism
7	Glyma04g02230.1	Pyridoxal-dependent decarboxylase family protein	4	0.00	0.08	0.06	0.03	0.01	0.02	0.02	0.05	0.08	amino acid metabolism
8	Glyma04g02271.1	Sterol methyltransferase 2	3	0.00	0.44	0.20	0.18	0.18	0.18	0.27	0.46	0.52	hormone metabolism
9	Glyma04g11550.1	Acetyl-CoA carboxylase 1	14	0.00	0.05	0.05	0.03	0.04	0.25	0.08	0.17	0.25	lipid metabolism
10	Glyma04g40580.1	O-methyltransferase 1	3	0.00	1.49	1.55	1.23	3.23	0.66	1.69	1.55	1.68	secondary metabolism
11	Glyma05g33930.1	Eukaryotic translation initiation factor 3C	5	0.00	0.27	0.24	0.27	0.12	0.04	0.41	0.05	0.26	protein
12	Glyma05g36600.1	Heat shock protein 70 (Hsp 70) family protein	11	0.00	0.41	0.15	0.01	0.08	0.46	0.35	0.49	0.55	stress
13	Glyma06g14220.1	O-methyltransferase 1	9	0.00	3.19	3.42	2.49	4.58	1.34	2.70	2.13	2.93	secondary metabolism
14	Glyma06g18800.1	Ribosomal protein L18e/L15 superfamily protein	2	0.00	0.12	0.04	0.08	0.16	0.03	0.03	0.06	0.19	protein
15	Glyma06g19000.1	ATPase- AAA-type- CDC48 protein	8	0.00	0.07	0.01	0.01	0.01	0.09	0.30	0.07	0.17	cell
16	Glyma06g38540.1	PYR1-like 12	5	0.00	3.58	3.30	1.95	1.94	1.71	3.15	1.39	2.47	stress
17	Glyma06g45910.2	Peroxidase superfamily protein	10	0.00	3.88	3.18	2.37	4.93	1.48	5.31	1.01	0.98	stress
18	Glyma07g14870.1	Translation initiation factor 3B1	4	0.00	0.11	0.05	0.02	0.02	0.13	0.03	0.34	0.32	protein
19	Glyma07g18400.1	Beta glucosidase 11	2	0.02	2.17	3.14	1.91	3.46	1.22	1.94	1.13	2.60	miscellaneous
20	Glyma07g33950.1	Leucine-rich repeat (LRR) family protein	10	0.00	3.59	2.75	3.66	2.90	2.04	2.88	3.07	1.76	not assigned
21	Glyma08g04380.1	Aldehyde dehydrogenase 2C4	11	0.00	2.37	2.38	0.15	2.46	1.43	1.26	1.21	2.19	fermentation
22	Glyma08g08970.1	Urease accessory protein G	4	0.00	0.25	0.26	0.12	0.19	0.43	0.14	0.31	0.33	amino acid metabolism

23	Glyma08g21410.1	HAD superfamily- subfamily IIIB acid phosphatase	9	0.00	4.63	6.91	3.49	3.76	1.41	0.80	2.21	2.83	miscellaneous
24	Glyma0985s00200.1	Ribosomal L5P family protein	4	0.00	0.08	0.08	0.03	0.04	0.21	0.45	0.25	0.21	protein
25	Glyma10g30110.1	HXXXD-type acyl-transferase family protein	13	0.00	3.65	4.63	3.87	4.16	1.75	1.15	2.29	2.03	stress
26	Glyma11g03330.1	Stress-inducible protein- putative	9	0.00	0.01	0.03	0.01	0.00	0.14	0.21	0.16	0.25	stress
27	Glyma11g07490.2	Isoflavone reductase	4	0.00	2.52	3.03	2.82	4.21	3.95	4.29	2.87	5.49	secondary metabolism
28	Glyma11g07496.1	Isoflavone reductase	3	0.00	0.84	0.01	0.00	0.03	0.80	0.08	0.39	0.51	secondary metabolism
29	Glyma11g07516.1	Similar to phenylcoumaran benzylic ether reductase	9	0.01	2.39	2.50	2.42	3.12	0.73	1.17	0.84	0.95	secondary metabolism
30	Glyma11g07670.1	Peroxidase superfamily protein	7	0.00	3.61	4.69	2.22	5.49	1.35	3.15	3.95	4.12	miscellaneous
31	Glyma11g19070.2	NAD- ADP-ribosyltransferases	12	0.00	0.04	0.04	0.04	0.05	0.03	0.04	0.02	0.07	protein
32	Glyma12g10850.1	Peroxidase superfamily protein	5	0.01	2.07	2.46	1.34	1.76	2.15	10.31	4.14	8.78	stress
33	Glyma12g30600.1	Histone deacetylase 2C	2	0.00	0.12	0.12	0.06	0.17	0.19	0.24	0.19	0.31	RNA
34	Glyma13g04050.1	Glutathione Stransferase C-terminal-like elongation factor	8	0.00	1.89	1.94	0.91	1.47	1.32	1.68	1.06	1.26	protein
35	Glyma13g18830.1	Ribosomal protein L14p/L23e family protein	5	0.00	0.08	0.08	0.01	0.00	0.29	0.03	0.28	0.43	protein
36	Glyma13g33590.1	Glyoxalase II 3	8	0.00	2.07	1.73	0.72	0.64	2.74	0.76	1.07	1.36	biodegrad of xenob
37	Glyma13g40100.2	Plasma membrane intrinsic protein 2	5	0.02	2.06	2.72	2.15	2.42	1.67	2.05	2.06	2.30	transport
38	Glyma13g42310.1	Lipoxygenase 1	11	0.00	0.10	0.01	0.00	0.01	0.24	0.04	0.07	0.06	hormone metabolism
39	Glyma14g05250.1	Subtilisin-like serine endopeptidase family protein	11	0.00	4.26	4.51	3.82	5.85	0.30	0.82	2.20	2.26	protein
40	Glyma14g06160.1	Ferritin 4	3	0.00	0.38	0.24	0.08	0.10	0.60	0.25	0.18	0.17	metal handling
41	Glyma15g01370.1	Protein of unknown functionDUF642	6	0.00	0.38	0.34	0.05	0.02	0.41	0.45	0.19	0.28	not assigned
42	Glyma15g03030.1	Lipoxygenase 1	16	0.00	0.14	0.00	0.00	0.00	0.12	0.06	0.09	0.04	hormone metabolism
43	Glyma15g07040.1	Quinone reductase family protein	7	0.00	5.11	8.25	7.95	9.93	1.86	3.12	3.77	5.40	lipid metabolism
44	Glyma15g13550.2	Peroxidase superfamily protein	8	0.00	3.25	2.50	6.68	5.58	1.69	3.44	3.30	5.49	miscellaneous
45	Glyma15g15200.1	Glycosyl hydrolase superfamily protein	4	0.04	9.76	9.86	7.42	10.21	2.07	7.70	5.32	6.19	miscellaneous
46	Glyma15g17620.1	Peroxidase superfamily protein	2	0.00	4.67	5.23	2.52	5.06	2.30	4.35	1.79	2.13	mmiscellaneous
47	Glyma15g39370.2	Glyoxalase II 3	3	0.00	3.65	4.87	4.46	7.04	10.66	6.15	3.01	3.55	biodegrad of xenob
48	Glyma15g40860.1	Ribosomal protein S5/Elongation factor G/III/V fp	22	0.00	0.55	0.07	0.02	0.07	0.45	0.15	0.44	0.28	protein

49	Glyma15g41540.1	Phosphoglycerate kinase family protein	7	0.02	1.68	1.83	1.39	1.58	1.10	1.14	0.85	1.13	PS.calvin cycle
50	Glyma15g42140.1	ATP-citrate lyase A1	2	0.00	0.18	0.10	0.01	0.01	0.17	0.01	0.25	0.28	TCA /org transform
51	Glyma16g07750.1	Unknown	53	0.00	1.09	0.36	0.79	0.88	1.49	0.65	0.86	0.98	not assigned
52	Glyma16g08460.1	NADP-malic enzyme 4	21	0.00	3.09	3.25	2.99	3.65	1.72	2.41	1.65	2.20	TCA /org transform
53	Glyma16g29450.1	Phosphatase-related	4	0.00	0.29	0.15	0.07	0.14	0.26	0.22	0.25	0.28	protein
54	Glyma17g07050.1	P450 reductase 2	3	0.00	0.25	0.21	0.05	0.09	0.12	0.06	0.20	0.34	miscellaneous
55	Glyma17g09280.1	Ribosomal protein L18e/L15 superfamily protein	2	0.00	0.09	0.07	0.13	0.22	0.05	0.08	0.08	0.29	protein
56	Glyma17g17730.1	Root hair specific 19	14	0.00	3.98	4.48	2.34	4.35	1.70	1.94	2.14	3.08	miscellaneous
57	Glyma17g18800.1	S adenosyl L-methionine-dependent methyltransferase SFP	4	0.00	1.63	2.35	0.66	0.91	1.38	1.36	1.57	1.63	secondary metabolism
58	Glyma18g02970.1	Ribosomal protein L1p/L10e family	3	0.00	0.01	0.03	0.02	0.06	0.01	0.11	0.05	0.22	protein
59	Glyma18g06510.1	NAD(P) binding Rossmann fold superfamily protein	7	0.00	9.07	8.56	4.50	4.31	0.79	7.05	4.06	1.80	secondary metabolism
60	Glyma18g08220.1	Heat shock protein 81.4	14	0.00	0.03	0.02	0.02	0.01	0.05	0.06	0.16	0.20	stress
61	Glyma18g14826.1	ATPase- AAA-type- CDC48 protein	3	0.00	0.03	0.00	0.00	0.00	0.03	0.01	0.21	0.15	cell
62	Glyma18g20600.2	Pyruvate phosphate dikinase-PEP/pyruvate binding domain	2	0.00	0.01	0.02	0.01	0.01	0.05	0.06	0.05	0.07	major CHO metabolism
63	Glyma18g46390.1	Phosphomannomutase	3	0.00	0.31	0.48	0.18	0.32	0.62	0.06	0.35	0.33	cell wall
64	Glyma18g48620.1	Hyaluronan / mRNA binding family	4	0.00	0.06	0.01	0.01	0.02	0.13	0.23	0.16	0.07	RNA
65	Glyma18g53700.1	Aldolase-type TIM barrel family protein	13	0.00	2.94	2.08	2.12	2.76	1.30	1.76	1.53	0.30	OPP
66	Glyma19g01120.1	Oxidoreductase zinc-binding dehydrogenase family protein	10	0.04	4.11	3.64	2.57	3.12	1.16	1.73	1.22	1.79	miscellaneous
67	Glyma19g03520.1	Ribosomal protein S19e family protein	4	0.00	0.12	0.12	0.04	0.03	0.24	0.26	0.33	0.41	protein
68	Glyma19g28740.1	H/ACA ribonucleoprotein complex-subunit Gar1/Naf1 prot	2	0.00	0.07	0.06	0.01	0.04	0.27	0.22	0.28	0.17	protein
69	Glyma19g40810.1	S-adenosylmethionine synthetase 2	10	0.00	0.64	0.29	0.06	0.22	0.42	0.42	0.32	0.08	amino acid metabolism
70	Glyma19g41220.1	Germin-like protein 5	3	0.00	5.45	8.09	7.13	12.13	1.87	5.05	3.46	4.53	stress
71	Glyma20g01580.1	Leucine-rich repeat (LRR) family protein	2	0.01	6.94	8.46	4.16	6.32	1.82	6.46	5.04	6.37	not assigned
72	Glyma20g30970.1	Ribosomal protein S13/S18 family	2	0.00	0.03	0.06	0.04	0.08	0.09	0.39	0.09	0.14	protein
73	Glyma20g33270.1	Coatomer- alpha subunit	7	0.00	0.05	0.03	0.01	0.01	0.09	0.04	0.05	0.16	cell

Protein No, same as shown in figure 13; MP, matched peptides; OPP, oxidative pentose phosphate pathway; PS, photo system; biodegrad of xenob, biodegradation of xenobiotics; major CHO, major carbohydrate; TCA, tricarboxylic acid

Table 4. Identified proteins in soybean roots that changed significantly during recovery after 4 days of flooding stress.

Protein No.	Protein ID	Protein description	MP	P Value	Ratios (Control)				Ratios (Post-flooding recovery)				Functional category
					6(0)/2(0)	8(0)/2(0)	10(0)/2(0)	12(0)/2(0)	6(4)/2(0)	8(4)/2(0)	10(4)/2(0)	12(4)/2(0)	
101	Glyma03g03460.1	Plant invertase/pectin methylesterase inhibitor	7	0.00	3.40	2.95	0.97	2.45	4.06	3.43	2.00	0.52	cell wall
102	Glyma05g03300.1	SNF1 related protein kinase regulatory subunit gamma 1	3	0.00	5.85	11.30	6.63	12.32	17.18	7.78	4.11	6.88	cell wall
23	Glyma08g21410.1	HAD superfamily subfamily IIIB acid phosphatase	7	0.03	19.00	14.49	7.71	3.25	0.54	0.87	1.73	1.89	miscellaneous
109	Glyma08g24760.1	MLP-like protein 43	6	0.00	11.80	14.74	8.79	21.94	1.57	3.58	4.46	12.25	stress
104	Glyma09g30910.1	S-glucosidase 44	4	0.04	2.93	2.16	1.92	2.35	1.49	2.00	2.15	1.59	miscellaneous
105	Glyma10g33350.2	Arabidopsis thaliana Peroxygenase 2	2	0.00	0.15	0.07	0.01	0.13	0.19	0.03	0.01	0.01	development
27	Glyma11g07490.2	Isoflavone reductase	8	0.02	1.77	1.49	1.11	1.84	1.71	1.63	1.42	3.33	secondary metabolism
106	Glyma13g34520.1	D-mannose binding lectin protein	5	0.00	3.50	2.11	2.81	6.04	6.59	7.95	9.13	4.27	miscellaneous
107	Glyma13g35480.1	Aluminium induced protein with YGL and LRDR motifs	2	0.01	3.36	2.55	3.38	4.53	5.37	2.85	0.72	2.12	hormone metabolism
108	Glyma13g37610.1	Ribosomal protein 5B	4	0.00	0.36	0.31	0.22	0.39	0.23	0.39	0.15	0.08	protein
38	Glyma13g42310.1	Lipoxygenase 1	10	0.00	0.08	0.08	0.03	0.07	0.42	0.32	0.18	0.07	hormone metabolism
39	Glyma14g05250.1	Subtilisin-like serine endopeptidase family protein	11	0.00	5.82	4.05	4.78	9.12	2.27	5.58	6.96	13.03	protein
42	Glyma15g03030.1	Lipoxygenase 1	13	0.00	0.11	0.05	0.06	0.09	0.26	0.24	0.17	0.06	hormone metabolism
44	Glyma15g13550.2	Peroxidase superfamily protein	6	0.00	4.28	5.55	4.96	8.61	1.43	5.23	7.29	5.05	miscellaneous
46	Glyma15g17620.1	Peroxidase superfamily protein	2	0.00	4.54	3.52	4.40	4.27	1.64	3.51	4.13	3.96	miscellaneous
109	Glyma15g31520.1	MLP-like protein 43	10	0.00	1.57	0.41	0.70	2.81	1.43	3.70	4.85	5.20	stress
51	Glyma16g07750.1	Unknown	41	0.00	2.54	2.87	1.53	1.94	1.24	1.99	1.86	3.53	not assigned
110	Glyma16g27880.1	Peroxidase superfamily protein	5	0.00	2.74	3.23	1.55	3.86	1.17	1.96	2.49	2.38	miscellaneous
111	Glyma17g03600.1	Haem oxygenase-like- multi-helical	3	0.00	0.40	0.26	0.18	0.21	0.46	0.48	0.28	0.31	not assigned
56	Glyma17g17730.1	Root hair specific 19	14	0.00	5.76	5.55	4.62	5.32	1.31	3.00	3.01	1.35	miscellaneous
112	Glyma18g10120.1	Co-chaperone GrpE family protein	3	0.00	0.21	0.32	0.19	0.34	0.35	0.58	0.49	0.38	protein

Protein No., same as shown in figure 13; MP, matched peptides

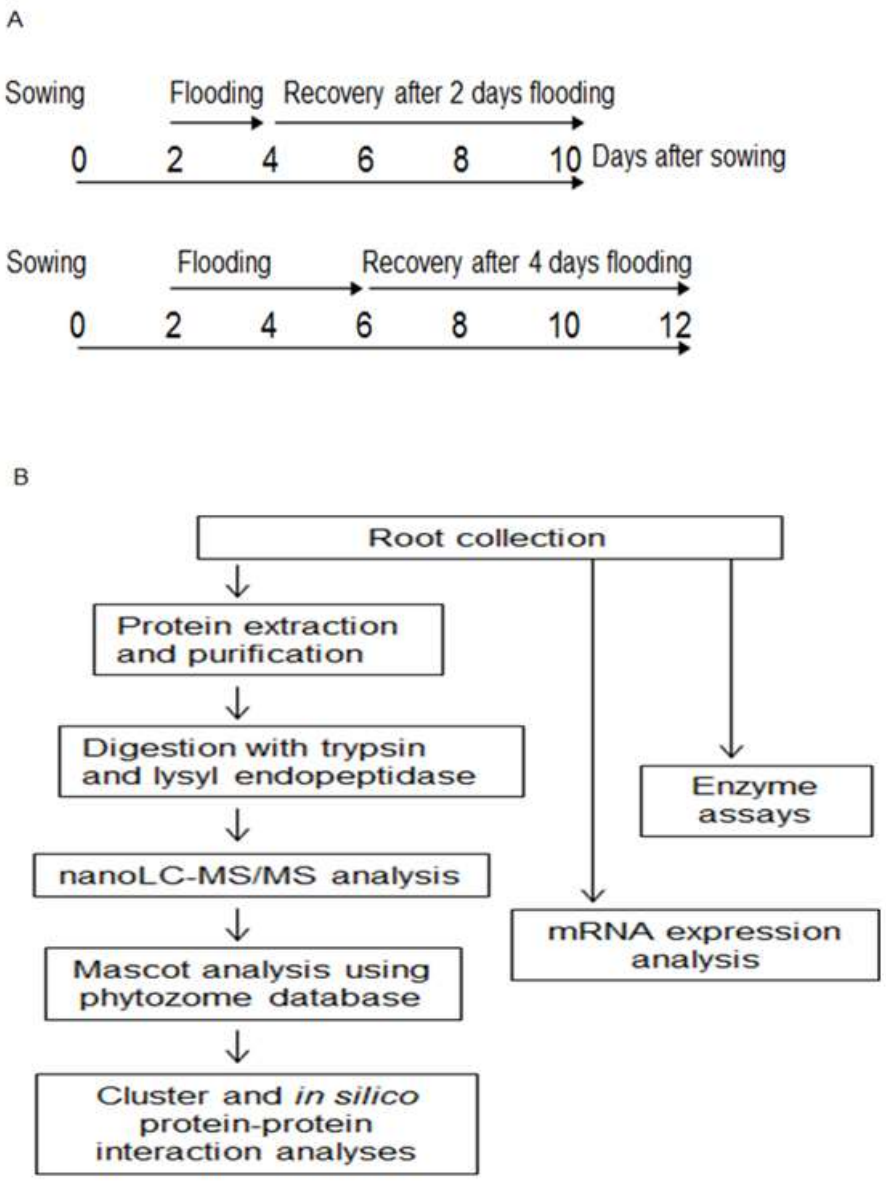
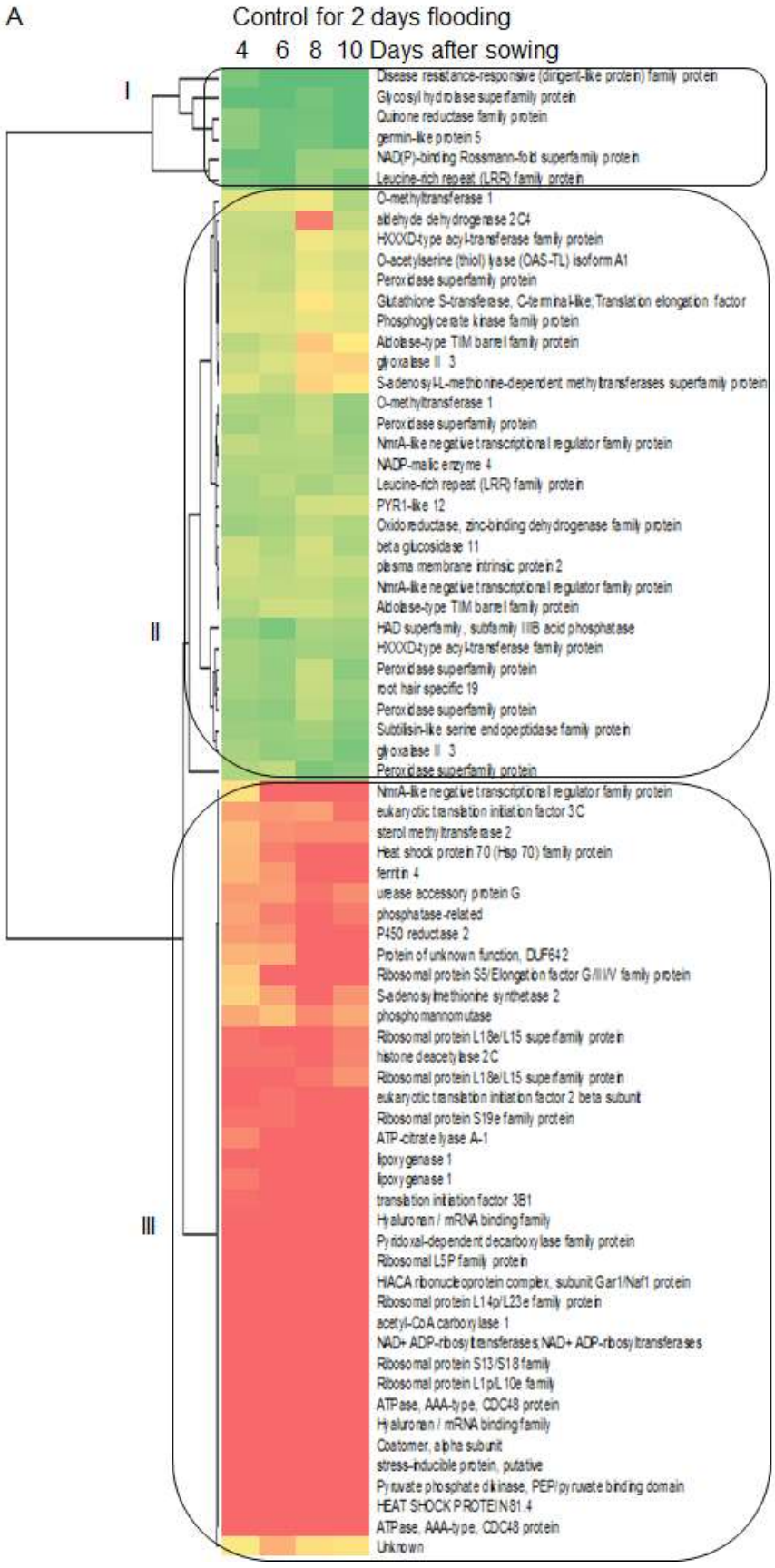
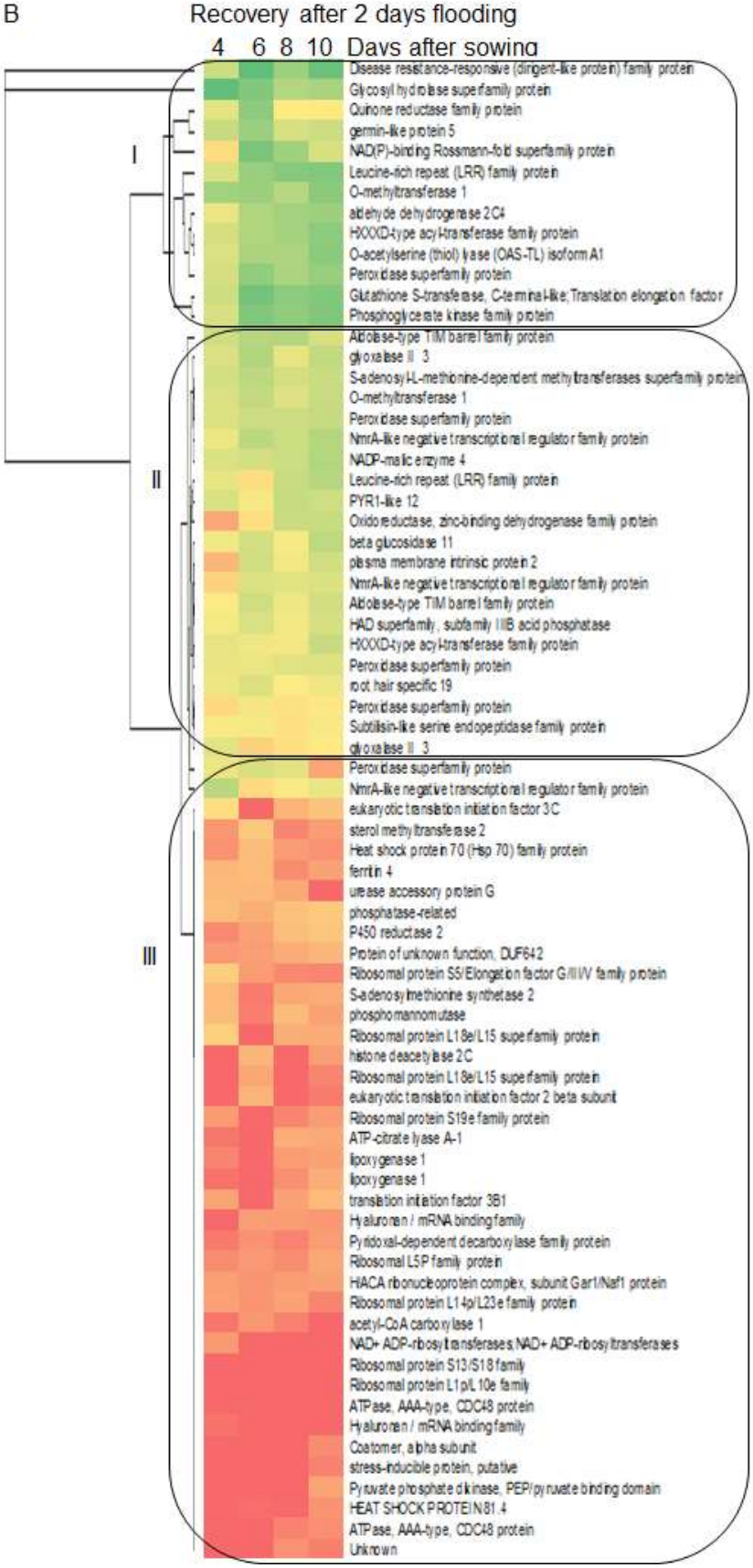


Figure 11. Experimental design used in the analysis of soybean root during post-flooding recovery. Two-day-old soybeans were flooded for 2 and 4 days. Roots were collected from the control and 2 days, 4 days flooded soybeans in the recovery phase (A). Proteins were extracted from roots and subjected to proteomic, transcript, and enzyme activity level analyses (B). Three independent biological replicates were performed for each experiment.


A
Control for 2 days flooding
4 6 8 10 Days after sowing
I
II
III
Disease resistance-responsive (dirigent-like protein) family protein
Glycosyl hydrolase superfamily protein
Quinone reductase family protein
germin-like protein 5
NAD(P)-binding Rossmann-fold superfamily protein
Leucine-rich repeat (LRR) family protein
O-methyltransferase 1
aldehyde dehydrogenase 2C4
HXXXD-type acyl-transferase family protein
O-acetylserine (thiol) lyase (OAS-TL) isoform A1
Peroxidase superfamily protein
Glutathione S-transferase, C-terminal-like; Translation elongation factor
Phosphoglycerate kinase family protein
Aldolase-type TIM barrel family protein
glyoxalase II 3
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
O-methyltransferase 1
Peroxidase superfamily protein
NmrA-like negative transcriptional regulator family protein
NADP-malic enzyme 4
Leucine-rich repeat (LRR) family protein
PYR1-like 12
Oxidoreductase, zinc-binding dehydrogenase family protein
beta glucosidase 11
plasma membrane intrinsic protein 2
NmrA-like negative transcriptional regulator family protein
Aldolase-type TIM barrel family protein
HAD superfamily, subfamily IIIB acid phosphatase
HXXXD-type acyl-transferase family protein
Peroxidase superfamily protein
root hair specific 19
Peroxidase superfamily protein
Subtilisin-like serine endopeptidase family protein
glyoxalase II 3
Peroxidase superfamily protein
NmrA-like negative transcriptional regulator family protein
eukaryotic translation initiation factor 3C
sterol methyltransferase 2
Heat shock protein 70 (Hsp 70) family protein
ferritin 4
urease accessory protein G
phosphatase-related
P450 reductase 2
Protein of unknown function, DUF642
Ribosomal protein S5/Elongation factor G/III/V family protein
S-adenosylmethionine synthetase 2
phosphomannomutase
Ribosomal protein L18e/L15 superfamily protein
histone deacetylase 2C
Ribosomal protein L18e/L15 superfamily protein
eukaryotic translation initiation factor 2 beta subunit
Ribosomal protein S19e family protein
ATP-citrate lyase A-1
lipoxygenase 1
lipoxygenase 1
translation initiation factor 3B1
Hyaluronan / mRNA binding family
Pyridoxal-dependent decarboxylase family protein
Ribosomal L5P family protein
HIACA ribonucleoprotein complex, subunit Gar1/Naf1 protein
Ribosomal protein L14p/L23e family protein
acetyl-CoA carboxylase 1
NAD+ ADP-ribosyltransferases; NAD+ ADP-ribosyltransferases
Ribosomal protein S13/S18 family
Ribosomal protein L1p/L10e family
ATPase, AAA-type, CDC48 protein
Hyaluronan / mRNA binding family
Coatomer, alpha subunit
stress-inducible protein, putative
Pyruvate phosphate dikinase, PEP/pyruvate binding domain
HEAT SHOCK PROTEIN 81.4
ATPase, AAA-type, CDC48 protein
Unknown


B
Recovery after 2 days flooding
4 6 8 10 Days after sowing
I
II
III
Disease resistance-responsive (dirigent-like protein) family protein
Glycosyl hydrolase superfamily protein
Quinone reductase family protein
germin-like protein 5
NAD(P)-binding Rossmann-fold superfamily protein
Leucine-rich repeat (LRR) family protein
O-methyltransferase 1
aldehyde dehydrogenase 2C4
HXXXD-type acyl-transferase family protein
O-acetylserine (thiol) lyase (OAS-TL) isoform A1
Peroxidase superfamily protein
Glutathione S-transferase, C-terminal-like; Translation elongation factor
Phosphoglycerate kinase family protein
Aldolase-type TIM barrel family protein
glyoxalase II 3
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
O-methyltransferase 1
Peroxidase superfamily protein
NmrA-like negative transcriptional regulator family protein
NADP-malic enzyme 4
Leucine-rich repeat (LRR) family protein
PYR1-like 12
Oxidoreductase, zinc-binding dehydrogenase family protein
beta glucosidase 11
plasma membrane intrinsic protein 2
NmrA-like negative transcriptional regulator family protein
Aldolase-type TIM barrel family protein
HAD superfamily, subfamily IIIB acid phosphatase
HXXXD-type acyl-transferase family protein
Peroxidase superfamily protein
root hair specific 19
Peroxidase superfamily protein
Subtilisin-like serine endopeptidase family protein
glyoxalase II 3
Peroxidase superfamily protein
NmrA-like negative transcriptional regulator family protein
eukaryotic translation initiation factor 3C
sterol methyltransferase 2
Heat shock protein 70 (Hsp 70) family protein
ferritin 4
urease accessory protein G
phosphatase-related
P450 reductase 2
Protein of unknown function, DUF642
Ribosomal protein S5/Elongation factor G/III/V family protein
S-adenosylmethionine synthetase 2
phosphomannomutase
Ribosomal protein L18e/L15 superfamily protein
histone deacetylase 2C
Ribosomal protein L18e/L15 superfamily protein
eukaryotic translation initiation factor 2 beta subunit
Ribosomal protein S19e family protein
ATP-citrate lyase A-1
lipoxygenase 1
lipoxygenase 1
translation initiation factor 3B1
Hyaluronan / mRNA binding family
Pyridoxal-dependent decarboxylase family protein
Ribosomal L5P family protein
HIACA ribonucleoprotein complex, subunit Gar1/Naf1 protein
Ribosomal protein L14p/L23e family protein
acetyl-CoA carboxylase 1
NAD+ ADP-ribosyltransferases; NAD+ ADP-ribosyltransferases
Ribosomal protein S13/S18 family
Ribosomal protein L1p/L10e family
ATPase, AAA-type, CDC48 protein
Hyaluronan / mRNA binding family
Coatomer, alpha subunit
stress-inducible protein, putative
Pyruvate phosphate dikinase, PEP/pyruvate binding domain
HEAT SHOCK PROTEIN 81.4
ATPase, AAA-type, CDC48 protein
Unknown

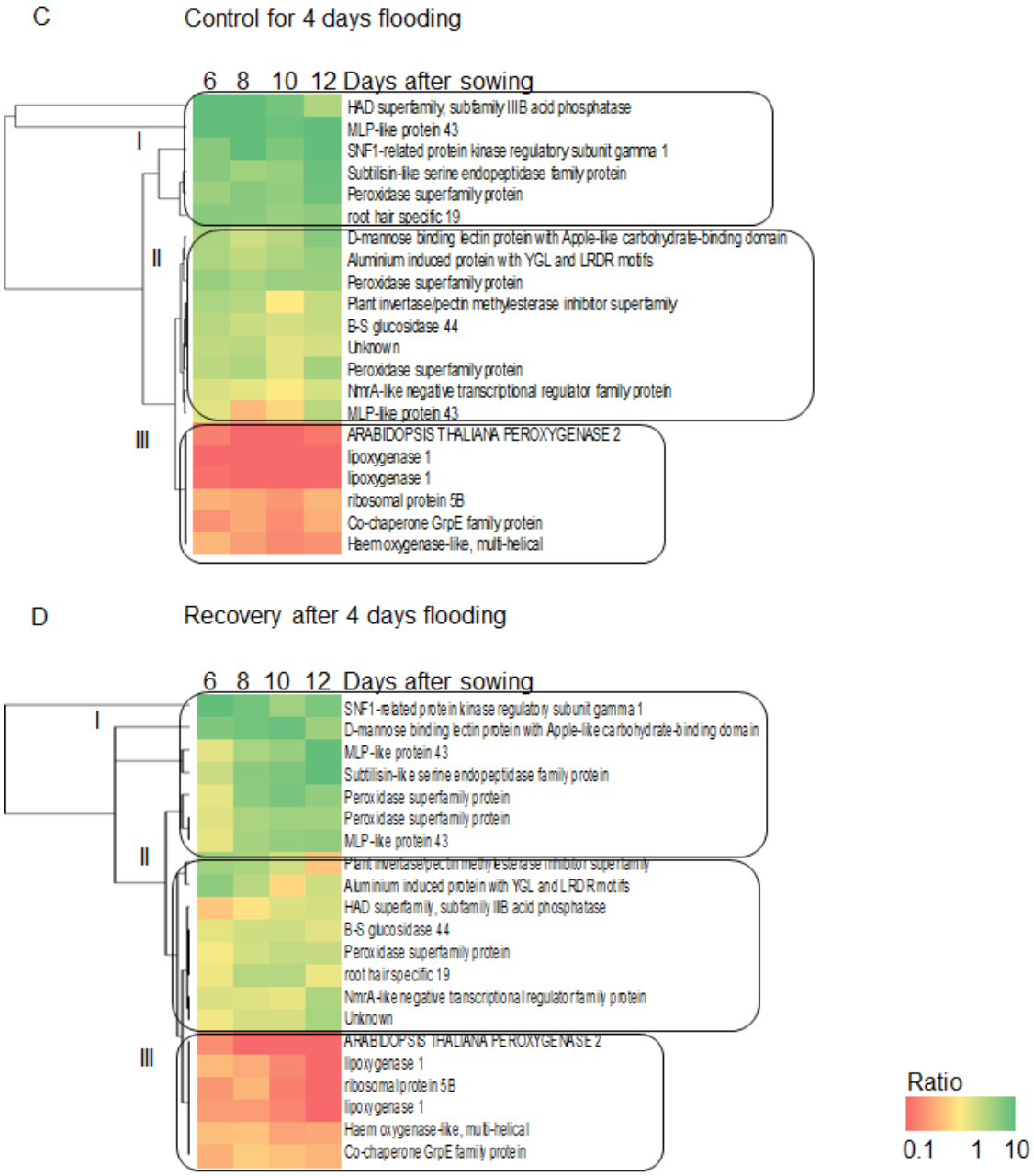


Figure 12. Cluster analysis of flooding recovery-responsive proteins in soybean root. Seventy three and 21 proteins changed during recovery after 2 (A, B) and 4(C, D) days flooding were analyzed by using the induction levels of the proteins. Clustering results of the proteins in control (A, C) and recovery (B, D) estimated based on time course data of the ratios when compared with 2-day-old soybeans.

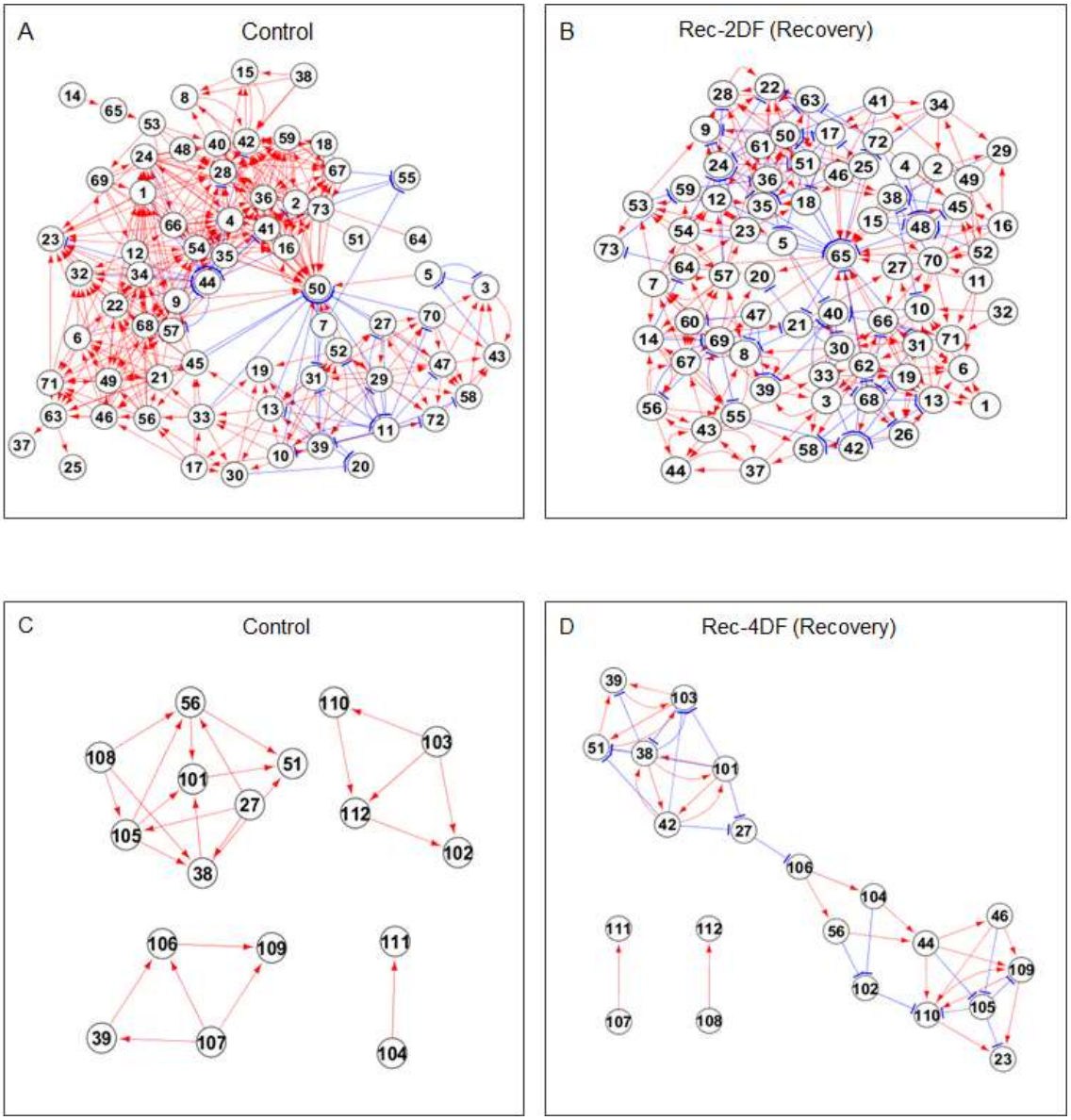


Figure 13. *In silico* protein-protein interaction analysis of flooding recovery-responsive proteins in soybean root. Protein interactions of the control and recovery after 2 days flooding (A, B; Rec-2DF) and 4 days flooding (C, D; Rec-4DF) estimated based on time course data of the ratios when compared with untreated 4-day-old soybeans and 2-days treated 4-day-old soybeans for 2 days treatment, and with untreated 6-day-old soybeans and 4-days treated 6-day-old soybeans for 4 days treatment. A red arrow shows an inductive interaction and a blue T-bar shows a suppressive interaction. Protein numbers are the same as in Tables 3 and 4.

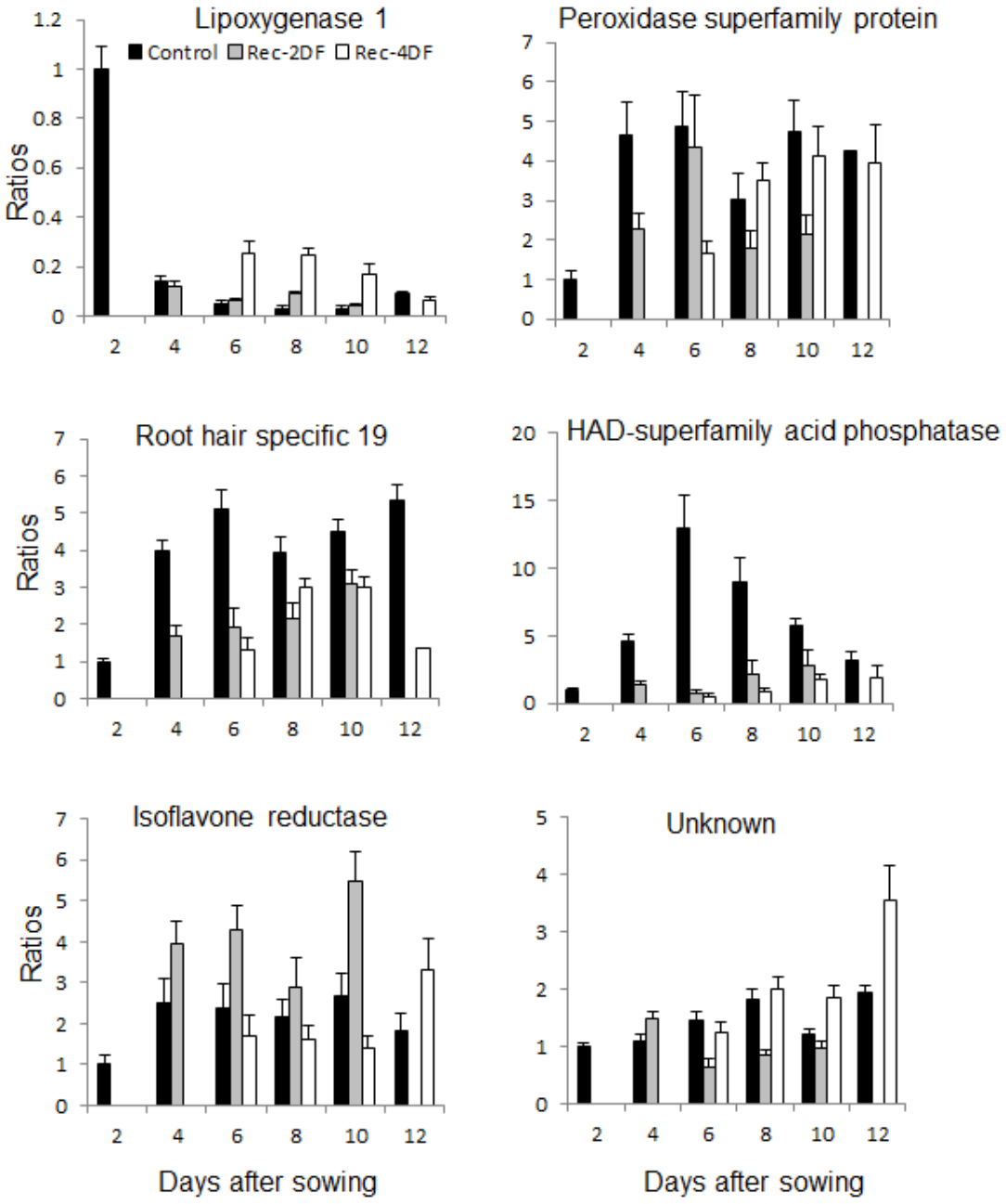


Figure 14. Changes in protein abundance in soybean root during recovery after flooding. Abundance changes of 6 common proteins between 2 (Rec-2DF) and 4 days (Rec-4DF) post-flooding recovery were calculated. Black, grey and white columns indicate abundance ratios of control, recovery following 2-days flooding, and recovery following 4-days flooding, respectively. Figure was prepared using protein ratios obtained after SIEVE analysis. Data are means ± SE from three independent biological replicates (n=3).

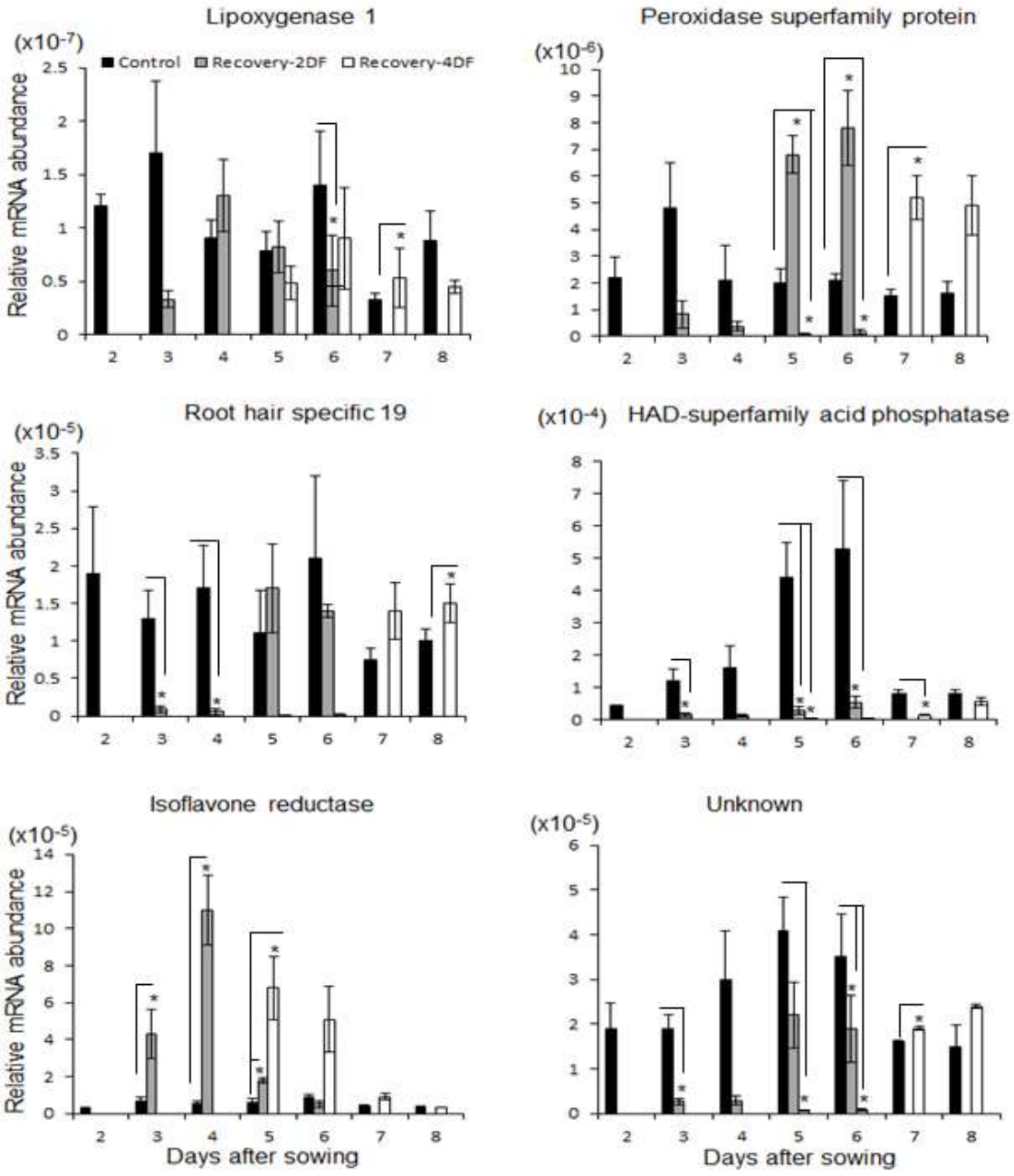


Figure 15. mRNA expression levels of genes encoding selected proteins during recovery after flooding in soybean root. The mRNA abundance of selected 6 genes was analyzed by qRT-PCR. The expression data was normalized using 18S rRNA. Black, grey and white columns indicate changes in mRNA abundance of control, recovery following 2-days flooding (Rec-2DF) and recovery following 4-days flooding (Rec-4DF), respectively. Data are means ± SE from three independent biological replicates (n=3). Asterisk (*) mark above the error bars shows significant changes between age-matched control and treated seedlings as determined by Student's *t*-test.

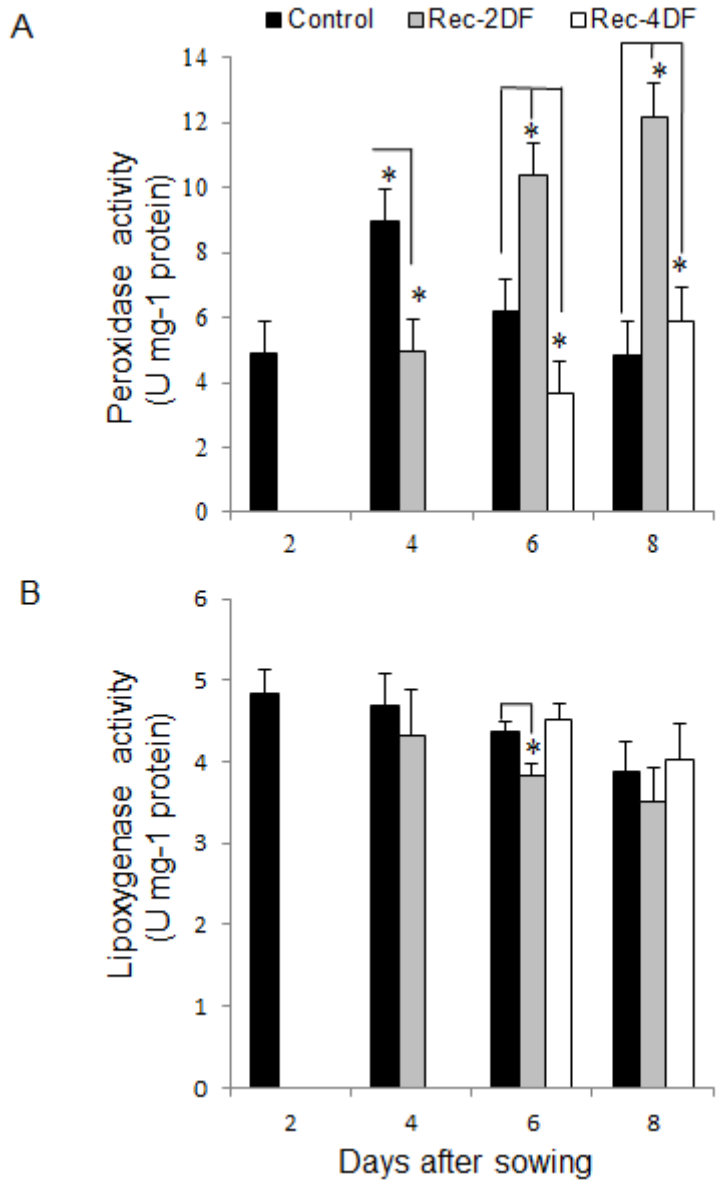


Figure 16. Peroxidase and lipoxygenase activities during recovery after flooding in soybean root. Two-day-old soybeans were flooded for 2 and 4 days and roots were collected under flooding and during recovery after 2 days flooding (Rec-2DF) and 4 days flooding (Rec-4DF). Activities of peroxidase (A) and lipoxygenase (B) were analyzed during flooding and post-flooding recovery. Black, grey and white columns indicate enzyme activities in control, recovery following 2-days flooding, and recovery following 4-days flooding, respectively. Data are means ± SE from three independent biological replicates. Asterisk (*) mark above the error bars shows significant changes between age-matched control and treated seedlings as determined by Student's *t*-test.

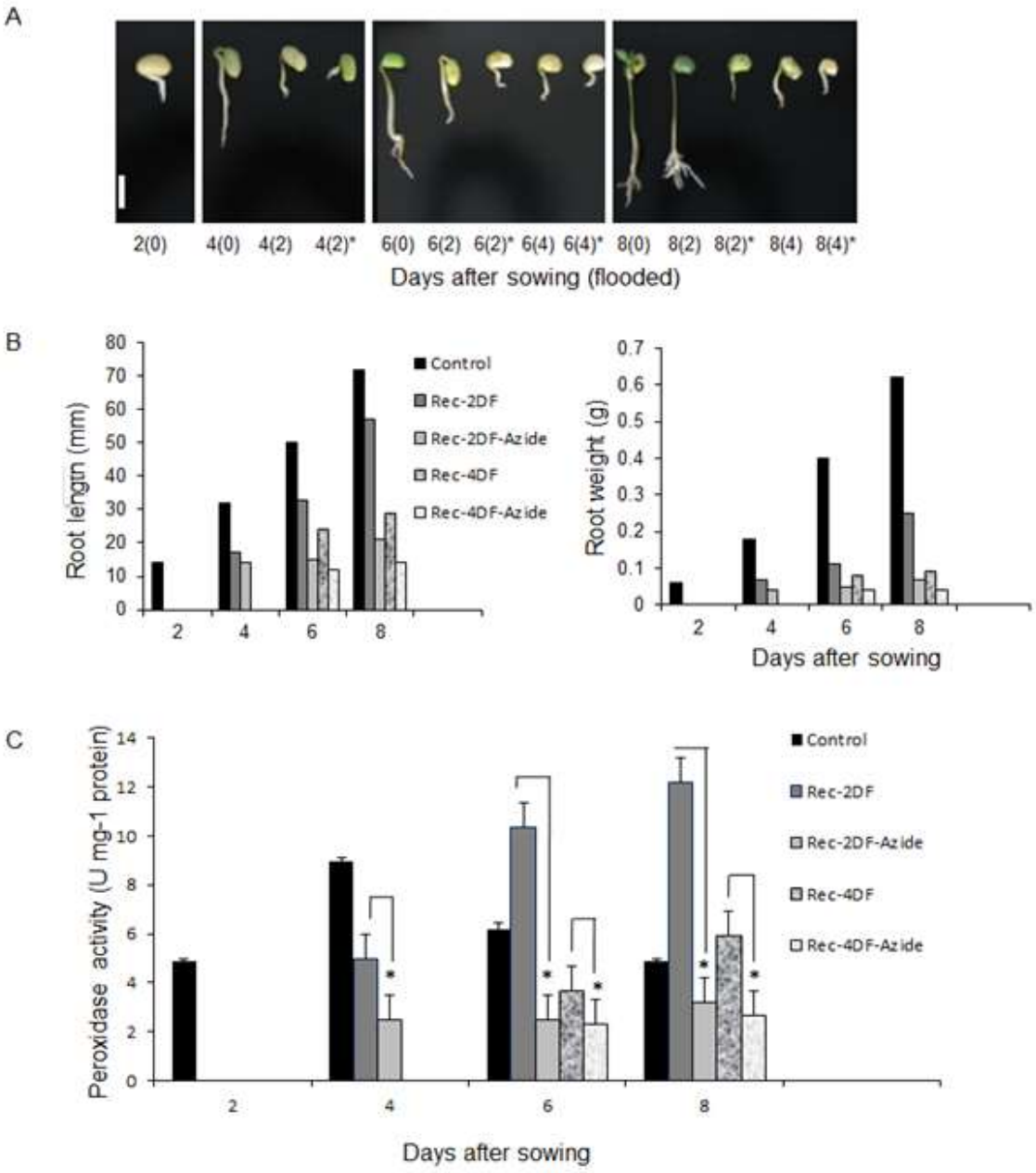


Figure 17. Peroxidase activity inhibition assay during recovery after flooding in soybean root. Two-day-old soybeans were flooded for 2 and 4 days with or without sodium azide and roots were collected under flooding/sodium azide treated, and after water removal during recovery stage. Activity of peroxidase was analyzed during treatment and recovery stages. Data are means ± SE from three independent biological replicates (n=3). Asterisk (*) mark in A indicates sodium azide treated seedlings while above the error bars in C, it shows significant changes between flooded without or with sodium azide as determined by Student's *t*-test. Bar indicates 10 mm.

CHAPTER 4
CHARACTERIZATION OF POST-FLOODING RECOVERY-RESPONSIVE PROTEINS IN SOYBEAN

4.1. Introduction

Soybean attempts to recover following short-term flooding as few proteomic studies shed light on the post-flooding recovery. Proteomic analysis of post-flooding recovery in soybean revealed that long chain fatty acids synthesizing beta-ketoacyl reductase and nucleotides metabolism related nucleotidyl transferase were significantly induced in hypocotyl during the recovery (Chapter 2). The beta-ketoacyl reductases are the enzymes involved in the biosynthesis of complex fatty acids and lipids such as waxes. These enzymes were involved in microsomal fatty acid elongation in *Arabidopsis* (Beaudoin et al., 2009) and in fatty acid elongation in maize development (Dietrich et al., 2005). Nucleotidyl transferases such as adenylyl transferases play vital role in normal growth as well as enhance tolerance against salt and osmotic stresses (Rubio et al., 2008). These reports suggested involvement of the mentioned enzymes in abiotic stress responses, though post-stress recovery has yet to be investigated.

Proteomic analysis of post-flooding recovery in soybean revealed that peroxidase and haloacid dehalogenase-acid phosphatase were significantly changed in soybean root (Chapter 3). Peroxidases are involved in a variety of physiological functions such as lignin and suberin formation, cross-linking of cell wall components, and metabolism of ROS (Almagro et al., 2009; Herrero et al., 2013). Plant peroxidases possess antioxidant activity and were induced by flooding (Kausar et al., 2012), drought (Kausar et al., 2012), metallic (Jouili et al., 2011), and cold stresses (Nourredine et al., 2015). Acid phosphatases catalyze the hydrolysis of phosphate monoesters at acidic pH (Vincent et al., 1992). Purple acid phosphatases play significant role in phosphorus foraging and recycling in *Arabidopsis* (Tran et al., 2010). Acid phosphatases were associated with abiotic stress responses such as flooding (Komatsu et al., 2009a), drought/salinity (Ehsanpour and Amini, 2003), heat (Babu et al., 2008), and cold (Hu et al., 2010). Soybean post-flooding recovery research is a new avenue and the understanding of the underlying mechanisms behind post-flooding recovery may provide knowledge about responsive proteins that can form basis for development of tolerant varieties.

Global environmental changes are transforming weather patterns that lead to frequent flooding events, extreme drought, heat, cold, salinity and other abiotic stresses (Versulues et al., 2006). Abiotic stress acts as a main limiting factor that affects plant

distribution (Chaves et al., 2003), disturbs plant vegetation (Bray et al., 2000), and impairs growth and yield of crops around the world (Mittler and Blumwald, 2010). Plant responds to abiotic stress at molecular, cellular, physiological, and biochemical levels (Yamaguchi-Shinozaki and Shinozaki, 2006). Phytohormones such as jasmonate and salicylate play important roles in the regulation of developmental processes and signaling networks in plants under abiotic stresses (Yoon et al., 2009; Gemes et al., 2011). Jasmonate and methyl jasmonate are growth regulators in higher plants (Komatsu et al., 2013a). Jasmonate accumulated rapidly after wounding (Creelman et al., 1992), triggering its biosynthesis from linolenic acid (Farmer and Ryan, 1990), suggesting that jasmonate is an important molecule in stress signaling in plants. Salicylate plays role in plant responses to abiotic stresses including osmotic stress, drought, salt, heat, and UV stress (Horvath et al., 2007; Nazar et al., 2011). In order to get more insight into involvement of these 4 enzymes in stress responses and post-stress recovery, the activity changes of these enzymes to flooding, drought, and cold stresses as well as with jasmonate and salicylate treatments were analyzed in soybean.

4.2. Materials and methods

4.2.1. Plant materials and treatments

Materials and growth conditions are same as mentioned in section 1.2.1. Two-day-old soybeans were treated for 2 days and root and hypocotyl were collected under treatment (4 days) and then at recovery point (6 days) after removal of the stress (Figure 18). Flooding stress was applied by complete submerging the tray containing seedling cases. Drought stress was applied by withholding the water supply and cold stress was applied by growing at 4°C. Jasmonate and salicylate treatments in the concentrations of 100 μM were applied to the flooded seedlings in glass tubes, each containing 4 seedlings in 120 mL water. Same-aged seedlings without treatment were grown as control. Three independent biological replicates were performed for each experiment.

4.2.2. Enzyme activity assays

Peroxidase assay: Same as described in section 3.2.10.

Beta-ketoacyl reductase assay: Same as described in section 2.2.10.

Nucleotidyl transferase assay: Same as described in section 2.2.10.

Acid phosphatase assay: A portion (125 mg) of root and hypocotyl was grounded separately at 4°C in 1 mL of extraction buffer that consisted of 50 mM Na_3PO_4 (pH7.0), 10 mM EDTA, 0.1 % Triton X-100, 1 % SDS, and 10 mM mercaptoethanol. Supernatant was collected by centrifugation at 17,970 × g at 4°C for 15 min, and used as enzyme extract. The reaction mixture for determining the activity consisted of 335 µL of 0.1 M citrate buffer (pH 4.8), 165 µL of substrate mix consisting of 100 µL *p*-nitrophenyl phosphate dissolved in 200 µL of 0.1 M citrate buffer, and mixed with 100 µL of the enzyme extract. This mixture was incubated at 37°C for 5 min. The reaction was stopped by adding 335 µL NaOH, and followed by absorption measurement at 405 nm (Suen et al., 2015).

4.2.3. Protein concentration measurement

Same as mentioned in section 2.2.2.

4.2.4. Statistical analysis

The results of the activity assays were analyzed for statistical significance using Tukey's HSD test.

4.3. Results

4.3.1. Protein abundance changes in the selected proteins during recovery after flooding stress

The proteomics of post-flooding recovery in soybean root and hypocotyl has been analyzed recently as discussed in chapters 2 and 3. In the root, 2 proteins that is peroxidase superfamily protein and haloacid dehalogenase-acid phosphatase were significantly changed during the post-flooding recovery stage (Fig. 19A, B). In hypocotyl, 2 proteins that is beta-ketoacyl reductase and nucleotidyl transferase were significantly changed during the post-flooding recovery stage (Fig. 19C, D). Peroxidase was decreased under flooding and increased during post-flooding recovery in roots (Fig. 19A). Haloacid dehalogenase-acid phosphatase was clearly decreased under flooding but gradually increased during the recovery (Fig. 19B). In hypocotyl, protein

abundances of beta-ketoacyl reductase (Fig. 19C) and nucleotidyl transferase (Fig. 19D) were increased under flooding but decreased gradually as the post-flooding recovery proceeded. Based on the changes in protein abundances during post-flooding recovery, these 4 enzymes were characterized using their enzyme activity patterns under flooding, drought, and cold stresses, as well as phytohormones like jasmonate and salicylate treatments.

4.3.2. Enzyme activities changes under flooding, drought, and cold stresses

The pattern of activity change of peroxidase was different under various stresses in root (Fig. 20A) and hypocotyl (Fig. 20B). Peroxidase activity decreased under flooding stress in both root and hypocotyl, but increased during the post-flooding recovery, although increase in activity during the recovery was still less than that of control plants. The activity of peroxidase increased noticeably under drought and decreased during post-drought period in both root and hypocotyl. The enzyme activity was highly suppressed under cold stress, but increased significantly during the post-cold stage in root and hypocotyl.

The enzyme activity of acid phosphatase was decreased significantly under flooding stress and slightly increased during recovery in root (Fig. 20C). In hypocotyl, it was significantly decreased by flooding and further decreased during the recovery stage. Fig. 20D). In root under drought stress, the enzyme activity was almost similar to age-matched control seedlings, but decreased during the post-drought recovery (Fig. 20C). In hypocotyl, activity was decreased under drought (Fig. 20D). The activity was suppressed by cold treatment but recovered in root (Fig. 20C); however, in hypocotyl, opposite trend was evident (Fig. 20D).

The enzyme activity of beta-ketoacyl reductase remained unchanged under flooding in root (Fig. 20E), however, it decreased in hypocotyl under flooding and increased slightly during the recovery stage (Fig. 20F). The activity of the enzyme trended towards increase in both organs during the post-drought and post-cold recovery stages (Fig. 20E, F).

Flooding stress significantly increased the activity of nucleotidyl transferase which was further increased during recovery stage in root (Fig. 20G). In hypocotyl,

activity decreased under flooding but recovered (Fig. 20H). The enzyme activity increased during drought stress and post-drought stages in root (Fig. 20G), however, in hypocotyl, the activity was much lower than the age-matched control seedlings (Fig. 20H). The activity was decreased in root by cold treatment but recovered (Fig. 20G). In hypocotyl activity did not recover to the level of untreated seedlings (Fig. 20H).

4.3.3. Enzyme activity changes by jasmonate and salicylate treatment

Treatment of flooding-stressed soybean with phytohormones indicated activity changes in the analyzed enzymes. Jasmonate application significantly suppressed peroxidase activity, which further decreased during the post-flooding stage in both root and hypocotyl (Fig. 21A, B). The activity of acid phosphatase was significantly suppressed by jasmonate in both organs, however, it trended clearly towards increase during recovery stage (Fig. 21C, D). The enzyme activity of beta-ketoacyl reductase was slightly changed by jasmonate treatment in root while it was significantly suppressed in hypocotyl (Fig. 21E, F). The enzyme activity of nucleotidyl transferase was highly enhanced by jasmonate addition in root (Fig. 21G); however, in hypocotyl, it was decreased under flooding stress (Fig. 21H).

The salicylate treatment decreased the activity of peroxidase in both root and hypocotyl (Fig. 21A, B). The activity of acid phosphatase was significantly suppressed by salicylate in root; however, it trended clearly towards increase during the post-stress stage (Fig. 21C). In hypocotyl, salicylate decreased the enzyme activity and could not recover (Fig. 21D). Salicylate stress slightly decreased the activity of beta-ketoacyl reductase in root (Fig. 21E); however, in hypocotyl, activity was more decreased by salicylate treatment (Fig. 21F). Salicylate addition increased the activity of nucleotidyl transferase in root that further increased significantly during the recovery stage (Fig. 21G). In hypocotyl, it decreased the activity significantly that could not recover (Fig. 21H).

4.4. Discussion

4.4.1. Recovery responsive enzymes respond to flooding, drought and cold stresses in soybean

All the 4 analyzed enzymes depicted significant differential changes in enzyme activities when analyzed under flooding, drought, and cold stresses and during post-stress stages. Peroxidase decreasaed under flooding stress, but could not recover during recovery. Earlier, as mentioned in chapter 3, peroxidase activity was increased during the recovery stage. Peroxidases actively participate in ROS metabolism and cell wall structure (Almagro et al., 2009; Herrero et al., 2013). Peroxidases were decreased under flooding stress in soybean (Kausar et al., 2012), wheat (Csiszar et al., 2012), and maize (Meisrimler et al., 2014). Kausar et al. (2012) reported increase in peroxidase under drought stress. Varga et al. (2012) stated fall in ascorbate peroxidase activity in cereals during cold winter. However, activity was raised in Medicago under cold stress (Nourredine et al., 2015). The involvement of peroxidases in ROS metabolism and cell wall structure might have helped the soybean seedlings to recover from stress-induced damage by scavenging toxic ROS and strengthening cell wall.

Acid phosphatase activity was decreased under flooding and cold stresses and increased significantly in root during the recovery stages. Drought did not change the activity in root. Acid phosphatase gene was down-regulated in soybean root including hypocotyl under flooding stress (Komatsu et al., 2009a), and protein abundance was also decreased in flooding-stressed soybean root (Chapter 3). In Medicago sativa, acid phosphatase activity was increased under drought stress (Ehsanpour and Amini, 2003). Inositol-5-phosphatase increased the tolerance against drought in Arabidopsis (Perera et al. 2008). An overexpression of maize protein phosphatase imparted cold tolerance in tobacco (Hu et al., 2010), indicating vital role of the phosphatase in stress tolerance. Tran et al. (2010) reported involvement of acid phosphatases in phosphorus foraging and recycling. Overexpression of trehalose-6-phosphatase genes conferred tolerance against cold treatment in rice (Ge et al., 2008). Current study indicates that acid phosphatase respond to stress and post-stress recovery changes in soybean, possibly by phosphorus foraging and recycling.

Beta-ketoacyl reductase activity did not change in soybean root under flooding stress; however, it was significantly decreased in hypocotyl. The activity was significantly suppressed under drought and cold stresses, but increased during the post-cold stage. In hypocotyl, the activity was suppressed under drought and cold stresses

and did not recover. The enzyme activity of beta-ketoacyl reductase was slightly decreased under flooding and progressively increased in hypocotyl during recovery stage (Chapter 2). This enzyme is involved in the synthesis of complex long chain fatty acids for the synthesis of waxes in the cell wall, as the suppression in the activity resulted in decreased cuticle wax (Beaudoin et al., 2009). Long-chain fatty acids containing lipids were deposited on primary plant surfaces to act as a barrier for pathogens (Jenks et al., 1994). Waxes serve as protective barrier for plant cells and a decrease in beta-ketoacyl reductase under flooding and other stresses indicates the weakening of cuticle. These results suggest that the decrease in activity under the stresses and relative increase during the recovery stage indicated changes in the waxes content of the cuticle during the stress and post-stress stages, which might have facilitated recovery.

The activity of nucleotidyl transferase was significantly increased in soybean root under flooding stress; however, it was decreased in hypocotyl. Nucleotidyl transferases are a big family of enzymes that include important enzymes like adenylyl transferases and guanylyl transferases. These enzymes catalyze vital chemical reactions related to nucleotide metabolism. Nicotinamide mononucleotide adenylyltransferase catalyzes NAD biosynthesis from nicotinamide mononucleotide and ATP. In *Arabidopsis,* pyrophosphohydrolase maintains NAD^+ levels under oxidative stress by recycling nucleotides and regulates defense mechanisms against oxidative DNA damage (Ishikawa et al., 2009). Rubio et al. (2008) reported that phosphopantetheine adenylyltransferase plays a vital role in growth and osmotic stress resistance in *Arabidopsis*. These reports and current study concise vital roles of nucleotidyl transferases in stress response and recovery through changes in nucleotide metabolism. In chapters 2 and 3, the protein abundances of these 4 proteins were not shown in both organs. The protein abundances changed in both organs, but due to statistical limitations, beta-ketoacyl reductase and nucleotidyl transferase were removed from root proteomics data and peroxidase and acid phosphatase were removed from hypocotyl proteomics data.

4.4.2. Jasmonate and salicylate significantly affect activities of recovery-responsive

enzymes in soybean

Jasmonate suppressed the activities of the peroxidase and acid phosphatase in soybean root and hypocotyl under flooding stress; however, acid phosphatase increased significantly during the recovery stage in root. The decrease in activity of beta-ketoacyl reductase by jasmonate in hypocotyl and no change in root points towards organ-specific responses. Jasmonate enhanced the activity of nucleotidyl transferase in root, but opposite happened in hypocotyl, indicating organ-specifc responses to jasmonate addition in soybean under flooding stress. Phytohormones have been reported to play essential roles in stress responses. Jasmonate has a central role in plant development and reproduction (Creelman and Mullet, 1995; Browse, 2009), and has been linked in responses to salt, drought, and chilling stresses (Santino et al., 2013). An increase in the level of jasmonate was reported from salt-treated hairy roots, indicating its involvement in the response to salt (Kramell et al., 2000). Methyl jasmonate improved survival ratio in rice seedlings under chilling stress (Lee et al., 1997). Komatsu et al. (2010) reported that jasmonate synthesis is decreased in soybean under flooding stress. In this study, jasmonate-treated seedlings recovered following flooding removal. Results of the current study suggest that jasmonate-treated soybeans recovered following stress removal, indicating its role in post-flooding recovery.

Salicylate suppressed the activities of peroxidase, acid phosphatase, and beta-ketoacyl reductase, which could not recover. However, salicylate treatment significanatly enhanced the activity of nucleotidyl transferase in root during recovery, but it was suppressed in hypocotyl. Salicylate is involved in thermogenesis, stomatal closure or flowering (Raskin et al., 1992). It plays a role as signal in the activation of defense responses against microbial pathogens (Garcion and Metraux, 2006). Salicylate operates along with other phytohormones, such as jasmonate or ethylene and is part of a signaling network with crosstalk and synergisms that determine an optimal response to a single or combination of stresses (Fujita et al., 2006; Koorneef and Pieterse, 2008; Spoel and Dong, 2008). Global expression-profiling studies provided evidence that salicylate, jasmonate, and ethylene pathways interact either positively or negatively (Glazebrook et al., 2003; de Vos et al., 2005). Salicylate suppressed the activities of all enzymes except nucleotidyl transferase that increased more than control in recovery stage, indicating the

involvement of this enzyme in recovery through altering the nucleotide metabolism.

4.5. Conclusions

The proteomic analyses of soybean hypocotyl and root invoked the need of characterizing significantly changed indicator proteins during post-flooding recovery. The beta-ketoacyl reductase, nucleotidyl transferase, peroxidase, and acid phosphatase were further characterized under flooding, drought, and cold stresses, as well as with phytohormones treatments. In this study, phytohormones depicted mixed effects on the activities of the analyzed enzymes under flooding stress and during the recovery stage. Jasmonate suppressed the activities of peroxidase and acid phosphatase when compared with age-matched control plants. Salicylate effects on root were following the trend observed in jasmonate treatment, but activity suppression was severe. Jasmonate treatment clearly enhanced the activity of nucleotidyl transferase in the root under flooding that decreased during recovery stage. Salicylate treatment increased the nucleotidyl transferase activity during recovery stage.

Jasmonate was linked to signaling and defense responses in various abiotic stresses such as drought, salinity, chilling, and flooding (Santino et al., 2013; Komatsu et al., 2010a). A crosstalk between jasmonate and salicylate responds to stresses in a synergetic or antagonistic way. Tsai et al. (2014) suggested that ethylene and jasmonate are important in the revival of homeostasis following oxygen deprivation. Nucleotidyl transferases are involved in nucleotide metabolism and amino acids activation. Increase in the activity of this enzyme by jasmonate under flooding stress and in recovery by salicylate indicates induction of these pathways during the post-flooding recovery in soybean. These results suggest that jasmonate may play a positive important role in the post-flooding recovery in root of soybean by bringing about the re-adjustments in the nucleotide metabolism.

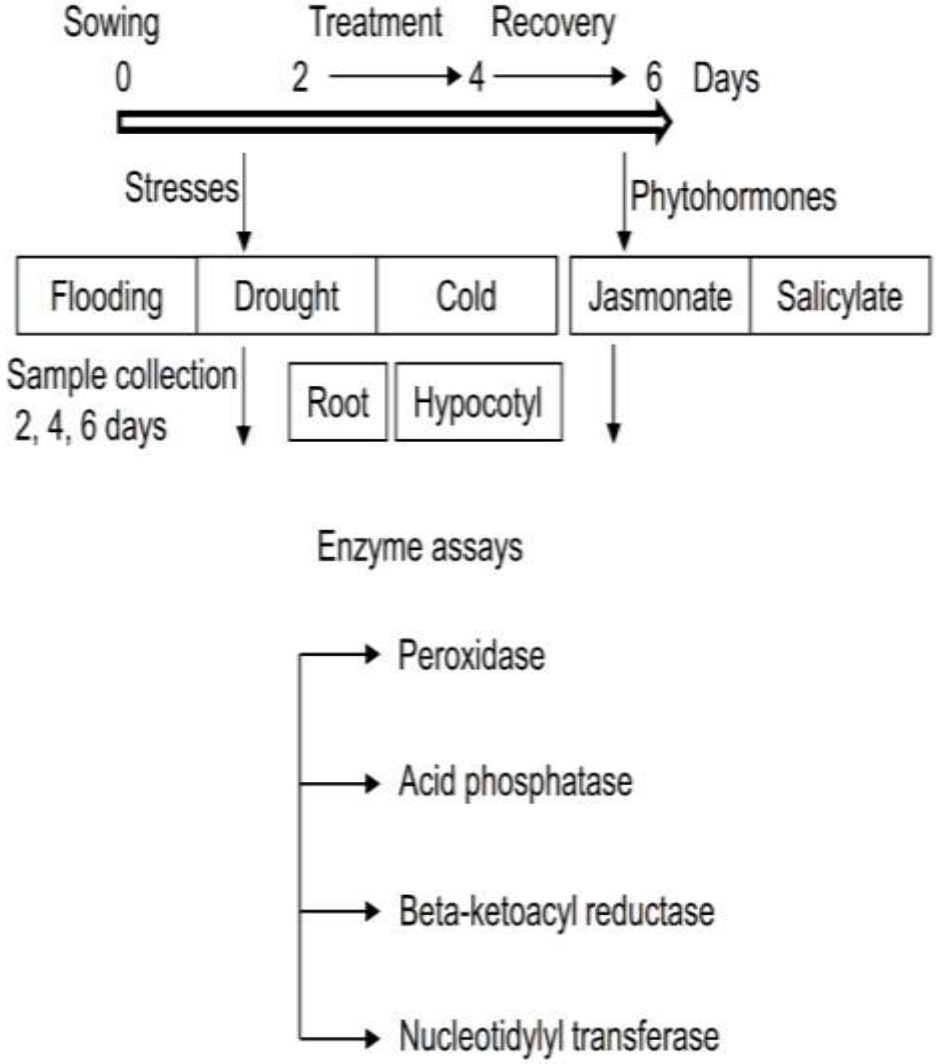


Figure 18. Experimental design used for the organ/stress specifc enzyme activity assays. Soybeans were grown for 2 days. Two-day-old soybeans were treated with flooding, drought, and cold stresses and/or with jasmonate and salicylate for 2 days. The treated seedlings were allowed to recover for 2 days following stress removal. Root and hypocotyl were collected. Age-matched untreated soybean seedlings were used as control plants. Enzyme activities of peroxidase, acid phosphatase, beta-ketoacyl reductase, and Nucleotidyl transferase were analyzed.

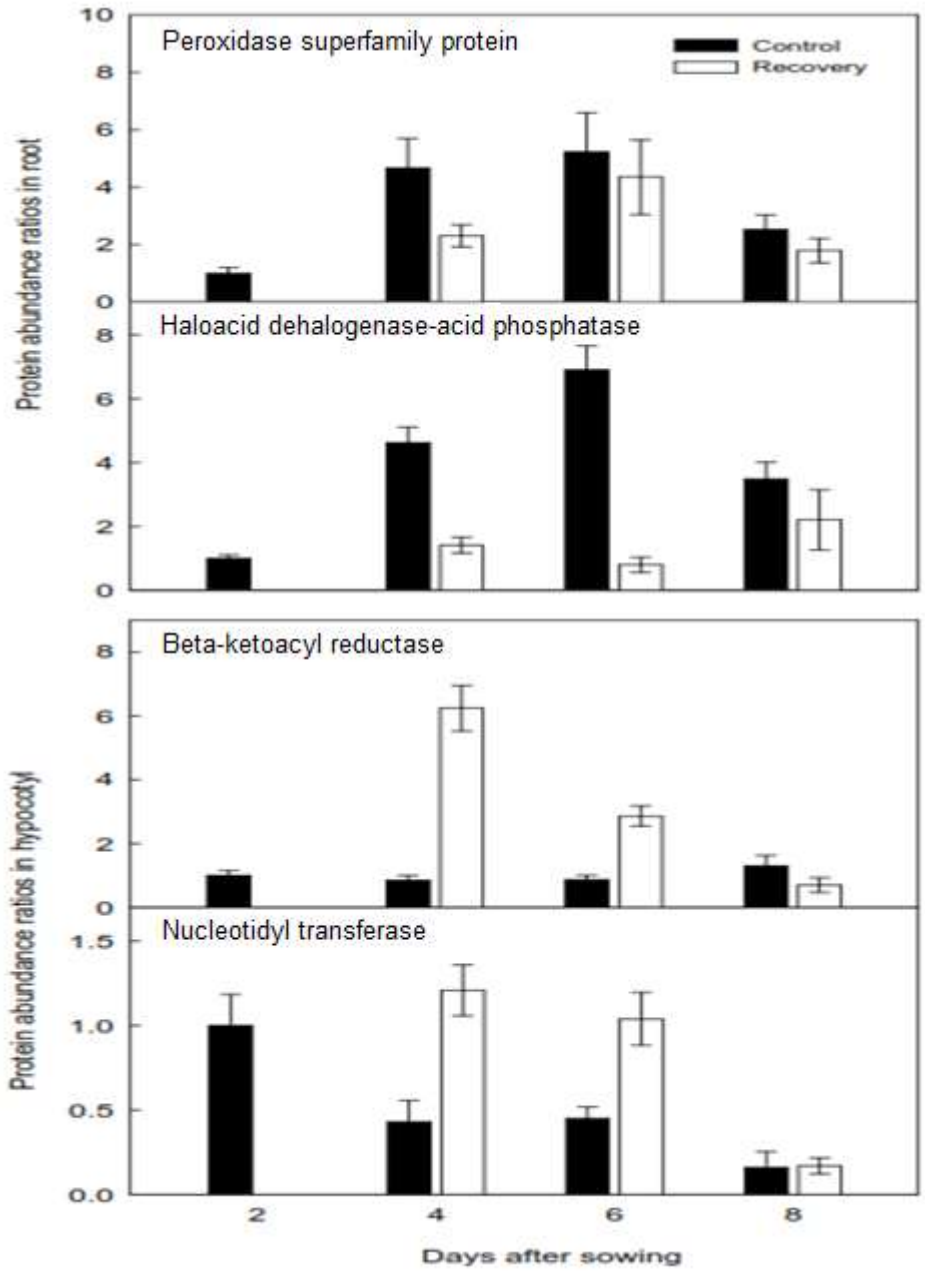


Figure 19. The change in protein abundance in soybean during recovery stage. Two-day-old soybeans were flooded for 2 days and allowed to recover following stress removal. Root and hypocotyl were collected. Proteomic analysis was performed for recovery (white column) and age-matched untreated control (black column). Protein abundance changes were calculated for peroxidase and haloacid dehalogenase-acid phosphatase in root, and beta-ketoacyl reductase and nucleotidyl transferase in hypocotyl. Data obtained after SIEVE analysis are means ± SE from 3 independent biological replicates (n=3).

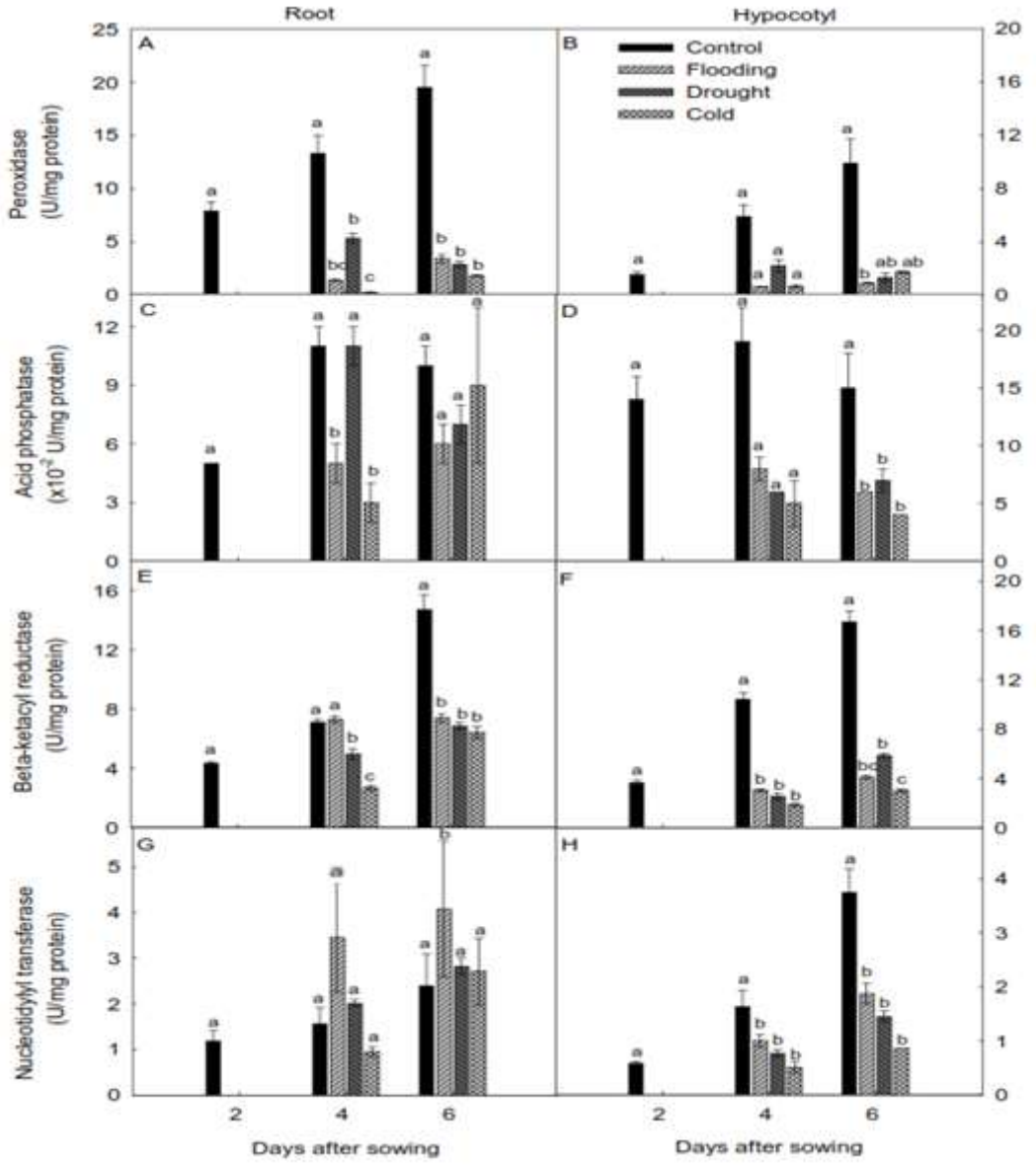


Figure 20. Changes in enzyme activities of responsive proteins in soybean root and hypocotyl during recovery after flooding, drought, and cold stresses. Two-day-old soybeans were treated with flooding, drought, and cold stresses for 2 days and allowed to recover for 2 days following stress removal. Root (A, C, E, G) and hypocotyl (B, D, F, H) were collected. The enzyme activities of peroxidase (A, B), acid phosphatase (C, D), beta-ketoacyl reductase (E, F), and nucleotidyl transferase (G, H) were analyzed. Columns indicate enzymatic activities in control (black), flooding (forward diagonal), drought (cross-hatched), and cold-stressed seedlings (reverse diagonal). In case of treated seedlings, 2 days indicate starting point of stress, 4 days indicate 2 days stressed plant, and 6 days indicate 2 days stressed and 2 days recovered plant. Data are means ± SE from 3 independent biological replicates (n=3). Different letters above the columns indicate significant changes in mean values as determined by Tukey's HSD test ($p < 0.05$).

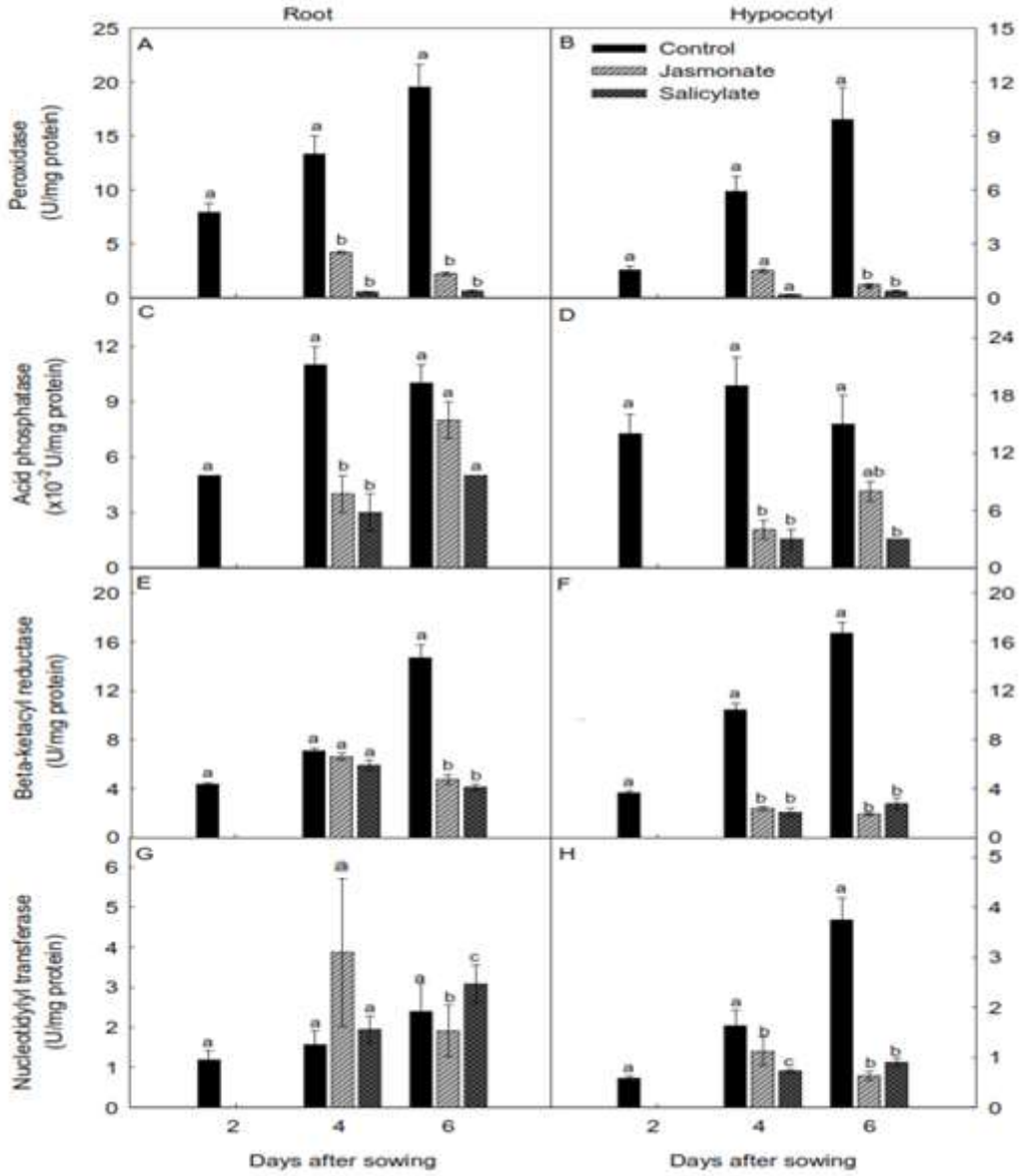


Figure 21. Changes in enzyme activities of responsive proteins in soybean root and hypocotyl during post-flooding recovery in response to jasmonate and salicylate phytohormones. Two-day-old soybeans were treated with jasmonate and salicylate separately for 2 days and allowed to recover for 2 days following removal of treatment. Root (A, C, E, G) and hypocotyl (B, D, F, H) were collected. The enzyme activities of peroxidase (A, B), acid phosphatase (C, D), beta-ketoacyl reductase (E, F), and nucleotidyl transferase (G, H) were analyzed. Columns indicate activities in control (black), jasmonate (lined), and salicylate-treated seedlings (cross-hatched). In case of treated seedlings, 2 days indicate starting point of treatment, 4 days indicate 2 days treated plant, and 6 days indicate 2 days treated and 2 days recovered plant. Data are means ± SE from 3 independent biological replicates (n=3). Different letters above the columns indicate significant changes in mean values as determined by Tukey's HSD test ($p < 0.05$).

CONCLUSION AND FUTURE PROSPECTS

Global climatic changes are major concern for the agricultural crops that are exposed to abiotic stresses (Lobell and Gourdji, 2012; Mittler and Blumwald, 2010). Many important plants are vulnerable to various kinds of abiotic stresses (Ahmad and Prasad, 2012). Soybean, which is rich source of oils and proteins, is prone to flooding stress that reduce its growth and yield (Githiri et al., 2006). The effects of flooding on soybean are manifold and exhibited at morphological, biochemical, and molecular levels (Komatsu et al., 2015). Flooding reduces the oxygen supply to the tissues and shifts the aerobic to anaerobic metabolism (Armstrong and Drew, 2002; Gibbs and Greenway, 2003). Flooding stress affected the morphology of soybean that was recovered when the stress was removed (Chapter 1). Length and weight of hypocotyl and root were efficiently increased during post-flooding recovery stage. Based on the morphological changes, to investigate the post-flooding recovery mechanism in soybean, hypocotyl and root proteins were analyzed using gel-free/label-free proteomic technique. The current study will form the basis for pin-pointing the changes in indicator proteins for developing flooding-tolerant soybean.

The proteomic studies of flooding stress response mechanism in soybean have been reviewed (Komatsu et al., 2012a; Komatsu and Hossain, 2013; Hossain and Komatsu, 2014; Komatsu et al., 2015). The previous studies carried provided knowledge about molecular changes in soybean under flooding stress. Glycolysis, fermentation, and tricarboxylic-acid-cycle-related proteins are increased under flooding stress (Komatsu et al., 2010b; Nanjo et al., 2012). The oxidative phosphorylation cannot take place due to oxygen deficiency and glycolysis and fermentation remain the ways to partially compensate for the energy losses. Cell wall, cell membrane, and membranes of organelles are damaged, and intra-cellular ionic homeostasis is disturbed (Komatsu et al., 2015). Plants attempt to recover following flooding removal; however, what happens to these changes in the post-flooding stage, has remained a less answered question. The proteomic changes, which occur during this transition from flooding to post-flooding stage, may provide important information regarding surviaval and recovery. Salavati et al., (2012) reported that soybean brings about re-arrangements in

its cytoskeleton and cell wall structure that help the plant recover following stress removal. The study came out with important results; however, the use of gel-based technique resulted in the loss of many low-abundance proteins. In the current study, gel-free proteomics was utilized to unravel the response mechanisms in the hypocotyl and root of soybean during post-flooding recovery stage.

Hypocotyl plays vital roles in plant growth and development by growing exclusively through cell expansion (Derbyshire et al., 2007). The *Arabidopsis* hypocotyl has been used as model organ for secondary growth studies in plants (Ragni and Hardtke, 2014). In current study, to unravel the mechanism involved in post-flooding recovery in soybean, hypocotyl proteins were analyzed in transition from flooding to post-flooding recovery stage. Proteins identified in hypocotyl (Chapter 2) suggest that delayed use/degradation of development-related proteins and decreased abundance of proteases might have helped soybean hypocotyl to recover during the post-flooding stage. Most importantly in hypocotyl, pyruvate kinase, nucleotidyl transferase, and beta-ketoacyl reductase were increased under flooding and during initial stage of recovery but decreased progressively as the recovery proceeded. Increase in pyruvate kinase indicated elevation in glycolysis frequency (Nanjo et al., 2012) as to partially compensate for the energy deficiency under flooding stage that was normalized during the recovery stage. Nucleotidyl transferase was increased in protein abundance under flooding and initial recovery, but its enzyme activity was elevated during the post-flooding recovery stage. Nicotinamide mononucleotide adenylyl transferase, a nucleotidyl transferase, catalyzs NAD biosynthesis from nicotinamide mononucleotide and ATP, and imparted tolerance against ROS in *Arabidopsis* (Hashida et al., 2010). Beta-ketoacyl reductases were increased under flooding and initial recovery stage at protein abundance level. The activity of the enzyme depicted an increasing trend during the recovery stage. These enzymes are involved in the synthesis of very long chain fatty acids that take part in cuticular wax formation deposited on the cell surfaces of plants to act as protective barrier (Beaudoin et al., 2009). These results suggest that identified indicator enzymes involved in energy generation, nucleotide metabolism, and complex fatty acids formation coordinate the cellular responses to protect against flooding-induced damages and help the hypocotyl to recover during post-flooding stage.

Two enzymes that changed significantly during post-flooding recovery in hypocotyl, were further characterized at organ-specific and stress-specific enzyme activity levels, and also with phytohormone treatment. The beta-ketoacyl reductase did not change in root under flooding; however, it was decreased in hypocotyl and increased slightly during recovery stage. It was decreased in both organs under drought and cold stresses, but trended towards increase during the recovery stage. Nucleotidyl transferase was increased in root under flooding and drought stresses, but decreased in hypocotyl. Additionally, jasmonate and salicylate affected the activities of beta-ketoacyl reductase and nucleotidyl transferase in both organs. Nucleotidyl transferase activity was elevated by jasmonate and decreased progressively during recovery; however, it was suppressed by salicylate under flooding but increased during the recovery in root (Figure 22). These results suggest that jasmonate associated changes in nucleotidyl transferase may facilitate soybean root during recovery stage after flooding stress.

In the current study, root proteins were also analyzed to search for the mechanism of post-flooding recovery in soybean. Protein abundances of large number of proteins were significantly changed during the recovery stage. Proteins identified in root (Chapter 3) suggest that stress-related dirigent-like and germin-like proteins play essential role in recovery following flooding in soybean. In the current study of root proteins, peroxidase, lipoxygenase, isoflavone reductase, and HAD-acid phosphatase were significantly changed during the post-flooding recovery stage. Peroxidase was decreased under flooding stress and increased during the post-flooding recovery stage in root of soybean. Peroxidase reported to decrease in rice under flooding stress (Ismail et al., 2009). Peroxidases are involved in scavenging of toxic ROS like peroxides, and also in cell wall lignification (Herrero et al., 2013). Lipoxygenase slightly decreased during the recovery stage. This enzyme is involved in jasmonate synthesis, but also cause peroxidation of membranes under stress conditions. The decrease in the activity of this enzyme indicates less membrane lipid damage during the recovery stage. Secondary metabolism related isoflavone reductase was increased during the post-flooding recovery stage. Khatoon et al., (2012a) reported decrease in this protein in soybean under flooding stress. Isoflavobe reductase is involved in the metabolism of flavonoids and lignins (Dixon, 2001), as well as defence against oxidative stress (Kim et al., 2010).

These results suggest that ROS scavenging and cell wall-lignin-related functions of the peroxidase and isoflavone reductase might be directly involved in post-flooding recovery in soybean root.

Two enzymes that changed significantly during post-flooding recovery in root, were further characterized at organ-specific and stress-specific enzyme activity levels, and also with phytohormone treatment. Peroxidase was decreased under flooding and cold stresses and was increasing during recovery stage. Acid phosphatase was suppressed in root under flooding and cold stresses and slightly increased during recovery; however, opposite happened in hypocotyl. Additionally, jasmonate and salicylate suppressed the activities of peroxidase and acid phosphatase in both organs (Figure 22). Lipoxygenases involved in biosynthesis of jasmonate were decreased in protein abundance in soybean roots under flooding stress when compared to pre-flooding stage. However, the protein abundance of the lipoxygenases was decreased further gradually as post-flooding recovery stage proceeded. Jasmonic acid acts as growth regulators in higher plants (Komatsu et al., 2013a). Lipoxygenase activity was progressively decreased in soybean roots passing through post-flooding recovery stage. Kamal and Komatsu (2016) reported that proteins related to stress, glycolysis, fermentation, signaling, secondary and hormone metabolism, cell wall and cell organization were induced by jasmonate and salicylate treatments in soybean under flooding stress. Results of the current study and previous reports are in agreement for the involvement of jasmonate and salicylate in flooding stress response in soybean. The overall outcome of the study has been summarized (Figure 23).

Post-stress recovery in plants is relatively less investigated area with only few studies appeared in this regard. Studies on roots of germinating *Phaseolus vulgaris* in response to chilling stress and recovery (Badowiec and Weidner, 2014), changes of protein abundance in *Pisum sativum* roots under chilling stress and recovery (Badowiec et al., 2013), and calcium chloride involvement in post-drought recovery in *Camellia sinensis* (Upadhaya et al., 2011) have been conducted. The understanding of the post-flooding/post-stress stages can pin-point indicator proteins and provides better ways to manipulate the relevant genes that might confer tolerance to the plant species against the stresses. Overexpression of the genes of identified indicator proteins may increase plant

tolerance to flooding stress and thereby increase crop yields. Future research needs to put a stronger focus on the recovery stages of flooding and other abiotic stresses. It will ultimately help to understand the recovery mechanisms used by different plants under various kinds of stresses. The research on post-flooding recovery in soybean could further be extended to organelle-specifc level to unravel the changes at organelle level. The results of this study will form the basis for further understanding of the post-flooding recovery mechanism.

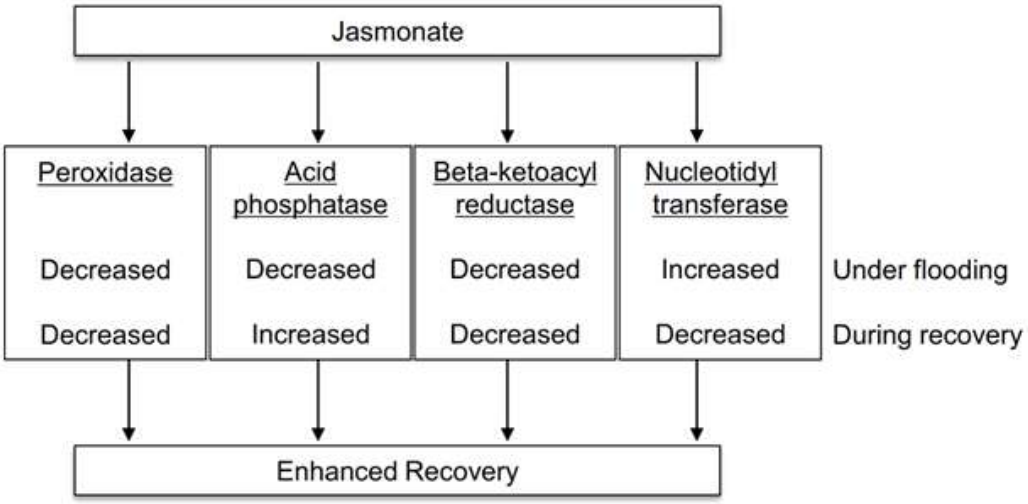


Figure 22. An overview of enzyme activity changes in root of soybean under flooding with jasmonate and during recovery. The enzyme activities of peroxidase, acid phosphatase, beta-ketoacyl reductase, and nucleotidyl transferase were analyzed in response to jasmonate treatment in soybean during post-flooding recovery stage.

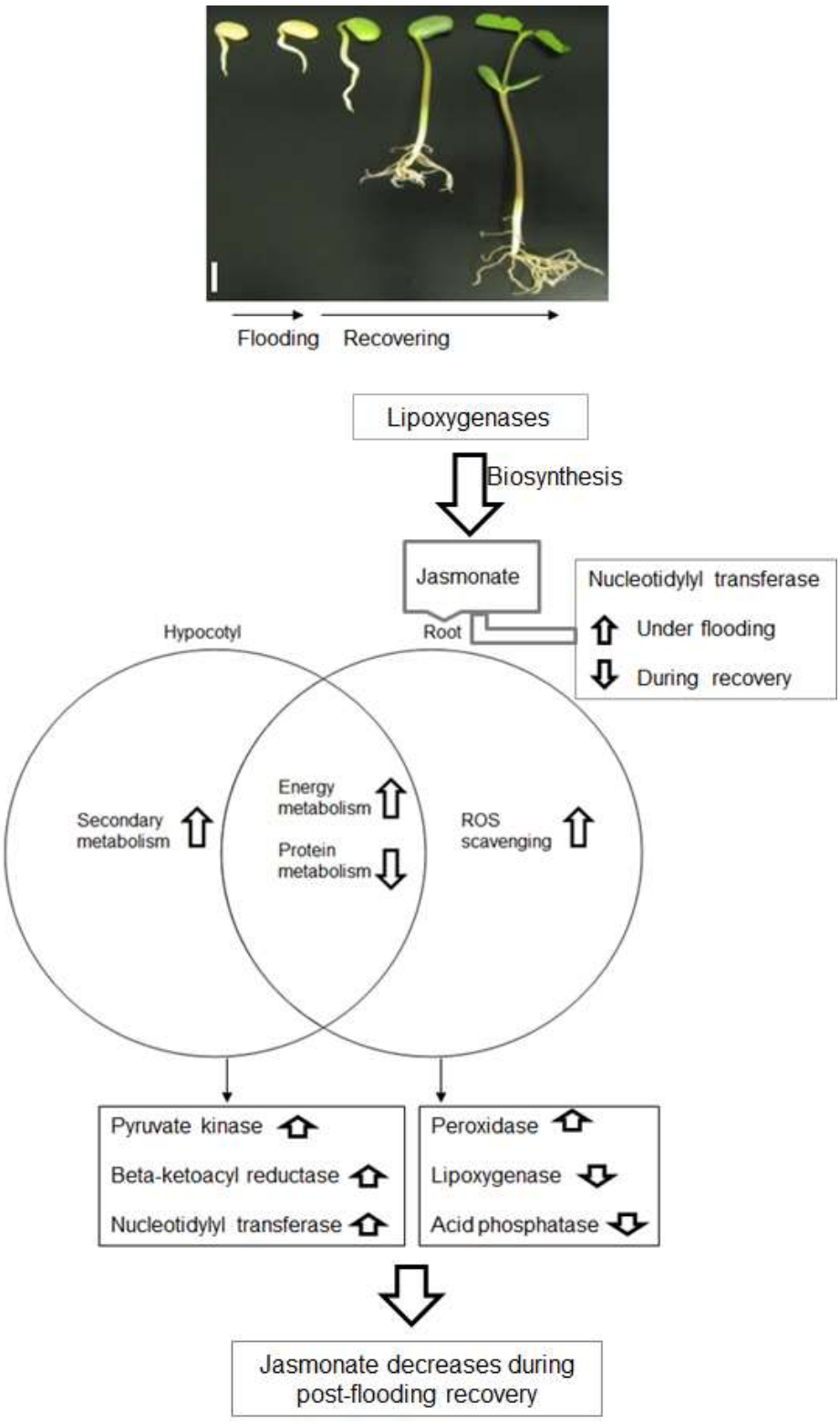


Figure 23. The overall response in soybean hypocotyl and root during post-flooding recovery stage. The figure shows transition from flooding to post-flooding stage involving common and organ-specific mechanisms. Up and down arrows indicate increased and decreased proteins, respectively.

Supplemental Table 1. List of qRT-PCR primers used in the study.

Gene Name	Protein ID	Direction	Sequence of Primer
Nucleotidyl transferase superfamily protein	Glyma03g25740.1	Forward	5′- ACGCGTATGAGAGGGGAGAT -3′
		Reverse	5′- CAAGTCTTCGGCACTCGTCT -3′
Pyruvate kinase family protein	Glyma10g34490.1	Forward	5′- GGGAGAGCCAATGAGCCATT -3′
		Reverse	5′- AAGCACCGGTGAATGTCCAT -3′
Beta-Ketoacyl reductase 1	Glyma11g37560.1	Forward	5′- TTGCCTGAGTTTGTCGTCGA -3′
		Reverse	5′- AATCAGGAACGCAGCAGGAA -3′
Lipoxygenase 1	Glyma15g03030.1	Forward	5′-CAAGTGAGACACCAGCTCCA-3′
		Reverse	5′-CCCCCTACGAGGATAAGGAA-3′
Peroxidase superfamily protein	Glyma15g17620.1	Forward	5′-TGACCCTCAATGCAGAAACA-3′
		Reverse	5′-GAGGCCATGGAAGCTAAACA-3′
Root hair specific 19	Glyma17g17730.1	Forward	5′-TCTCTGCTCAGCTCAGTCCA-3′
		Reverse	5′-GGTGATCCTTCTCTGCTTGG-3′
HAD-superfamily protein	Glyma08g21410.1	Forward	5′-CATTCCTGAAGAGTGCGTTG-3′
		Reverse	5′-ACGGTATCTGATCGTCTTCG -3′
Isoflavone reductase	Glyma11g07490.2	Forward	5′-AAAGCAGTGGATGACCCAAG-3′
		Reverse	5′-AGTGGCCTAATGCCAACATC-3′
Unknown	Glyma16g07750.1	Forward	5′-AAGACGAAGCAGGAGACGAA-3′
		Reverse	5′-TGATGATGGTGGTGTCTTGG-3′
18S rRNA	X02623.1*	Forward	5′-TGATTAACAGGGACAGTCGG -3′
		Reverse	5′-ACGGTATCTGATCGTCTTCG -3′

Protein ID, according to the Phytozome database; *Genebank accession number

SUMMARY

Soybean is an important legume crop that is rich source of protein and oils for diet, but is sensitive to flooding stress that reduces its growth and yield. Flooding reduces the light and oxygen availability for the plant tissues resulting in shifting of oxidative phosphorylation to anaerobic metabolism. Post-flooding recovery is important in understanding the post-stress responses in the soybean that may provide useful information regarding recovery efficiency as well as key factors involved in it. To unravel the mechanisms involved in post-flooding recovery in soybean, hypocotyl and root proteins were analyzed using gel-free proteomic technique. Two-day-old soybeans were flooded for 2, 4, and 6 days and allowed to recover following flooding removal. Hypocotyl and root were collected and lengths and weights were measured. Soybean seedlings managed to recover after 2 and 4 days flooding; however, 6 days flooding proved lethal for seedlings. Morphological analysis revealed that growth suppression was more severe with increasing flooding duration. Based on the morphological results, both hypocotyl and root were analyzed to reveal changes in their protein profiles.

In proteomic analysis of soybean hypocotyl, 20 proteins were significantly changed in recovering soybeans at all time points. Based on proteomic, clustering, and *in-silico* protein-protein interaction analyses, 3 proteins were further analyzed for enzyme activity assays. Pyruvate kinase was increased under flooding, but gradually decreased during post-flooding recovery period at protein-abundance and enzyme-activity levels. Nucleotidyl transferase was increased in protein abundance under flooding and initial recovery, but opposite trend was observed in its enzyme activity. Beta-ketoacyl reductase was increased under flooding and decreased during recovery at protein-abundance level, but its enzyme activity gradually increased during the post-flooding recovery period. These results suggest that pyruvate kinase, nucleotidyl transferase, and beta-ketoacyl reductase play key roles in post-flooding recovery in soybean hypocotyl by promoting glycolysis for the generation of ATP and regulation of secondary metabolic pathways.

Proteomic analysis of soybean root resulted in identification of 73 and 21 proteins during the recovery stages following 2- and 4-day flooding, respectively. Based on the

proteomic and bioinformatic analyses, 6 proteins were commonly changed in recoveries following 2- and 4-day flooding. Lipoxygenase 1 and HAD-acid phosphatase were decreased at protein abundance level during the recovery period. Lipoxygenase is involved in jasmonate biosynthesis, while acid phosphatase adjusts changes in phosphate foraging. Peroxidase superfamily protein, isoflavone reductase, root hair specific 19, and an unknown protein increased in abundance during the course of recovery. Consistent with these findings, the enzymatic activity of peroxidase was decreased under flooding stress, but increased significantly during the recovery stage. These results suggest that peroxidases and acid phosphatases might play key roles in post-flooding recovery in soybean roots through the scavenging of toxic radicals as well as re-adjusting the phosphate foraging.

Based on the results from the proteomic analysis of hypocotyl and root in soybean, enzyme activities of 4 enzymes with organ/stress specificity and time-dependency were further analyzed. The activities of the 4 enzymes were analyzed under flooding, drought and cold stresses. The activities of acid phosphatase, beta-ketoacyl reductase, and nucleotidyl transferase were changed in an organ-specific and stress-specific pattern. Furthermore, jasmonate and salicylate suppressed the activities of peroxidase and acid phosphatase in both organs under flooding stress; however, acid phosphatase increased during recovery. Beta-ketoacyl reductase slightly changed; whereas, nucleotidyl transferase activity was highly elevated by jasmonate in root and decreased progressively during recovery. These results suggest that jasmonate-associated changes in nucleotidyl transferase may facilitate soybean root recovery after flooding stress.

In the overall recovery response in hypocotyl and root, energy metabolism-related proteins and protein metabolism-related proteins were increased and decreased respectively, in the initial stages of post-flooding recovery. The organ-specific changes reveal that in root, proteins involved in toxic-radicles-scavenging like peroxidases are induced for reducing the oxidative damage caused by flooding. In hypocotyl, proteins involved in secondary-metabolism-related nucleotidyl transferases and beta-ketoacyl reductases are induced during post-flooding recovery. Post-stress recovery analysis can pin-point the indicator proteins whose respective genes may be utilized for attaining tolerant varieties.

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