Comprehensive Analysis of Initial-Response Mechanism in Soybean under Flooding Stress

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Comprehensive Analysis of Initial-Response Mechanism in Soybean under Flooding Stress

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Abbreviations

ABA	Abscisic acid
CID	Collision-induced dissociation
IMAC	Immobilized metal affinity chromatography
KEGG	Kyoto encyclopedia of genes and genomes
LC	Liquid chromatography
MS	Mass spectrometry
NAC	Nascent polypeptide associated complex
PolyMAC	Polymer-based metal-ion affinity capture
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
ROS	Reactive oxygen species
SCX	Strong cation exchange
QTL	Quantitative trait loci

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INTRODUCTION

Climate change affects the environment of earth including temperature, precipitation, and hydrological cycles (Grimm et al., 2013; Merilä and Hendry, 2014). Climate change is largely caused by both natural factors such as volcanic activities/crustal movements (Grimm et al., 2013) and artificial factors such as the increase in the concentration of greenhouse gases/aerosol (Merilä and Hendry, 2014). The increasement in the frequency and intensity of heat waves and other extreme events reduces agricultural production (Anwar et al., 2013). All of these climate changes lead to effects of various abiotic stresses such as drought (Choat et al., 2012), salinity (Parvaiz and Satyawati 2008), cold (Williams et al., 2015), and high temperature (Tubiello et al., 2008). Plant productivity was decreased under these abiotic stresses, resulting in price increases for agricultural crops (Wheeler and von Braun, 2013). In addition, global climate change severely limits the distribution of wild crop relatives and leading to fragmentation of the distribution and even extinction (Howden et al., 2007). These changes of climatic conditions lead to the alterations of plant living environment and suppress the development of agriculture in farmland.

Global warming, which refers to the increasement of greenhouse gas such as carbon dioxide concentration, becomes a megatrend (Portmann et al., 2013). Global warming leads to significant environmental changes including rising sea levels due to thermal expansion and melting of glaciers/ice sheets (Mimura, 2013). In addition, human-induced world climate change increased the frequency of precipitations of higher magnitude as well as tropical cyclone activity (Knutson et al., 2010). As a result, the occurrence of flooding events, which is typically caused by heavy or continuous rainfall in areas with poorly drained soil, is expected to be higher (Perata et al., 2011). Flooding is one of the most severe environmental stresses that negatively impacts the productivity of arable farmland and is responsible for major economic losses in the agricultural industry (Lin, 2011). A large amount of crop producing area in the worldwide was affected by the flooding or waterlogging (Ahmed et al., 2012). To avoid the economic loss caused by flooding, developing the flooding tolerant cultivars is necessary.

Flooding is a complex stress that imposes many constraints to plant growth. The severe constraint is starvation of oxygen and carbon dioxide, which is caused by extremely slow rates of diffusion through the water compared to that in air (Jackson and Colmer, 2005). Gas exchange between the atmosphere and the plant tissue is reduced by flooding. Gas such as oxygen diffusion is much slower in water than in air (Armstrong, 1979). The limitation of oxygen availability in flooded soil causes hypoxic and anoxia conditions for plant root (Sauter, 2013). Continuously, respiration of plant root system, which provides substantial reduction in energy status, is inhibited by hypoxic and anoxia conditions (Ashraf, 2012). The capacity for ATP production and energy transformation through mitochondrial oxidative phosphorylation was restricted by lower oxygen concentration in flooded plants (Gibbs and Greenway, 2003). To overcome the problem, anaerobic pathways including glycolysis and fermentation were activated (Bailey-Serres and Voesenek 2008). On the other hand, the photosynthesis was suppressed in plants by flooding stress (Voesenek et al., 2006; Wang et al., 2014). Furthermore, flooding also leads to change in soil chemical characteristics including soil pH and redox potential (Dat et al., 2004), which limits the availability of soil nutrients and leads to the accumulation of phytotoxins (Pezeshki and DeLaune, 2012). Theses results indicated that plant growth was suppressed under flooding stress through suppressing photosynthesis, decreasing energy/nutrients supply, and accumulating toxic metabolites (Figure 1).

Soybean is one of the most widely cultivated crop in the world and it provides an abundant source of protein and edible vegetable oil for human consumption (Ainsworth et al., 2012). The soybean production and consumption are gradually increased (Lee and Khor, 2015). Meanwhile, global trade of soybeans and soybean products are also gradually increased worldwide (Lee and Khor, 2015). The production of soybean increased from 219 to 320 million metric tons from 2007 to 2015 and the usage of soybean also increased from 229 to 312 million metric tons (Figure 2). The exports of soybean increased 9.7% each year and ending stocks of soybean almost did not change from 2007 to 2015 (Figure 2). As one of the major agricultural crops, soybean is particularly sensitive to flooding stress. The plant growth and grain yields are markedly reduced in flooded soil (Githiri et al., 2006). When soybean was treated with flooding at

the vegetative growth stage or the reproductive stage, its seed yield and quality were much reduced compared to untreated soybean (Oosterhuis et al., 1990). In addition, grain yield of soybean was estimated to be reduced to one fourth because of flooding injuries in the regions of the world where soybean is rotated with rice in paddy fields (Sullivan et al., 2001). These findings indicate that development of flooding tolerant soybean is desirable to promote its planting in wide area.

To develop the flooding tolerant plants, uncovering function of root system is one key point. The plant root system is important for nutrient uptake from the surrounding soil (Lynch, 2007). In addition, the root system is an important region for plant development and it contains differentiation zone, elongation zone, and apical meristem zone (Figure 3). Root tip is comprised of actively dividing cells that develop into primary and lateral roots (Mathesius et al., 2011). In soybean, the root tip is characterized by an open meristem and quiescent center, which is located below the meristem and is mainly composed of a pool of stem cells that are important for root development (Sun et al., 1957). The root tip plays key roles in the development of root structure and responses to environmental stresses, particularly flooding (Komatsu et al., 2012a). Based on the above reasons, the study of soybean root tip might provide insight into plant responses to flooding stress.

In order to survive under flooding condition, plants evolve the adaption characters. The rate of elongation growth in completely submerged rice was rapidly increased to help its leaves remain above the water surface to maintain gas exchange between the atmosphere and the plant body (Hirano et al., 2014). On the other hand, the internode elongation rate of deepwater rice was promoted by flooding condition (Voesenek and Bailey-Serres, 2009). In the hyponastic growth of the semiaquatic plant *Rumex palustirs*, the leaf angle rapidly increased upward to keep a portion of the leaves above water under flooding condition (Peeters et al., 2002). In contrary, leaf angle altered downward in tomato plants responded to flooding for reduction of the light absorption by the leaves and subsequent transpiration and uptake of water by the roots (Calvo-Polanco et al., 2014). To sustain the integrity of gas exchange routes, constitutive air space called aerenchyma is developed in the roots and shoots of rice (Steffens et al., 2011). In wheat, lysigenous aerenchyma is formed in the roots to help

plant survive under waterlogged conditions (Haque et al., 2011). In soybean, secondary aerenchyma is formed in nodules, stems, hypocotyls, and adventitious roots to transport oxygen from the shoot to the roots under flooding stress (Shimamura et al., 2010). Plants survive and escape from flooding condition through these adaptive evolutions.

To explore the adaptive mechanism for flooding stress in plants, many related genes were identified. In rice, the submergence1 locus isolated from a submergence-tolerant landrace (FR13A) (Xu et al., 2006) and the snorkel locus from a deepwater landrace (C9285) (Mickelbart et al., 2015) were related to flooding tolerance. Submergence1 controls the tolerance of complete submergence by dampening underwater growth through storaging carbohydrates in reserve until favorable growth conditions return (Ismail et al., 2012), snorkel controls the avoidance of submergence by promoting underwater elongation growth (Fukao and Xiong, 2013). Submergence1 locus on chromosome 9 mainly contributes to the tolerance of complete submergence of vegetative plants (Xu et al., 2006). Recently, a finding indicated that submergence1 A promoted photosynthetic and metabolic recovery from submergence damage in rice leaves (Alpuerto et al., 2015). In *Arabidopsis*, hypoxiaresponsive unknown protein genes were low-oxygen responded in other species, and overexpression of several unknown protein genes enchanced tolerance to submergence (Lee et al., 2011). These results provide useful candidate genes for uncovering flooding tolerant mechanism in rice and *Arabidopsis*.

In soybean, flooding stress impairs plant growth by inhibiting root elongation and reducing hypocotyl pigmentation (Hashiguchi et al., 2009). To understand the underlying mechanisms of soybean responses to flooding stress, proteomic techniques were applied and many results have been reviewed (Komatsu and Hossain, 2013; Komatsu et al., 2012a, 2013a, 2015a, 2015b; Hossain et al., 2013). Proteins involved in glycolysis and fermentation such as enolase and alcohol dehydrogenase were clearly increased under flooding stress, indicating that anaerobic respiration was activated to provide nessary energy in soybean (Komatsu et al., 2011a). Another aspect was that cell wall loosening and synthesis related proteins were increased and decreased, respectively, under flooding stress, suggesting that cell wall was damaged in soybean by flooding stresss (Komatsu et al., 2013a). Continuously, proteins involved in active

oxygen species (ROS) scavenging such as ascorbate peroxidase, were obviously decreased in soybean under flooding stress (Hossain et al., 2013) (Figure 4). In addition, proteins involved in protein synthesis were decreased under flooding stress in soybean (Komatsu et al., 2015a). These results indicate that energy metabolism, cell wall, active oxygen scavenging, and protein synthesis related proteins involve in flooding stress in soybean.

Proteins involved in plant recovery from flooding provide essential clues in better understanding the flooding injury. Salavati et al. (2012) reported that the proteins associated with cell wall modification and *S*-adenosylmethionine synthesis were involved in recovery mechanism. Khan et al. (2014) reported that peroxidase superfamily protein continuously increased in protein abundance, mRNA expression level, and enzyme activity during the course of recovery stages, indicating peroxidases played key roles in post-flooding recovery in soybean roots through scavenging of toxic radicals. Khan et al. (2015) also reported that pyruvate kinase, nucleotidyly transferase, and beta-ketoacyl reductase played key roles in post-flooding recovery in soybean hypocotyl by promoting glycolysis for the generation of ATP and regulation of secondary metabolic pathways. These results indicate that cell wall modification, ROS scavenging, and energy supply related pathways play key roles in soybean during recoverying from flooding.

In rice, flooding-responsive mechanisms were regulated by phytohormones (Almeida et al., 2003). To confirm the function of phytohormones in soybean under flooding stress, proteomic techanique was used. Oh et al. (2014a) reported that secondary metabolism, cell, and protein degradation/synthesis realted proteins were decreased; however, they were increased by gibberellic acid supplementation under flooding in soybean (Oh et al., 2014a). Komatsu et al. (2009) reported that ethylene biosynthesis related genes were up-regulated in soybean under flooding condition. Another finding is that abscisic acid (ABA) enchanced flooding tolerance of soybean through controling energy conservation processes (Komatsu et al. 2013b). Furthermore, calcium-related signal transduction played important roles in regulating soybean growth under flooding stress (Oh et al., 2014b). Additionally, phosphorylation status of proteins played key role in phytohormones signaling pathways in plants (Chen et al.,

2010a). These results suggest that phytohormones related signal pathways are involved in flooding response in soybean through regulation of phosphoproteins.

Reversible modification of proteins by phosphorylation is a common signaling event that occurs in response to both abiotic and biotic stresses. Phosphorylation leads to protein structural changes, which can directly regulate protein activity and induce changes in interaction partners or subcellular localization (Jørgensen and Linding, 2008). To study signaling events, which are regulated by phosphorylation in plants, mass spectrometry (MS) based phosphoproteomics including enrichment, detection, and quantification of phosphopeptides has been developed in recent decade. For phosphopeptides enrichment, the basic principle is that takes advantage of the affinity binding between negatively charged phosphate and positively charge metalions (Fíla and Honys, 2012). Immobilized metal affinity chromatography (IMAC) coupled with strong cation exchange (SCX) is firstly used for positively charge metalions and the first reported SCX-IMAC application in plants resulted in identification of 283 phosphopeptides (Nühse et al., 2003). A variant of IMAC, polymer-based metal-ion affinity capture (PolyMAC), exhibited high selectivity. It was reported that 5386 unique phosphopeptides were identified using PolyMAC-titanium and PolyMAC-zirconium ion affinity chromatography (Wang et al., 2013) (Table 1). These techniques greatly promote the development of phosphoproteomic research for plants.

For phosphopeptides detection, stable isotope labeling and label free quantitation methods have been applied in plant phosphoproteomics. There are two main methods in label free quantitation. The first is based on precursor ion peak intensity/area and the second is based on the number of MS/MS spectra acquired for a given peptide (Reiland et al., 2011; Engelsberger and Schulze, 2012; Wang et al., 2013). The phosphoproteomics has been applied in *Arabidopsis* (Xue et al., 2013), rice (Ye et al., 2015), wheat (Zhang et al., 2014a), cotton (Fan et al., 2014), and soybean (Nguyen et al., 2012). In *Arabidopsis*, 1850 phosphopeptides were quantified and 468 phosphopeptides representing 497 phosphosites showed significant changes under osmotic stress using library-assisted extracted ion chromatogram (Xue et al., 2013). In rice, DNA synthesis, RNA splicing, and RNA-directed DNA methylation pathways were extensively affected by protein phosphorylation at the stage of developing anthers

(Ye et al., 2015). In wheat, 61 phosphoproteins, which are mainly related to signal transduction and embryo/endosperm development of grains, showed significant changes in phosphorylation under water-deficit conditions (Zhang et al., 2014a). A total of 167 phosphoproteins, which are involved in signal transduction, RNA metabolism, intracellular transport, were responded to sodium nitroprusside in cotton leaf (Fan et al., 2014). A complex network of kinase substrate and phosphatase-substrate interactions in response to rhizobial inoculation was revealed in soybean using quantitative phosphoproteomic technique (Nguyen et al., 2012). These results suggest that many biological processes are regulated by phosphorylation and these studies provide important resources for functional studies of protein phosphorylation in plant growth and development.

Many cellular events, such as hormonal signaling, transcriptional control, glucose degradation/sucrose accumulation, alcohol fermentation, gamma aminobutyric acid shunts, suppression of active oxygen scavenging, mitochondrial impairment, ubiquitin/proteasome-mediated proteolysis, and cell wall loosening, have been identified in soybean under flooding stress (Komatsu et al., 2012a, 2013a, 2015b). However, the initial flooding response mechanism has not been clearly elucidated in soybean. To uncover the molecular mechanisms of soybean affected by initial flooding stress and identify the candidate genes for cultivating flooding tolerant soybean, gel-free/label-free proteomic and sequencing-based transcriptomic techaniques are applied. Firstly, to explore the initial flooding responsive events, cellular proteins including phosphoproteins are characterized in soybean root tip at initial flooding stress. Secondly, to uncover the mechanism controlling those flooding responded proteins, nuclear proteins including nuclear phosphoproteins are analyzed in soybean root tip at initial flooding stress. Thirdly, to identify the initial flooding-tolerance mechanisms in soybean, flooding-tolerant mutant (Komatsu et al., 2013c) and ABA-treated soybean, which exhibits flooding-tolerant phenotype (Komatsu et al., 2013b), are used. Through this study, the initial flooding responsive events are explored by identification of cellular and nuclear proteins. Initial flooding-tolerance mechanisms in soybean are uncovered with identified proteins and genes.

Table 1. Enrichment method for phosphopeptide and phosphotprotein.

Enrichment approach	Principle	Numbers of phosphopeptides or proteins	Findings	References
Immunoaffinity enrichment	Specific antibodies connect with pTyr, pSer, and pThr containing peptides.	459 P-Tyr proteins	Multiple antibodies were suitable for detection of all possible P-Tyr proteins in a sample.	Bergström Lind et al., 2008
IMAC	Negatively charged phosphate groups on the phosphorylated amino acids interact with positively charged metal ions such as Ni^{2+}, Fe^{3+}, Ga^{3+}, Zr^{4+}, and Ti^{4+} which are chelated with silica or agarose through nitriloacetic acid or iminodiacetic acid.	4470 phosphopeptides	Pre-enrichment step prior to peptide-based IMAC allowed a manageable timetable in carrying out global analyses of phosphorylation events involved in the signal transduction cascades.	Cantin et al., 2008
MOAC	The phosphate groups on the amino acids interact with positively charged metal oxides.	8 phosphopeptides	Phosphoproteins, especially those present at low concentrations were detected.	Gates et al.,2010
Phos-Tag chromatography	Uses 1,3-bis [bis(pyridine-2-ylmethyl) amino] propan-2-olatodizinc (II) complex as a selective phosphate binding tag in aqueous solution at neutral pH.	135 phosphopeptides	Phosphorylation of ubiquitin ligase ATL31 controled plant nutrient response by targeting 14-3-3 proteins for degradation.	Yasuda et al., 2014
PolyMAC	It is similar to IMAC.	5348 phosphopeptides	Central metabolisms and cell signaling related proteins were phosphorylated.	Wang et al., 2013
SAX	The phosphopeptides are separated based on the number of phosphorylated residues.	305 phosphopeptides	Phosphopeptides were separated under gradient elution based on their different interaction with SAX adsorbent.	Han et al., 2008
SCX	Tryptic peptides with charge of +2 are eluted early in the chromatography.	10000 phosphopeptides	Ultra acidic strong cation exchange was especially valuable for the research related to basophilic kinases.	Hennrich et al., 2012
HILIC	Phosphopeptides with polar phosphate groups are strongly retained on the HILIC stationary phase, resulting in separation from non-phosphorylated species.	968 phosphopeptides	The transduction of signals and the expression of CDWEs and antioxidant enzymes were activated in the pathogen to obtain nutrients from the host during the biotrophic growth.	Yang et al., 2013

IMAC, immobilized metal affinity chromatography; MOAC, metal oxide affinity chromatography; SAX, strong anion exchange; SCX, strong cation exchange; PolyMAC, polymer-based metal-ion affinity capture; HILIC, hydrophilic interaction chromatography.

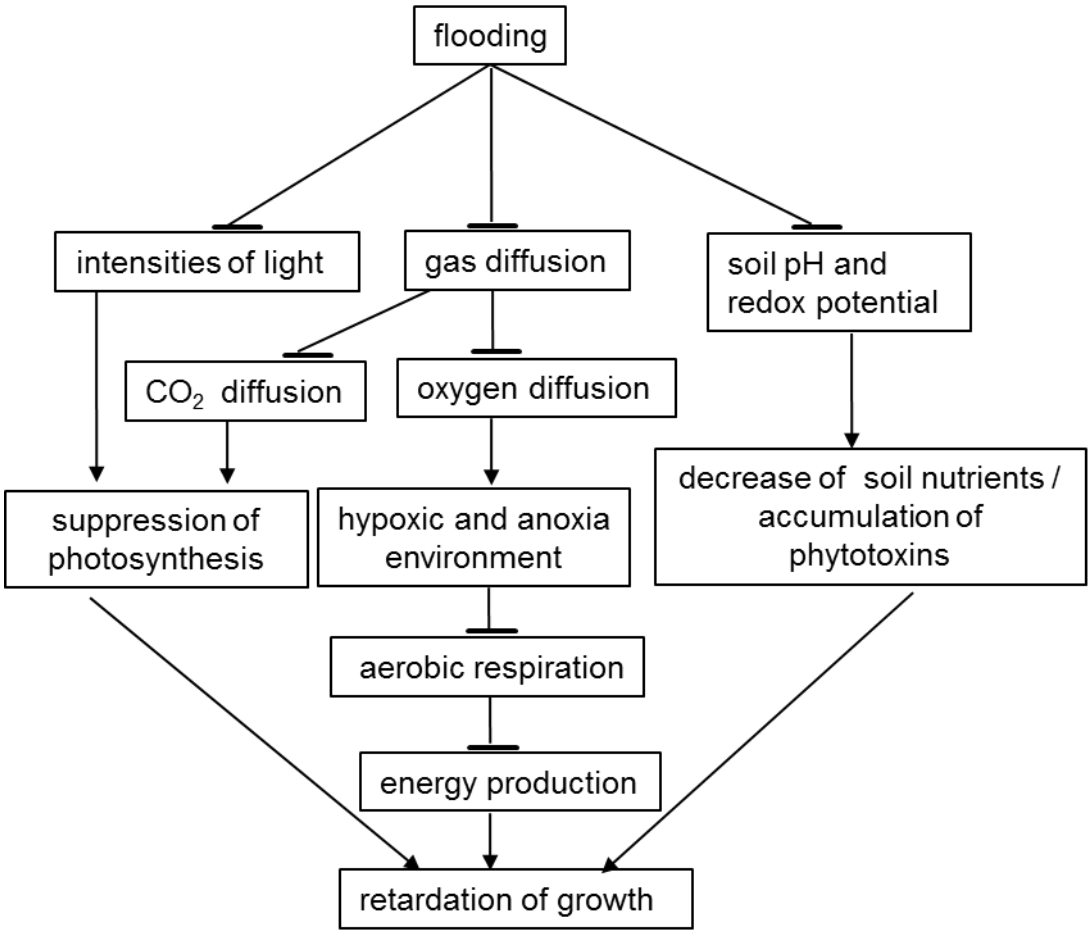

Figure 1. The physiological mechanism in plants under flooding stress. Firstly, flooding decreased intensities of light, rate of gas diffusion, and redox potential of soil. Secondly, flooding decreased concentrations of oxygen and carbon dioxide. Thirdly, flooding suppressed photosynthesis, soil nutrients, and aerobic respiration. Finally, these events led to retardation of plant growth under flooding condition. T-bars and arrows indicate negative and positive effects, respectively.

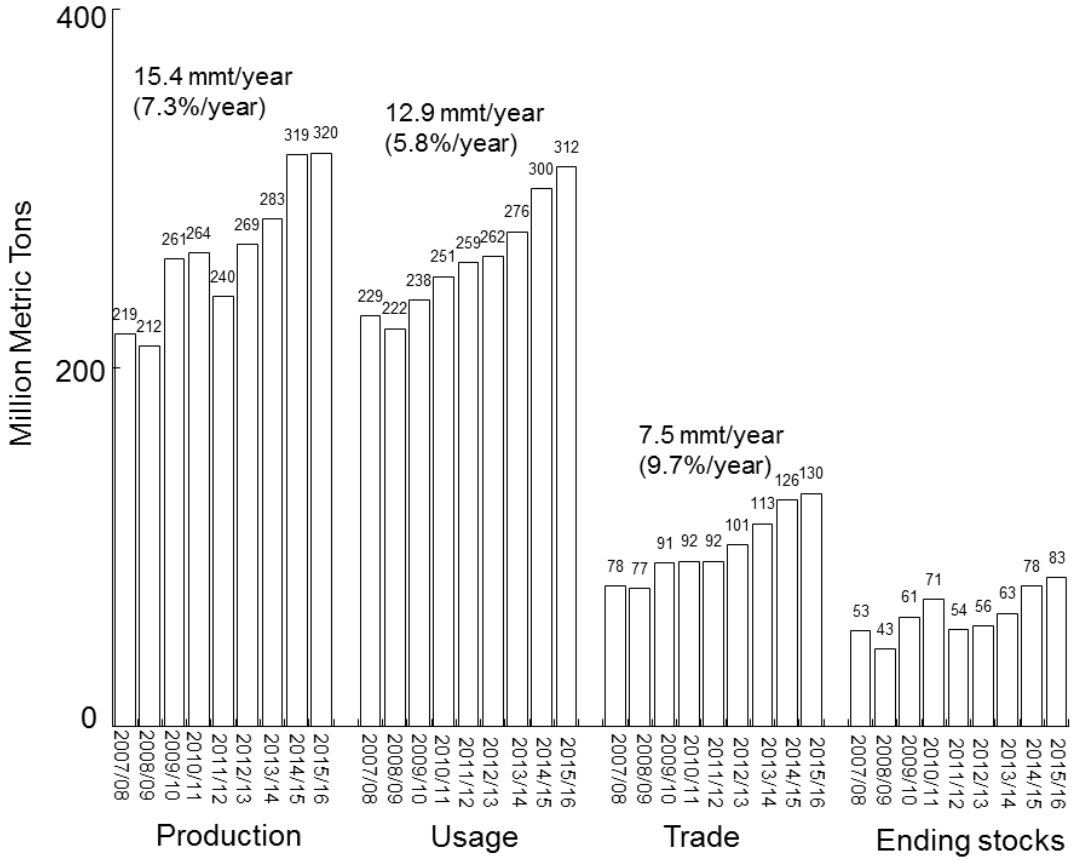

Figure 2. Production, usage, trade, and ending stocks of soybean in the world during the past 9 years. The production, usage, and trade of soybean in the world were gradually increased from 2007 to 2015. The average of increased percentage of soybean production, usage, and trade arrived at 7.3%, 5.8%, and 9.7%, respectively, during the past 9 years (modified from World Soybean Usage & Ending Stocks. http://www.agmanager.info/marketing/outlook/newletters/Soybeans.asp).

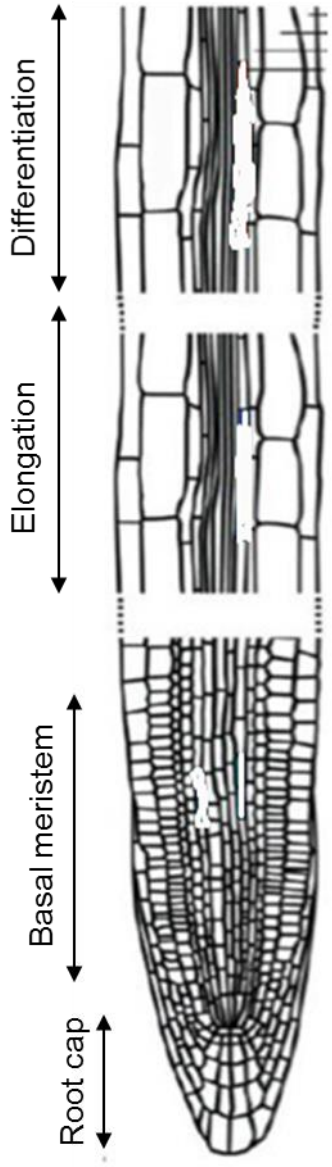

Figure 3. Schematic shape of the primary root. This figure shows the basical structure of primary root in plant. Longitudinal organization of the primary root in the younger region of the root (close to the root tip) contains the different zones of differentiation, elongation, meristem, and root cap (modified from Bensmihen, 2015).

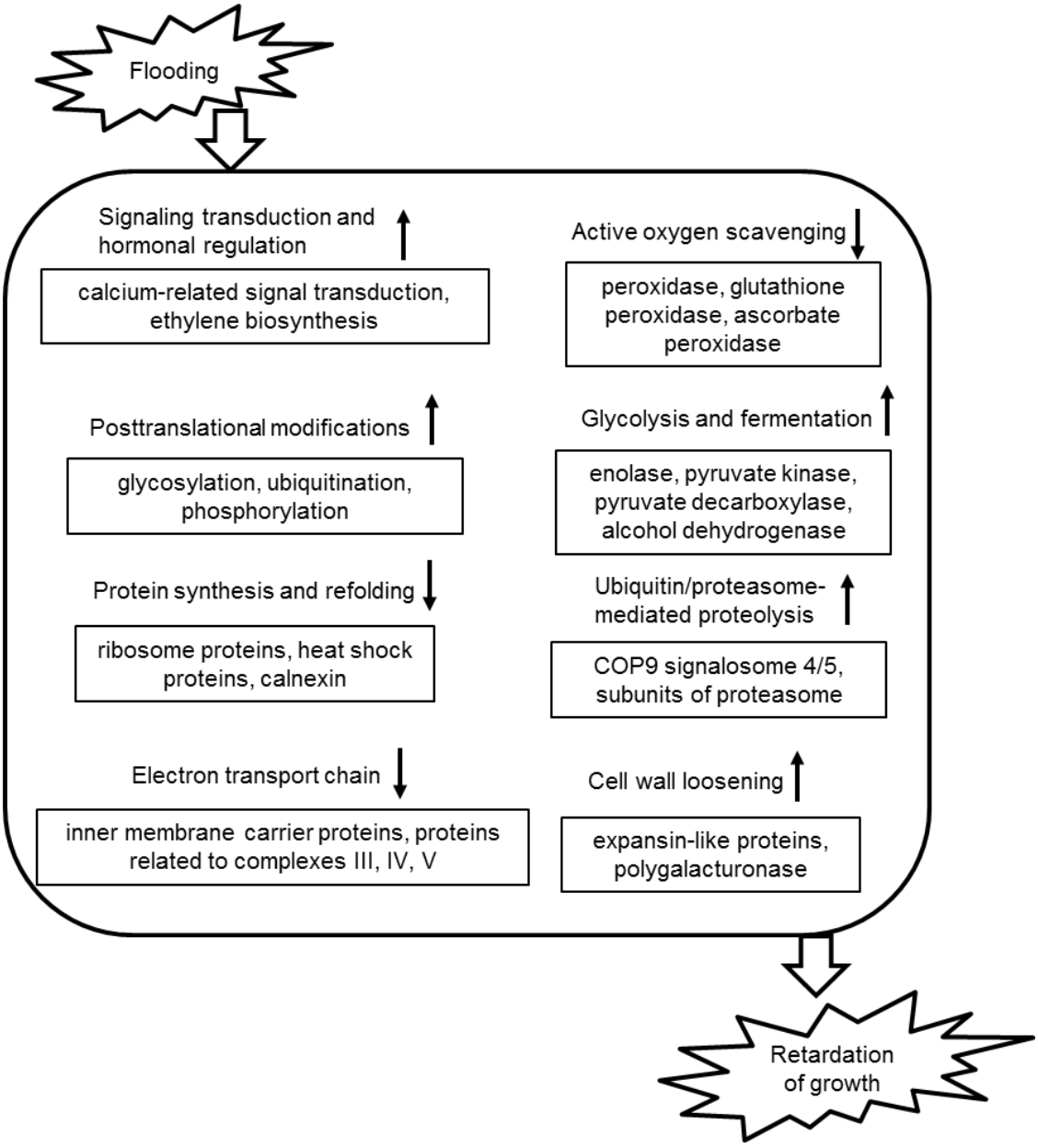

Figure 4. The cellular events in the soybean affected by flooding stress. The cellular events affected by flooding stress in soybean were explored using proteomic technique. The important results were summarized in this figure.

CHAPTER 1

CHARACTERIZATION OF RESPONSE MECHANISM IN SOYBEAN UNDER INITIAL FLOODING STRESS

1.1. Introduction

Soybean is a flooding-intolerant crop and the elongation of soybean roots under flooding stress was suppressed within the first 24 h (Hashiguchi et al., 2009). Genes associated with alcohol fermentation, ethylene biosynthesis, pathogen defense, and cell wall loosening were significantly up-regulated; and proteins involved in ROS scavengers and chaperons were changed under 12 h of flooding stress (Komatsu et al., 2009). In addition, genes related to glycolysis and ubiquitin-mediated protein degradation were up-regulated; however, cell wall synthesis- and chromatin structure synthesis-related genes were down-regulated at the first 6 h of flooding stress (Nanjo et al., 2011a). Based on these findings, it is suggested that key responses and signal transduction events are rapidly activated at the initial flooding stress in soybean.

In root tip, Oh et al. (2014c) performed a nuclear proteomic analysis under flooding stress and 94 differentially abundant nuclear proteins, which were predominantly involved in protein synthesis, transcription, RNA processing, DNA synthesis, and chromatin structure, were identified under flooding stress. Additionally, proteins involved in protein synthesis, post-translational modification, and protein folding were decreased by flooding stress in endoplasmic reticulum (Komatsu et al., 2012b). Recently, Wang et al. (2016) reported that fermentation and protein synthesis/degradation were essential in root tip under flooding stress. In addition, biotin/lipoyl attachment domain containing protein and Class II aminoacyl tRNA/biotin synthetases protein were repressed in root tip during time-course flooding stress (Wang et al., 2016). Theses results suggest that root tip is sensitive to flooding stress because cellular and subcellular events are activated.

A number of proteins related to folding and synthesis of protein were dephosphorylated under flooding conditions and changes in translational and post-translational levels affected the balance of several metabolic pathways, including carbohydrate metabolism (Nanjo et al., 2011b). Nanjo et al. (2011c) reported that the ATP content in soybean root tip was decreased after 24 h of flooding stress and that energy production was regulated through protein phosphorylation. Additionally, the levels of ATP were closely correlated with regions of active growth/cell expansion, cytoplasmic calcium gradients, and ROS activity (Kim et al., 2006). Although many

proteins/phosphoproteins, which responded to flooding stress, were identified in root tip of soybean, the initial flooding responded proteins/phosphoproteins have not been characterized completely in soybean.

In this study, to understand the initial response mechanism of flooding stress in soybean, cellular proteins were analyzed using gel-free proteomics. To explore the signaling transduction pathway of initial flooding in soybean, phosphoproteomics was performed. Phosphopeptides from root tip were enriched using PolyMAC technique. ATP content was measured in soybean root tip under initial flooding stress. In addition, to identify the key cellular proteins and phosphoproteins involved in the soybean flooding response, *in silico* protein-protein interactions between differentially changed proteins were analyzed using hierarchical clustering based on S-system differential equations.

1.2. Materials and methods

1.2.1. Plant material and treatments

Seeds of soybean (*Glycine max* L.) cultivar Enrei were sterilized with 1% sodium hypochlorite solution, rinsed in water, and sown on 500 mL of silica sand moistened with 125 mL of water in a plastic case (180 mm × 140 mm × 45 mm). Soybeans were grown in a growth chamber illuminated with white fluorescent light (160 μmol m^{-2} s^{-1}, 16 h light period/day) at 25°C. Two-day-old soybeans were treated without (control) or with flooding for 3, 6, 12, and 24 h. Additionally, for checking effect of ethylene on soybean under flooding stress, 2-day-old soybeans were transferred to column tubes (38 mm ID x 130 mm) and treated with water, 300 ppm ethephon, and 5 mM silver nitrate under darkness at room temperature for 0, 3, 6, and 12 h. Three independent experiments were performed as biological replicates.

1.2.2. Measurement of ATP content

The content of ATP was measured according to Mohanty et al. (1993) with some modifications. A portion (250 mg) of each collected soybean root tip was ground in liquid nitrogen with a mortar and pestle and was then mixed with 1 mL of ATP extraction buffer, which consisted of 510 mM trichloroacetic acid and 17 mM EDTA.

After sonication for 10 min, the sample was centrifuged at 20000×g for 5 min, and the resulting supernatant was collected for measurement of the ATP content. For the reaction, 100 μL of the supernatant sample was added to 900 μL of reaction solution, consisting of 280 mM HEPES-NaOH (pH 7.7), 4 mM $MgCl_2$, 2 mM glucose, 2 mM NAD, and 4 U/mL glucose-6-phosphate dehydrogenase, and the absorbance at 340 nm was immediately measured using a spectrophotometer (DU730, Beckman, Indianapolis, IN, USA). Hexokinase (4 U/mL) was added to the reaction solution, which was then incubated at 25ºC for 30 min, and the absorbance at 340 nm was again measured. ATP content was determined from the increase in absorbance at 340 nm.

1.2.3. Protein extraction for proteomic analysis of cellular proteins

A portion (500 mg) of sample was ground to powder in liquid nitrogen using a mortar and pestle. The powder was transferred into a solution of 10% trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone and vortexed. The resulting suspension was sonicated for 10 min and then incubated for 60 min at -20°C. After incubation, the suspension was centrifuged at 9000×g for 20 min at 4°C. The resulting supernatant was discarded, and the pellet was washed twice with 0.07% 2-mercaptoethanol in acetone. The final pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and resuspended by vortexing for 60 min at 25°C in lysis buffer consisting of 8 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine.The suspension was centrifuged at 20000×g for 20 min at 25°C and the supernatant was collected as protein extract. Protein concentration was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

1.2.4. Protein extraction for proteomic analysis of phosphoproteins

A portion (500 mg) of sample was ground in liquid nitrogen with a mortar and pestle and then mixed with extraction buffer, containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, phosphatase inhibitor mixture, and protease inhibitor mixture. After centrifugation at 12000×g for 20 min, the obtained supernatant was diluted with 3 volumes of cold acetone containing 0.07% 2-mercaptoethanol and then incubated at -20°C for 2 h. The resulting precipitate was collected and washed 3

times with an acetone solution containing 0.07% 2-mercaptoethanol. Finally, the protein pellets were dried in a Speed-Vac and resuspended in lysis buffer.

1.2.5. Protein clean up and digestion

The extracted proteins (150 μg) were cleaned up as follows. Protein samples were disovled in 150 μL of lysis buffer, 600 μL of methanol was added, and the resulting solution was thoroughly mixed. Subsequently, 150 μL of chloroform was added to the solution and mixed again. To induce phase separation, 450 μL of water was added to the solution, and the resulting mixture was vortexed and centrifuged at 20000×g for 5 min. The upper aqueous phase was discarded, and 450 μL of methanol was added to the organic phase. The samples were centrifuged at 20000×g for 5 min, the resulting supernatants were discarded, and the pellets were dried. The dried pellets were resuspended in 50 mM NH_4HCO_3.

For cellular protein, the purified samples were then reduced, alkylated, and digested using trypsin and lysyl endopeptidase at a 1:100 enzyme/protein concentration at 37°C for 16 h. The digested peptides were acidified with 2 μL of 100% formic acid to pH lessen than 3, desalted with a C18-pipette tip (Nikkyo Technos, Tokyo, Japan), and analyzed by nano liquid chromatography (LC)-MS/MS. For phosphoprotein, the purified samples were reduced, alkylated, and digested using trypsin at a 1:100 enzyme/protein concentration at 37°C for 16 h. The resulting peptides were desalted with a MonoSpin C18 column (GL Science, Tokyo, Japan).

1.2.6. Phosphopeptide enrichment

PolyMAC phosphopeptide enrichment reagent (Tymora, West Lafayette, IN, USA) was used to enrich phosphopeptides. Desalted peptides (150 μg) were resuspended in 100 μL of loading buffer, consisting of 100 mM glycolic acid, 1% trifluoroacetic acid, and 50% acetonitrile, to which 10 μL of PolyMAC-Ti reagent was added. After the resulting mixture was shaken for 30 min, 200 μL of 300 mM HEPES (pH 7.7) was added to increase the pH of the sample above 6.3. After brief vortexing, 50 μL of magnetic capture beads was added to the sample, which was then shaken vigorously for 10 min. The beads were then separated from the solution using a magnetic separator

rack (Invitrogen Dynal AS, Oslo, Norway). Captured beads were washed once in loading buffer and twice in washing buffer, consisting of 100 mM acetic acid, 1% trifluoroacetic acid, and 80% acetonitrile. Phosphopeptides were then eluted by washing the beads twice with 100 μL of 400 mM NH_4HCO_3.

1.2.7. Mass spectrometry analysis

Peptides in 0.1% formic acid were loaded onto an Ultimate 3000 nano-LC system (Dionex, Germering, Germany) equipped with a C18 PepMap trap column (300 μm ID× 5 mm, Dionex). The peptides were eluted from the trap column and separated using 0.1% formic acid in acetonitrile at a flow rate of 200 nL/min on a C18 Tip column (75 μm 1D × 120 mm, Nikkyo Technos, Tokyo, Japan) with a spray voltage of 1.8 kV. The peptide ions in the spray were detected, and the peptides were analyzed on a nanospray LTQ XL Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) operated in data-dependent acquisition mode with the installed Xcalibur software (version 2.0.7, Thermo Fisher Scientific). Full-scan mass spectra were acquired in the Orbitrap MS over 400-1500 m/z with a resolution of 30000. A lock mass function was used to obtain high mass accuracy (Olsen et al., 2005). As the lock mass, the ions including $C_{24}H_{39}O_4$+ (m/z 391.28429), $C_{14}H_{46}NO_7Si_7$+ (m/z 536.16536), and $C_{16}H_{52}NO_8Si_8$+ (m/z 610.18416) were used. Values for ion isolation window were set as follows: activation type was collision-induced dissociation (CID), default charge state was 2, isolation width was 2.0 m/z, normalized collision energy was 35.0 eV. Values for dynamic exclusion were determined as follows: repeat count was 2, repeat duration was 30.0 sec, exclusion duration was 90.0 sec, and exclusion mass width was ±1.5 Da. The 3 most intense precussor ions above a threshold value of 500 were selected for collision-induced fragmentation. The acquired MS spectra were used for protein identification. Data of MS were deposited with the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno et al., 2013) and the identifiers of dataset were PXD 000689 and PXD 000898.

1.2.8. Protein identification using acquired mass spectrometry data

Protein identification was performed using the Mascot search engine (version 2.3.0.2, Matrix Science, London, UK) and a soybean peptide database (54,175 sequences) obtained from the soybean genome database (Phytozome version 9.0, http://www.phytozome.net/soybean) (Schmutz et al., 2010). DTA files were generated from acquired raw data files and then converted to Mascot generic files using Proteome Discoverer software (version 3.3.1, Thermo Fisher Science). The parameters used in the Mascot search were as follows: carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine was set as a variable modification. For phosphopeptide, carbamidomethylation of cysteine was set as a fixed modification, and phosphoST, phosphoY, and oxidation of methionine were set as variable modifications. For peptides after phosphopeptide enrichment, 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 charges were 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 to improve the accuracy and sensitivity of peptide identification (Brosch et al., 2009). False discovery rates for peptide identification of all searches were less than 1.0%. Peptides with a percolator ion score of more than 13 ($p < 0.05$) were used for protein identification. The Mascot search results were exported in XML format for analysis of differentially abundant proteins.

1.2.9. Analysis of differentially abundant proteins

Using SIEVE (version 2.1, Thermo Fisher Scientific), the relative abundances of peptides and proteins were compared between the control and treated groups. For the analysis, the chromatographic peaks detected by MS were aligned, and the peptide peaks were detected as a frame using a frame time width of 5 min and a frame m/z width of 10 ppm, and produced frames for all parent ions were scanned by MS/MS. Chromatographic peak areas of each sample within a single frame were compared, and the ratios between two sample groups in each frame were determined. The frames detected in the MS/MS scan were matched to the imported Mascot search results. The ratio of peptides between samples was determined from the variance-weighted average of the ratios in frames, which matched the peptides in the MS/MS spectrum. The ratios

of peptides were further integrated to determine the ratio of the corresponding protein. In the differential analysis of protein abundance, total ion current was used for normalization. The minimum requirements for identification of a protein were a minimum of two matched peptides. For phosphoproteins, the minimum requirement for the identification of a protein was a minimum of one matched phosphopeptide. Significant changes in the abundance of proteins between the control and treated samples were analyzed ($p < 0.05$).

1.2.10. Categorization of protein function

Function of identified protein was categorized using MapMan bin codes software (http://mapman.gabipd.org/) (Usadel et al., 2005).

1.2.11. Cluster analysis of protein abundance and *in silico* protein-protein interaction analysis

Ratios of protein abundance at different time points of flooding stress were used for cluster analysis by a hierarchical clustering method (a centroid linkage method based on a Euclidean distance metric). Interacting proteins were identified by comparisons of changes in abundance ratios over time. Differential equations were used to describe the amount of gene products (Sima et al., 2009). The S-system differential equation was used to mathematically model protein-protein interactions (Voit, 2000) and applied to fitting to the time course of protein abundance and analyzing for protein interactions (Tanaka et al., 2005). Each *in silico* protein-protein interaction was evaluated on the basis of a goodness-of-fit test, which indicates how well the S-system differential equation simulates the expression of the corresponding target protein. Interactions with r^2 (coefficient of determination) values of >0.9 were considered as candidate interactions and were further evaluated as model interactions.

1.2.12. RNA extraction

A portion (100 mg) of sample was ground into powder in liquid nitrogen using a sterilized mortar and pestle. Total RNA was extracted using an RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Briefly, the

grounded tissue powder was immediately transferred into 450 μL of buffer RLT and vortexed vigorously. The lysate was transferred to spin column and centrifuged at 12000×g for 2 min. The supernatant was transferred to a new microcentrifuge tube and half volume of ethanol was added to the cleaned lysate. The mixture was transferred to an RNeasy spin column and centrifuged at 8000×g for 15 sec. The RNeasy spin column was washed using 350 μL of buffer RW1. The DNase I incubation solution was directly added to the RNeasy spin column and sustained for 15 min. After treatment, 350 μL of Buffer RW1 was added to the RNeasy spin column and centrifuged at 8000×g for 15 sec. RNeasy spin column was washed 2 times using 500 μL of buffer RPE. RNA was eluted from the spin column using 40 μL of RNase-free water. The isolated RNA was reverse-transcribed using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, the reaction system containing 800 ng of RNA template was used. The reaction conditions were as follows: 25°C for 5 min, followed by 42°C for 30 min and 85°C for 5 min.

1.2.13. Quantitative reverse transcription-polymerase chain reaction analysis

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed in a 10 μL reaction mixture using SsoAdvanced SYBR Green Supermix (Bio-Rad) and 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. Gene expression was normalized using 18S rRNA as an internal control. The primers were designed using the Primer3 web interface (http://frodo.wi.mit.edu) (Supplemental Table 1). Primer specificity was checked by BLASTN searches against sequences in the soybean genome database (Phytozome) with the designed primers as queries by melt curve analysis.

1.2.14. Statistical analysis

The statistical significance of the results was evaluated by the Student's *t*-test when only two groups were compared. The statistical significance of comparisons between multiple groups was evaluated with one-way ANOVA with Tukey's multiple comparison. A p value of less than 0.05 was considered as statistically significant. All

calculations were performed using SPSS software (version 12.0J; IBM. Armonk, NY, USA).

1.3. Results

1.3.1. Physiological and biochemical changes during initial stage of flooding stress in soybean

To understand the changes affected by initial flooding stress in soybean, several physiological and biochemical characters were analyzed. Two-day-old soybean was treated without or with flooding stress for 3, 6, 12, and 24 h (Figure 5) in 3 independent biological replicates. The elongation, fresh weight, dry weight, and ATP content of soybean were measured. As results, the elongation of soybean root was suppressed by flooding stress (Figure 6A). The fresh weight and dry weight of flooded soybeans almost did not change after 3 h of flooding stress; however, they were constantly increased in control plants (Figure 6B and 6C). In addition, the dry weight of soybean root tip did not change during the early stages of flooding stress (Figure 6D). Furthermore, the ATP content of root tip significantly decreased under flooding stress, especially at the first 3 h of flooding stress compared with control plants ($p < 0.01$) (Figure 6E).

1.3.2. Time-dependent analysis of identified cellular proteins

To identify the initial flooding stress responded proteins in soybean root tip, gel-free/label-free proteomic technique was used. Proteins were extracted from the root tip of soybean and analyzed using nanoLC-MS/MS. Comparative analysis of the protein profiles in the flooding-treated root tip showed that the abundances of 71 proteins significantly changed with time course manner (Table 2). Among 71 significantly changed proteins, numerous isozymes were included. To obtain a better understanding of the initial flooding stress responded proteins in soybean root tip, the differentially changed proteins were functionally categorized using MapMan bin codes. Using this approach, 71 significantly changed proteins were mainly involved in amino acid metabolism, glycolysis, tricarboxylic acid cycle, hormone metabolism, stress, and protein synthesis (Figure 7).

1.3.3. Cluster analysis of significantly changed cellular proteins

To further analyze the profile of significantly changed cellular proteins identified in soybean root tip, the cluster analysis with hierarchical clustering method was performed. Based on this approach, 5 clusters (I–V) of differentially changed proteins were recognized (Figure 6). Cluster I contained proteins that increased in seedlings treated with flooding for 3 and 6 h, but decreased after 12 h, and then increased again after 24 h compared to the untreated control seedlings. Cluster II was comprised of two proteins increased at 3, 6, and 12 h, but decreased after 24 h of flooding stress, and nine proteins decreased 6 h after flooding. Clusters III and V were comprised of proteins that showed an upward trend in abundance levels under flooding condition with time course manner. Cluster IV included early responsive proteins that were decreased in seedlings in response to flooding. Forty-seven proteins of the 71 proteins were grouped in Cluster I (Figure 8).

To better understand the physiological processes that happend in flooding-stressed soybean, the interactions among these significantly changed cellular proteins were analyzed using the changes in the abundances of proteins during the first 24 h of flooding stress (Figure 9). The in- and out promotive numbers of interacting proteins were constructed with an S-system modeling approach. One potential interacting protein network was comprised of 10 proteins, which contained the mostly abundant interactions, was detected belonging to cluster I (Figures 9 and 8). These 10 interacting proteins were: sugar isomerase (No 52), cobalamin independent synthase (No 50), acetyl-CoA carboxylase 1 (No 1), enolase (No 13), clathrin_ heavy chain (No 2), alpha_xylosidase 1 (No 24), phosphoglucomutase (No 9), citrate lyase b (No 15), heat shock protein 70 (No 21), and calreticulin 1b (No 22) (Figure 9). To confirm whether these 10 proteins were responded to initial flooding stress on mRNA express level, the genes corresponding to these 10 proteins were analyzed using qRT-PCR techanique (Figure 10). As results, *ATP-citrate lyase*, *alpha xylosidase*, and *heat shock protein 70* were down-regulated in root tip of soybean under flooding stress; whereas, *calreticulin* was up-regulated. The mRNA expression levels of other 6 genes were not significantly changed under flooding condition (Figure 10).

1.3.4. Identification of phosphoproteins in root tip of soybean under flooding stress

To understand the role of protein phosphorylation at the initial flooding stress response in soybean, a quantitative phosphoproteomic technique was used in this study. Two-day-old soybeans were treated without or with flooding stress for 3, 6, 12, and 24 h, and proteins were extracted from the root tip at each time point. Phosphopeptides were enriched and analyzed using gel-free proteomic technique (Figure 6). To identify the flooding responsive phosphoproteins in root tip of soybean, phosphoproteins were compared between the untreated soybean and flooding stressed soybean using SIEVE software. Using this approach, a total of 34 significantly changed phosphoproteins were identified at initial 3 h of flooding stress (Figure 11). Among of 34 phosphoproteins, 27 and 7 were increased and decreased, respectively (Table 3). Several proteins related to ribosomal proteins (6/34) and major CHO metabolism (4/34) were increased; however, protein synthesis-related proteins (3/34) were decreased. In addition, 4 and 2 cell wall-related proteins were increased and decreased, respectively (Table 3). To understand the function of these initial flooding stress responded phosphoproteins, the identified phosphoproteins were functionally categorized using MapMan bin codes (Figure 11) and they were mainly categorized in protein metabolism and cell wall.

1.3.5. Cluster and bioinformatic-based analyses of protein-protein interactions of significantly changed phosphoproteins under flooding stress

To explore the change pattern of these initial flooding responded phosphoproteins, chluster analysis was performed (Figure 12). According to the descriptions of 34 significantly changed phosphoproteins (Table 3), 22 phosphoproteins (Table 4) were selected. Under flooding stress, 3 clusters (I, II, and III) of differentially changed proteins were recognized in soybean. Cluster I contained one protein that increased at each point of flooding stress; Cluster II was comprised of proteins that decreased at 3 and 6 h of flooding stress, but remained unchanged in following stress; and Cluster III encompassed only one protein that sustantly decreased with less than 10-fold by exposure to flooding stress (Figure 12B).

To determine the function of these 22 phosphoproteins, the interactions among

them were analyzed with an S-system based modeling approach using the changes in the abundances of proteins during the first 24 h of flooding stress (Figure 13). As results, 3 sucrose phosphate synthase 1F enzymes (Nos. 8, 9, and 10) were positioned in the center of the protein interaction network in both untreated and flooding stress treated soybean. Aluminum-induced protein with YGL and LRDR motifs (No. 1), DEAD-DEAH box RNA helicase (No. 18), and SART1 (No. 20) were not included in either protein interaction network. UDP-glucose 6-dehydrogenase (No. 5), regulatory particle non-ATPase 10 (No. 14), Ypt/Rab GAP domain of gyp1p (No. 16), and nucleotide/sugar transporter (No. 17) were included in the protein interaction network formed under flooding stress. However, eukaryotic translation initiation factor 3C (No. 2), RED family protein (No. 12), cellulose synthase-like D3 (No. 13), and global transcription factor group B1 (No. 15) were not integrated into the protein interaction network under flooding stress conditions. Notably, eukaryotic translation initiation factor 4G (No. 22) was on the edge of the network under non stressed conditions; however, it was located in the center of the protein interaction network under flooding stress (Figure 13).

1.3.6. Effects of ethylene on soybean growth under flooding stress

Eukaryotic translation initiation factor 4G was shown to have a MA3 domain that shares similarity with programmed cell death 4 protein (Cheng and Gallie, 2013), which increases ethylene sensitivity in *Arabidopsis* when its expression is reduced (Lei et al., 2011). To confirm whether ethylene promotes the growth of soybean at the initial flooding stress through regulation of eukaryotic translation initiation factor 4G, soybean plants were treated with an ethylene releasing agent (ethephon) and ethylene receptor antagonist (silver nitrate) during flooding stress for 3, 6, and 12 h (Figure 14A). The fresh weight of soybeans increased gradually in the control. In contrast, the fresh weight of soybean significantly increased after the initial 3 h of flooding stress and then drastically slowed, as before, with continued stress exposure. However, the fresh weight of soybean significantly increased with ethephon treatment compared to control plants, even after 12 h of flooding stress, whereas the weight of plants was dramatically and significantly decreased by silver nitrate under flooding conditions at all examined time points (Figure 14B).

1.4. Discussion

1.4.1. Soybean growth is suppressed by initial flooding stress

Two-day-old soybeans were treated without or with flooding stress for 3, 6, 12, and 24 h (Figure 5). The elongation of root of flooded soybean was suppressed and the fresh weight and dry weight of flooded soybeans almost did not change after 3 h of flooding stress (Figure 6). Hashiguchi et al. (2009) reported that the growth of soybean seedling was significantly suppressed in the first 24 h of flooding stress. Flooding is complex stress that involves hypoxic, water, and light stresses (Jackson and Colmer, 2005). The effect of flooding was a result of various related factors such as limited gas diffusion/reduced irradiance and these factors led to slow down photosynthesis under flooding condition (Das et al., 2009). The decreased photosynthesis severely affect the growth of plants. Combined our present results, they clearly demonstrate that growth of soybean is suppressed at an initial phase during flooding stress treatment.

1.4.2. Fermentation is activated by initial flooding stress in soybean

Among 71 commonly changed proteins, the abundances of 2 alcohol dehydrogenases (Glyma14g27940.1 and Glyma 06g12780.1) were markedly increased with time course manner (Table 2). Oxygen was markedly reduced under flooding condition and plants have to adapt to low oxygen status through increasing anaerobic energy production (Voesenek et al., 2006). Alcohol fermentation is a major pathway for anaerobic energy production and alcohol dehydrogenase works as a key enzyme (Smith and ap Rees). In the present study, alcohol dehydrogenase was increased within 24 h after flooding stress. This result indicated that alcohol dehydrogenase was increased to strengthen alcohol fermentation and provide energy to soybean under flooding condition. Proteomic and transcriptomic analyses of flooding responsive proteins and genes showed that the expression of alcohol dehydrogenase increased remarkably in response to flooding stress during the early stages of soybean growth (Komatsu et al., 2009). Further gene expression analysis in soybean roots also showed that the gene of alcohol dehydrogenase was markedly up-regulated after 6 h of flooding stress (Komatsu et al., 2011a). Together with our present findings, these results indicate that alcohol

fermentation in root is activated to promote anaerobic energy production for soybean to adapt to the initial phase during flooding stress.

1.4.3. Heat shock protein, xylosidase, ATP-citrate lyase, and calreticulin are responded to initial flooding stress in soybean

Heat shock protein is involved in many cellular processes, including protein folding, protein translocation across membranes, and protein degradation (Wang et al., 2004). Komatsu et al. (2013d) reported that abundance of heat shock protein 70 in the cotyledons and roots of soybean increased in response to flooding stress, and the increase of heat shock protein 70 was associated with calcium content in cotyledon. The level of heat shock protein 70 was reported to increase in soybean roots under flooding stress, but decrease in the root tip (Nanjo et al., 2011 a,b). In the present study, heat shock protein 70 was also decreased at 12 h and 3 h at the protein and mRNA levels, respectively. In addition, it was also found to be interacted with several other proteins in soybean root tip exposed to flooding stress. These results suggest that heat shock protein 70 is sensitive to flooding stress and has organ specificity. Furthermore, heat shock protein 70 might play role at initial stage of flooding response in soybean root tip through regulating other proteins.

Xylosidase plays an important role in xylem remodeling during the process of cell wall maturation (Sunna and Antranikian, 1997). Proteomic research on exudates from germinating *Lupinus albus* seeds indicated that xylosidase was involved in protecting the spermosphere environment and acted as a first defense against pathogens (Scarafoni et al., 2013). Here, because xylosidase was decreased at both the protein and mRNA level at the initial flooding stress, the formation of cell wall might be suppressed without enough effective xylosidase. This result indicated that the susceptibility of soybean to flooding-induced damage might be due to impairment of cell wall development, which would reduce plant defense against environmental stress. ATP-citrate lyase catalyzes the formation of acetyl-CoA/oxaloacetate from citrate/CoA with the concomitant hydrolysis of ATP (Elshourbagy et al., 1990). Reverse genetic analyses indicated that ATP citrate-lyase was required for the normal growth and development of *Arabidopsis* (Fatland et al., 2005). Langlade et al. (2002) reported that expression of the

ATP-citrate lyase gene was up-regulated in roots of maize and was positively correlated with the plant's ability to cope with adverse soil conditions. In the present study, ATP citrate-lyase was decreased at both the protein and mRNA level in soybean root tip at the initial flooding stress, indicating that flooding stress might restrain growth of soybean through decreasing level of ATP citratelyase and this enzyme might be taken as marker for the normal growth of soybean.

Calreticulin is a major calcium-sequestering protein found in the lumen of the endoplasmic reticulum (Corbett et al., 1999). In rice, calreticulin plays a role in the signaling pathway involved in cold stress response (Li et al., 2003). Komatsu et al. (2007) reported that accumulation of calreticulin binding protein 1 in a cold tolerant rice variety was higher than that in rice varieties with intermediate cold tolerance. Previous proteomic analysis of soybean roots revealed that calreticulin was decreased in response to flooding stress (Komatsu et al., 2009). In the present study, calreticulin was increased and interacted with other flooding responsive proteins in the initial stages of flooding stress. The findings from these past and present studies in rice and soybean indicate that calreticulin is involved in response to various types of stresses. Flooding stress might cause a change of calcium level, and further activate calcium-related signal transduction pathways. The transcriptional and translational regulation of calreticulin in root tip may control the responses of soybean to the initial stages of flooding stress. Furthermore, because calreticulin was interacted to other proteins, posttranslational regulation with calcium might play an important role in root tip of soybean in initial stage of flooding.

1.4.4. Energy production and protein synthesis are regulated through protein phosphorylation in root tip of soybean in the initial stages of flooding stress

In the results of cellular proteins study, several kinases including pfkB-like carbohydrate kinase and phosphoglycerate kinase, were significantly changed under the initial flooding stress (Table 2). In addition, calreticulin was increased at 24 h at the protein and mRNA levels and interacted with other proteins in the initial stages of flooding stress. Although protein kinases were involved in the process of phosphorylation (Komatsu et al., 2007) prior to the present study, it was unclear whether protein phosphorylation plays a role in plant responses to flooding stress. In this study,

the ATP content of soybean root tip decreased in response to flooding, particularly during the initial stages of flooding stress (Figure 6), indicating that phosphorylation is involved in regulating plant responses to flooding stress.

To better understand the role of phosphorylation in the initial stages of flooding stress of soybean, the phosphorylation status of differentially changed proteins in flooding-stressed plants was analyzed. The identified phosphoproteins were predominantly related to protein synthesis and modification in the root tip of early-stage soybean plants (Figure 12). Nanjo et al. (2011b) reported that the ATP content in root tip of soybean was decreased after 24 h of flooding stress and energy production was regulated through flooding-stress induced changes in protein phosphorylation. These results indicate that protein synthesis and modification-related proteins contain phosphorylation sites and are regulated through phosphorylation. Furthermore, the production of ATP by mitochondrial oxidative phosphorylation is restricted by flooding-induced oxygen deprivation (Gibbs and Greenway, 2003). Komatsu et al. (2011b) reported that flooding stress damaged the electron transport chain, although the tricarboxylic acid cycle increased in mitochondria. These findings suggest that the decrease of ATP content might be caused by oxygen deprivation. The decrease of ATP and protein synthesis is important cellular events that might be modulated by protein phosphorylation during the initial stages of flooding stress in root tip of soybean.

1.4.5. Protein synthesis and cell wall-related proteins are rapidly phosphorylated and dephosphorylated in root tip of soybean in response to flooding stress

In the present study, a total of 34 phosphoproteins were significantly changed during the early stage of flooding stress.These differentially changed proteins were mainly predicted to function as ribosomal, cell wall, and protein synthesis-related proteins (Table 3). Among the 6 identified cell wall-related proteins, 4 were increased and 2 were decreased, and out of 3 protein synthesis-related proteins, 1 was increased and 2 were decreased (Table 3). These results indicate that phosphorylation affects protein synthesis and cell wall formation in soybean under flooding stress. Previous proteomic research indicated that flooding mainly affects protein synthesis in the endoplasmic reticulum of soybean root tip (Komatsu et al., 2012b). Research on seed

germination indicated that translational activation of stored mRNA was important for seed germination and the regulation of protein abundance was a critical regulatory mechanism controlling germination (Rajjou et al., 2004). Proteomic analysis of the flooding tolerance mechanism in soybean indicated that proteins related to protein synthesis were significantly changed in wild-type plants under flooding stress, but did not markedly differ in a flooding tolerant mutant (Komatsu et al., 2013c). In the present study, several protein synthesis related proteins, including eukaryotic translation initiation factor, were shown to be regulated by phosphorylation. In maize, the phosphorylation of eukaryotic translation initiation factor played a role in the specific regulation of the nuclear export of eukaryotic translation initiation factor-bound mRNAs (Lewandowska-Gnatowska et al., 2011). These results suggest that the export and activation of mRNAs are important for protein synthesis and might be regulated by the phosphorylation of eukaryotic translation initiation factors in soybean roots exposed to flooding stress.

Cell wall proteomic analysis in soybean indicated that formation of the cell wall was suppressed in response to flooding through decreasing the abundance of proteins related to the production of precursors of cell wall synthesis (Komatsu et al., 2010). Subsequent study in soybean under flooding stress confirmed that cell wall related proteins were significantly affected by flooding stress (Nanjo et al., 2011b). Here, the cell wall-related proteins including cellulose synthase were shown to be regulated by phosphorylation. In *Arabidopsis*, the phosphorylation of cellulose synthase alters microfibril structure in the primary cell wall and leads to defective anisotropic cell expansion (Chen et al., 2010b). The findings from these past and present studies indicate that the suppression of cell wall formation is a critical plant response to flooding stress and the phosphorylation of cellulose synthase might play an important role in the suppression of cell wall formation in flooded soybean.

1.4.6. Ethylene involves in soybean responses to initial flooding stress through protein phosphorylation

Eukaryotic translation initiation factor 4G increased and was located at the edge of a predicted protein network in control plants; however, it was decreased and positioned

in the center of a protein network under flooding stress conditions (Figures 12 and 13). It was reported that the phosphorylation of eukaryotic translation initiation factors affected mRNA cap binding in wheat (Khan and Goss, 2004). Eukaryotic translation initiation factor 4G has an MA3 domain that shares similarity with programmed cell death 4 protein (Cheng and Gallie, 2013). In *Arabidopsis*, the decreased expression of a protein containing an MA3 domain improved ethylene sensitivity (Lei et al., 2011). Plant ethylene levels increased approximately 20-fold in the initial stages of waterlogging in *Rumex palustris Sm* and *Rumex acetosella L* (Banga et al., 1996), and ethylene has been shown to promote the hyponastic growth of submerged *Arabidopsis* seedlings (Millenaar et al., 2005). Because ethylene might be related to the poor growth of soybean under flooding stress, the effect of ethylene on soybean growth was analyzed in this study (Figure 14). The fresh weight of soybean plants increased under flooding conditions in the presence of an ethylene-releasing agent, but significantly decreased compared to untreated control plants by treatment with an ethylene receptor antagonist, even under flooding stress conditions. These results suggest that an ethylene-responsive signaling pathway might be involved in soybean responses to initial flooding stress through affecting the phosphorylation status of regulatory proteins.

1.5. Conclusion

The present proteomic study characterized the changes of cellular proteins including phosphoproteins in soybean at initial flooding stress. The main findings are as follows: (i) The fresh weight and dry weight of soybeans were almost not changed after 3 h of flooding stress and ATP content of root tip was significantly decreased by flooding stress. (ii) Fermentation, cell wall formation, and calcium-related pathways were promotly affected by initial flooding stress in soybean. (iii) Protein synthesis/modification- and cell wall-related proteins such as sucrose phosphate synthase 1F, 60S acidic ribosomal protein, and UDP-glucose 6-dehydrogenase, were phosphorylated and dephosphorylated, respectively, in response to flooding stress. (iv) The decrease of dephosphorylated eukaryotic translation initiation factor 4G, which increases ethylene sensitivity, is the central early event in the soybean response to flooding stress; and (v) treatment of flooded soybean with ethylene dramatically

increases plant growth. Taken together, these results suggest that flooding stress in soybean induces complex responsive processes including alteration of energy metabolism, suppression of cell wall formation, and activation of signal transduction and these events are regulated through phosphorylation. Furthermore, ethylene signaling pathway might participate in plant response mechanisms to initial flooding stress through protein phosphorylation in the root tip of soybean.

Table 2. Identification of flooding-responsive proteins in soybean root tip with time course manner using a gel-free proteomic technique

Protein No.	Protein ID[a]	Description	MP[b]	Ratio[c]					*p*-value	Function[d]
				48(0)	51(3)	54(6)	60(12)	72(24)		
1	Glyma04g11550.1	acetyl_CoA carboxylase 1	28	1	1.38	1.18	0.63	1.37	0.0274	Lipid metabolism
2	Glyma18g02960.1	clathrin_ heavy chain	25	1	1.19	1.16	0.50	1.23	0.0114	Cell
3	Glyma07g00900.1	lipoxygenase 1	24	1	1.51	1.25	0.73	1.77	0.0002	Hormone metabolism
4	Glyma13g42320.1	lipoxygenase 1	21	1	1.20	1.33	0.76	1.81	0.0024	Hormone metabolism
5	Glyma15g03030.1	lipoxygenase 1	21	1	1.09	1.31	0.49	1.00	0.0124	Hormone metabolism
6	Glyma06g48360.1	methylenetetrahydrofolate reductase 2	18	1	1.64	1.04	0.65	1.33	0.0031	C1-metabolisme
7	Glyma19g03490.1	glycosyltransferase_ family 35	17	1	1.44	1.40	0.85	1.58	0.0494	Major CHO
8	Glyma15g40860.1	ribosomal protein S5	17	1	1.30	1.24	0.63	1.44	0.0116	Protein.synthesis.
9	Glyma08g04890.1	phosphoglucomutase/phosphomannomutase	16	1	1.50	1.24	0.68	1.55	0.0261	Glycolysis
10	Glyma20g01010.1	phosphofructokinase family protein	16	1	1.49	1.25	0.72	1.38	0.0055	Glycolysis
11	Glyma18g52430.2	oxoglutarate dehydrogenase_ E1 component	16	1	1.11	1.14	0.42	1.41	0.0326	Tricarboxylic acid cycle
12	Glyma15g03050.1	lipoxygenase 1	15	1	1.34	1.12	0.71	1.37	0.018	Hormone metabolism
13	Glyma19g37520.1	enolase	15	1	1.42	1.22	0.24	1.45	0.0001	Glycolysis
14	Glyma13g19331.1	heat shock cognate protein 70_1	14	1	1.47	1.12	0.62	1.22	0.0119	Stress.abiotic.
15	Glyma09g04000.2	ATP_citratelyase B_1	14	1	1.43	1.12	0.51	1.17	0.0105	Tricarboxylic acid cycle
16	Glyma14g36850.1	aldolase superfamily protein	14	1	1.26	1.27	0.84	1.67	0.0114	Glycolysis.
17	Glyma13g42310.1	lipoxygenase 1	13	1	0.92	1.41	0.42	1.14	0.032	Hormone metabolism
18	Glyma16g08460.1	NADP_malic enzyme 4	13	1	1.69	1.32	0.95	2.07	0.0358	Tricarboxylic acid cycle
19	Glyma17g33050.1	aspartate aminotransferase 5	12	1	1.19	1.43	0.80	1.79	0.0292	Amino acid metabolism
20	Glyma02g13330.1	reversibly glycosylated polypeptide 3	12	1	1.05	1.26	0.70	0.43	0.0076	Cell
21	Glyma05g36600.1	heat shock protein 70	12	1	1.38	1.18	0.63	1.37	0.0274	Stress.abiotic.
22	Glyma20g23080.1	calreticulin 1b	11	1	1.19	1.16	0.50	1.23	0.0114	Signaling
23	Glyma06g19820.1	aldehyde dehydrogenase 10A8	11	1	1.51	1.25	0.73	1.77	0.0002	Secondary metabolism
24	Glyma01g20460.1	alpha_xylosidase 1	11	1	1.20	1.33	0.76	1.81	0.0024	Misc
25	Glyma14g00760.1	thiolase family protein	11	1	1.09	1.31	0.49	1.00	0.0124	Amino acid metabolism
26	Glyma18g48280.1	ATPase_ V1 complex_ subunit B protein	11	1	1.64	1.04	0.65	1.33	0.0031	Transport
27	Glyma17g37770.1	tubulin beta_1 chain	11	1	1.44	1.40	0.85	1.58	0.0494	Cell
28	Glyma16g19364.1	cytosol aminopeptidase family protein	11	1	1.44	1.27	0.82	1.75	0.0398	Protein.degradation
29	Glyma12g11130.1	beta_amylase 5	11	1	1.48	1.74	0.98	2.50	0.0458	Major CHO
30	Glyma19g32990.1	actin 7	11	1	1.61	1.28	0.75	1.61	0.0441	Cell
31	Glyma15g40110.1	TCP_1/cpn60 chaperonin family protein	10	1	1.66	1.15	0.61	1.33	0.0241	Protein.folding
32	Glyma13g41120.2	triosephosphateisomerase	10	1	1.46	0.52	0.94	1.61	0.0003	Glycolysis
33	Glyma14g01100.1	heat shock protein 89.1	10	1	1.51	1.15	0.62	1.35	0.0376	Stress.abiotic.
34	Glyma17g17730.1	root hair specific 19	10	1	1.64	1.18	0.70	2.01	0.0067	Miscellaneous
35	Glyma20g29840.1	tubulin beta 8	10	1	1.57	1.27	0.64	1.32	0.031	Cell
36	Glyma19g40810.1	S_adenosylmethioninesynthetase 2	9	1	1.19	0.96	0.10	0.84	0.02	Amino acid metabolism

37	Glyma04g36870.3	glyceraldehyde_3_phosphate dehydrogenase C subunit 1	9	1	1.40	1.24	0.81	1.73	0.0097	Glycolysis
38	Glyma17g04210.1	lipoamide dehydrogenase 2	9	1	1.76	1.50	0.03	1.31	0.001	Tricarboxylic acid cycle
39	Glyma14g27940.1	alcohol dehydrogenase 1	9	1	1.42	1.72	1.50	3.49	0.0182	Fermination ADH
40	Glyma13g15140.1	cobalamin_independent synthase family protein	9	1	0.85	0.94	0.40	1.60	0.0119	Amino acid metabolism
41	Glyma13g34290.1	gamma_glutamyl hydrolase 1	8	1	1.52	1.63	0.94	2.40	0.0414	Protein.degradation
42	Glyma18g52250.1	NAD(P)_linked oxidoreductase superfamily protein	8	1	1.02	1.07	0.27	0.84	0.0418	Secondary metabolism
43	Glyma19g07240.3	GTP binding Elongation factor Tu family protein	8	1	0.86	0.16	0.27	1.10	0.0013	Protein.synthesis
44	Glyma07g38790.1	NAD(P)_binding Rossmann_fold superfamily protein	8	1	1.19	1.61	0.65	1.85	0.0312	Miscellaneous
45	Glyma08g25950.1	cytochrome P450 family 72 subfamily A polypeptide 15	7	1	1.01	0.65	0.20	0.36	0.0121	Miscellaneous
46	Glyma15g31520.1	MLP_like protein 43	7	1	1.55	1.49	1.39	3.13	0.0298	Stress.abiotic
47	Glyma17g14750.1	glycosyl hydrolases family 32 protein	7	1	1.85	0.99	0.65	0.91	0.0092	Major CHO
48	Glyma18g49240.1	HXXXD_typeacyl_transferase family protein	7	1	1.81	1.16	0.58	0.93	0.0494	Secondary metabolism.
49	Glyma06g46190.2	aconitase 3	7	1	0.78	0.05	0.15	0.48	0.0003	Tricarboxylic acid cycle
50	Glyma05g10840.3	cobalamin_independent synthase family protein	7	1	1.46	1.26	0.38	1.41	0.0035	Amino acid metabolism.
51	Glyma10g00920.1	lactate/malate dehydrogenase family protein	7	1	0.79	0.87	0.49	1.23	0.0263	Tricarboxylic acid cycle
52	Glyma04g03490.1	sugar isomerase (SIS) family protein	7	1	1.59	1.29	0.27	1.51	0.0395	Glycolysis.
53	Glyma06g08380.1	non_ATPase subunit 9	7	1	1.09	1.13	0.26	1.06	0.0382	Protein.degradation.
54	Glyma05g10840.3	cobalamin_independent synthase family protein	7	1	1.31	0.86	0.61	0.96	0.0414	Amino acid metabolism.
55	Glyma14g39140.1	UDP_glucosepyrophosphorylase 2	7	1	0.95	1.28	0.55	1.26	0.0425	Glycolysis.
56	Glyma15g11530.1	glyoxalase I homolog	6	1	1.23	1.26	0.29	1.64	0.0446	Biodegradation
57	Glyma06g12780.1	alcohol dehydrogenase 1	6	1	1.50	0.71	1.24	0.17	0.0028	Fermination ADH
58	Glyma08g45531.1	kunitz trypsin inhibitor 1	6	1	1.35	0.01	0.99	1.31	0.0047	Stress.abiotic
59	Glyma16g32960.1	enolase	6	1	1.40	1.92	0.97	4.28	0.0033	Glycolysis
60	Glyma10g07410.1	embryonic cell protein 63	6	1	0.19	0.21	0.12	1.26	0.028	Development
61	Glyma10g31590.1	methionine gamma_lyase	5	1	2.10	0.84	0.02	0.60	0.0011	Amino acid metabolism
62	Glyma01g41990.1	glycosyl hydrolases family 32 protein	5	1	1.57	0.58	0.50	0.58	0.0045	Major CHO metabolism
63	Glyma12g09940.2	FAD/NAD(P)_binding oxidoreductase family protein	5	1	1.78	1.64	1.28	3.81	0.0323	Not assigned
64	Glyma07g33950.1	leucine_rich repeat (LRR) family protein	5	1	1.31	1.42	1.33	3.93	0.0471	Not assigned
65	Glyma03g03270.1	arginase/deacetylase superfamily protein	5	1	3.30	1.50	1.95	4.15	0.022	Amino acid metabolism
66	Glyma11g20570.2	ribosomal protein L11 family protein	4	1	1.21	0.06	0.83	1.55	0.0108	Protein synthesis
67	Glyma16g33710.1	kunitz family trypsin and protease inhibitor protein	4	1	1.61	0.36	0.74	1.64	0.0016	Stress.abiotic
68	Glyma04g02230.1	pyridoxal_dependent decarboxylase family protein	3	1	0.66	0.24	0.004	0.05	0.0146	Amino acid metabolism
69	Glyma13g10360.1	methionine gamma_lyase	3	1	1.67	0.72	0.02	0.62	0.0243	Amino acid metabolism
70	Glyma08g20250.1	lipoxygenase 1	3	1	1.34	0.01	0.54	1.44	0.0036	Hormone metabolism
71	Glyma17g23730.1	cobalamin_independent synthase family protein	2	1	1.24	0.18	0.75	1.22	0.0001	Amino acid metabolism

a) Protein is ID according to the Phytozome database. b) MP means matched peptides; No. means different significantly changed proteins; c) Ration indicates ratio of quantities of protein, it was calculated using treatments divide control. *p*-value was got by one-way ANOVA with Tukey's multiple comparison. d) Functional category obtained from MapMan bin codes.

Table 3. Identification of protein abundances of phosphoproteins in soybean root tip under flooding stress for 3 h

NO	Protein ID[a]	Description	MP[b]	Ratio[c]	SD	*p*-value	Function[d]
1	Glyma08g26520.1	UDP_glucose 6_dehydrogenase family protein	6	38565.92	0.44	< 0.001	Cell wall
2	Glyma18g50000.1	UDP_glucose 6_dehydrogenase family protein	6	38565.92	0.44	< 0.001	Cell wall
3	Glyma03g07460.1	AMP deaminase/myoadenylate deaminase	5	38517.27	0.5	< 0.001	Nucleotide metabolism
4	Glyma08g45425.1	eukaryotic translation initiation factor 4G	4	0.89	0.87	0.046	Protein.synthesis
5	Glyma17g11820.1	sucrose phosphate synthase 1F	4	37421.83	0.61	< 0.001	Major CHO metabolism
6	Glyma01g45710.1	DNAJ heat shock N_terminal domain_containing protein	3	0.13	1.23	< 0.001	Stress
7	Glyma08g24663.1	SART_1 family	3	69914.78	0.59	< 0.001	Development
8	Glyma13g23060.2	sucrose phosphate synthase 1F	3	39747.58	0.63	< 0.001	Major CHO metabolism
9	Glyma05g04580.1	plant VAP homolog 12	3	6.53	0.94	0.049	Protein.targeting
10	Glyma17g15010.1	plant VAP homolog 12	3	6.53	0.94	0.049	Protein.targeting
11	Glyma14g14360.1	nucleotide/sugar transporter family protein	2	0.11	1.30	0.049	Not assigned
12	Glyma04g07180.3	DEAD-DEAH box RNA helicase family protein	2	78865.24	0.89	< 0.001	DNA
13	Glyma02g29910.1	sucrose phosphate synthase 1F	2	12.53	0.81	0.026	Major CHO metabolism
14	Glyma13g23060.1	sucrose phosphate synthase 1F	2	12.53	0.81	0.026	Major CHO metabolism
15	Glyma01g44281.1	cellulose synthase_like D3	2	55202.33	0.75	< 0.001	Cell wall
16	Glyma11g01230.1	cellulose synthase_like D3	2	55202.33	0.74	< 0.001	Cell wall
17	Glyma0169s00200.3	60S acidic ribosomal protein family	2	51471.25	0.72	0.002	Protein.ribosomal protein
18	Glyma03g35080.1	60S acidic ribosomal protein family	2	51471.25	0.72	< 0.001	Protein.ribosomal protein
19	Glyma10g07850.2	60S acidic ribosomal protein family	2	51471.25	0.72	0.002	Protein.ribosomal protein
20	Glyma12g30800.3	60S acidic ribosomal protein family	2	51471.25	0.72	0.002	Protein.ribosomal protein
21	Glyma13g39490.2	60S acidic ribosomal protein family	2	51471.25	0.72	0.002	Protein.ribosomal protein
22	Glyma19g37780.1	60S acidic ribosomal protein family	2	51471.25	0.72	0.002	Protein.ribosomal protein
23	Glyma05g33930.1	eukaryotic translation initiation factor 3C	1	0.12	2.14	0.045	Protein.synthesis
24	Glyma08g05740.1	eukaryotic translation initiation factor 3C	1	0.12	2.14	0.045	Protein.synthesis
25	Glyma09g08650.1	RED family protein	1	77500.92	0.88	< 0.001	Not assigned
26	Glyma15g20260.1	RED family protein	1	77500.92	0.88	< 0.001	Not assigned
27	Glyma02g05250.1	regulatory particle non_ATPase 10	1	99999.9	1.00	< 0.001	Protein.degradation
28	Glyma11g01430.2	P_loop containing nucleoside triphosphate hydrolases	1	75627.21	0.87	< 0.001	RNA
29	Glyma13g31580.1	aluminium induced protein with YGL and LRDR motifs	1	99999.9	0.71	< 0.001	Hormone metabolism
30	Glyma15g07720.2	aluminium induced protein with YGL and LRDR motifs	1	99999.9	0.71	< 0.001	Hormone metabolism
31	Glyma19g30560.1	global transcription factor group B1	1	71304.47	0.84	< 0.001	RNA
32	Glyma20g38810.1	Ypt/Rab_GAP domain of gyp1p superfamily protein	1	79141.36	0.63	< 0.001	Signalling
33	Glyma13g06050.1	UDP_glucose 6_dehydrogenase family protein	2	0.08	1.32	0.05	Cell wall
34	Glyma19g03500.1	UDP_glucose 6_dehydrogenase family protein	2	0.08	1.32	0.05	Cell wall

a) Protein ID is according to the Phytozome database; b) MP means matched peptides; c) Ratio indicates ratio of quantities of protein and it was calculated using treatments divide control. *p*-value was calculated by *t*-test. d) Functional category obtained from MapMan bin code.

Table 4. Abundances of significantly changed phosphoproteins in soybean under flooding stress with time-dependent manner change

No	Description	Protein ID[a]	Ratio[b] in flooded group					Ratio[b] in control group				
			48(0)	51(3)	54(6)	60(12)	72(24)	48(0)	51(0)	54(0)	60(0)	72(0)
1	aluminium induced protein with YGL and LRDR motifs	Glyma13g31580.1 Glyma15g07720.2	1	55.63	105.03	222.48	389.67	1	1	1	1	1
2	eukaryotic translation initiation factor 3C	Glyma05g33930.1 Glyma08g05740.1	1	1.17	0.53	0.56	1.1	1	0.28	0.16	0.94	0.59
3	P_loop containing nucleoside triphosphate hydrolases superfamily	Glyma11g01430.2	1	1.13	0.65	1.35	1.13	1	0.55	0.29	1.46	0.93
4	60S acidic ribosomal protein family	Glyma0169s00200.3 Glyma10g07850.2 Glyma12g30800.3 Glyma13g39490.2 Glyma19g37780.1 Glyma03g35080.1	1	0.79	0.74	1.09	1.03	1	0.41	0.39	1.43	2.06
5	UDP_glucose 6_dehydrogenase family protein	Glyma08g26520.1 Glyma18g50000.1	1	0.62	0.55	1.33	1.79	1	0.24	0.29	0.13	1.77
6	UDP_glucose 6_dehydrogenase family protein	Glyma13g06050.1	1	0.33	0.27	0.63	0.68	1	38.39	59.81	22.1	28.1
7	UDP_glucose 6_dehydrogenase family protein	Glyma19g03500.1	1	0.33	0.27	0.63	0.68	1	40.59	65.39	22.79	29.24
8	sucrose phosphate synthase 1F	Glyma02g29910.1 Glyma13g23060.1	1	0.74	0.49	1.13	1.09	1	0.36	0.35	1.05	0.91
9	sucrose phosphate synthase 1F	Glyma13g23060.2	1	0.73	0.47	1.09	1.07	1	0.35	0.35	1.04	0.92
10	sucrose phosphate synthase 1F	Glyma17g11820.1	1	0.69	0.51	1.22	1.09	1	0.37	0.39	1.08	1.02
11	plant VAP homolog 12	Glyma05g04580.1 Glyma17g15010.1	1	0.51	0.50	1.02	0.99	1	0.18	0.26	1.16	0.62
12	RED family protein	Glyma09g08650.1 Glyma15g20260.1	1	0.87	0.69	0.63	1.06	1	0.48	0.26	1.08	0.68
13	cellulose synthase_like D3	Glyma01g44281.1 Glyma11g01230.1	1	0.71	0.74	0.74	1.08	1	0.28	0.25	1.67	1.34
14	regulatory particle non_ATPase 10	Glyma02g05250.1	1	0.86	0.34	2.18	0.96	1	1	1	1	1

15	global transcription factor group B1	Glyma19g30560.1	1	0.78	0.41	1.00	0.76	1	0.44	0.36	1.41	1.41
16	Ypt/Rab_GAP domain of gyp1p superfamily protein	Glyma20g38810.1	1	0.71	0.78	1.35	1.18	1	0.75	0.49	2.08	1.84
17	nucleotide/sugar transporter family protein	Glyma14g14360.1	1	0.48	0.46	0.79	0.95	1	1.31	0.59	1.60	0.89
18	DEAD-DEAH box RNA helicase family protein	Glyma04g07180.3	1	0.33	0.28	0.21	0.30	1	0.34	0.14	0.48	0.60
19	DNAJ heat shock N_terminal domain_containing protein	Glyma01g45710.1	1	0.67	0.69	1.47	1.94	1	3.18	0.71	1.93	1.29
20	SART_1 family	Glyma08g24663.1	1	0.58	0.53	1.11	0.32	1	1.10	0.36	1.36	1.71
21	AMP deaminase_ putative / myoadenylate deaminase	Glyma03g07460.1	1	0.67	0.56	1.26	1.15	1	0.17	0.17	1.60	1.79
22	eukaryotic translation initiation factor 4G	Glyma08g45425.1	1	0.61	0.51	0.99	1.09	1	3.37	0.89	1.87	1.78

a) Protein ID is according to the Phytozome database. b) Ratio indicates ratio of quantities of protein, calculated using treatments divided by control.

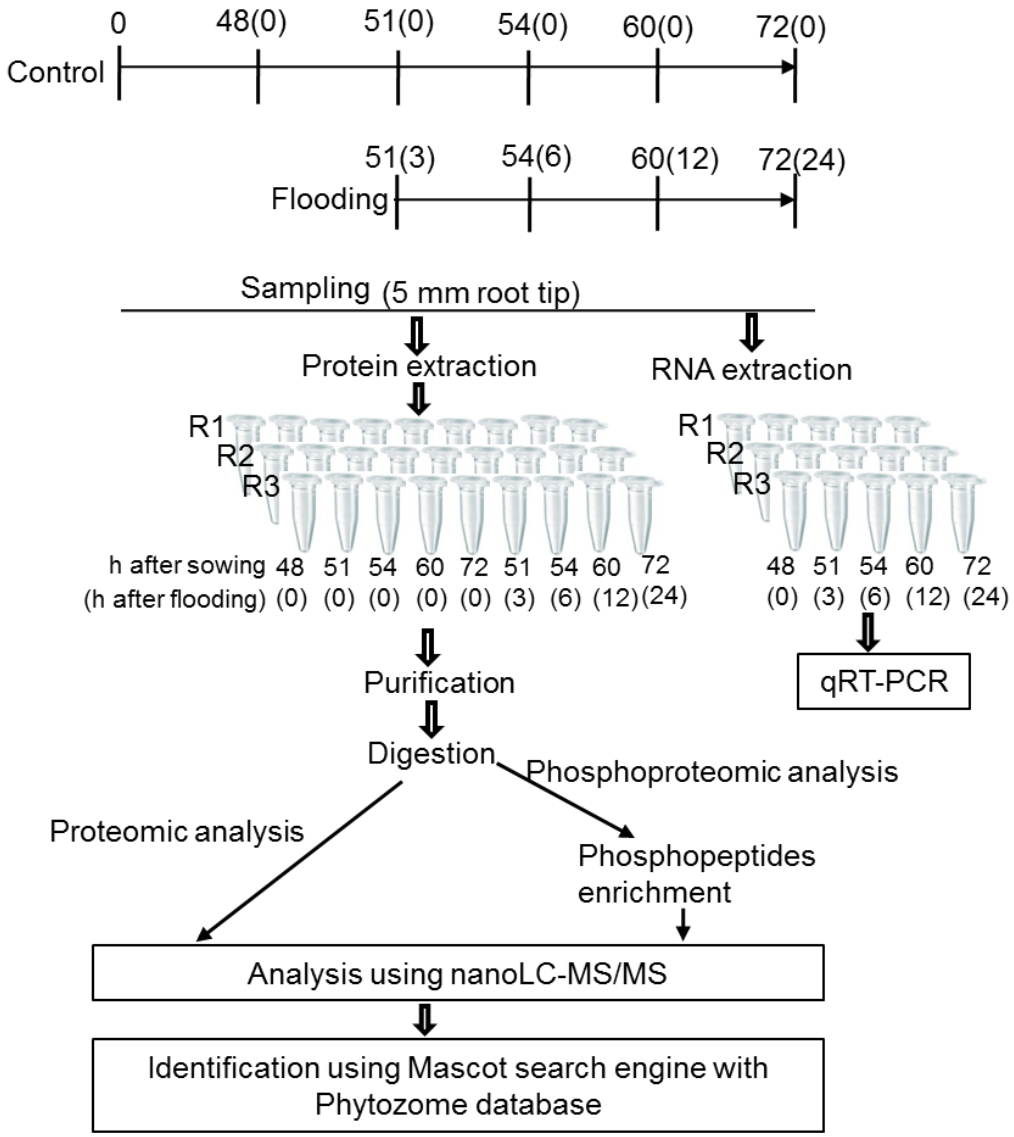

Figure 5. Experimental design of cellular protein study. Two-day-old soybeans were treated without or with flooding stress for 3, 6, 12, and 24 h. Proteins were extracted from root tip and used for gel-free proteomic and phosphoproteomic analyses. For proteomics of cellular protein, the digested peptides were directly applied for nanoLC-MS/MS analysis; for proteomics of phosphoprotein, phosphopeptides were enriched from digested peptides and applied for nanoLC-MS/MS analysis. Three independent experiments were performed as biological replicates.

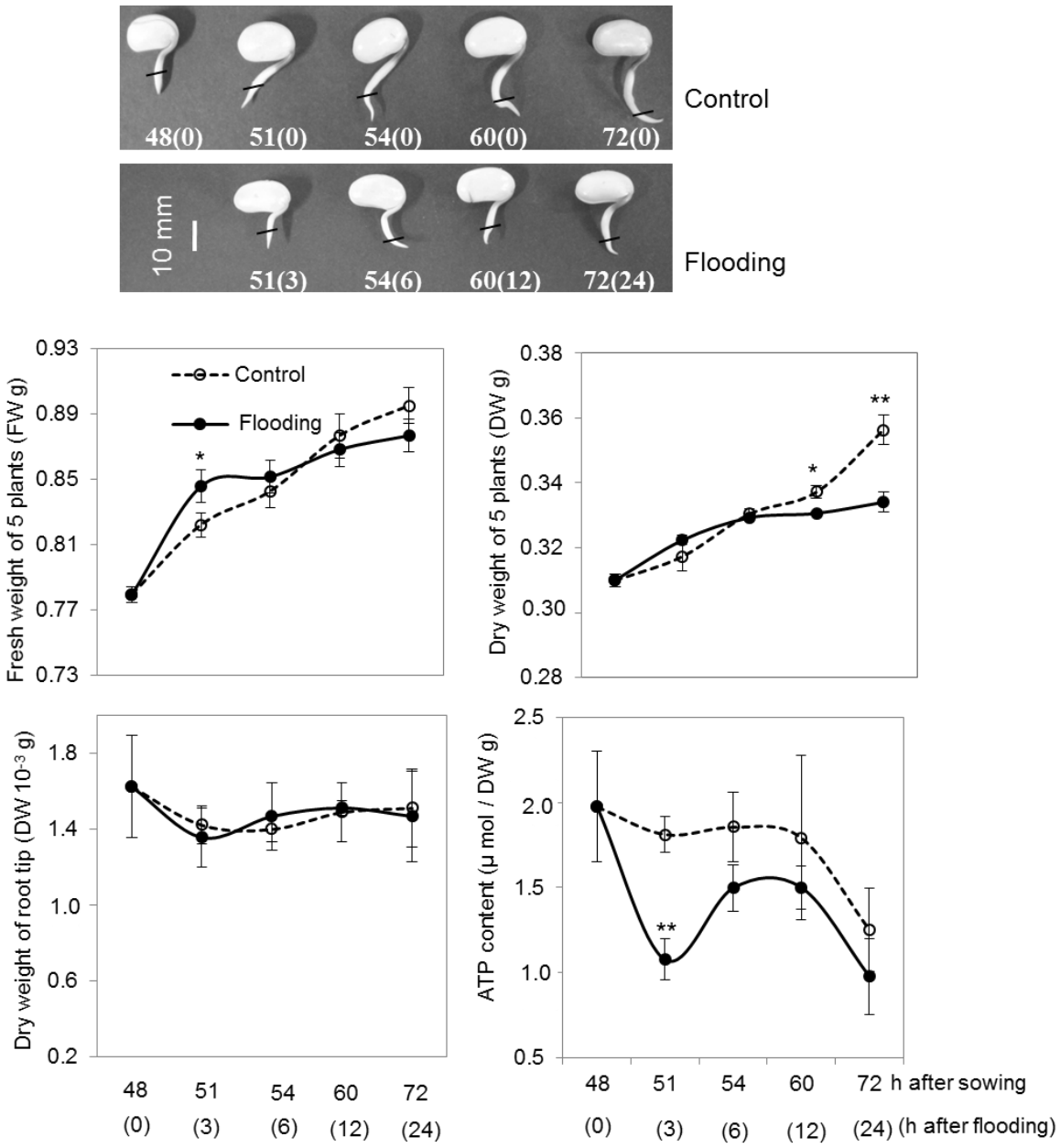

Figure 6. Morphological and biochemical changes in soybean during the early stages of flooding stress. Soybean seeds were germinated for 48 h and treated without (open circles) or with (closed circles) flooding stress for 3, 6, 12, and 24 h. Photographs of soybean were taken at each time point (A). The fresh weight of soybean was measured at each time point (B). Changes in the dry weight of soybean (C) and soybean root tip (D) under flooding stress were measured at each time point, and ATP content of soybean root tip (E) under flooding stress was measured at each time point. Numbers indicate the time (h) after sowing, and the numbers in parentheses indicate the duration (h) of flooding stress. Data are means ± SD from 3 independent biological replicates. Student's *t*-test analysis was used to compare values between the control and flooding stress. Asterisks indicate significant change (*$p < 0.05$, **$p < 0.01$). The scale bar indicates 10 mm.

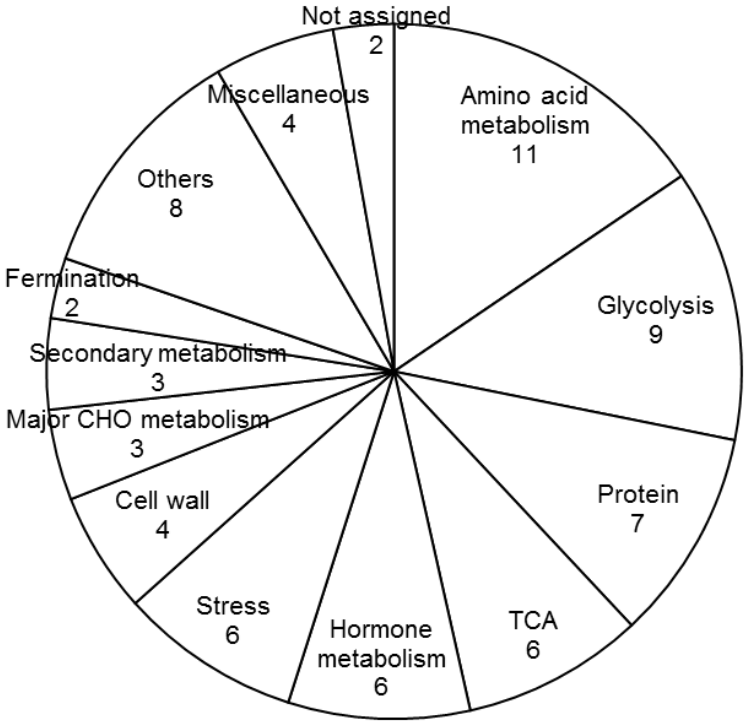

Figure 7. Functional distribution of significantly changed cellular proteins in 2-day-old soybean under flooding stress with time course manner. The abundances of cellular proteins in root tip treated with flooding stress were compared with those 48 h after sowing. Functional classification of the 71 identified cellular proteins in flooding-treated soybean was performed according to MapMan bin codes. TCA, tricarboxylic acid cycle.

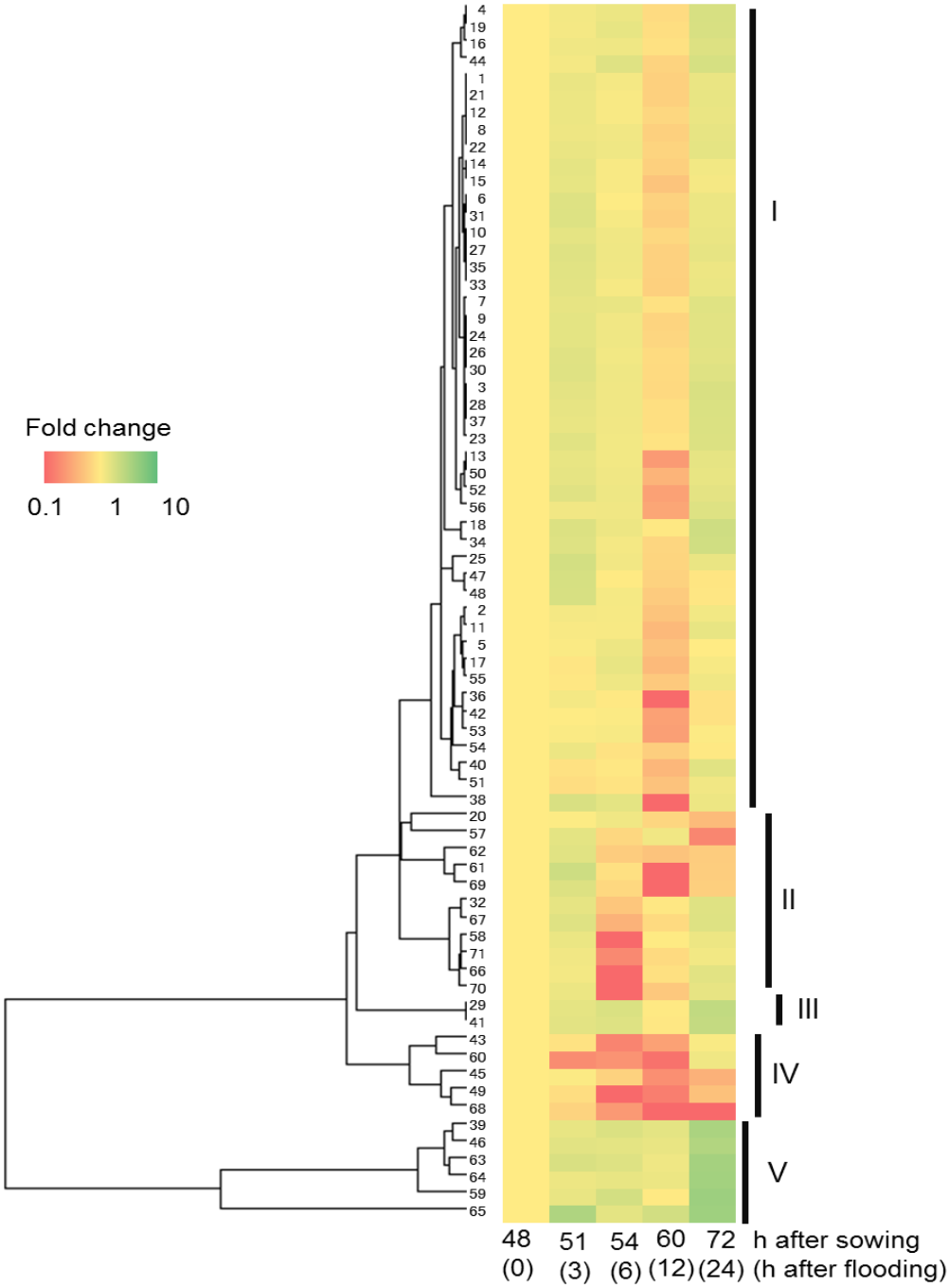

Figure 8. Clusters of significantly changed proteins in soybean root tip under flooding stress. Clusters are indicated by black rectangles. Abundance patterns of individual proteins are indicated based on the color legend for control and flooding-stress treated samples at 3, 6, 12, and 24 h (from left to right). Red and green indicate decreased and increased protein abundances, respectively, yellow means not changed. The temporal expression profiles of the 71 differentially changed proteins were analyzed. The number to the left corresponds to the protein number in Table 2.

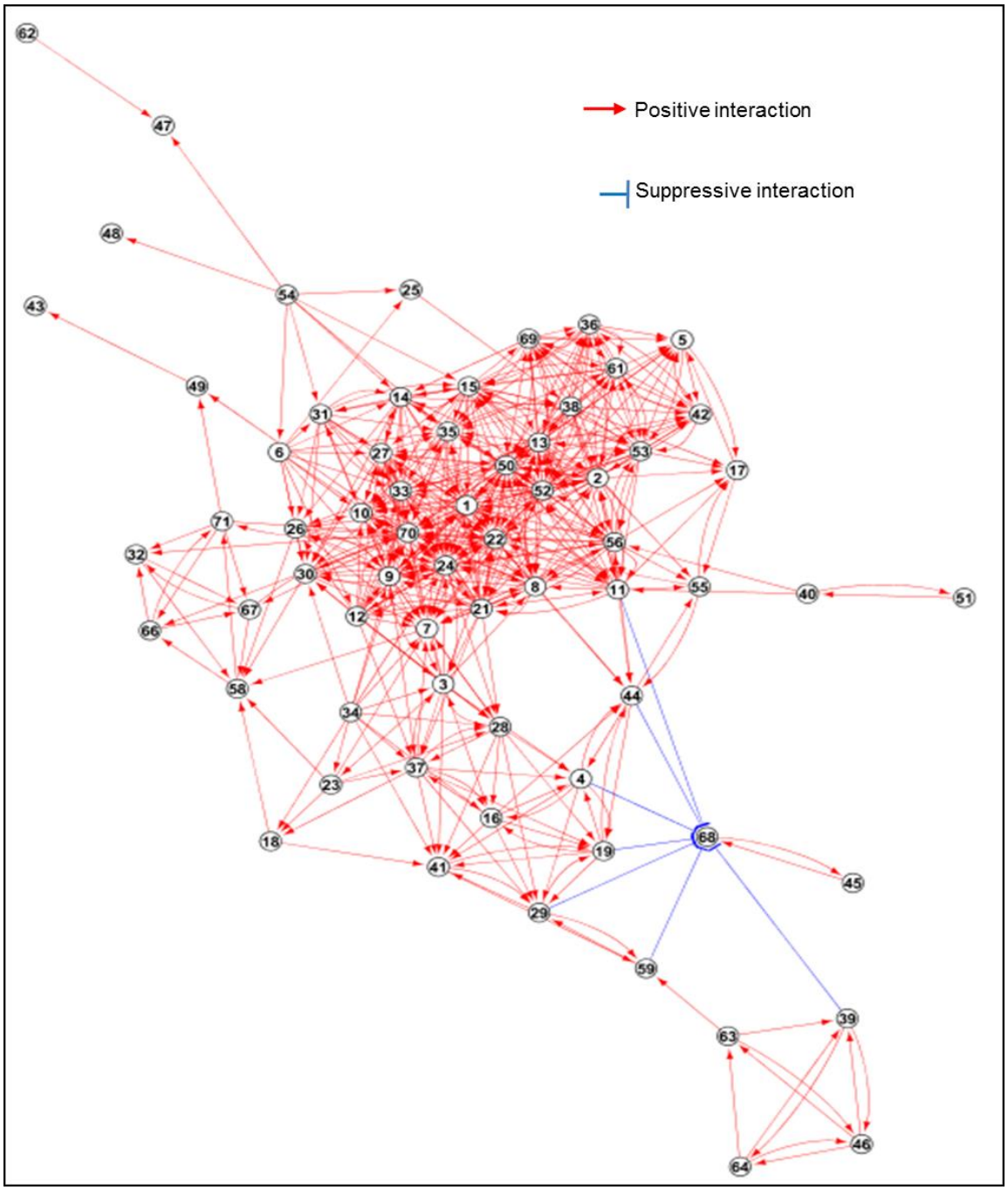

Figure 9. Network map of *in silico* protein–protein interactions regulated by flooding stress in soybean root tip. Interacting proteins were estimated based on time course change analysis. This figure showns a network of proteins for which associated proteins are connected. Red arrows show inductive interactions and blue T-bars show a suppressive interaction. The functions and names of proteins are shown in Table 2 according to these digits.

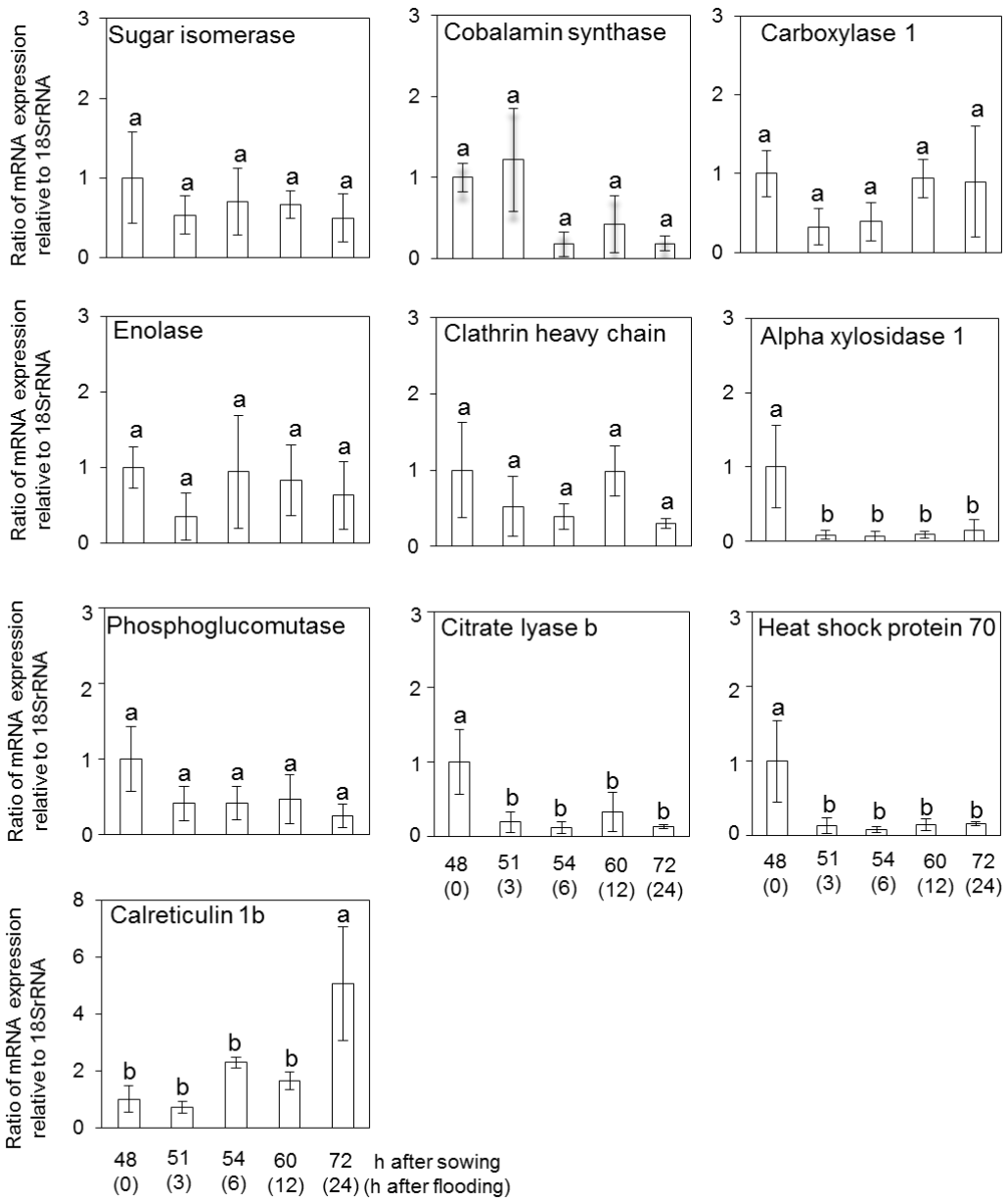

Figure 10. Effects of flooding on the mRNA abundance of proteins identified in the gel-free proteomic analysis.Two-day-old soybeans were treated without or with flooding stress for 3, 6, 12, and 24 h, and RNAs extracted from root tip were analyzed by qRT-PCR. Relative mRNA abundances of 10 genes were normalized according to the abundance of 18S rRNA. The name and ID of genes are as follows: *sugar isomerase* (Glyma04g03490.1), *cobalamin independent synthase* (Glyma05g10840.3), *acetyl CoA carboxylase 1* (Glyma04g11550.1), *enolase* (Glyma19g37520.1), *Clathrin heavy chain* (Glyma18g02960.1), *alpha xylosidase 1* (Glyma01g20460.1), *phosphoglucomutase* (Glyma08g04890.1), *ATP-citrate lyase b* (Glyma09g04000.2), *heat shock protein 70* (Glyma05g36600.1), and *calreticulin 1b* (Glyma20g23080.1). Data are shown as means ± SD from 3 independent biological replications. Means with the same letter were not significantly different from control levels based on one-way ANOVA with Tukey's multiple comparison ($p < 0.05$).

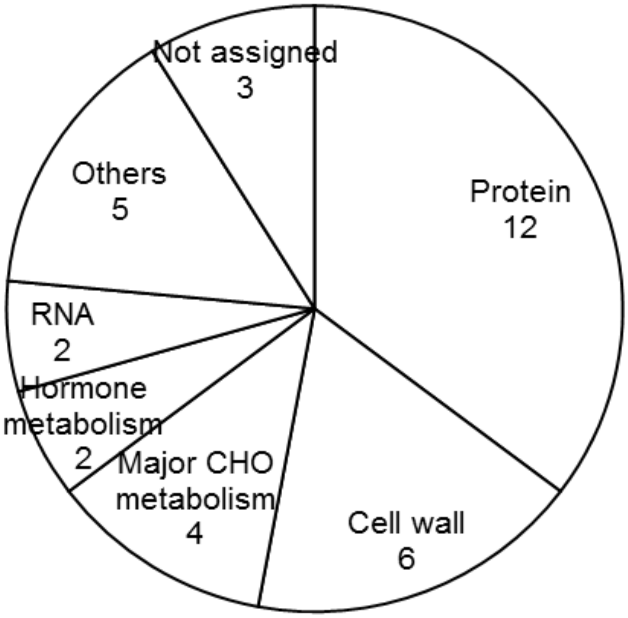

Figure 11. Functional distribution of significantly changed phosphoproteins in 2-day-old soybean under flooding stress for 3 h. The abundances of phosphoproteins in root tip treated with flooding stress were compared with those without flooding stress. Functional classification of the 34 identified phosphoproteins in flooding-treated soybean was performed according to MapMan bin codes.

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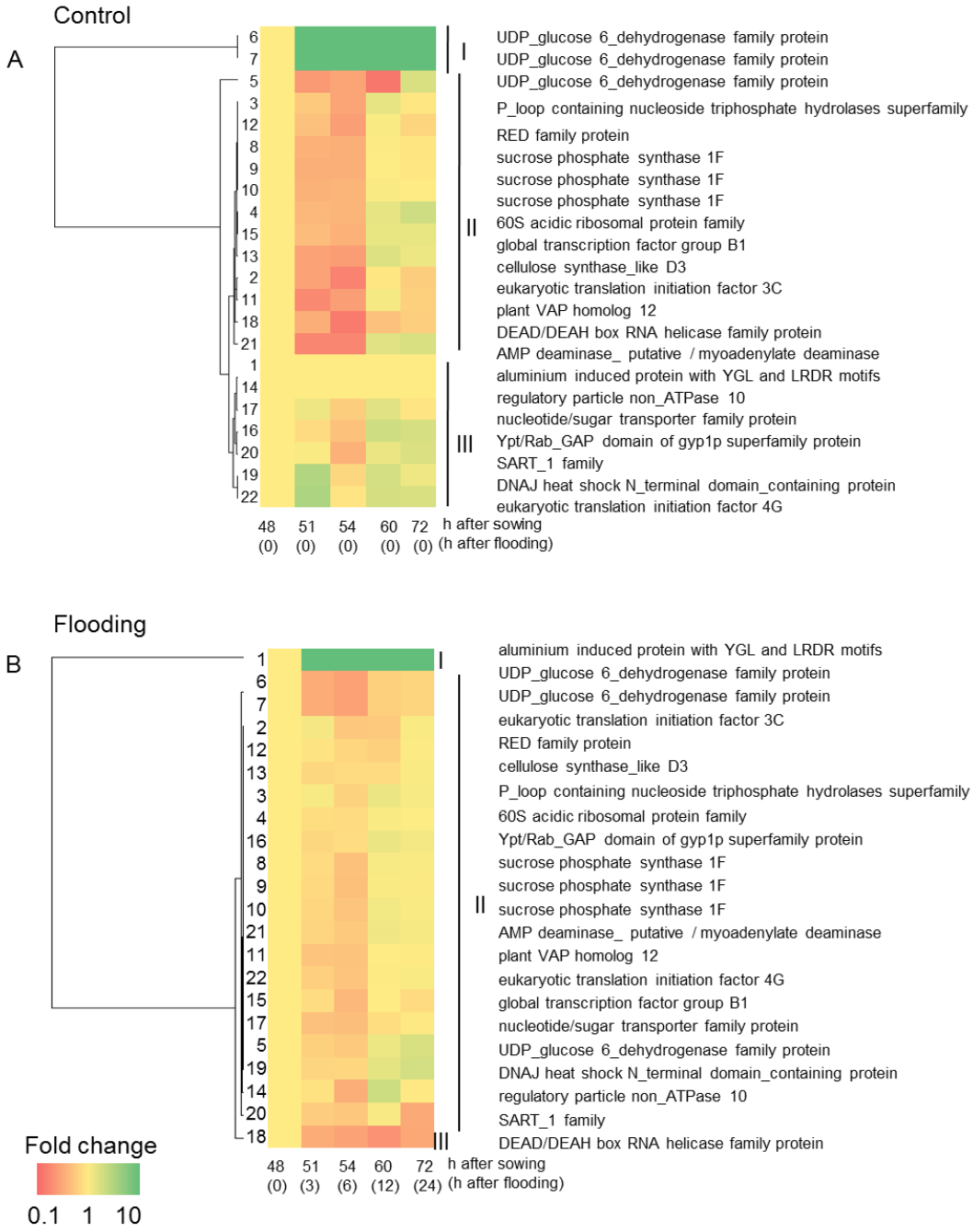


Figure 12. Clusters of significantly changed phosphoproteins in root tip of soybean under flooding stress. Clusters are indicated by black rectangles. Abundance patterns of individual protein are indicated based on the color legend (lower left) for control (A) and flooding-stressed (B) soybeans after 3, 6, 12, and 24 h of treatment. Red and green colors indicate decreased and increased protein abundances, respectively, and yellow means that the protein abundance was unchanged. The temporal profiles of protein abundance of the 22 differentially changed phosphoproteins in Table 4 were used for the analysis.

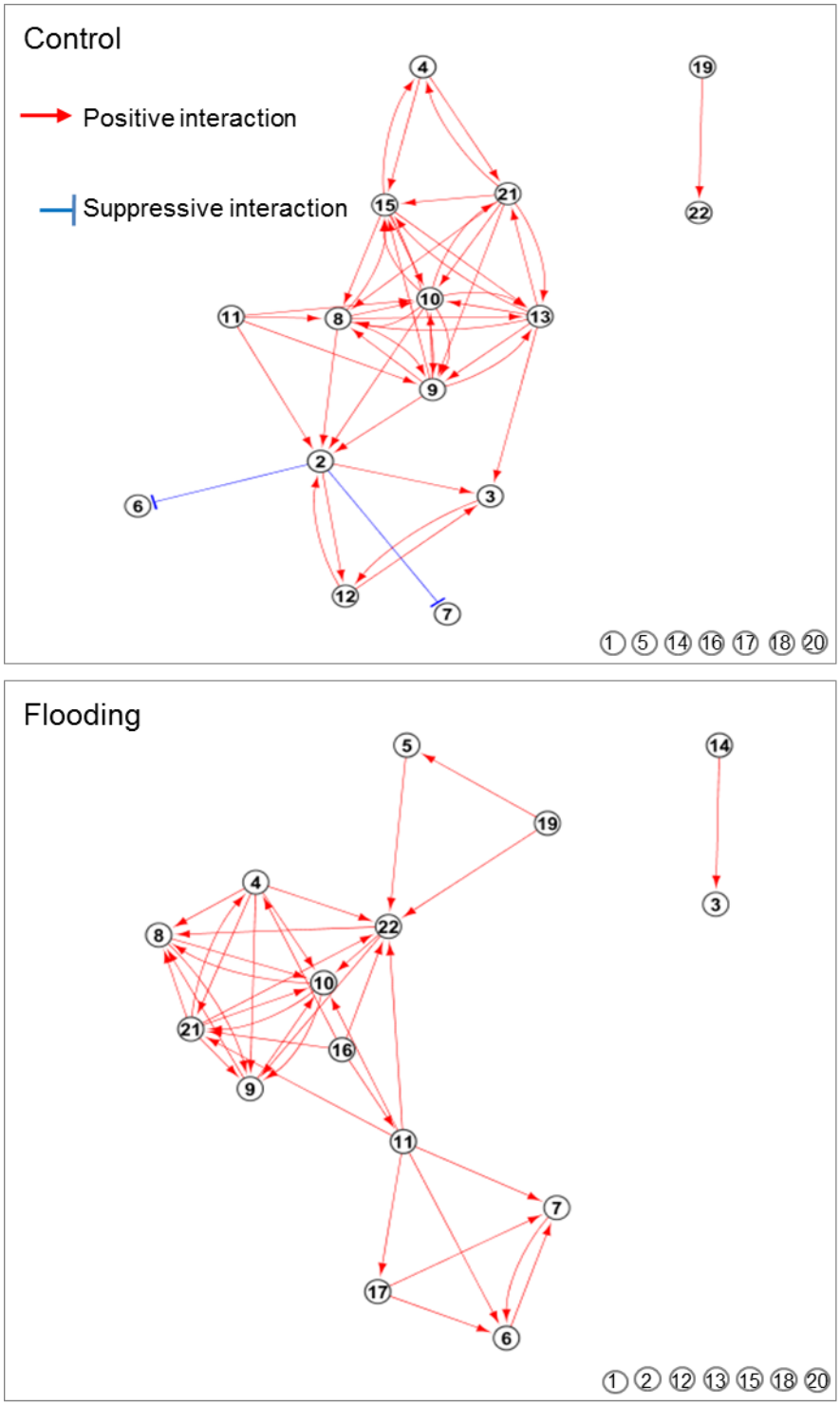


Figure 13. Network map of *in silico* protein–protein interactions regulated by flooding stress between phosphoproteins in root tip of soybean under flooding stress. Interacting proteins in each network were estimated based on time-course differential analysis. Red arrows show inductive interactions and blue T-bars show suppressive interactions. The protein numbers correspond to the proteins listed in Table 4.

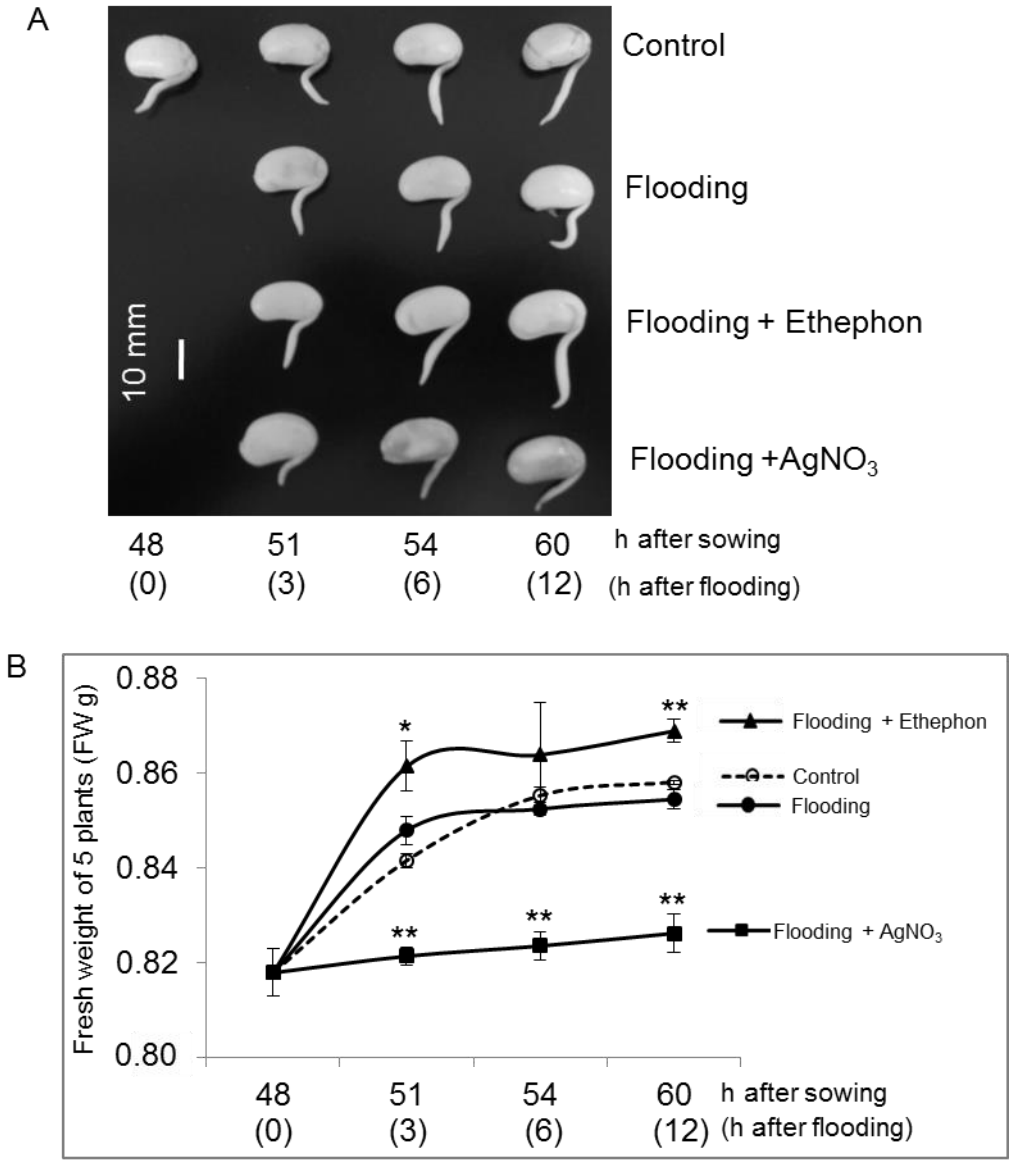


Figure 14. Effects of ethylene on soybean weight under flooding stress. Soybean seeds were germinated for 48 h and transferred to column tubes containing 120 mL of water, 300 ppm ethephon, and 5 mM silver nitrate ($AgNO_3$) for 0, 3, 6, and 12 h. Photographs of soybean plants were taken at each time point (A). The fresh weight of soybeans was measured at each time point (B). Data are means ± SD from 3 independent biological replicates. The asterisks indicate a significant difference compared to controls as assessed using the Student's *t* test (*$p < 0.05$, **$p < 0.01$). The scale bar indicates 10 mm.

CHAPTER 2

CHARACTERIZATION OF RESPONSE MECHANISM IN NUCLEUS OF SOYBEAN UNDER INITIAL FLOODING STRESS

2.1. Introduction

The nucleus is the most important cellular organelle due to it contains nearly all of the cellular genetic information, which is organized into chromosomes that provide sites for DNA replication and transcription (Maniotis et al., 1997). The nucleus is composed of the inner/outer nuclear membranes, nuclear pore complexes, and nuclear lamina (Hetzer et al., 2005). In nucleus, phospholipid-rich membrane contains sensitive ion channels and pores for the shuttling of various biomolecules across the nuclear membrane through conformational and morphological transformations (Dahl et al., 2008). The major activities of the cell, including protein synthesis, cell division/growth, and differentiation were controlled by nucleus in plants (Wilson and Dawson, 2011). To perform the study of nuclear proteins, the isolation and purification of nuclei is a key step. Although a number of different techniques for nuclei isolation have been developed, most of the purification methods contain similar sequential steps including disruption, filtration, centrifugation, solubilization and separation.

Nuclei were first successfully isolated from suspensions of cultured cells through steps containing disruption, filtration, and centrifugations at different speeds (Willmitzer and Wagner, 1981). Continuously, nuclei were purified from rice embryos using 30% percoll gradients after the disruption, filtration, and centrifugation steps (Yamaguchi et al., 1992). To purify nuclei from specific cell and phases of the cell cycle. The optimized protocols using streptavidin-coated magnetic beads (Deal and Henikoff, 2011) and flow cytometry methodology (Silva et al., 2010) were successfully applied. Till now, nuclear proteomic analysis has been performed in many plant species including *Arabidopsis* (Bae et al., 2003), *Medicago* (Repetto et al., 2008), rice (Choudhary et al., 2009), chickpea (Pandey et al., 2008), tomato (Wang et al., 2014), maize (Guo et al., 2014), wheat (Bancel et al., 2015), and soybean (Cooper et al., 2011). The reults from those studies indicate that complex regulatory networks exist in nuclei of plants.

Phosphorylation of nuclear proteins involved in active metabolism in plants (Ranjeva and Boudet, 1987). It was reported that activities happened in nucleus were affected by phosphorylation of histone H3; for example, phosphorylation at serine 10/serine 28 by the kinase *At*Aurora3 was involved in chromosome segregation and

metaphase/anaphase transition (Kurihara et al., 2006). The phosphorylation at threonine 3 by *At*Haspin contributed to embryonic formation in *Arabidopsis* (Ashtiyani et al., 2011). Cho et al. (2014) reported that auxin signaling output was potentiated through phosphorylation of auxin response factor 7/19 by brassinosteroid-insensitive 2 during lateral root organogenesis. Similarly, two closely related transcriptional repressors, homeo-domain-leucine zipper protein 1/3, which bind to a conserved homeodomain binding site in the promoters of several brassinosteroid-repressed genes, were stabilized through phosphorylation by brassinosteroid-insensitive 2 (Zhang et al., 2014b). Based on the findings from these studies, the phosphorylation of nuclear proteins appears to play an important role in many plant biological processes, including cell division, transcriptional activation, and hormone responses.

In chapter 1, energy metabolism, cell wall formation, and signal transduction were affected in soybean at initial flooding stress. In this chapter, to explore the upstream mechanisms controling those flooding-responsive events in the early stages of soybean growth, a gel-free nuclear proteomic approach was used. To uncover the flooding signaling transduction pathway in nucleus, nuclear phosphoproteomics was performed. Nuclei were isolated from root tip of soybean and nuclear proteins were extracted from enriched nuclei fraction. The nuclear phosphopeptides were successfully enriched from digested nuclear peptides. The digested peptides including phosphopeptides were further identified using nanoLC-MS/MS. The results from the nuclear proteomics were analyzed using bioinformatics and confirmed on mRNA expressions and protein abundance such as Western blot and proteomics of crude protein extract.

2.2. Materials and methods

2.2.1. Plant material and treatments

Seeds of soybean (*Glycine max* L.) cultivar Enrei were used as material. The growth conditions are described in 1.2.1 of Chapter 1. For initial flooding stress, 2-day-old soybean was treated without or with flooding stress for 3 h. For continuous flooding stress, 2-day-old soybean was treated without or with flooding stress for 3, 6, and 24 h. After treatment, root tips were collected as samples for protein and mRNA extraction. For ABA effects checking, 2-day-old soybeans were flooded for 3 h with 500 mL water

supplemented without or with 100 μM ABA. Three independent experiments were performed as biological replicates for all treatments.

2.2.2. Nuclei isolation and protein extraction

Nuclei were isolated according to the manufacture's instructions of Plant Nuclei Isolation/Extraction Kit (Sigma, St. Louis, MO, USA) with some modifications (Figure 16). Briefly, a portion (2.0 g) of samples was ground with buffer. The homogenates were filtered through a double layer of Filter Mesh and centrifuged at 1300 xg for 10 min at 4°C. The resulting pellet was resuspended in Nuclei Isolation buffer containing protease inhibitor mixture (Roche, Werk Penzberg, Germany), and layered on top of cushions containing 60% percoll prepared in 1xNuclei Isolation buffer and 2.3 M sucrose. After centrifugation at 3200xg for 30 min at 4°C, the middle layer was collected and washed with Nuclei Isolation buffer containing protease inhibitor mixture to remove percoll and sucrose. The enriched nuclei fraction was vortexed and sonicated with extraction buffer. After sonication, the homogenate was centrifuged at 12000xg for 30 min at 4°C and the supernatant was collected as nuclear proteins.

2.2.3. Extraction of crude proteins

Protein extraction is described in 1.2.3 of Chapter 1.

2.2.4. Western blot analysis

Proteins were extracted using SDS sample buffer containing 60 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol. The protein concentration was measured with a Pierce 660 nm Protein Assay Kit with Ionic Detergent Compatibility Reagent (Thermo Fisher Scientific, Rockford, IL, USA). Proteins were separated on a 12% SDS–polyacrylamide gel electrophoresis gel and then transferred onto a polyvinylidene difluoride membrane using a semidry transfer blotter. The blotted membrane was incubated overnight at 4°C in blocking buffer consisting of 20 mM Tris–HCl (pH 7.5), 500 mM NaCl, and 5% skim milk (Difco, Sparks, MD, USA). After blocking, the membrane was incubated with a 1:8000 anti-histone H3 antibody (Abcam, Cambridge, UK) for 1 h at room temperature. Anti-rabbit IgG conjugated with

horseradish peroxidase (Bio-Rad) was used as the secondary antibody. After 1 h incubation with the secondary antibody, signals were detected using an ECL plus Western blotting detection kit (Nacalai Tesque, Kyoto, Japan) following the manufacturer's protocol, and the signals were visualized using a LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). Coomassie brilliant blue staining was used as loading control. The relative intensities of bands were calculated using Quantify One software (version 4.5; Bio-Rad).

2.2.5. Enzyme activity analysis

Proteins were extracted using buffer containing 50 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 1mM EDTA, 2% polyvinylpyrrolidone, 0.1% Triton X-100, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. For alcohol dehydrogenase assay, the reaction solution was composed of 50 mM MES-NaOH, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM NADH, and 4% acetaldehyde. NADH oxidation was monitored at 340 nm at 25°C for 5 min. The enzyme activity was calculated with formula: μnits/mL = (ΔA340 x total volume x dilution factor)/(6.22 x sample volume) (Komatsu et al., 2013d).

For catalase activity, the reaction buffer was composed of 50 mM potassium phosphate and 15 mM H_2O_2. The subsequent decomposition of hydrogen peroxide was measured at 240 nm at 25°C for 5 min. The enzyme activity was calculated with formula: μnits/mL = (ΔA240 x total volume x dilution factor)/(40 x sample volume) (Kato and Shimizu, 1987).

For fumarase activity, the reaction buffer was consisted of 70 mM KH_2PO_4-NaOH, 0.05% Triton X-100, and 50 mM malic acid. The reaction was directly measured at 340 nm. The enzyme activity was calculated with formula: μnits/mL = (ΔA340 x total volume x dilution factor)/(2.55 x sample volume) (Huang and Jacoby, 1999).

For NADH-cytochrome *c* reductase activity, the reaction buffer contained 20 mM potassium phosphate (pH 7.2), 0.2 mM NADH, 0.02 mM cytochrome *c*, and 30 mM NaN_3. The reduction of cytochrome *c* was followed spectrophotometrically as the absorbance increase at 550 nm. The enzyme activity was calculated with formula: μnits/mL = (ΔA550 x total volume x dilution factor)/(21.1 x sample volume) (Hasinoff,

1990).

2.2.6. Protein clean up and digestion

The clean up and digestion of proteins is same as described in 1.2.5 of Chapter 1.

2.2.7. Phosphopeptide enrichment

The phosphopeptide enrichment is described in 1.2.6 of Chapter 1.

2.2.8. Mass spectrometry analysis

MS analysis is described in 1.2.7 of Chapter 1. Data of MS have been deposited with the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno et al., 2013) and the identifiers of dataset were PXD 0002429 and PXD 001486.

2.2.9. Protein identification using acquired mass spectrometry data

Protein identifications of acquired mass spectrometry data is described in 1.2.8 of Chapter 1.

2.2.10. Differential analysis of the identified proteins

Differential analysis of the identified proteins is described in 1.2.9 of Chapter 1.

2.2.11. Analyses of protein localization and functional categorization

Protein localization was queried using the intracellular targeting prediction programs of YLoc (http://abi.inf.unituebingen.de /Service/YLoc/webloc.cgi) (Briesemeister et al., 2010), WoLF PSORT (http://www.genscript.com/wolf-psort.html) (Horton et al., 2007), Plant-mPLoc (http://www.csbio.sjtu.edu.cn/ bioinf/plant-multi) (Chou and Shen, 2007; 2008; 2010), and NucPred (http://www.sbc.su.se/maccallr/nucpred/) (Brameier et al., 2007). Pathway mapping of identified proteins was performed using the Kyoto encyclopedia of genes and genomes (KEGG) database (http://www.genome.jp/kegg/) (Kanehisa and Goto, 2000). Function of identified proteins was categorized as described in 1.2.10 of Chapter 1. Cluster

analysis was performed as described in 1.2.11 of Chapter 1.

2.2.12. RNA extraction

RNA extraction is described in 1.2.12 of Chapter 1.

2.2.13. Quantitative reverse transcription polymerase chain reaction analysis

qRT-PCR analysis is described in 1.2.13 of Chapter 1. The primers were designed using the Primer3 web interface (http://frodo.wi.mit.edu) (Supplemental Table 1). Primer specificity was checked by BLASTN searches against sequences in the soybean genome database (Phytozome) with the queries of designed primers by melt curve analysis.

2.2.14. ATP content

The measurement of ATP content is described in 1.2.2 of Chapter 1.

2.2.15. Statistical analysis

Statistical analysis is described in 1.2.14 of Chapter 1.

2.3. Results

2.3.1. Western blot, enzyme activity, and proteomic analyses of nuclear proteins from root tip of soybean

To identify the upstream events controlling flooding-responsive proteins in root tip of soybean, nuclear proteomic analysis, which can detect low abundant proteins, was performed. For the analysis, nuclei were isolated from the root tip of 2-day-old soybean (Figure 15), and the purity of the enriched nuclei fraction was confirmed by immunoblotting and enzymatic assays with organelle-specific antibodies and enzymes, respectively (Figure 16). In the immunoblotting analysis, anti-histone H3 antibody, which was used as a marker for nuclear protein, cross-reacted with a 17-kDa protein (Figure 16). The relative intensity of the corresponding signal band was significantly increased in the nuclear protein fraction compared to the cellular protein fraction (Figure 16).

To assess protein contamination from other organelles, the enzyme activities of several subcellular markers in the cellular protein and enriched nuclei fractions (N1 and N2) were examined (Figure 16). Alcohol dehydrogenase, catalase, fumarase, and NADH-cytochrome *c* reductase were used as marker enzymes of cytosolic, peroxisome, mitochondrial, and endoplasmic reticulum contamination, respectively (Figure 16). The cellular protein fraction had significantly higher activities of alcohol dehydrogenase, catalase, fumarase, and NADH-cytochrome *c* reductase compared with the two enriched nculei fractions (N1 and N2). In the final enriched nuclei fraction, the activities of these four enzymes were also lower than those in the cellular protein fraction. Based on the immunoblotting and enzyme activity analyses of subcellular-specific proteins, the purified nuclei fraction was confirmed to be highly enriched for nuclear proteins and was not contaminated with proteins from the cytosol, peroxisomes, mitochondria, or endoplasmic reticulum. To further evaluate the purity of the obtained nuclear proteins, the subcellular localization of the proteins was predicted using Plant-mPLoc (Chou and Shen, 2007; 2008; 2010), WoLF PSORT (Horton et al., 2007), YLoc (Briesemeister et al., 2010), and NucPred software (Brameier et al., 2007). Among the 399 identified proteins, 256 (64%) were predicted to be localized in the nucleus (Figure 17). These results further confirmed that nuclear proteins were highly enriched and predominant in the fractions obtained from root tip of soybean.

2.3.2. Identification of flooding-responsive nuclear proteins in root tip of soybean

To identify flooding-responsive nuclear proteins in soybean, a gel-free proteomic technique was used to analysis root tip proteins from 2-day-old soybeans treated without or with flooding stress for 3, 6, and 24 h. The extracted proteins were digested and analyzed using nanoLC-MS/MS (Figure 15), and the identified proteins were compared between 2-day-old soybean and samples collected from flooding-stressed and control plants at each time point using SIEVE software. A total of 273 (Figure 18) and 365 (Figure 18) proteins significantly changed in abundance in control and flooded soybean, respectively, compared to 2-day-old soybean.

To better understand the function of the identified proteins, functional categorization using MapMan bin codes was performed (Figure 18). The detected

proteins were mainly categorized in the protein synthesis, RNA, and DNA functional categories (Figure 18A). Protein synthesis-related proteins were further subcategorized as having functions related to ribosomes, ribosome biogenesis, elongation, and initiation; RNA-related proteins were functionally subcategorized into RNA processing, transcriptional regulation, RNA binding, and transcription; and DNA-related proteins were subcategorized into chromatin structure, DNA repair, and unspecific functions (Figure 18B). Notably, chromatin structure-related proteins were increased in control plants and decreased in flooded soybeans.

To determine the biological processes that are mediated by the identified nuclear proteins in soybean at the initial flooding stress, the significantly changed nuclear proteins were analyzed using the KEGG database. The analysis indicated that the significantly changed nuclear proteins were predominantly involved in pre-mRNA processing and pre-ribosome biogenesis (Figure 19, Table 5). Proteins related to Prp 19 complex were decreased in both control and flooded soybean, whereas exon-junction complex-related proteins, including ACINUS, Y14, MEGOH, and THOC, were increased in control plants and decreased in flooding-stressed soybean (Figure 19). In addition, NOP1 and NOP56, which are positioned upstream of 60S pre-ribosome biogenesis, and NUP155, which forms part of the spoke complex, were increased in control plants, but decreased at the initial flooding stress (Figure 19). These results indicated that mRNA processing and pre-ribosome biogenesis were affected at the initial flooding stress in the root tip of soybean.

2.3.3. Suppression of ribosome function in soybean exposed to flooding stress

The nuclear proteomic results indicated that the abundance of nuclear proteins involved in mRNA processing and 60S pre-ribosome biogenesis were changed in root tip of soybean in the initial stage of flooding stress (Figure 18). To confirm that the mRNA expression level of flooding-responsive nuclear proteins was also altered under flooding stress, qRT-PCR analysis was performed. NOP56 and NOP1, which function upstream of pre-ribosome biogenesis, exhibited down-regulated mRNA expression after 1.5 and 6 h of flooding stress. The mRNA expression levels of THOC4 and MAGOH, which play important roles in mRNA transport processes, were also significantly down-

regulated in root tip exposed to flooding stress for 3 h (Figure 20A).

To further confirm that ribosome function was affected by sustained flooding stress, proteomic analysis of cellular proteins extracted from root tip of soybean exposed to flooding stress for 24 h was performed using nanoLC-MS/MS. Proteins that significantly changed in abundance were identified and functionally categorized using MapMan bin codes. The analysis revealed that the levels of 101 protein synthesis-related proteins were altered in response to flooding stress. These proteins were further subcategorized into 25 decreased and 9 increased 60S ribosome-related proteins, 13 decreased and 10 increased 40S ribosome-related proteins, 18 decreased and 9 increased protein synthesis/initiation-related proteins, and 17 decreased protein synthesis/elongation-related proteins (Figure 20B). These findings indicated that ribosome function was affected at the initial stage of flooding stress in early-stage soybean plants.

2.3.4. Decrease of histone H1 and H3 in root tip of soybean exposed to flooding stress

In the root tip of soybean, numerous chromatin structure-related proteins, which mainly consisted of histones H1, H2A, H2B, H3, and H4, were decreased in response to flooding stress (Figure 18, Table 5). To confirm that these histone proteins were affected during the initial stages of flooding stress, qRT-PCR and Western blot analyses were performed. The genes encoding histones H1, H2A, H2B, H3, and H4 were selected for qRT-PCR analysis, and anti-histone H3 antibody was used for Western blot analysis. Among the 5 targeted histone proteins, the mRNA expression of *histones H1, H3, H2A, and H4* was significantly down-regulated during the first 6 h of flooding stress (Figure 21). In contrast, the mRNA expression level of *histone H2B* was nearly unchanged in the initial stages of flooding stress in early-stage soybean (Figure 21). In the Western blot analysis, the level of histone H3 did not appear to change during the germination stage; however, a significant decrease in histone H3 was observed under flooding stress (Figure 21). These results indicated that histones H1 and H3 were affected by flooding stress in soybean.

2.3.5. Identification of flooding-responsive nuclear phosphoproteins in root tip of

soybean

To identify flooding-responsive nuclear phosphoproteins, a gel-free proteomic technique was used. Two-day-old soybeans were treated without or with flooding stress for 3 h. Phosphopeptides were enriched from the nuclear protein fraction and analyzed using nanoLC–MS/MS. A total of 14 phosphoproteins, which consisted of 10 increased and 4 decreased proteins, significantly changed in root tip of soybean in response to flooding stress (Table 6). The subcellular localization of the identified phosphoproteins was predicted using WoLF PSORT, YLoc, and NucPred. Based on these analyses, 10 proteins were predicted to be localized in the nucleus: zinc finger/BTB domain-containing protein 47, 2 glycine-rich proteins (Glyma01g41250.1 and Glyma11g04170.1), ribosomal protein L1p/L10e, rRNA processing protein Rrp5, U3 small nucleolar RNA-associated protein MPP10, eukaryotic translation initiation factor 4G, calmodulin binding transcription activator, ribosomal protein S24/S35, and DEAD-DEAH box RNA helicase (Table 6). Out of them, 5 proteins were categorized as protein synthesis using MapMan bin codes (Table 6).

2.3.6. Comparison of mRNA expression level, nuclear protein abundance, and phosphorylation

To confirm the phosphorylation status of these 9 nuclear phosphoproteins, they were further compared on mRNA expression, protein abundance, and phosphorylation levels. The ratio of mRNA to protein for zinc finger/BTB domain containing protein 47, glycine-rich protein, and calmodulin binding transcription activator did not change in response to flooding; however, their phosphoprotein abundances were clearly increased. In particular, the ratio of phosphorylated zinc finger/BTB domain-containing protein 47 and glycine rich protein increased by more than 20 fold. Similarly, although rRNA processing protein Rrp5 and DEAD-DEAH box RNA helicase had decreased mRNA expression level and protein abundance under flooding conditions, they exhibited increased phosphorylation. In contrast, U3 small nucleolar RNA-associated protein MPP10, eukaryotic translation initiation factor 4G, and ribosomal protein S24/S35 displayed a decreased mRNA to protein ratio, and phosphorylation, whereas ribosomal protein L1p/L10e did not show any changes in these 3 experimental parameters in

response to flooding. These results indicated that zinc finger/BTB domain containing protein 47, glycine-rich protein, calmodulin-binding transcription activator, rRNA processing protein Rrp5, and DEAD-DEAH box RNA helicase were regulated by phosphorylation in the nuclei of soybean in response to flooding stress (Figure 22).

2.3.7. Effect of ABA on mRNA expression, ATP content, and fresh weight in root tip of soybean under flooding stress

In *Arabidopsis*, zinc finger-containing protein and glycine-rich protein affect seed germination and seedling growth in an ABA-dependent manner (Kim et al., 2007; Long et al., 2013). rRNA processing protein Rrp5, which is related to ribosome biosynthesis, was also shown to be differentially regulated during the germinating stage (Nakagami et al., 2010). To investigate the role of these proteins in the initial responses of plants to flooding stress, the effect of ABA on mRNA expression, ATP content, and fresh weight of soybean under flooding conditions was examined. Two-day-old soybeans were flooded without or with 100 μM ABA for 24 h. To understand the relationship between ABA and identified proteins, the mRNA expression levels of *zinc finger/BTB domain-containing protein 47, glycine-rich protein, and rRNA processing protein Rrp5* were analyzed using qRT-PCR (Figure 23A). The mRNA expression levels of *zinc finger/BTB domain-containing protein 47 and glycine-rich protein* were up-regulated by flooding stress; however, expression of these genes was down-regulated in flooded soybeans treated with ABA. In contrast, the mRNA expression level of *rRNA processing protein Rrp5* was down-regulated by flooding stress, and was further down-regulated with the addition of ABA (Figure 23A). These results indicated that the mRNA expression of *zinc finger/BTB domain-containing protein 47, glycine-rich protein*, and *rRNA processing protein Rrp5* was influenced by ABA under flooding stress.

To understand the relationship with phosphorylation, ATP content was measured. The ATP content of root tip continuously decreased during the germination stage in control (Figure 23B). After treatment of soybean with flooding stress without or with ABA, the ATP content markedly decreased in the first 6 h, but did not change with continued flooding exposure. The ATP content was lower in flooded and ABA-treated soybean root tip compared to that in untreated soybean root tip (Figure 23B). Finally,

the effect of ABA on fresh weight was analyzed. The fresh weight of soybeans increased gradually under control (non-flooding) conditions (Figure 23C), but exhibited a significant increase within the initial 3 h of flooding stress without ABA. The increase in fresh weight then slowed with continued flooding stress exposure (from 6 to 24 h) (Figure 23C). Similarly, when soybean was treated with 100 μM ABA under flooding conditions, the fresh weight of seedling increased after the initial 3 h of flooding stress and then slowed dramatically between 6 and 24 h (Figure 23C).

2.4. Disscussion

2.4.1. Percoll and sucrose cushions are suitable for nuclei isolation from root tip of soybean and enzyme activity assay is useful to confirm the purity of nuclei

In the present study, the nuclei were purified using percoll and sucrose cushions (Li et al., 2015). Using proteomic technique, 399 nuclear proteins were identified;, 256 proteins out of them were predicted to be localized in the nucleus. Komatsu et al. (2014, 2013b) and Oh et al. (2014c) reported that 39, 65, and 95 nuclear proteins were identified without using percoll and sucrose cushions in soybean. Among them, 7, 13, and 27 nuclear proteins were commonly identified in the present study. On the other hand, previous reports (Komatsu et al., 2013b, 2014; Oh et al., 2014c) only applied Western blot to confirm the purity of nuclei. In the present study, the results from Western blot and enzyme activity assays clearly indicated that the nuclear proteins were highly enriched and contamination from other subcellular proteins was reduced (Figure 16). The present finding indicates that the purity of isolated nuclei is improved through using percoll and sucrose cushions in root tip of soybean and enzyme activity assays of different subcellular markers are more useful approach to confirm the purity of nuclei.

2.4.2. Protein translation is altered by flooding stress through suppression of mRNA transport and 60S pre-ribosome biogenesis

In the present study, nuclear proteomic analysis was performed to explore the mechanisms controlling flooding-responsive proteins in root tip of soybean at initial flooding stress. Among the identified proteins, four exon-junction complex-related proteins were differentially accumulated in untreated and flooded soybean root tip. In

particular, NOP1 and NOP56, which function upstream of 60S pre-ribosome biogenesis, were increased and decreased in untreated and flooded soybean root tip, respectively (Figure 19). The mRNA expression of *NOP1, NOP56, MEGOH*, and *THOC* was down-regulated in root tip in response to flooding stress (Figure 20). The exon-junction complex was involved in mRNA export and nonsense-mediated decay/mRNA surveillance (Le Hir et al., 2001), and six components of the complex have been identified in *Arabidopsis* nuclei (Pendle et al., 2005). The nuclei of M. truncatula were found to contain numerous ribosomal proteins required for the intense protein synthesis that occurs during the seed-filling stage, which is important for seed germination and subsequent plant growth (Repetto et al., 2008). These findings, together with the present results, indicate that the nuclei of root tip in early-stage soybean contain numerous proteins related to pre-ribosome biogenesis and mRNA processing, and these proteins are decreased in abundance during the initial stages of flooding stress.

To confirm that the function of cytosolic ribosomes is adversely affected by flooding stress, protein synthesis-related proteins were analyzed using a proteomic technique. Among the identified proteins, 60S ribosome -, protein synthesis initiation-, and protein synthesis elongation-related proteins were significantly decreased in soybean root tip exposed to flooding stress (Figure 20). This result indicates that the function of cytosolic ribosomes is rapidly suppressed by flooding stress. In maize, protein synthesis was depressed in root tip during hypoxic acclimation (Chang et al., 2000). In addition, the proteomic analysis of endoplasmic reticulum proteins of soybean root tip also indicated that protein synthesis was affected by flooding stress (Komatsu et al., 2012b). These findings, together with the present results, suggest that flooding stress might affect protein synthesis in ribosome by altering the regulation of pre-ribosome biogenesis and mRNA processing-related proteins, which are stored in the nuclei of soybean root tip.

2.4.3. Flooding stress affects chromatin structure through regulation of histone proteins

Among the chromatin structure-related nuclear proteins identified in the present study, histones H1, H2A, H2B, H3, and H4 were increased in untreated soybean and decreased in flood-stressed soybean. Furthermore, histones H1 and H3 were

significantly decreased in abundance at the initial flooding stress in the root tip of soybean (Figure 21), indicating that flooding stress might promptly trigger active response in nuclei and activate signal transduction through regulation of histones. A large number of histone variants, including 4 common core histones, were identified among chromatin-associated proteins of rice (Tan et al., 2007). In addition, specific histone variants in the nucleosome were associated with distinct chromosomal domains involved in the regulation of gene expression (Bernstein et al., 2006). These results suggest that the nuclear fractions were highly purified and flooding stress might affect gene expression in root tip of soybean by altering the level of histone variants.

Histone H1 played an important role in the regulation of chromatin structure and gene activity (Izzo et al., 2008), and was reported to accumulate in a drought-tolerant genotype of tomato (Trivedi et al., 2012). In *Arabidopsis*, histone H1 was specifically induced by salt, drought, and ABA (Han and Wagner, 2014). Furthermore, osmotic stress induced the phosphorylation of histone H3 at threonine 3 in pericentromeric regions of *Arabidopsis* (Wang et al., 2015). In this study, both histones H1 and H3 were significantly decreased at the protein and mRNA expression level in root tip of soybean during the initial stages of flooding stress. These results suggest that histones H1 and H3 may alter chromatin structure in response to multiple abiotic stresses including flooding, and may also play important roles in mediating tolerance to flooding stress through the regulation of gene expression and protein modification in root tip of soybean.

2.4.4. Protein synthesis-related phosphoproteins in nuclei of soybean root tip are responded to flooding stress

Nuclear phosphoproteomic analysis on flooding-treated soybean revealed that the levels of 14 phosphoproteins were significantly changed at the initial stages of flooding stress. Using WoLF PSORT, YLoc, and NucPred, 10 phosphoproteins, which included 5 protein synthesis-related proteins, were predicted to be localized in nuclei (Table 6). It was reported that the levels of a number of protein synthesis related proteins were significantly changed in wild-type soybean under flooding stress, but did not markedly differ in a flooding-tolerant mutant (Komatsu et al., 2013c). Previous research also

demonstrated that protein synthesis in the endoplasmic reticulum was affected by flooding stress (Komatsu et al., 2012b). Phosphoproteomic study on soybean indicated that the phosphorylation status of protein synthesis-related proteins was affected by flooding stress (Nanjo et al., 2011b). In this study, 5 identified protein synthesis related proteins were predicted to function upstream of protein synthesis and included 3 proteins that are associated with pre-ribosome biosynthesis. The present results, combined with those previous findings, suggest that flooding stress adversely affects protein synthesis by controlling the abundance and phosphorylation status of protein synthesis-related proteins in the nuclei of soybean root tip.

The present analyses also demonstrated that the novel rRNA processing protein Rrp5 decreased in both mRNA expression and protein abundance, but displayed increased phosphorylation under flooding stress (Figure 23). Notably, the mRNA expression level of *Rrp5* was further down-regulated in soybean treated with ABA under flooding conditions (Figure 23). rRNA processing protein Rrp5 plays an important role in pre-ribosomal RNA synthesis by binding to multiple cleavage sites and also coordinates pre-rRNA processing and assembly (Lebaron et al., 2013). In *Arabidopsis*, 40S ribosome biogenesis co-factors including Rrp5, are essential for gametophyte and embryo development (Missbach et al., 2013). Based on these results, protein synthesis-related proteins appear to be important for the resistance of soybean against flooding stress, which may adversely affect protein synthesis by disrupting ribosome biogenesis in the nuclei of soybean root tip.

2.4.5. Zinc finger/BTB domain-containing protein 47 and glycine-rich protein are phosphorylated in nuclei of soybean root tip treated with ABA under flooding stress

To better understand the responsive mechanism of the 10 identified nuclear phosphoproteins in soybean-root tip facing against the initial flooding stress, the mRNA expression levels, abundance, and phosphorylation of these proteins were compared. The analyses demonstrated that several nuclear phosphoproteins, which are zinc finger/BTB domain-containing protein 47 and glycine-rich protein, are increased on phosphorylation level; however, decreased on protein and mRNA expression level (Figure 23), indicating that these proteins are phosphorylated in nuclei of soybean root

tip in the initial stages of flooding stress and strengthen phosphorylation status might be negative feedback for their protein synthesis. These two proteins have not been previously identified as flooding-responsive proteins and the corresponding genes were significantly down-regulated in soybean root tip treated with ABA (Figure 23). These findings indicate that these two nuclear phosphoproteins might be differentially regulated specifically in the nuclei of soybean root tip under flooding stress and may be downstream of the ABA signaling pathway.

Zinc finger/BTB domain-containing protein 47 belongs to the zinc finger and BTB/POZ domain-containing transcription factor family, whose members commonly contain a DNA binding zinc finger and a transcription-repressing BTB/POZ domain (Lee and Maeda, 2012). In *Arabidopsis*, a zinc finger-containing protein was shown to negatively impact the germination and seedling growth with ABA dependant manner (Kim et al., 2007). Previous proteomic analysis of soybeans indicated that zinc finger proteins and related mRNAs were down-regulated by ABA supplementation under flooding conditions (Komatsu et al., 2013b). The present findings supported these previous results, as the mRNA expression and protein levels of zinc finger/BTB domain containing protein 47 were decreased in soybean by the addition of ABA under flooding stress. Notably, however, the phosphorylation of zinc finger/BTB domain-containing protein 47 increased under flooding stress. Taken together, these results suggest that under flooding conditions, nuclear-localized zinc finger/BTB domain-containing protein 47 is an ABA-responsive protein in the root tip of soybean and phosphorylation of this protein might increase signal transduction activity to protect plants against the initial stages of flooding stress.

In plants, glycine-rich proteins are characterized by the presence of semi-repetitive glycine-rich motifs, and the expression of these genes is modulated by both biotic and abiotic factors (Sachetto-Martins et al., 2000). In *Arabidopsis*, a glycine-rich RNA-binding protein appears to negatively impact seed germination and seedling growth in an ABA dependent manner (Kim et al., 2007). It was reported that a novel glycine-rich protein was induced by salt stress, and overexpression of this protein in *Arabidopsis* increased plant sensitivity to ABA, as demonstrated by retarded seed germination and seedling growth after salt and ABA treatment (Long et al., 2013).

These findings indicate that glycine-rich proteins are involved in plant responses to various stresses, including osmotic and flooding stresses. In the present study, the fresh weight of soybean decreased, and the mRNA expression level of glycine-rich protein was downregulated in the initial stages of flooding stress in soybean additionally treated with ABA. Komatsu et al. (2013b) reported that ABA enhanced the flooding tolerance of soybean by controlling energy conservation and slowing plant growth. Together with the present findings, these results suggest that ABA may affect the flooding tolerance of early stage-soybean through the regulation of nuclear-localized genes, including glycine-rich protein, and the phosphorylation of nuclear proteins.

2.5. Conclusion

To identify the upstream events regulating cellular responses to flooding stress, nuclear proteomic and nuclear phosphoproteomic analyses of soybean root tip were performed. The results obtained were illustrated as follows: (i) Flooding stress led to the decrease of exon-junction complex, Box C/D snoRNPs, and histone variants related proteins. (ii) mRNA export and pre-ribosomal biogenesis were suppressed and chromatin structure was changed by initial flooding stress. (iii) Protein synthesis in cytoplasm was suppressed in soybean at initial flooding stress. (iv) Phosphorylation status of protein synthesis and ribosome biogenesis related nuclear proteins was affected by flooding stress in soybean; and (v) ABA influenced the initial flooding response in soybean through the regulation of nuclear-localized proteins, such as glycine-rich protein, zinc finger/BTB domain-containing protein 47, and rRNA processing protein Rrp5. These results suggest that the suppression of protein synthesis at initial flooding stress is caused by inhibition of pre-ribosome biogenesis and mRNA transport in nucleus.

Table 5. Changed nuclear proteins in soybean root tip at initial 3 h of flooding stress

Protein ID[a]	KO[b]	Abbreviation	Description	Ratio[c] 48(0)	51(0)	51(3)	Function[d]
pre-mRNA processing							
Glyma04g43500.1	K13025	EIF4A3	EIF4A3; ATP-dependent RNA helicase	1	1.961	1.650	RNA.processing.splicing
Glyma11g15040.3	K12890	SR	SR; splicing factor, arginine/serine-rich 1/9	1	1.848	1.165	RNA.RNA binding
Glyma01g38120.1	K12881	THOC4	THOC4; THO complex subunit 4	1	1.441	0.779	RNA.processing.splicing
Glyma15g10420.1	K12877	MAGOH	MAGOH; protein mago nashi	1	1.266	ND	development
Glyma11g02100.1	K12876	Y14	Y14; RNA-binding protein 8A	1	1.155	0.744	cell.cycle
Glyma15g17060.2	K12875	ACINUS	ACINUS; apoptotic chromatin condensation inducer in the nucleus	1	1.139	0.738	protein.synthesis.ribosome biogenesis
Glyma19g39850.1	K12874	AQR	AQR; intron-binding protein aquarius	1	1.134	0.733	RNA.processing.splicing
Glyma11g01770.1	K12872	RBM22	RBM22; pre-mRNA-splicing factor RBM22/SLT11	1	1.096	0.724	RNA.RNA binding
Glyma08g18490.1	K12870	ISY1	ISY1; pre-mRNA-splicing factor ISY1	1	ND	0.712	DNA.synthesis/chromatin structure
Glyma18g02310.1	K12869	Syf	Syf; crooked neck	1	1.054	0.686	RNA.regulation of transcription
Glyma20g10260.1	K12867	SYF1	SYF1; pre-mRNA-splicing factor SYF1	1	ND	0.645	RNA.regulation of transcription
Glyma15g00980.1	K12862	PLRG1	PLRG1; pleiotropic regulator 1	1	ND	0.585	RNA.processing.splicing
Glyma14g02940.1	K12860	CDC5L	CDC5L; pre-mRNA-splicing factor CDC5/CEF1	1	1.004	0.577	RNA.processing
Glyma20g18440.3	K12857	SNRNP40	SNRNP40; Prp8 binding protein	1	0.958	0.561	RNA.processing
Glyma17g07460.1	K12856	Prp8	Prp8; pre-mRNA-processing factor 8	1	0.909	0.553	RNA.processing
Glyma10g30870.1	K12854	Bn2	Bn2; pre-mRNA-splicing helicase BRR2 [EC:3.6.4.13]	1	0.889	0.551	RNA.regulation of transcription
Glyma01g01190.2	K12852	Snu114	Snu114; 116 kDa U5 small nuclear ribonucleoprotein component	1	0.862	0.540	DNA.synthesis/chromatin structure
Glyma20g38460.1	K12837	U2AF2	U2AF2; splicing factor U2AF 65 kDa subunit	1	0.853	ND	RNA.processing.splicing
Glyma14g38030.1	K12830	SF3B3	SF3B3; splicing factor 3B subunit 3	1	0.835	0.539	RNA.processing.splicing
Glyma20g18440.3	K12828	SF3B1	SF3B1; splicing factor 3B subunit 1	1	ND	0.538	RNA.processing
Glyma13g44290.1	K12825	SF3A1	SF3A1; splicing factor 3A subunit 1	1	0.821	0.498	RNA.processing.splicing
Glyma17g11410.1	K12820	Prp43	Prp43; pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	1	0.800	0.484	protein.synthesis.elongation
Glyma08g18490.1	K12816	Prp17	Prp17; pre-mRNA-processing factor 17	1	0.770	0.468	DNA.synthesis/chromatin structure
Glyma02g36700.1	K11097	Sm	Sm; small nuclear ribonucleoprotein E	1	0.763	0.455	stress
Glyma13g03760.1	K11094	U2B"	U2B"; U2 small nuclear ribonucleoprotein B"	1	0.750	0.419	RNA.regulation of transcription
Glyma20g00260.1	K11093	U1-70K	U1-70K; U1 small nuclear ribonucleoprotein 70kDa	1	0.716	ND	not assigned
Glyma20g21330.1	K11092	U2A'	U2A'; U2 small nuclear ribonucleoprotein A'	1	0.626	0.399	RNA.processing.splicing
Glyma19g29230.1	K11086	Sm	Sm; small nuclear ribonucleoprotein B and B'	1	0.597	0.368	not assigned
Glyma20g34010.2	K10599	Prp19	Prp19; pre-mRNA-processing factor 19	1	0.552	0.365	RNA.processing
Glyma09g01020.1	K06063	SKIP	SKIP; SNW domain-containing protein 1	1	0.521	0.282	RNA.processing
Glyma19g40600.1	K03283	HSP73	probable pre-mRNA-splicing factor ATP-dependent RNA helicase-like isoform 1	1	0.509	0.269	RNA.processing.RNA helicase
Nuclear pore complex							
Glyma08g05070.1	K14312	NUP155	NUP155; nuclear pore complex protein Nup155	1	2.258	0.798	protein.targeting.nucleus
Glyma13g44070.1	K14309	NUP93	NUP93; nuclear pore complex protein Nup93	1	0.712	0.607	protein.targeting.nucleus
Glyma05g09015.1	K14314	gp210	gp210; nuclear pore complex protein Nup210	1	0.261	1.253	protein.targeting.nucleus
pre-Ribosome biogenesis							

Glyma12g36480.1	K14573	NOP4	NOP4; nucleolar protein 4	1	1.806	ND	RNA.RNA binding
Glyma11g10520.1	K14568	EMG1	EMG1; rRNA small subunit pseudouridine methyltransferase Nep1	1	1.672	ND	not assigned
Glyma03g37300.1	K14567	UTP14	UTP14; U3 small nucleolar RNA-associated protein 14	1	1.519	1.243	RNA.processing.splicing
Glyma11g12200.1	K14565	NOP58	NOP58; nucleolar protein 58	1	1.511	1.228	protein.synthesis.ribosome biogenesis
Glyma17g13770.1	K14564	NOP56	NOP56; nucleolar protein 56	1	1.412	0.946	RNA.regulation of transcription
Glyma18g02340.1	K14563	NOP1	NOP1; rRNA 2'-O-methyltransferase fibrillarin	1	1.253	0.734	protein.synthesis.ribosome biogenesis
Glyma11g21290.1	K14561	IMP4	IMP4; U3 small nucleolar ribonucleoprotein protein IMP4	1	0.741	0.734	protein.synthesis.ribosome biogenesis
Glyma20g30110.1	K14560	IMP3	IMP3; U3 small nucleolar ribonucleoprotein protein IMP3	1	0.678	0.731	protein.synthesis.ribosomal protein
Glyma05g02340.1	K14559	MPP10	MPP10; U3 small nucleolar RNA-associated protein MPP10	1	0.617	0.716	protein.synthesis.ribosome biogenesis
Glyma09g10290.1	K14558	PWP2	PWP2; periodic tryptophan protein 2	1	0.532	ND	not assigned
Glyma17g01570.1	K14557	UTP6	UTP6; U3 small nucleolar RNA-associated protein 6	1	0.504	0.710	not assigned
Glyma19g37051.1	K14556	DIP2	DIP2; U3 small nucleolar RNA-associated protein 12	1	0.479	ND	protein.synthesis.ribosome biogenesis
Glyma05g02240.1	K14555	UTP13	UTP13; U3 small nucleolar RNA-associated protein 13	1	0.449	0.697	protein.synthesis.ribosome biogenesis
Glyma08g47440.1	K14554	UTP21	UTP21; U3 small nucleolar RNA-associated protein 21	1	0.435	0.688	protein.synthesis.ribosome biogenesis
Glyma13g32840.1	K14553	UTP18	UTP18; U3 small nucleolar RNA-associated protein 18	1	0.379	0.660	protein.synthesis.ribosome biogenesis
Glyma02g45960.2	K14550	UTP10	UTP10; U3 small nucleolar RNA-associated protein 10	1	0.356	0.641	protein.synthesis.ribosome biogenesis
Glyma10g02745.2	K14549	UTP15	UTP15; U3 small nucleolar RNA-associated protein 15	1	0.343	0.606	RNA.transcription
Glyma17g35220.2	K14548	UTP4	UTP4; U3 small nucleolar RNA-associated protein 4	1	0.338	0.591	signalling
Glyma17g03310.1	K14546	UTP5	UTP5; U3 small nucleolar RNA-associated protein 5	1	0.317	0.572	not assigned
Glyma11g07340.1	K14544	UTP22	UTP22; U3 small nucleolar RNA-associated protein 22	1	0.309	0.559	not assigned
Glyma08g15130.2	K14521	NAT10	NAT10; N-acetyltransferase 10	1	0.292	0.555	not assigned
Glyma15g06160.2	K11131	DKC1	DKC1; H/ACA ribonucleoprotein complex subunit 4	1	0.280	0.533	protein.synthesis.ribosome biogenesis
Glyma16g04570.1	K11128	GAR1	GAR1; H/ACA ribonucleoprotein complex subunit 1	1	0.246	0.422	protein.synthesis.ribosome biogenesis
Glyma07g37311.1	K11130	NOP10	NOP10; H/ACA ribonucleoprotein complex subunit 3	1	ND	0.492	not assigned.no ontology
Glyma15g06160.2	K11108	RCL1	RCL1; RNA 3'-terminal phosphate cyclase-like protein	1	ND	0.418	protein.synthesis.ribosome biogenesis
chromatin structure		No.					
Glyma20g33320.1		1	Histone superfamily protein	1	ND	0.654	DNA.synthesis/chromatin structure.histone.core.H2B
Glyma19g42760.1		2	gamma histone variant H2AX	1	ND	0.766	DNA.synthesis/chromatin structure.histone.core.H2A
Glyma19g33360.1		3	gamma histone variant H2AX	1	1.492	ND	DNA.synthesis/chromatin structure.histone.core.H2A
Glyma18g22940.1		4	P_loop containing nucleoside triphosphate hydrolases superfamily protein	1	0.363	0.533	DNA.synthesis/chromatin structure
Glyma17g37480.2		5	Histone superfamily protein	1	ND	0.556	DNA.synthesis/chromatin structure.histone.core.H3
Glyma17g31120.1		6	histone H2A protein 9	1	2.020	0.642	DNA.synthesis/chromatin structure.histone.core.H2A
Glyma17g06170.1		7	nucleosome assembly protein 1	1	2.123	1.472	DNA.synthesis/chromatin structure
Glyma15g04805.1		8	Histone superfamily protein	1	2.196	0.661	DNA.synthesis/chromatin structure.histone.core.H4
Glyma14g39600.2		9	high mobility group A	1	1.766	1.001	DNA.synthesis/chromatin structure
Glyma13g40890.1		10	histone H2A 12	1	1.975	0.922	DNA.synthesis/chromatin structure.histone.core.H2A
Glyma13g36190.1		11	histone H2A 10	1	1.429	0.945	DNA.synthesis/chromatin structure.histone.core.H2A
Glyma13g28720.1		12	chromatin remodeling factor17	1	0.383	0.432	DNA.synthesis/chromatin structure
Glyma13g16500.1		13	nucleosome assembly protein 1;2	1	ND	1.698	DNA.synthesis/chromatin structure
Glyma12g08800.1		14	Histone superfamily protein	1	1.961	0.673	DNA.synthesis/chromatin structure.histone.core.H2B

Glyma12g07050.1	15	Histone superfamily protein	1	1.308	ND	DNA.synthesis/chromatin structure.histone.core.H2A
Glyma12g05910.1	16	Histone superfamily protein	1	1.625	0.538	DNA.synthesis/chromatin structure.histone.core.H3
Glyma12g01630.1	17	DNA topoisomerase I alpha	1	1.026	ND	DNA.synthesis/chromatin structure
Glyma11g37961.1	18	Histone superfamily protein	1	ND	0.550	DNA.synthesis/chromatin structure.histone.core.H3
Glyma11g15060.1	19	Histone superfamily protein	1	ND	0.654	DNA.synthesis/chromatin structure.histone.core.H2A
Glyma10g07120.1	20	histone H4	1	2.299	0.677	DNA.synthesis/chromatin structure.histone.core.H4
Glyma10g03840.1	21	histone H1-like protein	1	2.386	0.750	DNA.synthesis/chromatin structure.histone.H1
Glyma09g35730.3	22	DNA topoisomerase 1 beta	1	1.038	ND	DNA.synthesis/chromatin structure
Glyma08g18490.1	23	U5 small nuclear ribonucleoprotein helicase_ putative	1	0.770	0.712	DNA.synthesis/chromatin structure
Glyma08g05710.1	24	histone H1-like protein	1	ND	0.880	DNA.synthesis/chromatin structure.histone.H1
Glyma07g06310.1	25	Histone superfamily protein	1	1.182	0.506	DNA.synthesis/chromatin structure core H3
Glyma07g03530.3	26	DEAH box RNA helicase family protein	1	ND	0.717	DNA.synthesis/chromatin structure
Glyma06g07280.2	27	DEAH box RNA helicase family protein	1	1.169	ND	DNA.synthesis/chromatin structure
Glyma03g30440.1	28	Histone superfamily protein	1	1.512	0.610	DNA.synthesis/chromatin structure.histone.core.H2A
Glyma02g41230.2	29	high mobility group A	1	2.125	ND	DNA.synthesis/chromatin structure
Glyma02g16150.1	30	histone H2A 11	1	ND	0.642	DNA.synthesis/chromatin structure.histone.core.H2A
Glyma02g15910.2	31	histone H1-like protein	1	2.491	0.746	DNA.synthesis/chromatin structure.histone.H1
Glyma01g01190.2	32	chromatin protein family	1	0.862	0.551	DNA.synthesis/chromatin structure

a) Protein ID is according to the Phytozome database; b) KO is the KEGG Orthology number; c) Ratio is calculated using treatments divide control; d) Function is obtained from MapMan bin codes. ND means not identified.

Table 6. Identification of flooding-responsive nuclear phosphoproteins in soybean root tip at initial 3 h of flooding stress

Protein ID[a]	Accession number	Name	MP[b]	MPP[c]	Frames	Hits	Ratio	*p*-value	WoLF PSORT	YLoc	NucPred	Function[d]
Glyma01g41250.1	AT4G17620.1	glycine_rich protein	1	1	2	4	44.080	0.000	Plas	Nucl	0.21	not assigned
Glyma11g04170.1	AT4G17620.1	glycine_rich protein	1	1	2	4	44.080	0.000	Nucl	Nucl	0.290	not assigned
Glyma15g05880.1	AT5G09400.1	K^+ uptake permease	1	1	2	2	27.935	0.000	Plas	Cyto	0.050	transport
Glyma02g26710.1	AT3G11964.1	RNA binding rRNA processing protein Rrp5	3	3	4	5	26.743	0.001	Nucl	Nucl	0.580	not assigned
Glyma14g08160.1	XP_008220798	zinc finger and BTB domain-containing protein 47	1	1	2	2	20.732	0.000	Nucl	Nucl	0.760	not assigned
Glyma06g01750.2	AT4G35300.1	tonoplast monosaccharide transporter2	5	5	7	15	11.128	0.002	Plas	ND	0.060	transport
Glyma07g37090.2	AT4G16150.1	calmodulin binding transcription regulators	2	2	4	7	3.197	0.002	Nucl	Cyto	0.650	RNA.regulation
Glyma06g00220.1	AT4G35300.1	tonoplast monosaccharide transporter2	3	3	4	11	2.399	0.001	Plas	ND	0.060	transport
Glyma14g02750.1	AT5G54910.1	DEA(D/H)_box RNA helicase	5	5	10	26	1.650	0.012	Nucl	Nucl	0.890	protein.synthesis
Glyma08g18460.1	AT3G58660.1	Ribosomal protein L1p/L10e	8	8	17	33	1.028	0.012	Nucl	Nucl	0.550	protein.synthesis
Glyma09g34760.5	AT3G49010.1	breast basic conserved 1	3	2	5	16	0.404	0.000	Chlo	Cyto	0.530	protein.synthesis
Glyma08g45425.1	AT3G60240.4	eukaryotic translation initiation factor 4G	4	4	3	21	0.261	0.025	Nucl	Nucl	0.960	protein.synthesis
Glyma05g27040.1	AT3G18240.1	Ribosomal protein S24/S35	3	3	11	26	0.092	0.001	Chlo	Nucl	0.570	protein.synthesis
Glyma17g09590.1	AT5G66540.1	U3 small nucleolar RNA-associated protein MPP10	11	11	19	49	0.006	0.000	Cyto	Nucl	0.830	protein.synthesis

a) Protein ID is according to the phytozome database; b) MP means matched peptides; c) MPP means matched phosphopeptides; d) Function obtained from MapMan bin code. Accession number, accession number according to the NCBI database. Localization predicted by WoLF PSORT, YLoc, and NucPred; Cyto, cytosol; Nucl, nuclear; Chlo, chloroplast; ND, not determined. For results from NucPred, the consensus score is basically the fraction of predictors which vote ‘nuclear’. For a discrete prediction, the user has to decide on a score threshold (preferably 0.8). Sequences, which score is greater than or equal to this threshold, are predicted to in the nucleus.

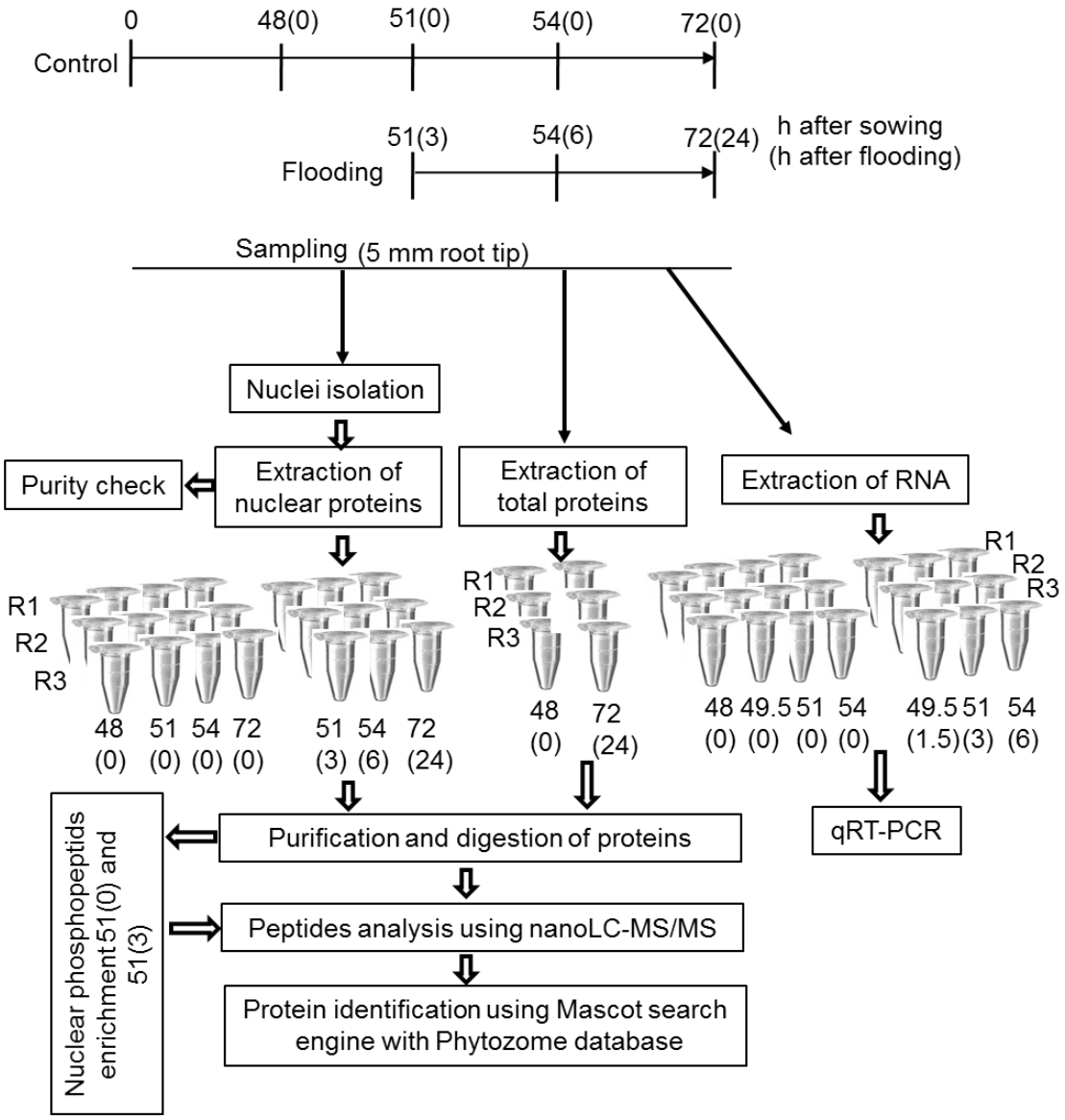


Figure 15. Experimental design of nuclear proteomic study. Two-day-old soybeans were treated without or with flooding stress for 3, 6, and 24 h. Proteins were extracted from purified nuclei and analyzed using gel-free proteomic and phosphoproteomic studies. For proteomics of nuclear protein, the digested peptides were directly applied for nanoLC-MS/MS analysis; for proteomics of nuclear phosphoprotein, phosphopeptides were enriched from digested peptides and applied for nanoLC-MS/MS analysis. Three independent experiments were performed as biological replicates.

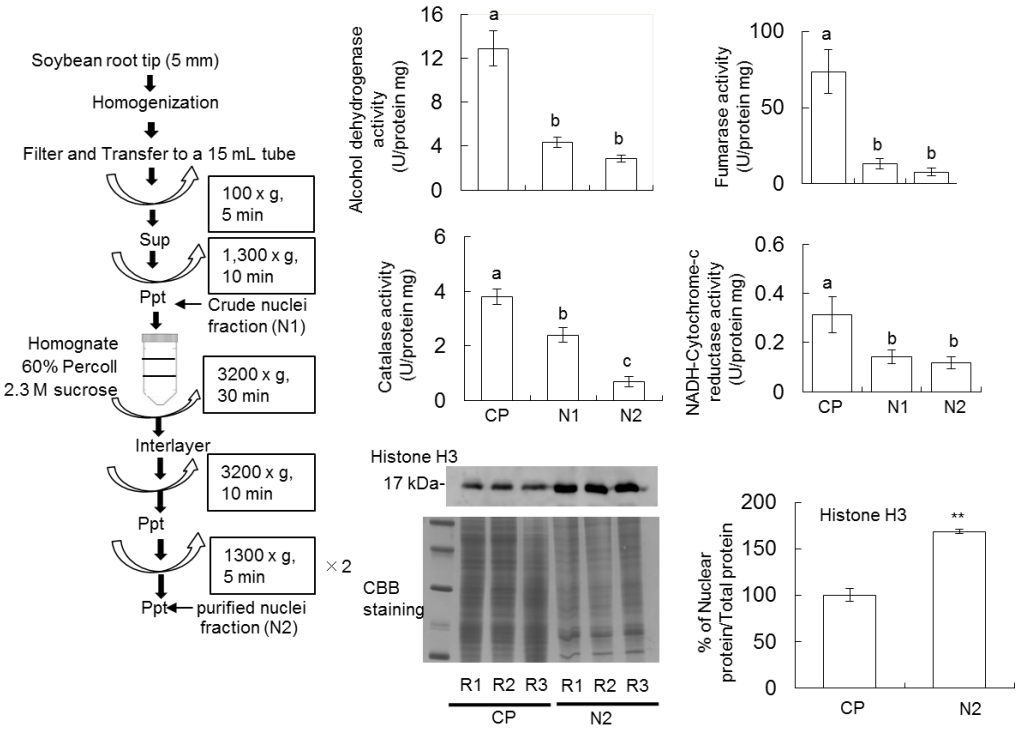


Figure 16. Purity of nuclear fractions obtained from root tip of soybean. Cellular protein (CP), crude nuclear protein fraction (N1), and purified nuclear protein fraction (N2) were extracted and analyzed. The obtained fractions were analyzed. Alcohol dehydrogenase, catalase, fumarase, and NADH-cytochrome c reductase were used as marker enzymes for the cytosol, peroxisomes, mitochondria, and endoplasmic reticulum, respectively. As a marker protein for the nucleus, histone accumulation was analyzed using Western blot analysis. Data are shown as means ± SD from 3 independent biological replicates (R1, R2, and R3). Means with the same letter were not significantly different among the 3 samples based on one-way ANOVA with Tukey's multiple comparison (p <0.05). Asterisks indicate significant changes in the relative intensity of signal band in the nuclear fraction compared to total protein according to Student's t-test (**p < 0.01).

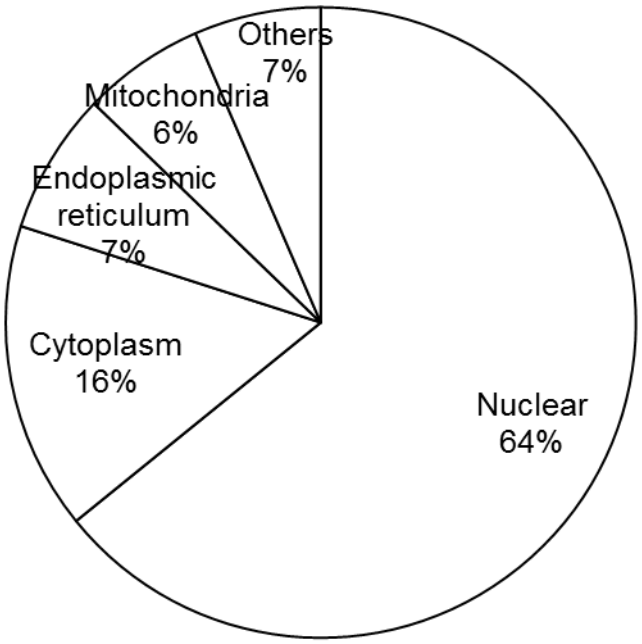


Figure 17. The subcellular localization of the 399 nuclear proteins identified in 2-day-old soybean. The localization of 399 proteins was predicated using Plant-mPLoc, WoLF PSORT, YLoc, and NucPred softwares.

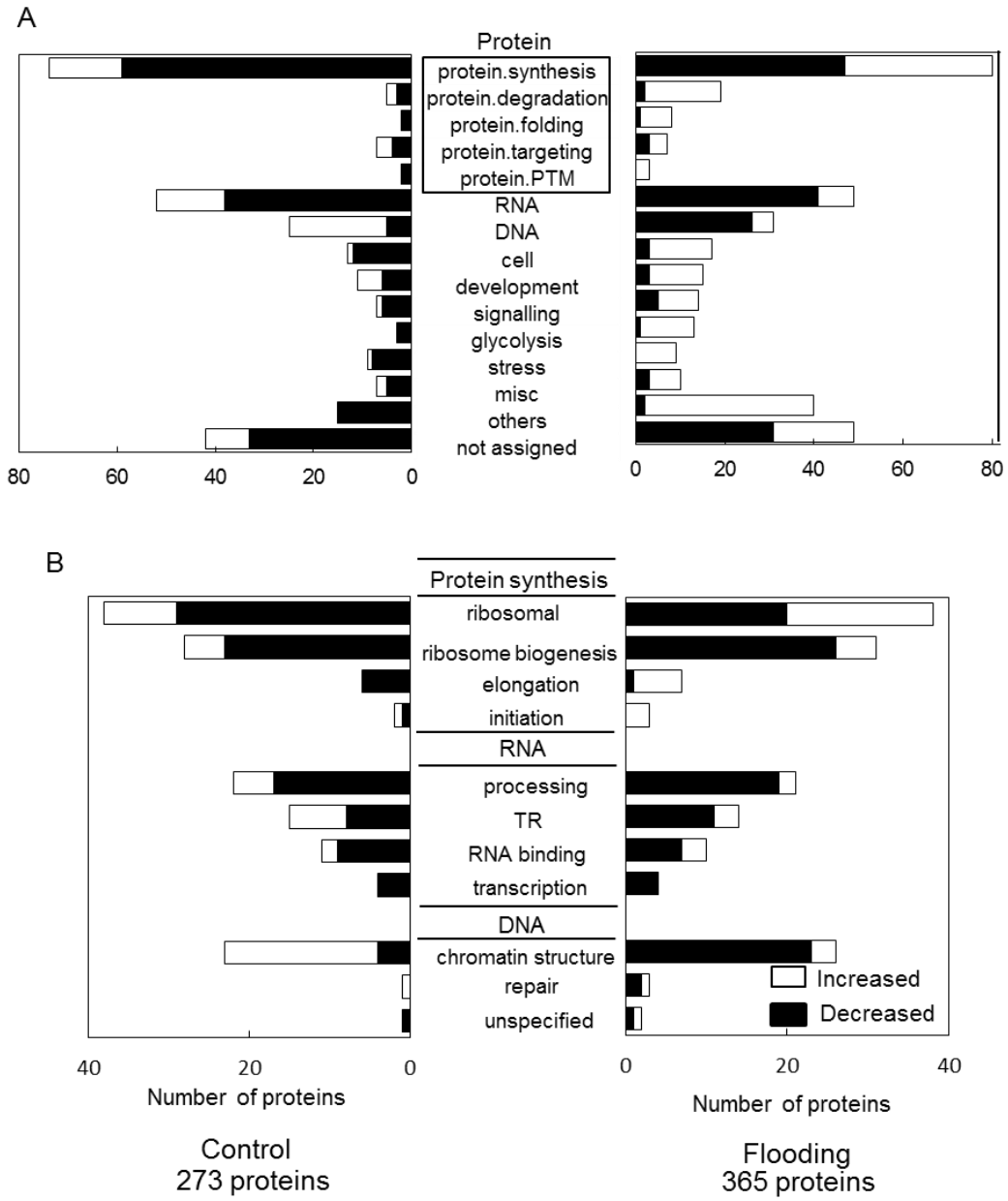


Figure 18. Functional distribution of significantly changed nuclear proteins in 2-day-old soybean treated without or with flooding stress for 3 h. The abundances of nuclear proteins in root tip treated without or with flooding stress were compared with those 48 h after sowing. Functional classification of the 273 and 365 identified nuclear proteins in control and flooding-treated soybean, respectively, was performed according to MapMan bin codes (A). Subcategorization of the proteins classified into protein synthesis, RNA, and DNA was performed (B). Abbreviations: PTM, post-translational modification; TR, transcriptional regulation.

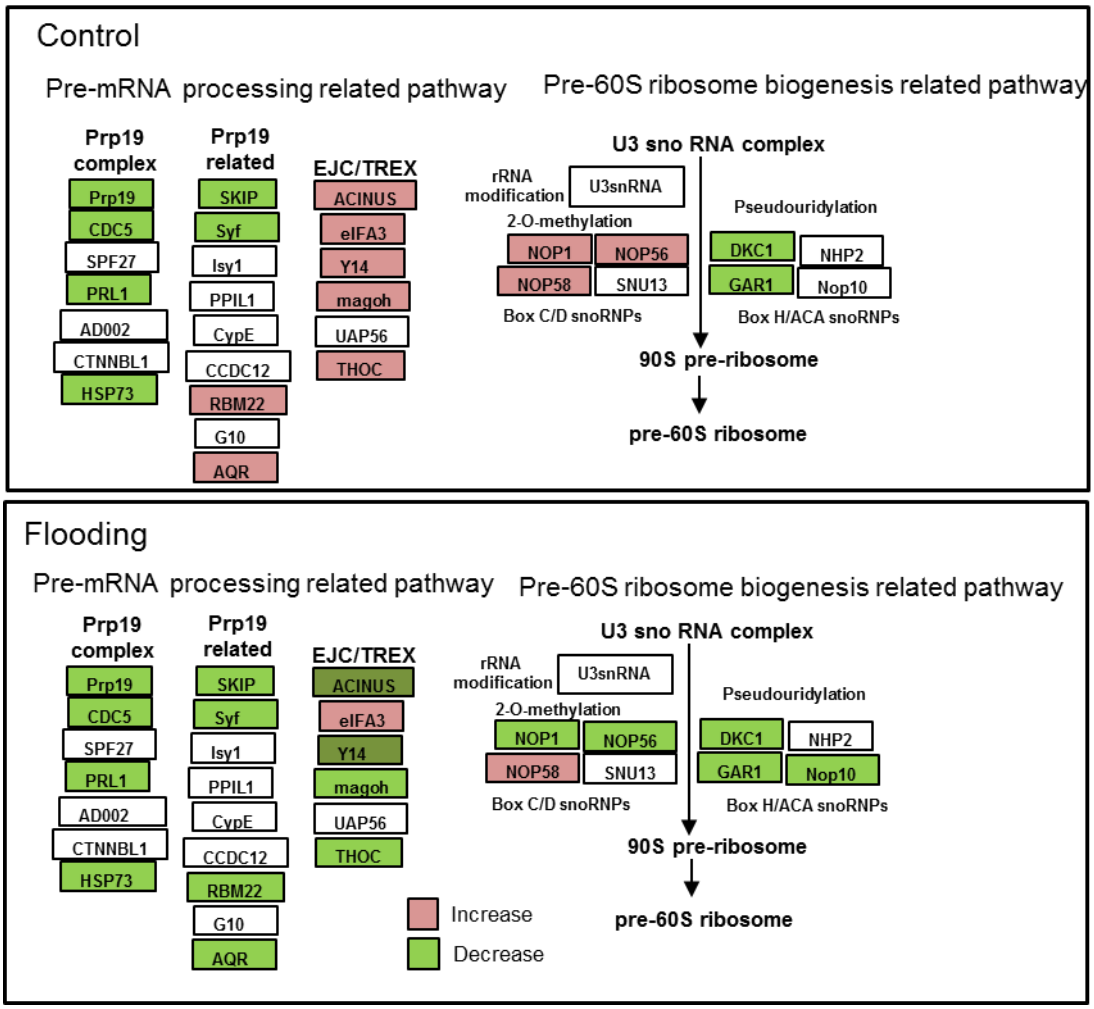


Figure 19. Mapping of pre-mRNA processing- and pre-ribosome biogenesis-related proteins. The significantly changed nuclear proteins in the initial 3 h of flooding stress were mapped using the KEGG pathway database. Pathways for pre-mRNA processing and pre-ribosome biogenesis in soybean were selected. Increases and decreases in protein abundance are indicated by red and green colors, respectively. Detailed information of these nuclear proteins is listed in Table 5.

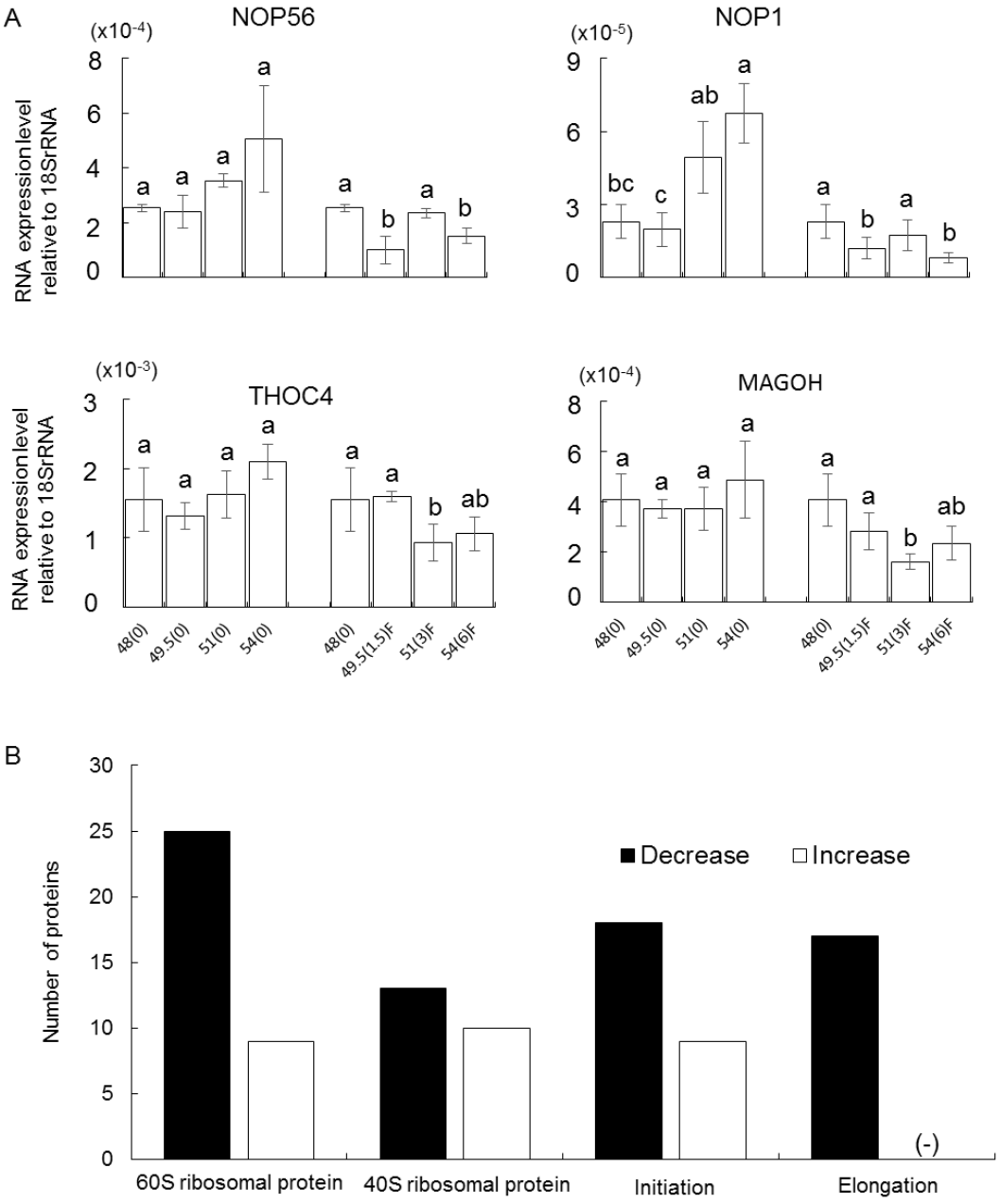


Figure 20. Effects of flooding stress on function of ribosome in soybean. For mRNA expression analysis, 2-day-old soybeans were flooded for 1.5, 3, and 6 h. Pre-mRNA processing- and pre-ribosome biogenesis-related proteins (Figure 19) were selected for qRT-PCR analysis. Data are shown as means ± SD from 3 independent biological replicates. Means with the same letter were not significantly different among the 4 examined time points based on one-way ANOVA with Tukey's multiple comparison (p <0.05) (A). For proteomic analysis, 2-day-old soybeans were flooded for 24 h. Cellular proteins were extracted from root tip of soybean and identified using nanoLC-MS/MS. The abundance of proteins was compared with that determined 48 h after sowing. Functional classification of protein synthesis-related proteins was performed according to MapMan bin codes (B).

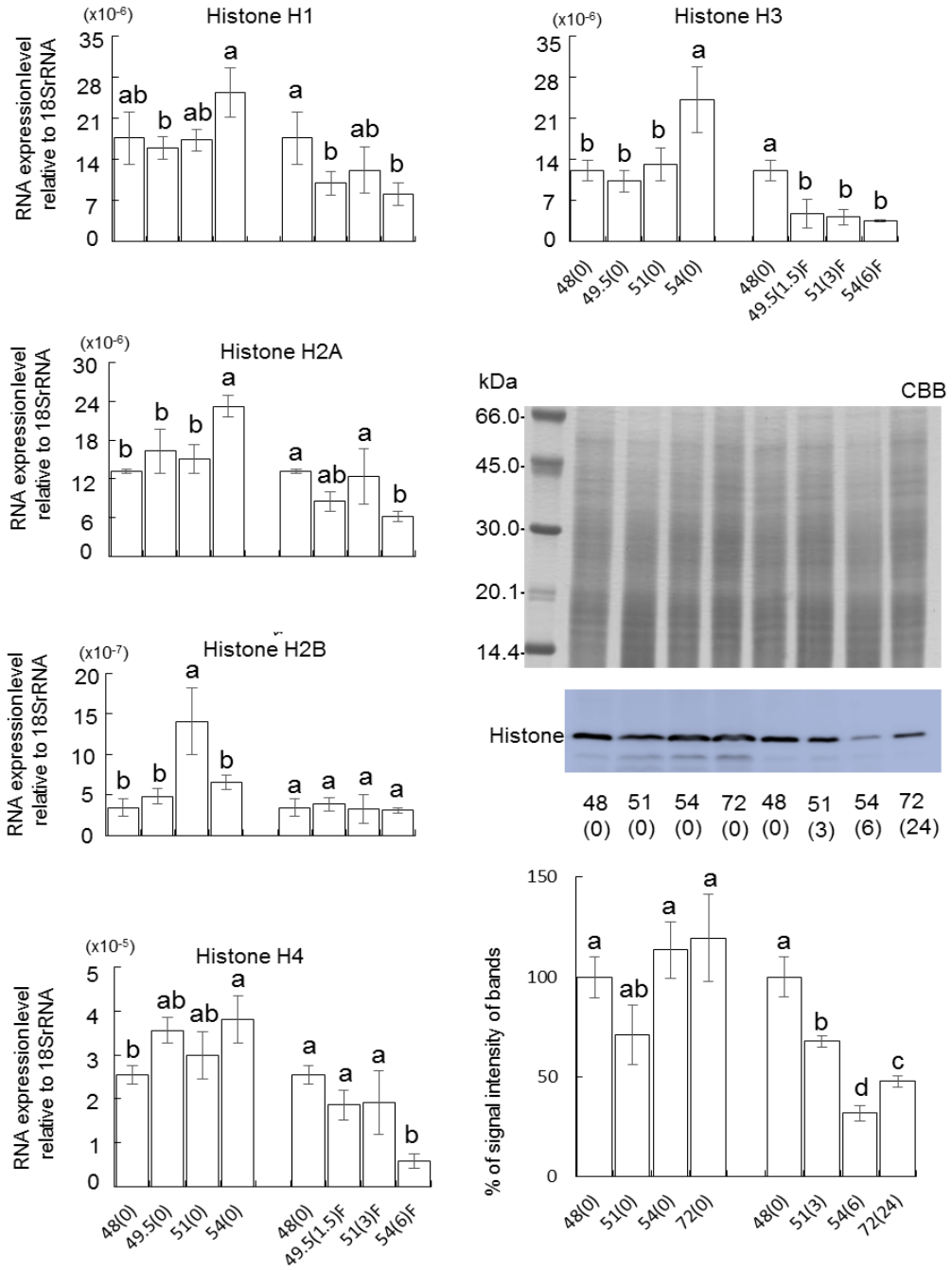


Figure 21. Effect of flooding stress on histones in soybean. For mRNA expression analysis, 2-day-old soybeans were flooded for 1.5, 3, and 6 h. The chromatin structure-related proteins such as histones H1, H2A, H2B, H3, and H4 were selected for qRT-PCR analysis. For protein abundance analysis, 2-day-old soybeans were flooded for 3, 6, and 24 h. Anti-histone H3 antibody was analyzed by Western blotting as a measure of histone accumulation. Data are shown as means ± SD from 3 independent biological replicates. Means with the same letter were not significantly different among the 4 examined time points based on one-way ANOVA with Tukey's multiple comparison (p <0.05).

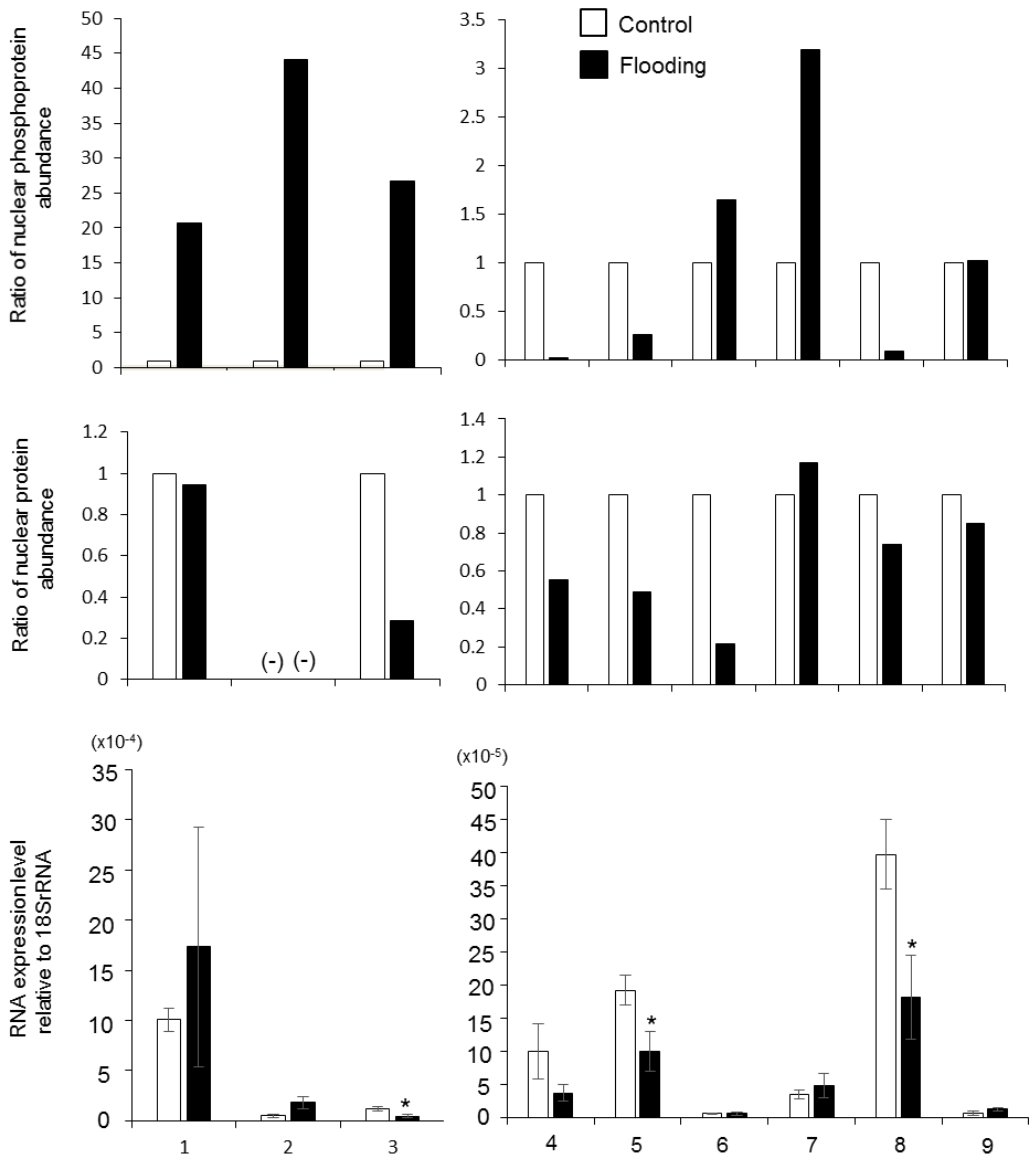


Figure 22. Comparison of ratios of nuclear phosphoprotein and nuclear protein abundance, and mRNA expression levels in flooding-stressed soybeans. Two-day-old soybeans were treated without (control, white column) or with (flooded, black column) flooding stress for 3 h, and root tip was collected. Phosphoprotein abundance was calculated using matched phosphopeptides. Protein abundance was calculated according to peptides identified in the total nuclear protein fraction. Relative mRNA expression level of 9 genes was normalized according to the abundance of 18S rRNA. The name of genes are as followings: 1, *zinc finger and BTB domain-containing protein 47*; 2, *glycine rich protein*; 3, *rRNA processing protein Rrp5*; 4, *U3 small nucleolar RNA-associated protein MPP10*; 5, *eukaryotic translation initiation factor 4G*; 6, *DEA(D/H)_box RNA helicase*; 7, *calmodulin binding;transcription activator*; 8, *Ribosomal protein S24/S35;* 9, *Ribosomal protein L1p/L10e.* Asterisks indicate significant changes between control and flooding conditions according to the Student's *t*-test (*$p < 0.05$).

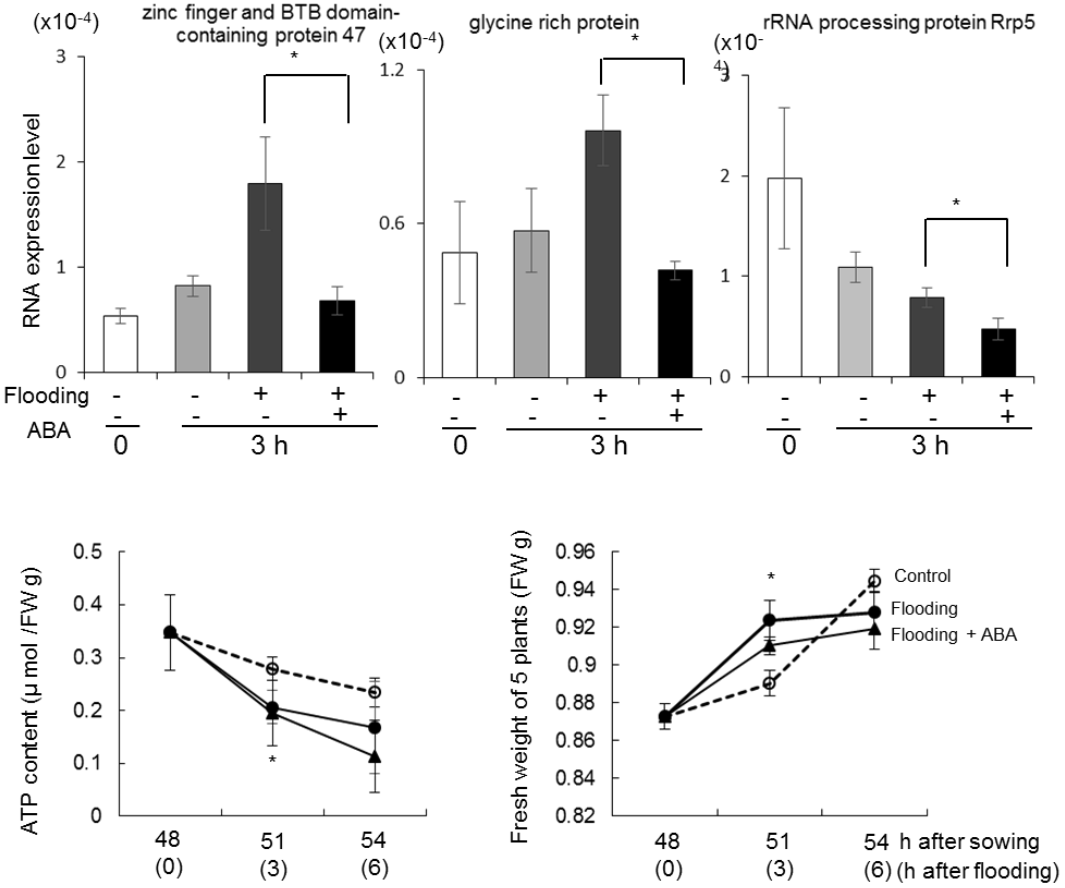


Figure 23. Effect of ABA on mRNA expression, ATP content, and fresh weight of soybean under flooding stress. For mRNA expression, 2-day-old soybeans were flooded without (flooding) or with 100 μM ABA for 3 h, and root tip was collected for qRT-PCR analysis (A). For the analysis of ATP content, 2-day-old soybeans were flooded without (flooding) or with 100 μM ABA for 3, 6, and 24 h, and root tip was collected as samples (B). For the measurement of fresh weight, 2-day-old soybeans were flooded without (flooding) or with 100 μM ABA for 3, 6, and 24 h, and soybeans were measured (C). Data are shown as means ± SD from 3 independent biological replications. Student's *t*-test analysis was used to compare values between the control and treated samples. Asterisks indicate a significant change (*$p < 0.05$)

CHAPTER 3

CHARACTERIZATION OF TOLERANT MECHANISM IN MUTANT LINE AND ABSCISIC ACID -TREATED SOYBEAN UNDER INITIAL FLOODING STRESS

3.1. Introduction

To promote the development of soybean, it is necessary to uncover the mechanism underlying flooding tolerance in soybean. In response to several days of flooding exposure, root elongation and lateral root development of soybean were suppressed (Komatsu et al., 2012a). Komatsu et al. (2013c) developed a flooding-tolerant soybean mutant line through gamma-ray irradiation and demonstrated that root development was not delayed in the mutant under flooding conditions. Notably, the mutant line survived under flooding stress for 6 days and grew well after the excess water was removed. A comparison of flooding-responsive proteins between the mutant and wild-type indicated that suppression of glycolysis and programmed cell death were important for the acquisition of flooding tolerance (Komatsu et al., 2013c). Komatsu et al. (2013b) also reported that the survival ratio was improved by treatment with ABA under flooding condition. Analysis of proteins from flooding-treated soybean with supplemental ABA indicated that ABA enhanced flooding tolerance by controlling energy conservation through the regulation of several transcription-related proteins (Komatsu et al., 2013b). These two findings provide a suitable material and a treatment of soybean to study the flooding-tolerant mechanism.

In plant breeding field, irradiation of gamma ray is an important approach to induce mutation (Nakagawa, 2009). Gamma ray belongs to one of ionizing radiations and it provides high energetic form of electromagnetic radiation (Wi et al., 2007). Gamma-ray leads to produce free radicals based on the interaction with atoms or molecules in the cell (Kovacs and Keresztes, 2002) and it is used to induce alteration on phenotypical and physiological levels (Kitano et al., 2015). For example, characters including reduced rate of germination and discoloration of cotyledons were observed when seeds were treated with irradiation through gamma ray; and metabolome analysis indicated that oxalate contents were decreased in the leaves of plants from gamma ray-irradiated seeds (Kitano et al., 2015). In addition, gamma ray also leads to aberrations of mitosis/chromosome and mutation of bases (Shirasawa, et al., 2016). These results suggest that irradiation with gamma ray is useful approach for crop breeding.

Transcriptomics is the quantification of the transcriptome, which contains complete set of transcripts in a cell (Wang et al., 2009). It provides the ability to identify

quantitative and qualitative differences in gene expression for a specific developmental stage or physiological condition (Wang et al., 2009). Transcriptomic studies on flooding or hypoxic stress have been performed in *Arabidopsis* (Banti et al., 2010; Mustroph et al., 2010), rice (Narsai et al., 2009), maize (Zhang et al., 2006), and soybean (Komatsu et al., 2009; Nanjo et al., 2011a). Gel-free/label-free proteomics is a central technology that has realized great progress toward the identification, quantification, and characterization of proteins (Bensimon et al., 2012). In addition, a joint analysis of the transcriptomic and proteomic data provides useful insights that can not be explored from individual analysis of mRNA or protein changes (Haider and Pal, 2013). These results suggest that novel results might be uncovered through comparative analysis using transcriptomic and proteomic techniques.

In this study, to explore the flooding-tolerance mechanisms of soybean, particularly at the initial flooding stress, flooding-tolerant mutant line (Komatsu et al., 2013c) and ABA-treated soybean (Komatsu et al., 2013b), which exhibited the flooding tolerance, were used as materials. To identify the marker proteins and genes for initial flooding tolerance in soybean, gel-free/label-free proteomic and RNA-sequencing based transcriptomic techniques were performed. Furthermore, bioinformatic, transcriptomic, enzymatic, and physiological analyses were performed to determine the function of the identified flooding tolerance related proteins and genes.

3.2. Materials and methods

3.2.1 Plant material and treatments

Seeds of soybean (*Glycine max* L.) cultivar Enrei were used as material. The growth conditions are described in 1.2.1 of Chapter 1. For initial flooding stress, 2-day-old soybeans were treated with water for 3 h. For ABA treatment, 10 μM ABA was supplied at the same time with flooding stress (Komatsu et al., 2013b). As mutant line, flooding tolerant mutant (Komatsu et al., 2013c) was used after backcross with wild type soybean. For proteomic analysis, root was collected as sample. For transcriptomic analysis, root including hypocotyl was collected as samples. Three independent experiments were performed as biological replicates for all experiments.

3.2.2. Extraction of proteins

Protein extraction is described in 1.2.3 of Chapter 1.

3.2.3. Clean up and digestion of proteins

The clean up and digestion of proteins is described in 1.2.5 of Chapter 1.

3.2.4. Mass spectrometry analysis

MS analysis is described in 1.2.7 of Chapter 1. Data of MS were deposited with the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno et al., 2013) and the identifier of dataset was PXD 003329.

3.2.5. Protein identification using acquired mass spectrometry data

Protein identifications of acquired MS data is described in 1.2.8 of Chapter 1.

3.2.6. Differential analysis of the identified proteins

Differential analysis of the identified proteins is described in 1.2.9 of Chapter 1.

3.2.7. RNA extraction

RNA extraction is described in 1.2.12 of Chapter 1.

3.2.8. Library construction and sequencing

The library construction and sequencing were carried out by Beijing Genomics Institute (Shenzhen, China). Briefly, mRNA was isolated using magnetic beads with Oligo (dT). First-strand cDNA was synthesized from mRNA fragments by reverse transcriptase using random hexamers (Invitrogen, San Diego, CA, USA) and second-strand cDNA was synthesized using reaction buffer, dNTPs, RNase H and DNA polymerase I (Amersham Biosciences, Piscataway, NJ, USA). The short fragments were purified and resolved with EB buffer for end reparation and addition of the poly (A) tail. After that, the short fragments were connected with adapters. Through agarose gel electrophoresis, the suitable fragments were selected for the PCR amplification. During

the quality control steps, Agilent 2100 Bioanaylzer (Agilent Technologies, Santa Clara, CA, USA) and ABI Step OnePlus Real-Time PCR system (Illumina, San Diego, CA, USA) were used in quantification and qualification of the sample library. Finally, the library was sequenced using Illumina HiSeqTM 2000 system.

3.2.9. Differential analysis of the identified genes

The clean reads were mapped to soybean reference genome, which was downloaded from National Center for Biotechnology Information (NCBI) (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF_000004515.4_Glycine_max_v2.0). Gene expression was quantified by calculating fragments per kilobase of exon per million fragments mapped (FPKM) values using RSEM software package (version: 1.2.12) (BioMed Central). The differentially expressed genes in each replication were analyzed using poisson distribution referring to method described by Audic and Claverie (1997). Correction for false positive and false negative errors were performed according to Benjamini and Yekutieli (2001) ($p < 0.05$, FDR< 0.05). The correlations between different replications were analyzed based on FPKM values of genes (Trapnell et al. 2009). The commonly significantly changed genes among three replications were selected for further comparison using student *t*-test ($p < 0.05$) to determine significant differences in gene expression between control and experimental groups. After comparison, the distribution of data were analyzed by MA plot using R software (R 3.2.4).

3.2.10. Quantitative reverse transcription polymerase chain reaction analysis

qRT-PCR analysis is described in 1.2.13 of Chapter 1. The primers were designed using the Primer3 web interface (http://frodo.wi.mit.edu) (Supplemental Table 1). Primer specificity was checked by BLASTN searches against sequences in the soybean genome database (Phytozome) with the queries of designed primers by melt curve analysis.

3.2.11. Categorization of function of significantly changed proteins and differentially expressed genes

Categorization of function of identified proteins and genes is described in 1.2.10 of Chapter 1.

3.2.12. Statistical analysis

Statistical analysis is described in 1.2.13 of Chapter 1.

3.3. Results

3.3.1. Identification of flooding stress-responsive proteins in flooding-tolerant mutant line and ABA-treated soybean

To investigate the initial tolerant mechanism of soybean to flooding, 2-day-old plants of flooding-tolerant mutant line (Komatsu et al., 2013c) and soybean treated with 10 μM ABA, which exhibited flooding tolerance (Komatsu et al., 2013b), were exposed to flooding stress for 3 h. To identify specific proteins that were changed in the mutant and ABA-treated plants in response to flooding stress, proteomic technique was used. Proteins extracted from the roots were analyzed using nanoLC-MS/MS (Figure 24) and protein abundance was compared with the flooding-stressed wild-type soybean. A total of 1045 (Figure 25) and 384 proteins (Figure 25) were significantly changed in the roots of mutant line and ABA-treated soybean, respectively. Among these identified proteins, 146 proteins were commonly changed between the mutant line and ABA-treated soybean (Figure 25).

To better understand the function of the identified proteins in soybean at the initial flooding stress, functional categorization was performed using MapMan bin codes (Figure 25). The significantly changed proteins in the mutant line were categorized as protein synthesis, signaling, cell, amino acid metabolism, transport, and stress-related proteins; whereas, the identified proteins in ABA-treated soybean were predominantly related to protein synthesis, RNA regulation, redox, development, and stress. The commonly changed flooding-responsive proteins between the mutant line and ABA-treated soybean were categorized as protein synthesis and RNA regulation proteins, and exhibited similar change tendency (Figure 25). Among the protein synthesis-related proteins, ribosomal proteins were identified and decreased in response to flooding stress, with the exception of nascent polypeptide associated complex (NAC)

and chaperonin 20 (Figure 27). These comparative proteomic results indicated that protein synthesis- and RNA regulation-related proteins were involved at the initial flooding stress in the mutant line and ABA-treated soybean.

3.3.2. Primary metabolic pathways in soybean at the initial flooding stress

To determine the soybean metabolic pathways involved in the initial flooding tolerance, the functional visualization of significantly changed proteins was performed using MapMan software (Figure 26). Mapping of the identified flooding-responsive proteins from the mutant line and ABA-treated soybean on the known metabolic pathways indicated that cell wall, fermentation, minor CHO metabolism, C1 metabolism, and nucleotide metabolism were clearly activated in the roots of both plants. The secondary and amino acid metabolic pathways were specifically activated in the roots of the flooding-tolerant mutant line (Figure 26).

3.3.3. Organ-specific expression of genes related to protein synthesis and RNA regulation

A number of protein synthesis- and RNA regulation-related proteins were significantly and commonly changed in abundance in the roots of the flooding-tolerant mutant line and ABA-treated soybean (Figure 25). To confirm the functions of these proteins at the initial flooding stress, the specific gene expression of these proteins in the roots and cotyledons was analyzed by qRT-PCR. Among the protein synthesis-related functional categories, most of the proteins were decreased in abundance with the exception of NAC and chaperonin 20. Consistent with this increase at the protein abundance, the mRNA expression level of *NAC* and *chaperonin 20* was up-regulated in the roots of both the mutant line and ABA-treated soybean compared to that in control, at the initial flooding stress. In the cotyledons, the mRNA expression level of *chaperonin 20* was up-regulated in the mutant line in response to flooding stress (Figure 27).

Among 7 RNA regulation-related proteins, the mRNA expression level of *THO complex subunit 4*, *glycine-rich RNA-binding protein 3*, and *eukaryotic aspartyl protease family protein* was up-regulated in the root of both the mutant line and ABA-

treated soybean at the initial flooding stress; whereas, *KH domain-containing protein* and *glycine-rich protein 2B* were up-regulated only in the root of the mutant line. The mRNA expression level of *RNA-binding* (*RRM/RBD/RNP motifs*) and *cold circadian rhythm/RNA binding 2* did not change. In addition, the mRNA expression profiles of these genes were in accordance with the patterns of protein abundance level except for THO complex subunit 4 (Figure 28). In the cotyledons, the mRNA expression level of *KH domain containing protein*, *glycine-rich RNA binding protein 3*, and *cold circadian - rhythm RNA binding 2* was up-regulated in the mutant line; whereas, *eukaryotic aspartyl protease family protein* was up-regulated in ABA-treated soybean compared to the wild type soybean under flooding stress. The expression of these genes was lower in the cotyledons compared to those in the roots (Figure 28). These results indicated that genes encoding protein synthesis- and RNA regulation-related proteins were specifically expressed in the roots of soybean at the initial flooding stress.

3.3.4. RNA-sequencing and global detection of differentially expressed genes

To identify the key genes involved in flooding tolerance, Illumina RNA-sequencing based transproteomics was performed in mutant line and ABA-treated soybean. The qualities of data generated from constructed libraries were analyzed in Table 7. In total, a sequencing depth greater than 47 million raw reads was obtained from each library. After filtration, more than 45 million clean reads were obtained from each sequenced libaray. The numbers of clean reads in control, mutant line, and ABA treated soybean were as follows: 45633515, 46683118, and 46648746 (Table 7). The clean reads were mapped to the soybean genome in NCBI. As results, more than 71% of the clean reads were mapped uniquely to the genome and 3% of them were mapped multiply to the genome (Table 8).

In mutant line, 729 differentially expressed genes including 332 up-regulated and 397 down-regulated genes were identified at initial flooding stress compared to wild type soybean. In ABA-treated soybean, 255 differentially expressed genes including 127 up-regulated and 128 down-regulated genes were identified at initial flooding stress compared to control. Additionally, 31 genes were commonly changed between mutant line and ABA-treated soybean (Figure 29).

3.3.5. Functional classification of differentially expressed genes

The functions of differentially expressed genes were clarified using MapMan bin codes. Functional categories including protein metabolism, RNA regulation, transport, signaling, stress, cell wall, amino acid metabolism, and hormone metabolism were recognized. The differentially expressed genes were mainly involved in categories of protein and RNA regulation (Figure 29). The commonly changed genes were mainly involved in RNA regulation, protein metabolism, and signaling (Figure 29).

3.3.6. mRNA expression of differentially expressed genes

Out of 31 commonly changed genes, change tendency of 12 genes was same between mutant line and ABA-treated soybean at initial flooding stress.To confirm the differentially expressed genes on mRNA level, the commonly changed 12 genes with same tendency between mutant line and ABA-treated soybean were selected for qRT-PCR analysis (Figure 30). *Nodulin-26*, *protein nuclear fusion defective 4-like*, and *zinc finger protein constans-like* were up-regulated; however, *zinc finger CCCH domain-containing protein 53*, *ATPase family AAA domain-containing protein 1*, *cytochrome P450 77A1-like*, *receptor-like protein kinase HSL1*, *matrix metalloproteinase*, *glucose-6-phosphate isomerase 1*, and *BTB/POZ domain-containing protein* were down-regulated in mutant line and ABA-treated soybean compared to wild type at initial flooding stress (Figure 30).

In order to find out flooding tolerance related genes, the mRNA expression of these 10 significantly changed genes was checked using qRT-PCR analysis. Compared to untreated wild type, *zinc finger CCCH domain-containing protein 53 like*, *BTB/POZ domain-containing protein*, and *receptor-like protein kinase HSL1* were down-regulated; however, *nodulin-26* and *protein nuclear fusion defective 4-like* were up-regulated in mutant line and ABA-treated soybean at initial flooding stress. Interestingly, *ATPase family AAA domain-containing protein 1*, *cytochrome P450 77A1-like*, *matrix metalloproteinase*, and *glucose-6-phosphate isomerase 1* were up-regulated by flooding stress in wild type soybean, but they were recovered in mutant line and ABA-treated soybean (Figure 31), indicating that these 4 genes contributed to flooding

tolerance in soybean.

3.4. Discussion

3.4.1. Protein synthesis-related proteins are involved in tolerance at initial flooding stress in soybean root

To uncover the tolerance mechanisms of soybean at the initial flooding stress, a flooding-tolerant mutant line and ABA-treated soybean were analyzed using a gel-free/label-free proteomic approach. The commonly changed proteins between the mutant line and ABA-treated soybean at the initial flooding stress were mainly related to protein synthesis and RNA regulation (Figure 25). In particular, NAC and chaperonin 20, which are related to protein synthesis, were significantly increased at both the protein and mRNA expression levels in response to flooding stress (Figure 27). NAC protects nascent polypeptides from proteolysis (Kogan and Gvozdev, 2014) and is also involved in the assembly and transport of newly synthesized proteins, thereby functioning as a modulator of protein synthesis and homeostasis in the cell (Preissler and Deuerling, 2012). Chaperones function in the correct refolding of proteins to produce functional structures (Ellis, 1990) and also play a crucial role in protecting plants against stress by reestablishing normal protein conformation and promoting cellular homeostasis (Wang et al., 2004). In the present study, many ribosomal proteins were decreased in abundance, indicating that flooding stress leads to the suppression of protein synthesis in soybean. This result indicates that NAC and chaperonin 20 might be involved in mediating soybean tolerance at the initial flooding stress by repairing and/or recovering newly synthesized proteins.

In tobacco, deletion of the NAC gene preferentially affected the development and physiology of chloroplasts and mitochondria by reducing the translocation efficiency of nascent proteins into organelles (Yang et al., 2007). Similarly, overexpression of a NAC gene led to improve tolerance to salinity and drought in transgenic *Arabidopsis* (Karan and Subudhi, 2012). In rice, deletion of a gene downstream of stress-responsive NAC1 led to reduce ROS-scavenging enzyme activity and decreased osmotic and oxidative stress tolerance (You et al., 2014). In plants, chaperonin 20 is involved in protein refolding under stress conditions and functions as an iron chaperone for superoxide

dismutase (Kuo et al., 2013). In the present study, redox-related proteins, including superoxide dismutase 1, were increased in mutant line and ABA-treated soybean roots at initial flooding stress. These results indicate that NAC and chaperonin 20 are involved in mediating and enhancing the initial flooding tolerance in soybean by promoting the assembly and transport of newly synthesized proteins and increasing the abundance of ROS-scavenging enzymes.

3.4.2. RNA regulation-related proteins are involved in tolerance at initial flooding stress in soybean root

Glycine-rich RNA-binding protein 3 and eukaryotic aspartyl protease, which are categorized as RNA regulatory proteins, were significantly increased at both the protein and mRNA expression levels at initial flooding stress. In contrast, THO complex subunit 4 was significantly decreased in protein abundance, but increased at the mRNA expression level at the first 3 h of flooding stress (Figure 28). The THO complex is required for mRNA export from the nucleus (Meier, 2012). In *Arabidopsis*, HPR1, a component of the nuclear THO complex, is involved in disease resistance and ethylene-mediated senescence (Pan et al., 2012). Xu et al. (2015) revealed that HPR1 affected the ethylene-signaling response through the transcriptional control of the reversion-to-ethylene sensitive gene in *Arabidopsis*. In soybean, ethylene sensitivity is enhanced at initial flooding stress through decreasing phosphorylation of eukaryotic translation initiation factor 4G (Chapter 1). Although the mRNA expression of THO complex subunit 4 was up-regulated in 2-day-old seedlings exposed to flooding stress in the present study, its protein abundance was decreased (Figure 28), because a number of protein synthesis-related proteins, such as ribosomal proteins, were decreased under flooding conditions. These results indicate that the ethylene-signaling cascade might be suppressed at initial flooding stress due to the decreased abundance of THO complex subunit 4 in flooding tolerant soybeans.

Aspartyl protease is one of the eukaryotic protease that catalyzes peptide substrates using aspartate residue (Parra-Ortega et al., 2009). In *Arabidopsis*, overexpression of a gene encoding an apoplastic aspartic protease led to dwarfing and resistance to *Pseudomonas syringae* (Xia et al., 2004). Furthermore, aspartic protease

gene overexpression conferred drought tolerance by increasing the activity of several antioxidases, including superoxide dismutase and catalase in *Arabidopsis* (Yao et al., 2012). In the present study, eukaryotic aspartyl protease was significantly increased in both the protein and mRNA levels at the initial flooding stress. In addition, the protein abundance of several antioxidases, such as peroxidase and superoxide dismutase, was also increased in the roots of the mutant line and ABA-treated soybean at the initial flooding stress. These results suggest that eukaryotic aspartyl protease might be involved in initial flooding tolerance in soybean by increasing abundances of antioxidases.

Glycine-rich proteins, which are characterized by the presence of semi-repetitive glycine-rich motifs, are modulated at the transcriptional level by both biotic and abiotic factors (Sachetto-Martins et al., 2000). Glycine-rich RNA-binding proteins are involved in the regulation of gene expression at the post-transcriptional level (Glisovic et al., 2008). In *Arabidopsis*, overexpression of glycine-rich RNA-binding protein retarded germination and seedling growth compared to wild-type plants in an ABA-dependent manner under salt or dehydration stress (Kim et al., 2007a; Long et al., 2013). In the present study, glycine-rich RNA-binding protein 3 was increased in both protein abundance and mRNA expression level in flooding tolerant mutant line and ABA-treated soybean (Figure 28); however, it was decreased in wild type soybean at the initial flooding stress (Chapter 2). It was reported that mitochondrial respiration and catalase/peroxidase activities were affected by expression of glycine-rich RNA-binding protein in *Arabidopsis* under cold stress (Kim et al., 2007b). Furthermore, ABA treatment enhanced the flooding tolerance of soybean by promoting energy conservation (Komatsu et al., 2013b). These results suggest that glycine-rich RNA-binding proteins might be involved in mediating initial flooding tolerance by affecting activities of ROS scavenging related enzymes and regulating energy conservation in an ABA-dependent manner.

3.4.3. Protein synthesis and RNA regulation related genes involved in initial flooding tolerance in soybean

To further explore the initial flooding tolerance-related genes in soybean, RNA-

sequencing based transcriptomic analysis was performed in flooding-tolerant mutant line and ABA-treated soybean. The commonly differentially expressed genes between the mutant line and ABA-treated soybean at the initial flooding stress were mainly related to protein synthesis and RNA regulation (Figure 29). In proteomic study, it has demonstrated that protein synthesis and RNA regulation related proteins had great contributions to initial flooding tolerance in soybean (Figures 25, 27-28). Previous microarray analysis of soybean indicated that RNA processing and initiation of protein synthesis related genes were up-regulated in tolerant plants (Nanjo et al., 2011a). In nuclear proteomic study, the pre-ribosomal biogenesis and mRNA transport related nuclear proteins were decreased in wild type soybean at initial flooding stress (Chapter 2). Proteomic analysis of endoplasmic reticulum proteins from root tip of soybean suggested that protein synthesis was affected by flooding stress (Komatsu et al., 2012b). Furthermore, drought stress also led to suppression of protein synthesis in root tip (Gil-Quintana, et al., 2013; Wang et al., 2016). These results suggest that protein synthesis and RNA regulation related genes might play great role in triggering and producing initial flooding tolerance in soybean.

3.4.4. Genes encoding cytochrome P450 77 A1, matrix metalloproteinase, glucose-6-phosphate isomerase, and ATPase family AAA domain-containing protein 1 contribute to initial flooding tolerance in soybean

Among the commonly changed genes between mutant line and ABA-treated soybean, 4 genes, which are *cytochrome P450*, *matrix metalloproteinase*, *glucose-6-phosphate isomerase,* and *ATPase family AAA domain-containing protein 1* were up-regulated by flooding stress in wild type soybean, but they were recovered in mutant line and ABA-treated soybean (Figure 31). Cytochrome P450s represent the largest family of enzymes, which are involved in plant metabolism including hormone biosynthesis and synthesis of primary/secondary metabolites (Nelson and Werck-Reichhart, 2011). Matrix metalloproteinase is involved in promoting degradation of plant extracellular matrix (Maidment et al., 1999). Glucose-6-phosphate isomerase is involved in the process of glycolysis through regulating glucose-6-phosphate and fructose-6-phosphate (Kim and Dang, 2005). ATPase family AAA domain-containing

protein identified in this study shared similar sequences with microtubule-severing protein and involved in cell expansion (Sedbrook and Kaloriti, 2008). Komatsu et al. (2009) reported that these 4 genes were up-regulated in wild type soybean by flooding stress compared to untreated soybean and this result was confirmed in current study (Figure 31). These results suggest that the 4 genes identified in this study contribute to obtain initial flooding tolerance in soybean might be through controlling biosynthesis of metabolites, formation of cell wall, process of glycolysis, and expansion of cell.

3.5. Conclusion

To identify the initial flooding-tolerance mechanisms in soybean, a flooding-tolerant mutant line (Komatsu et al., 2013d) and ABA-treated soybean, which exhibited a flooding-tolerant phenotype (Komatsu et al., 2013b), were analyzed. The main findings from the proteomic and transcriptomic analyses in the mutant line and ABA-treated soybean under flooding stress were as follows: (i) The significantly changed flooding tolerance related proteins were mainly categorized into protein synthesis and RNA regulation; (ii) NAC and chaperonin 20, which are involved in protecting newly synthesized proteins, were increased in protein abundance and mRNA expression at the initial flooding stress; (iii) glycine-rich RNA binding protein and eukaryotic aspartyl protease, which are involved in enhancing the activities of antioxidases, were increased in both the protein and mRNA levels at the initial flooding stress; (iv) transcriptomic analysis indicated that the significantly changed flooding tolerance related genes were also mainly involved in protein synthesis and RNA regulation; (v) matix metalloproteinase, which is involved in promoting degradation of plant extracellular matrix, was up-regulated in control; however, it was down-regulated in mutant line and ABA-treated soybean at initial flooding stress. These results suggest that protein synthesis- and RNA regulation-related proteins and genes play a key role in triggering tolerance to the initial flooding stress in soybean through protecting newly synthesized proteins and enhancing ability to remove ROS. Furthermore, *matrix metalloproteinase* might involve in inhibition of cell wall loosening and contribute to tolerance at initial flooding stress in soybean.

Table 7. Statistics of each sequenced sample

Sample name	Raw read (Average)	Clean reads (Average)	Clean bases (Average)	Genome map rate (Average)	Q30 (%) (Average)	Q20 (%) (Average)	GC (%) (Average)
Wild type	48203303	45633515	4.56G	74%	91.77	96.38	44.78
Mutant line	47148157	46683118	4.67G	76%	95.02	97.93	44.91
ABA treated	47147853	46648746	4.67G	76%	95.01	97.92	44.64

Q20, the percentage of bases with a Phred value > 20; Q30, the percentage of bases with a Phred value > 30.

Table 8. Summary of clean reads mapped to the reference genome

Sample name	Total read (Average)	Total mapped (Average)	Unique Match (Average)	Perfect Match (Average)	Mismatch (Average)	Multi-position Match (Average)	Total Unmapped Reads (Average)
Wild type	45633515	33803574(74.06%)	32477577(71.15%)	26258705.67(57.55%)	7544868.333(16.51%)	1325997(2.91%)	11829941(25.94%)
Mutant line	46683118	35281012(75.57%)	33924867.67(72.67%)	28710939(61.50%)	6570072.667(14.07%)	1356144(2.90%)	11402106.33(24.43%)
ABA treated	46648746	35256498(75.58%)	33678952(72.20%)	28692879(61.51%)	6563619(14.07%)	1577546(3.38%)	11392248(24.42%)

Clean reads were mapped to soybean genome in NCBI.

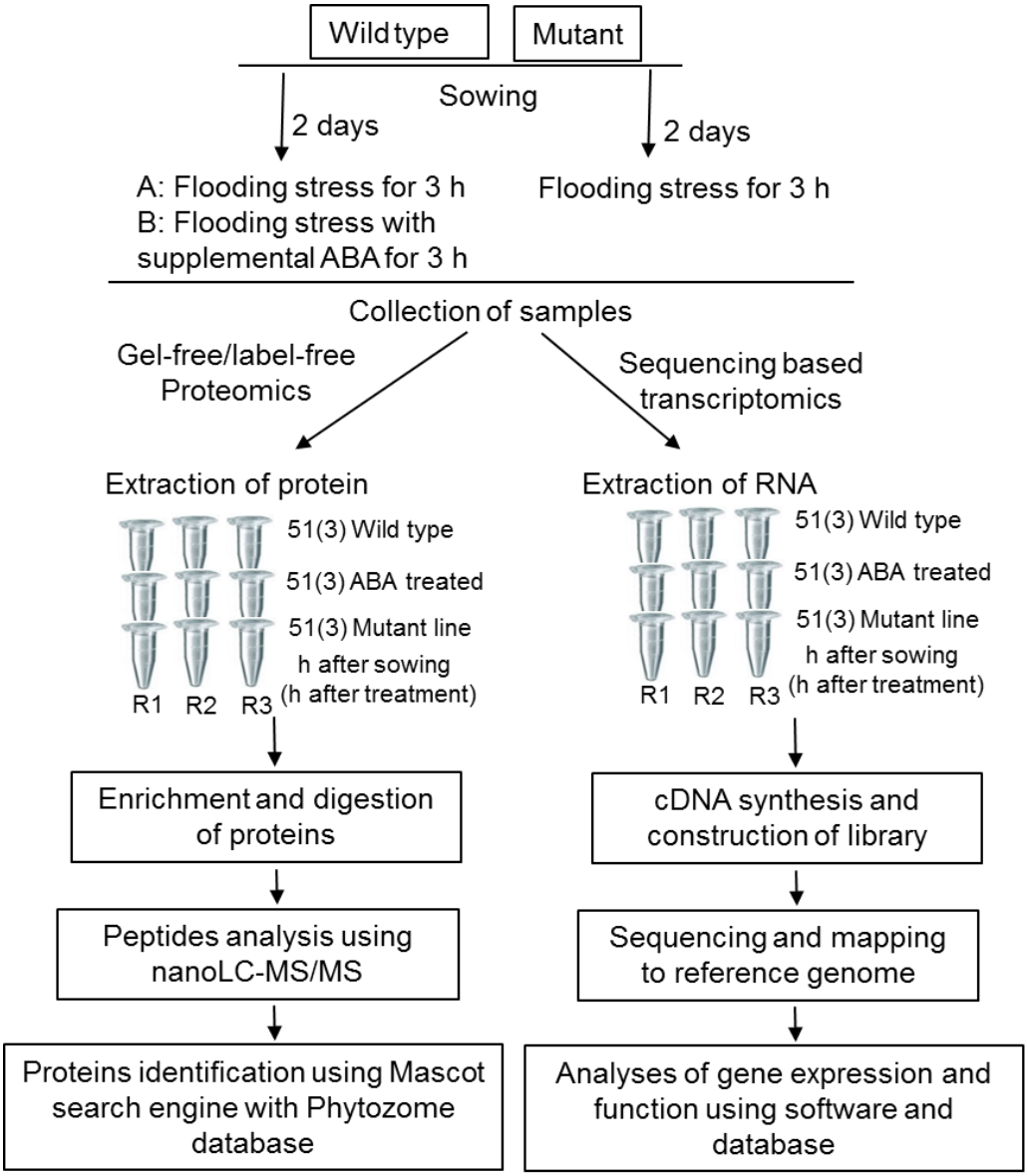


Figure 24. Experimental design for the proteomic and transcriptomic analyses of flooding-tolerant mutant line and ABA-treated soybean. Two-day-old soybeans were flooded for 3 h. For ABA treatment, 10 μM ABA was added to the water used for flooding. The mutant line was only exposed to flooding stress. Root was collected as samples for proteomic analysis. Root including hypocotyl was collected as sample for transcriptomic analysis. Proteins were extracted from the collected tissues, digested, and analyzed using nanoLC-MS/MS. RNA was isolated and used for cDNA synthesis. The transcripts were sequenced and idenfied using reference genome. Three independent experiments were performed as biological replicates for all experiments.

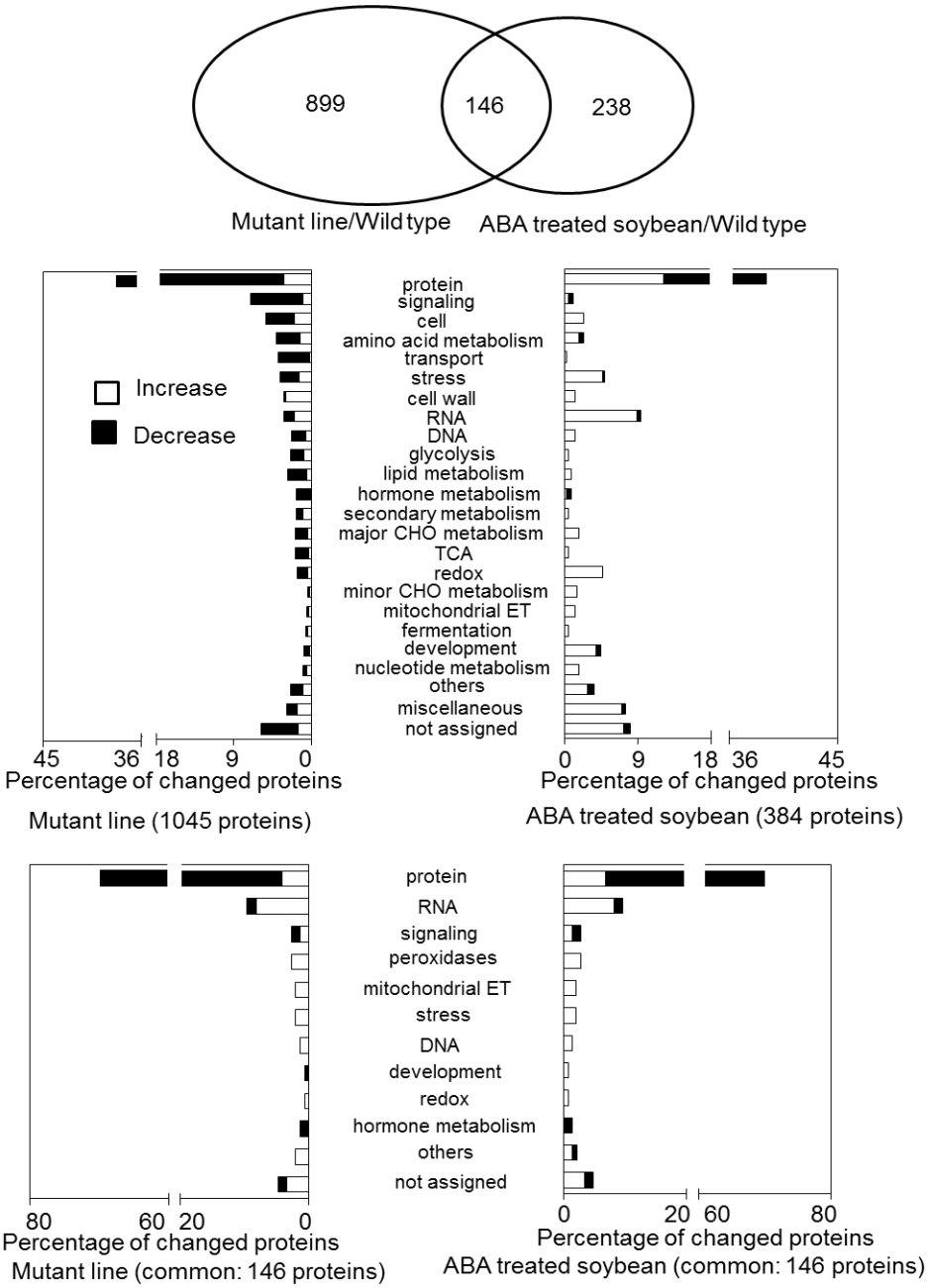


Figure 25. Functional distribution of proteins identified in the roots of the flooding-tolerant mutant line and ABA-treated soybean at the initial flooding stress. Two-day-old soybeans were flooded for 3 h and proteins were extracted from the roots and analyzed using nanoLC-MS/MS. For ABA treatment, 10 μM ABA was added to the water used for flooding. For mutant line, only flooding stress was applied. Functional classification of the differentially abundant proteins was performed according to MapMan bin codes. A total of 1045 and 384 proteins from the mutant line and ABA-treated soybean, respectively, were categorized based on functional distribution. A total of 146 common proteins were further categorized. Abbreviations: CHO, carbohydrates; ET, electron transport; TCA, tricarboxylic acid cycle; others, containing Biodegradation of Xenobiotics, C1-metabolism, metal handling, and polyamine metabolism.

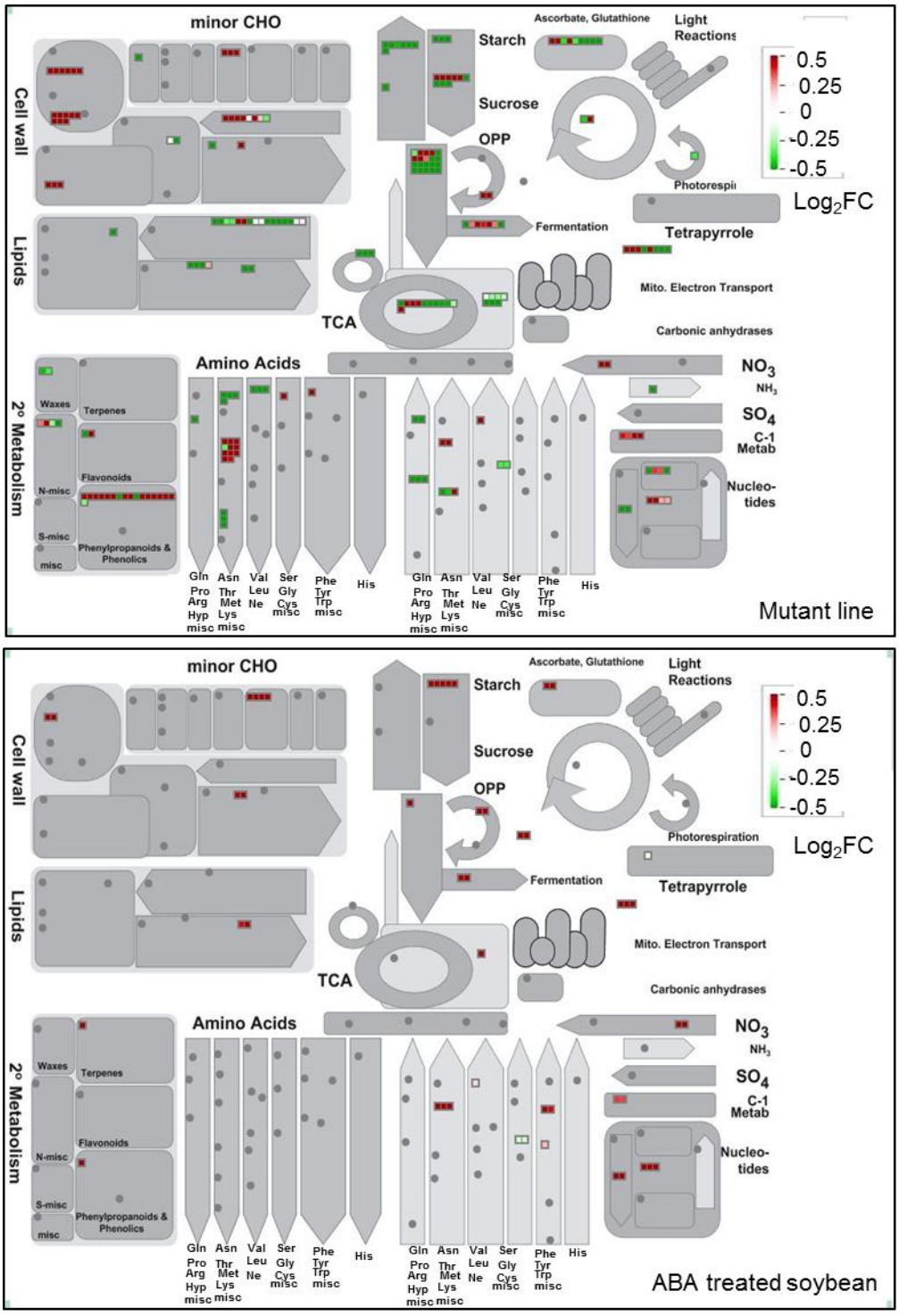


Figure 26. Mapping of proteins identified in the roots of the flooding-tolerant mutant line and ABA-treated soybean at the initial flooding stress. The 1045 and 384 flooding-responsive proteins identified in the mutant line and ABA-treated soybean, respectively, at the initial flooding stress were mapped on known metabolic pathways of soybean using MapMan software. MapMan visualization showing changes in protein abundance based on the Log_2FC of the differential protein ratio. Red and green colors indicate increased ($Log_2FC > 0$) and decreased ($Log_2FC < 0$) proteins, respectively.

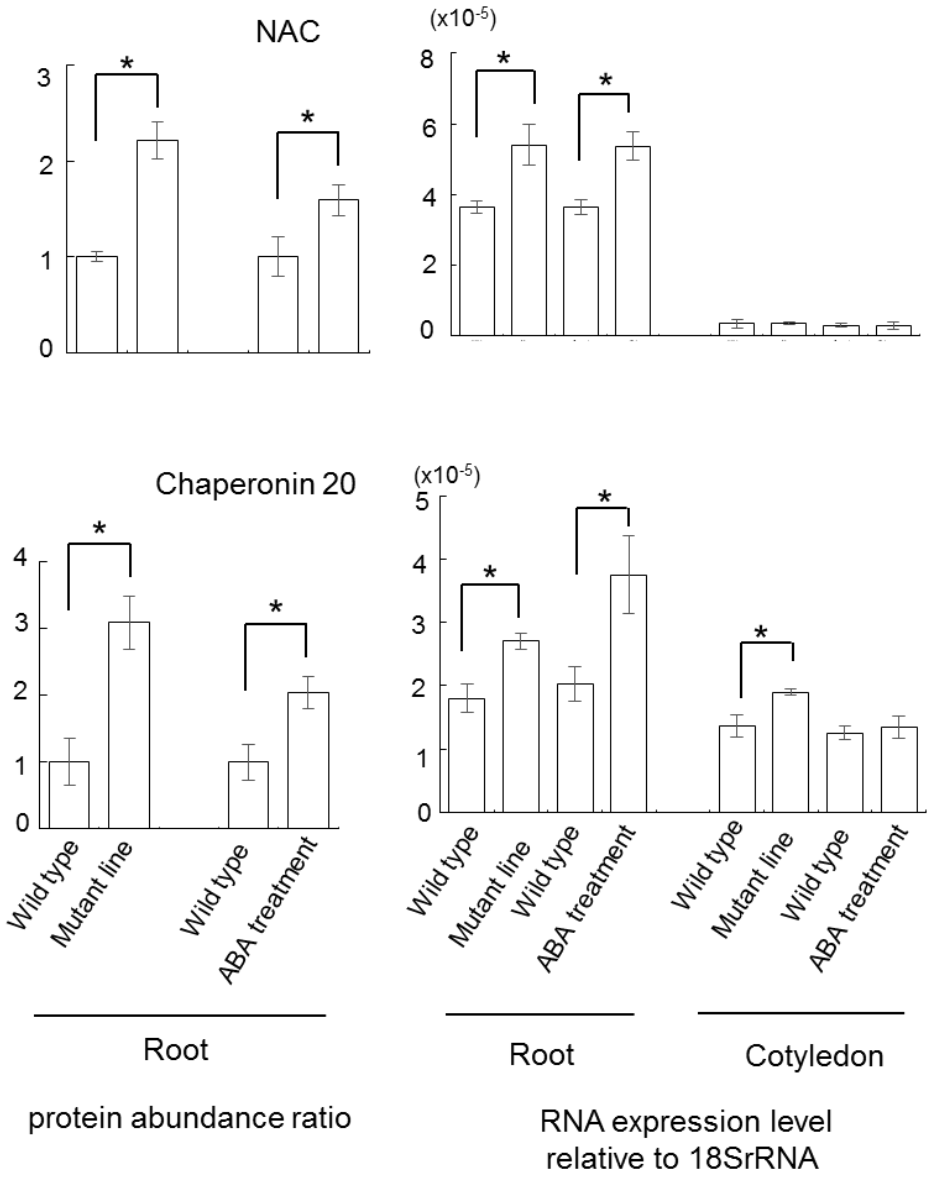


Figure 27. Organ-specific mRNA expression levels of genes related to protein synthesis in soybean under flooding stress. Two-day-old soybeans were flooded for 3 h and total RNA was extracted from the roots and cotyledons of wild-type, flooding-tolerant mutant line, and ABA-treated soybean. For ABA treatment, 10 μM ABA was added to the water used for flooding. The mRNA expression level of *NAC* (Glyma03g27580.1) and *chaperonin 20* (Glyma15g19970.1) was analyzed by qRT-PCR. Relative mRNA expression levels were normalized according to the abundance of 18S rRNA. Data are means ± SD from 3 independent biological replicates. The significance was analyzed using the Student's *t*-test (* $p < 0.05$) between wild type and mutant line, and/or wild type and ABA treated soybean.

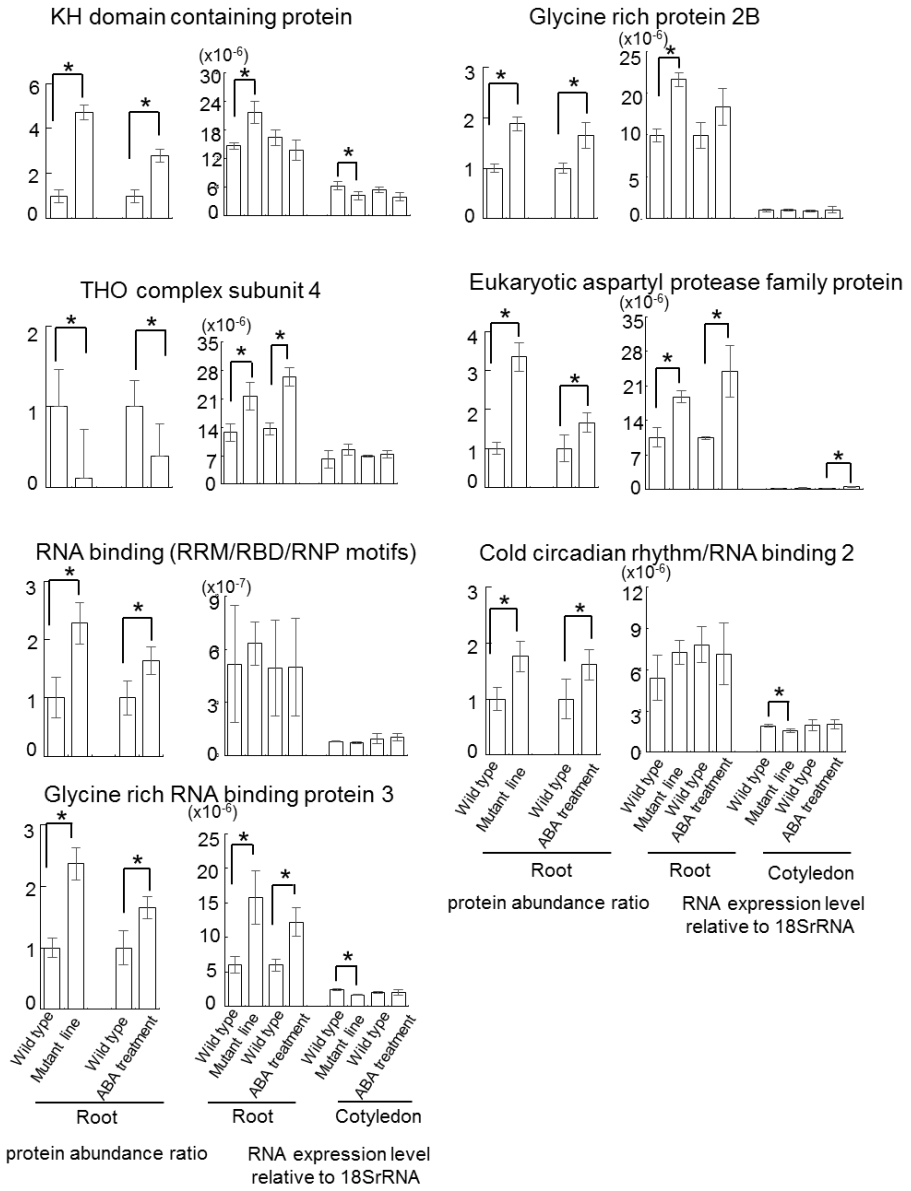


Figure 28. Organ-specific mRNA expression levels of genes related to RNA regulation in soybean under flooding stress. Two-day-old soybeans were flooded for 3 h and total RNA was extracted from the roots and cotyledons of wild-type, flooding-tolerant mutant line, and ABA-treated soybean. For ABA treatment, 10 μM ABA was added to the water used for flooding. For mutant line, only flooding stress was applied. The mRNA expression level of *KH domain-containing protein* (Glyma17g34850.1), *THO complex subunit 4* (Glyma18g04530.2), *RNA binding* (*RRM/RBD/RNP motifs*) (Glyma06g33940.2), *glycine-rich RNA-binding protein 3* (Glyma17g08630.1), *glycine-rich protein 2B* (Glyma12g03470.1), *eukaryotic aspartyl protease family protein* (Glyma11g25650.1), and *cold circadian rhythm/RNA binding protein* (Glyma04g01421.1) was analyzed by qRT-PCR. Relative mRNA expression levels were normalized according to the abundance of 18S rRNA. Data are means ± SD from 3 independent biological replicates. The significance was analyzed using the Student's *t*-test (* $p < 0.05$) between wild type and mutant line, and/or wild type and ABA treated soybean.

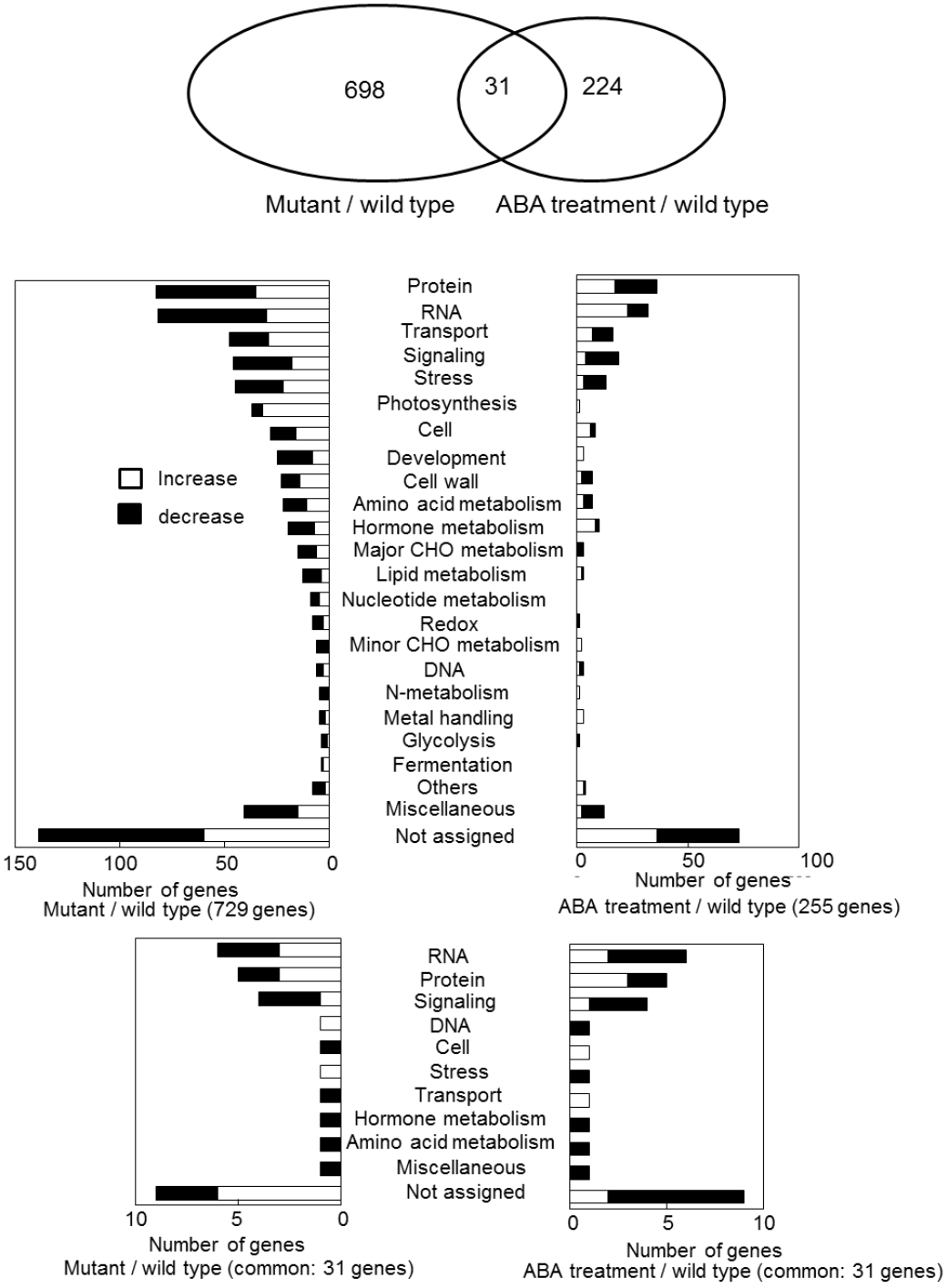


Figure 29. Functional categorization of differentially expressed genes in mutant line and ABA-treated soybean. Functional classification of the 729 and 255 differentially expressed genes in mutant line and ABA-treated soybean, respectively, were performed according to MapMan bin codes. Others contain C1-metabolism, TCA, gluconeogenesis, and polyamine metabolism.

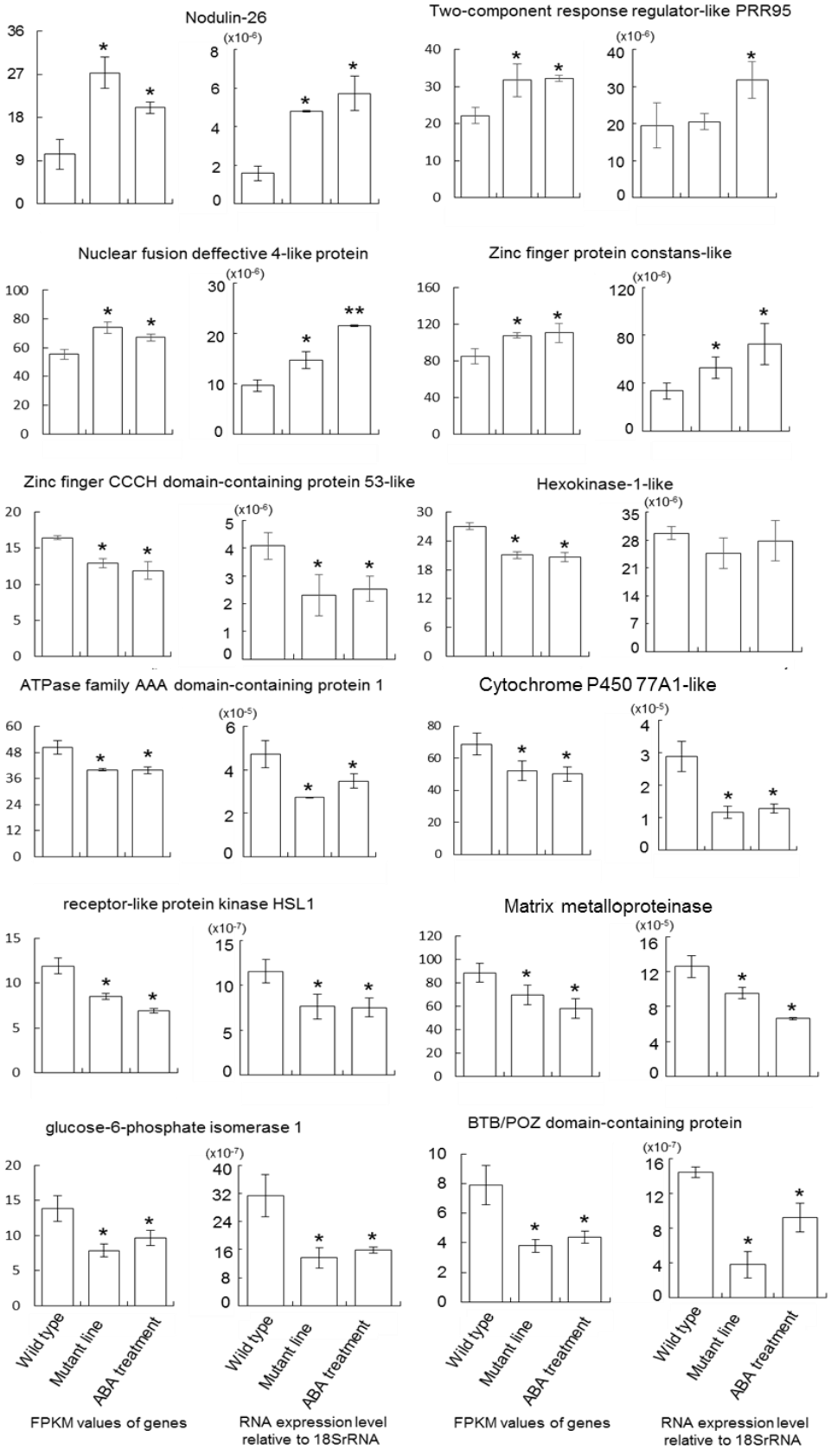

Nodulin-26
Two-component response regulator-like PRR95
Nuclear fusion deffective 4-like protein
Zinc finger protein constans-like
Zinc finger CCCH domain-containing protein 53-like
Hexokinase-1-like
ATPase family AAA domain-containing protein 1
Cytochrome P450 77A1-like
receptor-like protein kinase HSL1
Matrix metalloproteinase
glucose-6-phosphate isomerase 1
BTB/POZ domain-containing protein
Wild type
Mutant line
ABA treatment
FPKM values of genes
RNA expression level relative to 18SrRNA

Figure 30. The mRNA expression level of differentially expressed genes. Two-day-old soybeans were flooded for 3 h and total RNA was extracted from the roots including hypocotyl of the flooding-tolerant mutant line, wild-type, and ABA-treated soybean. For ABA treatment, 10 μM ABA was added to the water used for flooding. For mutant line, only flooding stress was applied. The mRNA expression of *nodulin-26* (Glyma13g40820), *two-component response regulator-like PRR95* (Glyma16g02050), *protein nuclear fusion defective 4* (Glyma16g17240), *zinc finger protein constans-like 1* (Glyma13g37970), *zinc finger CCCH domain-containing protein 53* (Glyma09g35980), *hexokinase-1-like* (Glyma01g01060), *ATPase family AAA domain-containing protein 1* (Glyma10g02410), *cytochrome P450 77A1* (Glyma17g01870), *receptor-like protein kinase HSL1* (Glyma13g30830), *matrix metalloproteinase* (Glyma02g03230), *glucose-6-phosphate isomerase 1* (Glyma04g09800), and *BTB/POZ domain-containing protein* (Glyma11g11100) was analyzed by qRT-PCR. Relative mRNA expression levels were normalized according to the abundance of 18S rRNA. Data are means ± SD from 3 independent biological replicates. The significance was analyzed using the Student's *t*-test (* $p < 0.05$) between wild type and mutant line, and/or wild type and ABA treated soybean.

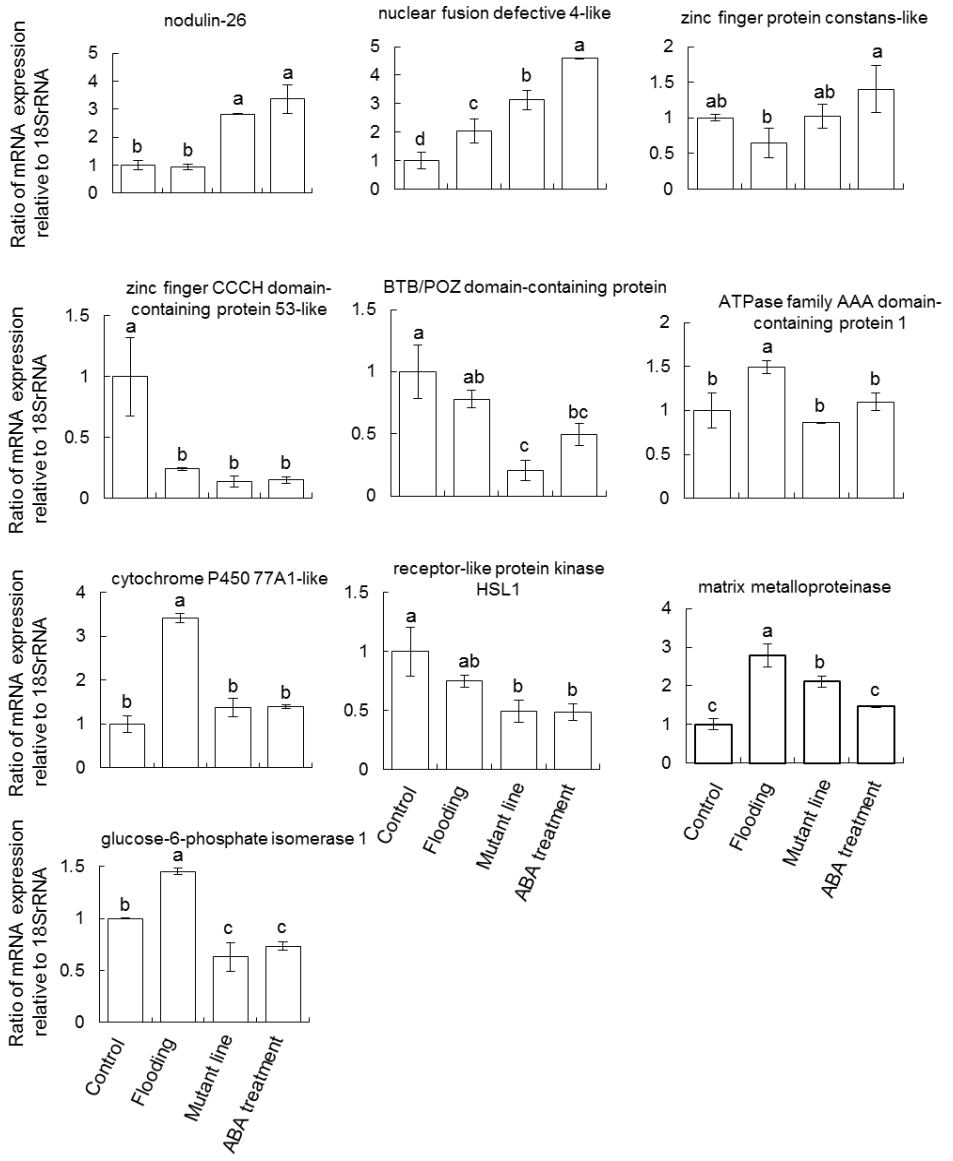


Figure 31. The mRNA expression level of flooding tolerance related genes in mutant line and ABA-treated soybean. Two-day-old soybeans were flooded for 3 h and untreated wild type was used as control. Total RNA was extracted from the root including hypocotyl of control, wild type, mutant line, and ABA-treated soybean. qRT-PCR was performed and relative mRNA expression levels were normalized according to the abundance of 18S rRNA. The analyzed genes were *nodulin-26* (Glyma13g40820), *protein nuclear fusion defective 4* (Glyma16g17240), *zinc finger protein constans-like 1* (Glyma13g37970), *zinc finger CCCH domain-containing protein 53 like* (Glyma09g35980), *BTB/POZ domain-containing protein* (Glyma11g11100), *ATPase family AAA domain-containing protein 1* (Glyma10g02410), *cytochrome P450 77A1-like* (Glyma17g01870), *receptor-like protein kinase HSL1* (Glyma13g30830), *matrix metalloproteinase* (Glyma02g03230), and *glucose-6-phosphate isomerase 1* (Glyma04g09800). Data are means ± SD from 3 independent biological replicates. The significance was analyzed using one-way ANOVA test with Tukey's multiple comparison ($p < 0.05$) and means with different letters indicate significantly changed.

CONCLUSION AND FUTURE PROSPECTS

Climate change around the worldwide affects the earth's ecosystem and causes high frequency of abiotic stresses including drought, flooding, salinity, cold, and high temperature. The abiotic stresses lead to great threat for agricultural crops. For example, a large amount of crop producing area was affected by the flooding or waterlogging (Ahmed et al., 2012) and with an annual damage exceeding 60 billion euro (Voesenek and Sasidharan, 2013). Flooding suppressed the plant growth through decreasing the availability of light, oxygen, and nutrients for the plant tissues. Soybean is one of the most important crop because its seed contains abundant protein and ediable oil (Gui et al., 2008). The trade of soybean products was increased in the worldwide during the past years. But, soybean is very sensitive to flooding stress and its growth and yield are markedly reduced by flooding stress (Githiri et al., 2006). Flooding severely limits the planting area of soybean (Rosenzweig et al., 2001). To solve this problem, many research work have been focused on exploring the mechanism of soybean responses to flooding stress. However, the mechanism of initial flooding signal perception and response in soybean has not yet uncovered compeletely.

Under flooding stress, root elongation of soybean is suppressed within 24 h (Hashiguchi et al., 2009). ATP content was decreased within 24 h (Nanjo et al., 2011b). In this study, the elongation of soybean root was suppressed and fresh/dry weight of soybean almost did not change after 3 h of flooding stress. Additionally, the ATP content of root tip significantly decreased under flooding stress, especially at the first 3 h of flooding stress compared with control plants (Chapter 1). These results indicate that flooding signal is triggered promptly and phenotype/biochemical changes happen within 3 h in soybean. Towards explore the mechanism of initial flooding response in soybean, protein from root tip was analyzed using gel-free/label-free proteomic techaniques. Previous 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) and indicated that energy metabolism, cell wall formation, active oxygen scavenging, and protein synthesis related proteins were affected by flooding stress.

In current study, proteomic analysis of cellular protein from root tip demonstrated that 2 alcohol dehydrogenases were increased within 24 h; xylosidase and heat shock protein 70 were decreased; however, calreticulin was increased on both protein abundance and mRNA expression levels and positioned in the center of in silico protein-protein interaction network (Chapter 1). Previous research indicated that flooding decrased the available oxygen for plant tissuees and anaerobic metabolism was enhanced to overcome the problem of energy shortage (Vartapetian and Jackson, 1997). In soybean, it was reported that alcohol fermentation related key gene including *alcohol dehydrogenases* were up-regulated under flooding stress for 12 h (Komatsu et al., 2009). Furthermore, the *alcohol dehydrogenase 2* overexpressed mutant line exhibited flooding tolerant phenotype (Tougou et al., 2012). These results suggest that energy metabolism is shifting from oxidative phosphorylation to anaerobic metabolism at initial flooding stress in soybean and alcohol dehydrogenases play key role in this process.

Heat shock protein, which is responsible for protein folding, assembly, translocation, and degradation, plays a key role in protecting plants against stress through re-establishing cellular homeostasis of normal proteins (Wang et al., 2004). Previous proteomic studies in soybean revealed that the abundance of heat shock protein 70 was decreased in cotyledon/root (Komatsu et al., 2013d) and increased in root tip (Nanjo et al., 2011b) under flooding stress. Additionally, the level of heat shock protein was related to calcium content in cotyledon of soybean (Komatsu et al., 2013d) and functioned through interacting with other proteins (Wang et al., 2004). In current results, heat shock protein 70 was decreased and interacted with other flooding responsive proteins (Chapter 1). These findings suggest that heat shock proteins involve in soybean responses to initial flooding stress might be through affecting the cellular homeostasis of stress responsive proteins.

Calreticulin, which has key role in ER as a molecular chaperone and Ca^{2+} signalling molecule (Johnson et al., 2001). It worked as molecular chaperones to newly synthesized unfolded proteins or glycoproteins and regulated the Ca^{2+} homeostasis in the ER lumen (Johnson et al., 2001). In *Arabidopsis*, calreticulin was involved in regulating plant defense against biotrophic pathogens and pathogen invasion (Qiu et al., 2012a,b). In rice, calreticulin played a role in cold stress response related pathway (Li

et al., 2003). In soybean, proteomic analysis revealed that calreticulin was decreased in root including hypocotyl under flooding stress (Komatsu et al., 2009). In the present study, calreticulin was increased and found to be part of an interacting multi-protein network (Chapter 1). These results suggest that calcium related signal transduction might be activated in soybean by initial flooding stress though the increase of calreticulin.

To explore the flooding signal transduction pathway, phosphoproteomic analysis of cellular proteins was performed. As results, the differentially changed phosphoproteins were mainly involved in protein synthesis and cell wall (Chapter 1). Previous proteomic studies in soybean indicated that protein synthesis in endoplasmic reticulum was servely affected by flooding stress (Komatsu et al., 2012b); however, they were not markedly different in flooding tolerant soybean under flooding stress (Komatsu et al., 2013c). In nucleus, many important compontents positioned in upstream of protein synthesis were suppressed and phosphorylated by initial flooding stress (Chapter 2). Theses results suggest that the process of protein synthesis might play great role in soybean responses to initial flooding stress and it is regulated by changing status of phosphorylation.

In soybean, proteins involved in producing precursors of cell wall synthesis were turned out to be decreased under flooding stress using cell wall proteomics (Komatsu et al., 2010). Cell wall loosening related proteins were highly increased in soybean under flooding stress (Nanjo et al., 2013). In this study, xylosidase, which plays an important role in xylem remodeling during the process of cell wall maturation (Sunna and Antranikian, 1997), was decreased in soybean at initial flooding stress (Chapter 1). Additionally, gene encoding matrix metalloproteinase, which is involved in promoting degradation of extracellular matrix proteins, was up-regulated in wild type soybean; however, it was down-regulated in flooding tolerant soybeans at initial flooding stress (Chapter 3). These results suggest that cell wall formation was affected by initial flooding stress and intergrity of cell wall is important for soybean to produce flooding tolerance.

Although many flooding responsive events including energy metabolism, cell wall formation/modification, and protein synthesis were identified in Chapter 1, the

upstream mechanism controlling these events under flooding stress is not clear. To uncover this question, nuclear proteomics was performed. It is known that nucleus contains nearly all of cellular genetic information, which is exisited in chromosomes providing sites for DNA replication and transcription (Maniotis et al., 1997). In the present study, mRNA export related proteins including MEGOH/THOC, pre-ribosomal biogenesis related proteins including NOP1/NOP56, and chromatin structure related proteins including histones H1, H2A, H2B, H3, and H4 were inhibited by initial flooding stress in soybean (Chapter 2), indicating that mRNA export from nucleus to cytoplasm, pre-ribosomal biogenesis, and chromatin structure were affected by initial flooding stress.

MEGOH and THOC belongs to components of exon-junction complex and this complex functioned in mRNA transport and nonsense-mediated decay/mRNA surveillance (Le Hir et al., 2001). In rice, the exon-junction complex related protins have essential role in plant growth, development, and reproduction (Gong and He, 2014). In *Arabidopsis*, six components of exon-junction complex were identified and their association was confirmed according to GFP-fusion protein localization (Pendle et al., 2005). NOP1 and NOP56 are two important compontents of snoRNPs (Woolford and Baserga, 2013). NOP1 involved in methylation of rRNA in the upstream of pre-60 ribosome biogenesis (Singh et al., 2008). NOP1 and NOP56 involved in the process of pre-ribosome biogenesis (Woolford and Baserga, 2013). In this study, these nuclear proteins were decreased by initial flooding stress (Chapter 2). Furthermore, pre-ribosome biosynthesis related nuclear phosphoproteins such as RNA processing protein Rrp5 was decreased and phosphorylated at initial flooding stress in soybean (Chapter 2). Previous proteomic analysis of endoplasmic reticulum indicated that protein synthesis was suppressed by flooding stress (Komatsu et al., 2012b). These results suggest that the decrease of mRNA export and pre-ribosome biogenesis related nuclear proteins play roles in inhibition of protein synthesis in soybean and they might be regulated through phosphorylation under flooding stress.

In addition to mRNA export and pre-ribosome biogenesis related nuclear proteins, chromatin structure related histone proteins, especially, histone H1 and H3 were decreased in soybean at initial flooding stress (Chapter 2). Izzo et al. (2008)

demonstrated that histone H1 played great role in regulating of chromatin structure and gene activity. In tomato, histone H1 was accumulated in a drought-tolerant genotype of tomato under water stress (Trivedi et al., 2012). In *Arabidopsis*, histone H1 was responded to abiotic stresses such as salt and drought (Han and Wagner, 2014). In addition, phosphorylation of histone H3 was induced by osmotic stress at threonine 3 in pericentromeric regions of *Arabidopsis* (Wang et al., 2015). These results suggest that histones H1 and H3 are responded to multiple abiotic stresses including flooding, and they might be involved in initial flooding stress in soybean through the regulation of gene expression and protein modification.

In this study, zinc finger/BTB domain-containing protein 47 and glycine-rich protein were phosphorylated under flooding stress and their corresponding genes were significantly down-regulated in soybean root tip treated with ABA (Chapter 2). Zinc finger/BTB domain-containing protein is characterized as a DNA binding zinc finger and a transcription-repressing BTB/POZ domain (Lee and Maeda, 2012). In *Arabidopsis*, seed germination and seedling growth were negatively affected by a zinc finger-containing protein with ABA dependent manner (Kim et al., 2007). In soybean, zinc finger proteins and related mRNAs were down-regulated by ABA supplementation under flooding conditions (Komatsu et al., 2013b). In mutant line and ABA-treated soybean, which exhibited flooding tolerant phenotype, gene encoding BTB domain protein was down-regulated by initial flooding stress (Chapter 3). These results suggest that nuclear-localized zinc finger/BTB domain-containing proteins are involved in flooding stress with ABA dependent manner and they might play great role in protecting soybean against flooding through regulation of phosphorylation.

In the present study, the fresh weight of soybean was decreased and increased with additional treatment with ABA (Chapter 2) and ethylene (Chapter 1), respectively, under flooding stress. Ethylene sensitivity was improved through decreasing eukaryotic translation initiation factor 4G and ATP content was decreased in soybean under flooding stress (Chapter 1). Previous proteomic analysis indicated that ABA enhanced the flooding tolerance of soybean by controlling energy conservation and slowing plant growth (Komatsu et al., 2013b). These results suggest that ethylene might weaken flooding tolerance of soybean through promoting seedling weight and energy consume;

however, ABA enhanced flooding tolerance of soybean through retarding plant growth and controlling energy conservation.

In current study, proteomic and transcriptomic analyses in flooding tolerant mutant line and ABA-treated soybean indicated that the significantly changed proteins and genes were mainly related to protein synthesis and RNA regulation (Chapter 3). Especially, NAC and chaperonin 20 were significantly increased in flooding tolerant soybeans in response to initial flooding stress. NAC constributes to assembly and transport of newly synthesized proteins, and protects nascent polypeptides from proteolysis (Kogan and Gvozdev, 2014). It plays great role for protein synthesis and homeostasis in the cell (Preissler and Deuerling, 2012). Chaperones involved in correct refolding of proteins and producing functional structures (Ellis, 1990). Wang et al. (2014) reported that chaperones functioned in protecting plants against stress through reestablishing normal protein conformation and promoting cellular homeostasis. The results from Chapter 1 and Chapter 2 indicated that protein synthesis in wild type soybean was suppressed by initial flooding stress. These results indicate that NAC and chaperonin 20 might be involved in mediating flooding tolerance in soybean at the initial flooding stress by repairing and/or recovering newly synthesized proteins.

The mechanisms of soybean response at early stage and recovery stage of flooding have been uncovered well (Komatsu et al., 2013a, 2015b; Khan et al., 2014, 2015). However, the mechanisms of initial flooding response and tolerance in soybean are few reported. In this study, the mechanisms of initial flooding response and tolerance in soybean were explored and they were summarized in Figures 32 and 33. This study provides the basic knowledge of the mechanism of soybean response to initial flooding stress on protein level. Through this study, many initial responsive proteins were identified and initial flooding tolerance relate proteins/genes were explored. The identified proteins such as NAC, chaperone 20, glycine-rich RNA binding protein, and eukaryotic aspartyl protease would be used as the indicators for initial flooding study in plants and benefit to rapidly selection of flooding tolerant crop. The current results of this study provide a basic step for helping us to understand the mechanisms of initial flooding resonse and tolerance in plants.

In soybean, genetic analysis was performed to reveal the mechanism of flooding

tolerance. Flooding tolerance related quantitative trait loci (QTL) including *Sft1*, *Sft2*, *Sft3* and *Sft4* was identified by Sayama et al. (2009) using recombinant inbred lines. van Toai et al. (2001) identified a single QTL, which was linked to the SSR marker Sat 064, was associated with improved soybean growth and yield under flooding stress in recombinant inbred lines. These QTL analyses provide useful information to find flooding tolerance related loci on chromosomes. In this study, the location of identified flooding response and tolerance related genes on chromosomes was analyzed in soybean genome database (Phytozome) (Figure 34). The location of these genes on chromosomes would be used as useful indicators for genetic analysis. Combined these results, it helps to uncover flooding response and tolerance mechanisms and contributes to select flooding tolerant soybean.

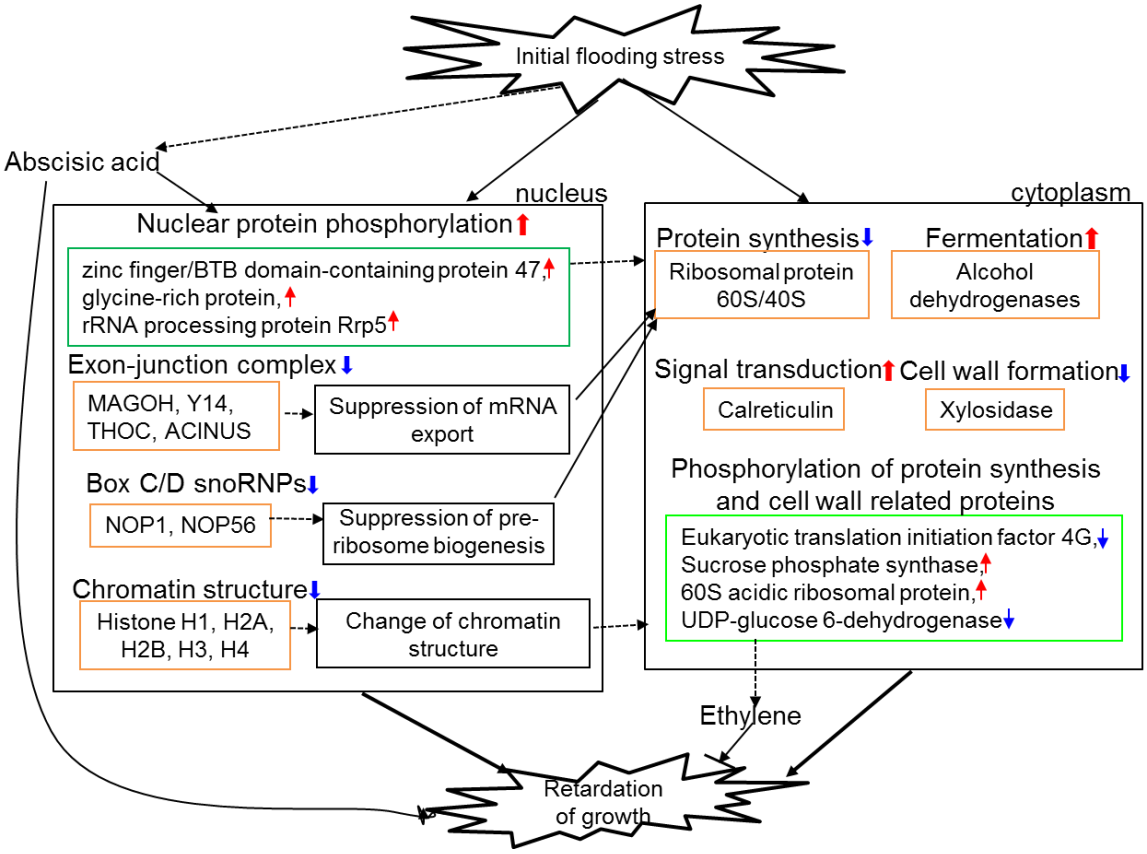


Figure 32. The model of cellular responses affected by initial flooding stress in soybean root tip. Flooding stress led to the decrease of exon-junction complex related proteins, Box C/D snoRNPs related proteins, and histone variants. Secondly, mRNA export and pre-ribosomal biogenesis were suppressed, chromatin structure was changed. These events happened in nucleus caused inhibition of protein synthesis in cytoplasm. In addition, ABA and ethylene involved in initial flooding response in soybean through regulation of protein phosphorylation. Through these processes, the growth of soybean was retarded by flooding stress. The solidline means clarified points and dotted line means speculative aspects. The upward red arrows mean increased and downward blue arrows mean decreased.

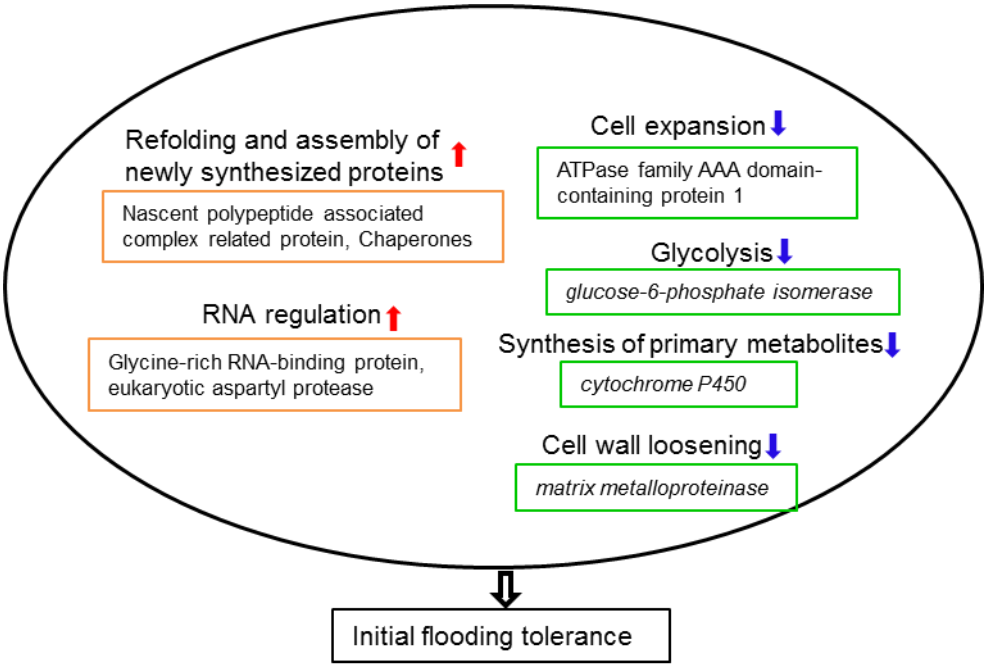
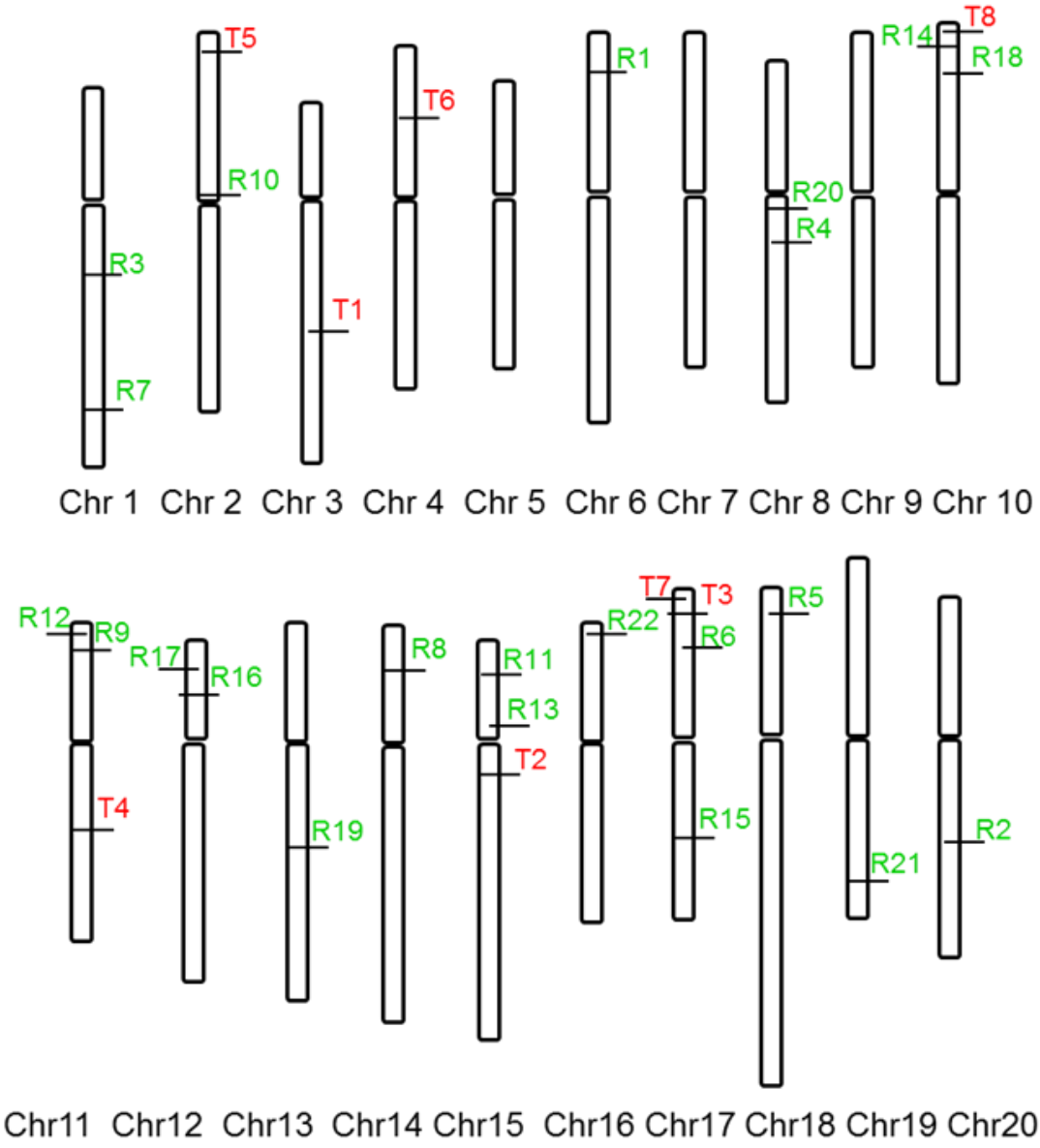


Figure 33. The mechanism of triggering and producing initial flooding tolerance in soybean. Correct refolding and assembly of newly synthesized proteins and transcriptional regulation of important genes play key role in producing initial flooding tolerance for soybean. The upward red arrows mean increased or up-regulated; downward blue arrows mean decreased or down-regulated.


T5
R10
R3
R7
T1
T6
R1
R14
T8
R18
R20
R4
Chr 1
Chr 2
Chr 3
Chr 4
Chr 5
Chr 6
Chr 7
Chr 8
Chr 9
Chr 10
R12
R9
R17
R16
T4
R19
R8
R11
R13
T2
T7
T3
R22
R6
R15
R5
R21
R2
Chr11
Chr12
Chr13
Chr14
Chr15
Chr16
Chr17
Chr18
Chr19
Chr20

R1: Alcohol dehydrogenase (Glyma06g12780) 9979155 - 9982400 (strand : +)

R2: Calreticulin 1b (Glyma20g23080) Chr20:33012502 - 33015990 (strand : +)

R3: Xylosidase 1 (Glyma01g20460) Chr01:24945870 - 24950893 (strand : -)

R4: Eukaryotic translation initiation factor 4G (Glyma08g45425) Chr08:45660611-45678386

R5: NOP1 (Glyma18g02340) Chr18:1473742 - 1476768 (strand : -)

R6: NOP56 (Glyma17g13770) Chr17:10553658 - 10557090 (strand : -)

R7: THOC (Glyma01g38120) Chr01:50277071 - 50282016 (strand : +)

R8: Zinc finger and BTB domain-containing protein 47 (Glyma14g08160) Chr14: 6174025 - 6174945 (strand : -)

R9: Glycine rich protein (Glyma11g04170) Chr11: 2770695 - 2775580 (strand : +)

R10: rRNA processing protein Rrp5 (Glyma02g26710.1) Chr2: 27723600 - 27763855 (strand : +)

R11: MAGOH (Glyma15g10420.1) Chr15: 7547614 - 7551072 (strand : -)

R12: Y14 (Glyma11g02100.1) Chr11: 1288717 - 1293475 (strand : -)

R13: ACINUS (Glyma15g17060.2) Chr15: 13354389 - 13358881 (strand : -)

R14: Histone H1 (Glyma10g03840.1) Chr10: 2882096 - 2883607 (strand : +)

R15: Histone H2A (Glyma17g31120.1) Chr17: 34272515 - 34274863 (strand : -)

R16: Histone H2B (Glyma12g08800.1) Chr12: 6524733 - 6525712 (strand : -)

R17: Histone H3 (Glyma12g05910.1) Chr12: 3993783 - 3994490 (strand : -)

R18: Histone H4 (Glyma10g07120.1) Chr10: 5837270 - 5837581 (strand : -)

R19: sucrose phosphate synthase (Glyma13g23060.2) Chr13: 26519997 - 26526041 (strand : -)

R20: UDP_glucose 6_dehydrogenase (Glyma08g26520.1) Chr8: 20855111 - 20857946 (strand : -)

R21: 60S acidic ribosomal protein (Glyma19g40080.1) Chr19: 46544712 - 46546719 (strand : +)

R22: 40s ribosomal protein (Glyma16g01460.1) Chr16: 1035408 - 1037504 (strand : +)

T1: NAC (Glyma03g27580) Chr03: 35308136 - 35310427 (strand : -)

T2: Chaperonin 20 (Glyma15g19970) Chr15:17483353 - 17486847 (strand : +)

T3: Glycine rich RNA binding protein 3 (Glyma17g08630) Chr17: 6382483 - 6385680 (strand : -)

T4: Eukaryotic aspartyl protease family protein (Glyma11g25650) Chr11: 24346293 - 24349967 (strand : -)

T5: Matrix metalloproteinase (Glyma02g03230) Chr02: 2494913 - 2496440 (strand : +)

T6: Glucose-6-phosphate isomerase 1 (Glyma04g09800) Chr04: 8071329 - 8076871 (strand : -)

T7: Cytochrome P450 77A1-like (Glyma17g01870) Chr17: 1157898 - 1159736 (strand : -)

T8: ATPase family AAA domain-containing protein 1 (Glyma10g02410) Chr10: 1677321 - 1689455 (strand : -)

Figure 34. The location of identified flooding response and tolerance related genes on chromosomes. The location of identified flooding response and tolerance related genes was analyzed in soybean genome database (Phytozome) based on their glyma numbers. Green color (R) means flooding response related factors and red color (T) means flooding tolerance related factors.

Supplemental Table 1. The primer sets of genes selected for qRT-PCR

Chapters	Protein ID[a]	Description	Sequence of primers
Chapter 1	Glyma04g03490.1	Sugar isomerase (SIS) family protein	F: 5'- TCATACATCAGGGGCGTGTA-3'
			R: 5'- CGCTGAATGTCTTGTGAGGA-3'
	Glyma05g10840.3	Cobalamin_independent synthase family protein	F: 5'- CTTCCCACAAGGCAGTGAAT-3'
			R: 5'- ACGAAGCACCAGCTTCCTTA-3'
	Glyma04g11550.1	Acetyl_CoA carboxylase 1	F: 5'- TGCAAACTGGAGAGGCTTTT-3'
			R: 5'- CTGTCAACAACAACCCATGC-3'
	Glyma19g37520.1	Enolase	F: 5'- AACAATGATGGCTCCCAGAG-3'
			R: 5'- ACCCTCTTGGGGTTGGTAAC-3'
	Glyma18g02960.1	Clathrin_ heavy chain	F: 5'- AGAAACGTGATCCCACCTTG-3'
			R: 5'- TTGTTCAGGGCTCTTGCTTT-3'
	Glyma01g20460.1	Alpha_xylosidase 1	F: 5'- GCTAGAGCGACACCCTTCAC-3'
			R: 5'- CTTGCCCTCTTGGACTTCTG-3'
	Glyma08g04890.1	Phosphoglucomutase/phosphomannomutase family protein	F: 5'- TGAGGATGGATCACGATTGA-3'
			R: 5'- TTCAACGCAACTTCCACAAG-3'
	Glyma09g04000.2	ATP-citrate lyase B_1	F: 5'- ACAAGCAACTTCCCATCCAG-3'
			R: 5'- AGCTGCTTCAACGGTAGCAT-3'
	Glyma05g36600.1	Heat shock protein 70 (Hsp 70) family protein	F: 5'- AGCTCGGTTTGAGGAGTTGA-3'
			R: 5'- TTGTTTGGCTCCTTTCCATC-3'
	Glyma20g23080.1	Calreticulin 1b	F: 5'- GCCAAGAAGCCTGAAGATTG-3'
			R: 5'- TTTCACCTGCCACAATTCAA-3'
Chapter 2	Glyma11g04170.1	Glycine rich protein	F: 5'-TTGCTTTTCAGGAGCCTTGT-3'
			R: 5'-ATCACCAAATCCCTGTGAGC-3'
	Glyma08g18460.1	Ribosomal protein L1p/L10e	F: 5'-TCCTCAAGCTCTCCAAGCTC-3'
			R: 5'-CCTTCCAGTTCCCCTTCTTC-3'
	Glyma02g26710.1	RNA binding rRNA processing protein Rrp5	F: 5'-ACAAAGGGCAGACATTCCAC-3'
			R: 5'-TCAGCAGCCCTTATTTGCTT-3'
	Glyma14g08160.1	Zinc finger and BTB domain-containing protein 47	F: 5'-AGAGCAAGGAGGAGCACAAG-3'
			R: 5'-TGCTCTTTCTGGGTTTGCTT-3'
	Glyma07g37090.2	Calmodulin binding transcription activator	F: 5'-CTGAGAACTAAGGCGGTTGC-3'
			R: 5'-GCTTCCGAACTTGAAAGCAC-3'
	Glyma14g02750.1	DEA(D/H) box RNA helicase family protein	F: 5'-GAAGGAGTTTCGCAAGCAAC-3'
			R: 5'-CGTCCTTGGTTTTCTTCGAG-3'
	Glyma05g27040.1	Ribosomal protein S24/S35	F: 5'-CAAGGAGTGCGAGGAGATTC-3'
			R: 5'-TGTCCAGGTATTCTGCCACA-3'
	Glyma08g45425.1	Eukaryotic translation initiation factor 4G	F: 5'-AATGCCAACACCTCCAGTTC-3'
			R: 5'-CATTGGCATTGACATTCCAG-3'
	Glyma17g09590.1	U3 small nucleolar RNA-associated protein MPP10	F: 5'-CCACTGGACCAACTCCTTGT-3'
			R: 5'-TCCTCATCCAATTCCTCGTC-3'

	Glyma17g13770.1	Nucleolar protein 56	F: 5'- CAACGTCCATCAATTTGCAC-3'
			R: 5'- AGCATGGGAAATCAAACGAG-3'
	Glyma18g02340.1	NOP1	F: 5'- ATAGGATGCTGGTCGGAATG-3'
			R: 5'- ATGGTTCAAGGGTGACTTGC-3'
	Glyma01g38120.1	THO complex subunit 4	F: 5'- ATCGTGGTAGGCAAAACCAG -3'
			R: 5'- TCTCTCTGAGCGCCGATATT -3'
	Glyma15g10420.1	Protein mago nashi	F: 5'- CGCGAACAACTCCAACTACA -3'
			R: 5'- CTCGTTCCCCATCACAATCT -3'
	Glyma10g03840.1	Histone H1	F: 5'- TCCCACTCATCCTTCCTACG -3'
			R: 5'- TGGTAGGTGGGAGCTTGAAC -3'
	Glyma17g31120.1	Histone H2A	F: 5'- CAAGAGCCGGTCTTCAGTTC -3'
			R: 5'- GGATGGCGAGCTGTAGATGT -3'
	Glyma12g08800.1	Histone H2B	F: 5'- TGGGAATTTCTTCCAAGTGC -3'
			R: 5'- GCATGCTTTCCAAGTTCTCC -3'
	Glyma12g05910.1	Histone H3	F: 5'- GCACCGAGCTTCTCATAAGG -3'
			R: 5'- ACGAGCAAGCTGAATGTCCT -3'
	Glyma10g07120.1	Histone H4	F: 5'- GGATCACAAAACCTGCGATT -3'
			R: 5'- GGTTTTGCCCTGTCTCTTGA -3'
Chapter 3	Glyma17g34850.1	KH domain_containing protein	F: 5'- CTGGTTATGGTGGTGCAGTG-3'
			R: 5'- CCATCATAGGCTCCACGAGT-3'
	Glyma18g04530.2	THO complex subunit 4-like isoform X1	F: 5'- GGCCGTCATCGTATGCTATT -3'
			R: 5'- GCAAAACGCTTCAACTCTCC -3'
	Glyma06g33940.2	RNA_binding (RRM/RBD/RNP motifs)	F: 5'- GCCGCATTGATAGATCCTGT -3'
			R: 5'- ATTAGGAGGCATCCCCATTC -3'
	Glyma17g08630.1	Glycine_rich RNA_binding protein 3	F: 5'- TTGGTGATGCCGGTAGTGTA -3'
			R: 5'- AACCCTCATCTCCATTGCTG -3'
	Glyma12g03470.1	Glycine_rich protein 2B	F: 5'- ACTGTGGTGAATCGGGACAT -3'
			R: 5'- ACTTGATGGGCAATCTCTGG -3'
	Glyma11g25650.1	Eukaryotic aspartyl protease family protein	F: 5'- CATGTGTTCAGCCCATGTTC -3'
			R: 5'- CCAAGAGCAAGGTTTGTGGT -3'
	Glyma04g01421.1	Cold circadian rhythm and RNA binding 2	F: 5'- ACGCAATCGAAGGAATGAAC -3'
			R: 5'- GGTTATAGCCACCTCCACCA -3'
	Glyma03g27580.1	Nascent polypeptide_associated complex (NAC)	F: 5'- ACTGCTCAGACCCAAGAGGA -3'
			R: 5'- CCTTGCGACTCTTCTTTTCG -3'
	Glyma15g19970.1	Chaperonin 20	F: 5'- GTTGTTGCCCCCAAATACAC -3'
			R: 5'- CTGTCTTTCCTTCCCCAACA -3'
	Glyma13g40820	Nodulin-26	F: 5'- AACACCAGCAGGGTTAGTGG-3'
			R: 5'- GCCGAGTAACTGAGCAATCC-3'
	Glyma16g02050	Two-component response regulator-like PRR95	F: 5'- TATGCCATGATGCTGCAAAT -3'
			R: 5'- TTCCGCTTCAGTCGAAACTT -3'
	Glyma16g17240	Nuclear fusion deffective 4-like protein	F: 5'- GGAAATGGATGATCCTGGTG -3'
			R: 5'- ACAACAGAAACCGGAAGGTG -3'
	Glyma13g37970	Zinc finger protein constans-like 1	F: 5'- AACCACCGAACAAGGTCAAG -3'

Glyma09g35980	Zinc finger CCCH domain-containing protein 53-like	R: 5'- AATGTGGTCGAAACCTCGTC -3' F: 5'- GTTGCAGGTTCCTTCATGGT -3'
Glyma01g01060	Hexokinase-1-like	R: 5'- CATGAGCTGAGAAGCAGCAG -3' F: 5'- GATGCGGTTGGAGAAGATGT -3'
Glyma10g02410	ATPase family AAA domain-containing protein 1	R: 5'- AGAAGACCATGCCATTTTGG -3' F: 5'- TGGAAGCAATCAGACAGCAC -3'
Glyma17g01870	Cytochrome P450 77A1-like	R: 5'- GCCAAGAACTAAGCGAATGC -3' F: 5'- AAGGGTTCGTCCAAGTGATG -3'
Glyma13g30830	Receptor-like protein kinase HSL1	R: 5'- CGGAGTGAAAACCGGTAAAA -3' F: 5'- CGCTCAACCTCTCCTTCAAC -3'
Glyma02g03230	Matrix metalloproteinase	R: 5'- GAGAAGTCCAAGACGCGAAG -3' F: 5'- TCTGGGCCATTTAACGATTC -3'
Glyma04g09800	Glucose-6-phosphate isomerase 1	R: 5'- TTCGGGAAGAAAACCGTATG -3' F: 5'- GGAGATACGTGGGTTGGCTA -3'
Glyma11g11100	BTB/POZ domain-containing protein	R: 5'- AGGGTCCCTCAGCCAATAGT -3' F: 5'- TATCCCCTGACCACAACCTC -3'
18S rRNA	X02623.1[b]	R: 5'- TGCAACCCTTGGAGGTATTC -3' F: 5'-TGATTAACAGGGACAGTCGG-3' R: 5'-ACGGTATCTGATCGTCTTCG-3'

a) Protein ID according to the Phytozome database; b) is according to Genebank.

SUMMARY

Climate change causes abiotic stresses such as cold and flooding, which lead to great threat for crops. Soybean is very sensitive to flooding stress, which markedly reduces its growth and yield. In particular, morphological and biochemical changes such as decrease of ATP content happen in soybean under flooding stress for 3 h. To uncover the response mechanism of soybean at initial flooding, proteins were analyzed using gel-free/label-free proteomic technique.

To identify the initial flooding-responsive proteins, proteomic analysis of cellular proteins including phosphoproteins was performed. The cellular events such as calcium-related signal transduction, fermentation, protein refolding/assembly, and cell wall formation, were promptly affected by initial flooding stress in soybean. Especially, alcohol dehydrogenases were markedly increased at initial flooding stress, indicating that energy supply shifted from aerobic to anaerobic metabolism in soybean. Analysis of phosphoproteins indicated that protein synthesis/modification, cell wall formation, and energy metabolism were regulated through phosphorylation in response to flooding. Out of them, eukaryotic translation initiation factor 4G, which involved in ethylene signal pathway, was decreased and positioned in the center of *in silico* protein-protein interaction network in the soybean response to flooding stress. The fresh weight of soybean was increased after treatment with ethylene under flooding condition. These results suggest that energy metabolism, cell wall formation, and protein synthesis are affected at initial flooding stress in soybean. Furthermore, ethylene might be involved in initial flooding response in soybean through regulating protein phosphorylation.

To explore the upstream mechanisms controlling the flooding-responsive events identified in proteomic analysis of cellular protein, proteomic analysis of nuclear proteins including phosphoproteins was performed. Functional analysis of identified nuclear proteins indicated that mRNA export/pre-ribosome biogenesis and chromatin structure were affected by flooding stress through decreasing exon-junction complex/pre-ribosomal biogenesis and histone variants related proteins. These events happened in nucleus caused inhibition of protein synthesis in soybean at initial flooding stress. Nuclear phosphoproteomic analysis confirmed that abscisic acid (ABA) involved in these processes through regulation of nuclear-localized phosphoproteins, such as glycine-rich protein, zinc finger/BTB domain-containing protein 47, and rRNA processing protein Rrp5. These results suggest that inhibition of pre-ribosome biogenesis and mRNA transport in nucleus is reason for suppression of protein synthesis in cytoplasm. Furthermore, ABA might be involved in initial flooding response in

soybean by altering the phosphorylation of nuclear-localized proteins.

To explore the initial flooding-tolerance mechanism in soybean, flooding-tolerant mutant line and ABA-treated soybean, which exhibited flooding tolerant phenotype, were analyzed using gel-free/label-free proteomic and RNA-sequencing based transcriptomic techniques. A total of 146 proteins and 31 genes were commonly changed between mutant line and ABA-treated soybean at initial flooding stress, which were mainly related to protein synthesis and RNA regulation. The initial flooding tolerance in soybean was regulated by the identified 140 proteins and 12 genes, which had same change tendency between mutant line and ABA-treated soybean. Out of them, nascent polypeptide-associated complex/chaperonin 20 and glycine-rich RNA binding protein/eukaryotic aspartyl protease, which are involved in protecting newly synthesized proteins and enhancing the activities of antioxidases, respectively, were increased in protein abundance and mRNA expression at the initial flooding stress. Among the commonly changed genes, *matrix metalloproteinase*, which is involved in promoting degradation of extracellular matrix proteins, was up-regulated in control; however, it was down-regulated in mutant and ABA-treated soybeans under initial flooding stress. These results suggest that initial flooding tolerance in soybean is produced might be through protecting newly synthesized proteins and enhancing activities of antioxidases to remove active oxygen species. Furthermore, *matrix metalloproteinase* might involve in inhibition of cell wall loosening and contribute to tolerance at initial flooding stress in soybean.

Taken together, in soybean, initial flooding stress was perceived and transmitted into nucleus and led to the decrease of mRNA transport/Box C/D snoRNPs and histone variants related proteins. The decrease of theses nuclear proteins causes suppression of mRNA export/pre-ribosomal biogenesis and change of chromatin structure. These changes happened in nucleus continuously regulate cytoplasmic events including inhibition of protein synthesis, alteration of energy metabolism, and suppression of cell wall formation. Ethylene weakened flooding tolerance of soybean through promoting seedling weight and energy consume; however, ABA enhanced flooding tolerance of soybean through retarding plant growth and controlling energy conservation. Furthermore, correct assembly of newly synthesized proteins, ROS scavenging, and inhibition of cell wall loosening might play key role in soybean for producing initial flooding tolerance.

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