Functional Analysis of Flooding and Drought Responsive Mechanism in Soybean using Gel-Free Proteomic Technique

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Biotechnology

(Doctoral Program in Life Science and Bioengineering)

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Abbreviations

ABA	Abscisic acid
APX	Ascorbate peroxidase
CBB	Coomassie brilliant blue
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
GA	Gibberellic acid
KEGG	Kyoto encyclopedia of genes and genomes
LC	Liquid chromatography
MS	Mass spectrometry
PCR	Polymerase chain reaction
qRT	Quantitative reverse transcription
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TCA	Tricarboxylic acid

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INTRODUCTION

Climate changes, which are naturally occurred or resulted from human activities, are major concern in the globe (Hashiguchi et al., 2010). These climate changes affect the frequency and magnitude of hydrological fluctuations, and as a result, lead to devastating events such as flooding and drought (Fukao et al., 2011). It is expected that the frequency of heat stress, drought, and flooding events is increasing (Mittler and Blumwald, 2010). High and low extremes in precipitation have increasingly limited food and forest production worldwide (Easterling et al., 2007). Based on predicting climate models, global surface temperature will be raised during next decades which will bring a drastic change in rainfall pattern and threat to plant vegetation worldwide. Severe weather events including flooding involved in climate change have increased in frequency over the past six decades (Bailey-Serres et al., 2012). Since the 1970s, global aridity has substantially increased and arid land also has increased from 17% to about 27% in 50 years (Dai, 2011). The change in rainfall and temperature variability has influence on nutritional quality and yield of crops (Porter and Semonov, 2005). Therefore, climate change is a potential threat to plant.

The majority of arable lands in the world are prone to unfavorable environmental conditions (Rockstrom and Falkenmark, 2000). Abiotic stress is regarded as a key limiting factor that disturbs plant vegetation (Bray et al., 2000) and impairs growth and yield of crop around the world (Mittler and Blumwald, 2010). Plant has processes of response and adaptation to abiotic stress at molecular, cellular, physiological, and biochemical levels (Yamaguchi-Shinozaki and Shinozaki, 2006). When plant encounters unfavorable conditions, plant develops the precise defense mechanisms to perceive and respond to abiotic stress, thereby being able to adjust these conditions (Rizhsky et al., 2004). For example, plant modulates the abundance of candidate proteins and either

increases or synthesizes novel proteins which involved in defense system in response to stress (Komatsu and Hossain, 2013). However, it is difficult to fully underline response mechanisms on these abiotic stresses because tolerance and sensitivity are complex phenomena that are quantitatively inherited and occur during different stages of plant growth/development (Chinnusamy et al., 2004). Thus, the development of crop which tolerants to abiotic stress is essential.

Flooding is a widespread phenomenon in soil being composed of an impermeable clay base or cracking grey clays with slow drainage and in areas of an extreme rainfall pattern or inadequate land planning (Voesenek et al., 2006). Flooding reduces gas exchange between the atmosphere and the plant tissue because gas such as oxygen diffusion is 10,000 times slower in water than in air (Armstrong, 1979). Flooding leads to change in soil chemical characteristics including soil pH and redox potential (Dat et al., 2004). The elevated level of water in soil limits oxygen availability causing hypoxic conditions, and as a result, plant root directly undergoes anoxia condition (Sauter, 2013). The respiration of plant root, which leads to substantial reduction in energy status, is inhibited by oxygen deficiency (Ashraf, 2012). This flooding-induced oxygen deprivation is the primary signal triggering the response as well as the main limiting factor for normal plant development (Saglio et al., 1988). In addition, energy production *via* mitochondrial oxidative phosphorylation is limited and toxic end-products *via* anaerobic metabolism are accumulated, resulting in growth inhibition and death in most crop species under flooding (Bailey-Serres and Voesenek, 2008). Thus, plant growth is severely affected by flooding due to limited oxygen level.

In plant, one of the primary responses to flooding is the reduction of stomatal conductance (Gomes and Kozlowski, 1980; Folzer et al., 2006). Flooding leads to the decrease of hydraulic conductivity by impaired root permeability (Clarkson et al., 2000).

Prolonged exposure of plant to flooding could result in root injuries, which in turn restrict photosynthetic capacity by inducing certain alteration in biochemical reactions of photosynthesis (Ashraf, 2012). Flooding-induced hypoxic condition shifts from aerobic pathway to alternative pathway, which is called anaerobic pathway, for energy generation (Gibbs and Greenway, 2003). Another common response to flooding is the production of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Jackson and Colmer, 2005). Flooding severely inhibits seminal root growth, decreases nutrient uptake, reduces fresh weight of seedlings, and results in premature senescence of lower leaves in wheat (Trought and Drew, 1980). Flooding also causes hormonal imbalance leading to stomatal closure (Jackson et al., 1996), decreases assimilation in root (Neuman and Smit, 1991), and reduces photosynthesis due to chlorophyll degradation (Ahsan et al., 2007a), and thus decreases growth of plant. The endogenous levels of nutrient are declined in different parts of plant under flooding (Ashraf et al., 2011). A marked decline in the selectivity of K^+/Na^+ uptake in the root zone and delay on the K^+ transport to the shoots are caused by oxygen deficiency resulted from flooding (Armstrong and Drew, 2002).

Proteomic analysis was performed to elucidate the response to flooding in various plants such as tomato (Ahsan et al., 2007a; Ahsan et al., 2007b), wheat (Kong et al. 2010; Haque et al., 2011), red/white clover (Stoychev et al., 2013), maize (Chen et al., 2014), cucumber (He et al., 2012), cacao (Bertolde et al., 2014), and soybean (Khatoon et al., 2012a; Salavati et al., 2012; Nanjo et al., 2013; Komatsu et al., 2014a) (Table 1, Figure 1). In maize leaves, proteins involved in energy metabolism and photosynthesis, programmed cell death, phytohormones, and polyamines were identified under flooding condition, suggesting that these proteins shed light on flooding tolerance and indicate ability of maize to restrict essential metabolites by reducing energy consumption as a

means of adaptation to hypoxia (Chen et al., 2014). Glycolysis/alcoholic fermentation, photosynthesis, protein metabolism, and oxidative stress related proteins were identified in cacao under flooding, indicating that the ability to maintain glycolysis and induce fermentation plays a main role in anoxia tolerance in cacao and may also serve to distinguish tolerant and susceptible genotypes in relation to this stressor (Bertolde et al., 2014). These previous observations indicate that energy metabolism is affected by flooding and involved in flooding adaptaion in plant.

Drought caused by prolonged water deficit in the soil is one of the widespread abiotic stresses because it has significant effect on the metabolic and physiological functions of a growing plant (Mahajan and Tuteja, 2005). Drought induces oxidative stress which results from overall reduction of photosynthetic electron transport chain by decreased carbon dioxide availability (Osmond and Grace, 1995), and at the same time, the decline of photosystem II activities is accompanied (Loreto et al., 1995). Drought significantly reduces photosynthesis, which resulted from decreased leaf expansion, impaired photosynthetic machinery, premature leaf senescence, and associated reduction in food production (Wahid and Rasul, 2005). Increase in the transpiration rate in response to drought tends to increase the pH of leaf sap, promotes abscisic acid (ABA) accumulation, and leads to reduced stomatal conductance (Davies et al., 2002; Wilkinson and Davies, 2002). It was well reported that the effects of drought stress in terms of physiological changes include stomatal closure, decrease of photosynthetic activity (Qureshi et al., 2007), increase of oxidative stress, alteration of cell wall elasticity (Caruso et al., 2009), and generation of toxic metabolites causing plant death (Ahuja et al., 2010). Each plant has different response mechanisms against drought stress. These findings indicate that physiological function is significantly affected by drought in plant.

Drought stress significantly affects the acquisition of nutrients by the root and their transport to shoots (Farooq et al., 2009). Accumulation of starch and dry matter in roots under drought stress involves in drought tolerance in perennial cotton (de Souza and da Silv, 1987). It was reported that dry matter partitioning into root accelerated by drought stress in chickpea (Leport et al., 1999). Reduction in the shoot/root biomass, photosynthesis, and root respiration rate is caused by severe drought stress (Liu and Li, 2005). Root growth is generally less affected by drought stress than shoot growth (Franco et al., 2011). The decreased shoot:root ratio, which results from either increased root growth or a relatively larger decrease in shoot growth compared to root growth, is commonly observed under drought stress (Franco et al., 2011). Under drought condition, plant root typically forms a large proportion of fine roots, which are capable of penetrating smaller soil pores and presumably optimize the exploratory capabilities of the root system as a whole, thereby playing an important role in survival against drought (Komatsu and Hossain, 2013).

There were several reports on proteomic analysis to characterize the response mechanism to drought in rice (Salekdeh et al., 2002; Mirzaei et al., 2012), sugar beet (Hajheidari et al., 2005), wheat (Caruso et al., 2009; Budak et al., 2013), maize (Vincent et al., 2005; Benesova et al., 2012), canola (Mohammadi et al., 2012a), barley (Kausar et al., 2013), tea plant (Zhou et al., 2014a), wild watermelon (Yoshimura et al., 2008), and soybean (Alam et al., 2010a; Mohammadi et al., 2012b; Toorchi et al., 2009) (Table 2, Figure 2). It was proposed that the inhibition of photosynthesis was caused by hypersensitive early stomatal closure and less efficient synthesis of protective/detoxification related proteins which confer to drought tolerance in maize (Benesova et al., 2012). Budak et al. (2013) reported on proteome changes in leaves of wheat under drought and suggested that different response to drought may rely on the

differential change pattern of protein between wild and modern genotypes of wheat. In barley, chloroplastic metabolism and energy related proteins might have an important function on the adaptation process of barley under drought (Kausar et al., 2013). Recently, proteomic analysis was used to evaluate the effect of ABA in tea plant under drought stress, indicating that ABA plays a critical role in response to drought by improving protein transport, carbon metabolism, and expression of defense proteins (Zhou et al., 2014a). These findings indicate that the regulatory mechanism of drought response is composed of complex trait.

Soybean is one of the main crops in the world, especially known as economically important legume crop (VandenBosch and Stacey, 2003). Soybean is a main source of high-quality protein and oil for human consumption and animal feed because it contains 40% protein and 20% oil (Liu, 2008). In addition to a major source of protein and oil, soybean also provides many kinds of secondary metabolites such as isoflavone (Sakai and Kogiso, 2008), saponin, phytic acid, oligosaccharid, goitrogen (Liener, 1994), and phytoestrogen (Ososki and Kennelly, 2003). Soybean is widely used to produce traditional foods including soy sauce, soy paste, and soymilk and is mainly processed into soybean meal and seed oil (Medic et al., 2014). Soybean has been considered as an alternative fuel gain by its oil (Candeia et al., 2009) and as a substitute of plastics maiden by its protein-based bio-degradable products (Song et al., 2011). The production of soybean has gradually increasing worldwide (Figure 3) due to increased crop yield and demand to meet the food and fuel needs of the growing world population. Furthermore, soybean enhances soil fertility (Cass et al., 1994; Tago et al., 2011) *via* nitrogen fixation from atmosphere by symbiotic nitrogen-fixing bacteria (Nagatani et al., 1971). However, abiotic stress such as flooding, drought, salt, and heavy metals (Deshmukh et al., 2014) greatly affects the growth, productivity, and seed quality of

soybean (Nguyen et al., 2012) because soybean is known as sensitive crop to abiotic stress. The improvement of tolerance against abiotic stress for soybean is an important challenge.

Flooding caused a decrease in nutrient uptake (Sallam and Scott, 1987), nitrogen fixation (Sung, 1993), and growth of soybean (Githiri et al., 2006). Total number of root, the length of the main, lateral, and adventitious roots, and the fresh weight of the root were significantly suppressed in soybean seedling under flooding stress for 3 days (Shi et al., 2008). van Toai et al. (2012) examined the effect of flooding on seed composition of soybean and found that the linoleic/linolenic acids, daidzein, genistein, and glycitein contents were significantly decreased under flooding stress. Root discoloration of soybean was increased by flooding compared to non-flooded soybean (Kirkpatrick et al., 2006). Flooding stress substantially resulted in reduction of biomass, taproot length, and pod number, inhibition of carbon/nitrogen content in root/nodule, decrease of nodule dry weight and number, and reduction of grain yield in soybean (Miao et al., 2012). These previous results indicate that flooding is one of the major constraints to growth of soybean.

Drought stress has detrimental impact on the symbiotic nitrogen fixation in soybean (Albrecht et al., 1984; Nandwal et al., 1991). During seed formation, drought reduces seed-calcium concentration (Smiciklas et al., 1989). Drought induces the increased accumulation of ROS and subsequent lipid peroxidation, and proline content in soybean (Alam et al., 2010a). It was reported that drought significantly inhibited the growth of roots and shoots, decreased pod number, carbon and nitrogen content in all organ, nodule number, and reduced grain yield in soybean at different stage (Miao et al., 2012). In drought-stressed soybeans, a low availability of photosynthate in leaves, coupled with an impaired ability of sucrose utilization by pods, leads to a reduced

carbohydrate flux from leaves to pods and a decreased hexose to sucrose ratio in pods, thus might be contribute to pod abortion (Liu et al., 2004). These findings indicate that drought results in severe damage for soybean growth and seed quality.

A variety of flooding-induced proteins were identified using proteomic technique, providing insight into flooding-responsive mechanisms in soybean. Subcellular proteomics was performed in plasma membrane (Komatsu et al., 2009), cell wall (Komatsu et al., 2010), mitochondrion (Komatsu et al., 2011a), endoplasmic reticulum (Komatsu et al., 2012), and nucleus (Komatsu et al., 2014a; Oh et al., 2014) of flooded soybean to understand the function and cellular processes of organelle in response to flooding. The effect of ABA (Komatsu et al., 2013a) was analyzed using proteomic technique in soybean root under flooding stress, suggesting that ABA played a role in enhacing flooding tolerance in soybean by controlling energy conservation *via* glycolytic system. Ethylene-releasing agent increased the weight of soybean under flooding, suggesting that ethlynene signaling pathway played a critical role in tolerance mechanism *via* protein phospholylation in soybean root tips under flooding (Yin et al., 2014). However, the effect of gibberellic acid (GA) on soybean response to flooding remained to be elucidated. There were various calcium signaling-related proteins identified in soybean under flooding stress (Komatsu et al., 2012). In soybean cotyledon, calcium played a role in signal transduction under flooding stress (Komatsu et al., 2013b). It was repoprted that exogenous calcium could improve the salt stress tolerance in germinating soybean *via* enriching signal transduction, energy pathway and transportation, and enhancing antioxidant enzymes (Yin et al., 2015). Howerer, flooding-responsive mechanism by exogenous calcium in soybean is poorly understood. These previous reports indicate that flooding-induced changes in protein level might play a role in the regulation of response mechanism in soybean.

Proteomic approach was used to clarify the drought-induced changes in soybean. Yamaguchi et al. (2010) used proteomic techanique to understand response of root elongation zone in soybean primary root under drought stress and founded that the abundance of proteins involved in isoflavonoid biosynthesis, lignin biosynthesis, and ferritin was changed, indicating that these proteins involved in inhibition of growth in root elongation region. Energy metabolism related proteins were more abundant in soybean root under drought stress (Toorchi et al., 2009; Alam et al., 2010a; Mohammadi et al., 2012b), suggesting that increased energy demand was one of responses to drought. Several ROS scavenger related proteins were induced during drought stress in soybean (Toorchi et al., 2009; Alam et al., 2010a; Mohammadi et al., 2012b), suggesting that these proteins played a role in protecting plant to drought-induced oxidative damage.

There were some reports on response of flooding and drought in several plants. For example, stomatal closure was involved in increased delivery of ABA from roots to the leaves in strawberry under drought and more rapidly induced by flooding, suggesting that it may be resulted from increased synthesis and release of ethylene under flooding, but not under drought in strawberry (Blanke and Cooke, 2004). The characterization of ascorbate peroxidase (APX), which is one of the antioxidant enzymes, was investigated in soybean under both flooding and drought stresses, indicating that they had completely opposite trend at the activity level and abundance change in soybean (Kausar et al., 2012). The activity of calatase, which is known as ROS scavenger, was decreased under flooding; while increased under drought in cowpea, suggesting that cowpea had varying ability to respond to oxidative stress by governing differential sensitivity to flooding and drought (El-Enany et al., 2013). Recently, Hossain and Komatsu (2014) summarized the major proteomic findings of soybean under flooding and drought stresses. They suggested that metabolic regulation

and quick activation of plant defense system are required to conquer the flooding stress; while, proteins involved in osmotic adjustment, defense signaling, and programmed cell death are critical to drought adaptation (Hossain and Komatsu, 2014). These results suggest that plant may independently respond and develop complex mechanism between flooding and drought.

Plant has the complex network in response to flooding and drought stresses. As mentioned above, flooding and drought were shown opposite morphology and their metabolic pathway. Despite the severity of flooding and drought stresses in soybean, the mechanisms at molecular level in response to flooding and drought have not been clearly elucidated. To contribute the improvement of tolerance in soybean against flooding and drought, the molecular mechanisms of soybean in response to flooding and drought was analyzed. In this study, proteomic technique was used to identify flooding and drought responsive proteins in soybean. Firstly, to get insight into soybean response to flooding supplemented with GA and calcium, a gel-free proteomic technique was used. Secondary, to get insight into response mehcanisms in soybean against flooding and drought, root proteins were identified using a gel-free proteomic technique. Thirdly, the mRNA expression, protein abundance, and activity of key enzymes, which were identified in soybean under flooding and drought by proteomic analysis, were analyzed. To characterize the function of key enzymes, further analysis was performed in time dependency, organ specificity, and stress specificity.

Table 1. Summary of published proteomic analyses in crops under flooding

Crops	Major findings	References
Tomato	Photosynthesis, disease resistance, stress/defense mechanisms, energy and metabolism, and protein biosynthesis related proteins	Ahsan et al., 2007a
	Secondary metabolite biosynthesis, programmed cell death, and disease/defense related proteins	Ahsan et al., 2007b
Wheat	Disease/defense and cell wall structure/modification related proteins	Kong et al., 2010
	Energy/redox status, defense responses, and cell wall turnover related proteins	Haque et al., 2011
	Pathogenesis-related proteins, ferrodoxin, and elongation factor 2	Haque et al., 2014
Rice	Tubulin α-1 chain, actin depolymerizing factor 4, glutathione *S*-transferase GSTU6, cytosolic APX, and glutatione *S*-trasferase GST27	Sadiq et al., 2011
Red/white clover	RuBisCO large/small subunits, ClpP protease subunints, and protease isoform	Stoychev et al., 2013
Maize	Energy metabolism, phosotynthesis, programmed cell death, phytohormones, and polyamines related proteins	Chen et al., 2014
	NADP-malic enzyme, glutamate decarboxylase, coproporphyrinogen III oxidase, glutathione *S*-transferase, glutathione dehydrogenase, and xyloglucan endotrasglycosylase 6	Yu et al., 2015
Cucumber	Glycolysis, TCA cycle, fermentative metabolism, nitrogen metabolism, and ROS defense related proteins	He et al., 2012
Cacao	Glycolysis/alcoholic fermentation, photosynthesis, protein metabolism, and oxidative stress related proteins	Bertolde et al., 2014
Soybean	Signal transduction, programmed cell death, RNA processing, redox homeostasis, and energy metabolism related proteins	Alam et al., 2010b
	Isoflavone reductase	Khatoon et al., 2012a
	Cell structure, cell wall metabolism, and cytoskeletal organization related proteins	Salavati et al., 2012
	Polygalacturonase inhibitor-like, expansin-like B1-like protein, cinnamyl-alcohol dehydrogenase, and cellulose synthase-interactive protein-like proteins	Nanjo et al., 2013
	Receptor for activated protein kinase C1	Komatsu et al., 2014a

Table 2. Summary of published proteomic analyses in crops under drought

Crops	Major findings	References
Rice	S-like RNase homologue, actin depolymerizing factor, rubisco activase, isoflavone reductase-like protein	Salekdeh et al., 2002
	Transport related proteins and oxidation-reduction related proteins	Mirzaei et al., 2012
	Coronatine-insentivie 1 protein, WD-40 repeat protein, and H-protein promoter binding factor 2a	Maksup et al., 2014
	Orthophosphate dikinase, glycine dehydrogenase, RuBisCO, glycine hydrozymethyltransferase, ATP synthase, superoxide dismutase [Cu-Zn], and dehydroascorbase reductase	Ji et al., 2012
Sugar beet	Redox regulation, oxidative stress response, signal transduction, and chaperone activites	Hajheidari et al., 2005
Wheat	Glycolysis and gluconeogenesis related proteins	Caruso et al., 2009
	RuBisCO large subunit, ferredoxin-NADP(H) oxidoreductase, glutathione transferase, Mn superoxide dismutase	Budak et al., 2013
	Lipoxygenase, plasma membrane proton ATPase, potassuium channel β subunit, monomeric G-protein, calnexin, and elicitor-induced protein	Alvarez et al., 2014
	Metabolic process, cell redox homeostasis, translation, and transport related proteins	Ford et al., 2011
Maize	Lignifications and flavonoid synthesis related proteins	Vincent et al., 2005
	Protective/detoxification related proteins	Benesova et al., 2012
	Redox homeostasis, cell rescue/defense, hormone regulation, and protein biosynthesis/degradation related proteins	Yang et al., 2014
	ROS metabolism, defense and detoxification, hydrolases, and carbohydrate metabolism related proteins	Zhu et al., 2007
Canola	V-type H+ ATPase, plasma-membrane associated cation-binding protein, HSP90, and elongation factor EF-2	Mohammadi et al., 2012a
Barley	Chloroplastic metabolism and energy related proteins	Kausar et al., 2013
Wild watermelon	Small G-protein family members	Yoshimura et al., 2008
Common bean	Carbonic anhydrase, chlorophyll a/b binding proteins, aldolase, glyceraldehyde-3-phosphate dehydrogenase, and oxidoreductase-like protein	Zadraznik et al., 2013
Sugarcane	Serine protease inhibitor, *S*-adenosylmethionine decarboxylase proenzyme, ubiquitin, and p18	Jangpromma et al., 2010
Soybean	Osmotic adjustment, defense signaling, and programmed cell death related proteins	Alam et al., 2010a
	Caffeoyl-CoA-*O*-methltransferase and 20S proteasome alpha subunit	Toorchi et al., 2009
	Heat shock protein 70, actin isoform, and methionine synthase	Mohammadi et al., 2012b

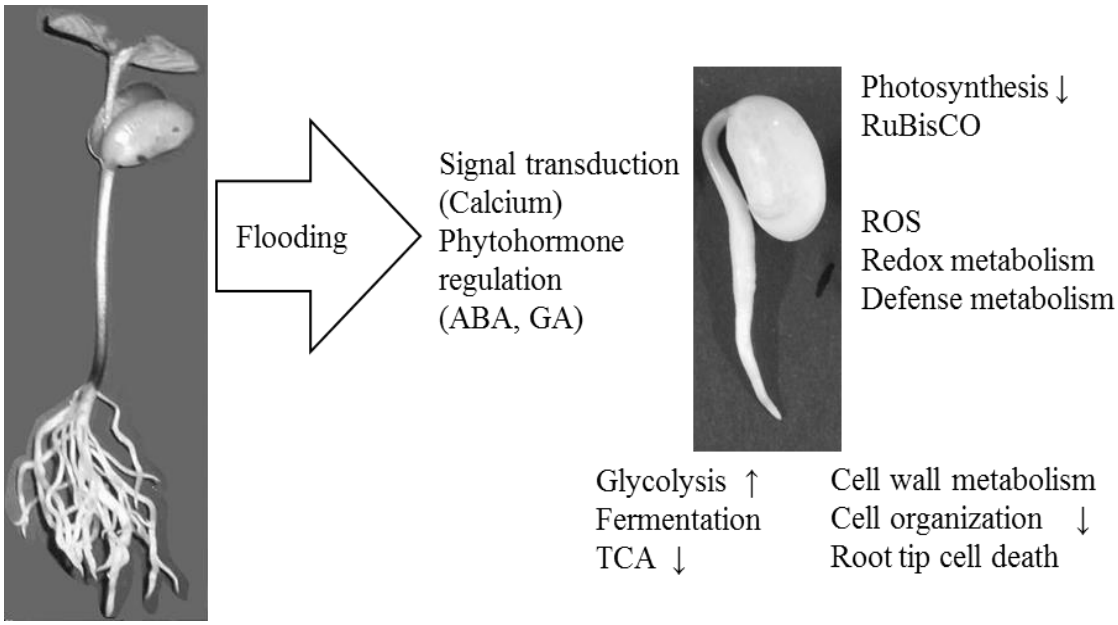

Figure 1. Overview of plant response to flooding stress. In response to flooding stress, plant leads to changes in signal transduction, phytohormone regulation, photosynthesis, ROS level, redox, defense/detoxification, energy metabolism such as glycolysis, fermentation and TCA, cell wall metabolism, cell organization, thereby causing cell death of root tip.

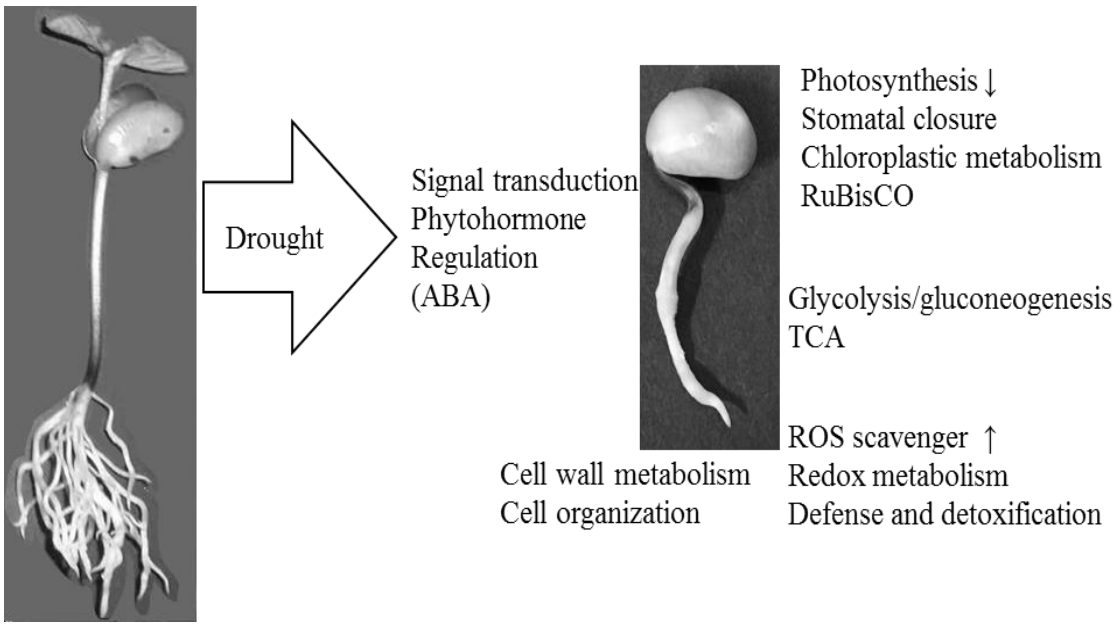

Figure 2. Overview of plant response to drought stress. In response to drought stress, plant leads to changes in signal transduction, phytohormone regulation, photosynthesis, stomatal closure, ROS scavenger, redox, defense/detoxification, energy metabolism glycolysis/gluconeogenesis and TCA, cell wall metabolism, and cell organization.

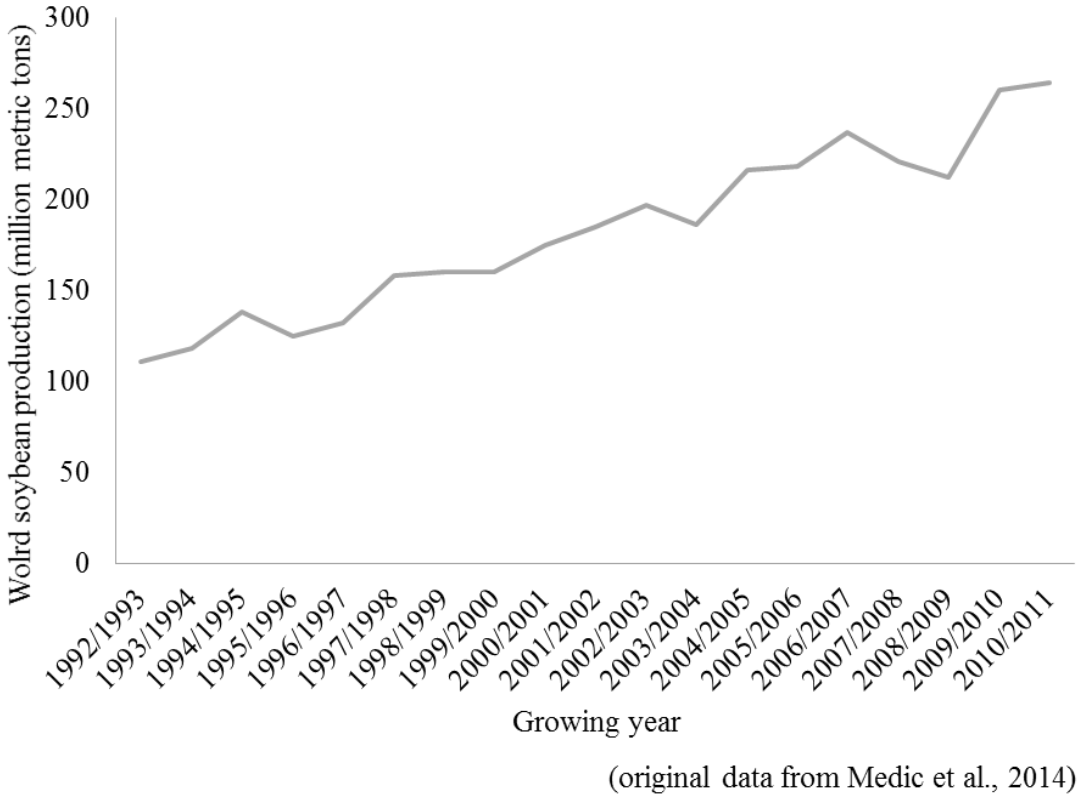

Figure 3. Historical soybean production. Its world production reached 263.7 million metric tons in 2010/2011, which has more than doubled in 1992/1993.

Chapter 1.

Characterization of root proteins in soybean affected by gibberellic acid and calcium under flooding

1.1. Introduction

Root is an important organ which is known as the first organ to recognize drought and flooding stresses. Plant initially responds to flooding by reducing root permeability, water absorption, and mineral uptake, followed by decreasing photosynthesis, altering hormonal balance, and developing aerenchyma and adventitious roots (Vartapetian and Jackson, 1997). It was reported that plant roots are also able to sense decreases in soil water content (Davies et al., 2002; Wilkinson and Davies, 2002). Recently, a number of protein synthesis and modification related proteins were found to be activated in the root tips of soybean during germination (Komatsu et al., 2014b). Yin et al. (2014) reported that the application of ethylene significantly promotes the soybean growth even under flooding stress condition. The initiation and elongation of lateral root are decreased under drought stress (Malamy, 2005; van der Weele et al., 2000; Xiong et al., 2006). Taken together, these observations indicate that root is an important organ for understanding the mechanisms by which soybean regulates abiotic stress response.

Flooding stress severely restricts growth and productivity of most terrestrial plants (Bailey-Serres and Voesenek, 2008) because most agriculturally important crops are sensitive to flooding (Setter and Waters, 2003). The physiological activities of several phytohormones, such as ethylene, ABA, and GA are affected by flooding in deepwater rice (Azuma et al., 2003; Choi, 2007), tomato (Vidoz et al., 2010), and soybean (Komatsu et al., 2013a). Differential network of phytohormone signaling pathways plays a role in regulation of root emergence and elongation, and adventitious root growth (Steffens et al., 2006). For example, in flooded tomato, adventitious root production to replace damaged root by flooding is strictly interconnected with ethylene and auxin (Vidoz et al., 2010). During sustained submergence, rice modulates the levels of ethylene and GA for survival (van der Straeten et al., 2010). Submergence-induced

root elongation is mediated by alteration of GA metabolism (Dubois et al., 2011). ABA plays a role in survival of soybean under flooding stress (Komatsu et al., 2013a). It was reported that exogenously added GA increased seedling growth of chickpea by enhancing endogenous GA content under drought stress (Kaur et al., 1998). In peanut, GA_3 alleviated drought stress by increasing carbohydrate content, amino-N, and total nitrogen (El-Meleigy et al., 1999). GA_3 application alleviated the decrease in the amount of nucleic acids by activating the synthesis of nucleic acids (Aliyev et al., 2000). These findings indicate that phytohormones are closely related to control root elongation of soybean under flooding stress.

Proteomic analysis was used to identify GA-responsive proteins in *Arabidopsis* (Gallardo et al., 2002) and rice (Tanaka et al., 2004). During germination, the abundance of α-2,4 tubulin, *S*-adenosylmethionine synthetase, and β-glucosidase was controlled by GA in *Arabiodopsis* (Gallardo et al., 2002). Signal transduction by GA regulated the accumulation of methylmalonate-semialdehyde dehydrogenase, which may play a role in root development and leaf sheath elongation in rice (Tanaka et al., 2004). Wen et al. (2010) reported that GA had a signicant effect on the abundance of salt-regualted proteins in rice under salt stress. The key enzymes of glycolytic pathway, aldolase and glyceraldehyde-3-phosphate dehydrogenase, were increased in GA-treated rice root, indicating that the glycolytic production of ATP *via* GA signaling may mediate root growth (Komatsu and Konishi, 2005). These results suggest that the elongation of root may be regulated *via* GA signaling and related proteins, and thereby respond to stress condition. However, GA-induced response to flooding in soybean has not been clearly understood.

Calcium is known as an essential plant nutrient for regulation of growth and development (Hepler, 2005) and prevention of ion leakage caused by biotic and abiotic

stresses (Lin et al., 2008). The cytosolic calcium concentration was increased by GA in barley aleurone cell at plasma membarane (Gilroy and Jones, 1992). Increased calcium transport activity, which is involved in endoplasmic reticulum and stimulated calcium flux across the plasma membrane, was also induced by GA (Bush et al., 1989; Bush et al., 1993). Previous study suggested that opimal amounts of antioxidative enzymes and antioxidants were regulated by calcium in antioxidative systeme of sweetpotato leaves under waterlogging stress (Lin et al., 2008). It was reported that tolerance to short-term hypoxic stress was enhanced by reduction of polyamine degradation, elevation of nutrient uptake, and accelerated synthesis of heat-stable proteins and polyamine mediated by calcium in muskmelon (Gao et al., 2011). Furthermore, there were reports on effect of exogenous calcium in soybean (Alam et al., 2011) and Zoysiagrass (Xu et al., 2013) under drought stress. The application of exogenous calcium was associated with increased relative water content, membrane stability, and chlorophyll content in soybean under drought stress (Alam et al., 2011). It was reported that tolerance to drought stress was improved to some extent when calcium was pretreated in Zoysiagrass by increasing biomass, chlorophyll content, and antioxidant enzymes (Xu et al., 2013). These observations suggest that calcium may play an important role in protecting soybean against stress *via* calcium-related signal transduction.

Calcium-related proteins in soybean cotyledon under flooding stress using proteomic technique were identified and indicated that calcium may be involved in flooding-induced signal transduction throught heat shock protein 70 (Komatsu et al., 2013b). He et al. (2012) reported that the level of enzymes involved in glycolysis, tricarboxylic acid (TCA) cycle, fermentative metabolism, nitrogen metabolism, and ROS defense was enhanced by exogenous calcium in cucumber under hypoxia. A number of calcium-related proteins, including annexin, calnexin, luminal-binding

protein, calcium ion-binding protein, and calcium-transporting ATPase 4, were identified in flooded soybean (Komatsu et al., 2012). Annexin, which is a calcium-dependent membrane-binding protein, was identified in soybean under flooding stress with ABA supplementation (Komatsu et al., 2013a). Three homologues of plasma membrane H^+-ATPase and calnexin were identified in soybean treated with polyethylene glycol (Nouri and Komatsu, 2010). These results suggest that calcium act as a key signaling factor in soybean under flooding stress. However, calcium-induced response to flooding in soybean remain to be eludicated.

Because phytohormone- and calcium-mediated mechanisms play an important role in regulatory response of plant under stress condition, several studies have examined the involvement of phytohormone and calcium in response mechanisms in plant. Phytohormone and calcium involve in the enhancement of tolerance to osmotic stress such as drought in soybean. However, flooding response of soybean by GA and calcium has not been elucidated. To understand the response mehanisms of soybean under flooding stress, flooding-responsive mechanism *via* GA and calcium was analyzed in soybean. In this chapter, a gel-free proteomic technique was used to identify into GA- and calcium-induced proteins in soybean under flooding stress. Also the role of GA and calcium in soybean under flooding and drought stresses was discussed.

1.2. Materials and methods

1.2.1. Plant material and treatments

Soybean seeds (*Glycine max* L. cultivar Enrei) were sterilized with 1% sodium hypochlorite solution for 2 min and subsequently rinsed twice with water. Soybeans were sown on 500 mL silica sand wetted with 125 mL water in a plastic case (180 mm x 140 mm x 45 mm). Soybeans were grown in a growth chamber under white fluorescent

light (160 μmol $m^{-2}s^{-1}$, 16 h light period/day) at 25°C and 70% relative humidity. Two-day-old soybeans were transferred to a glass tube (38 mm x 130 mm) containing 120 mL of tap water supplemented with 5, 10, and 15 μM GA_3 or 1, 5, 10, and 50 mM $CaCl_2$. For morphological experiment, 4-, 6-, and 8-day-old soybeans, which were treated for 2, 4, and 6 days, respectively, were collected and length and weight of root including hypocotyl were measured. For proteomic analysis, roots were collected from 4-day-old soybeans flooded with GA_3 or $CaCl_2$ for 2 days (Figure 4). Non-treated equivalent soybeans were collected as control. Three independent experiments were performed as biological replicates for all experiments.

1.2.2. Evans blue staining for assay of cell death

For assay of cell death in root tip of soybean, Evans blue staining was used. Soybeans were stained in a 0.25% aqueous solution of Evans blue for 15 min at room temperature. The stained sample was washed with water and immediately photographed. For quantitative assessment of staining, the terminal 5 mm of stained root tips was excised and immersed in 200 μL N, N-dimethylformamide for 24 h at 4°C. After incubation, the absorbance of Evans blue released from the root tip was measured at 600 nm.

1.2.3. Protein extraction for mass spectrometry analysis

A portion (500 mg) of frozen samples was ground into powder in liquid nitrogen using a mortar and pestle. The powder was transferred to 10% trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone and vortexed. The suspension was sonicated for 10 min and incubated for 60 min at -20°C followed by mixing every 15 min. After incubation, the suspension was centrifuged at 9,000 x *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 in lysis buffer consisting of 8 M urea, 2 M thiourea, 5% 3-[(3-Cholamidoprophyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 2 mM tributylphosphine by vortexing for 1 h at 25°C. The suspension was then centrifuged at 20,000 x *g* for 20 min at 25°C and the supernatant was collected as protein extracts. Protein concentration was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

1.2.4. Protein purification and digestion for mass spectrometry analysis

Extracted proteins (100 µg) were purified with methanol and chloroform to remove detergent from the sample. Samples were adjusted to total volume of 100 µL. Methanol (400 µL) was added to 100 µL of samples and then mixed. After mixing, 100 µL of chloroform and 300 µL of water were added to samples followed by mixed and centrifuged at 20,000 x *g* for 10 min to achieve phase separation. The upper phase was discarded and 300 µL of methanol was carefully added to lower phase. After centrifugation at 20,000 x *g* for 10 min, the supernatant was discarded and the remained pellet was dried. Dried samples were reduced with 50 mM dithiothreitol for 30 min at 56°C and alkylated with 50 mM iodoacetamide for 30 min at 37°C in the darkness. Alkylated proteins were digested with trypsin and lysyl endopeptidase (Wako, Osaka, Japan) at 1:100 enzyme/protein concentration at 37°C for 16 h. Resulting tryptic peptides were acidified with 10 µL of 20% formic acid to pH < 3, desalted with C18-pipette tip (Nikkyo Technos, Tokyo, Japan). Samples were subjected to nano liquid chromatography (LC) mass spectrometry (MS)/MS.

1.2.5. Protein identification by mass spectrometry

The purified peptide samples were separated using an Ultimate 300 nano LC system (Dionex, Germering, Germany). The peptide ions were detected using a nanospray LTQ Orbitrap discovery mass spectrometry (Thermo Fisher Scientific, San Jose, CA, USA) in data-dependent acquision mode with the installed Xcalibur software (version 2.0.7, Thermo Fisher Scientific). The peptides were loaded onto a C18 PepMap trap column (300 μm ID x 5 mm, Thermo Fisher Scientific) with 0.1% formic acid and eluted from the trap column with a linear acetonitrile gradient in 0.1% formic acid at a flow rate of 200 nL/min. The eluted peptides from trap column were separated and loaded onto a C18 NANO HPLC NTTC-360/75-3 capillary tip column (75 μm ID x 120 mm, Nikkyo Technos) with a spray voltage of 1.5 kV. Full-scan mass spectra were acquired in the MS over 400-1,500 *m/z* with a resolution of 30,000. The top ten most intense precursor ions were selected for collision-induced fragmentation in the linear ion trap at normalized collision energy of 35%. Dynamic exclusion was employed within 90 sec to prevent the repetitive selection of peptides (Zhang et al., 2009).

1.2.6. Data acquisition by mass spectrometry

Protein identification was performed by Mascot search engines (version 2.4.1, Matrix Science, London, UK) with a soybean peptide database (55,787 sequences) constructed from the soybean genome database (Phytozome version 9.1, http://www.phytozome.net/soybean) (Schmutz et al., 2010). The acquired raw data files were processed using Proteome Discoverer software (version 1.4, Thermo Fisher Scientific). The parameters used in the Mascot searches were as follows:

carbamidomethylation of cystein set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Peptide mass tolerance was set at 10 ppm, fragment mass tolerance was set at 0.5 Da, and peptide charge was set at +2, +3, and +4. An automatic decoy database search was performed as part of the search. Mascot results were filtered with Percolator function to improve the accuracy and sensitivity of peptide identification. The acquired Mascot results were imported for further analysis using SIEVE software (version 2.1, Thermo Fisher Scientific).

1.2.7. Analysis of differential abundant proteins using acquired mass spectrometry data

For differential analysis of relative abundances of peptides and proteins between control and experimental groups, a commercial label-free quantification package SIEVE software was used. The chromatographic peaks detected by MS were aligned, and the peptide peaks were detected as a frame on all parent ions scanned by MS/MS using the following settings: 5 min of frame time width and 10 ppm of frame *m/z* width. Chromatographic peak areas of each sample within a frame were compared, and the ratios between two sample groups in a frame were determined. The frames detected in the MS/MS scan were matched to the imported Mascot results. The ratio of peptides between samples was determined from the variance-weighted average of the ratios in frames, which matched the peptides with 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 the identification of a protein were two matched peptides. Significant changes in the abundance of protein between control and

treatment were analyzed (*t*-test, $p < 0.05$).

1.2.8. Functional analysis of identified proteins

Protein functions were categorized using MapMan bin codes software (http://mapman.gabipd.org/) (Usadel et al., 2005).

1.2.9. Statistical analysis

The statistical significance of the results was evaluated with a Student's *t*-test when only 2 groups were compared. The statistical significance of the more than 3 groups was evaluated with one-way ANOVA followed by Turkey's multiple comparison test. Both calculations were performed using SPSS software (version 22.0, IBM, Armonk, NY, USA). A p value of < 0.05 was considered to be statistically significant.

1.3. Results

1.3.1. Effects of GA_3 and calcium on growth of soybean seedlings under flooding stress

To evaluate the effects of GA_3 on morphological characteristics in soybean seedlings under flooding stress, 2-day-old soybeans were subjected to flooding without or with 5, 10, and 15 μM GA_3 for 2, 4, and 6 days. After 6 days flooding, soybean seedlings were further grown for 6 days under normal condition. The overall growth of soybean seedlings was inhibited under flooding without GA_3 (Figure 5A). The root including hypocotyl under flooding with various concentrations of GA_3 for 2, 4, and 6 days elongated compared to that under flooding without GA_3 (Figure 5A). Although the length of root including hypocotyl of 6 days flooding with 5 μM GA_3 was similar to that

under flooding without GA_3, the root length under flooding with 10 and 15 μM GA_3 was significantly longer than that under flooding without GA_3. (Figure 5B). The length of root including hypocotyl under flooding with 10 μM GA_3 was similar to that under flooding with 15 μM GA_3. However, there were no significant differences in the weight of root including hypocotyl between flooded soybeans without and with GA_3 (Figure 5B).

To evaluate the effects of calcium on morphological characteristics in soybean seedlings under flooding stress, 2-day-old soybeans were exposed to flooding without or with 1, 5, 10, and 50 mM $CaCl_2$. The overall length of root including hypocotyl under flooding with various concentrations of $CaCl_2$ was clearly longer than that under flooding without $CaCl_2$ (Figure 6A). Despite no marked differences in root between flooded soybean without and with 1 mM $CaCl_2$, the root length under flooding with 10 and 50 mM $CaCl_2$ was significantly longer than that under flooding without $CaCl_2$ (Figure 6B). Consistent with this finding, the weight of root including hypocotyl was also higher in soybeans exposed to $CaCl_2$ during the treatment period (Figure 6B). The length and weight of root were gradually increased in 2, 4, and 6 days flooded soybean with 10 and 50 mM $CaCl_2$ (Figure 6). The soybean seedlings subjected to flooding were stained with Evans blue dye to evaluate the corresponding degree of cell death in the root tip (Figure 7). The degree of staining in the root tips was dependent on the $CaCl_2$ concentration and treatment period (Figure 7A). Cell death in the root tips was severely induced by flooding without $CaCl_2$ compared to that in the presence of 50 mM $CaCl_2$ (Figure 7B).

1.3.2. Protein profiles in soybean root under flooding stress affected by GA_3 using gel-free proteomics

To investigate the effects of GA_3 on root proteins in soybean under flooding stress, a gel-free proteomic technique was used. Based on the morphological experiments, 2-day-old soybeans were subjected to flooding stress without or with 10 μM GA_3 for 2 days and proteins were extracted from roots and analyzed using nanoLC-MS/MS. Proteins identified from 2-day-old soybeans were used for comparison because 2-day-old soybeans were considered as starting point of treatments. Out of 1,070 proteins, 307 proteins, among which 206 and 101 proteins were decreased and increased, respectevely, were significantly changed in 4-day-old soybeans (control). Out of 911 proteins, 324 proteins, among which 262 and 62 proteins were decreased and increased, respectively, were significantly changed in 2 days flooded soybeans without GA_3 (flooding). Out of 942 proteins, 250 proteins, among which 130 and 120 proteins were decreased and increased, respectively, were significantly changed in 2 days flooded soybeans with GA_3 (flooding with GA_3).

To determine which proteins were changed in response to flooding without or with GA_3, the each identified proteins were compared. A total of 162, 83, and 38 proteins were specifically changed in 4-day-old soybeans (control), 4-day-old soybeans subjected to 2 days flooding without GA_3 (flooding), and 4-day-old soybeans subjected to 2 days flooding with GA_3 (flooding with GA_3), respectively (Figure 8). Forty-seven proteins were common among 4-day-old soybeans (control), 4-day-old soybeans subjected to 2 days flooding without GA_3 (flooding), and 4-day-old soybeans subjected to 2 days flooding with GA_3 (flooding with GA_3) (Figure 8). The comparison between 4-day-old soybeans subjected to 2 days flooding without and with GA_3 indicated that 133 proteins were commonly identified (Figure 8, Table 3).

1.3.3. Protein profiles in soybean root under flooding stress affected by calcium using

gel-free proteomics

To investigate the effects of calcium on root proteins in soybean under flooding stress, a gel-free proteomic technique was used. Based on the morphological experiments, 2-day-old soybeans were subjected to flooding stress without or with 50 mM $CaCl_2$ for 2 days and proteins were extracted from roots and analyzed using nanoLC-MS/MS. Proteins identified from 2-day-old soybeans were used for comparison because 2-day-old soybeans were considered as starting point of treatments. Out of 294 proteins, 126 proteins, among which 116 and 10 proteins were decreased and increased, respectevely, were significantly changed in 4-day-old soybeans (control). Out of 1,343 proteins, 588 proteins, among which 502 and 86 proteins were decreased and increased, respectively, were significantly changed in 2 days flooded soybeans without $CaCl_2$ (flooding). Out of 895 proteins, 329 proteins, among which 196 and 133 proteins were decreased and increased, respectively, were significantly changed in 2 days flooded soybeans with $CaCl_2$ (flooding with $CaCl_2$).

To determine whether the altered proteins were different in flooded soybeans without or with calcium, the each identified proteins were compared. A total of 99, 375, and 122 proteins were specifically changed in 4-day-old soybeans (control), 4-day-old soybeans subjected to 2 days flooding without calcium (flooding), and 4-day-old soybeans subjected to 2 days flooding with calcium (flooding with $CaCl_2$), respectively (Figure 9). Nine proteins were commonly identified among 4-day-old soybeans (control), 4-day-old soybeans subjected to 2 days flooding without calcium (flooding), and 4-day-old soybeans subjected to 2 days flooding with calcium (flooding with $CaCl_2$) (Figure 9). The comparison between 4-day-old soybeans subjected to 2 days flooding without and with calcium indicated that 192 proteins were common (Figure 9, Table 4).

1.3.4. Functional categorization of proteins in soybean under flooding stress with GA_3 and calcium

To elucidate what types of biological processes were altered in response to flooding without or with GA_3, functional categorization was analyzed using MapMan bin codes. The number of glycolysis, fermentation, and cell wall related proteins were increased by flooding; however, these proteins were not affected by GA_3 supplementation (Figure 10). Proteins involved in protein degradation and synthesis were decreased in response to flooding; while, these proteins were recovered by GA_3 supplementation (Figure 10). The number of redox, secondary metabolism, and cell organization related proteins were increased when GA_3 was supplemented under flooding in soybean root (Figure 10).

To elucidate what kinds of biological processes were altered in response to flooding without or with calcium, functional categorization was analyzed using MapMan bin codes. The number of glycolysis and fermentation related proteins were increased by flooding; however, these proteins were not affected by calciulm supplementation under flooding (Figure 11). Proteins involved in hormone metabolism, DNA synthesis, cell wall, and protein degradation/synthesis/posttranslational modification were decreased under flooding; while, these proteins were increased under flooding with calcium (Figure 11). The number of development, signaling, and lipid metabolism related proteins were increased when calcium was supplementated under flooding in soybean root (Figure 11).

1.4. Discussion

1.4.1. Mophological charateritics of soybean affected by GA_3 and calcium under flooding stress

To understand the effects of GA_3 and calcium on morphological charaterictis in soybean under flooding, 2-day-old soybeans were subjected to flooding without or with GA_3 and calcium. In present study, the application of GA_3 improved the elongation of root in soybean under flooding stress. Kaya et al. (2006) reported that GA_3 supplementation improved water deficit tolerance by maintaining membrane permeability and enhancing chlorophyll, water, and macronutrient contets in maize leaves. It was also reported that application of GA_3 significanlty improved the depressed plant traits, such as plant height, internode length, and dry matter production, in maize under drought stress (Akter et al., 2014). In this study, exogenous calcium also improved the elongation of soybean root under flooding stress. Under drought stress, calcium treatment increased protection agaist membrane lipid peroxidation and stability of membranes, and thereby resulting in enhanced drought tolerance in rice seedlings (Lu et al., 1999). Li et al. (2003) also reported that application of calcium played an important role in protecting liquorice cells from the polyethylene glycol-induced damage by mitigating oxidative stress. Taken together, these results suggest that GA and calcium may alleviate to flooding-induced inhibition of growth in soybean.

1.4.2. Identification of flooding responsive proteins in soybean affected by GA_3 using proteomic technique

To understand the effects of GA_3 on protein profiles in soybean root under flooding stress, a gel-free proteomic technique was used. One hundred thirty-three proteins were commonly identified between flooded soybean without and with GA_3 (Fiugure 8, Table 3). These 133 changed proteins were mainly categorized in protein degradation/synthesis/posttranslational modification (30/133), stress (14/133), cell wall (13/133), and amino acid metabolism (11/133). Among cell wall related proteins,

polygalacturonase inhibiting protein 1 was incrased in both flooded soybean without and with GA_3. Polygalacturonase inhibiting protein, which is plant extracellular leucine-rich proteins, plays an important role in plant defence. It is known as plant cell wall glycoproteins which inhibits fungal endopolygalacturonases. Ferrari et al. (2003) reported that the overexpression of the genes *polygalactruronase inhibiting protein 1* and *2* in *Arabiodpsis* reduced disease symptoms. It was reported that *pepper polygalacturonase inhibiting protein* gene family was up-regulated in the pepper leaves by Phytophthora capcis, salicylic acid, methyl jasmonate, ABA, wounding, and cold treatment, suggesting that pepper polygalacturonase inhibiting proteins may be involved in plant defense response and play a ciritial role in a plant's resistance to disease (Wang et al., 2013). These result suggest that cell wall related proteins such as polygalacturonase inhibiting protein might be involved in defense system of soybean under flooding.

1.4.3. Identification of flooding responsive proteins in soybean affected by calcium using proteomic technique

To understand the effects of calcium on protein profiles in soybean root under flooding stress, a gel-free proteomic technique was used. One hundred ninety-nine proteins were commonly identified between flooded soybean without and with calcium (Figure 9, Table 4). These 192 changed proteins were mainly categorized in protein degradation/synthesis/posttranslational modification (45/192), RNA binding/processing (25/192), cell wall (18/192), and stress (18/192). Among RNA binding/processing realted proteins, AT hook motif nuclear localized proteins were identified in both flooding without and with calcium. AT hook motif is a small DNA-binding protein motif and, in plant, AT hook proteins play a role in developmental

processes, such as flowering transition and stress responses. The *rice AT hook motif DNA-binding protein* genes are exclusively localized in the nucleus and may act as architectural transcription factors to regulate expression of target genes involved in plant growth and development (Kim et al., 2011). Yun et al. (2012) proposed that AT hook motif nuclear localized protein 22 acted as a chromatin remodeling factor which modifies the architecture of chromatin by modulating both histone acetylation and methylation. These results suggest that RNA related proteins might be involved in development and growth under flooding.

1.4.4. Comparison of flooding and drought stresses affected by GA_3 and calcium

In present study, flooding stress decreased growth of soybean root; however, exogenous GA_3 and calcium supplementation alleviated flooding-induced damage (Figures 5 and 6). Xu et al. (2003) reported that combined effect of calcium and GA in rice seedling under drought stress, indicating that tolerance to drought was increased during germination and young seedlings soaked with calcium and GA. Redox metabolism is one of the important mechanism in response to abiotic stress, because accumulated ROS lead to oxidative damages in the apoplastic compartment, lipid peroxidation-induced damage in cellular membranes, and imbalance in ion homeostasis by interfering ion fluxes (Baier et al., 2005). Under flooding stress, the number of redox related proteins were decreased; however, when GA_3 and calcium was supplemented under flooding, the number of those proteins were increased (Figures 10 and 11). Under drought stress, the predominance of proteins involved in ROS handling underscored the importance of managing ROS and oxidative damage during senescence to protect stem and to sustain stem reserve remobilization in wheat (Ehdaie et al., 2006). In *Camellia sinensis*, increased contents of non-enzymic antioxidant and

enhanced activities of enzymic antioxidant reduced ROS accumulation and lipid peroxidation by exogenous calcium under drought recovery (Upadhyaya et al., 2011). It was reported that activities of APX and catalase were strongly induced in canola when calcium was supplemented under water deficit stress (Alam et al., 2011). These results indicate that responsive mechanism such as redox metabolism in soybean against flooding and drought is regulated by GA_3 and calcium signaling; however, differences between flooding and drought in soybean still remaine the elucidation.

1.4.5. Concluding remarks

In conclusion, proteins affected by GA_3 and calcium in flooded soybean were identified to better understand GA_3- and calcium-mediated flooding and drought response mechanisms. However, drought-responsive mechanism already reported (Xu et al., 2003). Here, the experiment focused on flooding stress in soybean. The length of root including hypocotyl was elongated when GA_3 and calcium were added under flooding condition. Secondary metabolism, cell, redox, and protein degradation/synthesis related proteins were decrased by flooding, and these proteins were recovered by GA_3 supplementation. Fermentation and cell wall related proteins were not affected by GA_3 supplementation. Cell wall, protein degradation/synthesis, hormone metabolism, redox, and DNA synthesis-related proteins were decrased under flooding stress, but these were increased by calcium supplementation. Fermentation and glycolysis related proteins were increased under flooding stress; however, these proteins were not affected by calcium supplementation. These results suggest that GA_3 and calcium may involved in enhancing the growth of soybean under flooding and drought by regulating the abundance of redox related proteins, because flooding and drought induce decreased and increased level of those proteins, respectively.

1.5. Summary

Soybean growth is affected by flooding stress because it is flooding sensitive crop. To understand morphological response in soybean under flooding affected by GA_3 and calcium, 2-day-old soybeans were flooded with GA_3 or calcium for 6 days. The length of root including hypocotyl was elongated in presence of GA_3 and calcium under flooding stress. The fresh weight of root including hypocotyl under flooding in presence of GA_3 and calcium was more than that under flooding in absence GA_3 and calcium. Furthermore, exogenous calcium suppressed the cell death of root tip in soybean under flooding stress. Based on morphological experiment, to get insight into flooding-responsive mechanism in soybean with GA_3 and calcium supplementation, a gel-free proteomic technique was used. And differences between flooding and drought stresses in soybean were discussed to further understand the stress responsive mechanism in soybean. The number of glycolysis and fermentation related proteins were increased under flooding stress; however, the number of these proteins were not affected by GA_3 and calcium under flooding stress. GA_3 supplementation increased the number of proteins involved in redox, secondary metabolism, and cell organization. Calcium supplementation increased the number of hormone metabolism, DNA synthesis, redox, and cell wall. Drought stress predominantly induced redox related proteins and GA3 and calcium enchanced the redox metabolism in response to flooding and drought stresses. These results suggest that GA_3 and calcilum affect the abundance of proteins involved in hormone/secondary metabolism, cell organization, cell wall metabolism, and redox in soybean roots under flooding and drought stresses, thereby enhancing growth of soybean in response to flooding and drought.

Table 3. Differentially abundant 133 proteins in soybean root between flooding without and with GA_3.

Protein ID	Description	M.P.	Ratio 4(2)F	*P*value 4(2)F	Ratio 4(2)F+GA	*P*value 4(2)F+GA	Function
Glyma06g02290.1	Pyridoxal dependent decarboxylase family protein	3	0.00	0.0000	0.03	0.0000	a. a. metabolism
Glyma04g02230.1	Pyridoxal dependent decarboxylase family protein	5	0.02	0.0000	0.01	0.0000	a. a. metabolism
Glyma06g07320.1	cellulose synthase 1	2	0.03	0.0239	0.03	0.0214	cell wall
Glyma06g17050.6	TUDOR SN protein 1	22	0.03	0.0001	0.03	0.0000	RNA
Glyma08g46520.1	cytochrome P450 family 93 subfamily D polypeptide 1	2	0.04	0.0001	0.07	0.0001	misc
Glyma14g38580.1	cinnamate 4 hydroxylase	3	0.04	0.0069	0.05	0.0001	secondary metabolism
Glyma15g42140.1	ATP citrate lyase A 1	6	0.04	0.0000	0.21	0.0000	TCA / org
Glyma18g08220.1	HEAT SHOCK PROTEIN 81.4	16	0.04	0.0000	0.10	0.0000	stress
Glyma05g38510.1	CLPC homologue 1	3	0.05	0.0011	0.20	0.0007	protein
Glyma11g12505.1	cold circadian rhythm and rna binding 2	3	0.05	0.0000	0.10	0.0000	RNA
Glyma14g37440.1	glutamine dependent asparagine synthase 1	3	0.05	0.0000	0.21	0.0000	a. a. metabolism
Glyma16g10730.1	rotamase FKBP 1	6	0.05	0.0000	0.16	0.0000	protein
Glyma17g13770.1	homolog of nucleolar protein NOP56	6	0.05	0.0000	0.09	0.0000	RNA
Glyma18g04745.1	Zn dependent exopeptidases superfamily protein	2	0.05	0.0149	0.16	0.0289	transport
Glyma13g38790.1	DNAJ homologue 2	5	0.06	0.0000	0.16	0.0000	stress
Glyma02g47210.1	HEAT SHOCK PROTEIN 81.4	3	0.07	0.0000	0.18	0.0000	stress
Glyma10g31590.1	methionine gamma lyase	4	0.07	0.0000	0.11	0.0001	a. a. metabolism
Glyma03g36470.1	eukaryotic translation initiation factor 3C	4	0.08	0.0000	0.19	0.0000	protein
Glyma06g19000.1	ATPase AAA type CDC48 protein	9	0.08	0.0000	0.19	0.0000	cell
Glyma08g20020.1	UDP glucose:glycoprotein glucosyltransferases	2	0.08	0.0085	0.25	0.0077	protein
Glyma11g12300.1	Ribosomal protein S25 family protein	3	0.08	0.0000	0.36	0.0000	protein
Glyma11g12471.1	cold circadian rhythm and rna binding 2	2	0.08	0.0000	0.09	0.0000	RNA
Glyma13g43130.1	NADP malic enzyme 4	2	0.08	0.0001	0.06	0.0002	TCA / org
Glyma20g28780.1	Ribosomal protein S3Ae	3	0.08	0.0000	0.16	0.0019	protein
Glyma13g10360.1	methionine gamma lyase	2	0.09	0.0000	0.16	0.0000	a. a. metabolism
Glyma18g43390.1	metallopeptidase M24 family protein	6	0.09	0.0000	0.22	0.0000	protein
Glyma03g07925.1	regulatory particle AAA ATPase 2A	2	0.11	0.0000	0.36	0.0000	protein
Glyma08g03500.1	Class II aminoacyl tRNA and biotin synthetases superfamily protein	2	0.11	0.0000	0.27	0.0000	protein
Glyma09g16690.1	Chaperone protein htpG family protein	3	0.11	0.0000	0.08	0.0000	stress
Glyma18g15100.1	O fucosyltransferase family protein	2	0.11	0.0000	0.13	0.0000	not assigned
Glyma04g40470.1	Ribosomal protein L6 family	2	0.12	0.0000	0.44	0.0048	protein
Glyma20g09810.1	phosphoenolpyruvate carboxylase 3	9	0.12	0.0000	0.26	0.0000	glycolysis
Glyma11g03330.1	stress inducible protein putative	8	0.13	0.0000	0.22	0.0000	stress
Glyma12g10150.1	DNAJ homologue 2	2	0.13	0.0000	0.13	0.0000	stress
Glyma17g37820.1	Chaperone protein htpG family protein	14	0.13	0.0000	0.22	0.0000	stress
Glyma17g09280.1	Ribosomal protein L18e/L15 superfamily protein	4	0.14	0.0000	0.42	0.0002	protein
Glyma08g20230.2	lipoxygenase 1	3	0.15	0.0290	0.25	0.0350	hormone metabolism
Glyma05g33930.1	eukaryotic translation initiation factor 3C	5	0.16	0.0004	0.29	0.0024	protein
Glyma19g35510.1	regulatory particle AAA ATPase 2A	3	0.16	0.0011	0.39	0.0021	protein
Glyma02g37080.1	Class II DAHP synthetase family protein	4	0.17	0.0000	0.43	0.0060	a. a. metabolism
Glyma04g02271.1	sterol methyltransferase 2	5	0.17	0.0000	0.28	0.0000	hormone metabolism
Glyma15g12780.1	farnesyl diphosphate synthase 1	2	0.17	0.0000	0.42	0.0002	secondary metabolism
Glyma08g00770.1	Protein kinase superfamily protein	2	0.18	0.0000	0.30	0.0002	protein
Glyma08g13130.1	evolutionarily conserved C terminal region 2	5	0.18	0.0000	0.12	0.0000	signaling
Glyma08g05570.1	Translation elongation factor EF1A	6	0.19	0.0000	0.38	0.0000	signaling
Glyma12g02520.4	N.D.*	3	0.20	0.0012	0.34	0.0003	not assigned
Glyma18g48620.1	Hyaluronan / mRNA binding family	3	0.20	0.0000	0.17	0.0000	RNA
Glyma13g24090.1	Apoptosis inhibitory protein 5 (API5)	4	0.21	0.0000	0.49	0.0000	development

Glyma16g04960.1	xyloglucan endotransglucosylase/hydrolase 5	3	0.21	0.0000	0.32	0.0000	cell wall
Glyma18g06840.4	glutamine dependent asparagine synthase 1	4	0.21	0.0000	0.15	0.0000	a. a. metabolism
Glyma07g06590.1	ribosomal protein L5 B	3	0.22	0.0000	0.43	0.0000	protein
Glyma17g23900.1	GTP binding Elongation factor Tu family protein	3	0.22	0.0000	0.32	0.0000	protein
Glyma03g07420.4	metallopeptidase M24 family protein	3	0.23	0.0004	0.34	0.0005	protein
Glyma12g03570.1	subtilisin like serine protease 2	3	0.23	0.0038	0.23	0.0159	protein
Glyma13g20680.1	ATPase AAA type CDC48 protein	3	0.23	0.0000	0.27	0.0000	cell
Glyma15g10210.1	Ribosomal protein S11 family protein	4	0.23	0.0000	0.39	0.0000	protein
Glyma07g40300.1	dynamin like 3	2	0.24	0.0005	0.50	0.0000	misc
Glyma11g09990.1	tRNA synthetase class I (I L M and V) family protein	2	0.24	0.0243	0.40	0.0275	protein
Glyma15g21890.1	S adenosylmethionine synthetase family protein	2	0.25	0.0000	0.41	0.0000	metal handling
Glyma06g13360.1	HSP20 like chaperones superfamily protein	2	0.26	0.0103	0.37	0.0226	cell
Glyma17g31280.1	Cytochrome bd ubiquinol oxidase 14kDa subunit	2	0.26	0.0000	0.31	0.0000	mito electron transport
Glyma18g12393.1	citrate synthase 2	9	0.26	0.0000	0.05	0.0000	gluconeogenesis
Glyma20g03060.1	Nuclear transport factor 2 (NTF2) family protein	3	0.26	0.0000	0.41	0.0001	protein
Glyma15g02230.1	NADP malic enzyme 4	7	0.27	0.0000	0.32	0.0000	TCA / org
Glyma15g13650.1	Ribosomal protein S27a / Ubiquitin family protein	2	0.27	0.0080	0.42	0.0000	protein
Glyma19g09960.1	O fucosyltransferase family protein	2	0.27	0.0059	0.25	0.0305	not assigned
Glyma05g27090.1	Protein of unknown function DUF2359 transmembrane	2	0.28	0.0001	0.39	0.0001	not assigned
Glyma15g01370.1	Protein of unknown function DUF642	6	0.28	0.0000	0.22	0.0000	not assigned
Glyma18g14826.1	ATPase AAA type CDC48 protein	2	0.28	0.0000	0.43	0.0000	cell
Glyma06g14360.1	Ribosomal protein L6 family	4	0.29	0.0000	0.42	0.0000	protein
Glyma09g16553.1	Ribosomal L22e protein family	2	0.29	0.0000	0.47	0.0028	protein
Glyma12g08410.2	ATPase AAA type CDC48 protein	2	0.29	0.0000	0.44	0.0006	cell
Glyma18g49189.1	Hyaluronan / mRNA binding family	2	0.29	0.0000	0.33	0.0000	RNA
Glyma19g28200.2	xyloglucan endotransglucosylase/hydrolase 5	5	0.29	0.0000	0.43	0.0000	cell wall
Glyma02g07610.1	xyloglucan endotransglucosylase/hydrolase 5	4	0.31	0.0000	0.49	0.0000	cell wall
Glyma06g48260.1	cellulose synthase like G1	2	0.31	0.0000	0.44	0.0009	cell wall
Glyma17g14370.1	ribosomal protein S13A	2	0.31	0.0000	0.49	0.0000	protein
Glyma17g20430.1	peptidyl prolyl cis trans isomerase	5	0.31	0.0000	0.46	0.0000	cell
Glyma19g21200.1	ATPase AAA type CDC48 protein	4	0.31	0.0000	0.28	0.0000	cell
Glyma19g36120.1	adenosine kinase	2	0.31	0.0000	0.47	0.0003	nucleotide metabolism
Glyma07g34440.1	Ribosomal S17 family protein	3	0.32	0.0045	0.43	0.0125	protein
Glyma19g40810.1	S adenosylmethionine synthetase 2	11	0.32	0.0000	0.45	0.0000	a. a. metabolism
Glyma08g05820.1	Pathogenesis related thaumatin superfamily protein	2	0.33	0.0019	0.27	0.0021	stress
Glyma08g15260.1	Small nuclear ribonucleoprotein family protein	2	0.36	0.0000	0.47	0.0000	RNA
Glyma17g04340.1	S adenosylmethionine synthetase family protein	3	0.36	0.0000	0.48	0.0000	metal handling
Glyma12g30600.1	histone deacetylase 2C	4	0.37	0.0000	0.49	0.0000	RNA
Glyma17g05340.2	histone deacetylase 2C	3	0.37	0.0000	0.45	0.0000	RNA
Glyma19g28740.1	H/ACA ribonucleoprotein complex subunit Gar1/Naf1 protein	2	0.39	0.0000	0.38	0.0000	protein
Glyma20g31790.1	ATP synthase D chain mitochondrial	3	0.40	0.0000	0.45	0.0000	mito electron transport
Glyma16g26300.1	aspartate kinase 1	2	0.41	0.0277	0.43	0.0322	a. a. metabolism
Glyma17g07050.1	P450 reductase 2	4	0.45	0.0000	0.50	0.0000	misc
Glyma11g02410.1	RNA binding Plectin/S10 domain containing protein	3	0.48	0.0000	0.44	0.0000	protein
Glyma04g03860.1	Serine protease inhibitor (SERPIN) family protein	2	2.03	0.0000	2.14	0.0062	protein
Glyma11g37360.1	glyceraldehyde 3 phosphate dehydrogenase C subunit 1	2	2.04	0.0011	2.78	0.0000	glycolysis
Glyma04g06210.1	NAD(P) binding Rossmann fold superfamily protein	2	2.05	0.0382	2.90	0.0000	not assigned
Glyma11g33160.1	UDP glucose pyrophosphorylase 2	6	2.07	0.0000	2.50	0.0000	glycolysis
Glyma19g02031.1	Sugar isomerase (SIS) family protein	2	2.12	0.0034	2.94	0.0000	glycolysis
Glyma20g01106.1	N. D.*	2	2.14	0.0000	4.47	0.0000	not assigned
Glyma02g38730.1	Aldolase superfamily protein	6	2.29	0.0000	2.88	0.0000	glycolysis

Glyma03g03400.1	Plant invertase/pectin methylesterase inhibitor superfamily	3	2.37	0.0008	2.95	0.0000	cell wall
Glyma14g11711.1	Class II aminoacyl tRNA and biotin synthetases superfamily protein	4	2.40	0.0000	3.06	0.0000	protein
Glyma14g13480.1	aspartate aminotransferase 5	2	2.50	0.0065	2.90	0.0001	a. a. metabolism
Glyma15g07720.5	Aluminium induced protein	2	2.58	0.0274	3.81	0.0044	hormone metabolism
Glyma15g17620.1	Peroxidase superfamily protein	3	2.78	0.0001	3.30	0.0000	misc
Glyma17g15690.1	expansin like B1	6	2.83	0.0000	2.56	0.0000	cell wall
Glyma09g29340.1	Kunitz family trypsin and protease inhibitor protein	2	2.88	0.0000	5.00	0.0000	stress
Glyma14g38170.1	Peroxidase superfamily protein	6	3.03	0.0000	2.26	0.0000	misc
Glyma04g39190.1	alcohol dehydrogenase 1	9	3.04	0.0000	4.07	0.0000	fermentation
Glyma19g43150.1	O acetylserine (thiol) lyase (OAS TL) isoform A1	7	3.23	0.0000	3.75	0.0000	a. a. metabolism
Glyma13g34520.1	D mannose binding lectin protein	4	3.54	0.0000	4.37	0.0000	misc
Glyma19g02370.4	SPFH/Band 7/PHB domain containing membrane associated protein family	3	3.76	0.0000	4.73	0.0000	not assigned
Glyma20g17440.1	uricase / urate oxidase / nodulin 35 putative	9	3.80	0.0000	4.18	0.0000	nucleotide metabolism
Glyma18g01510.1	beta ketoacyl reductase 1	9	3.95	0.0000	6.06	0.0000	secondary metabolism
Glyma05g03300.1	SNF1 related protein kinase regulatory subunit	2	4.18	0.0001	4.32	0.0000	cell wall
Glyma13g30490.1	pyruvate decarboxylase 2	10	4.26	0.0000	4.73	0.0000	fermentation
Glyma06g12780.1	alcohol dehydrogenase 1	9	4.27	0.0000	5.55	0.0000	fermentation
Glyma08g08381.1	polygalacturonase inhibiting protein 1	7	4.32	0.0000	5.81	0.0000	cell wall
Glyma18g43460.1	pyruvate decarboxylase 2	8	4.50	0.0000	4.57	0.0000	fermentation
Glyma11g07490.2	isoflavone reductase putative	6	4.79	0.0000	7.36	0.0000	secondary metabolism
Glyma13g34540.1	D mannose binding lectin protein	2	5.04	0.0000	6.11	0.0000	misc
Glyma12g23150.1	Aluminium induced protein	5	6.14	0.0000	7.46	0.0000	hormone metabolism
Glyma05g05420.1	expansin like B1	2	7.28	0.0000	6.73	0.0000	cell wall
Glyma15g07940.1	Rubber elongation factor protein (REF)	3	7.43	0.0000	9.09	0.0000	not assigned
Glyma08g45510.1	Kunitz family trypsin and protease inhibitor protein	2	7.59	0.0000	9.42	0.0000	stress
Glyma05g28310.2	xyloglucan endotransglucosylase/hydrolase 16	3	10.29	0.0000	13.25	0.0000	cell wall
Glyma07g18570.1	Thiamine pyrophosphate dependent pyruvate decarboxylase family protein	5	10.36	0.0000	12.16	0.0000	fermentation
Glyma05g05390.1	expansin like B1	4	11.01	0.0000	19.32	0.0000	cell wall
Glyma05g36310.1	ACC oxidase 1	3	12.01	0.0000	11.20	0.0000	hormone metabolism
Glyma09g29310.1	Kunitz family trypsin and protease inhibitor protein	6	16.36	0.0000	19.68	0.0000	stress
Glyma16g33790.1	Kunitz family trypsin and protease inhibitor protein	2	34.25	0.0000	37.50	0.0000	stress
Glyma11g20940.1	dehydration induced protein (ERD15)	2	35.77	0.0005	47.08	0.0104	stress
Glyma08g08360.1	polygalacturonase inhibiting protein 1	3	42.26	0.0000	36.36	0.0000	cell wall
Glyma09g29200.2	Kunitz family trypsin and protease inhibitor protein	2	63.22	0.0000	100.20	0.0000	stress

Protein ID, according to the Phytozome database; M.P., number of matched peptide; Ratio, relative abundance of protein; Function, functional categorization by MapMan bin codes; N.D.*, no description in the Phytozome database; 4(2)F, 4-day-old soybean subjected to flooding for 2 days; 4(2)F+GA, 4-day-old soybean subjected to flooding with GA_3 for 2 days.

Table 4. Differentailly abundant 192 protein in soybean root between flooding without and with calcium.

Protein ID	Description	M.P.	Ratio 4(2)F	*P*value 4(2)F	Ratio 4(2)F+Ca	*P*value 4(2)F+Ca	Function
Glyma11g15040.3	RNA binding (RRM/RBD/RNP motifs) family	3	0.03	0.0292	0.18	0.0000	RNA
Glyma19g21200.1	ATPase AAA type CDC48 protein	3	0.03	0.0001	0.23	0.0100	cell
Glyma18g14826.1	ATPase AAA type CDC48 protein	2	0.03	0.0236	0.24	0.0366	cell
Glyma01g00740.3	Ribosomal protein L31e family protein	2	0.05	0.0298	0.26	0.0017	protein
Glyma12g08410.2	ATPase AAA type CDC48 protein	8	0.05	0.0000	0.25	0.0009	cell
Glyma11g17930.1	DNAJ homologue 2	3	0.05	0.0091	0.15	0.0087	stress
Glyma12g10150.1	DNAJ homologue 2	3	0.05	0.0091	0.15	0.0087	stress
Glyma06g03050.1	Papain family cysteine protease	2	0.05	0.0016	0.05	0.0014	protein
Glyma05g04220.1	stress inducible protein putative	3	0.06	0.0024	0.28	0.0330	stress
Glyma17g14660.1	stress inducible protein putative	3	0.06	0.0024	0.28	0.0330	stress
Glyma20g35120.5	*S*-adenosyl-*L*-methionine dependent methyltransferases superfamily protein	3	0.06	0.0053	0.24	0.0192	stress
Glyma07g00620.1	*O*-fucosyltransferase family protein	7	0.06	0.0000	0.17	0.0000	not assigned
Glyma01g42010.3	stress inducible protein putative	9	0.06	0.0000	0.42	0.0002	stress
Glyma19g09960.1	*O*-fucosyltransferase family protein	3	0.07	0.0000	0.22	0.0000	not assigned
Glyma08g45425.1	eukaryotic translation initiation factor 4G	2	0.09	0.0032	0.18	0.0097	protein
Glyma02g47330.1	seed imbibition 1	3	0.09	0.0017	0.12	0.0011	minor CHO metabolism
Glyma09g08120.1	Subtilase family protein	2	0.10	0.0000	0.13	0.0001	protein
Glyma05g31830.1	copper ion binding	5	0.10	0.0000	0.26	0.0005	not assigned
Glyma08g15100.1	copper ion binding	5	0.10	0.0000	0.26	0.0005	not assigned
Glyma14g34990.1	copper ion binding	5	0.10	0.0000	0.26	0.0005	not assigned
Glyma05g37150.1	vacuolar H ATPase subunit E isoform 3	2	0.10	0.0164	0.19	0.0422	transport
Glyma05g37160.1	vacuolar H ATPase subunit E isoform 3	2	0.10	0.0164	0.19	0.0422	transport
Glyma05g37190.1	vacuolar H ATPase subunit E isoform 3	2	0.10	0.0164	0.19	0.0422	transport
Glyma08g02390.1	vacuolar ATP synthase subunit E1	2	0.10	0.0164	0.19	0.0422	transport
Glyma17g09120.1	tRNA synthetase beta subunit family protein	3	0.10	0.0018	0.23	0.0111	protein
Glyma14g01430.1	seed imbibition 1	4	0.10	0.0001	0.16	0.0002	minor CHO metabolism
Glyma04g02271.1	sterol methyltransferase 2	3	0.10	0.0087	0.24	0.0120	hormone metabolism
Glyma04g07220.1	cellulose synthase 1	3	0.11	0.0048	0.23	0.0302	cell wall
Glyma06g07320.1	cellulose synthase 1	3	0.11	0.0048	0.23	0.0302	cell wall
Glyma08g46520.1	cytochrome P450 family 93 subfamily D polypeptide 1	2	0.11	0.0444	0.33	0.0334	misc
Glyma18g48620.1	Hyaluronan / mRNA binding family	6	0.12	0.0000	0.18	0.0000	RNA
Glyma18g12660.1	rhamnose biosynthesis 1	10	0.12	0.0043	0.17	0.0001	cell wall
Glyma03g07420.4	metallopeptidase M24 family protein	5	0.13	0.0013	0.35	0.0066	protein
Glyma05g07640.3	tRNA synthetase beta subunit family protein	4	0.13	0.0007	0.30	0.0109	protein
Glyma09g37850.1	Hyaluronan / mRNA binding family	4	0.13	0.0002	0.15	0.0003	RNA
Glyma08g23770.1	*O*-fucosyltransferase family protein	5	0.13	0.0000	0.31	0.0000	not assigned
Glyma17g05340.2	histone deacetylase 2C	3	0.13	0.0001	0.14	0.0001	RNA
Glyma08g46090.2	rotamase FKBP 1	2	0.13	0.0065	0.12	0.0003	protein
Glyma18g32830.1	rotamase FKBP 1	2	0.13	0.0065	0.12	0.0003	protein
Glyma10g32471.1	*S*-adenosyl-Lmethionine dependent methyltransferases superfamily protein	4	0.14	0.0052	0.30	0.0154	stress
Glyma02g02140.1	Ribosomal L22e protein family	5	0.14	0.0000	0.49	0.0216	protein
Glyma10g02270.1	Ribosomal L22e protein family	5	0.14	0.0000	0.49	0.0216	protein
Glyma19g42090.2	Ribosomal L22e protein family	4	0.14	0.0000	0.39	0.0031	protein
Glyma01g29420.1	metallopeptidase M24 family protein	5	0.14	0.0001	0.32	0.0015	protein
Glyma02g40310.1	eukaryotic translation initiation factor 2 beta subunit	5	0.15	0.0001	0.26	0.0001	protein
Glyma09g16553.1	Ribosomal L22e protein family	2	0.15	0.0023	0.31	0.0126	protein
Glyma20g38080.1	Ribosomal L22e protein family	4	0.15	0.0000	0.40	0.0060	protein
Glyma12g30600.1	histone deacetylase 2C	3	0.16	0.0000	0.17	0.0000	RNA

Glyma16g19311.1	metallopeptidase M24 family protein	3	0.16	0.0025	0.35	0.0189	protein
Glyma14g38600.1	eukaryotic translation initiation factor 2 beta subunit	5	0.17	0.0000	0.26	0.0060	protein
Glyma03g01550.1	Remorin family protein	2	0.17	0.0222	0.35	0.0492	RNA
Glyma05g20930.1	Granulin repeat cysteine protease family protein	2	0.17	0.0025	0.10	0.0044	protein
Glyma16g16290.1	Granulin repeat cysteine protease family protein	2	0.17	0.0025	0.10	0.0044	protein
Glyma17g18440.1	Granulin repeat cysteine protease family protein	2	0.17	0.0025	0.10	0.0044	protein
Glyma04g03020.1	Papain family cysteine protease	2	0.18	0.0130	0.17	0.0131	protein
Glyma10g06480.1	ATPase AAA type CDC48 protein	20	0.18	0.0000	0.29	0.0000	cell
Glyma13g20680.1	ATPase AAA type CDC48 protein	20	0.18	0.0000	0.32	0.0000	cell
Glyma09g02160.1	glycosyl hydrolase 9A1	5	0.19	0.0003	0.48	0.0043	cell wall
Glyma13g05520.4	RNA binding KH domain containing protein	2	0.19	0.0018	0.32	0.0097	RNA
Glyma19g02840.2	RNA binding KH domain containing protein	2	0.19	0.0018	0.32	0.0097	RNA
Glyma04g08540.1	La protein 1	2	0.20	0.0012	0.41	0.0051	RNA
Glyma09g37070.3	RNA binding KH domain containing protein	3	0.20	0.0012	0.38	0.0189	RNA
Glyma18g49600.2	RNA binding KH domain containing protein	3	0.20	0.0012	0.38	0.0189	RNA
Glyma19g36740.1	ATPase AAA type CDC48 protein	21	0.21	0.0000	0.33	0.0000	cell
Glyma06g02290.1	Pyridoxal dependent decarboxylase family protein	11	0.22	0.0013	0.13	0.0018	a.a. metabolism
Glyma03g33990.1	ATPase AAA type CDC48 protein	21	0.22	0.0000	0.33	0.0000	cell
Glyma13g39830.1	ATPase AAA type CDC48 protein	16	0.24	0.0000	0.34	0.0001	cell
Glyma04g02230.1	Pyridoxal dependent decarboxylase family protein	10	0.24	0.0035	0.20	0.0361	a.a. metabolism
Glyma02g01170.1	*S*-adenosylmethionine synthetase 2	4	0.25	0.0000	0.22	0.0000	a.a. metabolism
Glyma07g36150.1	*S*-adenosylmethionine synthetase family protein	12	0.25	0.0000	0.25	0.0000	metal handling
Glyma17g04330.1	*S*-adenosylmethionine synthetase family protein	12	0.25	0.0000	0.25	0.0000	metal handling
Glyma17g04340.1	*S*-adenosylmethionine synthetase family protein	12	0.25	0.0000	0.25	0.0000	metal handling
Glyma11g20060.2	ATPase AAA type CDC48 protein	12	0.25	0.0000	0.35	0.0002	cell
Glyma16g26630.1	xyloglucan endotransglucosylase/hydrolase 5	4	0.25	0.0000	0.34	0.0000	cell wall
Glyma01g38320.1	Tyrosyl tRNA synthetase class Ib bacterial/mitochondrial	2	0.26	0.0017	0.43	0.0354	protein
Glyma12g30060.1	ATPase AAA type CDC48 protein	15	0.26	0.0000	0.38	0.0002	cell
Glyma1337s00200.1	*S*-adenosylmethionine synthetase 2	11	0.26	0.0000	0.27	0.0000	a.a. metabolism
Glyma17g00710.1	glycosyl hydrolase 9A1	4	0.26	0.0004	0.38	0.0027	cell wall
Glyma13g33960.1	rhamnose biosynthesis 1	14	0.26	0.0000	0.35	0.0001	cell wall
Glyma07g40090.1	glycosyl hydrolase 9A1	3	0.27	0.0006	0.34	0.0024	cell wall
Glyma07g13900.1	Hyaluronan / mRNA binding family	4	0.28	0.0002	0.42	0.0002	RNA
Glyma09g23330.1	UDP glucosyl transferase 88A1	2	0.28	0.0340	0.35	0.0468	misc
Glyma09g23600.1	UDP glucosyl transferase 88A1	2	0.28	0.0340	0.35	0.0468	misc
Glyma16g29340.1	UDP glucosyl transferase 88A1	2	0.28	0.0340	0.35	0.0468	misc
Glyma16g29370.1	UDP glucosyl transferase 88A1	2	0.28	0.0340	0.35	0.0468	misc
Glyma05g03140.1	adenylate kinase 1	6	0.28	0.0000	0.32	0.0000	nucleotide metabolism
Glyma13g20480.1	methionine adenosyltransferase 3	5	0.29	0.0000	0.37	0.0000	a.a. metabolism
Glyma17g13760.1	adenylate kinase 1	7	0.29	0.0000	0.33	0.0000	nucleotide metabolism
Glyma05g31950.2	Small nuclear ribonucleoprotein family protein	3	0.30	0.0149	0.40	0.0268	RNA
Glyma08g15260.1	Small nuclear ribonucleoprotein family protein	3	0.30	0.0149	0.40	0.0268	RNA
Glyma10g06170.1	methionine adenosyltransferase 3	5	0.30	0.0000	0.36	0.0000	a.a. metabolism
Glyma10g28500.1	*S*-adenosylmethionine synthetase 2	7	0.31	0.0000	0.26	0.0000	a.a. metabolism
Glyma20g21431.1	Ribosomal protein L14p/L23e family protein	2	0.31	0.0052	0.33	0.0116	protein
Glyma20g03060.1	Nuclear transport factor 2 (NTF2) family protein with RNA binding (RRM RBD RNP motifs) domain	7	0.31	0.0000	0.33	0.0000	protein
Glyma01g41990.1	Glycosyl hydrolases family 32 protein	8	0.31	0.0000	0.46	0.0014	major CHO metabolism
Glyma15g06020.1	3-deoxy-d-arabino heptulosonate 7 phosphate synthase	2	0.32	0.0034	0.46	0.0142	a.a. metabolism
Glyma15g27510.3	rhamnose biosynthesis 1	9	0.32	0.0000	0.38	0.0018	cell wall
Glyma10g35381.1	phenylalanine ammonia lyase 2	4	0.32	0.0033	0.35	0.0003	secondary metabolism

Glyma20g32135.1	phenylalanine ammonia lyase 2	4	0.32	0.0033	0.35	0.0003	secondary metabolism
Glyma16g34120.1	Translation initiation factor eIF3 subunit	5	0.33	0.0007	0.50	0.0028	protein
Glyma07g35200.1	Nuclear transport factor 2 (NTF2) family protein with RNA binding (RRM RBD RNP motifs) domain	4	0.33	0.0000	0.37	0.0000	protein
Glyma08g15680.1	rhamnose biosynthesis 1	8	0.33	0.0001	0.40	0.0029	cell wall
Glyma09g36870.1	Transducin/WD40 repeat like superfamily protein	4	0.34	0.0045	0.49	0.0167	protein
Glyma05g32670.3	*S*-adenosyl-L-methionine dependent methyltransferases superfamily protein	2	0.34	0.0440	0.37	0.0497	stress
Glyma08g00320.1	*S*-adenosyl-L-methionine dependent methyltransferases superfamily protein	2	0.34	0.0440	0.37	0.0497	stress
Glyma03g21690.1	rotamase FKBP 1	5	0.34	0.0181	0.35	0.0040	protein
Glyma20g04830.1	Ribonuclease T2 family protein	3	0.35	0.0350	0.39	0.0380	RNA
Glyma06g46350.1	SKU5 similar 5	7	0.35	0.0000	0.28	0.0000	not assigned
Glyma12g10420.1	SKU5 similar 5	6	0.36	0.0000	0.28	0.0000	not assigned
Glyma02g24818.1	Adenylate kinase family protein	2	0.36	0.0095	0.36	0.0078	nucleotide metabolism
Glyma03g33880.1	PHE ammonia lyase 1	5	0.36	0.0067	0.41	0.0012	secondary metabolism
Glyma03g33890.1	PHE ammonia lyase 1	5	0.36	0.0067	0.41	0.0012	secondary metabolism
Glyma15g01370.1	Protein of unknown function DUF642	7	0.38	0.0000	0.38	0.0000	not assigned
Glyma05g22060.1	Subtilase family protein	7	0.38	0.0000	0.47	0.0000	protein
Glyma18g49189.1	Hyaluronan / mRNA binding family	3	0.38	0.0037	0.47	0.0078	RNA
Glyma11g11410.1	subtilisin like serine protease 2	4	0.39	0.0446	0.35	0.0273	protein
Glyma13g43970.1	Protein of unknown function DUF642	8	0.39	0.0000	0.37	0.0000	not assigned
Glyma19g36620.1	PHE ammonia lyase 1	6	0.39	0.0066	0.42	0.0017	secondary metabolism
Glyma04g42460.1	2-oxoglutarate (2OG) and Fe(II) dependent oxygenase superfamily protein	4	0.39	0.0008	0.44	0.0202	hormone metabolism
Glyma05g33860.1	Pathogenesis related thaumatin superfamily protein	2	0.40	0.0163	0.46	0.0081	stress
Glyma08g05820.1	Pathogenesis related thaumatin superfamily protein	2	0.40	0.0163	0.46	0.0081	stress
Glyma17g17850.1	Subtilase family protein	9	0.41	0.0000	0.43	0.0000	protein
Glyma04g05460.1	N.D.*	2	0.41	0.0312	0.20	0.0088	not assigned
Glyma06g05490.1	N.D.*	2	0.41	0.0312	0.20	0.0088	not assigned
Glyma18g33150.1	Nuclear transport factor 2 (NTF2) family protein	3	0.43	0.0452	0.48	0.0424	protein
Glyma12g03070.1	nucleolin like 2	11	0.44	0.0000	0.44	0.0000	protein
Glyma08g11650.1	Chalcone and stilbene synthase family protein	4	0.44	0.0191	0.33	0.0004	secondary metabolism
Glyma14g17930.1	serine/arginine rich 22	2	0.45	0.0141	0.45	0.0107	RNA
Glyma17g29080.1	serine/arginine rich 22	2	0.45	0.0141	0.45	0.0107	RNA
Glyma04g40760.1	CTC interacting domain 11	4	0.46	0.0268	0.42	0.0003	RNA
Glyma06g14030.1	CTC interacting domain 11	4	0.46	0.0268	0.42	0.0003	RNA
Glyma08g11520.1	Chalcone and stilbene synthase family protein	5	0.46	0.0244	0.45	0.0013	secondary metabolism
Glyma04g40750.2	CTC interacting domain 11	4	0.47	0.0241	0.42	0.0004	RNA
Glyma06g14050.3	CTC interacting domain 11	4	0.47	0.0241	0.42	0.0004	RNA
Glyma13g43630.1	heat shock protein 91	24	0.48	0.0006	0.48	0.0000	stress
Glyma07g00540.1	D-3-phosphoglycerate dehydrogenase	7	0.48	0.0107	0.43	0.0122	a.a. metabolism
Glyma08g23860.1	D-3-phosphoglycerate dehydrogenase	7	0.48	0.0107	0.43	0.0122	a.a. metabolism
Glyma04g11160.3	N.D.*	3	0.49	0.0002	0.49	0.0402	not assigned
Glyma06g10930.2	N.D.*	3	0.49	0.0002	0.49	0.0402	not assigned
Glyma03g33830.1	Pyridoxal dependent decarboxylase family protein	6	0.49	0.0084	0.35	0.0009	a.a. metabolism
Glyma13g07220.1	glycosyl hydrolase family 81 protein	3	2.32	0.0149	2.30	0.0000	stress
Glyma13g05120.3	SPFH/Band 7/PHB domain containing membrane associated protein family	5	2.56	0.0152	2.84	0.0002	not assigned
Glyma09g04337.1	beta xylosidase 1	3	2.66	0.0052	5.11	0.0000	cell wall
Glyma15g15370.1	beta xylosidase 1	3	2.66	0.0052	5.11	0.0000	cell wall
Glyma01g34410.2	AT hook motif nuclear localized protein 1	3	2.66	0.0435	5.69	0.0421	RNA
Glyma03g02670.5	AT hook motif nuclear localized protein 1	3	2.66	0.0435	5.69	0.0421	RNA
Glyma15g08840.1	xylem bark cysteine peptidase 3	2	2.78	0.0046	5.80	0.0149	protein
Glyma17g03350.1	N.D.*	7	2.79	0.0226	3.62	0.0000	not assigned

Glyma09g40520.5	AT hook motif nuclear localized protein 1	2	2.89	0.0208	6.08	0.0324	RNA
Glyma07g11810.1	glutamine synthase clone R1	7	3.04	0.0441	5.10	0.0022	N-metabolism
Glyma10g28900.1	Adenine nucleotide alpha hydrolases	4	3.13	0.0318	3.67	0.0079	hormone metabolism
Glyma20g23090.1	Adenine nucleotide alpha hydrolases	4	3.13	0.0318	3.67	0.0079	hormone metabolism
Glyma19g02370.4	SPFH/Band 7/PHB domain containing membrane associated protein family	4	3.14	0.0151	4.57	0.0044	not assigned
Glyma08g08520.1	FAD binding Berberine family protein	7	3.60	0.0011	3.95	0.0000	misc
Glyma03g39440.1	Calcineurin like metallo phosphoesterase superfamily protein	2	3.82	0.0452	3.55	0.0021	protein
Glyma19g42050.1	Calcineurin like metallo phosphoesterase superfamily protein	2	3.82	0.0452	3.55	0.0021	protein
Glyma09g24410.1	heat shock protein 90.1	7	3.93	0.0026	8.46	0.0106	stress
Glyma16g29750.1	heat shock protein 90.1	7	3.93	0.0026	8.46	0.0106	stress
Glyma01g34770.1	xyloglucan endotransglucosylase/hydrolase 26	2	3.98	0.0446	2.42	0.0004	cell wall
Glyma09g32630.1	xyloglucan endotransglucosylase/hydrolase 26	2	3.98	0.0446	2.42	0.0004	cell wall
Glyma05g25540.1	FAD binding Berberine family protein	5	4.07	0.0004	3.95	0.0002	misc
Glyma13g34520.1	D-mannose binding lectin protein	6	4.13	0.0153	6.98	0.0000	misc
Glyma19g39270.2	Peroxidase superfamily protein	4	4.15	0.0051	5.27	0.0186	misc
Glyma15g15200.1	Glycosyl hydrolase superfamily protein	4	4.41	0.0011	7.25	0.0039	misc
Glyma13g00790.1	Peroxidase superfamily protein	9	4.59	0.0239	4.60	0.0003	misc
Glyma17g06890.1	Peroxidase superfamily protein	9	4.59	0.0239	4.60	0.0003	misc
Glyma09g16690.1	Chaperone protein htpG family protein	4	5.03	0.0120	18.18	0.0071	stress
Glyma01g04350.1	Matrixin family protein	3	5.89	0.0011	4.52	0.0067	protein
Glyma09g04191.1	Glycosyl hydrolase superfamily protein	2	6.08	0.0011	17.27	0.0359	misc
Glyma02g00490.1	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein	5	6.25	0.0033	3.54	0.0369	protein
Glyma08g28800.1	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein	5	6.25	0.0033	3.54	0.0369	protein
Glyma18g51660.1	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein	5	6.25	0.0033	3.54	0.0369	proteinL30
Glyma15g07940.1	Rubber elongation factor protein (REF)	4	6.80	0.0051	10.10	0.0093	not assigned
Glyma18g43460.1	pyruvate decarboxylase 2	11	6.87	0.0265	6.28	0.0000	fermentation
Glyma09g28100.1	Enolase	17	8.18	0.0000	6.30	0.0000	glycolysis
Glyma03g36610.1	Peroxidase superfamily protein	3	8.25	0.0046	8.03	0.0015	misc
Glyma07g18570.1	Thiamine pyrophosphate dependent pyruvate decarboxylase family protein	13	11.90	0.0027	8.10	0.0000	fermentation
Glyma17g07250.1	xyloglucan endotransglycosylase 6	5	12.45	0.0211	4.09	0.0001	cell wall
Glyma13g31390.1	Rubber elongation factor protein (REF)	2	12.85	0.0036	19.21	0.0109	not assigned
Glyma02g16710.1	Eukaryotic aspartyl protease family protein	7	13.70	0.0098	10.91	0.0000	protein
Glyma15g39370.2	glyoxalase II 3	4	13.71	0.0180	19.00	0.0001	biodegradation of Xenobiotics
Glyma11g35600.1	Eukaryotic translation initiation factor 2	5	13.75	0.0010	17.46	0.0029	protein
Glyma18g02820.1	Eukaryotic translation initiation factor 2	5	13.75	0.0010	17.46	0.0029	protein
Glyma05g28310.2	xyloglucan endotransglucosylase/hydrolase 16	4	13.98	0.0188	4.18	0.0001	cell wall
Glyma05g36310.1	ACC oxidase 1	6	17.65	0.0022	11.72	0.0000	hormone metabolism
Glyma08g08360.1	polygalacturonase inhibiting protein 1	6	17.68	0.0016	9.61	0.0000	cell wall
Glyma04g09670.1	Rhamnogalacturonate lyase family protein	6	17.72	0.0069	15.56	0.0002	cell wall
Glyma16g33760.1	Kunitz family trypsin and protease inhibitor protein	3	24.95	0.0327	12.27	0.0240	stress
Glyma12g23150.1	Aluminium induced protein with YGL and LRDR motifs	6	26.96	0.0066	14.12	0.0006	hormone metabolism
Glyma11g08260.1	Rhodanese/Cell cycle control phosphatase superfamily protein	3	42.21	0.0001	31.05	0.0334	protein
Glyma09g29330.1	Kunitz family trypsin and protease inhibitor protein	6	121.70	0.0037	46.15	0.0002	stress
Glyma09g27700.1	Concanavalin A like lectin protein kinase family protein	5	461.87	0.0001	406.84	0.0000	signaling

Protein ID, according to the Phytozome database; M.P., number of matched peptide; Ratio, relative abundance of protein; Function, functional categorization by MapMan bin codes; N.D.*, no description in the Phytozome database; 4(2)F, 4-day-old soybean subjected to flooding for 2 days; 4(2)F+Ca, 4-day-old soybean subjected to flooding with calcium for 2 days.

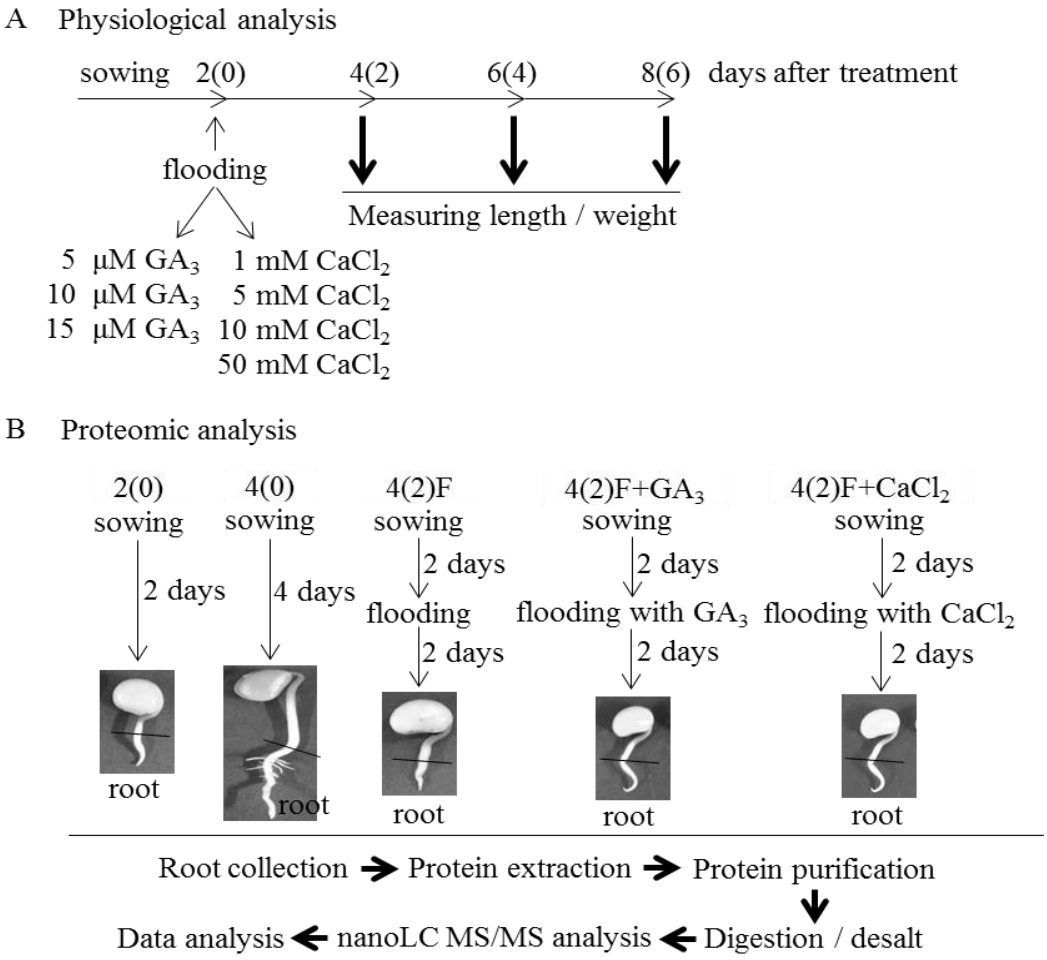

Figure 4. Experimental design for chapter 1. For physiological analysis, 2-day-old soybeans were flooded with GA_3 or $CaCl_2$ for 6 days (A). For proteomic analysis, 2-day-old soybeans were flooded with GA_3 or $CaCl_2$ for 2 days and root was collected (B). Untreated soybeans were collected as control. Three independent experiments were performed as biological replicates.

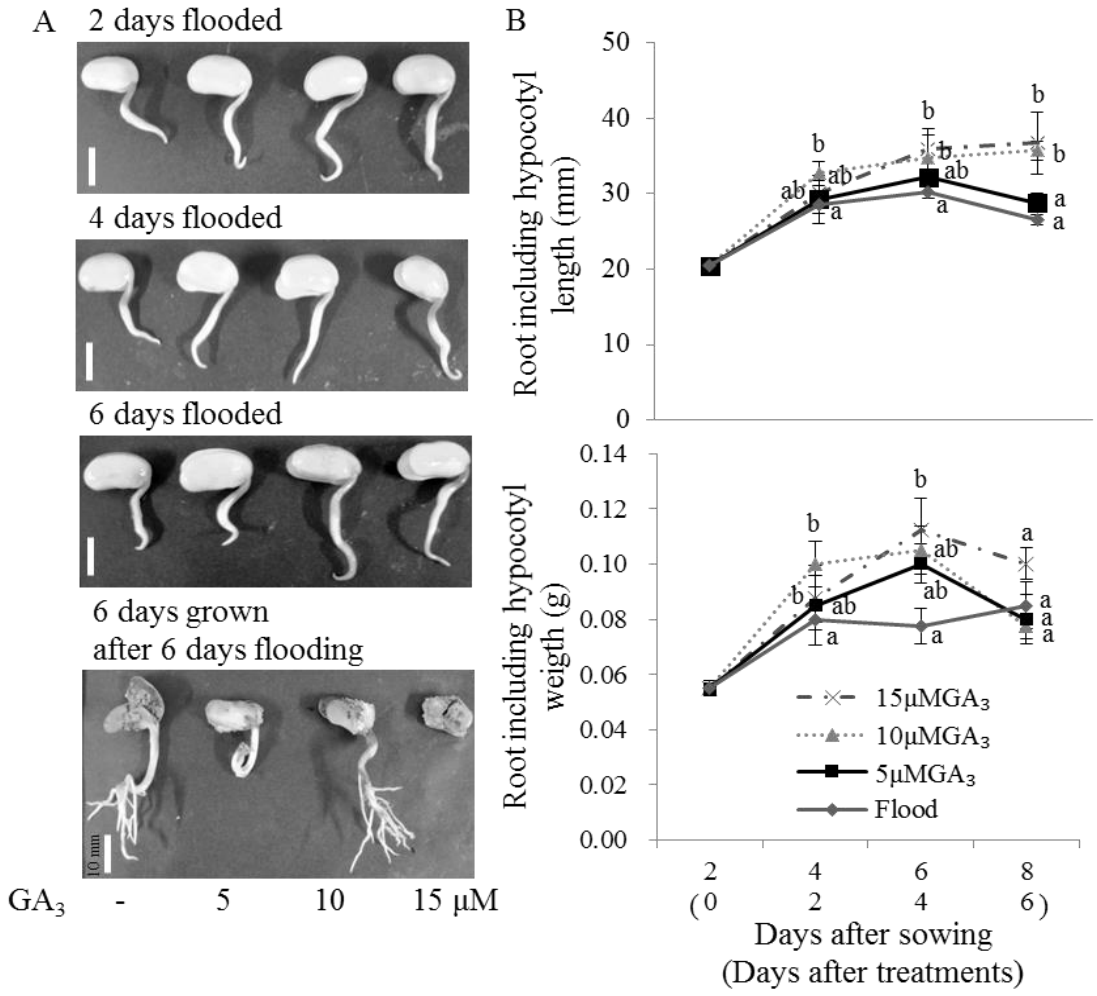

Figure 5. Effect of GA_3 on growth of soybean seedlings under flooding stress. Two-day-old soybeans were exposed to flooding stress in the absence and presence of 5, 10, and 15 μM GA_3. Photographs show soybean seedlings after 2, 4, and 6 days of flooding and then after an additional 6 days of growth under normal condition (A). Bars indicate 10 mm. Length and weight of roots including the hypocotyl were measured at the indicated time points (B). Data are means ± S.E. from 3 independent biological replications. Means with the same latter are not significantly different according to Tukey's multiple comparison test ($p < 0.05$).

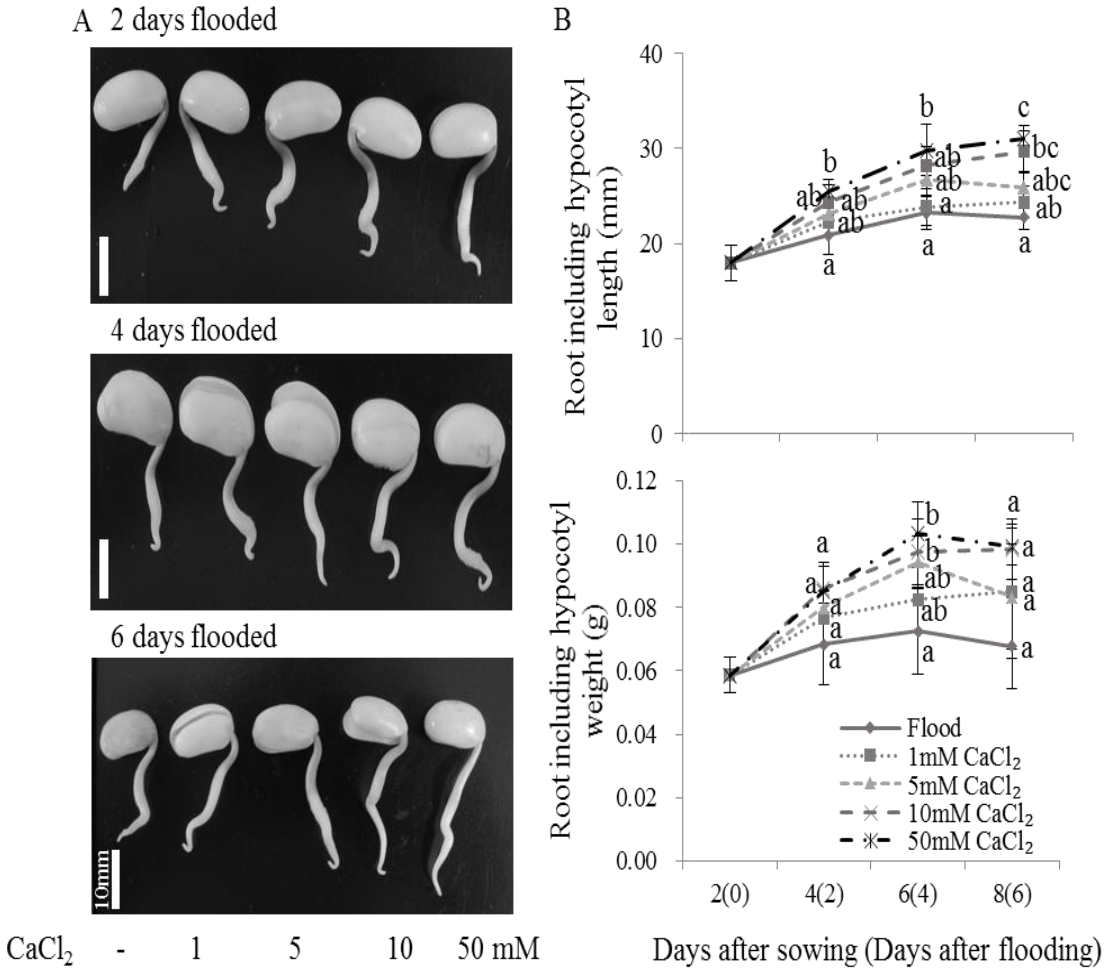

Figure 6. Effect of calcium on growth of soybean seedlings under flooding stress. Two-day-old soybeans were flooded without or with 1, 5, 10, and 50 mM $CaCl_2$ for 2, 4, and 6 days. Photographs show soybean seedlings after 2, 4, and 6 days of flooding (A). Bars indicate 10 mm. Length and weight of roots including the hypocotyl were measured at the indicated time points (B). Data are means ± S.E. from 3 independent biological replications. Means with the same latter are not significantly different according to Tukey's multiple comparison test ($p < 0.05$).

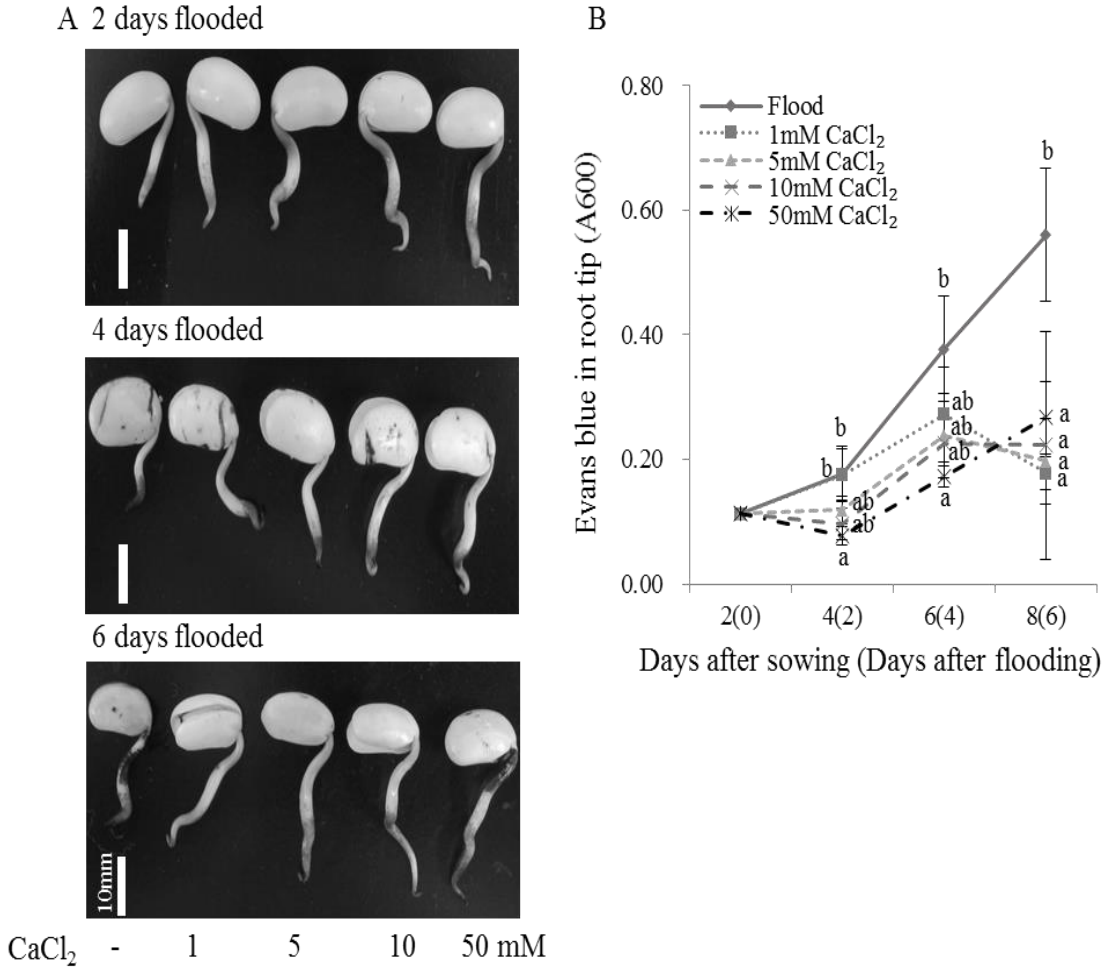

Figure 7. Evaluation of cell death in flooding-stressed soybean root tips treated with calcium. Two-day-old soybeans were flooded without or with 1, 5, 10, and 50 mM $CaCl_2$ for 2, 4, and 6 days. The roots were stained with 0.25% Evans blue dye, which was then extreacted, and measured spectroscopically at 600 nm. Photographs show soybean seedlings after 2, 4, and 6 days of flooding (A). Bars indicate 10 mm. Absorbance of Evans blue in root tips at the indicated time points (B). Data are means ± S.E. from 3 independent biological replications. Means with the same latter are not significantly different according to Tukey's multiple comparison test ($p < 0.05$).

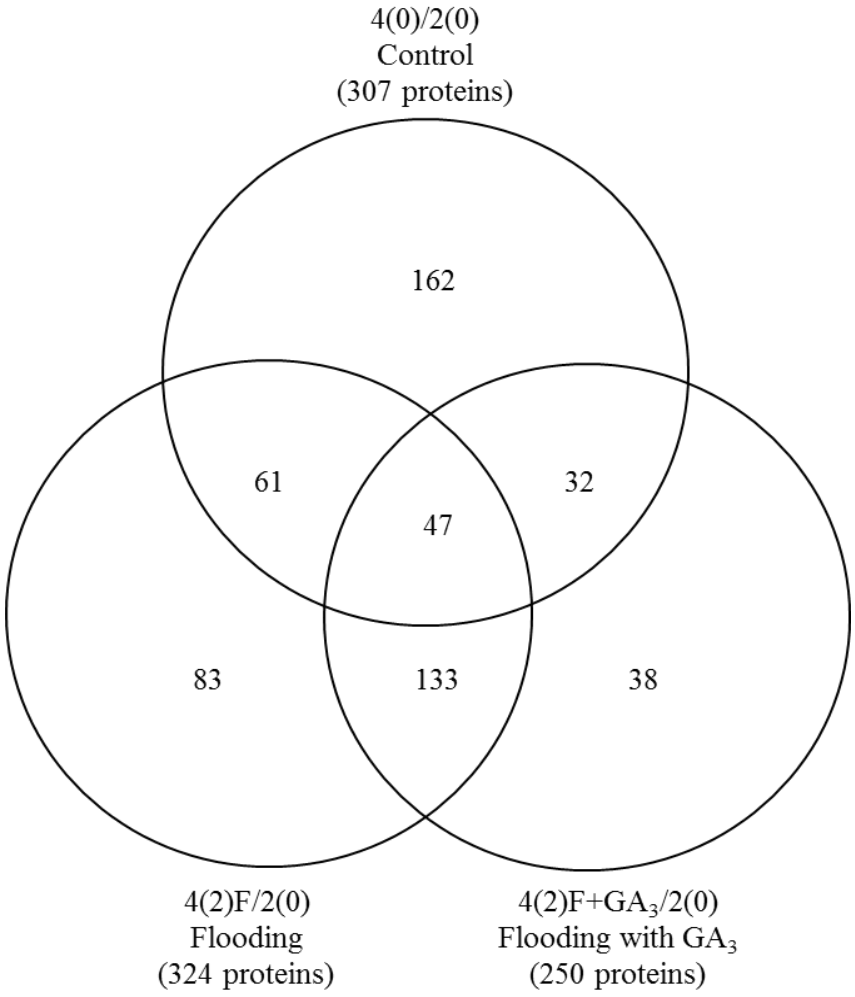

Figure 8. Venn diagram of changed proteins identified from soybean roots under flooding without or with GA_3. Two-day-old soybeans were flooded without or with 10 µM GA_3 for 2 days, and proteins were extracted from roots and analyzed by gel-free proteomics. The Venn diagram shows the number of differentially changed proteins in root of 4-day-old soybeans without treatment or with 2-day-flooding without and with GA_3. The overlapping proteins denote common proteins among untreated and flooded soybeans without or with GA_3. The numbers represent identified proteins in this study.

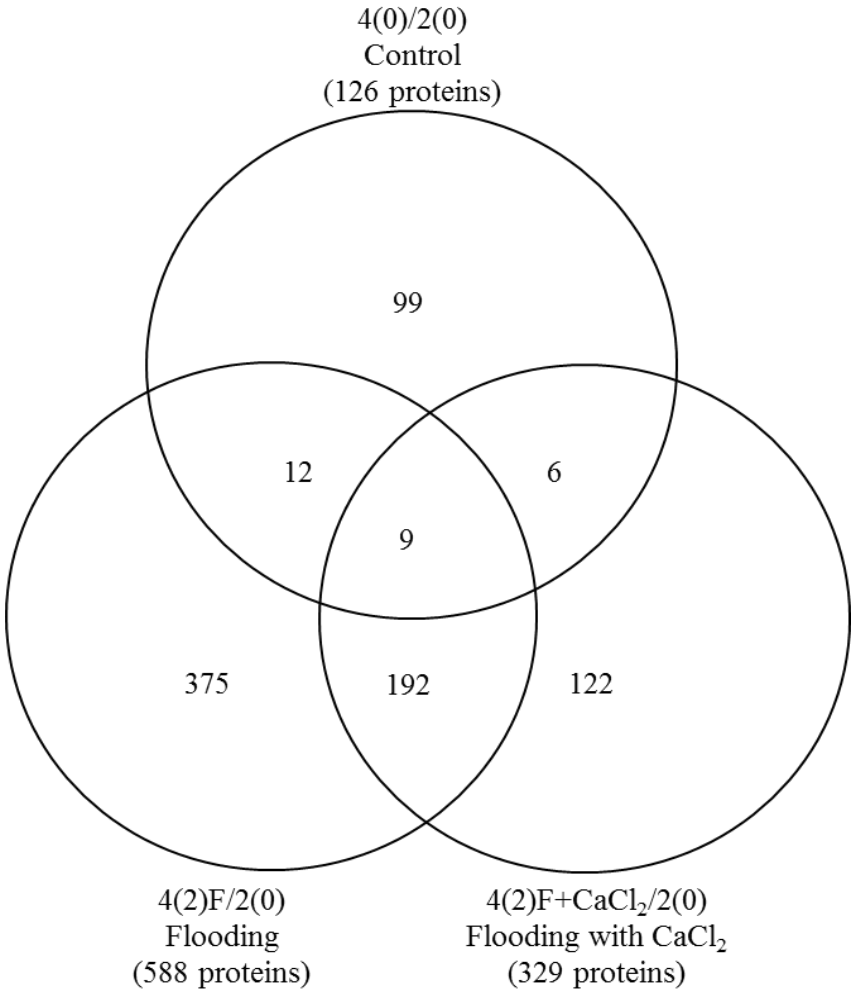

Figure 9. Venn diagram of changed proteins identified from soybean roots under flooding without or with calcium. Two-day-old soybeans were flooded without or with 50 mM $CaCl_2$ for 2 days, and proteins were extracted from roots and analyzed by gel-free proteomics. The Venn diagram shows the number of differentially changed proteins in root of 4-day-old soybeans without treatment or with 2-day-flooding without and with $CaCl_2$. The overlapping proteins denote common proteins among untreated and flooded soybeans without or with $CaCl_2$. The numbers represent identified proteins in this study.

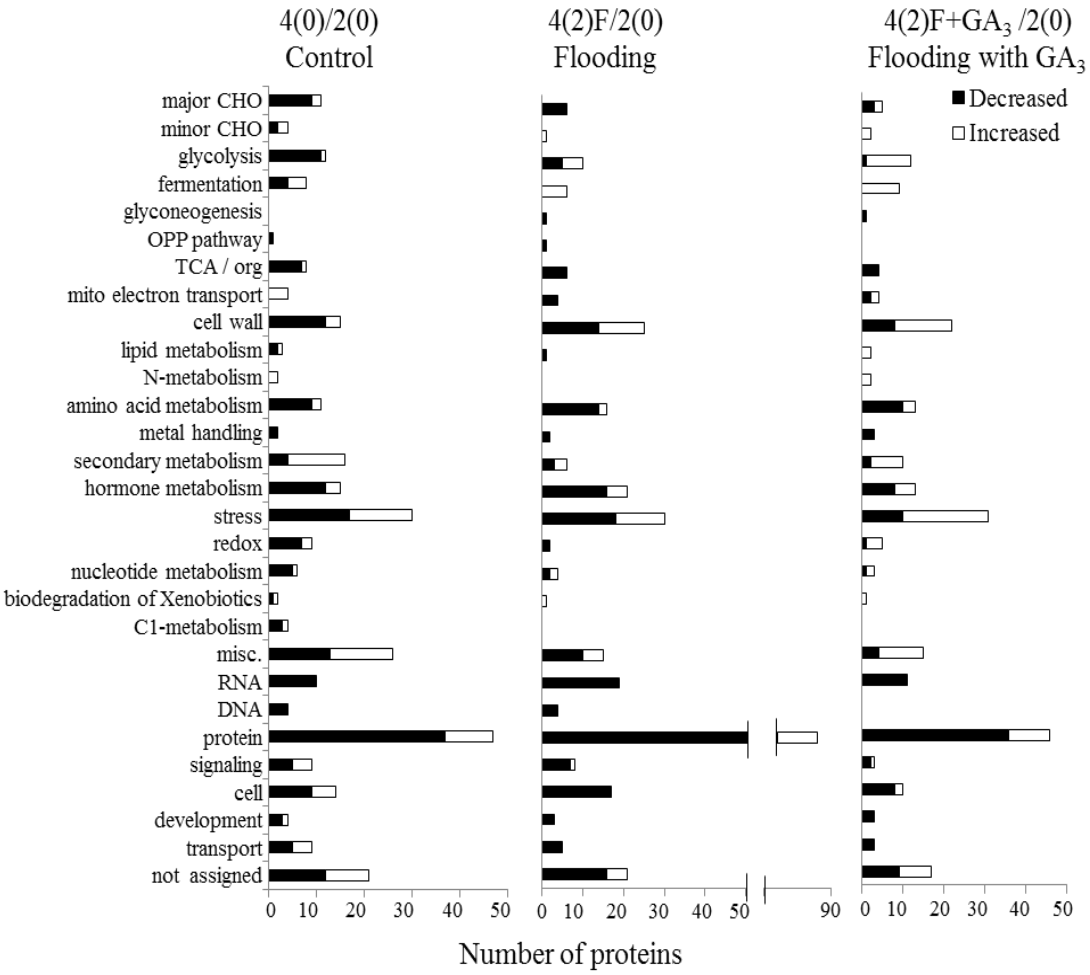

Figure 10. Functional categorization of identified proteins from soybean roots treated with GA_3. Identified proteins were categorized using MapMan bin codes: CHO, carbohydrates; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid; misc, miscellaneous; protein, protein synthesis/targeting/degradation/posttranslational modification; RNA, RNA processing/binding; DNA, DNA synthesis; C1, one carbon. Numbers of differentially abundant proteins are indicated on the x-axis in the graph. Black and white bars indicate decreased and increased proteins, respectively.

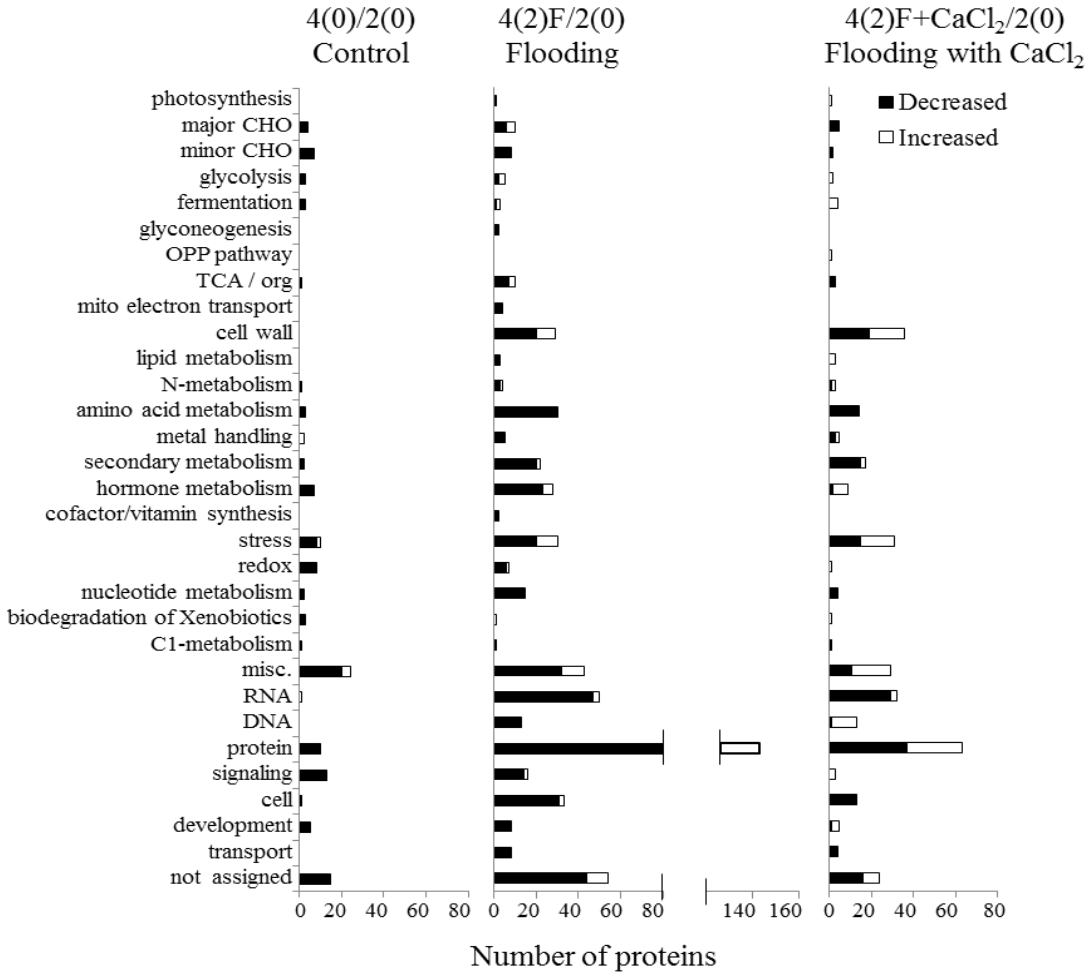

Figure 11. Functional categorization of identified proteins from soybean roots treated with calcium. Identified proteins were categorized using MapMan bin codes: CHO, carbohydrates; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid; misc, miscellaneous; protein, protein synthesis/targeting/degradation/posttranslational modification; RNA, RNA processing/binding; DNA, DNA synthesis; C1, one carbon. Numbers of differentially abundant proteins are indicated on the x-axis in the graph. Black and white bars indicate decreased and increased proteins, respectively.

Chapter 2.

Characterization of root proteins in soybean under flooding and drought stresses

2.1. Introduction

The frequency of flooding events caused by heavy rainfall has been increasing over the past six decades as a consequence of climate change in the globe (Bailey-Serres et al., 2012). It was estimated that flooding and/or waterlogging affected approximately 16% of crop-producing areas in the world (Boyer, 1982). Most terrestrial plants are severely affected by flooding because flooding limits the growth and survival ratio of plant (Bailey-Serres and Voesenek, 2008). Flooding mainly restricts the oxygen availability as gas slowly diffuses in water as compared to air (Dat et al, 2004). Flooding-induced hypoxic conditions restrict mitochondrial oxidative phosphorylation, which leads to energy crisis (Bailey-Serres and Voesenek, 2008), and accumulate ROS in plants (Colmer and Voesenek, 2009). Plant root is most subjected to flooding stress and initially suffers from oxygen deficiency (Sauter, 2013) as flooding influences the underground part of plant. Thus, flooding leads to the inhibition of root respiration and a marked reduction in the energy status of root cells (Ashraf, 2012). In addition to reduction of oxygen level, flooding results in the increases in carbon dioxide levels around the root system, and as a result, suppresses nitrogen fixation and assimilation (Bennett and Albrecht, 1984). These previous studies indicate that root system is severely affected by flooding due to altered energy metabolism in plant.

Drought is one of the important environmental stresses resulting from climate change and the water-supply shortage (Hu and Xiong, 2014). Drought directly leads to an average yield loss of more than 50% at the reproductive stage in crop (Boyer, 1982; Venuprasad et al., 2007). It is proposed that induction of root aerenchyma increased plant performance and improved carbon economy under drought in maize (Zhu et al., 2010). Increased root versus shoot growth leads to improved plant hydraulic status

under mild or moderate drought stress because of increased root to leaf surface, continuous production of new root tips, and enhanced plant capacity for acquiring water to support existing shoots (Hsiao and Xu, 2000). Partially dried root system results in decreased allocation to vegetative shoots (Dry et al., 2001). Because root is considered to be the only source for acquiring water from soil, density, proliferation, size, and growth of root are key responses to drought stress in plant (Kavar et al., 2008). Deep and prolific root systems play a role in enhanced avoidance of terminal drought stress in chickpea (Serraj et al., 2004). These previous findings confirm that the ability of drought adaptation is closely related to root traits in plant.

Proteomic technique was used to elucidate response of flooding and drought in plant root. It was reported that proteins involved in energy metabolism were increased and proteins involved protein folding and cell structure were decreased in soybean under flooding (Nanjo et al., 2012). The dephosphorylation of proteins by protein phosphatase which was identified specifically in flooded soybean root tip might be involved in flooding-induced cell death of root tip (Nanjo et al., 2013). Ghaffari et al. (2013) reported that changes in energy usage, water transport, and ROS scavenging are critical mechanisms to maintain root growth in sunflower under drought. Proteins involved in transcription, protein synthesis, transport-associating proteins, and cell structure were significantly changed in root of Picrorhiza under drought, suggesting that these proteins play a role in drought tolerance (Sanjeeta et al., 2014). These results indicate that flooding and drought affect a number of proteins in roots; however, the response mechanisms in root under these stresses still have been remained.

GA and calcium effect on flooding response in soybean was analyzed using a gel-free proteomic technique. Under flooding, the growth of root was inhibited; however, when GA_3 and calcium was added, the length of root in soybean was elongated.

Proteomic technique was used to get insight into flooding response mechanism affected by GA_3 and calcium in soybean. Proteins involved in secondary metabolism, cell, and protein degradation/synthesis were decrased by flooding, but these proteins were recovered by GA_3 supplementation. Proteins involved in protein degradation/synthesis, hormone metabolism, cell wall metabolism, and DNA synthesis related proteins were decrased by flooding; while, these proteins were recovered by calcium supplementation (Figure 11). Quantitative differences in the protein abundance of wheat root and its regulation by ABA under drought were analyzed and suggested that ABA treatment induced a reinforcement of the cell-wall lignification in the roots and the cell-wall structure differences were also involved in altered ABA responsiveness and contributed toward its drought sensitivity differences (Alvarez et al., 2014). It was reported that calcium involved in the salt stress tolerance of germinating soybeans *via* enriching signal transduction, energy pathway, and transportation, inhibiting proteolysis, redistributing storage proteins, enriching antioxidant enzymes and activating their activities, and accumulating secondary metabolites and osmolytes (Yin et al., 2015). These results suggest that response mechanism against flooding and drought stresses might be regulated by phytohormone and calcium.

As above mentioned, flooding and drought stresses are negatively affect root system and alter many kinds of root proteins involved in regulation of root growth and development. Despite several reports on the underlying mechanisms of plant responses to flooding (Khatoon et al., 2012b) and drought (Mohammadi et al., 2012c) using proteomic technique, the differences between flooding and drought response still need to clarify in soybean. In this chapter, to obtain insights into root responses to flooding and drought stresses in early stage of soybean, a gel-free proteomic technique was used.

2.2. Materials and methods

2.2.1. Plant material and treatments

Plant material is mentioned in section 1.2.1. in Chapter 1. Two-day-old soybeans were subjected to flooding by adding excess of water and drought by withholding watering. For morphological experiment, root including hypocotyl of 3-, 4-, and 5-day-old soybeans, which were treated for 1, 2, and 3 days, respectively, were collected. For proteomic analysis, roots were collected from 4-day-old soybeans subjected to flooding and drought for 2 days (Figure 12). Three independent experiments were performed as biological replicates for all experiments.

2.2.2. Protein extraction for mass spectrometry

Protein extraction is mentioned in section 1.2.2 in Chapter 1.

2.2.3. Protein purification and digestion for mass spectrometry

Protein purification and digestion is mentioned in section 1.2.3 in Chapter 1.

2.2.4. Protein identification by mass spectrometry

Protein identification by MS is mentioned in 1.2.4 in Chapter 1.

2.2.5. Data acquisition by mass spectrometry

Date acquisition by MS is mentioned in section 1.2.5 in Chapter 1.

2.2.6. Analysis of differential abundant proteins using acquired mass spectrometry data

Analysis of differential abundant proteins using acquired MS data is mentioned

in 1.2.6 in Chapter 1.

2.2.7. Analysis of function and localization

Protein functions were categorized using MapMan bin codes software (http://mapman.gabipd.org/) (Usadel et al., 2005). Protein localization was analyzed using the intracellular targeting predication programs of YLoc (http://abi.inf.unituebingen.de/Services/YLoc/webloc.cgi) (Briesemeister et al., 2010). Pathway mapping of identified proteins was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) (Kanehisa and Goto, 2000).

2.2.8. Statistical analysis

The statistical significance of the results was evaluated with a Student's *t*-test when only 2 groups were compared. The statistical significance of more than 3 groups was evaluated with one-way ANOVA followed by Tukey's multiple comparison test. Both calculations were performed using SPSS software (version 22.0). A p value of < 0.05 was considered to be statistically significant.

2.3. Results

2.3.1. Morphological characteristics of soybean under flooding and drought stresses

To understand the morphological characteristics in soybean under flooding and drought stresses, 2-day-old soybeans were subjected to flooding and drought for 1, 2, and 3 days. The overall growth of soybean seedlings was reduced by flooding and drought stresses. The elongation of soybean root including hypocotyl was significantly suppressed under flooding stress; however, that was remained long but induced fine

diameter under drought stress (Figure 13). The fresh weight of root including hypocotyl was measured to determine whether flooding and drought affect or not in soybean. Decreases in the fresh weight of root including hypocotyl were seen from 1 day after treatments with flooding and drought (Figure 13).

2.3.2. Identification of proteins in soybean under flooding and drought using gel-free proteomics

To identify flooding- and drought-responsive proteins in soybean root, a gel-free proteomic technique was used. Two-day-old soybeans were subjected to flooding and drought stresses for 2 days, and proteins were extracted from roots and analyzed using nanoLC-MS/MS. Proteins identified from 2-day-old soybeans were used for comparison because 2-day-old soybeans were considered as starting point of treatments. Out of 1,184 proteins, 99 proteins were significantly changed in 4-day-old soybeans (control), with 32 decreased and 99 increased proteins (Table 5). Out of 1,904 proteins, 97 proteins were significantly changed in 4-day-old soybeans treated with 2-day-flooding (flooding), with 26 decreased and 71 increased proteins (Table 6). Out of 592 proteins, 48 proteins were significantly changed in 4-day-old soybeans treated with 2-day-drought (drought), with 19 decreased and 29 increased proteins (Table 7).

2.3.3. Subcellular localization and functional categorization of identified proteins under flooding and drought stressess

To evaluate how differ the subcellular localization between flooding and drought, the predicted localization of the flooding and drought responsive proteins was compared. Because of the important role of intracellular compartments in response to stress, to understand interrelation between identified proteins and stress response, subcellular

localization was analyzed using the YLoc prediction program. Under flooding stress, 49 proteins (51%) were predicted to be localized in cytoplasm and 21 proteins (22%) were predicted to be localized in extracellular space (Figure 14). Vacuolar and nucleus localized proteins were decreased; whereas plasma membrane and peroxisome localized proteins were increased in response to flooding stress (Figure 14). On the other hand, the majority of proteins (69%) were localized in the cytoplasm under drought stress (Figure 14). Mitochondrion localized proteins were increased in response to drought stress (Figure 14).

To elucidate whether the biological processes of identified proteins are same or not in response to flooding and drought, functional categorization was analyzed using MapMan bin codes. The biological process of flooding- and drought-responsive proteins was compared (Figure 15). Glycolysis related proteins were increased in response to both flooding and drought stresses, although 1 protein was decreased by drought stress (Figure 15A). Proteins involved in protein synthesis and targeting were decreased under flooding; whereas they were increased under drought stress (Figure 15B). Protein degradation related proteins were increased by flooding and drought (Figure 15B). The number of proteins involved in fermentation, cell wall, and stress was increased in response to flooding; while, the number of proteins involved in redox and cell organization was increased by drought stress (Figure 15A).

2.3.4. Metabolic pathway mapping of identified proteins under flooding and drought stressess

Plant has the molecular responses against abiotic stresses involved in interactions and crosstalk with various molecular pathways. To obtain a better understanding of the key regulatory pathways of flooding and drought responses, the identified proteins were

mapped to KEGG *Glycine max* database. Based on functional categorization, glycolysis metabolism pathway including fermentation metabolism was used for mapping. Under flooding stress, glucose-6-phosphate isomerase, 6-phosphofructokinase, fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, enolase, pyruvate kinase, pyruvate decarboxylase, and alcohol dehydrogenase were increased (Figure 16). Under drought stress, glucose-6-phosphate isomerase, 6-phosphofructokinase, and fructose-bisphosphate aldolase were increased (Figure 16).

As proteins involved in protein synthesis were mainly changed in response to both flooding and drought stresses, these proteins related to mRNA surveillance and glutathione metabolism were mapped on the pathways (Figure 17). In the mRNA surveillance pathway, peptide chain release factor eRF subunit 1 was decreased in response to flooding; whereas leucyl aminopeptidase (PepA) and glutathione *S*-transferase in the glutathione metabolism pathway were increased (Figure 17).

2.3.5. Comparison of flooding- and drought-responsive proteins identified using gel-free proteomic technique

Each identified protein was compared to describe which proteins were commonly or specifically changed under flooding and drought. A total of 89, 93, and 36 proteins were specifically changed in 4-day-old (control), 4-day-old soybeans treated with 2-day-flooding (flooding), and 4-day-old soybeans treated with 2-day-drought (drought), respectively. Aldolase superfamily protein was common between control and flooding stress; whereas 9 proteins were common between control and drought stress. In flooding-treated and drought-treated soybean, 3 proteins were commonly identified with oppositely changed level in response to flooding and drought (Figure 18A). A total of 90 and 7 proteins were specifically changed in 4-day-old soybeans treated with 2-day-

flooding (flooding) and 4-day-old soybeans treated with 2-day-drought (drought), respectively, compared to 4-day-old soybeans (Figure 18B). Only 1 protein, which was represented as aminopeptidase P1, was commonly identified between flooding-treated and drought-treated soybeans (Figure 18B).

2.4. Discussion

2.4.1. Morphological difference between flooding and drought stresses

To understand morphological response to flooding and drought in soybean, 2-day-old soybeans were subjected to flooding and drought. Although root weight was suppressed by drought, root length was increased under drought stress in this study. Turner (1986) proven that plant increased root depth as a major mechanism to improve water uptake. It was reported that the weight and length of hypocotyl were decreased under 4-day-flooding but not drought stress in soybean (Kausar et al., 2012). Root weight was decreased in 4-day-flooding and 6-day-drought stressed soybean (Kausar et al., 2012). Mohammadi et al. (2012b) also reported that the weight of root, hypocotyl, and leaf was declined after 4 days drought and the length of root and hypocotyl was declined after 5 days drought in soybean. Similarly, the weight and length of root were decreased in response to flooding in soybean (Khatoon et al., 2012a). Together with previous studies, these results indicate that soybean growth was affected by flooding and drought stresses, but their morphological characteristics was different. To further elucidate the differences of flooding and drought response, a gel-free proteomic analysis was performed.

2.4.2. The role of flooding-specific proteins in soybean

Out of 93 flooding-specific proteins, proteins involved in fermentation (11/93),

which are represented as pyruvate decarboxylase and alcohol dehydrogenase, were markedly increased. When plant faces with oxygen deficient condition caused by flooding, plant stimulates the anaerobic pathway for energy production (Gibbs and Greenway, 2003). Alcohol fermentation, for which alcohol dehydrogenase is a key enzyme, is the most important source for anaerobic energy production in plant (Smith and ap Rees, 1979). It consists of a two-step conversion from pyruvate to ethanol; first step is the decarboxylation of pyruvate to acetaldehyde through pyruvate decarboxylase and second step is the reduction of acetaldehyde to ethanol through alcohol dehydrogenase (Perata and Alpi, 1993). Previous study was reported that the expression of *alcohol dehydrogenase 2* gene was remarkably up-regulated by 6 h flooding; however, that was not induced by drought in soybean root (Komatsu et al., 2011b). The *alcohol dehydrogenase 2* transgenic soybean had improved growth and greater tolerance to flooding stress (Tougou et al., 2012). As shown by up-regulation of sucrose synthase and alcohol dehydrogenase under flooding, continuous glycolysis was one of the important mechanisms in waterlogging tolerance in mung bean (Sairam et al., 2009). Flooding is considered as low oxygen stress because of slowed oxygen diffusion; however, drought does not considered. Furthermore, the activation of enzymes involved in fermentation is predominantly increased by hypoxia/anoxia conditions. Therefore, it was unexpected that the activity of fermentation related enzyme was higher in soybean under flooding than that under drought. These results suggest that fermentation metabolism plays a main role in adaptation to flooding in soybean.

2.4.3. The role of drought-specific protein

Out of 36 drought-specific proteins, protein involved redox (1/36), which is

represented as dehydroascorbate reductase, was increased. Dehydroascorbate reductase, which is a physiologically important reducing enzyme in the ascorbate-glutathione recycling reaction (Ushimaru et al., 1997), is responsible for regeneration of ascorbic acid from an oxidized state and regulates the cellular ascorbic acid redox state, which in turn affects cell responsiveness and tolerance to ROS (Chen and Gallie, 2006). Dehydroascorbate reductase was increased in drought tolerant rice cultivar under drought stress, indicating that dehydroascorbate reductase might play a role in antioxidant protection against damage by dehydration (Ji et al., 2012). Hossain et al. (2013) reported that the activity of dehydroascorbate reductase was increased in both heat pre-treated salt and drought stressed mustard. The oxidative stress defense related genes including dehydroascorbate reductase was more highly expressed in drought tolerant perennial grass cultivar than drought sensitive cultivar under drought, suggesting that the expression of genes for antioxidant defense could be play a critical role in adaptation to long-term drought stress in perennial grass (Zhou et al., 2014b). In contrast drought, flooding-induced hypoxic condition leads to restricted ability of antioxidant defense system. These results suggest that the drought-stressed soybean root might be increased the level of dehydroasobate reductase for protecting oxidative damage.

2.4.5. The change of energy metabolism related proteins under flooding and drought

Glycolysis related proteins were significantly increased between both conditions. Glycolysis oxidizes glucose to pyruvate and plays a critical role in the energy production and carbon skeletons formation for biosynthesis of primary/secondary metabolites (Plaxton, 1996). Pyruvate kinase, which plays an important role in the regulation of glycolysis, is involved in lactic and alcohol fermentation (van Dongen et

al., 2011). In the present study, pyruvate kinase was increased under flooding; however, that was not identified as differentially changed protein under drought in soybean (Figure 16). It was reported that the activity of pyruvate kinase was increased in soybean under flooding stress (Nanjo et al., 2012); whereas, the abundance of pyruvate kinase was decreased in grapevine under water deficit (Cramer et al., 2013), indicating that drought stress results in the stimulation of alternative glycolysis pathway which is considered to be a more energy efficient process for the replenishment of TCA cycle related metabolites (Plaxton, 1996; O'Leary et al., 2011; Tcherkez et al., 2012). These results suggest that energy metabolism pathways such as glycolysis were affected by both flooding and drought stresses in soybean; however, flooding might more severely affect energy production and usage than drought.

2.4.6. The change of protein synthesis related proteins in soybean under flooding and drought

Protein synthesis related proteins such as eukaryotic release factor 1 and glutathione *S*-transferase were decreased and increased in response to flooding and drought, respectively. Eukaryotic release factor 1 is required to complete protein synthesis *via* the release of completed peptides from ribosome and recognizes stop codons in mRNAs (Frolova et al., 1994). In *Arabidopsis, eukaryotic release factor 1 family* gene was mediated glucose and phytohormone responses during seed germination and early seedling development, indicating that eukaryotic release factor 1 plays a role in plant growth and development (Zhou et al., 2010). Glutathione *S*-transferase catalyzes the reduction of the disulfide linkages of proteins (Circu and Aw, 2012) and plays roles in detoxifying oxidative-stress metabolites and in leukotriene biosynthesis (Wilce and Parker, 1994; Sheehan et al., 2001). Link between glutathione

S-transferases and oxidative stress tolerance has been established by the finding that when expressed in yeast, a tau glutathione *S*-transferase from tomato can suppress apoptosis induced by the Bax protein (Kampranis et al., 2000), apparently by preventing oxidative damage (Dixon et al., 2002). These results suggest that flooding and drought might affect the maintenance of protein homeostasis including protein synthesis in soybean.

2.4.7. Concluding remarks

Soybean growth is adversely affected by flooding and drought. The activity and accumulation of the antioxidant enzyme, APX, were decreased under flooding, but increased under drought in soybean (Kausar et al., 2012). In this study, a proteomic technique was used to obtain insight into the response mechanisms of soybean to flooding and drought stresses. Tight metabolic regulation in consuming energy is needed to survive to flooding and proteins involved in osmotic adjustment, defense signaling, and programmed cell death play a role in adaptation to drought (Hossain and Komatsu, 2014). These previous studies suggested that flooding and drought have different effects on soybean and develop specific and complex response mechanisms. In this chapter, 3 *S*-adenosylmethionine synthetase were decreased and increased under flooding and drought stresses, respectively. These enzymes have pivotal function in tolerance to stress *via* up-regulation of polyamine oxidation (Guo et al., 2014). Taken together, these results suggest that response mechanisms to flooding and drought stresses are differentially controlled *via* polyamine oxidation and defense metabolism in soybean.

2.5. Summary

Flooding and drought affect soybean growth because soybean is stress-sensitive crop. To understand morphological response in soybean under flooding and drought, 2-day-old soybeans were exposed to flooding and drought until 3 days. The fresh weight of roots was markedly suppressed under both stresses, although the root morphology was clearly differed between two conditions. To understand the response mechanisms of soybean to flooding and drought stresses, a gel-free proteomic technique was used. A total of 97 proteins and 48 proteins were significantly changed in response to flooding and drought stresses, respectively. Of 97 proteins, 26 proteins were decreased and 71 proteins were increased under flooding stress. Of 48 proteins, 19 proteins were decreased and 29 proteins were increased under drought stress. Proteins involved in protein synthesis were decreased by flooding stress and increased by drought stress. Of them, eukaryotic release factor 1, which was involved in mRNA surveillance pathway, leucyl aminopeptidase, and glutathione *S*-transferase, which were related to glutathione metabolism pathway, were significantly changed under flooding and drought, respectively. Glycolysis related proteins were increased in roots both flooding and drought stresses. Fermentation, cell wall, and stress related proteins were increased in response to flooding; whereas, redox and cell organization related proteins were increased under drought stress. Among the identified proteins, 3 *S*-adenosylmethionine synthetases were commonly decreased and increased in response to flooding and drought stresses, respectively. These results suggest that *S*-adenosylmethionine synthetase is involved in the regulation of stress response because it was changed in response to flooding and drought stresses in soybean.

Table 5. List of identified proteins in 4-day-old soybean compared to 2-day-old soybean.

Protein ID	Description	M.P.	Ratio 4(0)	SD 4(0)	Pvalue 4(0)
Decreased					
Glyma17g33570.1	heat shock protein 81 2	2	0.0322	0.6718	0.0140
Glyma11g02630.1	glutathione peroxidase 6	2	0.0346	0.8668	0.0267
Glyma10g20880.1	heat shock protein 81 2	2	0.0356	0.6678	0.0167
Glyma20g14235.1	heat shock protein 81 2	2	0.0356	0.6678	0.0167
Glyma13g41120.2	triosephosphate isomerase	2	0.0407	0.6664	0.0000
Glyma01g42840.1	glutathione peroxidase 6	3	0.0411	0.7587	0.0152
Glyma07g38790.1	NAD(P) binding Rossmann fold superfamily protein	4	0.0676	0.3648	0.0000
Glyma04g37120.1	Translation elongation factor EF1B/ribosomal protein S6 family protein	2	0.1171	0.8936	0.0004
Glyma06g17930.5	Translation elongation factor EF1B/ribosomal protein S6 family protein	2	0.1171	0.8936	0.0004
Glyma01g30670.1	PEBP (phosphatidylethanolamine binding protein) family protein	2	0.1212	0.8265	0.0004
Glyma15g03030.1	lipoxygenase 1	6	0.1490	0.5108	0.0133
Glyma15g04290.1	triosephosphate isomerase	2	0.1863	0.5658	0.0028
Glyma18g44970.1	Protein of unknown function (DUF3411)	2	0.1991	0.8880	0.0399
Glyma09g25830.2	CAP160 protein	2	0.2078	0.7250	0.0001
Glyma11g04650.1	Peptidase M20/M25/M40 family protein	2	0.2269	0.9992	0.0413
Glyma11g19070.1	NAD ADP ribosyltransferases	5	0.2888	0.5522	0.0003
Glyma10g43990.1	Transketolase	2	0.3021	0.5096	0.0138
Glyma20g38720.1	Transketolase	2	0.3021	0.5096	0.0138
Glyma14g09300.1	poly(A) binding protein 2	2	0.3178	1.1962	0.0205
Glyma09g28440.1	Ribosomal protein S3 family protein	2	0.3194	0.5429	0.0063
Glyma10g26890.1	Ribosomal protein S3 family protein	2	0.3194	0.5429	0.0063
Glyma16g33230.1	Ribosomal protein S3 family protein	2	0.3194	0.5429	0.0063
Glyma16g33240.1	Ribosomal protein S3 family protein	2	0.3194	0.5429	0.0063
Glyma20g21190.1	Ribosomal protein S3 family protein	2	0.3194	0.5429	0.0063
Glyma07g39960.1	phosphofructokinase 2	3	0.3638	0.2332	0.0000
Glyma13g42320.1	lipoxygenase 1	8	0.3863	0.2174	0.0000
Glyma13g42310.1	lipoxygenase 1	2	0.4084	0.2822	0.0000
Glyma01g00730.4	*S*-adenosyl-*L*-methionine dependent methyltransferases superfamily protein	3	0.4303	0.7587	0.0275
Glyma06g21290.1	eukaryotic elongation factor 5A 1	2	0.4760	0.6376	0.0473
Glyma05g09310.2	Pyruvate kinase family protein	2	0.4782	0.4572	0.0217
Glyma19g00870.2	Pyruvate kinase family protein	2	0.4782	0.4572	0.0217
Glyma12g09390.1	NAD ADP ribosyltransferases	5	0.4885	0.5341	0.0150
Increased					
Glyma07g13710.1	Nucleoside diphosphate kinase family protein	3	2.1650	0.4122	0.0006
Glyma04g33750.1	NAP1 related protein 2	4	2.2902	0.3413	0.0000
Glyma06g20700.1	NAP1 related protein 2	4	2.2902	0.3413	0.0000
Glyma06g46190.2	aconitase 3	2	2.5304	0.6395	0.0145
Glyma12g10580.1	aconitase 3	2	2.5304	0.6395	0.0145
Glyma10g28890.1	calreticulin 1b	8	2.5514	0.2673	0.0340
Glyma15g12880.1	RAB GTPase homolog B1C	4	2.5761	0.5018	0.0192
Glyma15g41550.1	phosphoglycerate kinase	9	2.8126	0.2275	0.0239
Glyma08g17600.1	phosphoglycerate kinase	9	2.9625	0.2415	0.0209

Glyma18g52250.2	NAD(P) linked oxidoreductase superfamily protein	2	2.9965	1.0253	0.0424
Glyma18g16260.1	voltage dependent anion channel 1	3	3.0502	0.6166	0.0178
Glyma07g37240.1	MLP like protein 423	3	3.0706	0.6085	0.0050
Glyma02g11580.1	poly(A) binding protein 8	3	3.2876	1.0071	0.0404
Glyma07g33860.2	poly(A) binding protein 2	3	3.2876	1.0071	0.0404
Glyma17g02260.1	Copper amine oxidase family protein	15	3.8400	0.2410	0.0001
Glyma10g06480.1	ATPase AAA type CDC48 protein	6	6.3530	0.4801	0.0156
Glyma13g20680.1	ATPase AAA type CDC48 protein	6	6.3530	0.4801	0.0156
Glyma19g36740.1	ATPase AAA type CDC48 protein	6	6.3530	0.4801	0.0156
Glyma10g37960.1	tubulin beta 8	5	7.2808	0.4125	0.0441
Glyma20g29840.1	tubulin beta 8	5	7.2808	0.4125	0.0441
Glyma02g43040.1	ferritin 4	2	7.4089	0.6304	0.0137
Glyma14g06160.1	ferritin 4	2	7.4089	0.6304	0.0137
Glyma04g39930.1	manganese superoxide dismutase 1	3	9.4059	0.9376	0.0112
Glyma20g38440.1	dehydroascorbate reductase 2	7	9.9256	0.3957	0.0024
Glyma12g02250.1	NAD(P) binding Rossmann fold superfamily protein	2	11.2933	0.7238	0.0381
Glyma08g03690.2	heat shock protein 81 2	3	12.6253	0.6328	0.0286
Glyma05g03190.1	arginosuccinate synthase family	3	13.3615	0.3286	0.0357
Glyma05g03210.1	arginosuccinate synthase family	3	13.3615	0.3286	0.0357
Glyma17g13790.1	arginosuccinate synthase family	3	13.3615	0.3286	0.0357
Glyma08g07950.1	beta D xylosidase 4	2	13.9160	0.4475	0.0241
Glyma05g27840.1	urease	3	14.4729	0.2294	0.0137
Glyma13g10700.1	heat shock protein 70 (Hsp 70) family protein	2	15.1183	0.9233	0.0301
Glyma20g16070.1	heat shock protein 70 (Hsp 70) family protein	2	15.1183	0.9233	0.0301
Glyma10g07710.1	Aldolase superfamily protein	2	17.9669	0.4212	0.0000
Glyma13g21540.1	Aldolase superfamily protein	2	17.9669	0.4212	0.0000
Glyma05g37170.1	6 phosphogluconate dehydrogenase family protein	3	19.0335	0.5817	0.0327
Glyma08g02410.1	6 phosphogluconate dehydrogenase family protein	3	19.0335	0.5817	0.0327
Glyma08g45990.1	NAD(P) binding Rossmann fold superfamily protein	3	19.0767	0.2791	0.0294
Glyma08g44590.1	HEAT SHOCK PROTEIN 81.4	7	19.8495	0.4827	0.0077
Glyma14g01530.1	HEAT SHOCK PROTEIN 81.4	7	19.8495	0.4827	0.0077
Glyma18g08220.1	HEAT SHOCK PROTEIN 81.4	7	19.8495	0.4827	0.0077
Glyma02g47210.1	HEAT SHOCK PROTEIN 81.4	7	21.2989	0.4996	0.0219
Glyma02g38730.1	Aldolase superfamily protein	7	26.6215	0.3139	0.0000
Glyma06g08260.1	N terminal nucleophile aminohydrolases (Ntn hydrolases) superfamily protein	4	27.2914	0.6189	0.0417
Glyma04g08200.1	N terminal nucleophile aminohydrolases (Ntn hydrolases) superfamily protein	4	28.7950	0.6424	0.0282
Glyma02g07910.1	TCP 1/cpn60 chaperonin family protein	4	31.5755	0.5262	0.0312
Glyma16g26920.1	TCP 1/cpn60 chaperonin family protein	4	31.5755	0.5262	0.0312
Glyma03g34950.1	Aldolase superfamily protein	4	42.6718	0.3929	0.0019
Glyma07g37270.1	MLP like protein 423	4	49.9692	0.6234	0.0150
Glyma15g43180.1	Protein of unknown function DUF642	4	60.8605	0.6268	0.0074
Glyma05g29000.1	tubulin alpha 5	5	62.1498	0.4376	0.0456
Glyma08g12140.1	tubulin alpha 5	5	62.1498	0.4376	0.0456
Glyma10g11620.1	Protein of unknown function DUF642	3	71.3386	0.6804	0.0030
Glyma11g18710.1	N.D.*	2	71.7557	0.9596	0.0374

Glyma14g36850.1	Aldolase superfamily protein	9	72.9100	0.3156	0.0001
Glyma03g03200.1	Transketolase	8	86.0777	0.3318	0.0196
Glyma11g14360.1	RAB GTPase homolog A2B	4	90.0379	0.4885	0.0077
Glyma12g06280.2	RAB GTPase homolog A2B	4	90.0379	0.4885	0.0077
Glyma12g33550.1	RAB GTPase homolog A1F	3	92.6504	0.4980	0.0076
Glyma13g36910.1	RAB GTPase homolog A1F	3	92.6504	0.4980	0.0076
Glyma18g03760.1	RAB GTPase homolog A2B	3	92.6504	0.4980	0.0076
Glyma12g34000.1	Ras related small GTP binding family protein	2	97.3584	0.5113	0.0032
Glyma13g36530.1	Ras related small GTP binding family protein	2	97.3584	0.5113	0.0032
Glyma15g42150.1	40s ribosomal protein SA B	3	99.4218	0.6916	0.0309
Glyma09g04950.1	translationally controlled tumor protein	3	102.3687	0.6141	0.0334
Glyma15g15910.1	translationally controlled tumor protein	3	102.3687	0.6141	0.0334
Glyma08g17000.2	40s ribosomal protein SA B	2	125.6397	0.7782	0.0085

Protein ID, according to the Phytozome database; M.P., number of matched peptide; Ratio, relative abundance of protein; N.D.*, no description in Phytozome database; 4(0), 4-day-old soybean without any treatment.

Table 6. List of identified proteins in 2-day-flooding treated soybean compared to 2-day-old soybean.

Protein ID	Description	M.P.	Ratio 4(2)F	SD 4(2)F	*P*value 4(2)F	Function	Localization
Decreased							
Glyma16g04950.1	xyloglucan endotransglucosylase/hydrolase 5	3	0.1051	0.3330	0.0098	cell wall	Extracellular space
Glyma19g28220.1	xyloglucan endotransglucosylase/hydrolase 5	2	0.1067	0.3488	0.0141	cell wall	Extracellular space
Glyma12g04701.2	cold circadian rhythm and rna binding 2	4	0.1679	0.4077	0.0001	RNA	Cytoplasm
Glyma17g01500.1	Saposin like aspartyl protease family protein	2	0.2052	1.2535	0.0499	protein	Vacuole
Glyma14g00850.1	N.D.*	2	0.2747	0.8377	0.0271	not assigned	Nucleus
Glyma05g22060.1	Subtilase family protein	4	0.3504	0.3082	0.0016	protein	Vacuole
Glyma17g14750.1	Glycosyl hydrolases family 32 protein	7	0.3521	0.3301	0.0229	major CHO metabolism	Vacuole
Glyma17g17850.1	Subtilase family protein	6	0.3550	0.2863	0.0023	protein	Vacuole
Glyma09g29600.1	eukaryotic release factor 1 3	3	0.3662	0.3988	0.0297	protein	Cytoplasm
Glyma16g34180.1	eukaryotic release factor 1 3	3	0.3662	0.3988	0.0297	protein	Cytoplasm
Glyma03g28850.1	beta 1 3 glucanase 1	9	0.4038	0.1926	0.0000	misc	Vacuole
Glyma15g21890.1	*S*-adenosylmethionine synthetase family protein	4	0.4175	0.2899	0.0127	metal handling	Cytoplasm
Glyma03g38190.3	*S*-adenosylmethionine synthetase 1	3	0.4185	0.3348	0.0213	a. a. metabolism	Cytoplasm
Glyma19g40810.1	*S*-adenosylmethionine synthetase 2	3	0.4185	0.3348	0.0213	a. a. metabolism	Cytoplasm
Glyma11g17930.1	DNAJ homologue 2	4	0.4250	0.3539	0.0019	stress	Cytoplasm
Glyma12g10150.1	DNAJ homologue 2	4	0.4250	0.3539	0.0019	stress	Cytoplasm
Glyma12g31620.1	DNAJ homologue 2	4	0.4318	0.3850	0.0037	stress	Cytoplasm
Glyma13g38790.1	DNAJ homologue 2	4	0.4318	0.3850	0.0037	stress	Cytoplasm
Glyma02g07560.3	eukaryotic release factor 1 3	4	0.4448	0.3540	0.0432	protein	Cytoplasm
Glyma16g26600.1	eukaryotic release factor 1 3	4	0.4448	0.3540	0.0432	protein	Cytoplasm
Glyma18g00480.2	glycine rich RNA binding protein 2	2	0.4591	0.1956	0.0001	RNA	Mitochondrion
Glyma20g03060.1	Nuclear transport factor 2 (NTF2) family protein	4	0.4627	0.3245	0.0145	protein	Cytoplasm
Glyma11g36580.1	glycine rich RNA binding protein 2	2	0.4630	0.1812	0.0000	RNA	Mitochondrion
Glyma16g04890.1	eukaryotic release factor 1 3	2	0.4705	0.4679	0.0477	protein	Cytoplasm
Glyma18g06560.1	TBP associated factor 15B	2	0.4728	0.3578	0.0187	RNA	Nucleus
Glyma07g39540.1	N.D.*	3	0.4978	0.3824	0.0116	not assigned	Nucleus
Increased							
Glyma14g13480.2	aspartate aminotransferase 5	8	2.0065	0.1644	0.0006	a. a. metabolism	Chloroplast
Glyma02g38730.1	Aldolase superfamily protein	10	2.0523	0.1081	0.0000	glycolysis	Cytoplasm
Glyma20g33060.2	Pyruvate kinase family protein	4	2.0545	0.3087	0.0283	glycolysis	Peroxisome
Glyma06g09320.5	phosphofructokinase 3	6	2.1026	0.2208	0.0099	glycolysis	Golgi apparatus
Glyma05g36050.2	phosphofructokinase 3	4	2.1031	0.2501	0.0131	glycolysis	Cytoplasm
Glyma04g09180.1	phosphofructokinase 3	7	2.2424	0.1875	0.0021	glycolysis	Cytoplasm
Glyma18g01330.4	glyceraldehyde 3 phosphate dehydrogenase C2	4	2.2895	0.4398	0.0122	glycolysis	Cytoplasm
Glyma16g17190.1	Pectinacetylesterase family protein	4	2.3612	0.3760	0.0361	cell wall	Plasma membrane
Glyma20g34960.1	Plastid lipid associated protein PAP / fibrillin family protein	2	2.4963	0.6412	0.0316	cell	Chloroplast
Glyma01g10900.1	kunitz trypsin inhibitor 1	3	2.5632	0.1938	0.0039	stress	Extracellular space
Glyma07g01710.1	phosphofructokinase 3	3	2.5691	0.3295	0.0079	glycolysis	Cytoplasm
Glyma08g21370.1	phosphofructokinase 3	3	2.5691	0.3295	0.0079	glycolysis	Cytoplasm
Glyma10g16010.1	20S proteasome subunit PAA2	2	2.6715	0.6678	0.0413	protein	Cytoplasm
Glyma19g02031.1	Sugar isomerase (SIS) family protein	3	2.6803	0.5176	0.0325	glycolysis	Cytoplasm
Glyma19g43150.1	O acetylserine (thiol) lyase (OAS TL) isoform A1	7	2.7069	0.2925	0.0020	a. a. metabolism	Cytoplasm

Glyma13g34520.1	D mannose binding lectin protein with Apple	4	2.8680	0.3526	0.0039	misc	Extracellular space
Glyma13g27820.1	Eukaryotic aspartyl protease family protein	2	2.9937	0.3699	0.0053	protein	Plasma membrane
Glyma14g38170.1	Peroxidase superfamily protein	4	3.0380	0.4928	0.0059	misc	Extracellular space
Glyma15g30610.1	Calcium binding EF hand family protein	5	3.0711	0.3259	0.0160	signalling	Cytoplasm
Glyma06g44990.2	alpha/beta Hydrolases superfamily protein	2	3.1339	0.3937	0.0105	misc	Cytoplasm
Glyma12g12800.1	alpha/beta Hydrolases superfamily protein	3	3.2679	0.3879	0.0108	misc	Cytoplasm
Glyma01g29190.1	Thiamine pyrophosphate dependent pyruvate decarboxylase family protein	2	3.3917	0.3760	0.0372	fermentation	Cytoplasm
Glyma16g32960.2	Enolase	8	3.4718	0.1642	0.0000	glycolysis	Cytoplasm
Glyma03g07300.2	Thiamine pyrophosphate dependent pyruvate decarboxylase family protein	3	3.7614	0.3483	0.0032	fermentation	Cytoplasm
Glyma03g07380.1	Thiamine pyrophosphate dependent pyruvate decarboxylase family protein	3	3.7614	0.3483	0.0032	fermentation	Cytoplasm
Glyma16g33770.1	Kunitz family trypsin and protease inhibitor protein	2	3.8212	0.2571	0.0013	stress	Extracellular space
Glyma18g43460.1	pyruvate decarboxylase 2	4	3.8942	0.2631	0.0016	fermentation	Cytoplasm
Glyma03g40490.2	O acetylserine (thiol) lyase (OAS TL) isoform A1	9	3.9649	0.2703	0.0000	a. a. metabolism	Cytoplasm
Glyma16g32530.1	pfkB like carbohydrate kinase family protein	6	4.1059	0.2830	0.0409	major CHO metabolism	Cytoplasm
Glyma13g30490.1	pyruvate decarboxylase 2	7	4.5949	0.2531	0.0002	fermentation	Cytoplasm
Glyma20g17440.1	uricase / urate oxidase / nodulin 35 putative	6	4.8774	0.2919	0.0000	nucleotide metabolism	Peroxisome
Glyma17g15680.1	expansin like B1	5	4.9116	0.2482	0.0000	cell wall	Extracellular space
Glyma07g18570.1	Thiamine pyrophosphate dependent pyruvate decarboxylase family protein	4	5.3860	0.2650	0.0015	fermentation	Cytoplasm
Glyma06g12780.2	alcohol dehydrogenase 1	9	5.8148	0.1133	0.0000	fermentation	Cytoplasm
Glyma04g41990.1	alcohol dehydrogenase 1	13	5.8280	0.1042	0.0000	fermentation	Cytoplasm
Glyma10g23790.1	uricase / urate oxidase / nodulin 35 putative	7	5.9238	0.2563	0.0000	nucleotide metabolism	Peroxisome
Glyma09g29220.1	Kunitz family trypsin and protease inhibitor protein	2	6.8017	0.3975	0.0203	stress	Extracellular space
Glyma05g36310.1	ACC oxidase 1	4	7.0242	0.2203	0.0002	hormone metabolism	Cytoplasm
Glyma13g33590.1	glyoxalase II 3	5	7.1411	0.2141	0.0000	biodegradation of Xenobiotics	Plasma membrane
Glyma09g28100.1	Enolase	9	7.8314	0.1672	0.0000	glycolysis	Cytoplasm
Glyma14g27940.1	alcohol dehydrogenase 1	9	8.2838	0.1330	0.0000	fermentation	Cytoplasm
Glyma04g39190.1	alcohol dehydrogenase 1	4	8.3854	0.2629	0.0014	fermentation	Cytoplasm
Glyma05g05390.1	expansin like B1	3	8.4559	0.3151	0.0001	cell wall	Extracellular space
Glyma13g31390.1	Rubber elongation factor protein (REF)	2	8.9497	0.4039	0.0103	not assigned	Cytoplasm
Glyma15g07940.1	Rubber elongation factor protein (REF)	2	8.9497	0.4039	0.0103	not assigned	Cytoplasm
Glyma11g20940.2	dehydration induced protein (ERD15)	2	10.3434	0.6421	0.0074	stress	Nucleus
Glyma13g09530.1	alcohol dehydrogenase 1	2	10.4048	0.3619	0.0071	fermentation	Cytoplasm
Glyma11g18320.1	FAD/NAD(P) binding oxidoreductase family protein	3	11.3943	0.4098	0.0000	not assigned	Peroxisome
Glyma12g09940.2	FAD/NAD(P) binding oxidoreductase family protein	3	11.3943	0.4098	0.0000	not assigned	Peroxisome
Glyma08g08360.1	polygalacturonase inhibiting protein 1	3	12.3562	0.4296	0.0188	cell wall	Extracellular space
Glyma03g03460.1	Plant invertase/pectin methylesterase inhibitor superfamily	3	14.0406	0.5563	0.0229	cell wall	Extracellular space
Glyma11g37560.1	beta ketoacyl reductase 1	4	14.2282	0.5843	0.0189	secondary metabolism	Golgi apparatus
Glyma18g01510.1	beta ketoacyl reductase 1	4	14.2282	0.5843	0.0189	secondary metabolism	Golgi apparatus
Glyma16g33790.1	Kunitz family trypsin and protease inhibitor protein	2	16.4266	0.6547	0.0430	stress	Extracellular space
Glyma06g09770.1	Rhamnogalacturonate lyase family protein	4	21.5239	0.4344	0.0068	cell wall	Cytoplasm
Glyma09g29300.1	Kunitz family trypsin and protease inhibitor protein	2	24.0546	0.6784	0.0017	stress	Extracellular space
Glyma04g09670.1	Rhamnogalacturonate lyase family protein	3	24.0795	0.4674	0.0069	cell wall	Cytoplasm
Glyma05g25340.1	polygalacturonase inhibiting protein 1	4	25.0660	0.5564	0.0036	cell wall	Extracellular space
Glyma02g16710.1	Eukaryotic aspartyl protease family protein	3	41.3135	0.3730	0.0000	protein	Vacuole

Glyma12g35070.1	Aluminium induced protein with YGL and LRDR motifs	2	42.1048	0.4304	0.0095	hormone metabolism	Cytoplasm
Glyma13g35480.1	Aluminium induced protein with YGL and LRDR motifs	2	42.1048	0.4304	0.0095	hormone metabolism	Cytoplasm
Glyma09g29310.1	Kunitz family trypsin and protease inhibitor protein	3	44.0605	0.4248	0.0347	stress	Extracellular space
Glyma11g07490.1	NmrA like negative transcriptional regulator family protein	3	51.0567	0.4636	0.0058	secondary metabolism	Mitochondrion
Glyma12g23150.1	Aluminium induced protein with YGL and LRDR motifs	4	56.1539	0.4655	0.0314	hormone metabolism	Cytoplasm
Glyma01g37850.1	NmrA like negative transcriptional regulator family protein	2	59.5929	0.5149	0.0158	secondary metabolism	Mitochondrion
Glyma05g25360.1	polygalacturonase inhibiting protein 1	5	62.1986	0.4719	0.0036	cell wall	Extracellular space
Glyma05g25370.1	polygalacturonase inhibiting protein 1	5	62.4540	0.3361	0.0057	cell wall	Extracellular space
Glyma09g29330.1	Kunitz family trypsin and protease inhibitor protein	4	63.9983	0.4022	0.0035	stress	Extracellular space
Glyma09g29350.1	Kunitz family trypsin and protease inhibitor protein	4	93.5830	0.4569	0.0040	stress	Extracellular space
Glyma17g15640.1	expansin like B1	3	104.0757	0.2608	0.0001	cell wall	Extracellular space
Glyma17g15670.1	expansin like B1	3	104.0757	0.2608	0.0001	cell wall	Extracellular space

Protein ID, according to the Phytozome database; M.P., number of matched peptide; Ratio, relative abundance of protein; Function, functional categorization by MapMan bin codes; Localization, localization prediction using YLoc; N.D.*, no description in the Phytozome database; 4(2)F, 4-day-old soybean subjected to flooding for 2 days.

Table 7. List of identified proteins in 2-day-drought treated soybean compared to 2-day-old soybean.

Protein ID	Description	M.P.	Ratio 4(2)D	SD 4(2)D	*P*value 4(2)D	Function	Localization
Decreased							
Glyma19g32990.2	actin 7	3	0.0385	0.9474	0.0026	cell	Cytoplasm
Glyma02g29160.3	actin 1	2	0.0415	1.0849	0.0104	cell	Cytoplasm
Glyma03g30110.3	actin 11	2	0.0415	1.0849	0.0104	cell	Cytoplasm
Glyma09g17040.2	actin 1	2	0.0415	1.0849	0.0104	cell	Cytoplasm
Glyma10g03390.1	cupin family protein	2	0.0682	1.1676	0.0426	development	Golgi apparatus
Glyma01g45110.1	methylesterase PCR A	2	0.0732	1.4960	0.0440	cell wall	Vacuole
Glyma11g15870.1	RmlC like cupins superfamily protein	2	0.0761	0.5268	0.0008	development	ER
Glyma12g32000.1	aconitase 3	3	0.0813	0.8336	0.0034	TCA / org	Mitochondrion
Glyma13g38480.1	aconitase 3	3	0.0813	0.8336	0.0034	TCA / org	Chloroplast
Glyma08g29130.3	NAD(P) linked oxidoreductase superfamily protein	2	0.1130	0.8043	0.0024	hormone metabolism	Cytoplasm
Glyma14g03470.1	glutathione S transferase PHI 9	2	0.2275	1.0802	0.0372	misc	Cytoplasm
Glyma02g40806.1	UDP glucose pyrophosphorylase 2	3	0.2295	0.9001	0.0040	glycolysis	Cytoplasm
Glyma13g32940.1	dynamin related protein 3A	2	0.2544	0.9494	0.0399	misc	Cytoplasm
Glyma15g06380.1	dynamin related protein 3A	2	0.2544	0.9494	0.0399	misc	Cytoplasm
Glyma08g15480.1	actin 7	3	0.2886	0.9660	0.0349	cell	Cytoplasm
Glyma15g03030.1	lipoxygenase 1	5	0.3033	0.5347	0.0250	hormone metabolism	Cytoplasm
Glyma13g42320.1	lipoxygenase 1	8	0.3137	0.2558	0.0000	hormone metabolism	Cytoplasm
Glyma01g38580.1	Clathrin heavy chain	2	0.3455	0.8975	0.0477	cell	Plasma membrane
Glyma02g39360.1	Clathrin heavy chain	2	0.3455	0.8975	0.0477	cell	Cytoplasm
Increased							
Glyma18g31550.3	Insulinase (Peptidase family M16) protein	2	2.1742	0.9463	0.0006	protein	Mitochondrion
Glyma09g33960.1	RNA binding (RRM/RBD/RNP motifs) family protein	3	2.4882	0.3990	0.0002	RNA	Cytoplasm
Glyma16g00360.1	Translation elongation factor EF1B gamma chain	4	2.5151	0.3393	0.0244	protein	Cytoplasm
Glyma01g01950.1	RNA binding (RRM/RBD/RNP motifs) family protein	2	2.6279	0.4103	0.0362	RNA	Cytoplasm
Glyma07g13710.1	Nucleoside diphosphate kinase family protein	2	2.7182	0.9607	0.0069	nucleotide metabolism	Cytoplasm
Glyma10g07410.1	embryonic cell protein 63	2	3.3794	0.9324	0.0135	development	Nucleus
Glyma04g03490.1	Sugar isomerase (SIS) family protein	3	3.6454	0.7522	0.0336	glycolysis	Cytoplasm
Glyma03g03200.1	Transketolase	5	4.6249	0.6033	0.0366	PS	Chloroplast
Glyma11g33160.1	UDP glucose pyrophosphorylase 2	6	4.9888	0.6503	0.0297	glycolysis	Cytoplasm
Glyma08g40800.1	voltage dependent anion channel 1	2	5.0558	0.5774	0.0002	transport	Cytoplasm
Glyma14g36850.1	Aldolase superfamily protein	5	5.6113	0.5915	0.0460	glycolysis	Cytoplasm
Glyma03g34950.1	Aldolase superfamily protein	3	6.2785	0.7747	0.0470	glycolysis	Cytoplasm
Glyma08g07950.1	beta D xylosidase 4	2	7.7725	0.8015	0.0449	cell wall	Extracellular space
Glyma15g21890.1	*S*-adenosylmethionine synthetase family protein	4	9.0403	0.7228	0.0383	metal handling	Cytoplasm
Glyma08g18110.1	Ribosomal protein S5/Elongation factor G/III/V family	6	10.1610	0.5541	0.0353	protein	Cytoplasm
Glyma15g40860.1	Ribosomal protein S5/Elongation factor G/III/V family	6	10.1610	0.5541	0.0353	protein	Cytoplasm
Glyma03g38190.3	*S*-adenosylmethionine synthetase 1	4	10.6780	0.7492	0.0101	a. a. metabolism	Cytoplasm
Glyma19g40810.1	*S* adenosylmethionine synthetase 2	4	10.6780	0.7492	0.0101	a. a. metabolism	Cytoplasm
Glyma08g19420.3	actin 11	3	14.5690	0.5425	0.0288	cell	Cytoplasm
Glyma15g05570.1	actin 11	3	14.5690	0.5425	0.0288	cell	Cytoplasm
Glyma02g36530.1	Cytosol aminopeptidase family protein	4	16.2004	0.6957	0.0272	protein	Mitochondrion
Glyma17g08150.1	Cytosol aminopeptidase family protein	4	16.2004	0.6957	0.0272	protein	Mitochondrion

Glyma04g36870.3	glyceraldehyde 3 phosphate dehydrogenase C subunit 1	7	39.0706	0.3775	0.0391	glycolysis	ER
Glyma20g38440.1	dehydroascorbate reductase 2	4	41.6726	0.2260	0.0078	redox	Cytoplasm
Glyma10g43730.1	dehydroascorbate reductase 2	2	42.8452	0.2313	0.0039	redox	Cytoplasm
Glyma04g36860.1	glyceraldehyde 3 phosphate dehydrogenase C2	6	43.4007	0.3997	0.0303	glycolysis	Mitochondrion
Glyma13g42310.1	lipoxygenase 1	4	50.3627	0.3316	0.0049	hormone metabolism	Cytoplasm
Glyma06g18120.1	glyceraldehyde 3 phosphate dehydrogenase C subunit 1	5	58.6197	0.4665	0.0362	glycolysis	Mitochondrion
Glyma11g37360.1	glyceraldehyde 3 phosphate dehydrogenase C subunit 1	4	65.9735	0.4986	0.0312	glycolysis	Cytoplasm

Protein ID, according to the Phytozome database; M.P., number of matched peptide; Ratio, relative abundance of protein; Function, functional categorization by MapMan bin code; Localization, localization prediction using YLoc; 4(2)D, 4-day-old soybean subjected to drought for 2 days.

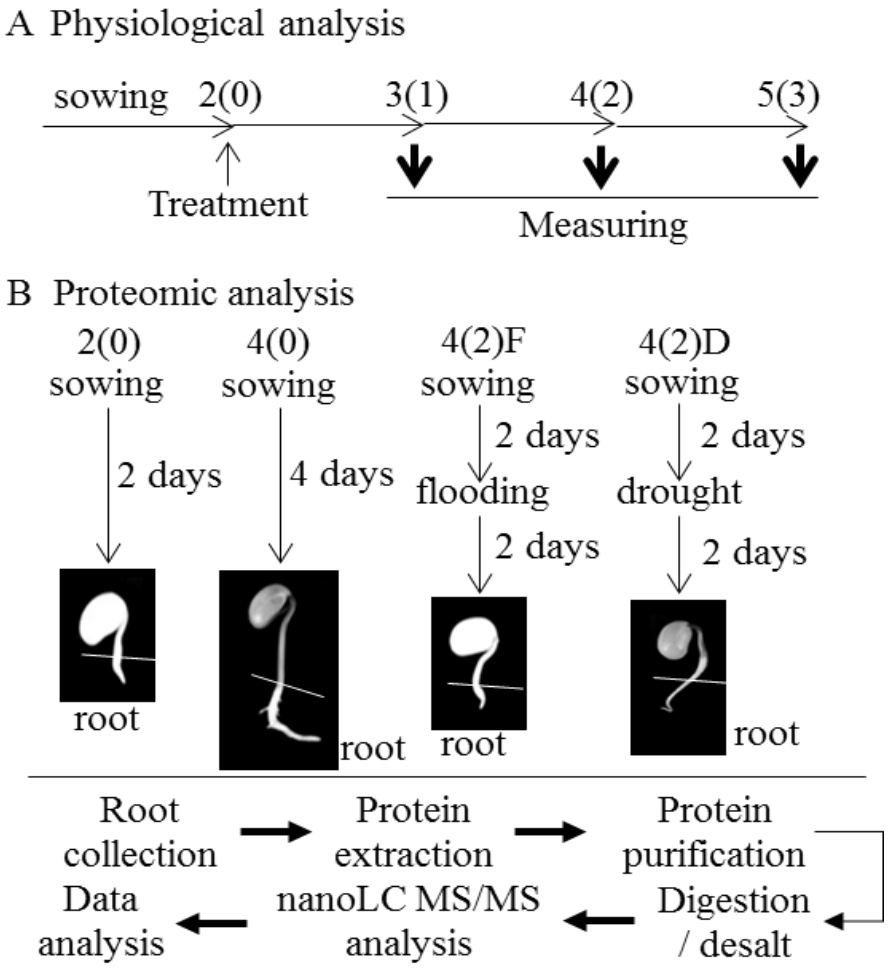

Figure 12. Experimental design for chapter 2. For physiological analysis, 2-day-old soybeans were treated with flooding and drought for 1, 2, and 3 days (A). For proteomic analysis, 2-day-old soybeans were treated with flooding and drougnt for 2 days and root was collected (B). Untreated soybeans were collected as control. Three independent experiments were performed as biological replicates.

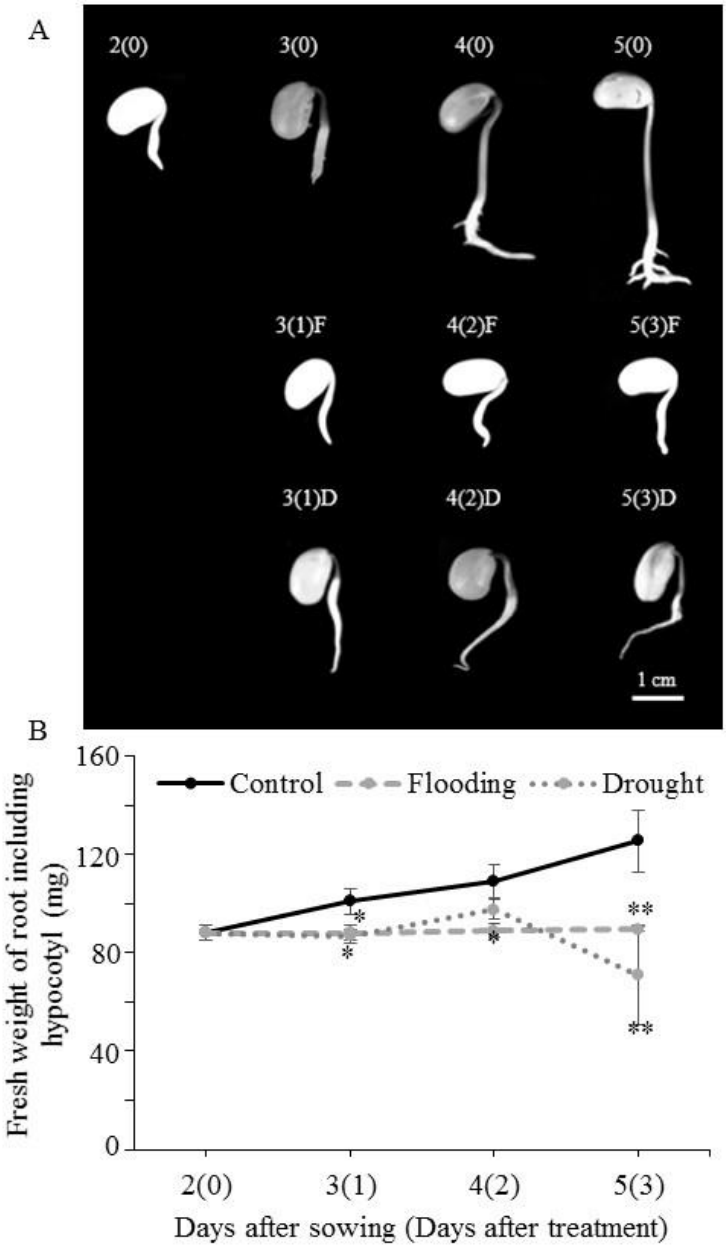

Figure 13. Effects of flooding and drought on growth of soybean seedlings. Two-day-old soybeans were treated with flooding or drought for 1, 2, and 3 days. Photograph shows the morphological differences between flooding and drought of 5-day-old soybeans (A). The fresh weight of root including hypocotyl was measured at indicated time points (B). Data are means ± S.E. of 3 independent biological replicates.

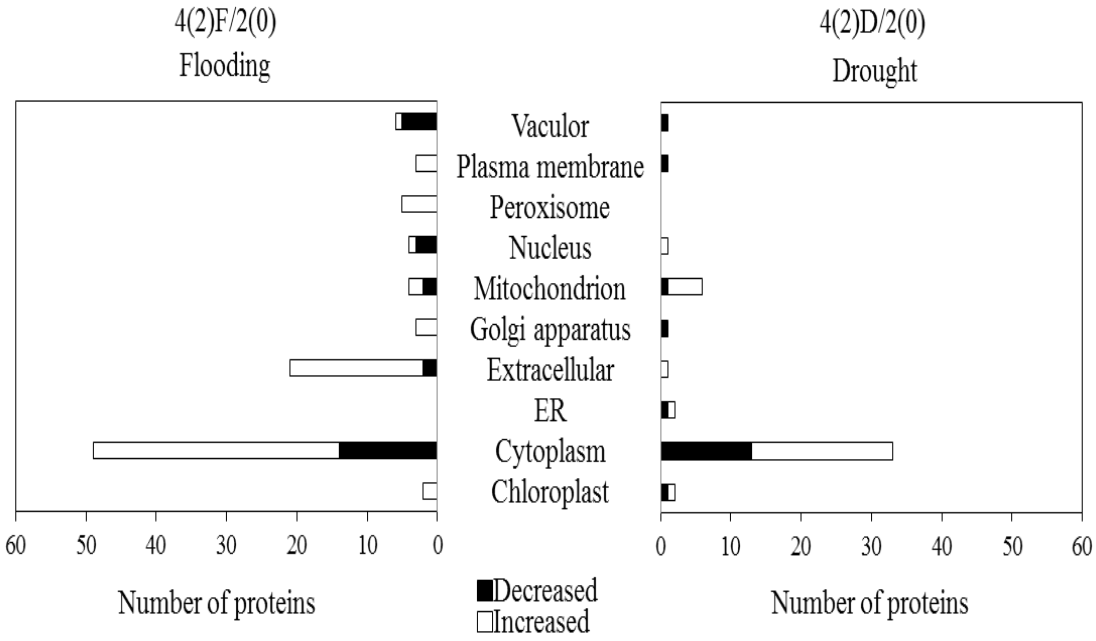

Figure 14. Predicted subcellular localization of flooding- and drought-responsive proteins in soybean roots. Localization of the identified proteins by nanoLC-MS/MS analysis was predicted using YLoc. The number of differentially abundant proteins is indicated on the x-axis of the graph. Black and white bars indicate decreased and increased proteins, respectively.

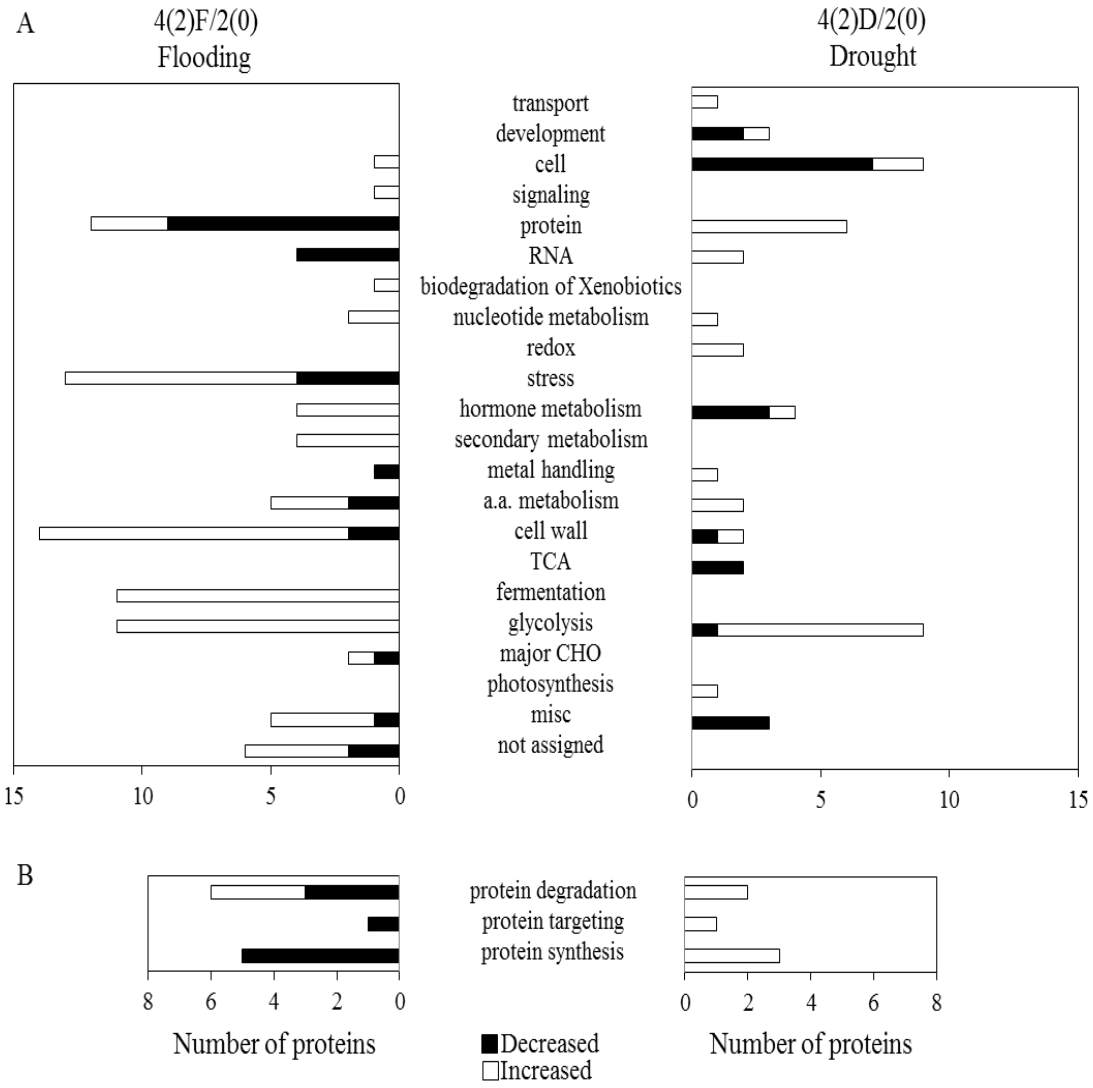

Figure 15. Functional categorization of flooding- and drought-responsive proteins in soybean roots. The tdentified proteins were categorized using MapMan bin codes: protein, protein degradation/synthesis/targeting; RNA, RNA binding/transcription; a.a. metabolism, amino acid metabolism; TCA, tricarboxylic acid; CHO, carbohydrates; and misc, miscellaneous enzyme families (A). Subcategorization of proteins in the "protein" category (B). Numbers of differentially abundant proteins are indicated on the x-axis in the graph. Black and white bars indicate decreased and increased proteins, respectively.

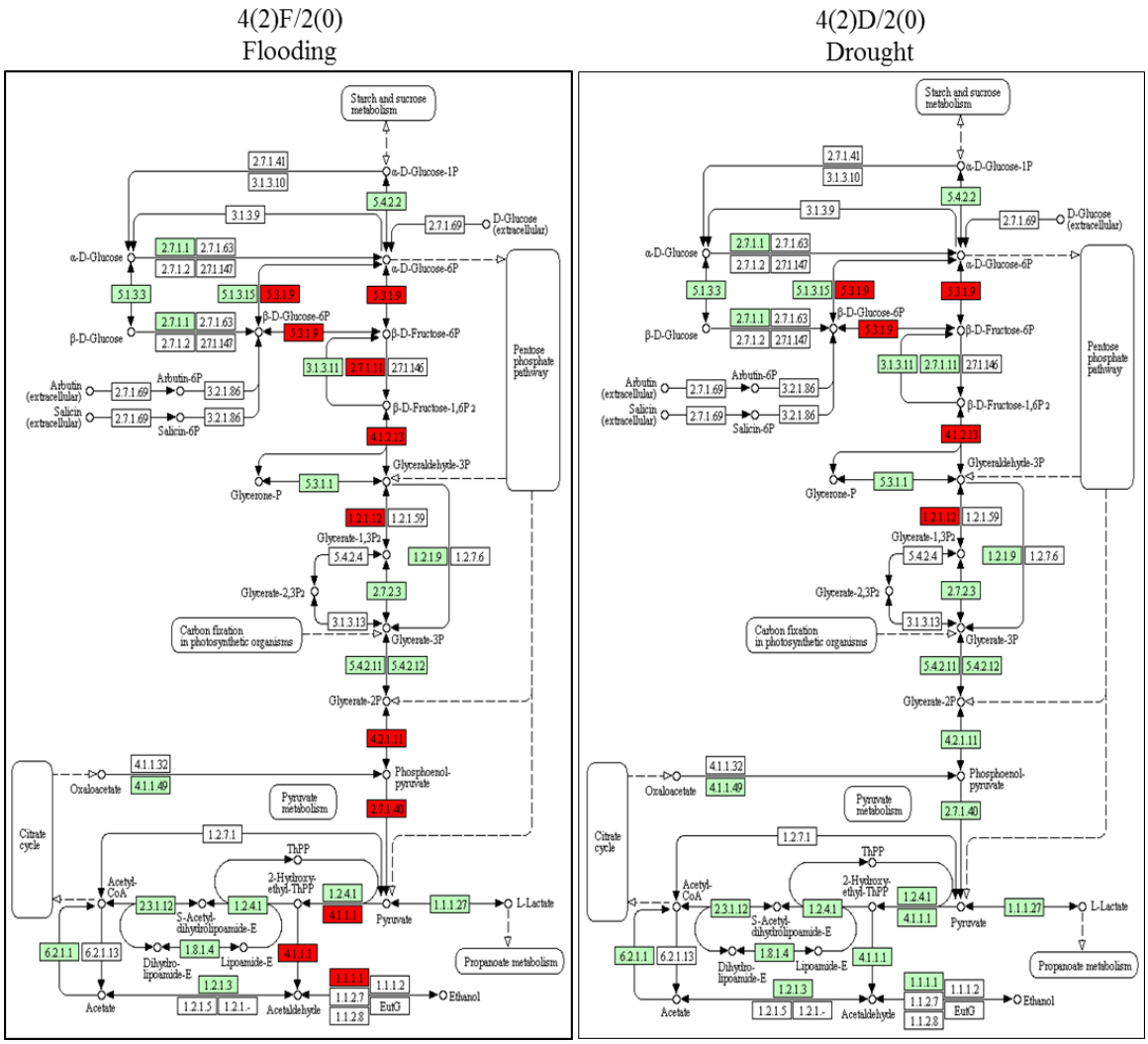

Figure 16. Mapping of identified flooding- and drought-responsive proteins on KEGG pathways for *Glycine max*. Glycolysis pathway was highlighted by the mapping of identified proteins. Numerous proteins involved in the glycolysis pathway were affected by both flooding and drought stresses. Enzymes in red represent increased proteins in this study. Enzymes in green were those found in the *Glycine max* KEGG database. EC numbers for the following proteins are: 1.1.1.1, alcohol dehydrogenase; 1.2.1.12, glyceraldehyde-3-phosphate dehydrogenase; 2.7.1.11, 6-phosphofructokinase; 2.7.1.40, pyruvate kinase; 4.1.1.1, pyruvate decarboxylase; 4.1.2.13, fructose-bisphosphate aldolase; 4.2.1.11, enolase; and 5.3.1.9, glucose-6-phosphate isomerase.

4(2)F/2(0)
Flooding
mRNA surveillance pathway

4(2)D/2(0)
Drought
Glutathione metabolism

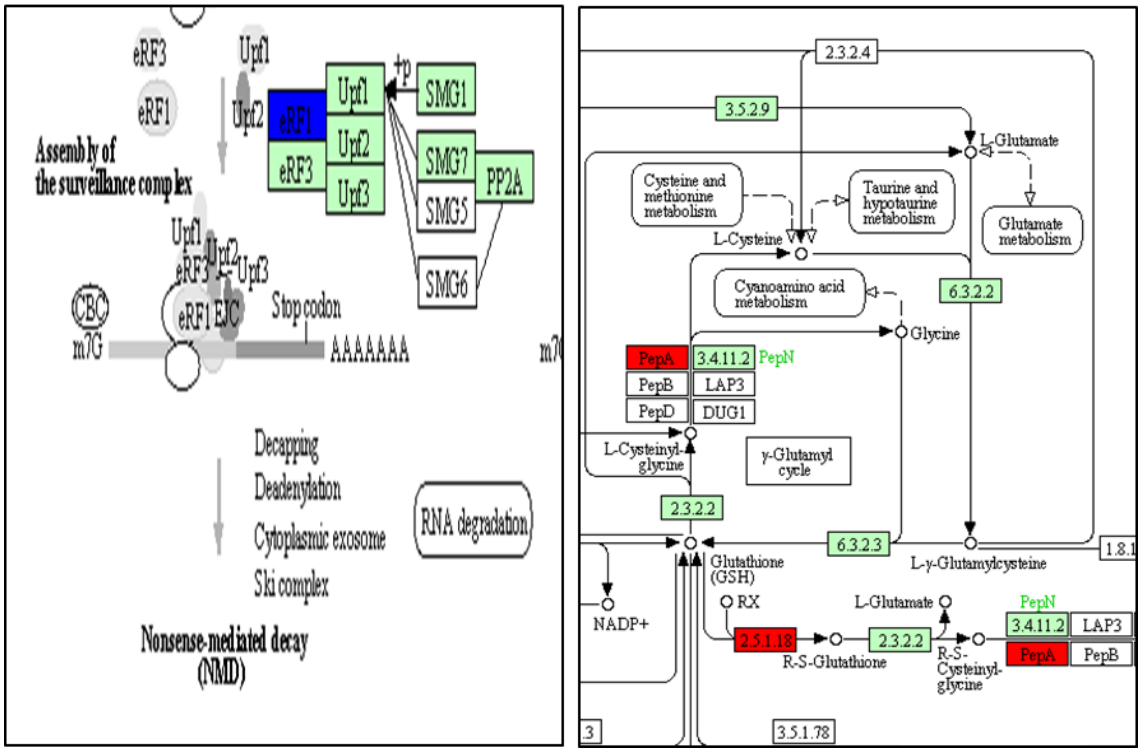

Figure 17. Mapping of identified flooding- and drought-responsive proteins on KEGG pathways for *Glycine max*. These pathways were highlighted by the mapping of identified proteins. Proteins involved in the mRNA surveillance and glutathione metabolism pathways were specifically induced by flooding and drought stress, respectively. Enzyme in blue was decreased under flooding, and those in red were increased under drought stress. Enzymes in green were those found in the *Glycine max* KEGG database. EC numbers for the following proteins are: 2.5.1.18, glutathione *S*-transferase; 3.4.11.1, leucyl aminopeptidase.

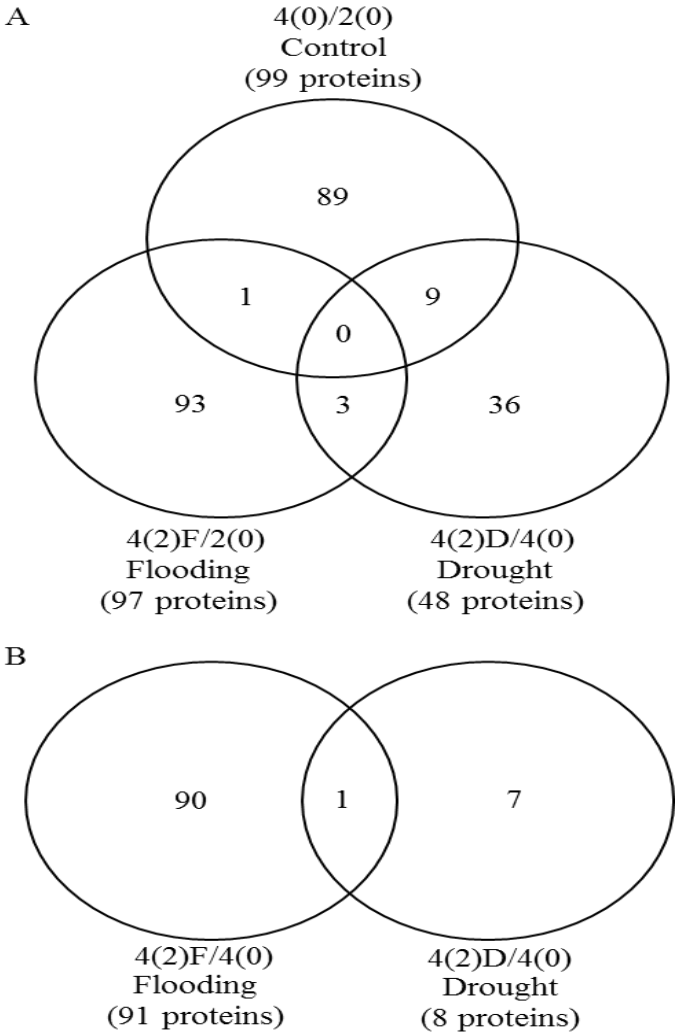

Figure 18. Venn diagram analysis of flooding- and drought-responsive proteins identified using gel-free proteomics. Two-day-old soybeans were treated with flooding or drought for 2 days, and proteins extracted from roots were analyzed using a gel-free proteomic technique. Venn diagram shows the number of identified proteins that overlapped among untreated, flooding-treated, and drought-treated soybeans (A). Venn diagram shows the number of identified proteins that overlapped between flooding- and drought-treated soybeans (B).

Chapter 3.

Charaterization of *S*-adenosylmethionine synthetase in soybean changed by under flooding and drought stresses

3.1. Introduction

Proteomic technique was used to understand organ specific response mechanism against flooding (Khatoon et al., 2012a) and drought (Mohammadi et al., 2012b) stresses in soybean, suggesting that differently changed level of proteins might lead to impair the growth of root, hypocotyl, and leaf of soybean under flooding and drought stresses. Kausar et al. (2012) reported that, in soybean, flooding and drought stresses were induced opposite activity and abundance change of APX, which is a ROS scavenger. The activity and abundance of APX were decreased under flooding stress; while, these were increased under drought stress in soybean (Kausar et al., 2012). In previous chapter, *S*-adenosylmethionine synthetase was decreased under flooding stress and increased under drought stress in soybean, indicating that *S*-adenosylmethionine synthetase might be important candidate factor for understanding response mechanism of soybean under stress conditions.

S-adenosylmethionine synthetase catalyzes the biosynthesis of *S*-adenosylmethionine, which has function as the donor for most biological methylation, including protein, RNA, DNA, and secondary metabolites of plant, and a precursor of polyamine and ethylene biosynthesis, from methionine and ATP (Tabor and Tabor, 1984; Anderson, 1990). *S*-adenosylmethionine is decarboxylated by *S*-adenosylmethionine decarboxylase to form decarboxylated *S*-adenosylmethionine for polyamine biosynthesis (Evans and Malmberg, 1989). In plant, *S*-adenosylmethionine act as a precursor for the synthesis of ethylene, which is one of the phytohormone involved in physiological processes and stress response (Yang and Hoffman, 1984; Abeles et al., 1992). *S*-adenosylmethionine serves as a methyl donor for pectin methylesterases and *O*-methyltransferase which are involved in pectin and lignin metabolism (Lamblin et al., 2001). These findings indicate that *S*-adenosylmethionine synthetase is seesential for

growth and development in plant.

It was reported that *S*-adenosylmethionine synthetase inhibited root growth mediated by ethylene and altered cell-wall structures and polymer in rice (Fukuda et al., 2007). Recently, tolerance to cold stress was enhanced by the overexpression of *S-adenosylmethionine synthetase* gene *via* promoting polyamine oxidation and improved hydrogen peroxide-induced antioxidant protection in blue alfalfa (Guo et al., 2014). The increase of *S*-adenosylmethionine synthetase was observed in rice under chilling (Cui et al., 2005), in lima bean by mechanical wounding (Arimura et al., 2002), in barley under salinity stress (Witzel et al., 2009), and in soybean by cotton worm feeding (Fan et al., 2012). In contrast, the decreased level of *S*-adenosylmethionine synthetase was shown in the flooded soybean (Hashiguchi et al., 2009; Nanjo et al., 2010). These results indicate that *S*-adenosylmethionine synthetases may be involved in the regulation of response mechanism in soybean under flooding and drought stresses.

The findings using proteomic technique in soybean under flooding and drought were summarized by Hossain and Komatsu (2014) to obtain a clear insight into complex mechanism of stress response due to their adverse effect on soybean. The majority of proteins, which were increased under flooding stress, were categorized in glycolysis, fermentation, cell wall metabolism, and nucleotide metabolism; while, proteins, which were decreased under flooding stress, were categorized in amino acid metabolism and cell organization. Flooding stress led to the decrease in ion transport related proteins and increase in cytoskeletal reorganization, cell expansion, and programmed cell death related proteins. The most of proteins, which were significantly changed by drought, were classified in redox regulation, oxidative stress response, signal transduction, protein folding, secondary metabolism, and photosynthesis. The abundance of proteins

involved in carbohydrate/nitrogen metabolism, cell wall modification, signal transduction, cell defense, and programmed cell death were highly affected by drought stress (Hossain and Komatsu, 2014).

However, this previous report provided with global mechanism in response to flooding and drought in soybean. In previous chapter, a gel-free proteomic technique was used to elucidate response mechanisms of soybean under flooding and drought. Based on this proteomic analysis, 3 *S*-adenosylmethionine synthetases were identified in both flooding and drought stress with oppositely changed level. In this chapter, to understand the role of *S*-adenosylmethionine synthetase in soybean, time dependency, organ specificity, and stress specificity were analyzed. To confirm the regulation of *S*-adenosylmethionine synthetase in soybean at mRNA expression level, protein abudance, and enzyme activity, analyses of quantitative reverse transcription polymerase chain reaction (qRT-PCR), immunoblot, and enzyme assay were performed.

3.2. Materials and Methods

3.2.1. Plant material and treatments

Plant material is mentioned in 1.2.1 in Chapter 1. Two-day-old soybeans were subjected to flooding (adding excess of water), drought (withholding water), salt (adding 200 mM NaCl), cold (keeping at 4°C), GA (supplying 10 μM GA_3), and calcium (supplying 50 mM $CaCl_2$). For time dependency, roots were collected from 2-, 3-, 4-, and 5-day-old soybeans, which were treated with flooding or drought for 1, 2, and 3 days. For organ specificity, root, hypocotyl, and cotyledon were collected from 4-day-old soybeans which were treated with flooding or drought for 2 days. For stress specificity, roots were collected from 4-day-old soybeans which were treated with flooding, drought, salt, cold, GA_3, or $CaCl_2$ (Figure 19). Non-treated equivalent

soybeans were collected as control. Three independent experiments were performed as biological replicates for all experiments.

3.2.2. Phylogenetic analysis

All *S*-adenosylmethionine synthetase sequences were retrived from Phytozome soybean genome database (version 9.1) (http://www.phytozome.net/soybean) (Schmutz et al., 2010). Phylogenetic analysis was conducted with Clustal W (version 2.1) (http://clustalw.ddbj.nig.ac.jp/top-j.html). Phylogenetic tree was visualized by TreeDyn program (Chevenet et al., 2006).

3.2.3. RNA extraction and analysis by quantitative reverse transcription polymerase chain reaction

A portion (100 mg) of frozen samples was ground into fine powder in liquid nitrogen using a sterilized mortar and pestle. Total RNA was extracted from the tissue powder using an RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). Extracted RNA was reverse-transcripted to cDNA using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) on a MyiQ Single-Color Real-Time PCR Detection system (Bio-Rad). The PCR conditions were as follows: 95°C for 30 sec, then 45 cycles of 95°C for 10 sec, and 60°C for 30 sec. Gene expression was normalized using 18S rRNA (X02623.1) as an internal control. The specific primers were designed using the Primer3 web interface (http://frodo.wi.mit.edu) (Rozen and Skaletsky, 2000) (Table 8). Specificity of primers was checked by BLASTN search against the soybean genome database with the designed primers as queries, by melt curve analysis, and by agarose gel electrophoresis of amplified fragments.

3.2.4. Protein extraction and immuno blot analysis

A portion (500 mg) of fresh samples was homogenized on ice in sodium dodecyl sulfate (SDS) sample buffer consisting of Tris, glycerol, SDS, and 2-mercaptoethanol (Laemmli, 1970) using a mortar and pestle. The homogenate was centrifuged at 20,000 x *g* for 10 min at 4°C. The supernatant was collected and re-centrifuged at 20,000 x *g* for 10 min at 4°C. The supernatant was used for gel electrophoresis. Protein concentration was determined using Pierce 660 nm Protein Assay Reagent (Thermo Fisher Scientific, Rockford, IL, USA) with bovine serum albumin as the standard. For immunoblot analysis, protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride membrane using a semi-dry transfer blotter. After blocking overnight at 4°C in buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 5% skim milk (Nacalai, Tokyo, Japan), the blotted membrane was incubated with anti-*S* adenosylmethionine antibody (prepared by Dr. Komatsu's Lab) for 1 h at room temperature. Anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) served as the secondary antibody. After 1 h incubation with the secondary antibody, immunoblot signals were detected using Chemi-Lumi One Super (Nacalai) according to the manufacturer's protocol and visualized using a luminescent image analyzer (LAS-3000; Fujifilm, Tokyo, Japan). Coomassie brilliant blue (CBB) was used as a loading control. The relative band intensities were calculated using Quantity One 1-D Analysis software (Bio-Rad).

3.2.5. Protein extraction and analysis of enzyme activity

A portion (100 mg) of fresh samples was homogenized in buffer containing 50 mM Tris-HCl (pH 7.6), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EDTA, and

2% polyvinylpyrrolidone using a mortar and pestle on ice. The homogenate was centrifuged at 10,000 x *g* for 15 min at 4°C twice. The supernatant was collected as enzyme extract and used for enzyme assay. Protein concentration was determined using the Bradford methiod (Bradford, 1976) with bovine serum albumin as the standard. The reaction mixture consisted of 100 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 150 mM KCl, 2 mM ATP, 5 mM dithiothreitol, and 1 mM methionine. One hundred micro liter of enzyme extract was added to reaction mixture and immediately measured. The activity of *S*-adenosylmethionine synthetase was assayed by specrophotometrically measured at OD_{340} (Kim et al., 1992).

3.2.6. Statisitical analysis

The statistical significance was evaluated with Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison ($p < 0.05$). All calculations were performed using SPSS software (version 22.0).

3.3. Results

3.3.1. Phylogenetic analysis of *S*-adenosylmethionine synthetases

Using proteomic analysis, 3 kinds of *S*-adenosylmethionine synthetses, which are Glyma15g21890.1 (*S*-adenosylmethionine synthetase family), Glyma03g38190.3 (*S*-adenosylmethionine synthetase 1), and Glyma19g40810.1 (*S*-adenosylmethionine synthetase 2), were identified in soybean under flooding and drought. These results revealed that these proteins were decreased and increased under flooding and drought stresses, respectively (Figure 20, Table 9). To understand relationship of soybean *S*-adenosylmethionine synthetase orthologous, analyses of phylogenetic distribution and amino acid sequence alignment were performed (Clustal W). In soybean, 14 *S*-

adenosylmethionine synthetases were existed in Phytozome soybean genome database (Figure 21). Soybean *S*-adenosylmethionine synthetase is a gene encoding peptide of 394-395 amino acid residues with an estimated molecular mass of 43,053 Da. Phylogenetic analysis using amino acid sequences predicted from homologs indicated that *S*-adenosylmethionine synthetase 1 and *S*-adenosylmethionine synthetase 2 were belonged to the same group; while *S*-adenosylmethionine synthetase family was belonged to different group (Figure 21) as shown amino acid sequence alignment (Figure 22). The amino acid sequence of *S*-adenosylmethionine synthetase 1 had similarity of 99.75% and 94.18% with *S*-adenosylmethionine synthetase 2 and *S*-adenosylmethionine synthetase family, respectively (Figure 22). The amino acid sequence of *S*-adenosylmethionine synthetase family had similarity of 94.43% with *S*-adenosylmethionine synthetase 2 (Figure 22).

3.3.2. Transcriptional level of *S-adenosylmethionine synthetases* in soybean with time dependent manner, organ specificity, and stress specificity.

To understand the change of *S*-adenosylmethionine synthetase in soybean at transcriptional level, the mRNA expression level of 3 *S-adenosylmethionine synthetase* gene was examined with time dependent manner, organ specificity, and stress specificity (Figure 23, Figure 24). For time dependent manner, 2-day-old soybeans were treated with flooding or drought for 1, 2, and 3 days. Untreated soybeans were used as control. In untreated soybean, the mRNA expression levels of *S-adenosylmethionine synthetase* genes were not significantly changed with time duration (Figure 23A). The mRNA expression levels of *S-adenosylmethionine synthetase family* and *2* were not significantly changed under flooding stress with time duration (Figure 23A). The mRNA expression levels of *S-adenosylmethionine*

synthetase family and *1* was significantly up-regulated at 3 days after drought stress (Figure 23A). However, *S-adenosylmethionine synthetase 2* were not significantly changed under drought stress (Figure 23A).

For organ specificity, 2-day-old soybeans were treated with flooding or drought for 2 days, and root, hypocotyl, and cotyledon were collected. Untreated soybeans were used as control. Under normal condition, the mRNA expression levels of *S-adenosylmethionine synthetase* genes were up-regulated in root than in hypocotyl and cotyledon at 2 days after sowing (Figure 23B). *S-adenosylmethionine synthetase family* was significantly expressed in hypocotyl than in root and cotyledon of 4-day-old soybean (Figure 23B). The mRNA expression level of *S-adenosylmethionine synthetase 1* was significantly up-regulated in cotyledon than in root and hypocotyl of 4-day-old soybean (Figure 23B). *S-adenosylmethionine synthetase 2* was not significantly changed in 3organ (Figure 23B). The mRNA expression level of *S-adenosylmethionine synthetase family* was up-regulated in root and hypocotyl; while, that was down-regulated in cotyledon under flooding stress (Figure 23B). The mRNA expression level of *S-adenosylmethionine synthetase 1* was not significantly changed in 3 organs under flooding stress; while, that of *S-adenosylmethionine synthetase 2* was significantly up-regulated in hypocotyl under flooding stress (Figure 23B). The mRNA expression level of *S-adenosylmethionine synthetase family* was up-regulated in root and hypocotyl than in cotylednon under drought stress (Figure 23B). The mRNA expression levels of *S-adenosylmethionine synthetase 1* and *2* were significantly up-regulated in root under drought stress (Figure 23B).

For stress specificity, 2-day-old soybeans were subjected to flooding, drought, salt, cold, GA_3, or $CaCl_2$ (Figure 24). The mRNA expression of *S-adenosylmethionine synthetase family* was not significantly changed under flooding stress (Figure 24). The

mRNA expression level of *S-adenosylmethionine synthetase 1* was significantly up-regulated by flooding stress. In contrast, the mRNA expression level of *S-adenosylmethionine syntheatse 2* was significantly down-regulated under flooding stress (Figure 24). The mRNA expression level of *S-adenosylmethionine synthetase family* was up-regulated under drought; while, that of *S-adenosylmethionine synthetase 2* was not significantly changed by drought stress (Figure 24). Furthermore, *S-adenosylmethionine synthetase family* was significantly up-regulated under cold, GA_3, and $CaCl_2$ stresses; however, the mRNA expression level of *S-adenosylmethionine synthetase 1* was not significantly changed any other stress conditions (Figure 24). The mRNA expression level of *S-adenosylmethionine synthetase 2* was not significantly changed by salt and cold; while, that was significantly down-regulated when GA_3 and $CaCl_2$ were treated in soybean root (Figure 24). Primer specificities were assessed by agarose gel electrophoresis (Figure 25).

3.3.3. Protein abundance of *S*-adenosylmethionine synthetase in soybean with time dependent manner, organ specificity, and stress specificity

To understand the abundance change of *S*-adenosylmethionine synthetase in soybean, immuno blot analysis was performed with time dependent manner, organ specificity, and stress specificity (Figure 26, Figure 27). For time dependent manner, 2-day-old soybeans were treated with flooding or drought for 1, 2, and 3 days. Untreated soybeans were used as control. In untreated soybean, *S*-adenosylmethionine synthetase was significantly changed with time duration (Figure 26A). The abundance of *S*-adenosylmethionine synthetase was significantly accumulated at 1 day after flooding and decreased at 2 and 3 days after flooding (Figure 26A). The abundance of *S*-adenosylmethionine syntheatase was not significantly changed under drought stress

with time duration (Figure 26A).

For organ specificity, 2-day-old soybeans were treated with flooding or drought for 2 days, and root, hypocotyl, and cotyledon were collected. Untreated soybeans were used as control. The abundance of *S*-adenosylmethionine synthetase was not significantly changed in 3 organ of untreated soybean (Figure 26B). The abundance of *S*-adenosylmethionine synthetase was not significantly changed under flooding as similar to untreated soybean (Figure 26B). There was no significant change on the abundance of *S*-adenosylmethionine synthetase under drought stress in 3 organ (Figure 26B).

For stress specificity, 2-day-old soybeans were subjected to flooding, drought, salt, cold, GA_3, or $CaCl_2$. The abundance of *S*-adenosylmethionine synthetase under flooding and drought stresses was similar to normal condition (Figure 27). Under salt stress, the abundance of *S*-adenosylmethionine synthetase was similar to that of normal condition (Figure 27). The *S*-adenosylmethionine synthetase was significantly accumulated under cold, GA_3, and $CaCl_2$ stresses (Figure 27).

3.3.4. The activity of *S*-adenosylmethionine synthetase in soybean with time dependent manner, organ specificity, and stress specificity

To understand the change of *S*-adenosylmethionine synthetase in soybean at enzyme activity, enzyme assay was performed with time dependent manner, organ specificity, and stress specificity (Figure 28, Figure 29). For time dependent manner, 2-day-old soybeans were treated with flooding or drought for 1, 2, and 3 days. Untreated soybeans were used as control. The activity of *S*-adenosylmethionine synthetase was not significantly changed with time duration in untreated soybean (Figure 28A). The activity of *S*-adenosylmethionine synthetase activity was decreased at 1 and 2 days after

flooding as compared to untreated soybean; whereas, the activity was increased under 3 days after flooding at a similar level as untreated soybean (Figure 28A). In soybean under 1 day after drought, the activity of *S*-adenosylmethionine synthetase was lower than that of untreated soybean; however, that was increased under 2 days after drought at a same level as untreated soybean (Figure 28A). Under 3 days after drought, the activity of *S*-adenosylmethionine synthetase was increased to higher level than that of untreated soybean (Figure 28A).

For organ specificity, 2-day-old soybeans were treated with flooding or drought for 2 days, and root, hypocotyl, and cotyledon were collected. Untreated soybeans were used as control. Under normal condition, the activities of *S*-adenosylmethionine synthetase were similar level in root and hypocotyl, but cotyledon had lower level of this enzyme compared to root and hypocotyl (Figure 28B). Although there was no significant changed in 3 organs under flooding stress, the pattern of *S*-adenosylmethionine synthetase activiy was similar to normal condition (Figure 28B). Under drought stress, the activity of *S*-adenosylmethionine synthetase was similar to normal condition, which had high level in root and hypototyl than in cotyledon (Figure 28B).

For stress specificity, 2-day-old soybeans were subjected to flooding, drought, salt, cold, GA_3, or $CaCl_2$. The activity of *S*-adenosylmethionine synthetase was decreased under flooding stress (Figure 29). Under drought stress, the activity of *S*-adenosylmethionine synthetase activity was similar to that of normal condtion (Figure 29). Salt stress was induced higher level of activity of *S*-adenosylmethionine synthetase; while, the activity of *S*-adenosylmethionine synthetase was not significantly changed by cold, GA_3, and $CaCl_2$ stresses in soybean (Figure 29).

3.4. Discussion

3.4.1. Soybean *S*-adenosylmethionine synthetase

S-adenosylmethionine synthetases, which were decreased and increased under flooding and drought stresses, respectively, were commonly identified in soybean (Chapter 2). To understand relationship of soybean *S*-adenosylmethionine synthetase orthologous, phylogenetic analysis was performed. Three *S*-adenosylmethionine synthetases were closely related isoforms (Figure 21), because of similarity of their amino acid sequence (Figure 22). In all plants, the amino acid sequence of *S*-adenosylmethionine synthetase contains two conserved *S*-adenosylmethionine synthetase motifs (Horikawa et al., 1990; Bairoch, 1993); first motif is a hexapeptide considered to bind to the adenine moiety of ATP (Pajares et al., 1991) and second motif is a glycine-rich nonapeptide consisted of a P-loop-like sequence, which involved in binding the triphosphate of ATP (Takusagawa et al., 1996). In this study, these *S*-adenosylmethionine synthetase motifs were existed in amino acid sequence of 3 soybean *S*-adenosylmethionine synthetases. These results indicate that soybean *S*-adenosylmethionine synthetase is highly conserved.

3.4.2. The role of *S*-adenosylmethionine synthetase on temporal change

In present study, the activity of *S*-adenosylmethionine synthetase was decreased until 2 days after flooding and increased at 3 days after flooding (Figure 28A). The enzyme activity of S-adenosylmethionine synthetase was significantly increased at 3 days after drought stress (Figure 28A). Time course response of *S*-adenosylmethionine synthetase in mature leaves of blue alfalfa to cold treatment was analyzed at transcript and protein levels, indicating that *S*-adenosylmethionine synthetase transcripts and proteins were highly induced at 8-48 h and 8-96 h after cold treatment, respectively

(Guo et al., 2014). RNA expression levels were analyzed to determine the developmental expression of barley *S-adenosylmethionine synthetase* genes with the kernels of 3 days before fertilization to 30 days after fertilization and found that the *S-adenosylmethionine synthetase* transcripts predominantly accumulated until 10 days after fertilization (Kim, 2013). Kim (2013) also found that barley *S-adenosylmethionine synthetase* genes were induced at 12 h of wounding and 48 h of NaCl, ABA, and spermidine treatments. It was reported that the expression level of ginseng *S-adenosylmethionine synthetase* was gradually up-regulated during salt stress until 12 h and then down-regulated at 24-72 h (Pulla et al., 2009). These results indicate that the change of *S*-adenosylmethione synthetase might be independent on time duration in soybean root in response to stress.

3.4.3. The role of *S*-adenosylmethionine synthetase in soybean organ

In present study, the activity of *S*-adenosylmethionine synthetase was increased in root and hypocotyl in untreated, flooding-, and drought-treated soybean; while, that was low in cotyledon in untreated, flooding-, and drought-treated soybean (Figure 28B). Organ specific expression patterns of *S-adenosylmethionine synthetases* in the periwinkle under salt stress were revealed that the radicula and hypocotyl had higher levels of these enzymes compared to the cotyledons, where the expression levels remained low (Schroder et al., 1997). In *Arabidopsis* stems, roots, and callus tissues, the *S-adenosylmethionine synthetase* gene was approximately 20-fold highly expressed than in leaves, seed pods, and inflorescences (Peleman et al., 1989a) and these were corresponded to 10- to 20-fold higher activity of *S*-adenosylmethionine synthetase in stems than in leaves (Peleman et al., 1989b). It was reported that the *S-adenosylmethionine synthetase 1* gene of lodgepole pine was specifically or highly

expressed in roots as compared to shoots (Lindroth et al., 2001). *S*-adenosylmethionine synthetase was specifically expressed ginseng root (Pulla et al., 2009). The transcripts of barley *S-adenosylmethionine synthetases* abundantly accumulated in grains, stems, and leaves than roots (Kim, 2013); however, in this study, the mRNA expression level and activity of *S*-adenosylmethionine synthetase was accumulated in root and hypocotyl than in cotyledon under normal condition (Figures 23B and 28B). These results indicate that *S*-adenosylmethionine synthetase might be specific to root and hypocotyl in soybean.

3.4.4. The role of *S*-adenosylmethionine synthetase in soybean under different stress conditions

In present study, the mRNA expression level of *S-adenosylmethionine synthetae family* was significantly up-regulated under drought, cold, GA_3, and $CaCl_2$ stresses; while, that of *S-adenosylmethionine synthetase 2* was significantly down-regulated under flooding stress in soybean root (Figure 24). The mRNA expression level of *S*-adenosylmethionine synthetase was specifically accumulated in response to NaCl, mannitol, or ABA treatments in tomato (Espartero et al., 1994). It was reported that all *S-adenosylmethionine synthetase* genes were significantly up-regulated in periwinkle under salt stress (Schroder et al., 1997). In addition to these reports, water stress led to the accumulation of *S*-adenosylmethionine synthetase in rice (van Breusegem et al., 1994). In jack pine and loblolly pine, *S-adenosylmethionine synthetase* was up-regulated under water deficit (Mayne et al., 1996; Chang et al., 1996). Similarly, in this study, the mRNA expression level of *S-adenosylmethionine synthetase family* was up-regulated under drought stress (Figure 24). It was reported that the mRNA expression level and protein abundance of *S*-adenosylmethionine synthetase were increased in

tomato leaves and roots during salinity stress (Sanchez-Aguayo et al, 2004). However, the expression of *S-adenosylmethionine synthetase* was not affected by heat shock, cold, and anoxia stresses (Mayne et al., 1996). In contrast, in this study, the mRNA expression level of *S-adenosylmethionine synthetase 2* was significantly down-regulated by flooding stress (Figure 24). Guo et al. (2014) reported that the expression of *S*-adenosylmethionine synthetase greatly induced and the abundance of *S*-adenosylmethionine synthetase was accumulated in cold-stresses blue alfalfa after ABA treatment. In present study, *S*-adenosylmethionine synthetase was induced by drought, cold, salt, GA_3, and $CaCl_2$ treatment; however, decreased by flooding stress in soybean root. It was reported that exogenously added jasmonic acid strongly induced *S-adenosylmethionine synthetase* gene in lima bean leaves (Arimura et al., 2012). In present study, the mRNA expression of *S-adenosylmethionine synthetase family* was significantly up-regulated and theire abundance and activity were increased in soybean when GA_3 was supplemented (Figures 24, 27, and 29). These results indicate that *S*-adenosylmethionine synthetase may be involved in different response to stress conditions and regulated by phytohormone such as GA_3 in soybean root.

3.4.5. Concluding remarks

In previous chapter, 3 *S*-adenosylmethionine synthetases were identified in soybean under flooding and drought stresses, which were decreased by flooding and increased by drought using proteomic analysis. Increased *S*-adenosylmethionine synthetase activity correlated with the number of lignified vessels in roots and shoots of tomato under salinity stress (Sanchez-Aguayo et al., 2004). *S*-adenosylmethionine synthetase was involved in stress responsive mechanism. In this chapter, to understand the characterization of *S*-adenosylmethionine synthetase in soybean under stress,

mRNA expression level, protein abundance, and enzyme activity analyses were performed (Table 10). The mRNA expression level of *S-adenosylmethionine synthetase 2* was down-regulated by flooding; while, that of *S*-adensosylmethionine synthetase family was up-regulated by drought stress. The mRNA expression levels of these genes were similar tendency of protomic analysis results. The activity of *S*-adenosylmethionine synthetase was decreased under flooding for 2 days; while, that was increased under drought for 3 days, indicating that *S*-adenosylmethionine synthetase was responded at early time of flooding and late time of drought. The activity of *S*-adenosylmethionine synthetase was increased in root and hypocotyl, indicating that these results were same as that of tomato under salinity stress (Sanchez-Aguayo et al., 2004). These results suggest that *S-adenosylmethionine synthetase family* and *2* might regulate drought and flooding responses, repectively, in soybean root. Further study will needed to elucidate how *S*-adenosylmethionine synthetase is involved in biosynthesis of lignin and ethylene and ascorbate-glutathionine pathway in soybean under flooding and drought stresses.

3.5. Summary

Flooding and drought affect soybean growth because soybean is stress-sensitive crop. In previous chapter, a gel-free proteomic technique was used to understand the responsive mechanisms of soybean under flooding and drought stresses. Among changed proteins, 3 *S*-adenosylmethionine synthetases, which were decreased and increased under flooding and drought stresses, respectively, were commonly identified in soybean. It was suggest that *S*-adenosylmethionine synthetase is involved in the regulation of stress response in soybean. In this chapter, to understand the

characterization of *S*-adenosylmethionine synthetase in soybean, time dependency, organ specificity, and stress specificity were analyzed at mRNA expression level, protein abundance, and enzyme activity. The mRNA expression level, protein abundance, and enzyme acitivity of *S*-adenosylmethionine synthetase were time-independently changed under flooding and drought stresses. The mRNA expression level, protein abundance, and enzyme activity of *S*-adenosylemthionine synthetase were more abundanted in root and hypocotyl than in cotyledon under flooding and drought stresses. The mRNA expression level of *S-adenosylmethionine synthetae family* was not affected by flooding; while, it was significantly up-regulated by drought stress. In contrast, the mRNA expression level of *S-adenosylmethionine syntheatse 2* was not affected by drought; while, it was significantly down-regulated by flooding stress. The activity of *S*-adenosylmethionine synthetase was decreased and increased in response to flooding and drought stresses, respectively; however, their protein abundances were not changed by flooding and drought stresses. In addition, *S*-adenosylmethionine synthetase was induced by salt, cold, GA_3, and $CaCl_2$ stresses at mRNA expression level, protein abundance, and enzyme activity level. These results suggest that the differential regulation of *S*-adenosylmethionine synthetase under flooding and drought might be involved in their different response in soybean root.

Table 8. List of qRT-PCR primers used in this study

Gene name	Protein ID	Sequence	
S-adenosylmethionine synthetase family protein	Glyma15g21890.1	Forward	5'-GTG CTT CTG GAA GTT AAA ATG G-3'
		Reverse	5'-TGA TCT CTC CGA AAA CCA TC-3'
S-adenosylmethionine synthetase 1	Glyma03g38190.3	Forward	5'-GAC TGG CAG TAT ATC CAG TTA CAG-3'
		Reverse	5'-GAT GCA AAA GAA GGG TGA T-3'
S-adenosylmethionine synthetase 2	Glyma19g40810.1	Forward	5'-CTG CTT CTT CAG CTT GAG AAA TG-3'
		Reverse	5'-CAA AGA CCA TGA CCA TGT TGG-3'
18S rRNA	X02623.1*	Forward	5'-TGA TTA ACA GGG ACA GTC GG-3'
		Reverse	5'-ACG GTA TCT GAT CGT CTT CG-3'

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

Table 9. List of 3 common proteins identified between 2-day-flooded soybean and 2-day-droughted soybean compare to 2-day-old soybean.

Protein ID	Description	M.P.	Ratio 4(2)F	*P*value 4(2)F	Ratio 4(2)D	*P*value 4(2)D	Function	Localization
Glyma15g21890.1	*S*-adenosylmethionine synthetase family protein	4	0.4175	0.0127	9.0403	0.0383	metal handling	Cytoplasm
Glyma03g38190.3	*S*-adenosylmethionine synthetase 1	3	0.4185	0.0213	10.6780	0.0101	a. a. metabolism	Cytoplasm
Glyma19g40810.1	*S*-adenosylmethionine synthetase 2	3	0.4185	0.0213	10.6780	0.0101	a. a. metabolism	Cytoplasm

Protein ID, according to the Phytozome database; M.P., number of matched peptide; Ratio, relative abundance of protein, Function, functional categorization by MapMan bin code; Localization, localization prediction using YLoc.

Table 10. Summary of mRNA expression level, protein abundance, and enzyme activity of *S*-adenosylmethionine synthetase

Gene description		Time dependency									Organ specificity												Stress specificity					
		3(0)	4(0)	5(0)	3(1)	4(2)	5(3)	3(1)	4(2)	5(3)	R	H	C	R	H	C	R	H	C	R	H	C	Flood	Drought	Salt	Cold	GA_3	$CaCl_2$
		Control			Flood			Drought			2(0)			4(0)			Flood			Drought								
mRNA expression level	*S-adenosylmethionine synthetase family*	D	D	U	x	x	x	x	x	U	U	D	D	D	U	D	U	U	D	U	U	D	x	U	x	U	U	U
	S-adenosylmethionine synthetase 1	D	D	U	x	x	x	x	x	x	U	D	D	D	D	U	x	x	x	U	D	D	U	x	x	x	x	x
	S-adenosylmethionine synthetase 2	x	U	D	x	x	x	x	x	x	U	D	D	x	x	x	U	U	D	U	D	D	D	x	x	x	D	D
Protein abundance		x	x	x	I	D	D	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	I	I	I
Enzyme activity		x	x	x	x	D	I	D	I	I	I	I	D	x	x	x	x	x	x	I	I	D	D	x	x	x	x	x

D, down-regulated or decreased; U, up-regulated; x, no changed; I, increased; 2(0), 2-day-old soybean; 3(0), 3-day-old soybean; 4(0), 4-day-old soybean; 5(0), 5-day-old soyubean; 3(1), 3-day-old soybean for 1 day treatment; 4(2), 4-day-old soybean for 2 days treatment; 5(3), 5-day-old soybean for 3 days treatment; R, root; H, hypocoty; C, cotyledon

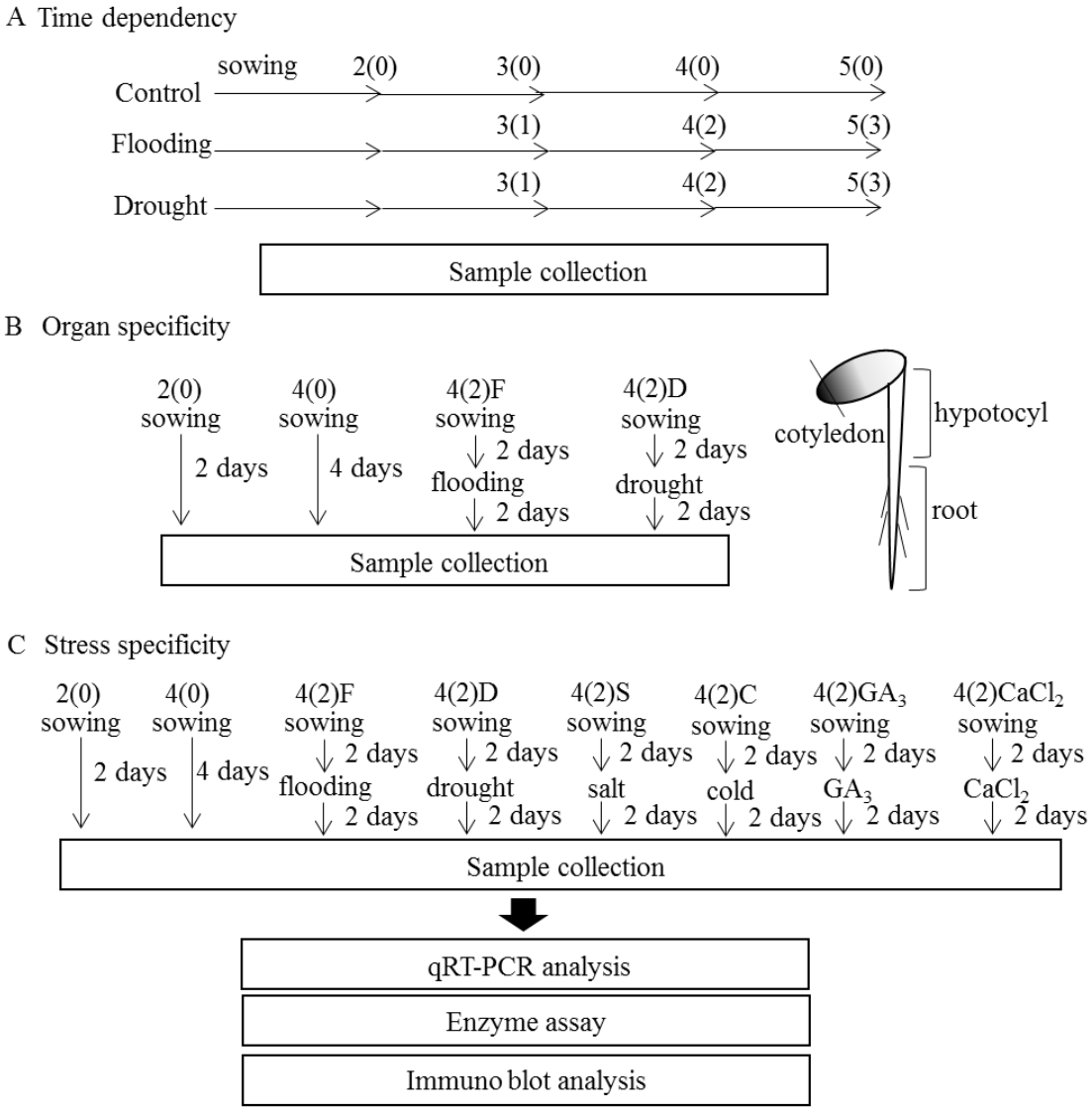

Figure 19. Experimental design for chapter 3. For time dependency, 2-day-old soybeans were treated with flooding or drought for 1, 2, and 3 days. Roots were collected as sample (A). For organ specificity, 2-day-old soybeans were treated with flooding or drougnt for 2 days. Root, hypocotyl, and cotyledon were collected as sample (B). For stress specificity, 2-day-old soybeans were treated with flooding, drought, salt, cold, GA_3, or $CaCl_2$ for 2 days. Roots were collected as sample (C). Untreated soybeans were collected as control. Three independent experiments were performed as biological replicates.

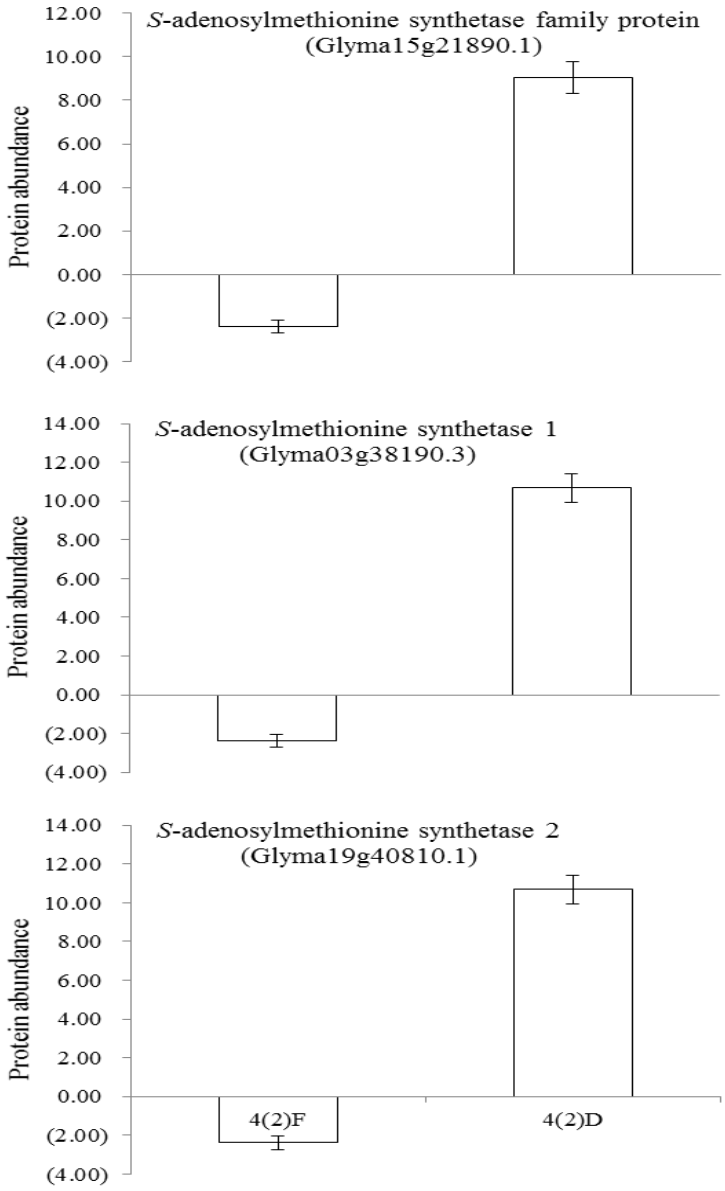

Figure 20. Protein abundance of 3 *S*-adenosylmethionine synthetases identified in soybean under flooding and drought stresses. Two-day-old soybeans were treated with flooding or drought stress for 2 days, and extracted proteins were analyzed using nanoLC-MS/MS. Protein abundance was determined by differential analysis using SIEVE software. Three *S*-adenosylmethionine synthetases were decreased and increased under flooding and drought stresses, respectively.

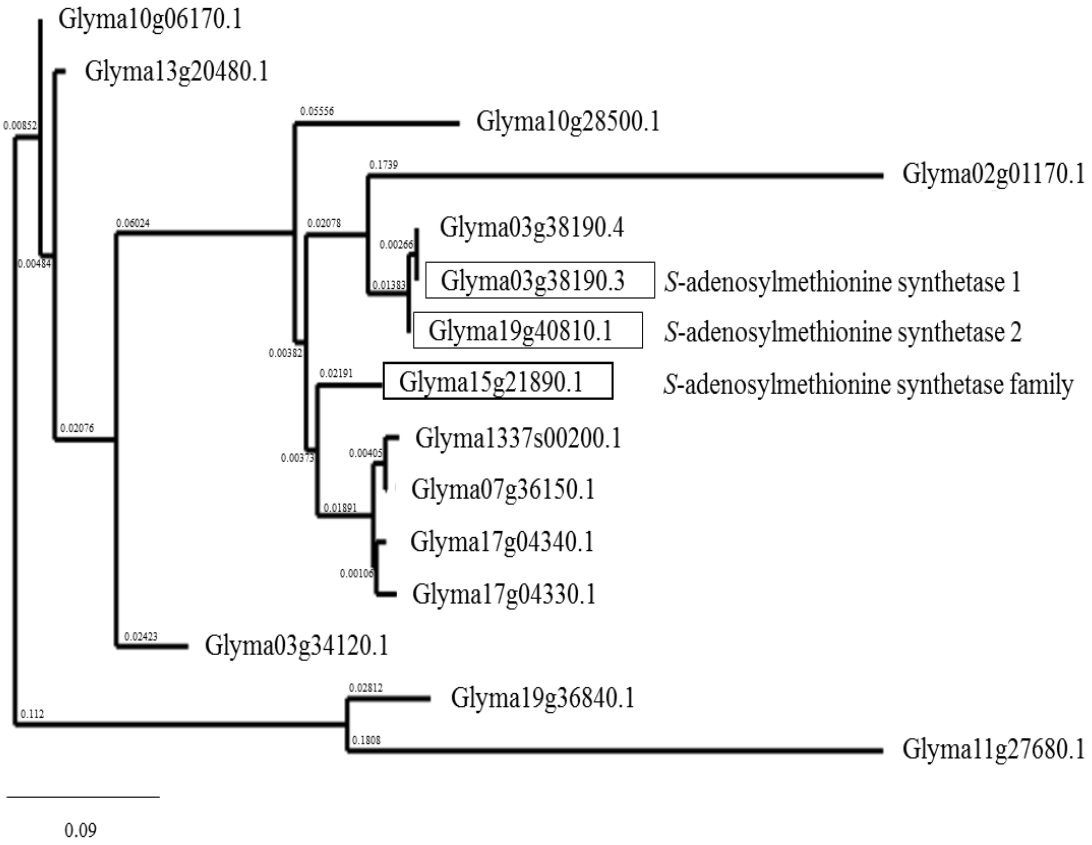

Figure 21. Phylogenetic distribution of *S*-adenosylmethionine synthetase sequences predicted from amino acid. Phylogenetic analysis was performed using Clustal W software. The scale bar corresponds to the number of amino acid substitutions per site. Rectangle indicats identified proteins in this study.

Figure 22. Amino acid sequence alignment of 3 types of *S*-adenosylmethionine synthetase proteins identified in flooding- and drought-treated soybean. Black rectangle indicates different parts of amino acid sequences among 3 *S*-adenosylmethionine synthetases.

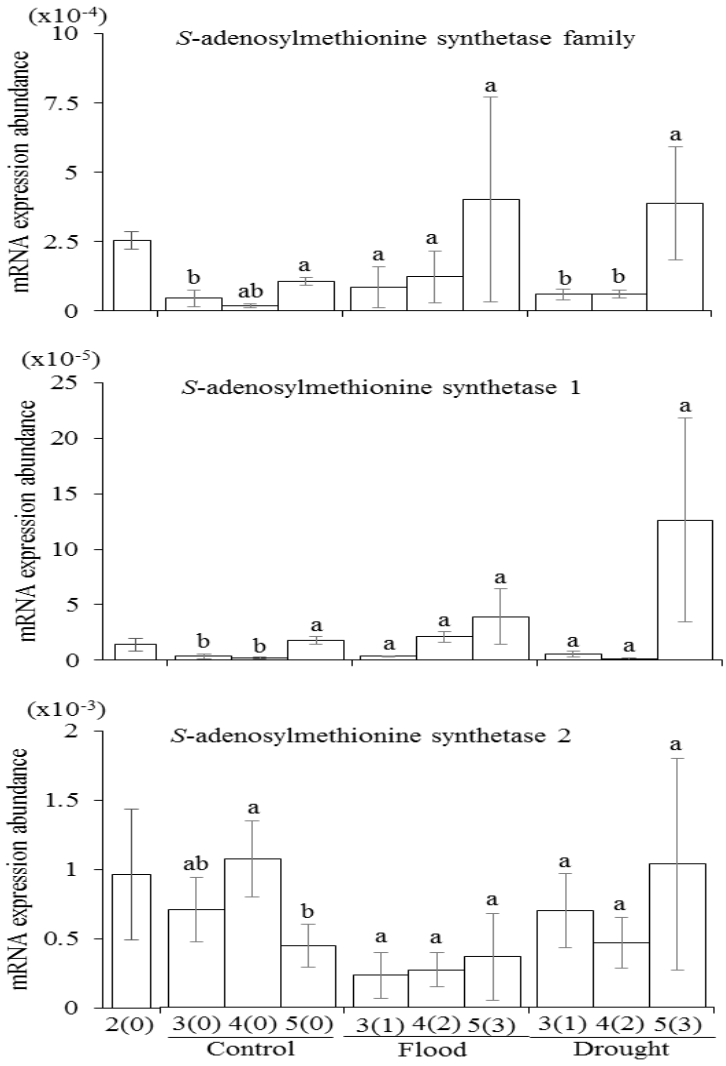

Figure 23A. The mRNA expression levels of *S*-adenosylmethionine synthetases in soybean root with time dependent manner under flooding and drought stresses. Two-day-old soybeans were treated with flooding or drought for 1, 2, and 3 days. Total RNA was extracted from roots after treatement and analyzed by qRT-PCR with specific primers. mRNA expression abundance was normalized against that of the 18S rRNA gene. Data are shown as means ± S.D. from 3 independent biological replicates. Means with the same letter indicate not wignificant changes according to Tukey's multiple comparion test ($p < 0.05$).

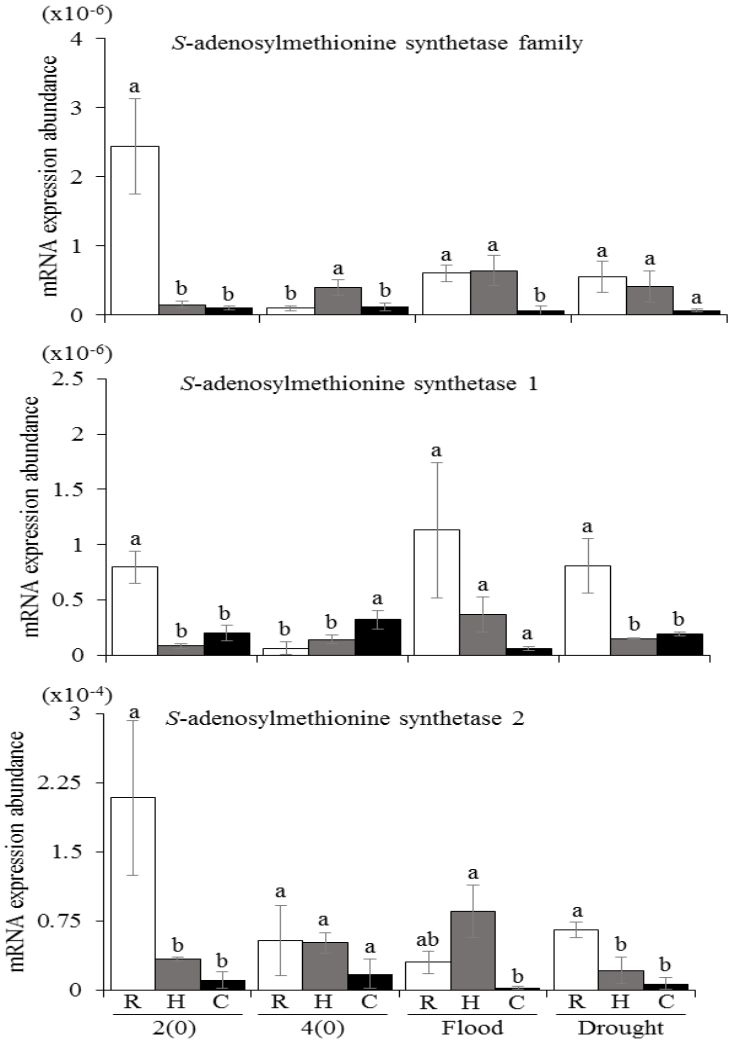

Figure 23B. The mRNA expression levels of *S*-adenosylmethionine synthetases in soybean organ under flooding and drought stresses. Two-day-old soybeans were treated with flooding or drought for 2 days. Total RNA was extracted from root, hypocotyl, and cotyledon after treatement for 2 days and analyzed by qRT-PCR with specific primers. mRNA expression abundance was normalized against that of the 18S rRNA gene. Data are shown as means ± S.D. from 3 independent biological replicates. Means with the same letter indicate not wignificant changes according to Tukey's multiple comparion test ($p < 0.05$).

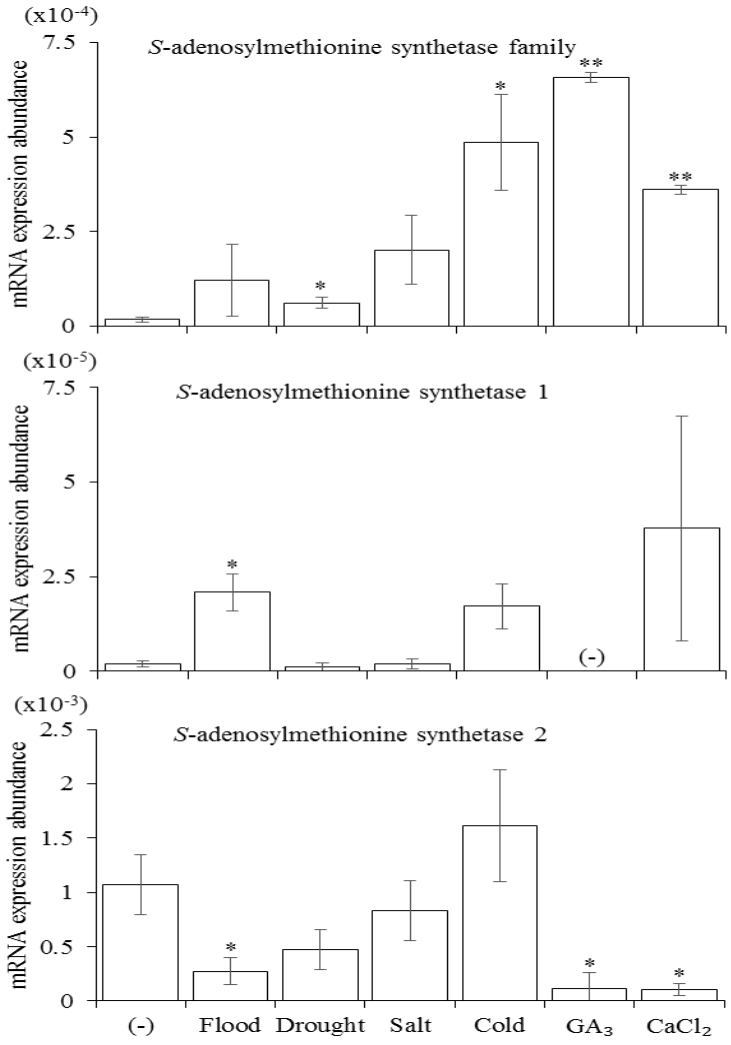

Figure 24. Effect of different stress conditions on the mRNA expression levels of *S*-adenosylmethionine synthetases in soybean root. Two-day-old soybeans were treated with flooding, drought, salt, cold, GA_3, or $CaCl_2$ for 2 days. Total RNA was extracted from roots after 2 days and analyzed by qRT-PCR with specific primers. mRNA expression abundance was normalized against that of the 18S rRNA gene. Data are shown as means ± S.D. from 3 independent biological replicates. Asterisks and double asterisks indicate significant changes in mRNA expression abundance between untreated and treated soybeans (Student *t*-test, * $p < 0.05$, ** $p < 0.01$).

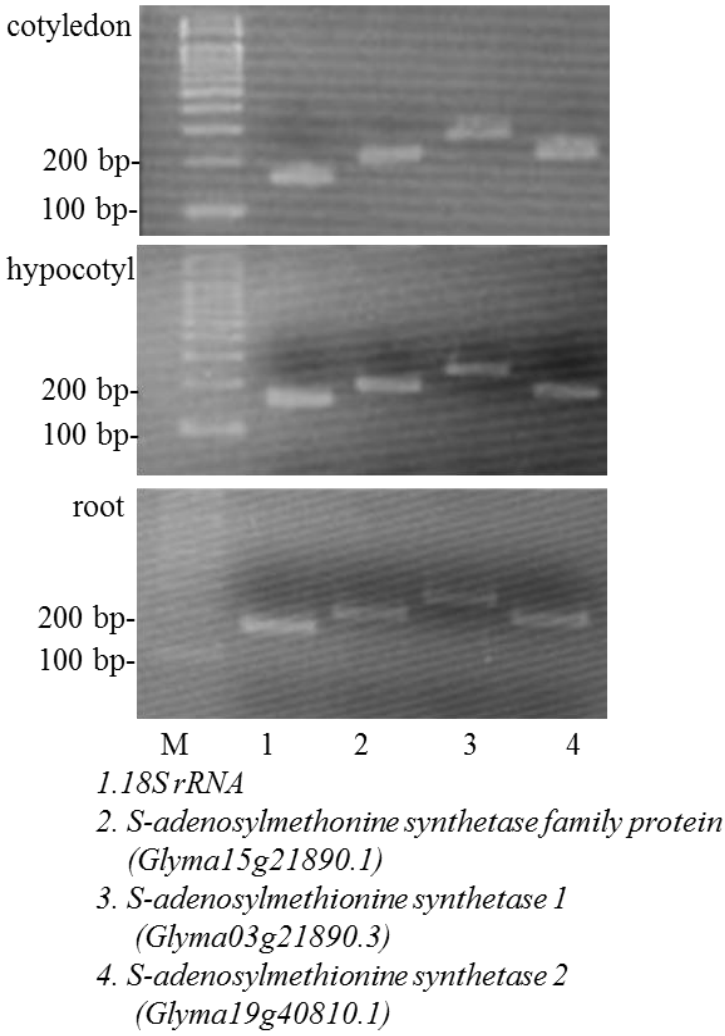

Figure 25. Agarose gel electrophoresis of qRT-PCR products. The products obtained with each primer set (Table 8) were visualized in agarose gel stained with ethidium bromide.

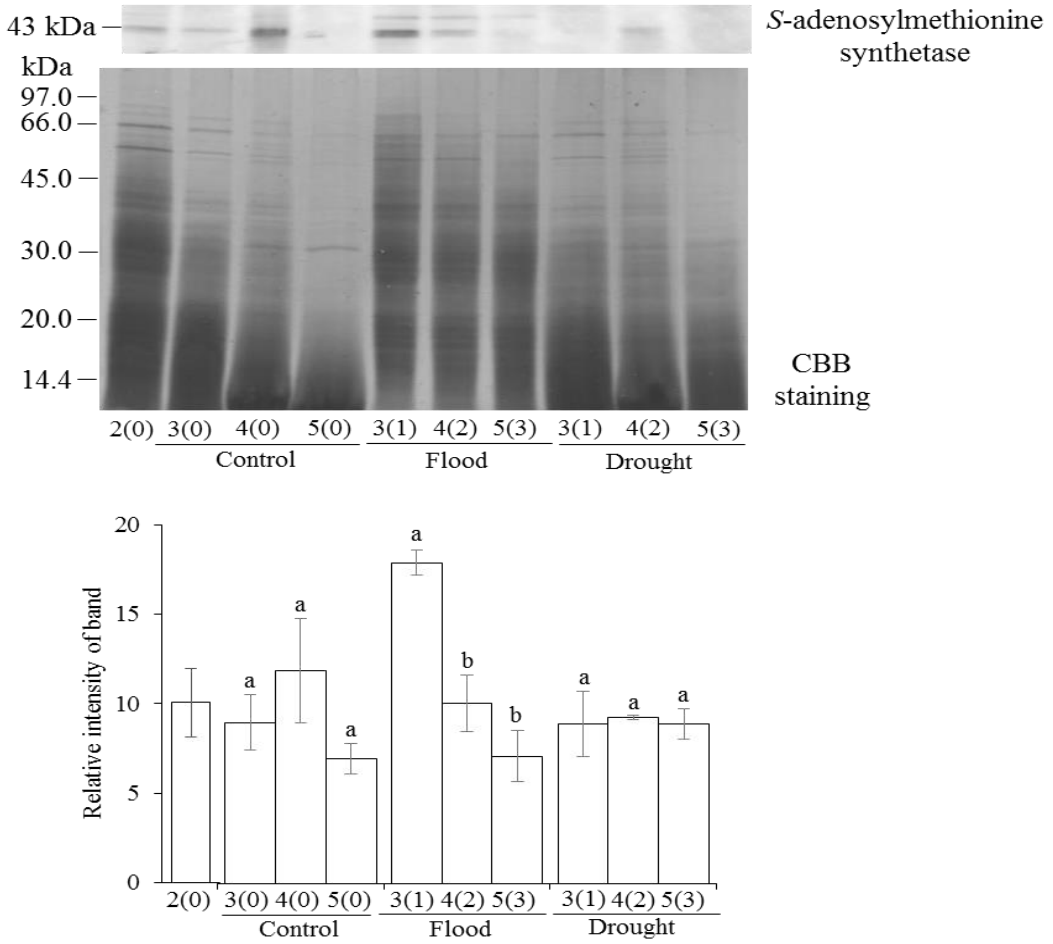

Figure 26A. Protein abundance of *S*-adenosylmethionine synthetase in soybean root with time dependent manner under flooding and drought stresses. Two-day-old soybeans were treated with flooding or drought for 1, 2, and 3 days. Extracted proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and reacted with anti-*S* adenosylmethionine antibody. Anti-rabbit IgG was used as the secondary antibody. Signals were detected using a Chemi-Lumi One Super kit. CBB staining was used as a loading control. The band intensities were calculated using Quantity One 1-D Anlysis software. Data are shown as means ± S.E. from 3 independent biological replicates. Means with the same letter indicate not significant changes according to Tukey's multiple comparion test ($p < 0.05$).

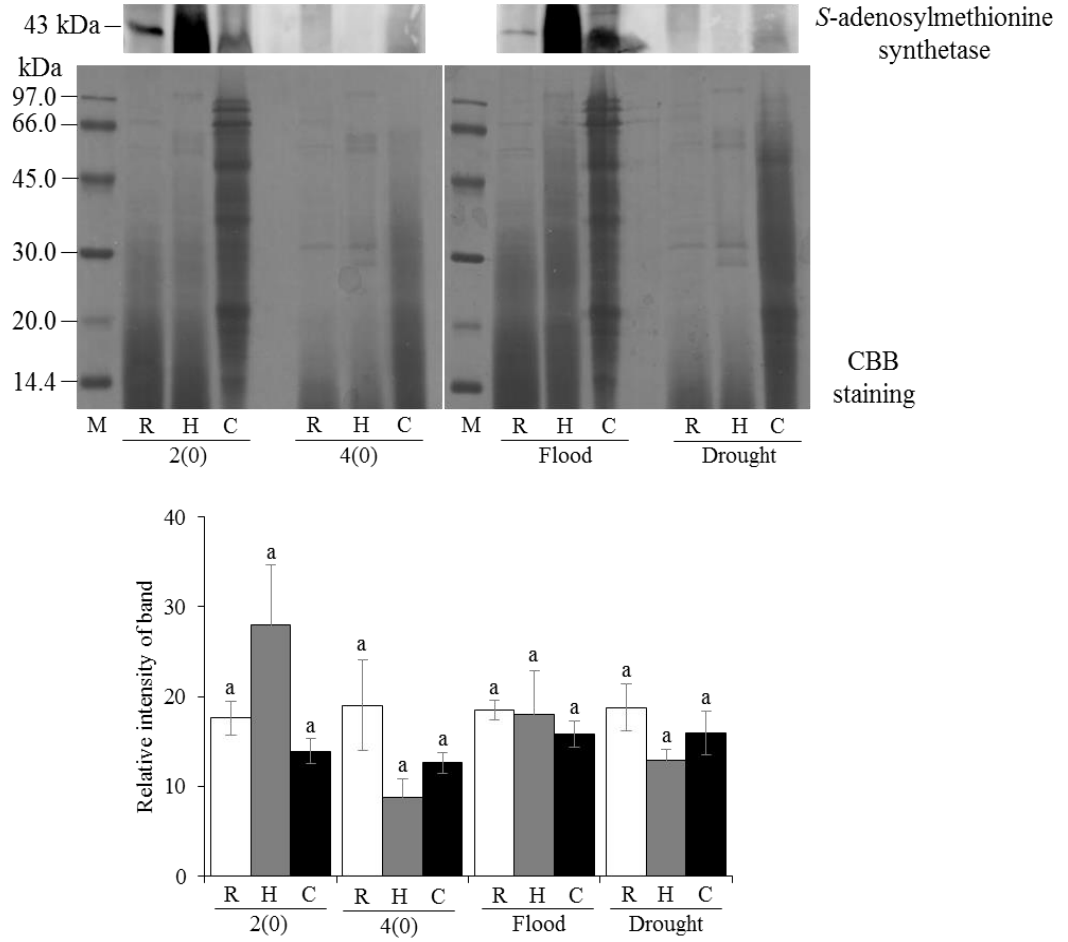

Figure 26B. Protein abundance of *S*-adenosylmethionine synthetase in soybean organ under flooding and drought stresses. Two-day-old soybeans were treated with flooding or drought for 2 days. Extracted proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and reacted with anti-*S* adenosylmethionine antibody. Anti-rabbit IgG was used as the secondary antibody. Signals were detected using a Chemi-Lumi One Super kit. CBB staining was used as a loading control. The band intensities were calculated using Quantity One 1-D Anlysis software. Data are shown as means ± S.E. from 3 independent biological replicates. Means with the same letter indicate not significant changes according to Tukey's multiple comparion test ($p < 0.05$).

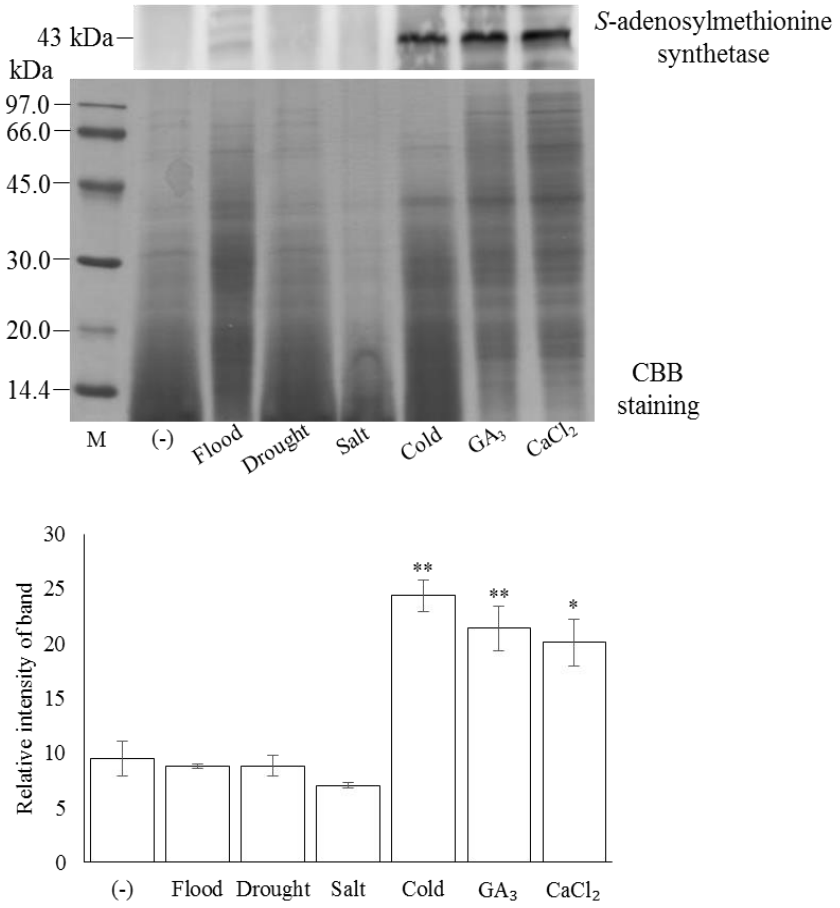

Figure 27. Protein abundance of *S*-adenosylmethionine synthetase in soybean root under different stress conditions. Two-day-old soybeans were treated with flooding, drought, salt, cold, GA_3, or $CaCl_2$ for 2 days. Extracted proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and reacted with anti-*S* adenosylmethionine antibody. Anti-rabbit IgG was used as the secondary antibody. Signals were detected using a Chemi-Lumi One Super kit. CBB staining was used as a loading control. The band intensities were calculated using Quantity One 1-D Anlysis software. Data are shown as means ± S.E. from 3 independent biological replicates. Asterisks and double asterisks indicate significant changes in mRNA expression abundance between untreated and treated soybeans (Student *t*-test, * $p < 0.05$, ** $p < 0.01$).

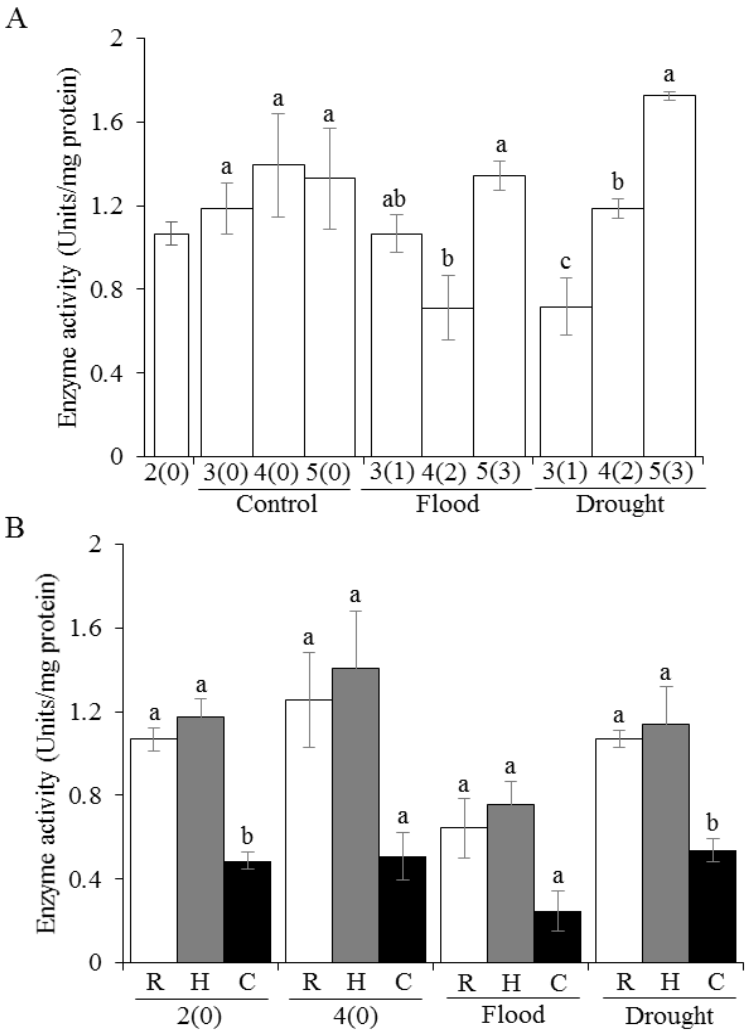

Figure 28. The activity of *S*-adenosylmethionine synthetase in soybean under flooding and drought stresses. For time dependency, 2-day-old soybeans were treated with flooding or drought for 1, 2, and 3 days and root was collected (A). For organ specificity, 2-day-old soybeans were treated with flooding or drought for 2 days and root, hypocotyl, and cotyledon were collected (B). Protein was extracted and activity was meaused spectrophotometrically at 340 nm. Data are shown as means ± S.E. from 3 independent biological replicates. Means with the same letter indicate not significant changes according to Tukey's multiple comparion test ($p < 0.05$).

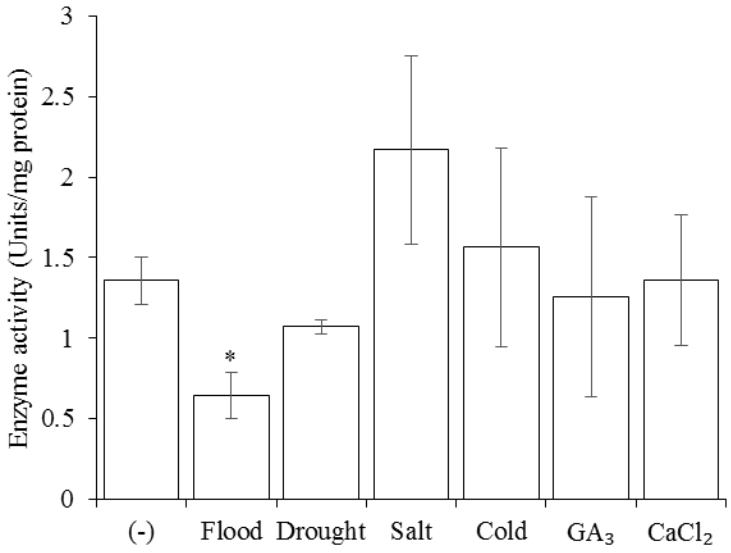

Figure 29. The activity of *S*-adenosylmethionine synthetase in soybean root under different stress conditions. Two-day-old soybeans were treated with flooding, drought, salt, cold, GA_3, or $CaCl_2$ for 2 days. Protein was extracted and activity was meaused spectrophotometrically at 340 nm. Data are shown as means ± S.E. from 3 independent biological replicates. Asterisks and double asterisks indicate significant changes in mRNA expression abundance between untreated and treated soybeans (Student *t*-test, * $p < 0.05$, ** $p < 0.01$).

CONCLUSION AND PROSPECTS

Soybean is an economically important crop follows rice, wheat, and maize; however, its cultivation is threatened by various abiotic streeses, which cause reduced growth and production (Mittler and Blumwald, 2010). Abiotic stress such as flooding and drought has gradually increasing due to severe climate change (Fukao et al., 2011). Flooding and drought are two major abiotic stresses leading to low oxygen or osmotic stress in agricultural crop (Sauter, 2013; Xiong and Zhu, 2002). Soybean provides human and animal with vegetable protein and oil, because it contains plenty of high-quality protein and oil (Liu, 2008). It was reported that soybean-based products is increasingly consumed in the world (Medic, 2014). Flooding and drought affect soybean growth and ultimately result in yield loss (Russell et al., 1990; Korte et al., 1983). It is important to improve soybean growth and productivity under adverse flooding and drought stresses. In order to accomplish development of tolerant soybean against stress, elucidation of responsive mechanism in soybean under stress is needed. In this study, to understand responsive mechanism of flooding and drought stresses in soybean, the effect of GA and calcium supplementation was investigated, proteomic analysis was performed, and the key marker identified by proteomic technique was characterized.

Previous proteomic study on effect of ABA in flooded soybean was indicated that ABA played an important role in tolerance mechanism in soybean under flooding stress (Komatsu et al., 2013a). Furthermore, ethylene signaling pathway participated in plant responsive mechanism through protein phosphorylation at the initial stages of flooding stress in the root tip of soybean (Yin et al., 2014). Proteomic analysis has been performed to understand responsive mechanism of soybean under flooding stress with GA_3 supplementation. The number of proteins which were significantly changed by

GA_3 under flooding was categorized in secondary metabolism, cell, protein degradation/synthesis, hormone metabolism, and cell wall metabolism in soybean. This result suggests that GA_3 affects the abundance of proteins involved in secondary metabolism, cell, protein degradation/synthesis, hormone metabolism, and cell wall metabolism. In present study, *S*-adenosylmethionine synthetase was increased by GA_3 treatment. Kim (2013) also reported that the RNA expression of barley *S-adenosylmethionine synthetase* was elevated in GA_3 and ethylene treatment at early time point. *S*-adenosylmethionin synthetase gene-specifically regulated the ethylene biosynthesis in mustard (Lim et al., 2002). Exogenous GA_3 might have effect on regulation of stress response *via* signaling pathway and improve soybean growth under various stresses.

GA_3 supplementation has effect on droguth as well as flooding in plant. It was reported that exogenous GA_3 increased calcium and potassium concentrations similar or close to normal condition and improved the water stress tolerance by enhancing chlorophyll concentration and some macro-nutrient concentrations in maize leaves under water deficit (Kaya et al., 2006). The number of proteins which were significantly changed by calcium under flooding was categorized in secondary metabolism, cell, protein degradation/synthesis, hormone metabolism, cell wall metabolism, and DNA synthesis in soybean. This result suggests that calcium affects the abundance of proteins involved in secondary metabolism, cell, protein degradation/synthesis, hormone metabolism, cell wall metabolism, and DNA synthesis. Gao et al. (2011) reported that enhanced tolerance to short-term hypoxia was induced by calcium-mediated reduction of polyamine degradation, elevation of nitrate uptake, and accelerated synthesis of heat-stable proteins and polyamines in muskmelon roots. In present study, *S*-adenosylmethionine synthetase was increased by calcium treatment.

These results suggest that calcium might be involved in improvement of stress tolerance by regulating the level of *S*-adenosylmethionine synthetase and related metabolites such as polyamine.

Gel-free proteomic technique was used to get insight into root responses to flooding and drought in soybean. A number of flooding- and drought-responsive proteins were identified in soybean. The number of glycolysis related proteins were increased in both flooding and drought stresses, indicating that flooding and drought affect energy metabolism such as glycolysis. In addition, the number of fermentation related proteins was increased under flooding stress, suggesting that these proteins might be important for soybean growth under flooding stress. It was reported that a strong alcoholic fermentation system was induced by exposure of anaerobic condition in rice (Bertani et al., 1980). Pyruvate decarboxylase, which is involved in the decarboxylation of pyruvate, is mainly incuced anaerobic condition in *Arabidopsis*. The activity of pyruvate decarboxylase was 9-fold increased in rice during anoxia stress period (Rivoal et al., 1997). The overexpression of *pyruvate decarboxylase* gene resulted in improved plant survival in *Arabidopsis*, suggesting that pyruvate decarboxylase controls ethanol fermentation (Ismond et al., 2003). The metabolic adjustment to low oxygen stress has included the down-regulation of energy-consuming metabolic pathways (Geigenberger, 2003), such as down-regulation of storage metabolism (Geigenberger et al., 2000; van Dongen et al., 2004) and energy-conserving shift from the invertase to the sucrose synthase route of sucrose degradation (Bologa et al., 2003; Huang et al., 2008), and thereby pyrophosphate is used than ATP (Gupta et al., 2009; Zabalza et al., 2009). These findings indicate that energy metabolism might be one of the regulation of stress response and involved in development and growth of soybean under flooding and drought stresses.

Three *S*-adenosylmethionine synthetases, which were representd as *S*-adenosylmethionine synthetase family, 1, and 2, respectively, have been identified as common proteins in soybean under flooding and drought stresses using a gel-free proteomic technique. These proteins were decreased and increased under flooding and drought stresses, respectively, indicating that *S*-adenosylmethionine synthetase might be important factor involved in the regulation of stress response in soybean. *S*-adenosylmethionine synthetase is involved in the biosynthesis of *S*-adenosylmethionine, which plays a role in a precursor of polyamine and ethylene biosynthesis (Evans and Malmberg, 1989; Yang and Hoffman, 1984). The mRNA expression level of S-adenosylmethionine synthetase 2 was down-regulated under flooding; while, that of *S-adenosylmethionine synthetase family* was up-regulated under drought stress. Similarly, the activity of *S*-adenosylmethionine synthetase was incrased under drought stress. In addition, salt and cold stresses increased *S*-adenosylmethionine synthetase level. It was reported that *S*-adenosylmethionine synthetase related to ethylene-meidated inhibition of root growth altered cell wall structure and polymer s in rice (Fukuda et al., 2007). Recently, tolerance to cold stress was enhanced by the overexpression of *S-adenosylmethionine synthetase* gene *via* promoting polyamine oxidation and improved hydrogen peroxide-induced antioxidant protection in blue alfalfa (Guo et al., 2014). The increase of *S*-adenosylmethionine synthetase was shown in rice under chilling (Cui et al., 2005), in lima bean by mechanical wounding (Arimura et al., 2002), in barley under salinity stress (Witzel et al., 2009), and in soybean by cotton worm feeding (Fan et al., 2012). These results indicate that *S*-adenosylmethionine synthetase may be involved in enhancement of soybean tolerance to flooding and drought stresses by regulating polyamine and ethylene metabolism.

A schematic diagram of soybean responses to flooding and drought is proposed in Figure 30. *S*-adenosylmethionine, is required for the biosynthesis of the phenylpropanoid constituents of cell wall in plant (Higuchi, 1981) and is a precursor for the biosynthesis of polyamines and ethylene (Kende, 1993). Under flooding stress, *S*-adenosylmethionine synthetase was decreased; while, it was increased to some extent by GA_3 supplementation. *S*-adenosylmethionine utilizes biosynthesis of cysteine which is required for synthesis of glutathionine. Under drought stress, *S*-adenosylmethionine synthetase was increased. Furthermore, under drought stress, glutathionine *S*-trasnferase and dehydrogenase ascorbate was increased which play a role in ROS scavenging through ascorbate-glutathionine pathway (Kumutha et al., 2009; Ushimaru et al., 1997). It is suggestd that *S*-adenosylmethionine synthetase might be involved in regulation of soybean growth *via* ethylene and ascorbate-glutathionine pathway.

Previous proteomic studies were found that the abundance of *S*-adenosylmethionine synthetases was significantly decreased during flooding (Hashiguchi et al., 2009, Nanjo et al., 2010). The increased abundance of *S*-adenosylmethionine synthetase was observed during post-flooding recovery stage, suggesting that *S*-adenosylmethionine synthetase was necessary to produce metabolites required to overcome the effects of flooding (Salavati et al., 2012). *S*-adenosylmethionine, which is catalyzed from methionine by *S*-adenosylmethionine synthetase is required for the biosynthesis of the phenylpropanoid constituents of cell wall in plant (Higuchi, 1981). Increased *S*-adenosylmethionine synthetase correlates with areas where lignification is occurring under normal condition (Peleman et al., 1989b), because *S*-adenosylmethionine synthetase is highly demanded for the methylation of lignin monomers prior to their polymerization (Yan et al., 2005). It was

reported that the organ and tissue specific expression of *S*-adenosylmethionine synthetase was closely involved in enhanced xylem development under salinity stress (Sanchez-Aguayo et al., 2004). These results suggest that *S*-adenosylmethionine synthetase may play an important role in stress alleviation and growth of soybean by regulating metabolites related to cell wall strengthen. *S*-adenosylmethionine synthetase might be useful marker enzyme in soybean.

In this study, the mRNA expression level of S-adenosylmethionine synthetase family was up-regulated under drought stress and that of *S-adenosylmethionine synthetase 2* was down-regulated under flooding stress. The activity of *S*-adenosylmethionine synthetase was decreased at early time of flooding stress and increased at late time of drought stress in soybean root. These results indicate that the regulation of *S*-adenosylmethionine synthetase is not same at transcriptionl level, translational level, and enzyme activity level. It must be elucidated which step is control the expression or abundance change of *S*-adenosylmethionine synthetase in soybean root *via* gene suppressing or expressing technique in the future. For improve the stress tolerance of soybean, further study will needed to how *S*-adenosylmethionine synthetase controls the biosynthesis of polyamine, ethylene, or lignin, and ascorbate-glutathionine pathway in soybean root under flooding and drought stresses.

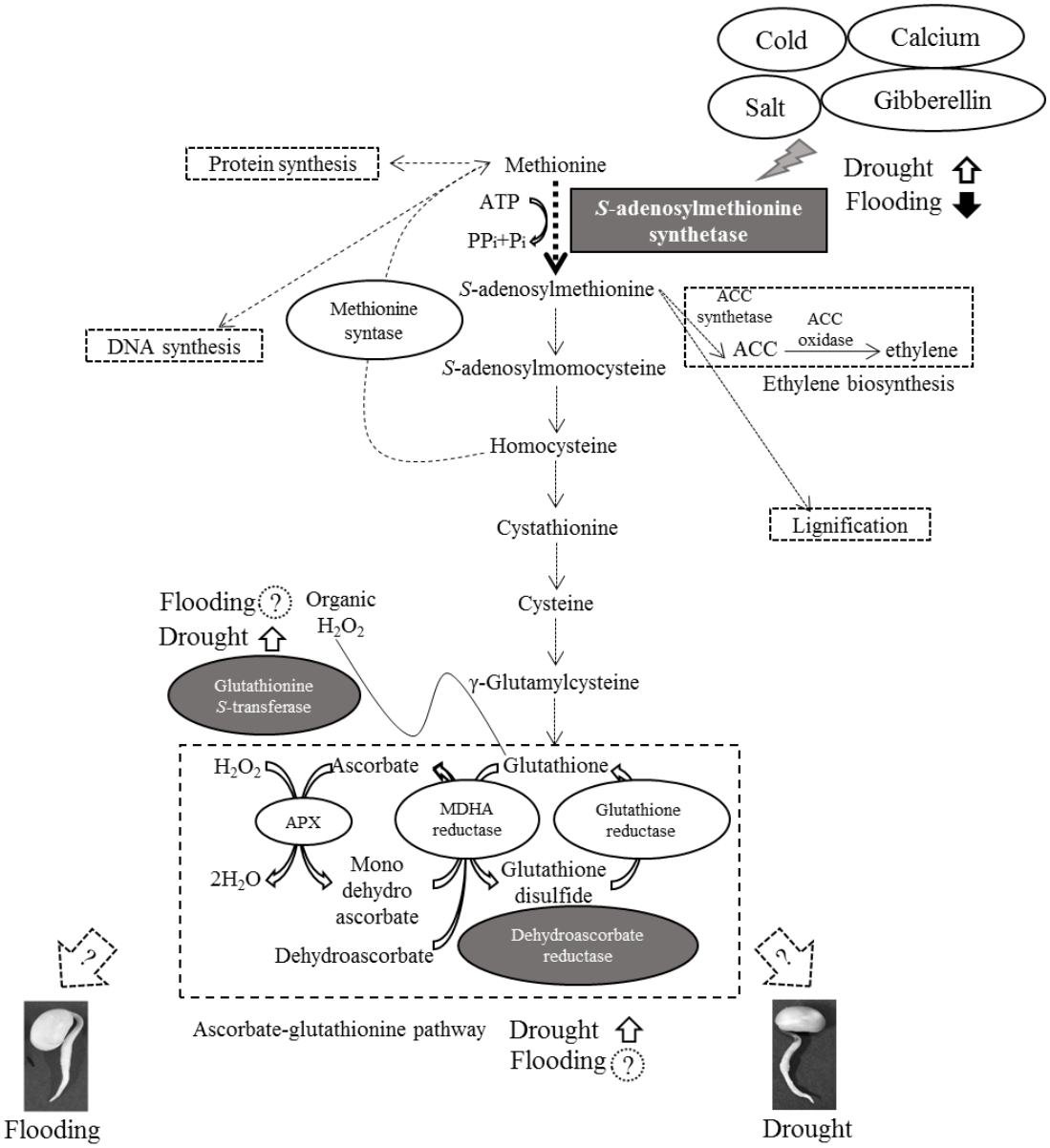

Figure 30. Schematic diagram of flooding and drought response mechanism in soybean in this study. Dotted arrow indicates predicted parts based on Wang et al. (2002) and Brown-Borg (2006). Gray circle and box indicate identified proteins in this study. *S*-adensoylmethionine synthetase involved in ethylene biosynthesis, lignification, and ascorbate-glutathionine pathway. ACC, 1-aminocyclopropane-1-carboxylic acid; MDHA, monodehydroascorbate.

SUMMARY

Abiotic stress such as flooding and drought negatively affects growth and grain yield of crop. Flooding and drought severely inhibits root/shoot growth and root diameter and results in oxygen deficiency and osmotic stress, respectively. Under flooding, the enzyme activity of reactive oxygen species scavengeing such as ascorbate peroxidase and catalase was decreased; while, under drought, it had higher activity in plant. In response to flooding and drought stresses, plant has completely opposite trend at physiological and biochemical levels. Soybean is one of the important major crop which provides protein and oil. Soybean is, however, known as flooding and drought sensitive crop. Out of comprehensive analyses, proteomic analysis was useful for elucidating molecular mechanisms in response to flooding and drought stresses in soybean. This study was performed to elucidate the molecular mechanisms of flooding and drought response in soybean. A gel-free proteomic technique was used to identify the flooding and drought responsive proteins in this study. The selected candidate proteins for understanding response to flooding and drought in soybean were further analyzed to confirm their mRNA expression levels.

To get insight into response mechanisms of soybean in response to flooding and drought, 2-day-old soybeans were subjected to flooding and drought for 2 days. The fresh weight of root including hypocotyl was significantly decreased under both conditions, although the root morphology is clearly differed between flooding and drought stress. The effects of GA and calcium on drought-responsive mechanism were well studied in soybean. Here, the effects of GA and calcium were focused on flooding stress, to understand flooding-responsive mechanism in soybean using a-gel free proteomic technique. The number of proteins involved in secondary metabolism, cell, protein degradation/synthesis were decreased by flooding stress; whereas, these proteins

were recovered by GA_3 supplementation under flooding. Proteins involved in protein degradation/synthesis, hormone metabolism, cell wall metabolism, and DNA synthesis were decreased under flooding; while, they were recovered by calcium supplementation. The number of glycolysis and fermantion related proteins were decreased under flooding; however, the number of those proteins was not changed by GA_3 and calcium supplementation. These results suggest that GA_3 and calcilum affect the abundance of proteins involved in secondary metabolism, cell, protein degradation/synthesis, hormone metabolism, and cell wall metabolism in soybean roots under flooding stress.

To further understand responsive mechanism of soybean root under flooding and drought, a gel-free proteomic technique was used. Proteins were extracted from roots and analyzed using a gel-free proteomic technique. A total of 97 and 48 proteins were significantly changed in response to flooding and drought, respectively. Under flooding, cytoplasm/extracellular space-localized proteins were increased and nucleus/vacuolar-localized proteins were decreased. Under drought, cytoplasm-localized proteins were mostly changed. Functional categorization indicated that glycolysis related proteins were significantly increased under both stresses. Protein synthesis and targeting related proteins were decreased by flooding and increased by drought stress in soybean. Under flooding, proteins involved in fermentation, cell wall, and stress were specifically changed; whereas, under drought, proteins involved in redox and cell organization were specifically changed. These results suggest that energy and redox metabolism may play critical role in flooding and drought responsive mechanisms in soybean root.

Among changed proteins, 3 *S*-adenosylmethionine synthetases, which were decreased and increased under flooding and drought stresses, respectively. They were commonly identified using proteomic technique, suggesting that *S*-adenosylmethionine synthetase is involved in the regulation of stress response in soybean. To understand the

role of *S*-adenosylmethionine synthetase in soybean, time dependency, organ specificity, and stress specificity were analyzed at mRNA expression level, protein abundance, and enzyme activity. The mRNA expression level of *S-adenosylmethionine synthetase family* was not significantly changed by flooding stress; while, that of *S-adenosylmethionine synthetase 2* was significantly down-regulated by flooding stress. In contrast, the mRNA expression level of *S-adenosylmethionine synthetase 2* was not significantly changed by drought stress; while, that of *S-adenosylmethionine synthetase family* was significantly up-regulated by drought stress. Protein abundance of *S*-adenosylmethionine synthetase was decreased and increased by flooding and drought, respectively, and their activities were similar to their protein abundance. The mRNA expression level and activity of *S*-adenosylmethionine synthetase were specificatlly induced in root and hypocotyl. Furthermore, *S*-adenosylmethionine synthetase was induced by salt, cold, GA_3, and $CaCl_2$ stresses at mRNA expression level and protein abundance. These results suggest that *S*-adenosylmethionine synthetase might be an important factor to understand the stress response in soybean.

Based on proteomic analysis, flooding and drought had influence on the abundance of proteins involved in redox, energy metabolism, and protein synthesis in soybean. *S*-adenosylmethionine synthetase were shown opposite tendency in response to flooding and drought stresses. These results indicate that responsive mechanisms of flooding and drought may regulate *via* energy and redox metabolism in soybean root and suggest that *S*-adenosylmethionine synthetase may be one of the useful marker proteins in soybean root under flooding and drought stresses.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my supervisor, Prof. Setsuko Komatsu, in National Institute of Crop Science at National Agriculture and Food Research Organization and in Graduate School of Life and Environmental Science at University of Tsukuba, who teaches me for all my research.

I want to express my thanks to Prof. Hiroshi Matsumoto, in Graduate School of Life and Environmental Science at University of Tsukuba, for his grate advice and supports for Ph. D. course.

I wish to acknowledge the helpful comments and advice of Prof. Takeo Usui and Dr. Yukari Sunohara in Graduate School of Life and Environmental Science at University of Tsukuba.

I am sincere grateful to Dr. Yohei Nanjo for his great help and support for MS analysis during Ph. D. course at National Institute of Crop Science. I wish to express my gratitude to Dr. Keito Nishizawa, Dr. Makoto Tougou, and Dr. Susumu Hiraga for their valuable comments and advices. I appreciate my colleagues Dr. Kamal Abu Hena Mostafa, Mr. Khan Mudassar Nawaz, Mr. Yin Xiaojian, Ms. Mustafa Ghazala, and Ms. Wang Xin for their valuable discussions.

I gratefully acknowledge the Japanese Government for supporting me during Ph. D. course under Monbukagakusho scholarship program and National Institute of Crop Science in Japan that I have performed my research in this institute.

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