Exploration of Physiological and Molecular Responses to Precipitation Extremes in Soybean and Nitrogen Fertility in Wheat

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Department of Crop and Soil Environmental Sciences

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(August 12th, 2016)
Blacksburg, VA

Keywords: flooding, drought, nitrogen-use-efficiency, *Glycine max*, *Triticum aestivum*,

Group VII Ethylene Responsive Factors, genome-scale gene expression analysis

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ABSTRACT (academic)

Soybean and wheat are important crop species due to their significance for human consumption, animal feed, and industrial use. However, increasing global population and worsening climate change have put a major strain on the production system of these crops. Natural disasters such as flooding and drought can severely impact growth and productivity of these crops. In addition, increased application of synthetic nitrogenous fertilizers to meet the global food demand has led to environment related issues. Therefore, with a goal of understanding mechanisms of flooding and drought tolerance in soybean and nitrogen-use-efficiency in wheat, we explored their physiological and transcriptomic regulation. We characterized the fundamental acclimation responses of soybean to flooding and drought and compared the metabolic and transcriptomic regulation during the stresses in a tissue-specific manner. We demonstrated the dynamic reconfiguration of gene expression and metabolism during flooding, drought, and recovery from these stresses. Our study displayed that flooding triggers more dramatic adjustments than drought at the transcriptional level. We also identified that the soybean genome encodes nine members of group VII ERF genes and characterized their responses in leaves and roots under flooding and drought. Based on the expression patterns, it is estimated that two of the nine genes are promising candidate genes regulating tolerance to submergence and drought. In addition, our genome-scale expression analysis

discovered commonly induced ERFs and MAPKs across both stresses (flooding and drought) and tissues (leaves and roots), which might play key roles in soybean survival of flooding and drought. In wheat, we evaluated the effect of three different nitrogen rates on yield and its components across four diverse soft red winter wheat genotypes. The cultivar Sisson displayed superior performance in grain yield and nitrogen use efficiency at low nitrogen levels. Our results suggested that improvement of nitrogen use efficiency in low nitrogen environments can be achieved through the selection of three components: grain number/spike, 1000-seed weight, and harvest index. Overall, this study has advanced our understanding of how plants respond to abiotic stresses such as flooding, drought, and nutrient limitation conditions.

ABSTRACT (public)

Soybean and wheat are commercially important crop throughout the world. Soybean is a major source of protein and oil for humans, livestock and industrial products including biofuel production. Similarly, wheat is a major source of food products such as bread, pasta, and cookies. However, increasing global population and worsening climate change have put a major strain on the production system of these crops. Natural disasters such as flooding and drought are on the rise, which have severely impacted soybean growth and productivity. In addition, increased application of synthetic nitrogenous fertilizers in wheat production to meet the global food demand has led to environment related issues. Therefore, a mechanistic understanding of flooding and drought tolerance in soybean and nitrogen use efficiency in wheat is of utmost importance. The knowledge obtained from these studies can aid in the development of new varieties in these crops. Here, we carried

out our study on soybean by imposing either complete submergence or restricting water supply to characterize the responses to these stresses in shoot and root systems. We found several compounds and genes that were altered distinctly under these two water-related stresses. In addition, we identified some promising genes that can significantly regulate tolerance in soybean to flooding and drought in soybean. To study wheat nitrogen use efficiency, we selected four diverse soft red winter wheat varieties and grew them under three nitrogen levels. We found that the cultivar Sisson has the lowest yield penalty among the four varieties resulting from low nitrogen conditions. In addition, we also demonstrated that three yield traits (grain number per spike, 1000-seed weight, and harvest index) are important selection targets to develop high nitrogen use efficiency varieties.

Acknowledgements

It gives me immense pleasure expressing my strong gratitude to Dr. Takeshi Fukao for his learned counsel, unstinted attention, valuable suggestions, scintillating support and regular supervision for all the research which made it possible to create this manuscript. He gave me the opportunity to grow my understanding of plant science and is an excellent mentor to work with.

I would like to express my deep sense of gratitude and appreciation to my committee members Dr. Saghai Maroof, Dr. Ruth Grene, Dr. Carl Griffey and Dr. Wade Thomason for their continuous guidance and support during my pursue of Ph.D. study.

My sincere gratitude extends to Dr. Song Li and Dr. Dhivyaa Rajasundarum for their support in data analyses. I learned a lot from interactions with them, and they were the ones who introduced me to the basics of bioinformatics.

I would like to thank my colleagues Fraz Hussain, Jasper Alpuerto, Kyle Brassier, Joseph Magliozzi and Peyton Scott who have always been there for encouragement, both as mentor and assistant and provided their learned tips and tricks of running bench works. Their presence in the lab really made the lab a fun-to-work place.

My sincere thanks go to all the staff members of Department of Crop and Soil Environmental Sciences and Translational Plant Science (TPS) society who provided me tuition scholarships, travel grants, and a conducive academic environment during my entire years of study.

Last but not the least, my appreciation goes to all my family members who showed me the path, supported and encouraged me to pursue a career in plant science.

Dedicated to farming communities all around the world

ATTRIBUTIONS

Chapter 2: Physiological and transcriptomic characterization of submergence and reoxygenation responses in soybean seedlings

Takeshi Fukao is an assistant professor in Department of Crop and Soil Environmental Sciences, Virginia Tech and corresponding author on the manuscript. He was the principal investigator of this project, and this study was supported by the grants from US Department of Agriculture (Grant number# 2011-04015 and Hatch grant) to him.

Joseph O. Magliozzi was an undergraduate student at Virginia Tech and is a co-author on this manuscript and who contributed to the biochemical assays for this study.

Saghai Maroof is a professor in the Department of Crop and Soil Environmental Sciences, Virginia Tech and a co-author on the manuscript. He generously provided soybean seeds for the study and contributed to the development of the manuscript.

Chapter 3: Crosstalk of transcriptomic and metabolic responses to submergence and drought in leaves and roots of soybean plant

Takeshi Fukao was the principal investigator of this study, and this work was supported by the Hatch grant from US Department of Agriculture and funds from Virginia Soybean Board to him.

Song Li is an assistant professor, and **Dhivyaa Rajasundaram** is his post-doctoral associate in Department of Crop and Soil Environmental Sciences, Virginia Tech. They assisted in computational analysis of RNASeq data for this study

Chapter 4: Differential responses of grain yield, grain protein content and their associated traits to nitrogen supply in soft red winter wheat

Takeshi Fukao was the principal investigator of this study. This study was supported by the grant from Virginia Small Grains Board to him.

Kyle Brasier is a graduate research assistant in Department of Crop and Soil Environmental Sciences, Virginia Tech in Dr. Carl Griffey's lab. He assisted in grain protein assays using XDS rapid content analyzer and combustion N analyzer.

Carl Griffey (Professor) and **Wade Thomason** (Associate Professor) from Department of Crop and Soil Environmental Sciences, Virginia Tech generously provided wheat seeds, assisted in the experimental setup and revised the manuscript.

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CHAPTER 1: INTRODUCTION

1.1 Soybean submergence and drought stress

Soybean [*Glycine max* (L.) Merr.] is an important legume crop used as protein and oil sources for human consumption, animal feed, and industrial products. It has an annual economic worth of ~$38 billion (*FAOSTAT*, 2013). However, trends of global climate change-induced precipitation extremes such as flooding and drought events are increasing (*Mittler*, 2006; *Bailey-Serres* et al., 2012), and deleteriously impacting agricultural productivity and sustainability. Thus, despite soybean's ability to grow in a wide range of climatic conditions (*Mutava* et al., 2015), its growth and development are affected by flooding and drought (*Komatsu* et al., 2012; *Mohammadi* et al., 2012). Moreover, these two abiotic stresses can occur simultaneously in a single crop season, further exacerbating crop losses.

Complete inundation upon direct seeding is a common environmental constraint for soybean production throughout the world, resulting in uneven stand establishment and seedling death. Soybean plants can encounter submergence in early vegetative stages as well. Submergence restricts normal gaseous exchange which limits the energy production via aerobic respiration. Therefore, plants have to switch to carbon-inefficient anaerobic metabolism while underwater (*Bailey-Serres* & *Colmer*, 2014). On the other hand, when flood-water subsides, plants have to endure a rapid influx of oxygen which causes oxidative damage to the cells through over-production of various reactive oxygen species (ROS) (*Fukao* et al., 2011). Hence, flooding events bring two sequential stresses that can be damaging to soybean.

The inability of plants to perform normal mitochondrial phosphorylation for adenosine triphosphate (ATP) generation during submergence is succeeded by many other downstream events orchestrated by the accumulation of gaseous phytohormone ethylene in tissues of

submerged plants. Active biosynthesis and entrapment of ethylene during stress triggers many acclimation responses such as shoot elongation, the formation of adventitious roots and carbohydrate metabolism (*Fukao & Bailey-Serres*, 2008). Ethylene also triggers an increase in endogenous gibberellic acid (GA) level and decrease in abscisic acid (ABA) content which causes internode elongation so that plants can push itself above water- a phenomenon observed in deep water rice (*Sauter*, 2000). It has been shown that accumulation of ethylene during desubmergence regulates gene transcripts involved in ROS detoxification, dehydration response and metabolic processes in *Rumex* and *Arabidopsis* (*Voesenek* et al., 2003; *Tsai* et al., 2014).

Partial or complete plant submergence in a soybean field can occur more frequently at the seedling establishment and early vegetative stages. *VanToai* et al. (1994, 2010) reported the existence of genetic variation among soybean cultivars to root waterlogging tolerance. Evaluation of quantitative trait loci (QTLs) for seed-flooding tolerance (*Sayama* et al., 2009), early vegetative (*Githiri* et al., 2006) and reproductive stage waterlogging tolerance (*VanToai* et al., 2001; *Reyna* et al., 2003; *Cornelious* et al., 2005; *Nguyen* et al., 2012) has identified several small-effect loci. As observed in other plant species, waterlogging stimulates the formation of aerenchyma and adventitious roots in soybean plants, facilitating transport of oxygen from non-submerged shoots (*Thomas* et al., 2005; *Rhine* et al., 2009; *Shimamura* et al., 2010; *Valliyodan* et al., 2014). Several studies have shown the alterations in genes and proteins during 2-3 d of submergence and recovery in hypocotyl and roots of seedling stage soybean (*Komatsu* et al., 2009; *Nanjo* et al., 2011, 2014; *Oh & Komatsu*, 2015; *Yin & Komatsu*, 2015; *Khan* et al., 2015). They recognized differential regulation of gene transcripts and proteins associated with carbohydrate catabolism, anaerobic fermentation, cell wall loosening, ROS detoxification, ethylene biosynthesis and formation of adventitious roots. However, detailed physiological and

molecular characterization of acclimation responses to prolonged submergence and subsequent reoxygenation has not been performed in the major legume species.

Drought conditions cause osmotic stress that negatively affects the normal growth and yield in crops. Osmotic stress is also caused when vegetative tissues suddenly undergo dehydration upon desubmergence (*Fukao* et al., 2011). Various adaptive responses to such stresses in plants include: osmotic adjustment and stomatal closure, prevention of water loss through transpiration, maintenance of photosynthetic activity, induction of root growth and inhibition of shoot growth, synthesis of osmolytes, protection against reactive oxygen species (ROS), regulation of aquaporin activity and inhibition of ethylene accumulation (*Roitsch*, 1999; *Sharp*, 2002; *DaCosta & Huang*, 2006; *Demmig-Adams & Adams*, 2006; *Lei* et al., 2006; *Ghannoum*, 2009; *Parent* et al., 2009; *Sade* et al., 2012; *Prince* et al., 2015). Numerous genes governing responses and tolerance to dehydration are induced when ABA, a key plant stress-signaling hormone is produced and accumulated (*Yamaguchi-Shinozaki & Shinozaki*, 2006). However, osmotic stress-responsive gene expression can be regulated via both ABA-dependent and ABA-independent pathways. ABA-dependent gene expression involves ABA-responsive element (ABRE), and ABRE-binding protein/ABRE-binding transcription factors (AREB/ABFs) while dehydration-responsive element/C-repeat (DRE/CRT), and DRE-/CRT-binding protein 2 (DREB2) transcription factors are key players in ABA-independent gene expression pathways (*Yoshida* et al., 2014).

Physiological studies have shown some common and specific responses to drought and flooding in soybean, sunflower (*Helianthus annus*) and maize (*Zea mays*) seedlings (*Wample & Thornton*, 1984; *Soldatini* et al., 1990; *Mutava* et al., 2015). Both stresses largely reduced chlorophyll content, net carbon dioxide concentration and stomatal conductance. However,

reductions in photosynthesis during drought versus flooding stress can be distinctly regulated. Photosynthesis during drought is negatively affected by the change in ABA-induced stomatal conductance while an accumulation of starch granules plays a key role in the reduction during flooding (*Mutava* et al., 2015).

A number of studies have demonstrated that the members of group VII Ethylene-Responsive-Factor (ERFVII) genes are pivotal regulators of acclimation responses to submergence and drought in rice and *Arabidopsis*. Genetic and molecular analyses revealed that *SUBMERGENCE1A* (*SUB1A*), a gene encoding ERFVII, coordinates economization of energy reserves in rice while underwater through transcriptional, hormonal and metabolic regulation in rice (*Fukao* et al., 2006, 2008; *Xu* et al., 2006; *Singh* et al., 2010). Interestingly, *SUB1A* also enhances recovery from dehydration stress as a consequence of increased expression of stress-responsive genes and enhanced responsiveness to ABA (*Fukao* et al., 2011). *Arabidopsis* genome encodes five *ERF VII* genes; two *HYPOXIA RESPONSIVE ERF* (*HRE*) genes, *HRE1* and *HRE2*, and three *RELATED TO AP2* (*RAP2*) genes, *RAP2.2*, *RAP2.3* and *RAP2.12* (*Nakano* et al., 2006; *Bailey-Serres et al*., 2012). Overexpression of each of these genes significantly enhances submergence and/or low oxygen tolerance (*Hinz* et al. 2010; *Licausi* et al. 2010, 2011; *Papdi* et al., 2015). On the contrary, *hre1hre2* and *rap2.12rap2.3* double knockouts and *rap2.12*, *rap2.2 and rap2.3* single knockout mutants show susceptibility to hypoxia. It was also revealed that inducible expression of all three RAPs confers tolerance to osmotic stress, and *rap2.12rap2.3* double knockout mutants showed hypersensitivity to both submergence and osmotic stress (*Papdi* et al., 2015). These studies show the significance of ERFVII in adaptation to both high and low extremes of water availability in plants.

Recent genetic and molecular studies have demonstrated that there are overlapping pathways coordinating responses to flooding and drought. Mapping of QTLs in oilseed rape (*Brassica napus*) using a double haploid population indicated that QTLs for both waterlogging-related and drought tolerance-related traits overlap significantly (*Li* et al., 2014). A divergent sunflower WRKY transcription factor was revealed to confer both dehydration and submergence tolerance in *Arabidopsis* transgenic plants (*Raineri* et al., 2015). Drought tolerance of these transgenic plants expressing *HaWRKY76* under 35S CaMV promoter was governed by ABA-independent mechanisms and associated with stomatal conductance. Submergence tolerance, on the other hand, was associated with carbohydrate preservation through the repression of fermentation pathways. Though the transcription factor conferred the tolerance to the two stresses distinctively, a common nexus between these two stresses was also stipulated to be related to higher cell membrane stability and chlorenchyma maintenance. However, a time-course study regarding soybean-specific crosstalk to submergence and drought has not been conducted previously.

Plants have evolved sophisticated mechanisms to cope with environmental perturbations. Studies show that these stresses trigger expression of specific genes governing diverse functions (*Zhu*, 2002) resulting in dynamic reconfiguration of global gene expression patterns. Therefore, employing transcriptomics as a genomics tool to get a snapshot of all the transcripts for particular time and tissue enables the study of the functional status for the entire genome. Recent advances in sequencing and availability of a near complete soybean genome (*Schmutz* et al., 2010) has made the global genome-wide transcriptome analyses possible.

Genomics has been employed successfully in many economically important crop species such as rice and soybean for the global transcriptome dynamics studies under flooding stress.

Genome-wide transcriptome comparisons by *Jung* et al. (2010) between submergence tolerant M202 (*Sub1*) and sensitive M202 rice after submergence highlighted transcriptional regulation of *Sub1A*-mediated tolerance and 13 pathways associated with acclimation to the stress. Study of transcriptional response during 6 h and 12 h of complete submergence in 2-d-old roots and hypocotyl of soybean seedlings was performed by *Nanjo* et al. (2011). This study used custom soybean 60-mer oligo microarray chips containing ~40 k oligonucleotides based on soybean full-length cDNA sequences and ESTs. They reported the upregulation of many photosynthetic-related genes, homologous genes of transcription factors with a WRKY domain and downregulation of genes involved in cell wall metabolism, secondary metabolism along with many others related to acclimation to flooding. Identification of SNPs in genes upregulated during drought stress in two soybean cultivars was performed by *Vidal* et al. (2012). They evaluated suppression subtractive libraries (SSH) from two contrasting cultivars upon water deprivation and identified more than 6,000 SNPs in 2,222 upregulated genes during drought. The study by *Fukao* et al. (2011) on publicly available transcriptomic data shows that a large number of submergence inducible genes (25.5%) are also induced during drought in rice. Exploring the global gene expression pattern during submergence, drought, and recovery from these stresses is, therefore, important for the elucidation of common and specific genes and pathways, which can ultimately aid in the development of soybean cultivars having tolerance to multiple stresses.

1.2 Wheat nitrogen use efficiency

A recent declaration from the 'World Summit on Food Security' has estimated that our current agricultural output will have to increase by 70 percent by 2050 in order to feed the expected global population of more than 9 billion (*FAO*, 2009). To achieve this, we must be able to add 44 million metric tons annually to our current global food production system (*Tester & Langridge*, 2010). The application of nitrogenous fertilizer is accordingly predicted to increase by approximately 3 fold by 2050 (*Good et al*., 2004). However, in the current context of global climate change, there are already concerns regarding the excessive use of agricultural inputs, especially those of nitrogenous fertilizers due to its association with greenhouse gas emissions and eutrophication of fresh and marine ecosystems. Therefore, crop scientists must be able to address these issues and improving nutrient-use-efficiency in major crops is one of the solutions.

Wheat (*Triticum aestivum* L.) is a major cereal that is grown on 219 million ha in the world (*FAOSTAT*, 2013). As observed in other non-legume crop species, the amount of nitrogen supplied is a major determinant of grain yield and quality in wheat (*Vilmus* et al., 2013). Hence, improvement of nitrogen-use-efficiency (NUE) in wheat not only economizes nitrogen fertilizers applied for production, but also decreases nitrogen runoff and denitrification from agricultural fields. NUE, as a function of multiple interacting genetic and environmental factors, has been considered inherently complex (*Dawson* et al., 2008). The definition of NUE has been provided in a number of scientific publications (*Moll* et al., 1982; *Huggins & Pan*, 1993; *Fageria* et al., 2008; *Liang* et al., 2014). In general, NUE is defined as yield per unit N available in the soil or applied to the crop. It is the product of NUpE (N uptake efficiency) and NUtE (N utilization efficiency) and is the optimal combination between the assimilation and remobilization of N (*Masclaux-Daubresse* et al., 2010). NUpE is the capacity of plant roots to acquire N from soil or

the percentage of fertilizer N acquired by the plant. Similarly, NUtE is the fraction of plant-acquired N to be converted to total plant biomass or grain yield (*Xu* et al., 2012).

Nitrogen is usually applied in the form of nitrate or ammonium. Studies have shown that nitrate uptake occurs through at least four different transport systems and transported by members of two families of genes encoding nitrate transporters (*Okamoto* et al., 2006; *Lea & Azevedo,* 2006). After nitrogen uptake, the assimilation requires reduction of nitrate into ammonium, followed by ammonium assimilation into amino acids. The reduction of nitrate is catalyzed by enzyme nitrate reductase in the cytoplasm followed by translocation to the chloroplast and conversion to ammonium by nitrite reductase (*Lea & Azevedo*, 2006). Ammonium can then initially be incorporated into glutamine and glutamate by the activity of glutamine synthase and glutamate synthase respectively, which are ultimately transferred to other amino acids and nucleic acids (*Forde & Lea,* 2007). Remobilization of N occurs during senescence via degradation of leaf proteins especially photosynthetic proteins (*Masclaux-Daubresse* et al., 2010). This process acts as a large source of N to the growing leaves and seed storage organs.

Several studies on crop species such as maize, rice and wheat have revealed that significant genetic variation for NUE exists among cultivars (*De Datta & Broadbent*, 1990; *Lopez-Bellido* et al., 2008; *Liu* et al., 2009; *Giambalvo* et al., 2010; *Giuliana* et al., 2011). QTL analyses in wheat have identified a number of genomic regions associated with traits linked to metabolism and response to N. For instance, *Guo* et al. (2012) studied wheat seedlings using a set of recombinant inbred lines under varying concentrations of N, P, and K nutrients. They identified 380 QTLs in at least one of 12 treatments and 24 traits related to morphological, nutrient content and nutrient utilization efficiency. *Cormier* et al. (2014) employed genome-wide

association study in 214 European elite wheat varieties using 23,603 SNP for 28 traits related to NUE in eight environments with two different N fertilization rates. They identified 1,010 significantly associated SNP which defined 333 chromosomal regions associated with at least one trait.

The level of available N has been shown to affect uptake and utilization efficiency in different crops (*Kamprath* et al., 1982; *Moll* et al., 1982; *Le Gouis* et al., 2000). Under high N regimes, the observed genotypic differences in NUE were due to differences in N utilization efficiency (NUtE). On the contrary, under low N regimes, alteration in N uptake efficiency (NUpE) was largely responsible for the observed genotypic differences. Studies conducted on high and low NUE cultivars under different N regime have shown that high NUE cultivars under low N can support their higher grain yield via larger and deeper roots, greater root oxidation activity, higher glutamine synthase activity, greater maintenance of sufficient chlorophyll fluorescence and chlorophyll content and higher photosynthetic rate even at lower Rubisco concentrations (*Ray* et al., 2003; *Thomsen* et al., 2014; *Ju* et al., 2015; *Vijayalakshmi* et al., 2015; *Yu* et al., 2015). Altogether, selection of high NUE genotypes and identification of key traits related to high NUE can be major goals for improving crop NUE.

1.3 OBJECTIVES

Soybean submergence and drought (Chapters 2 and 3)

- Determine physiological and transcriptomic responses to submergence and reoxygenation at the seedling establishment stage (Chapter 2).
- Identify genes encoding group VII Ethylene-Responsive-Factors (ERFs) in the soybean genome and analyze their response to submergence, reoxygenation, and ethylene (Chapter 2).
- Compare physiological and biochemical responses to submergence and drought in soybean leaves and roots (Chapter 3).
- Generate and explore global gene expression profiles during submergence, drought and recovery from these stresses in leaves and roots of soybean (Chapter 3).

Wheat nitrogen use efficiency (Chapter 4)

- Study the effect of N level on yield and its components in diverse wheat genotypes.
- Characterize morphological and metabolic traits associated with nitrogen use efficiency.

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CHAPTER 2

Physiological and transcriptomic characterization of submergence and reoxygenation responses in soybean seedlings

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Published in: Plant, Cell & Environment (2014), 37(10): 2350-2365. doi: 10.1111/pce.12277

ABSTRACT

Complete inundation at the early seedling stage is a common environmental constraint for soybean production throughout the world. As floodwaters subside, submerged seedlings are subsequently exposed to reoxygenation stress in the natural progression of a flood event. Here, we characterized the fundamental acclimation responses to submergence and reoxygenation in soybean at the seedling establishment stage. Approximately 90% of seedlings succumbed during 3 d of inundation under constant darkness, whereas 10 d of submergence were lethal to over 90% of seedlings under 12 h light/12 h dark cycles, indicating the significance of underwater photosynthesis in seedling survival. Submergence rapidly decreased the abundance of carbohydrate reserves and ATP in aerial tissue of seedlings although chlorophyll breakdown was not observed. The carbohydrate and ATP contents were recovered upon de-submergence, but sudden exposure to oxygen also induced lipid peroxidation, confirming that reoxygenation induced oxidative stress. Whole transcriptome analysis recognized genome-scale reconfiguration

of gene expression that regulates various signaling and metabolic pathways under submergence and reoxygenation. Comparative analysis of differentially regulated genes in shoots and roots of soybean and other plants define conserved, organ-specific and species-specific adjustments which enhance adaptability to submergence and reoxygenation through different metabolic pathways.

Keywords: Affymetrix microarray; flooding; genome-scale gene expression analysis; *Glycine max*; Group VII Ethylene Responsive Factors

2.1 INTRODUCTION

The number of flooding events has been increasing across the globe over the past six decades as a consequence of global climate change *(Bailey-Serres* et al., 2012a). Waterlogging and submergence cause growth inhibition and death in most crop species because of limitation of energy production via mitochondrial oxidative phosphorylation and accumulation of toxic end-products via anaerobic metabolism. Soybean is major sources of protein and oil for humans, livestock, and various industrial products, with the total grain production of about 242 million tons worldwide (*FAOSTAT*, 2012). However, soybean yields are adversely affected by a variety of environmental stresses including flooding. Even transient flooding caused by poor drainage after rainfall or irrigation restricts growth, development, and seed production (*Linkemer* et al., 1998; *Wuebker* et al., 2001).

In-depth studies of acclimation and tolerance to submergence and oxygen deprivation have been conducted in Arabidopsis, rice, and other crop species using a combination of genetic, genomic, physiological and molecular approaches (*Bailey-Serres & Voesenek*, 2010; *Bailey-Serres* et al., 2012a, b; *Fukao & Xiong*, 2013). These analyses have defined common acclimation responses including management of shoot elongation, development of adventitious roots and aerenchyma, transcriptomic and metabolic reconfiguration to optimize energy metabolism, cytosolic pH, and reactive oxygen species (ROS) accumulation. In rice, master regulators of submergence survival have been identified and characterized. Interestingly, two antithetical responses, tolerance versus escape, are governed by related *ETHYLENE RESPONSIVE FACTOR* (*ERF*)-type transcription factor genes that regulate gibberellic acid (GA)-mediated underwater elongation (*Xu* et al., 2006; *Hattori* et al., 2009). Submergence tolerance is conferred by the multigenic *SUBMERGENCE1* (*SUB1*) locus containing tandem paralogous group VII ERF

genes, *SUB1A*, *SUB1B*, and *SUB1C*, whereas escape response is also largely regulated by the locus that encodes duplicated paralogous ERFVII genes, *SNORKEL1* (*SK1*) and *SNORKEL2* (*SK2*).

Several studies in Arabidopsis have emphasized the significant role of group VII ERF in the regulation of submergence and low oxygen tolerance in plants. The Arabidopsis genome encodes five members of this subgroup, four of which are involved in survival of submergence and oxygen deprivation (*Hinz* et al., 2010; *Licausi* et al., 2010, 2011). For example, constitutive expression of one of ERFVII genes such as *HRE1*, *HRE2*, *RAP2.2*, or *RAP2.12* significantly enhances tolerance to submergence and/or low oxygen through regulation of core hypoxia-inducible genes. Conversely, *hre1hre2* double-knockout and *rap2.2* knockout mutants are more susceptible to oxygen deficiency. Recent biochemical and molecular studies have revealed that HRE1, HRE2, and RAP2.12 proteins are stably accumulated under oxygen deprivation, but are degraded under oxygen-replete conditions through the N-end rule pathway (*Gibbs* et al., 2011; *Licauci* et al., 2011).

Soybean can encounter two types of flooding stress: waterlogging (partial inundation) and complete submergence. It was previously shown that there is natural genetic diversity for tolerance of adult plants to root waterlogging among the US soybean cultivars (*VanToai* et al., 1994). Quantitative trait locus (QTL) analyses identified several small-effect loci that affect waterlogging tolerance at the early reproductive stage (*VanToai* et al., 2001; *Nguyen* et al., 2012). As observed in other plants, waterlogging stimulates the formation of aerenchyma and adventitious roots in soybean plants, facilitating transport of oxygen from non-flooded shoots (*Bacanamwo & Purcell*, 1999; *Thomas* et al., 2005, *Shimamura* et al., 2010). Complete submergence occurs more frequently at the seedling establishment and early vegetative stages. A

number of genes and proteins that are upregulated or downregulated within 2 d of complete submergence have been identified in hypocotyls and roots of the early stage of soybean seedlings (*Hashiguchi* et al., 2009; *Komatsu* et al., 2009; *Nanjo* et al., 2011; 2013). It has been reported that a subset of proteins is phosphorylated or dephosphorylated in response to complete submergence in hypocotyls, roots, and root tips (*Nanjo* et al., 2010; 2012). These analyses recognized differential regulation of gene transcripts and proteins that are associated with carbohydrate catabolism, anaerobic fermentation, cell wall loosening, ROS detoxification and stress responses at the early time point of submergence. However, detailed physiological and molecular characterization of acclimation responses to prolonged (lethal) submergence and subsequent reoxygenation has not been performed in the major legume species.

In this study, we investigated the fundamental morphological and physiological responses during the course of submergence and reoxygenation in soybean at the seedling establishment stage. We also identified genes encoding group VII ERFs in soybean, all of which were subjected to quantitative RT-PCR analysis to monitor alterations in mRNA abundance in response to submergence, recovery, and ethylene. Genome-scale gene expression analysis was conducted to characterize the transcriptomic adjustments to submergence and reoxygenation.

2.2 MATERIALS AND METHODS

2.2.1 Plant materials and growth conditions

Soybean (*Glycine max* (L.) Merr. cv. 'Williams 82') seeds were sterilized in 3% (v/v) sodium hypochlorite and 0.1% (v/v) Tween-20 for 10 min and rinsed thoroughly with deionized water. Seeds were placed on wet paper towels for 4 d at 25 ^{o}C in the light (50 µmol m^{-2} s^{-1}). For submergence treatment, 4-d-old seedlings were placed in a 1L plastic beaker containing 1 L of deionized water and incubated for up to 10 d at 25 ^{o}C in a growth chamber (relative humidity, 50%; light intensity, 50 µmol m^{-2} s^{-1}; 12 h light/12h dark). For 1-aminocyclopropane-1-carboxylic acid (ACC) treatment, 4-d-old seedlings were incubated on wet paper towels containing 0.1 or 1 mM ACC for 24 h in the light (50 µmol m^{-2} s^{-1}). All aerial tissue (cotyledons and hypocotyls) of each seedling were harvested at midday on the day of treatment specified, immediately frozen in liquid nitrogen and stored at -80 ^{o}C until use. For plant viability evaluation, submerged seedlings were transplanted into soil-containing pots and recovered for 7 d in a greenhouse (28 ^{o}C) under natural light conditions. Plants were scored as viable when one or more new leaves appeared during the recovery periods.

2.2.2 Chlorophyll measurement

Chlorophyll *a* and *b* contents were assayed from 100 mg of tissue in 3 ml of 100% methanol as described by *Porra* (2002). Following centrifugation at 4 ^{o}C for 20 min at 21,000*g*, the absorbance of the supernatant was quantified at 652.0 and 665.2 nm with a UV-Vis spectrophotometer (Evolution 220; Thermo Scientific, Walthman, MA, USA).

2.2.3 Carbohydrate assay

Aerial tissue (50 mg) was homogenized in 1 mL of 80% (v/v) ethanol and incubated at 80 ºC for 20 min. Following centrifugation for 10 min at 21,000 *g*, the supernatant was collected in a new tube. This extraction process was repeated twice. The three extracts were combined, dried under vacuum, and dissolved in 1 mL of water. Total soluble carbohydrate content was assayed by the anthrone method, with glucose as the standard (*Fukao* et al., 2006). The carbohydrate extract (100 µL) was mixed with 1 mL of 0.14% (w/v) anthrone solution in 100% H_2SO_4 and incubated at 100 ºC for 20 min. After cooling, the absorbance of the solution was measured at 620 nm. Starch content was quantified as described in *Fukao* et al. (2012). The pellet obtained after ethanol extraction was washed with water, resuspended in 1 mL of water containing 10 units of heat-resistant α-amylase (Sigma-Aldrich, St Louis, MO, USA), and incubated at 95 ºC for 15 min. After cooling, the suspension was adjusted to 25 mM sodium citrate (pH 4.8) and five units of amyloglucosidase (Sigma-Aldrich) were added. After incubation at 55 ºC for 30 min, the reaction mixture was centrifuged for 30 min at 21,000 *g* and glucose content in the supernatant was quantified by the anthrone method as described above. The reaction efficiency of starch assay was validated by analyzing known amount of starch.

2.2.4 SDS-PAGE

Total protein was extracted from 100 mg of tissue in 800 µL of an extraction buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 0.5% (v/v) IGEPAL CA-360, and 1 mM phenylmethylsulfonyl fluoride on ice. Protein sample (50 µl) was fractionated in a 12% (w/v) SDS-PAGE gel and stained with Coomassie brilliant blue R-250. Protein concentrations were determined by Coomassie Plus protein assay reagent following the

manufacture's protocol (Pierce, Rockford, IL, USA). Bovine Serum Albumin (BSA) was used as the standard.

2.2.5 ATP and ADP assays

Aerial tissue (50 mg) was homogenized in 0.5 mL of 5% (w/v) trichloroacetic acid on ice. Following centrifugation at 4 ^{o}C for 15 min at 21,000 *g*, the supernatant (10 µL) was mixed with 10 mL of water and the diluted solution (10 µL) was neutralized with 490 µL of 25 mM Tris-acetate buffer (pH 7.75). For ATP measurement, a 1.5 mL microcentrifuge tube containing the neutralized extract (10 µL) was placed in a luminometer (GloMax 20/20; Promega, Madison, WI, USA), where 100 µL of rLuciferase/Luciferin Regent (Promega) was injected and luminescence measured for a 10s integration period. ADP content was estimated after conversion of ADP to ATP. The neutralized extract (30 µL) was combined with 30 µL of ADP conversion buffer containing 12.5 mM HEPES-KOH (pH 7.75), 10 mM $MgCl_2$, 8 mM KCl, 100 µM phosphoenolpyruvate, and 0.9 units of pyruvate kinase. Following incubation at 25 ^{o}C for 10 min, luminescence was quantified for ATP.

2.2.6 Lipid peroxidation analysis

Lipid peroxidation was assayed with the thiobarbituric acid test, which quantifies malondialdehyde (MDA) as an end product of lipid peroxidation (*Hodges* et. al., 1999). MDA was extracted from aerial tissue (50 mg) in 1 mL of 80% (v/v) ethanol on ice. After centrifugation at 4 ^{o}C for 20 min at 21,000 *g*, the supernatant (0.5 mL) was mixed with 0.5 mL of 20% (w/v) trichloroacetic acid containing 0.65% (w/v) thiobarbituric aicd. The mixture was incubated at 95 ^{o}C for 30 min and then immediately cooled on ice. Following centrifugation at 4

^{o}C for 10 min at 10,000 *g*, A_{532} was measured, subtracting the value for non-specific absorption at 600 nm. The MDA concentration was calculated from the extinction coefficient 155 $mM^{-1}cm^{-1}$.

2.2.7 Phylogenetic analysis

Phylogenetic relatedness of ERF proteins was analyzed using MEGA 5.2.1 (*Tamura* et al., 2011). The full length of the coding region (amino acid sequences) was aligned using Clustal W. A phylogenetic tree was constructed by the neighbor-joining method with Poisson correction and pairwise deletion of gaps. The reliability of the output phylogeny was estimated through bootstrap analysis with 1,000 replicates.

2.2.8 RNA extraction and quantitative RT-PCR

Total RNA was extracted from 100 mg of tissue using the RNeasy Plant Mini kit (Qiagen, Venlo, Limburg, the Netherlands). Genomic DNA was eliminated by the on-column digestion method described in the manufacture's protocol. cDNA was synthesized from 2 µg of total RNA following the method of *Fukao* et al. (2006). Real-time PCR was conducted in a 15 µL reaction using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in the CFX Connect real-time PCR detection system (Bio-Rad). Amplification specificity was validated by melt-curve analysis at the end of each PCR experiment. PCR efficiency (95-105%) was verified by the method of *Schmittgen & Livak* (2008). Relative transcript abundance was determined using the comparative cycle threshold method (*Livak & Schmittgen*, 2001). *Ribosomal protein L30* (Glyma17g05270) and *Fbox* (Glyma12g05510) were used as normalization controls (*Le* et

al., 2012). Sequences and annealing temperatures of primer pairs are listed in Supplementary Appendix A1.

2.2.9 Enzymatic activity assay

Alcohol dehydrogenase (ADH; EC 1.1.1.1) activity was assayed as described in *Fukao* et al. (2006). Crude protein was extracted from tissue (50 mg) in an extraction buffer (0.4 mL) containing 100 mM Tris-HCl (pH 9.0), 20 mM $MgCl_2$, and 0.1% (v/v) 2-mercaptoethanol on ice. Following centrifugation at 4 ºC for 20 min at 21,000 *g*, the supernatant was subjected to the activity assay. The reaction mixture (1 mL) contained 50 µL extract, 50 mM Tris-HCl (pH 9.0), and 1 mM NAD^+. Ethanol (50 µM) was added to initiate the reaction. The conversion of NAD^+ to NADH was monitored at A_{340} at 25 ºC for 2 min. Protein was quantified using Coomassie Plus protein assay reagent (Pierce), with (BSA) as the standard.

2.2.10 Microarray analysis

Two independent biological replicate samples were used for microarray analysis. Total RNA was extracted from aerial tissue (100 mg) as described above. The quality and quantity of RNA were assessed using Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA synthesis, cRNA amplification, and conversion to sense strand cDNA were performed from 500 ng of total RNA using the WT Expression kit (Ambion, Austin, TX, USA). Sense strand cDNA (5.5 µg) was fragmented and end-labeled using the GeneChip WT Terminal Labeling kit (Affymetrix, Santa Clara, CA, USA). Labeled cDNA (5.2 µg) was hybridized against Soybean Gene 1.0 ST Array (Affymetrix) at 45 ºC for 16 h. CEL files from the microarrays were analyzed by use of the R program and Bioconductor package (*Gentleman* et. al., 2004). The robust multiarray

average (RMA) method was applied to normalize raw data (CEL files) with the “oligo” package (*Carvalho & Irizarry*, 2010). Differential expression analyses (pairwise comparisons of stress treatments versus non-submergence control) were performed to calculate the signal-to-log ratio (SLR) by linear models for microarray data (LIMMA) (*Smyth*, 2004). Adjusted P-values for multiple experiments were determined using the Benjamini and Hochberg method. The microarray data generated in this study are accessible from the Gene Expression Omnibus (GEO) database under accession number GSE51710 (Platform; GPL13674). The genes with significant adjusted P-values ($P < 0.05$) and more than twofold changes in expression ($SLR > 1$ or $SLR < -1$) were categorized into functional groups using the mapping file for soybean (Phytozome v9.0; Gmax_189.xls) and visualized using the MapMan (*Thimm* et al., 2004) and PageMan software (*Usadel* et al., 2006).

2.3 RESULTS

2.3.1 Time-course observation of soybean seedling growth and viability under submergence

Plant responses to submergence and oxygen deprivation are species-specific. For example, most rice varieties promote elongation of aerial tissue during submergence, allowing plants to escape from submergence when the water level gradually increases (*Fukao & Bailey-Serres*, 2004). By contrast, growth of shoots and roots is significantly restricted under low oxygen and waterlogging in maize, poplar, cotton and Arabidopsis (*Johnson* et al., 1989; *Kreuzwieser* et al., 2009; *Christianson* et al., 2010; *Hinz* et al., 2010). To discern the influence of submergence in seedling growth of soybean, 4-day-old soybean seedlings were exposed to complete inundation for up to 10 d (12h light/12 h dark) (Fig. 1a). Elongation of hypocotyls was not affected during 10 d of submergence (Fig. 1b), whereas root growth was completely inhibited by the stress (Fig. 1c). Under aerobic conditions, unifoliate leaves emerged at day 7, but new leaves were not formed during submergence (Fig 1a). Complete submergence also restricted development of lateral roots and root hairs.

Although submergence largely impedes gas exchange and light absorption by plants, underwater photosynthesis affects the survival of various terrestrial wetland plants (*Colmer* et al., 2011). To evaluate the significance of light in seedling viability, 4-d-old seedlings were completely submerged under 12h light/12 h dark cycles or constant darkness. Following stress treatment, seedlings were transplanted into soil-containing pots and recovered in a greenhouse under normal growth conditions for 7 d (Fig. 1d). When exposed to submergence under constant darkness, 90% of seedlings died after 3 d of submergence (Fig. 1e). By contrast, 93.3% of seedlings survived the same duration of stress under 12h light/12 h dark cycles, and 10 d of submergence were lethal to over 90% of seedlings. Fresh weight of aerial tissue after 7 d of

recovery was also in accordance with the observations of seedling viability under dark and light/dark conditions (Fig. 1f). These results suggest that underwater photosynthesis and energy management largely influence viability of soybean seedlings under submergence.

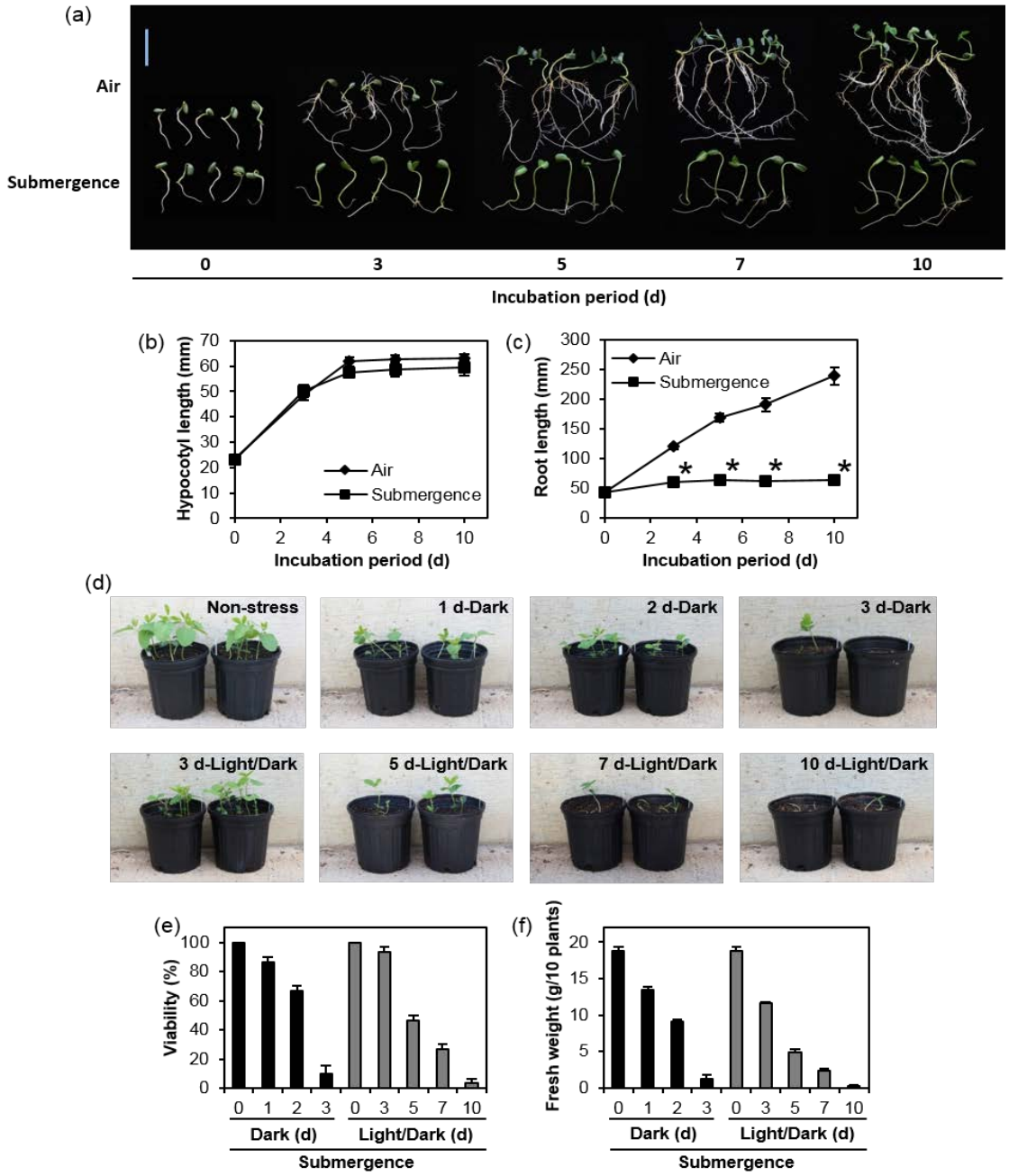

Figure 1. Phenotypes of soybean seedlings after submergence. (a) Soybean seedlings exposed to submergence. Four-day-old seedlings were completely submerged for up to 10 d (12 h light/12 h dark). Control seedlings were incubated under aerobic conditions (Air). Bar = 5 cm. (b), (c) Hypocotyl and root length of soybean seedlings submerged for up to 10 d (12 h light/12 h dark). The data represent means ± SE (n = 10). Asterisks indicate significant difference between air and submergence (P < 0.001). (d) Soybean plants recovered from submergence stress. Four-day-old seedlings were exposed to submergence under constant darkness or 12 h light/12 h dark cycles. Following stress treatment, seedlings were transplanted into soil-containing pots (five seedlings per pot) and recovered under regular growth conditions in a greenhouse for 7 d. (e) Viability of soybean plants after submergence. Plant viability was evaluated in the samples shown in (d). Plants were scored viable if new unifoliolate or trifoliolate leaves appeared during recovery. (f) Fresh weight of aerial tissue of soybean seedlings recovered from submergence stress. The entire aerial tissue of recovered plants (10 plants) was weighed in the samples shown in (d). In (e) and (f), the data represent means ± SE (n = 10 × 3).

2.3.2 Alternations in the abundance of energy reserves under submergence and reoxygenation in soybean seedlings

Submergence and low oxygen promote rapid degradation of energy reserves such as starch and soluble carbohydrates in various plants, which is necessary to support inefficient energy production through anaerobic metabolism (*Koch* et al., 2000; *Fukao* et al., 2006; *Branco-Price* et al., 2008). To characterize energetic responses to submergence in soybean, we monitored the abundance of chlorophylls, carbohydrates, protein, ATP, and ADP in aerial tissue of seedlings exposed to submergence for up to 7 d under 12h light/12h dark cycles (Fig. 2). The same metabolites were also quantified after a 1 d recovery period following 5 d of submergence, where approximately 50% of seedlings survived submergence stress (Fig. 1e). Minimal alterations in the level of chlorophyll *a* were observed over the course of submergence and reoxygenation (Fig. 2a), but these small differences are probably not biologically relevant. Similarly, the content of chlorophyll *b* remained steady under the stress. By contrast, starch was rapidly catabolized and decreased to 4.3% of the total starch content at day 0 after 5 d of submergence (Fig 2b), indicating that soybean seedlings are exposed to severe and prolonged starch starvation during submergence. After 5 d of submergence followed by 1 d of reoxygenation, the level of starch was recovered and increased to 31.5% of the total starch content at day 0. A similar trend was observed for the abundance of total soluble carbohydrates during submergence and reoxygenation, but the reduction in carbohydrates was less remarkable, which retained 31.1% of the total starch content at day 0 even after 7 d of submergence (Fig. 2c). The protein level gradually decreased to 51.5% of the total protein content at day 0 over the time course of stress treatment and was not recovered after 1 d of de-submergence (Fig. 2d & e). A rapid decline in the abundance of ATP was observed 1 d after submergence, and the level was quickly restored in

response to reoxygenation (Fig. 2f). Since the level of ADP remained relatively stable during the stress and recovery periods (Fig. 2g), a change in the ATP/ADP ratio was similar to that in the ATP content (Fig. 2h).

Various abiotic stresses trigger the excessive accumulation of ROS, resulting in oxidative damage of cellular components. To evaluate the degree of oxidative damage during submergence and reoxygenation, the accumulation of malondialdehyde (MDA), an end product of lipid peroxidation, was monitored in aerial tissue of soybean seedlings. The MDA content was unaltered over the submergence period, but was promptly elevated in response to re-aeration (Fig 2i), consistent with the observation in rice seedlings upon de-submergence (*Fukao* et al., 2011).

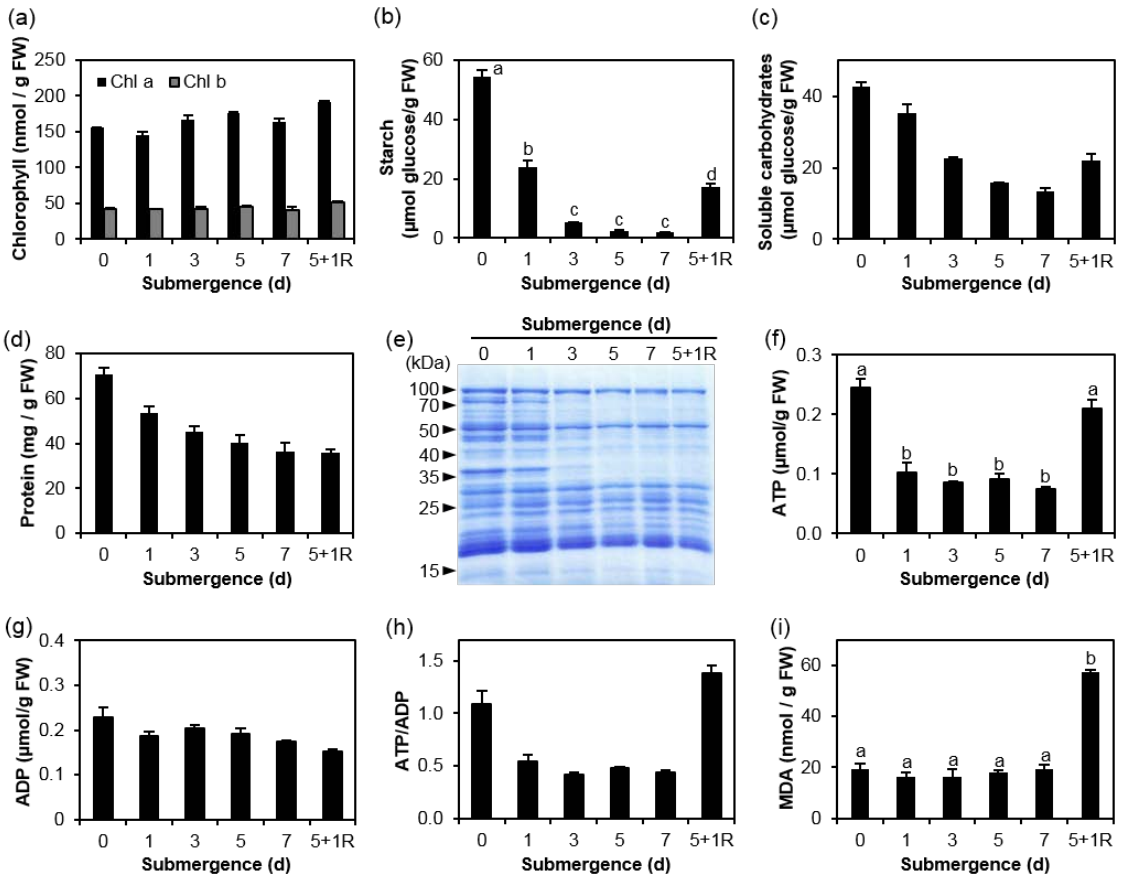

Figure 2. Physiological responses to submergence and reoxygenation in shoots of soybean seedlings. The contents of chlorophylls (a), starch (b), total soluble carbohydrates (c), total protein (d), ATP (f), ADP (g) and MDA (i) were analysed in aerial tissue of seedlings exposed to submergence for up to 7 d. For reoxygenation, seedlings submerged for 5 d were removed from water and incubated under aerial conditions for 1 d (5 + 1R). For (e), total protein was fractionated in a 12% (w/v) SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250. For (h), ATP/ADP ratios were calculated based on the data in (f) and (g). Data represent mean ± SE (n = 3). Bars not sharing the same letter are significantly different ($P < 0.05$).

2.3.3 The soybean genome does not contain *SUB1* and *SK1* orthologs, but soybean group VII ERFs are closely related to those in Arabidopsis

A number of genetic and molecular studies demonstrated that group VII ERFs are pivotal regulators of acclimation responses to submergence and oxygen deprivation in rice and Arabidopsis (*Hinz* et al., 2010; *Licausi* et al., 2010, 2011; *Gibbs* et al., 2011; *Bailey-Serres* et al., 2012a). To identify group VII ERFs in soybean, we analyzed the Williams 82 soybean genome sequence using BLAST. Comparative sequence analysis discovered nine ERFs in the group VII family that are closely related to Arabidopsis ERFVII such as HRE1, HRE2, RAP2.2, RAP2.3, and RAP2.12 (Fig. 3a). We designated these nine genes *ERFVII1-9*. All soybean ERFVII proteins contain a conserved N-terminal motif [NH_2-MCGGAII (A/S) D], which characterizes the group VII of ERF family (*Nakano* et al., 2006). To estimate the evolutionary relatedness of ERFVII proteins in soybean, Arabidopsis, and rice, a neighbor joining method of phylogenetic analyses were used (Fig. 3b). It appears that the soybean and Arabidopsis genomes do not contain any *SUB1* or *SK* orthologs as all of such rice proteins are clearly separated from soybean and Arabidopsis ERFVIIs. Soybean ERFVII proteins were divided into three distinct clades. Notably, each clade contained at least one Arabidopsis ERFVII with significant bootstrap values supporting the phylogeny, indicating high sequence conservation between soybean and Arabidopsis ERF VII proteins.

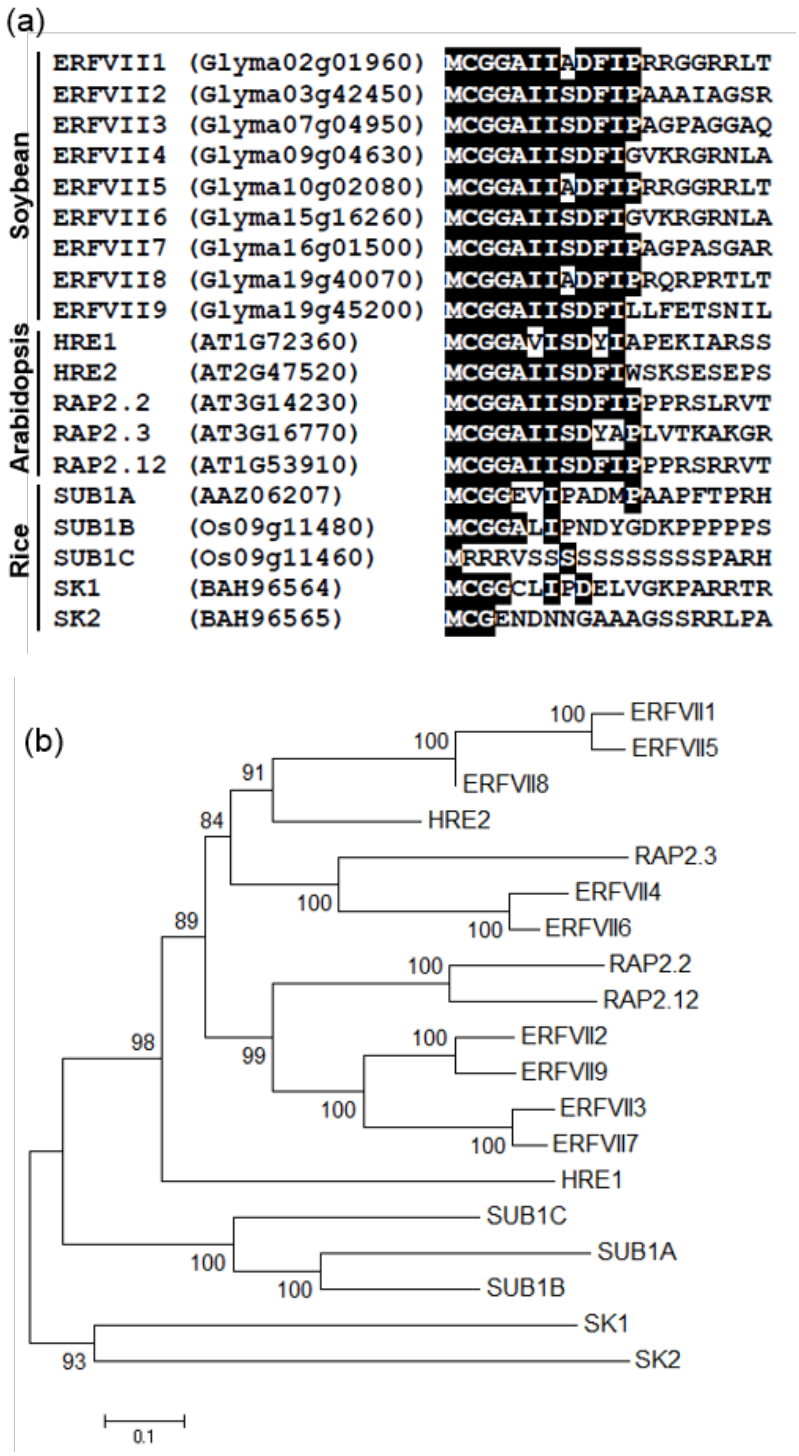

Figure 3. The group VII ERFs of soybean, Arabidopsis and rice. (a) N terminal alignment of soybean, Arabidopsis and rice group VII ERF proteins. The highly conserved signature motif is highlighted. (b) Phylogenetic tree based on amino acid sequences of soybean, Arabidopsis and rice group VII ERFs. The full length of the amino acid sequences was analyzed using the neighbour-joining method in MEGA, version 5.2.1. The length of each branch is proportional to sequence divergence. The numbers above branches represent bootstrap values from 1000 replicates.

2.3.4 Genes encoding group VII ERFs in soybean are induced in response to submergence reoxygenation, and/or ethylene

Master regulators of submergence tolerance and escape in rice, *SUB1A* and *SK*s are upregulated by submergence and ethylene (*Fukao* et al., 2006; *Hattori* et al., 2009). Arabidopsis ERFVII genes governing acclimation responses to submergence and oxygen deficiency, *HRE1*, *HRE2*, *RAP2.2*, and *RAP2.12* are also responsive to hypoxia and/or ethylene (*Hinz* et al., 2010; *Licausi* et al., 2010, 2011). To obtain the expression profile of soybean ERFVII genes during submergence, we monitored the abundance of nine ERFVII transcripts in aerial tissue of William 82 seedlings exposed to submergence by quantitative RT-PCR (Fig. 4a). Of the nine gene transcripts, *ERFVII1*, *ERFVII5*, *ERFVII6*, and *ERFVII8* mRNAs were highly accumulated in response to submergence, but dramatically decreased to the level at day 0 during 1 d of reoxygenation. The transcript abundance of *ERFVII4* was slightly elevated (~2-fold increase) at later time points. The levels of *ERFVII2*, *ERFVII3*, *ERFVII7*, and *ERFVII9* transcripts were not apparently altered (< 2-fold increase) during the course of submergence and reoxygenation. ADH is a rate limiting enzyme for ethanolic fermentation, which is activated under oxygen deprivation in various plants (*Russell* et al., 1990; *Andrew* et al., 1994; *Baxter- Burrell* et al., 2002; *Fukao* et al., 2003, 2006). Loss-of-function mutants of *adh* in maize, rice, and Arabidopsis succumb rapidly to oxygen deficiency and submergence, indicating that ethanolic fermentation through ADH is necessary for survival of the stress (*Schwartz*, 1969; *Rahman* et al., 2001; *Ismond* et al., 2003). Among six *ADH* genes in soybean, *ADH1* (Glyma14g27940) and *ADH3* (Glyma06g12780) are expressed in both cotyledons and hypocotyls (*Newman & VanToai*, 1992; *Komatsu* et al., 2011). We monitored the abundance of *ADH1* and *ADH3* transcripts in aerial

tissue of soybean seedlings exposed to submergence and reoxygenation (Fig. 4b). The level of *ADH1* mRNA rapidly increased in response to 1 d of submergence, but declined after 3 d of submergence. The transcript of *ADH3* was only slightly accumulated under submergence. Despite the clear induction of *ADH1* mRNA, the enzymatic activity of ADH was only slightly elevated during submergence (Fig. 4c).

To determine whether ethylene influences the accumulation of ERFVII gene transcripts, mRNA levels of nine ERFVII genes were monitored in aerial tissue of seedlings treated with ACC, an immediate precursor of ethylene (Fig. 5). The abundance of *ERFVII1*, *ERFVII4*, *ERFVII5*, and *ERFVII8* transcripts were clearly elevated (> 3-fold increase) in response to ACC. Application of ACC slightly induced (~2-fold increase) transcript accumulation of *ERFVII3*, *ERFVII*6 and *ERFVII7*. ACC-mediated accumulation of *ERFVII2* and *ERFVII9* mRNAs was minimal; these small differences are probably not biologically significant.

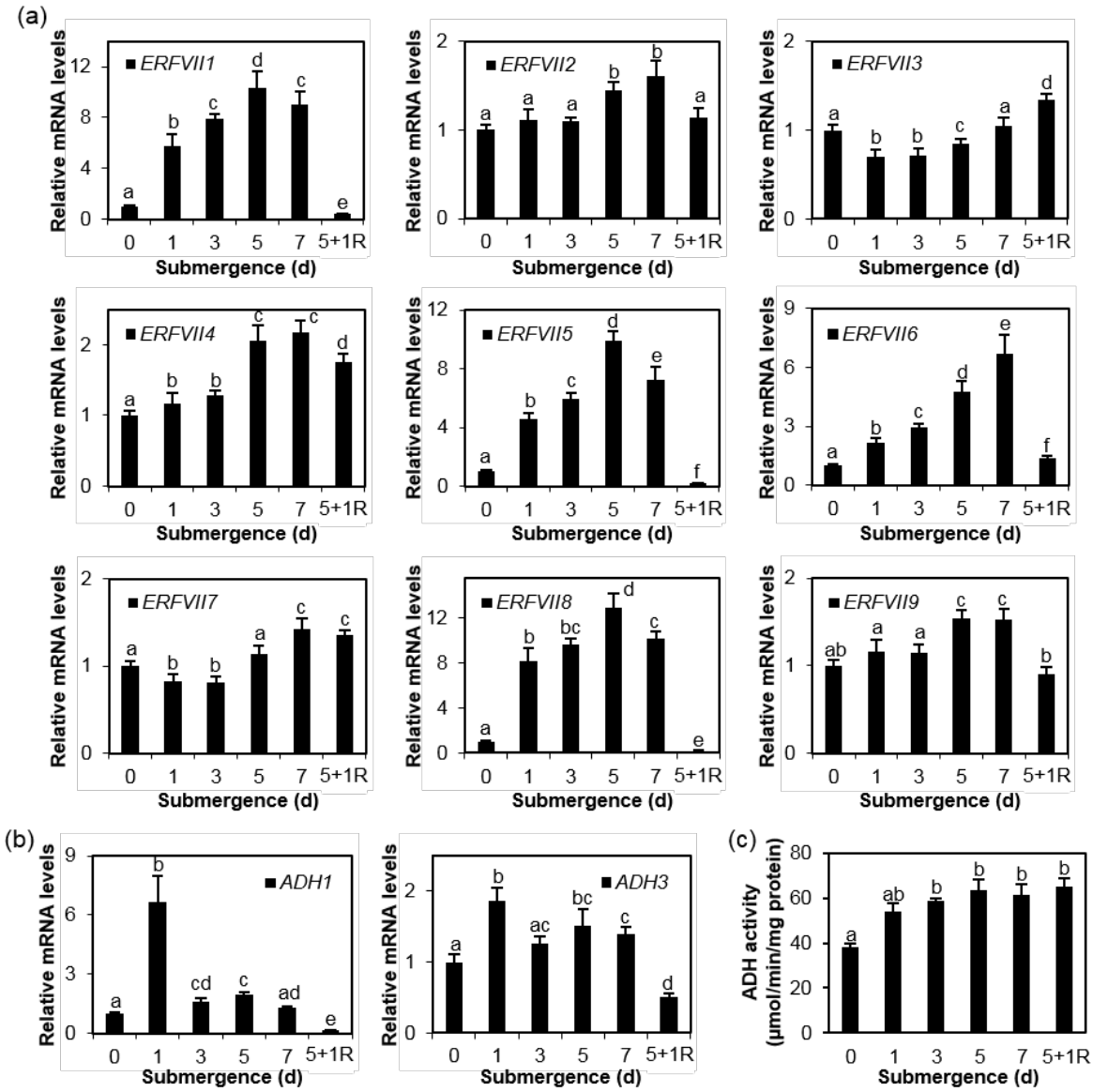

Figure 4. The effect of submergence and reoxygenation on mRNA accumulation of group VII ERFs and alcohol dehydrogenases in shoots of soybean seedlings. Relative mRNA levels of group VII ERF genes (a) and alcohol dehydrogenase (ADH) genes (b). Four-day-old seedlings were exposed to submergence for up to 7 d. For reoxygenation, seedlings submerged for 5 d were removed from water and incubated under aerial conditions for 1 d (5 + 1R). Aerial tissue was harvested at the time points specified and analyzed by quantitative RT-PCR. Relative level of each transcript was calculated by comparison to the non-stress control. (c) Specific activities of alcohol dehydrogenase during and after submergence. For (a), (b) and (c), data represent mean ± SE (n = 3). Bars not sharing the same letter are significantly different (P < 0.05).

2.3.5 Dynamic reconfiguration of global gene expression in response to submergence and reoxygenation

To characterize genome-scale adjustment of gene expression to submergence and reoxygenation, we performed whole-transcriptome analysis using aerial tissue of soybean seedlings exposed to 0, 1, and 5 d of complete submergence and 1 d of recovery following 5 d of submergence. Pairwise comparisons of stress treatments versus non-submergence control identified 5,124 gene transcripts with significant adjusted *P*-values ($P < 0.05$) and more than twofold changes in expression (SLR > 1 or SLR < -1) in at least one stress condition. In response to 1 d of submergence, the transcript abundance of 1,614 genes was significantly altered, of which 64% were downregulated. Prolonged duration of submergence considerably increased the number of differentially regulated genes, with 4,374 genes altered in their expression after 5 d of submergence. Of these genes, 66.4% was negatively regulated, consistent with the observation after 1 d of submergence. Reoxygenation significantly changed the abundance of 1,044 gene transcripts, of which 59.2% was downregulated.

To examine the overlap of differentially regulated genes during submergence and reoxygenation, Venn diagrams were constructed for genes upregulated or downregulated under stress conditions (Fig. 6a). Among 581 genes induced by 1 d of submergence, 409 genes (70.1%) were upregulated in response to 5 d of submergence. Similarly, 79.3% of genes negatively regulated by 1 d of submergence were also downregulated in response to 5 d of submergence. These results suggest that most genes differentially regulated at the early stage of submergence are also critical for adaptation to prolonged submergence in shoots of soybean seedlings. Submergence- and reoxygenation-responsive genes are also significantly overlapped; 41.8% of

reoxygenation-inducible genes were upregulated by 1 d or 5 d of submergence, whereas 69.1% of genes negatively regulated by reoxygenation were downregulated in response to submergence.

Genes commonly induced in response to brief (1 d) and prolonged (5 d) submergence included: pyruvate decarboxylase (PDC) (Glyma01g29190), aldehyde dehydrogenase (ALDH) (Glyma13g23950), aspartate aminotransferase (Glyma14g13480), invertase inhibitors (Glyma03g37410; Glyma17g04020; Glyma17g05180; Glyma19g40010), expansins (Glyma05g05390; Glyma05g05420; Glyma17g15640; Glyma17g15670; Glyma17g15680), xyloglucan endotransglucosylase (XET) (Glyma09g07070; Glyma13g01140; Glyma15g18360; Glyma17g07220), non-symbiotic hemoglobin (Glyma11g12980; Glyma14g20380), ACC oxidase (Glyma07g15480), gibberellin 2-oxidae (GA_2ox) (Glyma13g33300; Glyma20g27870), respiratory burst oxidases (Glyma10g29280; Glyma20g38000), lateral organ boundaries (LOB) domain containing protein (Glyma18g02780), and a number of genes associated with anaerobic respiration, stress responses and disease resistance, most of which are also upregulated in shoots and roots of Arabidopsis seedlings under hypoxia and in rosette leaves and roots of adult Arabidopsis plants under submergence (*Mustroph* et al., 2009; *Lee* et al., 2011). A variety of signaling components such as transcription factors, protein kinases/phosphatases, F-box proteins, and histone methyltransferase were also included by 1d and 5 d of submergence.

Previously, *Nanjo* et al. (2011) illustrated transcriptional responses to complete submergence (6 h and 12 h) in roots including hypocotyls of soybean seedling using Agilent 60-mer oligonucleotide arrays. Using Venn diagrams, we compared gene transcripts which are differentially regulated in shoots (cotyledons and hypocotyls) and roots (hypocotyls and roots) during submergence (Fig. 6b). In accordance with the observations in Arabidopsis seedlings and adult plants (*Mustroph* et al., 2009; *Lee* et al., 2011), we found only partial overlap in shoot- and

root-responsive genes under submerged conditions although the small overlap can also be attributed to distinct microarray platforms and growth/stress conditions between the two experiments. Actual overlap in shoots (cotyledons and hypocotyls) and roots may be smaller as both samples used for these microarray analyses contain hypocotyls. These results confirm that submergence triggers distinct signaling and metabolic pathways in shoots and roots at the transcriptional level, which coordinate diverse organ-specific acclimation responses to the stress.

Among 5,124 differentially-expressed genes, 3,562 were classified into functional groups based on the bin code for soybean (Phytozome v9.0) and the expression changes of 629 were visualized on the major metabolic pathway diagrams using MapMan. Genes associated with various metabolic pathways were altered in expression during 1 d of submergence (Supplementary Appendix A1), but prolonged submergence (5 d) considerably downregulated more of the genes involved in carbohydrate breakdown, lipid synthesis/degradation, cell wall synthesis/degradation, amino acid synthesis, photosynthesis and secondary metabolism including terpene, flavonoid, and phenylpropanoid synthesis/degradation (Fig 7a). Interestingly, 87% of these genes were not downregulated by 1 d of reoxygenation following 5 d of submergence (Fig. 7b), suggesting that a variety of pathways restricted under prolonged submergence is turned on upon re-exposure to atmosphere through dynamic transcriptomic reconfiguration. We also analyzed the alterations in mRNA accumulation of genes associated with protein metabolism using PageMan (Supplementary Appendix A2). Submergence increased the abundance of transcripts involved in protein degradation, but repressed expression of genes responsible for protein synthesis (Supplementary Appendix A2). Significant changes in expression of these genes were not observed during 1 d of reoxygenation. Introgression and manipulation of transcription factor genes have enhanced tolerance to various abiotic stresses including

submergence and low oxygen in plants (*Xu* et al., 2006; *Hinz* et al., 2010; *Hirayama & Shinozaki*, 2010; *Licausi* et al., 2010). Of 1,642 submergence-induced genes, 200 genes (12.2%) encode putative transcription factors. Submergence increased mRNA accumulation of specific genes in nearly all transcription factor (Fig. 8a). Expression patterns of genes encoding MYB and ERF transcription factors were compared during submergence and reoxygenation (Fig. 8b). In accordance with the results of quantitative RT-PCR analysis (Fig. 4a), most of these transcription factors genes including *ERFVII1*, *ERFVII5*, *ERFVII6*, and *ERFVII8* were further induced in response to 5 d of submergence, but declined in expression during 1 d of reoxygenation.

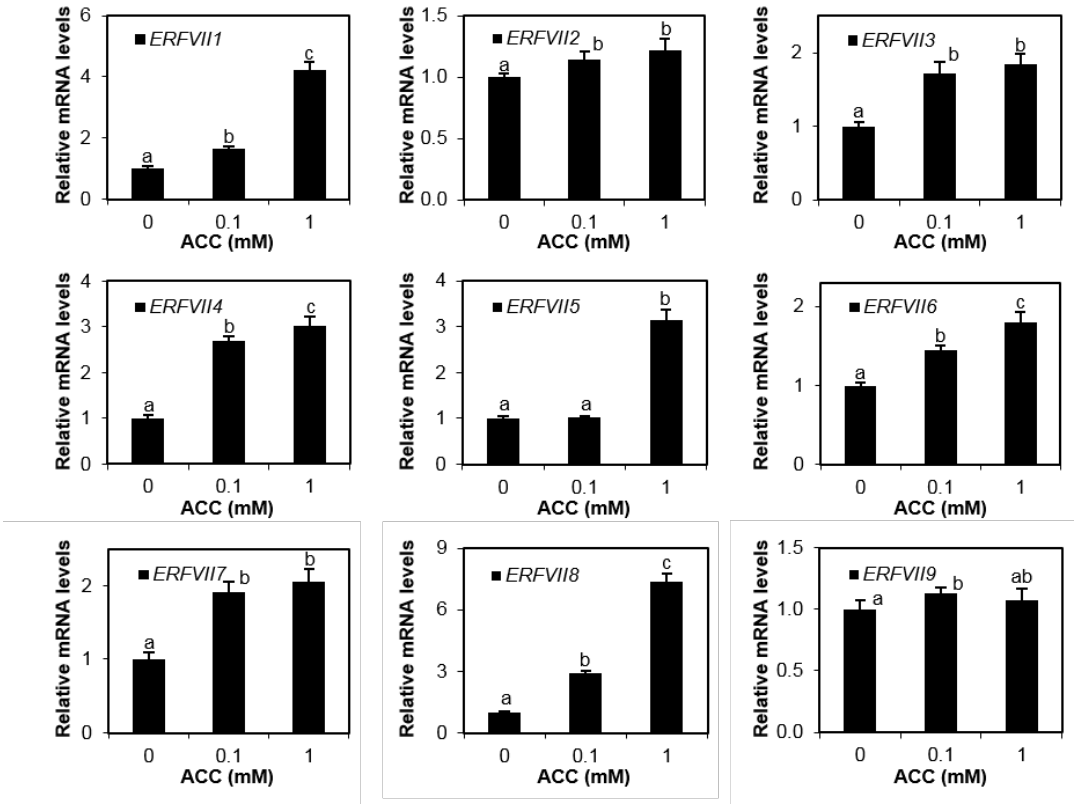

Figure 5. The effect of ethylene on mRNA accumulation of group VII ERFs in shoots of soybean seedlings. Four-day-old seedlings were treated with 0, 0.1 or 1 mM ACC, an immediate precursor of ethylene, for 24 h. Following treatment, aerial tissue was harvested and analyzed by quantitative RT-PCR. Relative level of each transcript was calculated by comparison to the mock control. Data represent mean ± SE (n = 3). Bars not sharing the same letter are significantly different (P < 0.05).

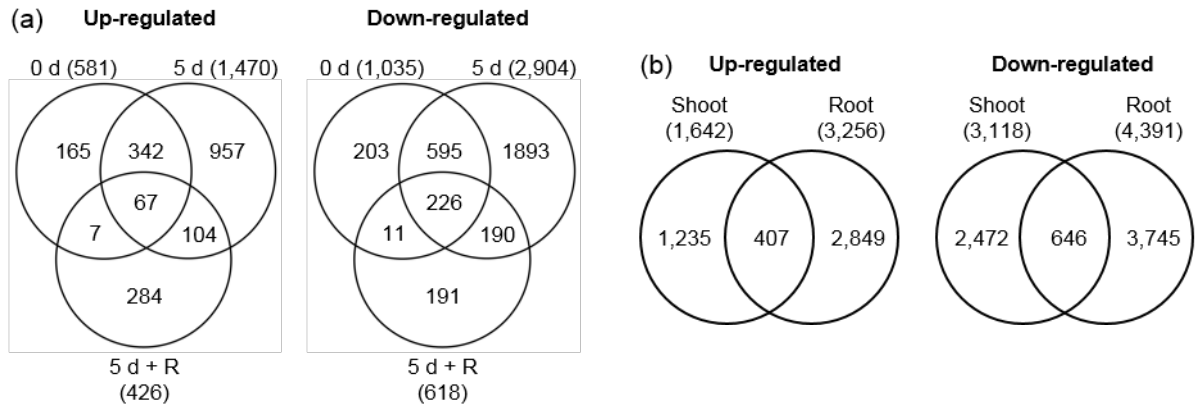

Figure 6. Venn diagrams representing gene transcripts which are differentially regulated in response to submergence and reoxygenation. (a) Overlap in mRNAs upregulated or downregulated in response to submergence (1 d and 5 d) and reoxygenation in shoots of soybean seedlings. Differentially upregulated (SLR >1 and adj. P < 0.05) and downregulated (SLR < −1 and adj. P < 0.05) genes are applied to construct the diagrams. R; 1 d of recovery. (b) Overlap in mRNAs upregulated or downregulated during submergence in shoots (cotyledons and hypocotyls) and roots (hypocotyls and roots). Differentially regulated genes in shoots (1 d or 5 d of submergence) and roots (6 h or 12 h of submergence) are applied to construct the diagrams. Microarray datasets in soybean roots were obtained from Nanjo *et al*. (2011).

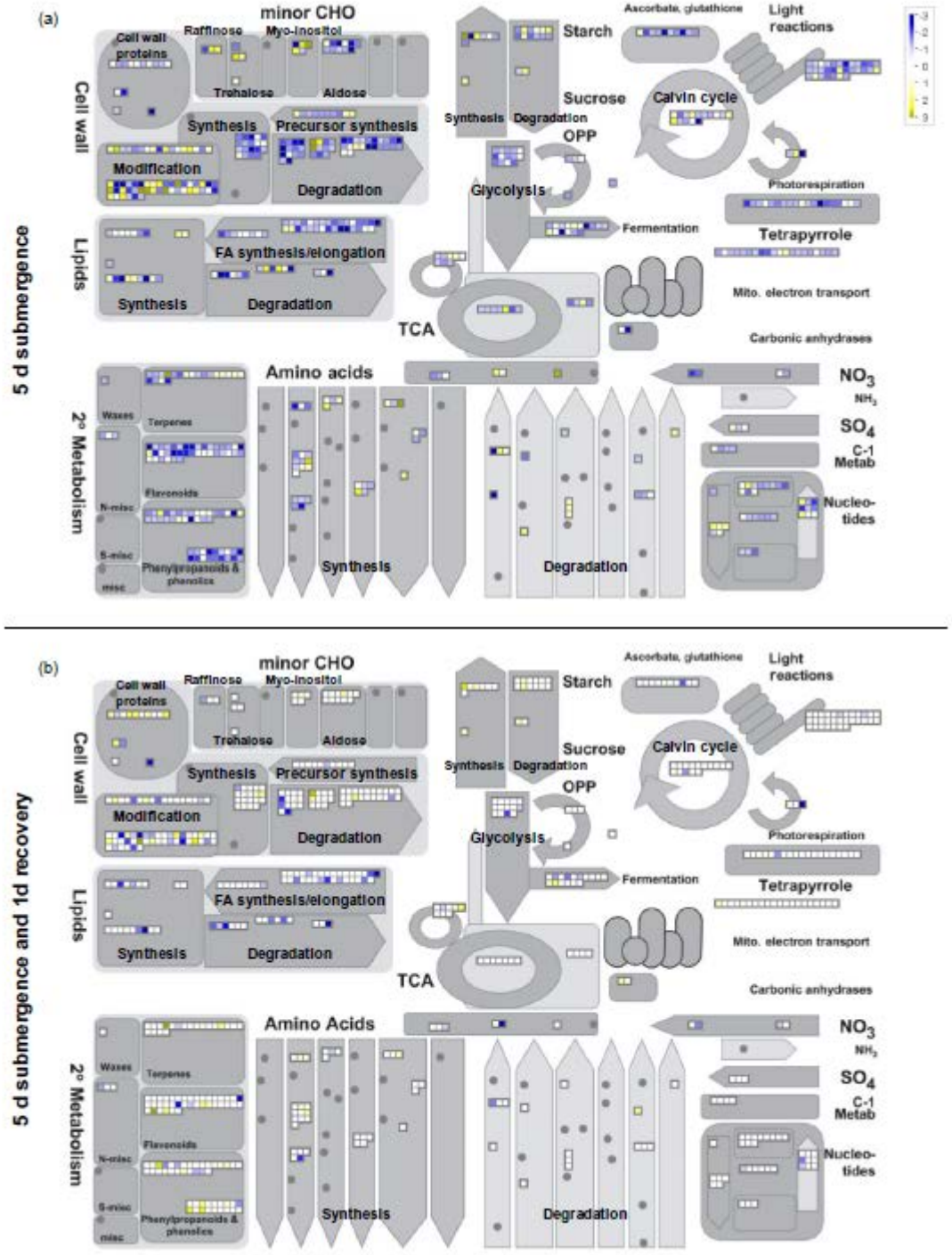

Figure 7. Expression changes of genes encoding enzymes involved in major metabolic pathways during submergence and recovery (a) 5 d of submergence. (b) 1 d of recovery following 5 d of submergence. Alterations in the abundance of mRNAs with significant adjusted *P*-values ($P < 0.05$) and more than twofold changes in expression (SLR > 1 or SLR< −1) were visualized on the metabolic pathway diagrams. The log2 values of fold changes are displayed using the colour code.

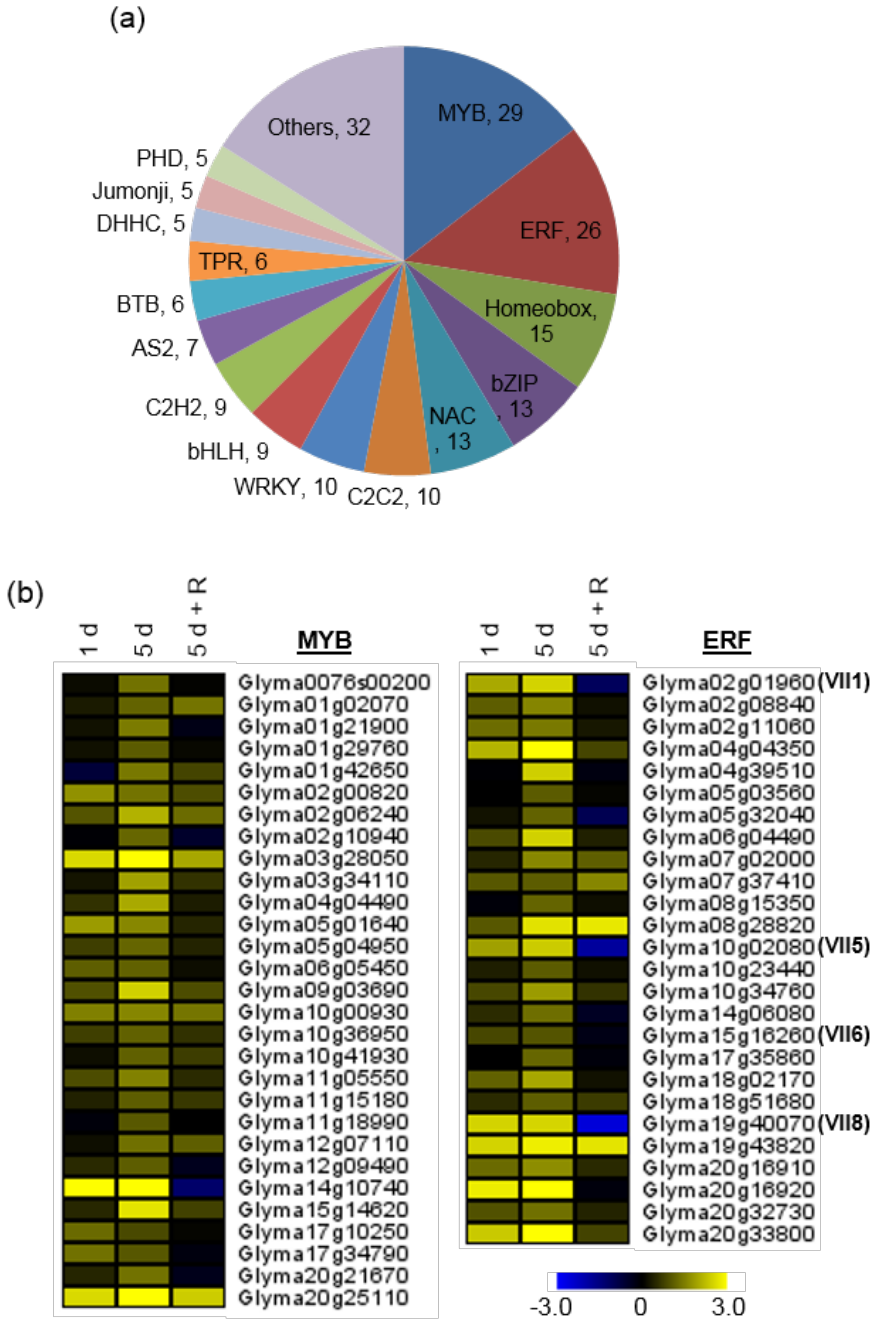

Figure 8. Genes encoding transcription factors which are differentially upregulated in response to submergence. (a) Numbers of genes belonging to transcription factor families that are significantly induced by 1 d and/or 5 d of submergence. Others; transcription factor families with fewer than five differentially upregulated genes. (b) Expression changes of genes encoding MYB and ERF transcription factors during submergence and reoxygenation. The log2 values of fold changes are displayed using the color code. R; 1 d of recovery.

2.4 DISCUSSION

We characterized the fundamental responses to complete submergence in aerial tissue of Williams 82 soybean seedlings at the morphological, physiological, and transcriptomic levels. It was previously shown that root tips are extremely susceptible to submergence and oxygen deficiency in maize, soybean and pea seedlings (*Andrew* et al., 1994; *Subbaiah & Sachs*, 2003; *Gladish* et al., 2006, *Nanjo* et al., 2013). When soybean seedlings were exposed to complete submergence, root growth was totally inhibited, presumably because of death of root tips (Fig 1c). Waterlogging (partial submergence) stimulates development of adventitious roots in various plants including soybean (*Subbaiah & Sachs*, 2003; *Thomas* et al., 2005; *Vidoz* et al., 2010; *Steffens* et al., 2012). However, we did not observe emergence of adventitious roots in soybean seedlings treated with complete submergence. This response may be a component of the quiescence survival strategy in submerged soybean seedlings, enabling economization of energy reserves under water where oxidative respiration and photosynthesis are largely limited.

We demonstrated that constant darkness considerably reduces tolerance to complete submergence in soybean seedlings as compared to 12h light/12h dark cycles (Fig. 1d, e, f). This observation emphasizes the significance of underwater photosynthesis in seedling survival under submergence although abrupt re-exposure to light following prolonged darkness may have an additional negative impact on seedling survival. In fact, the degree of photosynthesis significantly affects the survival of various terrestrial wetland plants under complete submergence (*Colmer* et al., 2011). Consistently, the volume of floodwater relative to seedlings positively correlates with the abundance of dissolved oxygen in water and the degree of submergence survival in soybean (*Nanjo* et al., 2013). It was also demonstrated that *SUB1A* enhances tolerance to submergence and prolonged darkness in rice through restriction of chlorophyll degradation and carbohydrate breakdown under stress conditions (*Fukao* et al., 2006,

2012). All together, these results indicate that management of energy production and consumption substantially influences survival of complete submergence in soybean and other plants.

Degradation of chlorophyll is a common response to prolonged submergence in aerial tissue of many terrestrial plants (*Huynh* et al., 2005; *Mommer* et al., 2005; *Fukao* et al., 2006; *Manzur* et al., 2009). However, we observed that the abundance of chlorophyll *a* and *b* remained nearly stable over the course of submergence treatment (Fig. 2a). The materials used for these experiments are aerial tissue of 4-d-old seedlings, which are actively synthesizing and accumulating chlorophyll in cotyledons. It may be possible that the rate of chlorophyll synthesis is balanced with that of degradation in young soybean seedlings under complete submergence. Alternatively, it is more likely that seed storage proteins in cotyledons are primarily degraded under energy-starved conditions such as submergence, thereby resulting in the avoidance of chlorophyll and chloroplast protein degradation during 7 d of submergence. This may be observed only in tissues which contain a large quantity of storage proteins such as legume cotyledons.

During submergence, considerable expenditure of carbohydrate reserves through glycolysis and fermentation is required to support ATP production as more efficient oxidative phosphorylation is not functional under anaerobic conditions (*Fukao & Bailey-Serres*, 2004; *Bailey-Serres* et al., 2012a). Our results showed that submergence promotes rapid degradation of starch, soluble carbohydrates and ATP in aerial tissue of soybean (Fig. 2b, c, f). Upon de-submergence, the levels of stored carbohydrates and ATP were recovered, presumably due to recommencement of photosynthesis and aerobic respiration. Re-exposure to atmospheric oxygen allows plants to recover from carbohydrate starvation and an energy crisis, but it also induces

oxidative stress as a post-submergence stress (*Fukao & Xiong*, 2013). Indeed, we observed a pronounced elevation of MDA, an indicator of oxidative stress, in response to 1 d of reoxygenation following 5 d of submergence (Fig. 2i). It is most likely that avoidance of oxidative stress through activation of antioxidant enzymes and metabolites is a critical component of submergence tolerance. In rice, the degree of submergence survival is positively correlated with the capacity for ROS detoxification, but negatively correlated with the level of MDA accumulation during reoxygenation (*Kawano* et al., 2002; *Ella* et al., 2003). Consistently, a variety of genes associated with ROS detoxification including superoxide dismutase, ascorbate peroxidase, glutathione S-transferase and metallothionein were significantly upregulated in response to 1 d of reoxygenation relative to 5 d of submergence (Supplementary Appendix A4).

A variety of genetic, biochemical, and molecular studies have demonstrated that group VII ERFs are key regulators for adaptation and tolerance to submergence and oxygen deficiency in rice and Arabidopsis, all of which are induced in response to submergence, low oxygen, and/or ethylene (*Xu* et al., 2006; *Hattori* et al., 2009; *Hinz* et al. 2010; *Licausi* et al., 2010, 2011). Our study recognized nine genes encoding group VII ERF on the soybean genome, four of which were highly induced during submergence, but markedly reduced upon de-submergence (Fig. 4a & 8b). Of these four genes, *ERFVII1*, *ERFVII5*, and *ERFVII8* also showed more than threefold induction in response to ACC (Fig. 5). Interestingly, phylogenetic analysis placed these ERFVII proteins and Arabidopsis HRE2 in the same clade with significant bootstrap values (Fig. 3b). It appears that these three genes are promising candidates for the regulation of acclimation responses and tolerance to submergence in soybean. A rapid reduction in mRNA abundance of these ERFVII genes upon de-submergence may be of importance for prompt recommencement of normal growth and development. In support of this possibility, constitutive expression of

SUB1A, one of rice ERFVII genes, significantly enhances survival of complete submergence through repression of GA-mediated underwater elongation and carbohydrate consumption, but it also negatively affects various GA-related developmental processes including seed germination, flowering, seed maturation, and grain production in rice even under non-stress conditions (*Fukao & Bailey-Serres*, 2008). Additionally, heterologous overexpression of rice SUB1A in Arabidopsis dampens GA responsiveness, resulting in the inhibition of seed germination, flowering and seed production (*Peña-Castro* et al., 2011). Arabidopsis group VII ERF proteins, HRE1, HRE2, and RAP2.12, are accumulated under oxygen deprivation, which triggers expression of core genes involved in adaptation to submergence/low oxygen (*Gibbs* et al., 2011; *Licauci* et al., 2011). However, reoxygenation promotes degradation of these proteins through the N-end rule pathway of targeted proteolysis. These results suggest that fine-tuning of ERFVII mRNA and/or protein accumulation is crucial for the adaptation to submergence and resumption of growth/development following de-submergence. Of nine *ERFVII* genes in soybean, *ERFVII2*, *ERFVII3*, *ERFVII7*, and *ERFVII9* were not clearly upregulated (< 2-fold changes) during submergence and reoxygenation (Fig. 4a). Phylogenetic analysis revealed that these ERFVII proteins are closely related with Arabidopsis RAP2.2 and RAP2.12 (Fig. 3b). These ERFVII genes could also be involved in submergence responses and tolerance in an N-end rule-dependent manner. Further genetic and molecular analyses are required to validate the functional importance of the inducible and constitutive ERFVII genes in adaptation to submergence in soybean.

Pronounced induction of *ADH* gene expression and enzymatic activity is a common response to submergence and oxygen deprivation in plants (*Andrew* et al., 1994; *Baxter-Burrell* et al., 2002; *Fukao* et al., 2003, 2006; *Mustroph* et al., 2010; *Lee* et al., 2011). It was also shown

that submergence significantly upregulates four to five *ADH* transcripts in tissue consisting of roots and hypocotyls of soybean seedlings (*Komatsu* et al., 2009; *Nanjo* et al., 2011). In addition, ADH activity showed sevenfold induction after 24 h of anoxia in roots of soybean seedlings (*Russell* et al., 1990). However, in aerial tissue of soybean seedlings, only *ADH1* was markedly induced (Fig. 4b) and the enzymatic activity of ADH showed only minor induction (~1.7-fold increase) during the course of submergence (Fig. 4c). Based on these results, it is feasible that submergence-mediated activation of *ADH* gene expression and enzymatic activity is organ-specific in soybean. Instead of ADH, several genes encoding aldehyde dehydrogenase (ALDH) were positively regulated during submergence and reoxygenation in aerial tissue of soybean seedlings. In rice seedlings, submergence enhances mRNA accumulation of *ALDH2a* and reoxygenation increases the abundance of ALDH2*a* protein and enzymatic activity of ALDH, resulting in the detoxification of acetaldehyde accumulated in response to re-aeration (*Nakazono* et al., 2000; *Tsuji* et al., 2003). Detailed biochemical analyses are required to determine the necessity and significance of fermentation and ALDH in metabolic adaptation to submergence in shoots and roots of soybean seedlings.

Switching between two distinct pathways that convert sucrose to hexose sugars may benefit the management of energy reserves during submergence and oxygen deprivation (*Fukao & Bailey-Serres*, 2004). The sucrose synthase pathway consumes one molecule of ATP to catabolize a molecule of sucrose, whereas the invertase pathway requires two molecules of ATP per molecule of sucrose. Hypoxia increases the enzymatic activity of sucrose synthase, but decreases invertase activity in roots of maize seedlings (*Zeng* et al., 1999). Additionally, sucrose synthase and invertase mRNAs are inversely regulated during submergence and oxygen deficiency in roots of maize, poplar and soybean (*Zeng* et al., 1999; *Kreuzwieser* et al., 2009;

Nanjo et al., 2011). In this study, none of the genes encoding sucrose synthase or invertase were differentially expressed during submergence (Supplementary Appendix A2). Instead, a number of genes encoding invertase inhibitors were upregulated in response to 1 d and/or 5 d of submergence. Re-evaluation of publicly available microarray datasets confirmed that submergence/low oxygen stimulate the accumulation of invertase inhibitor mRNAs in rice shoots, poplar roots and Arabidopsis seedlings (*Branco-Price* et al., 2008; *Kreuzwieser* et al., 2009; *Mustroph* et al., 2010). Based on these results, it is hypothesized that activation of invertase inhibitors may be an alternative pathway to regulate the balance between sucrose synthase and invertase activities, aiding in the economization of energy reserves under submerged and low oxygen conditions.

Comparison of submergence-responsive genes in shoots and roots allowed us to recognize conserved and organ-specific pathways which were differentially regulated in response to submergence. For example, activation of carbohydrate metabolism and fermentation pathways is prominent in tissue consisting of roots and hypocotyls (*Nanjo* et al., 2011), which is in accordance with the observations in Arabidopsis shoots and roots, rice shoots, poplar roots and cotton roots (*Kreuzwieser* et al., 2009; *Christianson* et al., 2010; *Mustroph* et al., 2010). However, in shoots of soybean seedlings, only a limited number of genes associated with starch degradation, glycolysis, and fermentation were induced in response to 1 d of submergence (Fig. S1). Furthermore, most of the genes involved in these pathways were significantly downregulated by prolonged submergence (Fig. 7) even though marked degradation of starch and soluble carbohydrates were observed over the course of submergence (Fig. 2b, c). These results suggest that the constitutive basal levels of transcripts associated with sugar catabolism and fermentation are sufficient for breakdown of carbohydrate reserves under submergence in

aerial tissue of soybean seedlings. On the other hand, pathways involved in cell wall synthesis/ degradation and secondary metabolisms were commonly downregulated in shoots and roots of soybean seedlings (Fig 7) (*Nanjo* et al., 2011). Restriction of these energy-consuming pathways may benefit the avoidance of carbohydrate starvation and an energy crisis under submergence. Organ-specific regulation of gene expression in shoots and roots reflects that distinct developmental and metabolic adjustments are demanded in the two functionally distinct organs under submerged conditions although the conserved responses are regulated by a core group of genes in shoots and roots.

In conclusion, we characterized the fundamental physiological and transcriptomic responses during the course of submergence and reoxygenation in aerial tissue of soybean seedlings. Comparative analysis of differentially regulated genes in shoots and roots of soybean and other plants highlights conserved, organ-specific and species-specific regulation of gene expression which enhances adaptability to submergence. Cotyledons of young soybean seedlings contain a large amount of nutrients including proteins, lipids, and carbohydrates, which may cause unique transcriptomic and metabolic responses to submergence. Time-course analyses of metabolic flow and integration of transcriptome data with metabolomic data will advance our understanding of the regulatory mechanisms underlying a variety of acclimation responses to submergence and reoxygenation in soybean.

2.6 REFERENCES

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CHAPTER 3

Crosstalk of transcriptomic and metabolic responses to submergence and drought in leaves and roots of soybean plant

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ABSTRACT

The number of flooding and drought events is on the rise throughout the world due to global climate change, which has already resulted in agricultural and economic damage. These precipitation extremes can occur sequentially within a single crop growing season or independently in the same fields in different years. Therefore, understanding the regulatory mechanisms underlying tolerance to both of these stresses in major crops such as soybean can largely contribute to the development of climate-smart crop production systems. In this study, we characterized the acclimation responses to submergence, drought and recovery from these stresses in soybean leaves and roots at the early vegetative stage. Plants submerged for up to 3 d or withheld from water for up to 6 d were able to recover completely from the stresses. During these stress and recovery periods, many of the amino acids were dynamically regulated displaying their importance in such stress conditions. Whole transcriptome analysis identified 15,074 gene transcripts that responded to these stresses among which 73 were AP2/ERF transcription factors and 199 were MAP kinase family genes. K-mean clustering and GO

enrichment analysis of the identified differentially expressed genes displayed several stress-specific, tissue-specific and overlapping pathways associated with metabolism, development, response to stress, signal transduction, cellular homeostasis and redox regulation. Our study demonstrated the dynamic metabolic and transcriptomic reconfiguration in soybean leaves and roots needed to acclimate during submergence and drought stresses, but a significant number of genes and pathways are commonly regulated under both of these water-related stresses.

Keywords: submergence; drought; mRNA sequencing, amino acids, *Glycine max*, Group VII Ethylene Responsive Factors, Mitogen-Activated Protein Kinases

3.1 INTRODUCTION

Global climate change has significantly altered the frequency and magnitude of precipitation, increasing the number of flooding and drought events throughout the world (*Fukao & Xiong*, 2013; *Syed* et al., 2015). In the US alone, the overall economic damage from flooding and drought in the last 3 decades is estimated at around $86 and $199 billion, respectively (*Suzuki* et al., 2014; *Smith & Matthews*, 2015). In agricultural environments, these precipitation extremes can occur subsequently during a single crop growing season or in different years at the same fields (*Michelbart* et al., 2015). This can significantly impact overall crop productivity and global food security. Hence, improvement of tolerance to both of these stresses in major crops can largely contribute to the stabilization of food production under changing climates.

Soybean [*Glycine max* (L.) Merr.] is an economically important crop and a major source of protein and oil used for food, feed and industrial applications (*Clemente & Cahoon*, 2009). However, soybean is sensitive to both flooding and drought (*Korte* et al., 1983; *Russell* et al., 1990), whose growth, development and seed production can be negatively affected by even transient flooding and moderate drought stresses. Flooding stress in soybean can largely decrease nitrogen fixation and inhibit nutrient uptake (*Sung*, 1993; *Sallam & Scott*, 1987), reduce mRNA translation of defense- and metabolic-related genes leading to impaired growth (*Khatoon* et al., 2012). It can suppress both vegetative and reproductive development which results in reduced yield (*Girithi* et al., 2006). Similarly, drought stress in soybean can reduce net photosynthesis, cell water potential, the rate of mitochondrial ATP synthesis (*Liu* et al., 2004; *Ribas-Carbo* et al., 2005), and overall seed yield by decreased vegetative growth and seed number (*Frederick* et al., 2001).

Detailed studies on acclimation and adaptation responses to flooding and drought have been conducted in *Arabidopsis*, rice and other crop species (*Bailey-Serres & Voesenek*, 2008, 2010; *Bailey-Serres* et al., 2012a; *Fukao & Xiong*, 2013; *Osakabe* et al., 2014). These analyses recognized common regulatory networks during flooding events. These include adaptive energy management and activation of fermentation pathway for ATP generation, the formation of thinner and larger leaves, development of adventitious roots and aerenchyma, metabolic and transcriptomic reconfiguration to adjust defense responses to altered cytosolic pH and reactive oxygen species (ROS) production. Similarly, tolerance mechanisms under drought stress include endogenous ABA biosynthesis. This event leads to altered turgor pressure of guard cells, stomatal closure, control of ion transport channels and regulation of genes encoding osmoprotectants, late embryogenesis abundant (LEA) and chaperone and ROS scavengers.

Comparison of transcriptomic studies has found several conserved responses to flooding or drought across species. For instance, *Mustroph* et al. (2010) performed a cross-kingdom (plantae, animalia, fungi and bacteria) comparison of transcriptomic reconfiguration in 21 organisms during low-oxygen stress. Many species in the study displayed an increase in mRNAs encoding enzymes associated with ROS networks and a decrease in mRNAs encoding proteins associated with ribosome biogenesis and cell wall synthesis in addition to core metabolic configuration to enhance substrate-level ATP production and NAD^+ regeneration. Likewise, another study by *Shaar-Moshe* et al. (2015) compared 17 microarray experiments of *Arabidopsis*, rice, wheat and barley (*Hordeum vulgare*) under drought and discovered 225 differentially expressed and overlapping genes among studies and species which were functionally categorized into groups involved in regulatory functions (protein degradation and transcription), metabolic processes (amino acid and carbohydrate metabolism) and response to

stimuli. Despite a large number of transcriptomic studies on flooding and drought tolerance, genome-scale gene expression responses to the two water-related stresses and recovery from these stresses has not been subjected to comparative analysis in plants including soybean.

Genetic and molecular studies have recognized several transcription factors that regulate both flooding and drought tolerance (*Tamang & Fukao*, 2015). In rice, SUB1A, a group VII member of ERF transcription factors, enhances tolerance to and recovery from complete submergence by limiting energy consumption, amino acid metabolism and elongation growth through transcriptional and hormonal regulation (*Fukao* et al., 2006; *Fukao & Bailey-Serres*, 2008; *Jung* et al., 2010; *Mustroph* et al., 2010, *Barding* et al., 2012, 2013; *Alpuerto* et al., 2016). In addition, it also enhances recovery from dehydration through enhanced responsiveness to ABA, elevated accumulation of mRNAs-associated acclimation to dehydration, and reduction of leaf water loss and lipid peroxidation (*Fukao* et al., 2011). Recently, it was reported that another ERFVII gene, EREBP1, is important for adaptation during both submergence and drought stress in rice (*Jisha* et al., 2015). The study revealed that genotypes with overexpressed *EREBP1* have enhanced recovery from submergence and drought with restricted underwater elongation, reduced ROS accumulation, and elevated mRNA accumulation of ABA biosynthetic genes and ABA content in leaves. *Arabidopsis* genome encodes five ERFVII genes; two HYPOXIA RESPONSIVE ERF (HRE) genes, HRE1 and HRE2, and three RELATED TO AP2 (RAP2) genes, RAP2.2, RAP2.3, and RAP2.12 (*Nakano* et al., 2006). It has been reported that all of these genes are positive regulators for plant survival under submergence (*Hinz* et al., 2010; *Licausi* et al., 2010, 2011; *Park* et al., 2011; *Seok* et al., 2014; *Gibbs* et al., 2015). Interestingly, three of ERF-VII genes, RAP2.2, RAP2.3, and RAP2.12 also confer tolerance to osmotic (dehydration) and oxidative stresses when overexpressed (*Papdi* et al., 2015). In sunflower

(*Helianthus annuus*), a WRKY transcription factor, HaWRKY76, plays a key role in conferring tolerance to submergence and drought (*Raineri* et al., 2015). When overexpressed in *Arabidopsis*, HaWRKY76 contributed to the conservation of carbohydrate reserves during submergence and maintenance of higher water content in leaves and production of more seeds under drought compared to the wild type. These studies have provided genetic evidence that there are overlapping regulatory pathways and processes governing acclimation responses to both flooding and drought in plants.

In the current study, we performed time-course, comparative analysis of physiological, metabolic and transcriptomic response to submergence, drought, and recovery from these stresses in leaves and roots of soybean at an early vegetative stage. RNASeq analysis and individual metabolite assays identified tissue-specific, stress-specific and overlapping responses to water extremes and recovery, providing beneficial information to elucidate the core regulatory processes underlying both flooding and drought tolerance in soybean.

3.2 MATERIALS AND METHODS

3.2.1 Plant material and growth conditions

Seeds of soybean [*Glycine max* (L.) Merr. cv. William 82] were sterilized using 3% (v/v) sodium hypochlorite and 0.1% (v/v) Tween-20 for 10 min followed by rinsing with deionized water. For germination, seeds were placed on wet paper towels under continuous light at 25^{o} C (50 μmol m^{-2} s^{-1}) for 4 d. Three germinated seedlings per pot were transplanted into soil-containing pots (14×14×16 cm) and grown until V1 stage under natural light conditions in a greenhouse.

3.2.2 Submergence and drought stress treatment

For submergence treatment, the V1 stage plants were completely submerged in a plastic tank (65×65×95 cm) filled with water (water depth: 90 cm) at 25^{o} C and incubated for up to 6 d followed by de-submergence with recovery period for up to 7 d. To observe growth response to submergence, plant height and dry biomass (root and shoot dried at 65 ^{o}C for 3 d) of control and submerged plants were measured 0, 3 and 5 d after submergence. For plant viability evaluation, plants submerged for up to 6 d were desubmerged and recovered for 7 d under regular growth conditions. The plants able to generate new leaves during recovery periods were scored as viable.

For drought treatment, plants at the V1 stage were exposed to dehydration by withholding water for up to 6 d followed by 24 h of recovery while saturating the control pots with water every day. Both visible leaf desiccation symptoms and soil moisture content (SMC) measured by soil moisture meter (HH2 Moisture Meter, Delta-T Devices, Cambridge, England) were monitored each day at midday to determine moderate and severe drought conditions. The moderate and severe drought symptoms were observed after 5 d and 6 d of withholding water with SMC of 4.5% and 2.7%, respectively.

The submergence tissue samples (roots and leaves) were collected at 0, 1, 2, 3 and 3 d + 24 h of desubmergence (total of 10 samples). Similarly, the drought tissue samples (roots and leaves) were collected at 5 d (moderate), 6 d (severe) and 6 d + 24 h (recovery) of re-watering and 7 d (control) (total of 8 samples). All tissues (18 samples) were harvested at midday, instantly flash-frozen in liquid nitrogen and stored in -80 ^{o}C until further use. Entire submergence, drought and recovery treatments were conducted in three independent biological replications (a total of 54 samples).

3.2.3 Relative water content (RWC) analysis

Leaf RWC was measured from the first trifoliate leaves of plants either submerged for 0, 3 and 3 d + 24 h recovery or withheld of water (drought treatment) for 5 d, 6 d and 6 d + 24 h recovery and 7 d control. Individual leaves were detached, and the fresh weight (FW) was immediately measured. Following the measurement, the leaves were soaked in water for 24 h, and the rehydrated leaves were re-weighed to record turgid weight (TW). Finally, the leaves were dried at 65 ^{o}C for 3 d, and the dry weight (DW) was recorded. Leaf RWC was computed following the equation: RWC (%) = (FW-DW)/ (TW-DW) × 100.

3.2.4 Lipid peroxidation measurement

For the quantification of malondialdehyde (MDA), an end-product of lipid peroxidation, the thiobarbituric acid assay was conducted (*Hodges* et al. 1999). MDA was extracted from 50 mg of homogenized tissues in 1 mL of 80% (v/v) ethanol on ice. After centrifugation at 4 ^{o}C for 20 min at 21,000 g, the supernatant (0.5 mL) was mixed with 0.5 mL of 20% (w/v) trichloroacetic acid containing 0.65% (w/v) thiobarbituric acid. The mixture was then incubated at 95 ^{o}C for 30 min

and cooled immediately on ice. Following centrifugation at 4 ^{o}C for 10 min at 10,000 g, absorbance at 532 nm was measured, and non-specific absorption at 600 nm was determined. Finally, MDA concentration was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}.

3.2.5 Individual amino acids assay

Fifty milligrams of homogenized tissue were used for individual amino acid quantification following the protocol described by *Field* et al. (2004). The tissue was mixed with 1 mL of 70% (v/v) methanol and incubated at 45 ^{o}C for 10 min followed by centrifugation for the collection of the supernatant. A 30 nmol of α-aminobutyric acid (AABA) was added as an internal control. This extraction process was repeated twice, and the supernatants from three extracts were pooled. The collected supernatant was filtered through 0.2 µm PTFE membrane, dried under vacuum and finally dissolved in 600 µL of 50mM HCl. The reconstituted sample (10 µL) was then mixed with 70 µL of AccQ Fluor Borate Buffer and 20 µL of AccQ Fluor reagent (Waters Corporation, Milford, MA, USA). Following incubation of the mixture at 55 ^{o}C for 10 min, the derivatized amino acids were analyzed using an Agilent 1260 Infinity Quaternary LC system (Agilent Technologies, Santa Clara, CA, USA) coupled with a fluorescence detector. Each amino acid was separated through an X-Terra MS C18 column (Waters Corporation) at 50 ^{o}C using a 40-min gradient of sodium acetate buffer, 140 mM sodium acetate (pH 5.05), 17 mM trimethylamine, 0.1% (w/v) EDTA, acetonitrile and water at a flow rate of 0.35 mL min^{-1}. Derivatized amino acids were detected by fluorescence with excitation at 250 nm and emission at 395 nm. Individual amino acids were identified and quantified using a calibration curve generated by the injection of standards of known concentrations.

3.2.6 RNA extraction and quantitative RT-PCR

Total RNA was extracted from 100 mg tissue using miRNeasy Mini kit (Qiagen, Hilden, Germany). Following manufacturer's protocol, genomic DNA was removed by the on-column digestion method. The cDNA was synthesized from 2 µg of extracted total RNA as described by *Fukao* et al. (2006). The qRT-PCR was conducted in a 15 µl reaction using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in the CFX Connect real-time PCR detection system (Bio-Rad). Transcripts of nine soybean ERF VIIs reported by *Tamang* et al. (2014) were amplified for expression profile and RNASeq data validation. *Ribosomal protein L30* (Glyma.13g318800) was used as normalization controls (*Le* et al., 2012). Amplification specificity was validated by melt-curve analysis at the end of each PCR experiment. The PCR efficiency (90-105%) was verified earlier in *Tamang* et al. (2014) by the method of *Schmittgen & Livak* (2008). Relative transcript abundance was determined using the comparative cycle threshold method (*Livak & Schmittgen*, 2001). Sequences and annealing temperature of primer pairs are given in Supplementary Appendix A3.

3.2.7 Stranded RNA-Seq library construction and RNA sequencing

Three independent biological replicates for each of 18 samples (a total of 54 samples) from submergence and drought experiments were used for mRNA sequencing. Total RNAs were extracted as described above. Library preparation and sequencing were conducted at Biocomplexity Institute, Virginia Tech. The RNA concentrations and RNA integrity numbers (RINs) were determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNAs with RIN $\geq$ 8.0 were used for library construction with Apollo 324 Next Generation Sample Preparation System (Wafergen, Fremont, CA, USA). The polyA RNAs were

isolated from total RNA (500 ng) using PrepX PolyA mRNA Isolation Kit (Wafergen). The purified polyA RNAs were converted into libraries of template molecules using PrepX RNA-Seq for Illumina Library Kit (Wafergen) for subsequent cluster generation and sequencing. Briefly, poly-A mRNAs were fragmented into smaller pieces (~140 nt) using the heat and divalent cation method. The cleaved RNA fragments were ligated with 3' and 5' adapters and then converted to first strand cDNA using reverse transcriptase, followed by second strand synthesis. The products were then purified and enriched with 13 cycles of PCR to create the final cDNA library. The libraries generated were validated using Agilent 2100 Bioanalyzer and quantitated using Quant-iT dsDNA HS Kit (Invitrogen, Carlsbad, CA, USA) and quantitative PCR. Six individually indexed cDNA libraries were pooled and sequenced on HiSeq 2500 (Illumina, San Diego, CA, USA) to get a minimum of 60 million paired-end reads. Libraries were clustered onto a flow cell using TruSeq PE Cluster Kit v3-cBOT-HS (Illumina) and sequenced (100 bp pair-end) using TruSeq SBS Kit v3-HS (Illumina). A total of 1.6 billion (2×100) pair-end reads were generated from 54 libraries. The detail information on sequencing result is presented in Supplementary Appendix B2.

3.2.8 RNA-Seq data and differential gene expression analysis

Statistics on sequencing data quality were obtained using FastQC software (version 0.11.4 http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The sequence reads were aligned to 'Williams 82' Soybean reference genome (assembly Glyma.Wm82.a.2.0, annotation v1.1) using STAR alignment program (*Dobin* et al. 2013). The mapped reads were used to estimate the gene expression value (fragments per kilobase of transcripts per million mapped reads- FPKM) for annotated genes using Subreads (*Liao* et al. 2013). Genes showing differential expression were

identified using the DESeq2, v 1.10.1 statistical tool (*Love* et al. 2014). Fold-changes for the submergence experiment were computed by dividing mean normalized gene expression values at 1, 2 and 3d by the value at 0 d and dividing 3d + 24h values by either 0d control or 3d submergence. Similarly, for drought experiment, fold-change values were calculated by dividing mean normalized gene expression values at 5 d and 6d by the value at 0 d and dividing 6d + 24h recovery by either 0d or 6d of withholding water. Significantly upregulated or downregulated genes were identified by Benjamini-Hochberg statistical method (*Benjamini & Hochberg*, 1995) at 10^{-4} false discovery rate, $\log_2$fold change of at least 1.5 and counts per million (CPM) value of at least 4.

3.2.9 Clustering, functional and pathway enrichment analysis

K-mean clustering of the 15,074 DEGs into 40 clusters (C1 through C40) was performed based on their changing patterns of FPKM across 18 different experimental conditions. Clusters with similar expression pattern were pooled together for GO enrichment analysis (Supplementary Appendix B3). Singular enrichment analysis (SEA) for GO annotation analysis was performed using AgriGo version 1.2 (*Du* et al., 2010) and overrepresented GO_Biological_Process were identified using Hypergeometric test with a significance threshold of 0.05 after Benjamini-Yekutieli correction (FDR ≤ 0.05) (*Benjamini & Yekutieli*, 2001) with "Glycine max Wm82.a2.v1" as the reference set. The enriched GO terms were further summarized in REVIGO online tool (*Supek* et al., 2011) with small similarity (0.5).

3.3 RESULTS

3.3.1 Soybean responses to submergence and drought at the early vegetative stage

To determine an optimal duration for the evaluation of tolerance to submergence and drought stress, V1 stage soybean plants were exposed to either complete inundation for up to 3 d or gradual water stress for up to 6 d under greenhouse conditions (Fig.1a, 2a). Complete submergence for 3 d did not affect whole plant viability, while 84% of the plants died within 6 d of submergence (Fig. 1b). Three days of submergence, as a non-lethal stress level, was also evidenced from the relative water content in V1 leaves after 3 d submergence followed by 24 h recovery during which the water content returned to the normal level (Fig. 1c). Growth responses to submergence are species-specific. For example, submergence stimulates elongation of aerial tissue in most rice accessions and *Rumex palustris* (*Jackson*, 2008; *Bailey-Serres* et al., 2012b), whereas growth of *Arabidopsis*, lowland tolerant rice, *Rumex acetosa* and *Rorippa sylvestris* is restrained when immersed in water (*Bailey-Serres* et al., 2012b). To characterize soybean responses to submergence, shoot elongation and biomass growth were monitored over 5 d of submergence. Plant height increased similarly under control and submerged conditions for 3 d, but the growth was significantly restricted after 5 d of submergence (Fig. 1d). Shoot and root biomass gradually increased under non-stress conditions for 5 d, but evident biomass growth was not observed under submerged conditions (Fig. 1e, f). It seems that submergence inhibits elongation growth and biomass accumulation in soybean as observed in *Arabidopsis* (*Vashisht* et al., 2011).

We also determined the recoverable period of drought stress in our experimental system. Soil water content in non-stressed pots was approximately 44.2%. Withholding of water for 5 d and 6 d decreased soil water content to 4.6% and 2.7%, respectively (Fig. 2b). Relative water

content of V1 leaves was 97.8% in non-stressed pots. After 5 d and 6 d of water withholding, leaf relative water content declined to 80.5 % and 60.7%, respectively (Fig. 2b). Although all leaves were shrunken after 6 d of water stress, the plants were apparently recovered within 24 h after re-watering (Fig. 2a). In addition, relative water content in V1 leaves was restored to the level in non-stressed plants during the recovery period (Fig. 2b). These results indicate that 6 d of water withholding was still sub-lethal to the plants. MDA is an indicator of lipid peroxidation (*Shulaev & Oliver*, 2006) which commonly occurs under drought stress due to increased accumulation of ROS (*de Carvalho*, 2008; *Fukao* et al., 2011). It appears that soybean plants experienced only moderate stress after 5 d of water deficit as demonstrated by insignificant induction of MDA in leaves and roots (Fig. 2c). On the other hand, the level of MDA was obviously induced by 6 d of water stress in roots, which was quickly reduced during 24 h of recovery (Fig. 2c). Significant induction of MDA was not observed in leaves, suggesting roots are exposed to more severe oxidative stress than leaves.

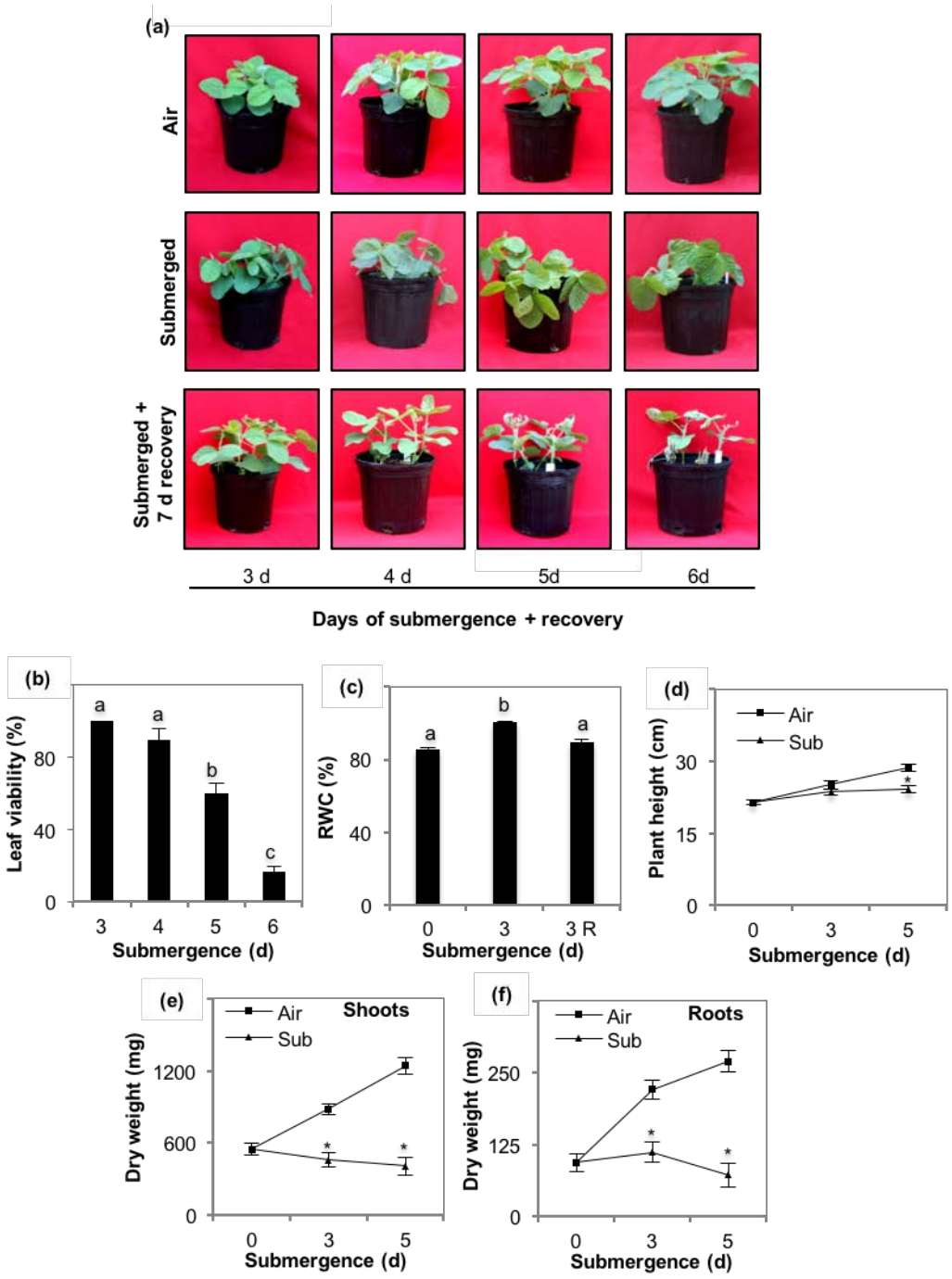


Figure 1. Growth responses and survival under submergence in soybean at the V1 stage. (a) Plants at the V1 stage were grown under air (top) or submerged conditions (middle) for up to 6 d. Only submerged plants were recovered under aerobic conditions for 7 d (bottom). (b) Viability of plants after up to 6 days + 7 d of recovery. (c) Relative water content of V1 leaves. (d) Plant height of soybean under air and submerged conditions. (e), (f) Dry weight of shoots and roots under air and submerged conditions. In (b), (c), (d), (e), (f) data represent mean ± SE (n = 15 × 3 for viability and relative water content, n = 25 for dry weight, n = 10 × 3 for plant height). Bars not sharing the same letter are significantly different (P < 0.05). * represents significance difference (P < 0.05).

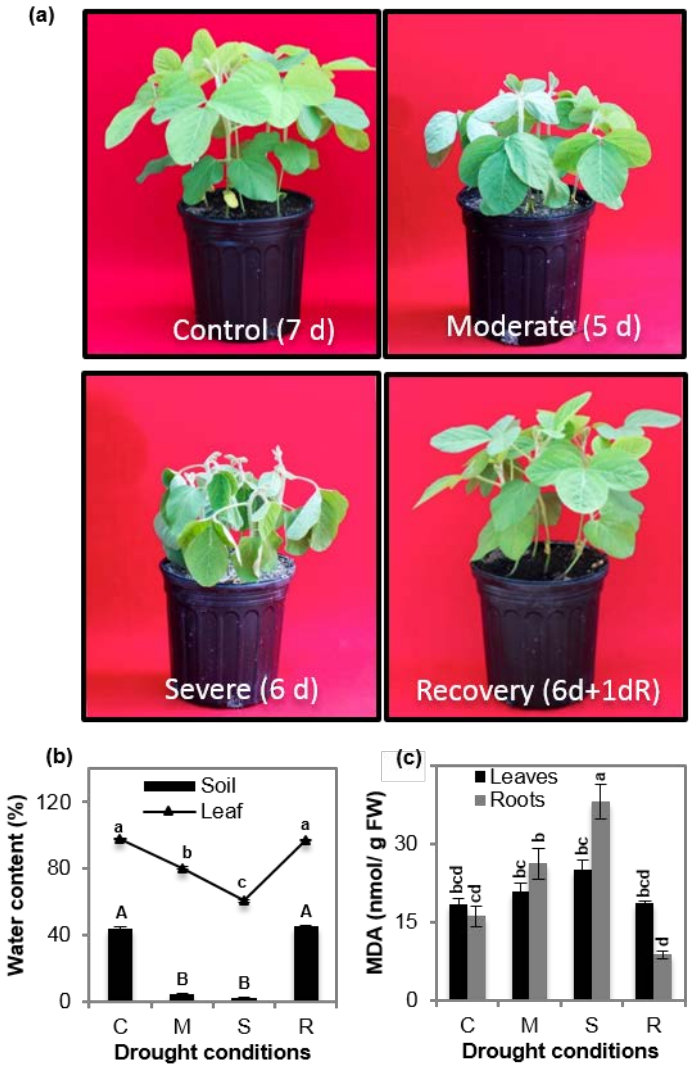


Figure 2. Drought responses of soybean at the V1 stage. (a) Soybean plants exposed to soil dehydration for up to 6 d and recovered for 1 d. (b) Relative water content of V1 leaves and their corresponding soil moisture content during drought treatment. (c) MDA levels in VI leaves and roots during drought treatment. C; control, M; moderate drought, S; severe drought, R; 1 d of recovery from 6 d of drought. Data represent mean ± SE (n = 15 for relative water content of V1 leaves, n = 5 for soil moisture content, n = 3 for MDA). Bars not sharing the same letter are significantly different ($P < 0.05$).

3.3.2 Alterations in the levels of amino acids in leaves and roots of soybean under submergence, drought, and recovery from these stresses

Plants have been known to accumulate specific metabolites under stressful conditions, amino acids in particular (*Hayat* et al., 2012). We monitored the levels of individual amino acids in leaves and roots of soybean submerged for up to 3 d, withheld from water for up to 6 d and recovered from these stresses for 1 d (Fig 3; Supplementary Appendix B1a, b). Duration of these stress treatments was at sub-lethal levels (Figure 1, 2). The levels of 5 amino acids (threonine, glutamate, alanine, tyrosine and GABA) were not significantly altered by submergence in the leaves. On the contrary, aspartate was ~2-4-fold downregulated during the entire submergence period in leaves and returned to the level at day 0 during 24 h recovery. Interestingly, glycine was upregulated specifically during recovery from submergence in leaves (~6-fold induction). Arginine (~46-fold during 3 d of submergence) had the most dramatic induction in leaves under submergence, which quickly declined to the levels below the detection limit during recovery. Similarly, valine had ~5-fold upregulation at day 3 of submergence in leaves but reached to the level at day 0 during recovery. In roots, 10 (arginine, isoleucine, leucine, phenylalanine, valine, glycine, glutamate, alanine, tyrosine and GABA) out of 15 amino acids were upregulated during submergence. Among them, alanine (~50-fold), tyrosine (~20-fold) and GABA (~15-fold) had the greatest induction. Interestingly, the levels of these 10 amino acids were still significantly higher (at least 2-fold) even after 1 d of recovery, suggesting their slower turnover rates. Amino acids which were induced during submergence but whose levels recovered to the values of control plants during 1 d recovery were lysine, tryptophan, and proline. Compared to 3 d submergence, aspartate concentration increased in leaves and roots during 24 h recovery period while the levels of threonine, glycine, phenylalanine and alanine were upregulated only in leaves.

Under drought stress, the abundance of arginine and phenylalanine in roots and glycine in both tissues was not altered (Fig. 3; Supplementary Appendix B1b). Lysine was the only amino acid whose level declined (~1.5-fold) during recovery in roots from drought. The levels of arginine, lysine, tyrosine and GABA were upregulated in leaves under both moderate (~2-fold) and severe drought (~3 to 7-fold) stress, but their levels quickly receded to the levels of control during 24 h recovery. In addition, tryptophan, isoleucine, leucine, phenylalanine, proline, and valine were constitutively induced in varying degrees during drought stress (~9 to 18-fold) and 24 h recovery (~4 to 18-fold). Compared to 6 d of severe drought, most of the amino acids were downregulated during 24 h of recovery in leaves and roots, but only alanine was upregulated in roots.

Of 15 amino acids identified and quantified, tryptophan, isoleucine, leucine, proline, and valine were induced in response to both submergence and drought in both leaves and roots, suggesting that common metabolic pathways involved in the biosynthesis and degradation of these amino acids are regulated under the antithetical water stresses.

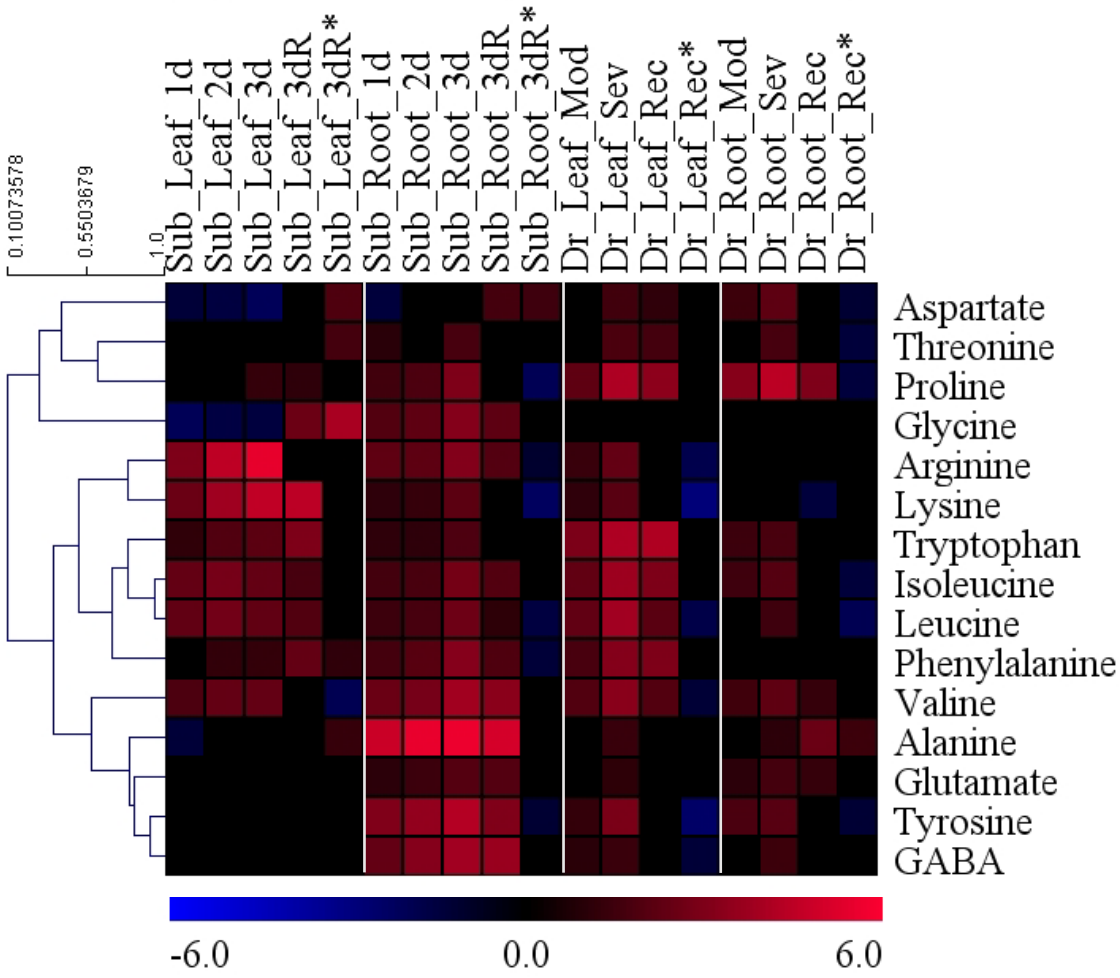


Figure 3. Heat map showing log_2 fold change in the abundance of 15 amino acids as compared to the levels in control plants in response to submergence, drought and recovery from these stresses. 3dR and 3dR* indicate log_2 fold changes calculated by dividing 3d submergence + 24 h recovery values by 0 d control and 3 d submergence, respectively. Rec and Rec* were also computed by the same method.

3.3.3 Transcriptomic responses to submergence and drought in soybean leaves and roots

Plants display many transcriptional changes to adjust their trade-offs between growth and defense response and acclimation to external stimuli (*Cramer* et al., 2011). To characterize transcriptional reprogramming during the course of submergence and drought stress and during the recovery from these stresses, we conducted genome-wide transcriptome analysis in leaves and roots of V1 stage plants. We constructed 54 libraries (5 time points x 2 tissue types x 3 biological replications for submergence; 4 time points x 2 tissue types x 3 biological replicates for drought) for mRNA sequencing and generated ~1.6 billion sequencing reads, among which 73% (~1.15 billion) were uniquely mapped to the annotated soybean William 82 genome assembly 2.0 (Supplementary Appendix B2). For the validation of RNASeq data, expression levels of nine soybean ERFVII genes, previously reported by *Tamang* et al., (2014) (Figure 9), were subjected to qPCR-assay. Correlation coefficient between the expression data generated from two platforms was computed (Fig. 4a). Pearson and Spearman coefficients were 0.97 and 0.92, respectively, suggesting a strong agreement across these platforms. In a principal component analysis (PCA) of gene expression across all experimental conditions, PCA1 and PCA2 explained 81% and 10% of the variance observed in our samples, respectively (Fig. 4b). Libraries generated from two different tissues (leaves and roots) were distinctly separated along PC1, whereas those generated from two stresses were differentiated along PC2, suggesting that a difference in tissue type is a major determinant of the variation in our data.

Pairwise comparisons of stress treatments versus non-stress controls identified 15,074 differentially regulated genes (DEGs) with significant adjusted *P*-values ($P \leq 10^{-4}$) and at least 1.5 $\log_2$ fold alteration in expression of transcripts level (CPM $\geq$4) in at least one of the 18 experimental conditions (5 time points x 2 tissue types for submergence; 4 time points x 2 tissue

types for drought). The effect of submergence on the number of DEGs was more dramatic as compared to drought (Fig. 5a). During submergence, the number of upregulated genes (1,450 unique genes) were less than that of downregulated genes (3,870 unique genes) in leaves across all time points. In roots, however, a greater number of upregulated genes (4,924 unique genes) were detected as compared to downregulated genes (2,152 unique genes) at all time-points. Under water deficit, a smaller number of genes were downregulated in both leaves and roots. Recovery from submergence and drought stresses drastically reduced the number of DEGs, but hundreds of genes were still significantly upregulated or downregulated relative to control levels (3dR and Rec in Fig. 5a).

To investigate stress-specific and overlapping DEGs, Venn diagrams were constructed for genes upregulated or downregulated under submergence and drought (Fig. 5b). Drought and drought recovery upregulated 2,293 genes in leaves, roots or both, 706 genes (30.7%) of which were also induced in response to submergence and submergence recovery. Similarly, of 3,759 downregulated genes under drought and drought recovery, 1,723 genes (49.5%) were also downregulated by submergence and submergence recovery. These results demonstrate that a significant number of genes and processes associated with drought tolerance and recovery are also involved in submergence tolerance and recovery.

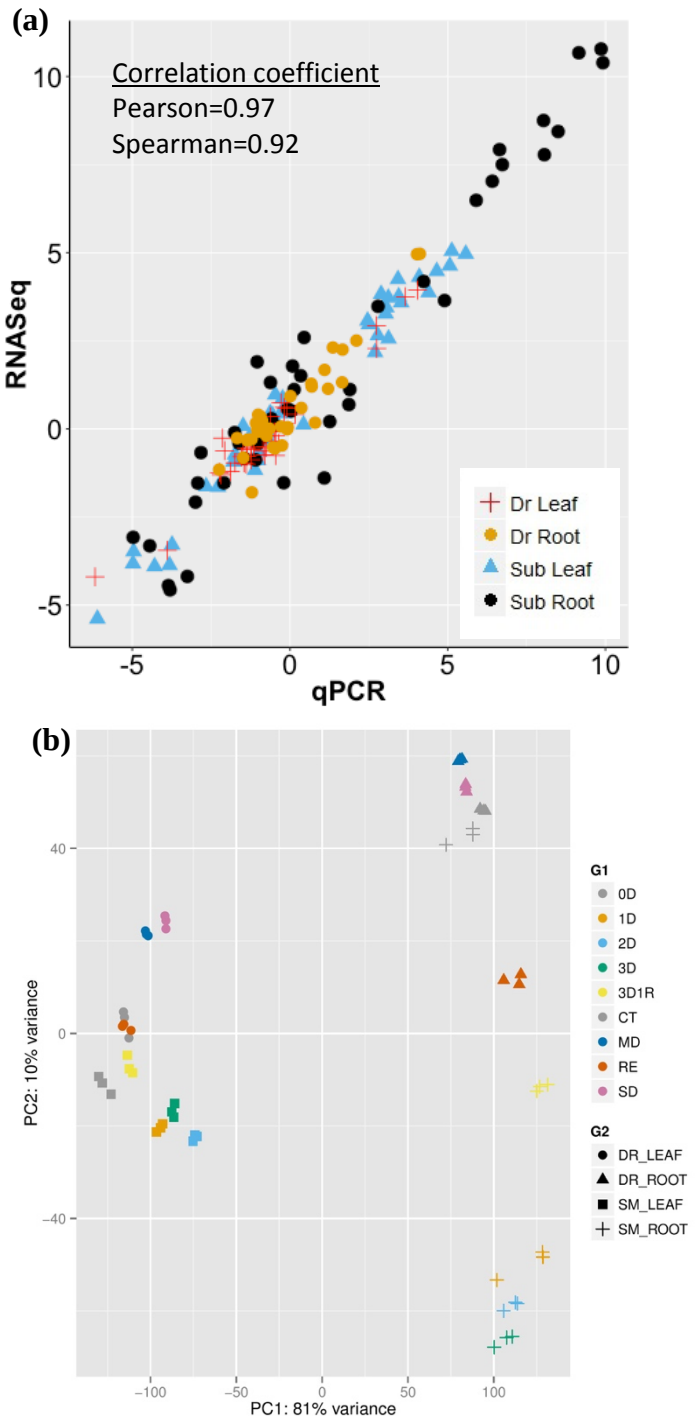


Figure 4. Validation of mRNA-Seq dataset. **(**a) Comparison of log_2 fold change estimates between qPCR and RNASeq platforms across all experimental conditions. (b) Principle component analysis plot explaining the variance across stress and tissue conditions.

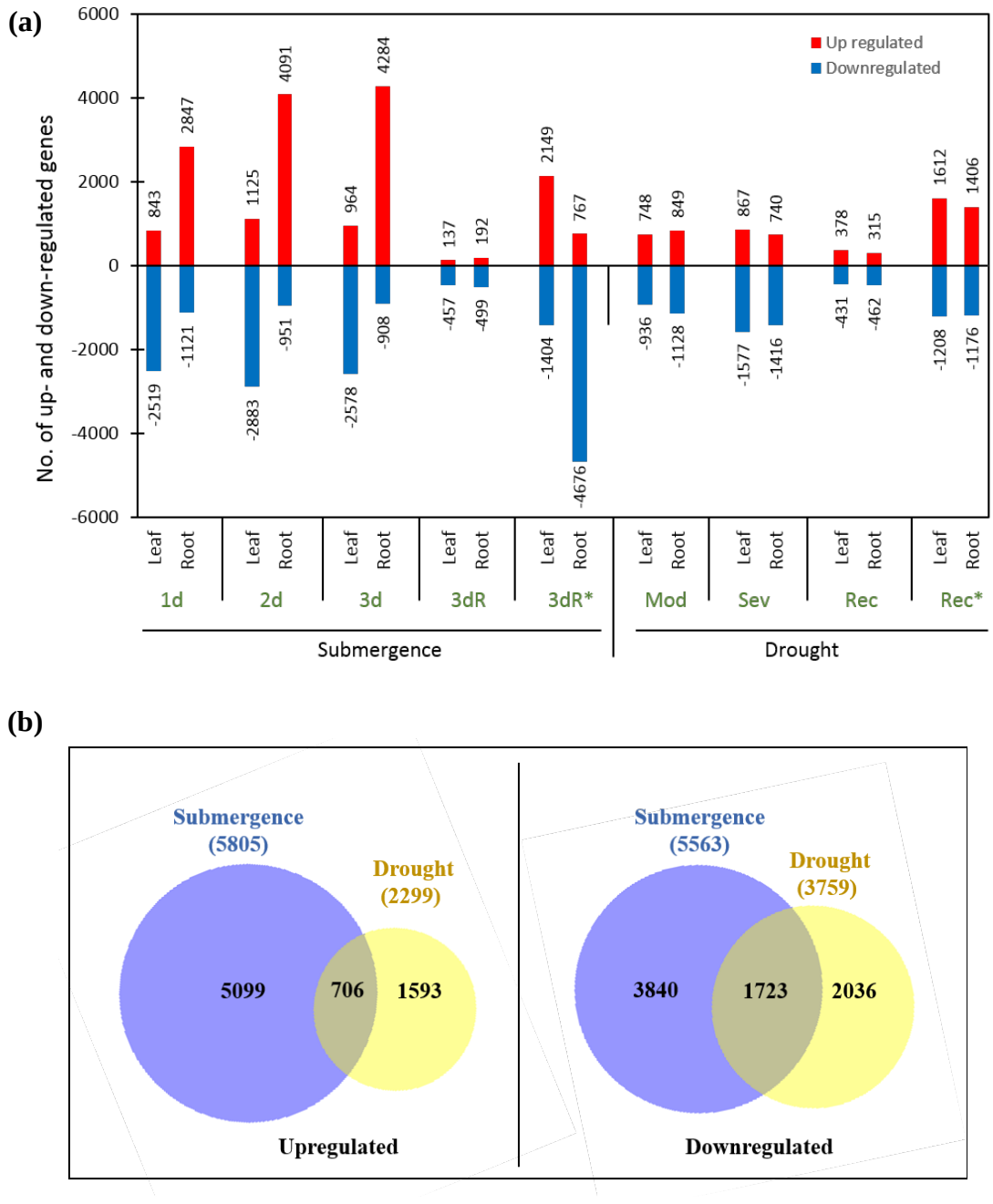


Figure 5. Transcriptomics response of soybean leaves and roots during submergence, drought and recovery from these stresses**.** (a) The number of upregulated or downregulated genes at each time point. 3dR and 3dR* indicate $\log_2$ fold changes calculated by dividing 3d submergence + 24 h recovery values by 0 d control and 3 d submergence, respectively. Rec and Rec* were also computed by the same method. (b) Venn-diagrams showing the number of overlapped genes upregulated or downregulated.

3.3.4 Clustering and functional enrichment analyses of DEGs in leaves and roots of soybean under submergence and drought

RNASeq analysis identified 15,074 DEGs across 18 experimental conditions. In order to categorize these DEGs into groups based on their differential expression pattern in both stresses, tissues, and time-point, k-means clustering analysis was performed (Fig. 6). Of 40 clusters, 27 clusters with similar expression patterns were pooled together into 5 groups and 13 subgroups for functional enrichment using Gene Ontology (Table 1). Pooling was conducted for clusters containing 1) genes regulated commonly under both stresses in both tissues (Group A), 2) genes regulated simultaneously under both stresses in leaves or roots (Group B), 3) genes regulated mutually in both tissues under submergence or drought (Group C), 4) genes regulated specifically in each tissue and stress (Group D), and 5) genes regulated commonly during recovery from both stresses in both tissues (Group E).

To estimate the functional importance of the classified DEGs during stress and recovery, we performed Singular Enrichment Analysis (SEA) for Gene Ontology (GO) using the web-based tool AgriGO. The enriched GO terms were further summarized using REVIGO. The numbers of genes and enriched GO terms in each pooled cluster are listed in Table 1. Of the 13 sub-groups of gene expression patterns, of particular interest are those which consists of genes upregulated or downregulated under both submergence and drought in both leaves and roots (Groups A1 and A2). GO enrichment analysis revealed that pathways involved in mRNA processing, mRNA metabolism, and translational initiation are upregulated under both stresses in both tissues (Table 2A; Supplementary Appendix B3A, B4). In contrast, GO terms representing energy demanding processes such as carbohydrate biosynthetic processes, carbohydrate metabolic process, and GTP metabolic process were downregulated by both stresses in both tissues (Table 2B; Supplementary

Appendix B3B). During recovery from both stresses in both tissues (Group E), biochemical pathways associated with rRNA and non-coding RNA (ncRNA) metabolism and ribosome biogenesis were enriched (Table 2C; Supplementary Appendix B3C), which are necessary components to initiate processes for cellular repair and regrowth. The greatest number of GO terms was detected in a subgroup that contains genes downregulated by both stresses in leaves (Group B2). In this group, pathways involved in metabolism and biosynthesis of amino acids, proteins, lipids, nucleic acids, pigments, secondary metabolites and other small molecules were downregulated along with processes associated with photosynthesis and redox homeostasis (Table 2D). In roots, however, less GO terms related to metabolism and biosynthesis were detected as downregulated pathways. Unique GO terms observed in roots include DNA replication, protein phosphorylation, microtubule-based movement, and responses to biotic stress and chemicals (Table 2E). Stress-specific and tissue/stress-specific GO terms (Groups C1, C2, C3, D1, D2, and D4) are listed in Supplemental Appendix B4. Among the 13 sub-groups, no GO terms were significantly enriched in B1 (genes upregulated under both submergence and drought in leaves) or D3 (genes upregulated under drought in leaves) presumably due to the below-threshold number of genes under these sub-groups.

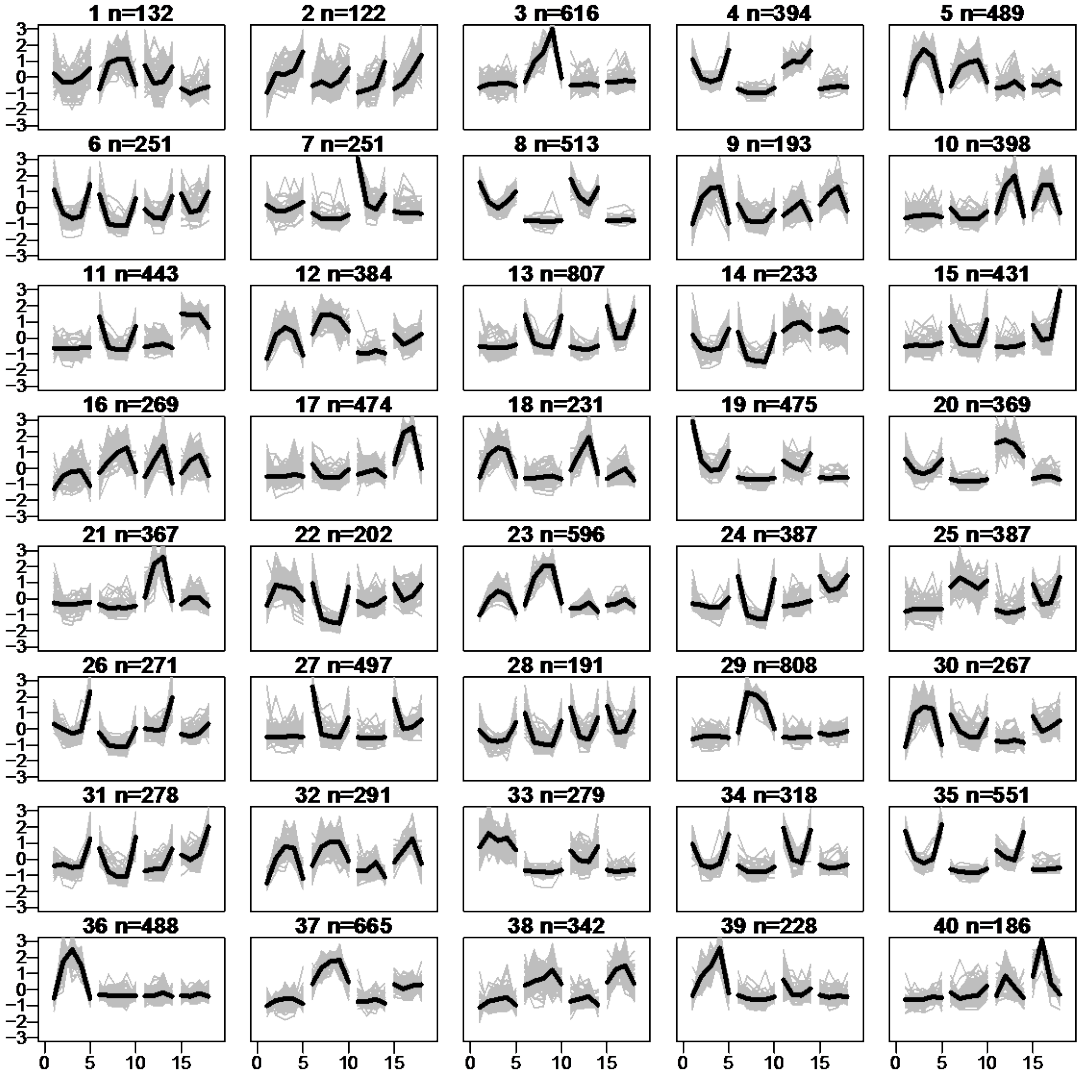


Figure 6. Clustering analysis of 15074 differentially expressed genes across 18 experimental conditions. Cluster ID number and number of genes contained in each cluster is provided above each cluster figure. The order of experimental conditions in the x-axis (from left to right) is as follows: Submergence_Leaf; 1=0d, 2=1d, 3=2d, 4=3d, 5=3dR; Submergence_Root; 6=0d, 7=1d, 8=2d, 9=3d, 10=3dR; Drought_Leaf; 11=Control, 12=Moderate, 13=Severe, 14=Recovery; Drought_Root; 15=Control, 16=Moderate, 17=Severe, 18=Recovery.

Table 1: Pooled clusters representing similar expression pattern across 18 experimental conditions

Groups	Cluster Number	Total gene number	No. of GO terms enriched
A. Common for both tissues and stresses			
1. Submergence + Drought in Leaf + Root; Upregulated	C16 C32	560	4
2. Submergence + Drought in Leaf + Root; Downregulated	C6 C28	442	4
B. Tissue-specific			
1. Submergence + Drought only in Leaf; Upregulated	C18	231	0
2. Submergence + Drought only in Leaf; Downregulated	C8 C19 C34 C35	1857	38
3. Submergence + Drought only in Root; Downregulated	C13 C15 C24 C27	2122	18
C. Stress-specific			
1. Submergence in Leaf + Root; Upregulated	C5 C12 C23	1469	10
2. Submergence in Leaf + Root; Downregulated	C14	233	1
3. Drought in Leaf + Root; Upregulated	C10 C40	584	2
D. Tissue and stress specific			
1. Submergence in Leaf; Upregulated	C36 C39	716	12
2. Submergence in Root; Upregulated	C3 C29 C37	2089	9
3. Drought in Leaf; Upregulated	C21	367	0
4. Drought in Root; Upregulated	C17	474	12
E. Recovery-specific			
1. Submergence + Drought recovery in Leaf + Root; Upregulated	C2	122	4

Table 2: Biological processes associated enriched GO terms

A. UPREGULATED GO terms during both stresses in both tissues (Group A1)

GO term	Biological Process	FDR
GO:0006397	mRNA processing	0.0018
GO:0006413	Translational initiation	0.040004
GO:0016071	mRNA metabolic process	0.0018
GO:0006396	RNA processing	0.019999

B. DOWNREGULATED GO terms during both stresses in both tissues (Group A2)

GO term	Biological Process	FDR
GO:0008152	Metabolic process	0.019999
GO:0016051	Carbohydrate biosynthetic process	0.00027
GO:0005975	Carbohydrate metabolic process	0.00011
GO:0046039	GTP metabolic process	0.01

C. UPREGULATED GO terms during recovery from both stresses in both tissues (Group E1)

GO term	Biological Process	FDR
GO:0016072	rRNA metabolic process	6.3E-06
GO:0042254	Ribosome biogenesis	7E-09
GO:0006396	RNA processing	1.1E-05
GO:0034660	ncRNA metabolic process	0.00061

D. DOWNREGULATED GO terms during both stresses in leaves (Group B2)

GO term	Biological Process	FDR
GO:0008152	Metabolic process	1.5E-20
GO:0009987	Cellular process	0.011
GO:0015979	Photosynthesis	1.7E-38
GO:0042440	Pigment metabolic process	5.8E-05
GO:0009058	Biosynthetic process	6.6E-06
GO:0015672	Monovalent inorganic cation transport	0.0033
GO:0006412	Translation	3.2E-08
GO:0019725	Cellular homeostasis	0.047995
GO:0006091	Generation of precursor metabolites and energy	2.2E-05
GO:0051186	Cofactor metabolic process	0.00012
GO:0019748	Secondary metabolic process	0.0037
GO:0044282	Small molecule catabolic process	9.4E-05
GO:0005996	Monosaccharide metabolic process	0.00011
GO:0034641	Cellular nitrogen compound metabolic process	1.8E-10
GO:0006629	Lipid metabolic process	0.0053
GO:0044237	Cellular metabolic process	5.2E-05
GO:0046148	Pigment biosynthetic process	2.7E-05
GO:0044281	Small molecule metabolic process	2E-08
GO:0033014	Tetrapyrrole biosynthetic process	4.6E-07

GO:0042180	Cellular ketone metabolic process	0.00062
GO:0006066	Alcohol metabolic process	0.0012
GO:0044106	Cellular amine metabolic process	0.002
GO:0046483	Heterocycle metabolic process	2E-08
GO:0009308	Amine metabolic process	0.0012
GO:0006399	tRNA metabolic process	0.0014
GO:0051188	Cofactor biosynthetic process	0.00062
GO:0033013	Tetrapyrrole metabolic process	1.2E-06
GO:0043038	Amino acid activation	0.002
GO:0055114	Oxidation-reduction process	7E-08
GO:0006082	Organic acid metabolic process	0.00059
GO:0018130	Heterocycle biosynthetic process	6.7E-08
GO:0006352	DNA-templated transcription, initiation	0.016998
GO:0008610	Lipid biosynthetic process	0.00093
GO:0009228	Thiamine biosynthetic process	0.0051
GO:0006546	Glycine catabolic process	0.0056
GO:0009152	Purine ribonucleotide biosynthetic process	0.0011
GO:0034660	ncRNA metabolic process	0.01
GO:0044271	Cellular nitrogen compound biosynthetic process	0.00055

E. DOWNREGULATED GO terms by both stresses in roots (Group B3)

GO term	Biological Process	FDR
GO:0006979	Response to oxidative stress	4.5E-07
GO:0007018	Microtubule-based movement	1.2E-08
GO:0008152	Metabolic process	0.0013
GO:0044092	Negative regulation of molecular function	0.0012
GO:0051258	Protein polymerization	0.0013
GO:0006073	Cellular glucan metabolic process	0.012001
GO:0007017	Microtubule-based process	4.8E-07
GO:0009154	Purine ribonucleotide catabolic process	0.0013
GO:0005975	Carbohydrate metabolic process	0.0023
GO:0006793	Phosphorus metabolic process	0.012001
GO:0055085	Transmembrane transport	0.0027
GO:0055114	Oxidation-reduction process	7.8E-07
GO:0006260	DNA replication	0.0013
GO:0015995	Chlorophyll biosynthetic process	0.0013
GO:0065009	Regulation of molecular function	0.047995
GO:0006468	Protein phosphorylation	0.021999
GO:0009607	Response to biotic stimulus	0.01
GO:0042221	Response to chemical	2.9E-05

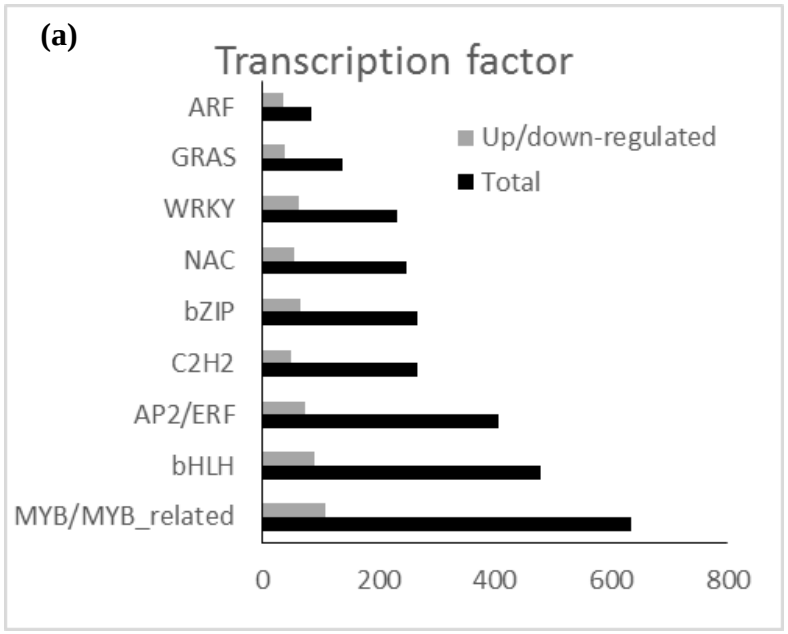
3.3.5 Expression profiles of transcription factors (TFs) and MAP kinases under submergence and drought in leaves and roots of soybean

Differentially expressed soybean TFs were identified using the soybean transcription factor gene list available at Plant Transcription Factor Database version 3.0. The soybean (cv. Williams 82) genome encodes 5,069 TFs, which are classified into 57 families (*Jin* et al., 2014). 1,005 differentially expressed TFs; MYB/MYB-related (109), bHLH (90) and AP2/ERF (73) were predominant classes of TFs (Fig. 7a). Of 154 TF genes upregulated during drought and recovery from the stress, 56 genes (36.4%) were also induced in response to submergence and recovery (Fig. 7b). In addition, 27.2% (72/265) of transcription factors downregulated during drought and recovery from the stress were also reduced by submergence and recovery. Several studies have found ERF transcription factors as key regulators governing tolerance to submergence and drought (*Tamang & Fukao*, 2015). In this study, we detected 73 AP2/ERF TFs significantly upregulated or downregulated under submergence, drought and/or recovery from these stresses (Fig. 8a; Supplementary Appendix B7a). Consistent with overall gene expression patterns shown in Fig. 5a, submergence upregulates a greater number of AP2/ERF genes in roots as compared to leaves. In leaves, no AP2/ERF genes were upregulated or downregulated during recovery from submergence and drought. In roots, however, three AP2/ ERF genes were upregulated during recovery from drought, two (Glyma.02G016100; ERFVII1 and Glyma.10G016500; ERFVII5) of which were also induced during recovery from submergence. Venn-diagrams revealed that 56.3% (9 of 16 genes) of ERFVII genes induced during drought and recovery were also upregulated in response to submergence and recovery (Fig. 8b). In addition, 42.9% (6 of 14 genes) of ERFVII genes downregulated by drought and recovery were reduced during submergence and recovery (Supplementary Appendix B7b).

Previously, *Tamang* et al. (2014) identified nine ERFVII genes in the soybean genome (Fig. 9). Eight out of the nine genes were included in the list of 73 AP2/ERF genes differentially expressed during submergence, drought and/or recovery from these stresses (Fig. 8a). We monitored the expression patterns of these nine soybean ERFVII genes in leaves and roots over the course of stress treatment and recovery by qPCR analysis (Fig. 9; Supplementary Appendix B6a-b). Among the nine gene transcripts, expression levels of ERFVII1, ERFVII5 and ERFVII8 were highly induced in both leaves and roots by submergence across all time points. During 24 h of recovery, the mRNA levels of these genes were dramatically declined in leaves, whereas they remained high (~3-6 $\log_2$ fold) in roots. Under drought conditions, ERFVII5 and ERFVII8 were induced in leaves (~3-4 $\log_2$fold) and declined during recovery. In roots, most ERF VII genes were either negatively regulated or negligibly induced during drought and recovery. Exceptionally, only ERFVII8 were highly induced (~4 $\log_2$fold) under 24 h of recovery. Based on these results, it is anticipated that ERFVII5 and ERFVII8 can be key regulators for submergence and drought responses in soybean.

The soybean genome encodes 2,166 putative protein kinases which represent 4.7% of all protein-coding genes (*Liu* et al., 2015). Among protein kinases, the mitogen-activated-protein kinase (MAPK) gene family is involved in a variety of cellular, physiological and abiotic stress signaling pathways (*Tena* et al., 2001; *Ichimura* et al., 2002). Earlier, *Neupane* et al. (2013) identified 199 MAPK family genes (150 MAPKKKs, 15 MAPKKs, and 39 MAPKs) in the soybean genome. Among the 199 MAPK family genes, 91 genes (68 MAPKKK, 4 MAPKK and 19 MAPK) that were differentially regulated by submergence, drought and/or recovery from these stresses in the current study (Fig. 10a; Supplementary Appendix B8a). A heat map illustrated that submergence upregulated or downregulated a much larger number of MAPK

family genes than drought. Tissue-specific upregulation or downregulation was also observed under both submergence and drought conditions (Fig 10b; Supplementary Appendix B8b). Venn diagram comparison revealed that 30.8% and 25.0% of genes upregulated or downregulated under drought and drought recovery were also upregulated or downregulated under submergence and submergence recovery; a smaller number of genes were overlapped between the two stresses as compared to the entire DEG and TF analyses.



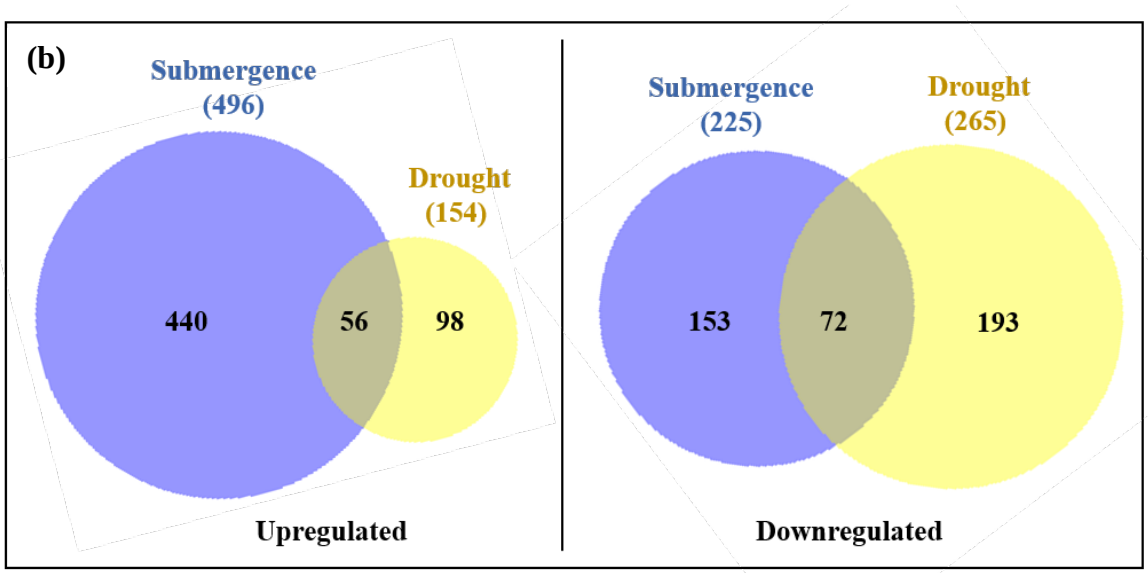


Figure 7. Differentially expressed transcription factor (TF) genes. (a) Top nine soybean TF families with the total number of genes in the soybean genome (black bar) and the number of differentially expressed TFs under submergence and drought (grey bar). (b) Venn-diagrams showing submergence- and drought-regulated genes.

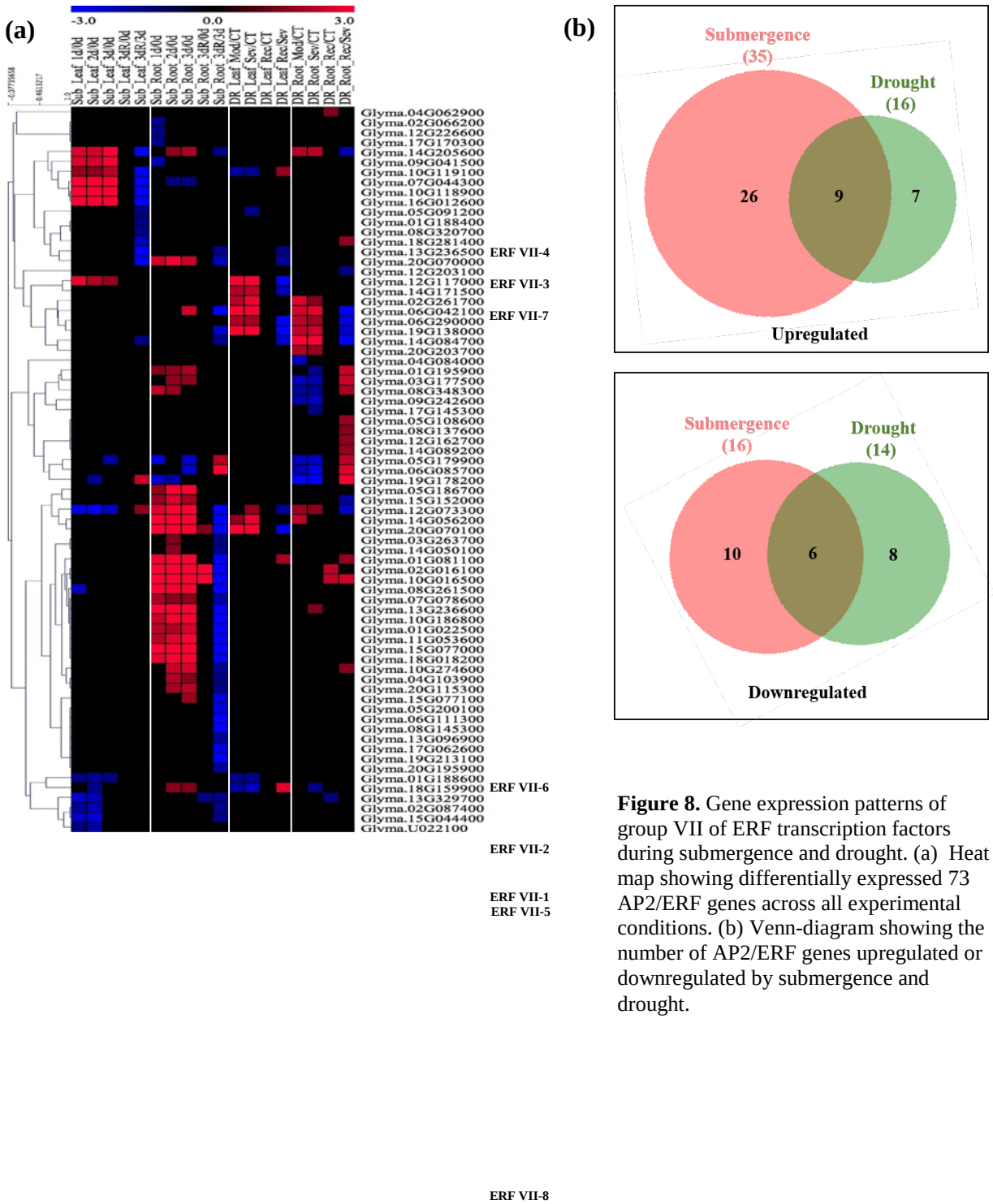


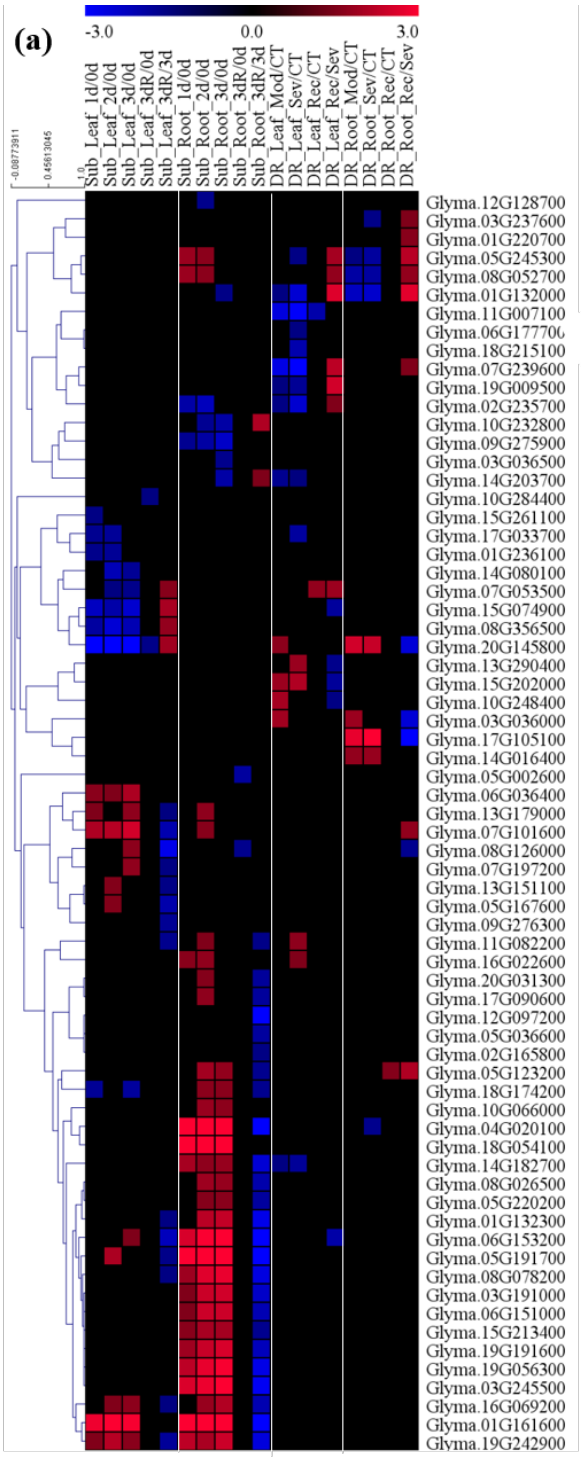
Figure 8. Gene expression patterns of group VII of ERF transcription factors during submergence and drought. (a) Heat map showing differentially expressed 73 AP2/ERF genes across all experimental conditions. (b) Venn-diagram showing the number of AP2/ERF genes upregulated or downregulated by submergence and drought.

		Submergence										Drought							
		Leaves					Root					Leaves				Root			
ERF ID	Gene ID	1d	2d	3d	3dR	3dR*	1d	2d	3d	3dR	3dR*	Mod	Sev	Rec	Rec*	Mod	Sev	Rec	Rec*
ERF VII-1	Glyma.02G016100	3.5	4.1	3.4	-2.7	-6.1	6.4	6.7	6.6	2.8	-3.9	-0.9	-0.2	-0.3	-0.1	0.7	0.0	1.7	1.7
ERF VII-2	Glyma.03G263700	-0.2	-0.2	-0.6	-1.7	-1.1	0.1	0.3	-0.6	-0.8	-0.2	-0.8	-0.5	-1.7	-1.2	-0.7	-1.1	-1.3	-0.3
ERF VII-3	Glyma.07G044300	3.1	3.0	2.5	-1.3	-3.7	-1.4	-2.1	-2.9	-1.0	1.9	-0.2	-0.6	-2.1	-1.5	-1.5	-2.2	-1.0	1.2
ERF VII-4	Glyma.09G041500	2.8	3.1	2.5	-1.8	-4.3	-3.0	-4.4	-5.0	-0.1	4.9	-1.4	-0.8	-2.2	-1.4	-1.2	-0.9	-1.4	-0.5
ERF VII-5	Glyma.10G016500	4.7	5.6	4.4	-0.6	-5.0	8.1	8.5	8.0	4.2	-3.8	3.7	4.0	-2.1	-6.2	-0.3	-0.7	1.4	2.1
ERF VII-6	Glyma.15G152000	-0.6	-0.2	-0.5	-1.5	-1.0	0.1	0.4	-1.0	0.0	1.1	-0.6	-0.4	-1.9	-1.5	1.1	0.7	-0.5	-1.2
ERF VII-7	Glyma16G012600	3.4	3.1	2.9	-0.9	-3.8	-1.6	-1.1	-2.8	-1.0	1.9	-0.5	-0.8	-2.2	-1.5	-1.0	-1.7	-0.9	0.8
ERF VII-8	Glyma.19G213100	5.1	5.1	2.7	-2.3	-5.0	9.9	9.9	9.2	5.9	-3.3	2.8	2.7	-1.1	-3.9	0.4	-0.1	4.0	4.1
ERF VII-9	Glyma.19g262700	-1.0	-0.7	-1.2	-0.7	0.4	-0.7	-0.6	-1.8	-0.5	1.3	-0.5	0.2	-1.2	-1.4	-0.8	-0.9	-1.0	-0.1

Color Scale

-10.00 -5.00 0.00 5.00 10.00

Figure 9. Log2-fold changes in the expression of nine soybean ERF-VII genes across all experimental conditions calculated by qRT-PCR analysis. 3dR and 3dR* indicate $\log_2$ fold changes calculated by dividing 3d submergence + 24 h recovery values by 0 d control and 3 d submergence, respectively. Rec and Rec* were also computed by the same method.


(a)
-3.0
0.0
3.0
Sub_Leaf_1d/0d
Sub_Leaf_2d/0d
Sub_Leaf_3d/0d
Sub_Leaf_3dR/0d
Sub_Leaf_3dR/3d
Sub_Root_1d/0d
Sub_Root_2d/0d
Sub_Root_3d/0d
Sub_Root_3dR/0d
Sub_Root_3dR/3d
DR_Leaf_Mod/CT
DR_Leaf_Sev/CT
DR_Leaf_Rec/CT
DR_Leaf_Rec/Sev
DR_Root_Mod/CT
DR_Root_Sev/CT
DR_Root_Rec/CT
DR_Root_Rec/Sev
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Glyma.11G007100
Glyma.06G177700
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Glyma.02G235700
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Glyma.15G261100
Glyma.17G033700
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Glyma.20G145800
Glyma.13G290400
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Glyma.03G036000
Glyma.17G105100
Glyma.14G016400
Glyma.05G002600
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Glyma.13G179000
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Glyma.08G126000
Glyma.07G197200
Glyma.13G151100
Glyma.05G167600
Glyma.09G276300
Glyma.11G082200
Glyma.16G022600
Glyma.20G031300
Glyma.17G090600
Glyma.12G097200
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Glyma.05G123200
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Glyma.05G220200
Glyma.01G132300
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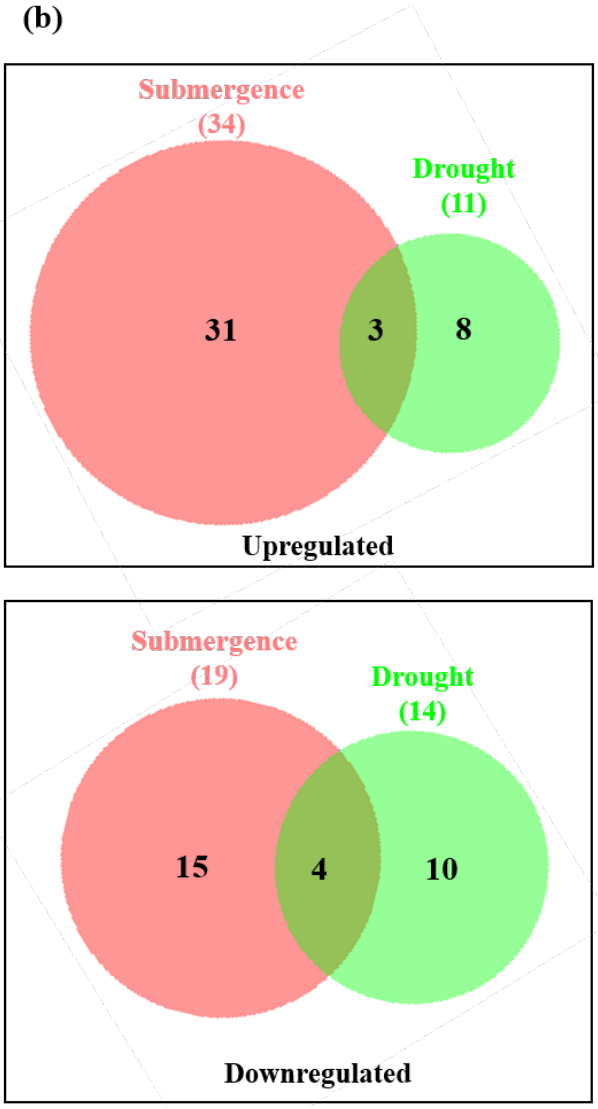
(b)
Submergence
(34)
Drought
(11)
31
3
8
Upregulated
Submergence
(19)
Drought
(14)
15
4
10
Downregulated

……...continued

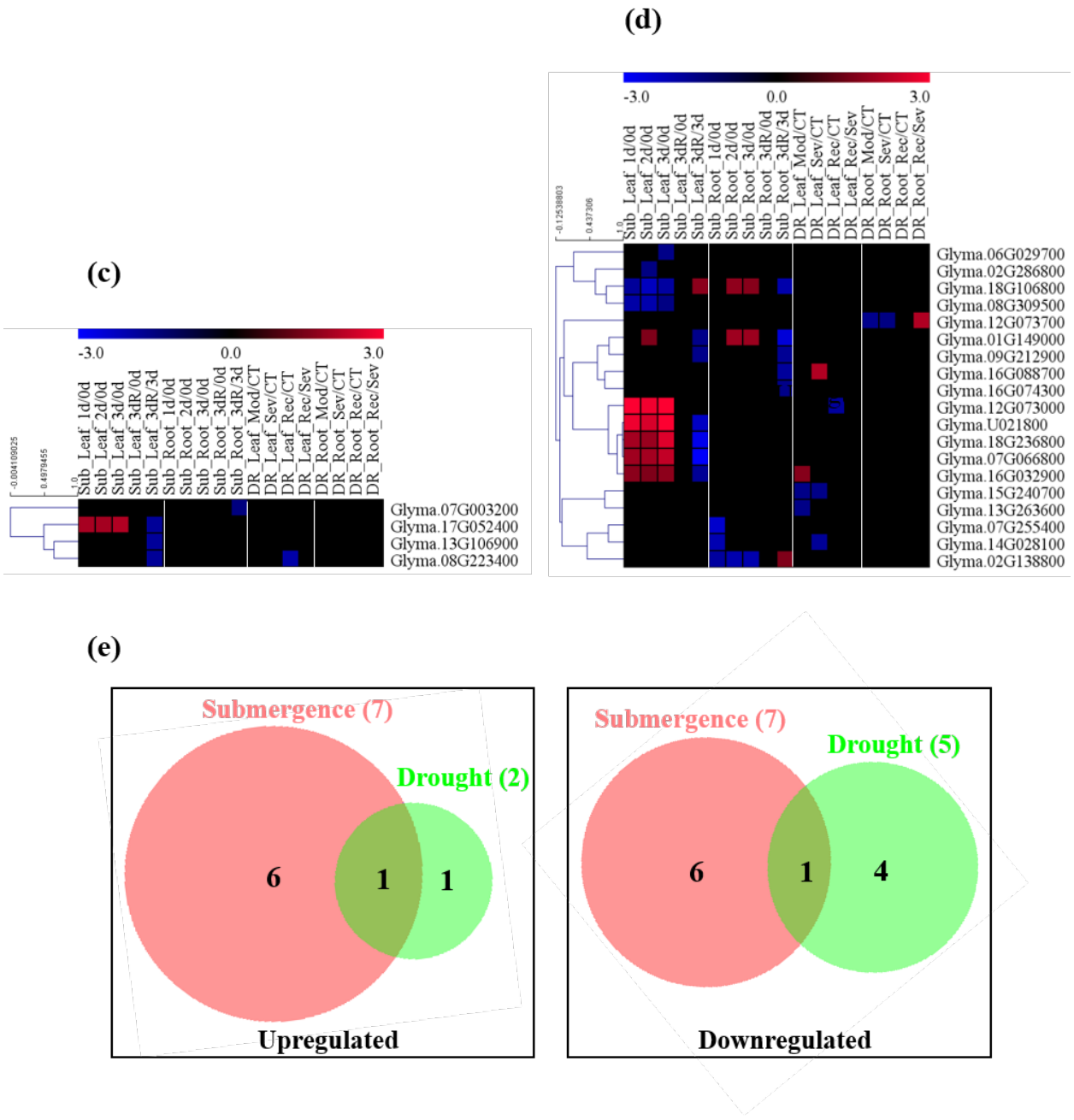

Figure 10. Expression pattern of genes associated with MAPK cascades during submergence and drought. (a) (c) (d) Heat maps showing differentially expressed 68 MAPKKK, 4 MAPKK and 19 MAPK genes across all experimental conditions, respectively. (b) (e) Venn-diagrams showing the number of MAPKKK and MAPK genes upregulated or downregulated by submergence and drought, respectively. A Venn diagram for MAPKK was not presented as only a single gene was differentially upregulated or downregulated by submergence and drought, respectively.

3.4 DISCUSSION

The responses of plants to abiotic stresses depend on the duration and magnitude of stress as well as the tissue-type (*Cramer* et al., 2011). Plants have evolved to endure flooding stress by either restricting their growth to avoid starvation or promoting rapid growth to circumvent the stress (*Voesenek* et al., 2006; *Bailey-Serres & Voesenek*, 2008; *Colmer &Voesenek*, 2009; *Bailey-Serres* et al., 2012a, b). We observed the former quiescence strategy in soybean where an increase in plant height and biomass growth were restricted while underwater; an energy saving strategy during the stress (Fig. 1a, d-f). It has been well recognized that a limited number of rice genotypes can survive under 14-16 d of complete submergence through the quiescence strategy (*Das* et al., 2005). However, unlike submergence-tolerant rice, the viability of soybean at the early vegetative stage declined after three days of submergence (Fig. 1b), suggesting that soybean is susceptible to submergence.

Under drought, ROS is inevitably produced that is linked with ABA accumulation, which then leads to stomatal closure (*de Carvalho* et al., 2008; *Noctor* et al., 2014). We observed the gradual accumulation of MDA in roots during 6 d of water limitation in soybean, which receded during 24 h of recovery (Fig. 2c). The data suggest quick scavenging of ROS after re-watering, consistent with the result of leaf relative water content analysis and the appearance of soybean plants after the recovery period.

Plants modulate metabolic processes to enhance adaptability to environmental perturbations such as submergence and drought. One of the most common responses to stress in plants is accumulation of low molecular weight, highly soluble compatible organic solutes that are non-toxic even at high concentrations (*Serraj & Sinclair*, 2002; *Ashraf & Foolad*, 2007). Our study demonstrated the accumulation of several amino acids during submergence and drought

(Fig. 3). *Magneschi & Perata* (2009) reviewed the importance of alanine and GABA induction in rice seedlings for the maintenance of osmotic potential under low oxygen conditions. Hypoxia also led to an increase in the endogenous levels of several amino acids, particularly alanine and GABA in soybean roots (*Oliveira & Sodek*, 2013) and rice coleoptiles (*Kato-Noguchi & Ohashi*, 2006). We observed that soybean roots accumulated ~50-fold alanine and ~15-fold GABA under 3 d of complete submergence. On the other hand, arginine had a large amount of leaf-specific accumulation. During recovery from hypoxia, accumulation of alanine is thought to be important for the maintenance of glycolytic flux and retaining of carbon and nitrogen resources within the cell (*Rocha* et al., 2010). Besides alanine, several amino acid levels were also still significantly higher in leaves and roots at the end of 24 h recovery. This might result from the slow turnover rates of these compounds. Interestingly, glycine showed recovery-specific induction in leaves after submergence. Glycine betaine, a compound which can be synthesized from glycine, is known to accumulate under abiotic stress in plants and is proposed to stabilize the oxygen-evolving PSII complex from photo-damage, prevent protein denaturation and restrict excessive ROS accumulation (*Chen & Murata*, 2008, 2011). Induction of glycine in leaves during recovery from submergence might contribute to the mitigation of post-submergence damage caused by sudden exposure to atmospheric oxygen and higher light by its conversion to glycine betaine.

Accumulation of proline is frequently observed under water deficit, which is important for the protection of cellular structures (*Lehmann* et al., 2010). It is also reported that proline is responsible for scavenging the ROS and other free radicals (*Hayat* et al., 2012). Indeed, we observed that the abundance of proline is elevated during drought and recovery from the stress in leaves and roots (Fig. 3). *Good & Zaplachinski* (1994) reported that the levels of alanine, arginine, asparagine, GABA, histidine, isoleucine, leucine, lysine, methionine, phenylalanine,

proline, serine, threonine and valine linearly increase during drought followed by a reduction in their concentrations upon rehydration in *Brassica napus* leaves. In this study, alanine, aspartate, GABA, glutamate, isoleucine, leucine, proline, threonine, tryptophan, tyrosine and valine are significantly upregulated in leaves and roots, during drought treatment (Fig. 3). These results suggest that accumulation patterns of individual amino acids are somewhat species-specific, but several amino acids such as alanine, GABA, isoleucine, leucine, proline, threonine and valine may play an important role in acclimation to dehydration stress. As observed during submergence, the magnitude and variety of amino acid accumulation are tissue-specific; more amino acids accumulate during drought and recovery from the stress in leaves than roots.

Of 15 amino acids surveyed, tryptophan, isoleucine, leucine, proline and valine are commonly induced in response to both submergence and drought in both leaves and roots. The role of these amino acids except proline in adaptation to these stresses is still not clear. Genetic and physiological analysis of transgenic and mutant lines over-producing or under-producing these amino acids will determine the cause and effect relationship between particular amino acids and stress tolerance.

Plants require transcriptomic adjustment to balance growth and defense under stress conditions. For instance, during 12 h flooding of 2-d-old soybean, 9,875 genes responded in the roots (*Nanjo* et al., 2011), whereas 4,374 genes were altered in 4-d-old soybean shoots during 5 d of complete submergence (*Tamang* et al., 2014). Similarly, under 6 d of drought stress (SMC ≤ 5%, 30% RWC), 3,040 and 3,506 gene transcripts were altered at the V6 and R2 stage soybean leaves, respectively (*Le* et al., 2012). In 14-d-old roots (V2 stage) of William 82 soybean, 741 genes responded under 10 h of air drying (drought) (18% RWC) (*Ha* et al., 2015). *Song* et al., (2016) observed the differential regulation of 3,957 genes in tap roots of soybean during 19 d of

drought stress (8.3% SMC). Distinct numbers of differentially expressed genes across these experiments can be attributed to the variation in growth stages, genotypes and experimental setup and platforms. In this study, we detected a greater number of DEGs during submergence and recovery from the stress than drought and its recovery (Fig. 5b). Submergence is a complex stress and plants face multiple external and internal challenges simultaneously or sequentially during and after submergence (*Tamang & Fukao*, 2015), thereby resulting in a plethora of adjustments at the transcript level.

Submergence-induced low oxygen conditions eventually lead to a decrease in ATP production and energy crisis in plants (*Drew*, 1997; *Bailey-Serres & Voesenek*, 2008). Submergence or low oxygen conditions have been shown to regulate a core set of genes in plants. For instance, results from microarray studies demonstrated that submergence/hypoxia induces 49 genes in *Arabidopsis*, many of which are involved in sucrose catabolism, ethanolic fermentation, regulation of ROS, gene transcription along with several unknown functions (*Mustroph* et al., 2009; *Lee* et al., 2011). Our GO enrichment analysis demonstrated that processes involved in protein and macromolecules modification, transcription initiation and vesicle-mediated transport are commonly induced in leaves and roots in response to complete submergence (Supplementary Appendix B5-A). These processes significantly contribute to the acclimation of soybean to submergence by providing cellular homeostasis. Interestingly, processes related to reproduction were induced only in vegetative tissues (leaves) under submergence (Supplementary Appendix B5-D). It is known that several abiotic stresses including low oxygen induce flowering response in plants (*Wada & Takeno*, 2010; *Takeno*, 2012; *Kazan & Lyons*, 2016). Submergence, in addition to low oxygen, is known to impose multiple stresses on plants such as low light and nutrient deficiency (*Tamang & Fukao*, 2015)

and the combination of these stresses might induce early flowering response to maximize the chances of reproduction under these diverse stress conditions in soybean.

Water stress influences a variety of biological processes leading to developmental and physiological changes. A common response to drought is the accumulation of endogenous ABA in vegetative tissues that promotes stomatal closure, induction of stress-related genes and metabolic adjustment to enhance adaptability (*Seki* et al., 2007). During drought, thousands of genes associated with signal transduction, photosynthesis, carbohydrate metabolism and stress endurance are regulated (*Fukao & Xiong*, 2013). Water stress in our study induced protein folding and regulation of transcriptional processes in both the tissues of soybean (Supplementary Appendix B5-C) that is important for metabolic and transcriptional reprogramming as a response to the stress. Although none of the GO terms were significantly enriched in leaves under drought, 50% of the enriched processes in roots are associated with biosynthesis of several metabolites such as amines and aromatic amino acids, organic and carboxylic acids and various nitrogen compounds (Supplementary Appendix B5-F). Root growth is increased under drought stress (*Gargallo-Garriga* et al., 2014) and therefore several biosynthetic processes are upregulated specifically in roots. This result is in agreement with our amino acid result where more amino acids are active in roots compared to leaf tissue (Fig. 3).

Among several processes, downregulation of photosynthesis in leaves is a common phenomenon under both stresses (Table 2D). This is caused by limited gas exchange and low light levels under submergence and reduced stomatal conductance under drought. *Mutava* et al. (2015) demonstrated that lower rates of photosynthesis under flooding is also a result of starch granule accumulation in leaves as the phloem transport machinery gets disrupted. Similarly, several metabolic and biosynthetic processes are downgraded in leaves (Table 2D) and roots

(Table 2E) due to submergence and drought which is required for an energy saving strategy under stress and is a common acclimation response in species following quiescence strategy of stress tolerance. Overlapping responses to both submergence and drought in both tissues include upregulation of mRNA processing and translational initiation and downregulation of carbohydrate biosynthesis/metabolism and GTP metabolism (Table 2 A, B). Close observation of the specialized GO child terms in the GO hierarchy reveals that these genes are associated with splicing factors, nucleic acid binding family, chromatin protein family, translation initiation factors and novel cap-binding proteins (Supplementary Appendix B4). It seems that acclimation responses to the two opposite water-related stresses are commonly coordinated at mRNA processing and transcriptional initiation stages. During recovery from both stresses, pathways associated with transcriptional and translational regulation are enriched in both leaves and roots (Table 2C). These processes are necessary for repair of organelles and cells as well as vegetative regrowth during the recovery period.

A number of genetic and molecular studies have identified TF families which are involved in response and tolerance to submergence and drought. These include members of MYB, bHLH, AP2/ERF, WRKY, bZIP, HSP, NAC and LOB-domain proteins (*Garg* et al., 2013; *Thirunavukkarasu* et al., 2013; *Tamang* et al., 2014; *Mohanty* et al., 2016). Among the 57 families of TFs in soybean *(Jin* et al., 2014), MYB, bHLH and AP2/ERF are major TF families that contain differentially expressed TF genes (Fig. 7a). Detailed studies in rice and *Arabidopsis* have characterized members of group VII AP2/ERF family and demonstrated their role in governing tolerance to submergence, drought, and recovery (*Tamang & Fukao*, 2015). The soybean genome contains nine ERFVII gene members (*Tamang* et al., 2014) and our transcriptomic study demonstrated that among 73 differentially expressed AP2/ERF genes under

submergence and drought, eight are members of group VII ERF (Fig 7a). A qPCR assay of the nine soybean ERFVII genes suggests that ERFVII5 and ERFVII8 might be important for soybean tolerance to and recovery from submergence and drought because their mRNA levels were highly induced under these stresses or recovery (Fig. 9). Previously, *Tamang* et al., (2014) indicated the potential positive role of these genes under submergence and reoxygenation. Recently, it was discovered that two out of five group VII ERF members in *Arabidopsis* (RAP2.2 and RAP2.12) redundantly regulate 39 out of the 49 core hypoxia responsive genes by binding to the conserved 12-bp *cis*-regulatory motif (*Gasch* et al., 2016). Further functional characterization and identification of upstream and downstream components of these genes should elucidate their potential roles during submergence and drought, and provide a mechanistic understanding of the tolerance in soybean.

Mitogen-Activated-Protein-Kinase (MAPK) cascade is an evolutionarily conserved signal transduction network which plays a key role in the regulation of various abiotic stress responses (*Tena* et al., 2001; *Sinha* et al., 2011). The cascade is composed of three classes of protein kinases: MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAPK; MAPKKK phosphorylates MAPKK and activated MAPKK phosphorylates MAPK (*Hamel* et al., 2012; *Danquah* et al., 2013). Our study identified 68 MAPKKK, 4 MAPKK and 19 MAPK differentially expressed during submergence, drought, and recovery from the stresses. Among them, 4 and 5 kinases are commonly upregulated or downregulated, respectively, during both stresses (Supplementary Appendix B8b), which might be key kinases for the regulation of responses to submergence and drought. A study by *Ning* et al. (2010) suggested that DSM1, a MAPKKK in rice, functions as an early signaling component under drought stress and regulates scavenging of ROS. Recently, *Singh & Sinha* (2016) demonstrated that MPK3 physically

interacts and phosphorylates SUB1A protein, a master regulator of submergence tolerance in rice, thereby activating SUB1A-mediated acclimation responses. Further investigation of the MAP kinases identified in our current study is required to dissect their role during submergence and drought stress. The two ERFVIIs commonly regulated under submergence and drought in our study (Figure 9) might be candidate substrates of the MAP kinases.

3.5 REFERENCES

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CHAPTER 4

Differential responses of grain yield, grain protein content and their associated traits to nitrogen supply in soft red winter wheat

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Submitted to: Journal of Plant Nutrition and Soil Science on June 2nd 2016

ABSTRACT

Increased application of nitrogen fertilizers has significantly raised grain yield and protein content in wheat. However, only 30-50% of applied fertilizer nitrogen is currently utilized by the plant. Nitrogen not absorbed by plants can be discharged to water bodies and atmosphere, resulting in detrimental impacts on environment and human health. Previously, it was reported that there is wide genetic variation in nitrogen use efficiency for grain yield (NUEY) and grain protein content (NUEP) within 281 U.S. soft red winter wheat accessions. In this study, we evaluated the effect of three different nitrogen fertilizer regimes (high, medium and low) on grain yield, grain protein content, NUEY, NUEP and their associated traits in four soft red winter wheat genotypes (IL07-4415, MD05W10208-11-8, Sisson, and OH06-150-57) selected from the previously tested 281 accessions. Among the four genotypes, a high yielding cultivar, Sisson, exhibited superior performance in terms of grain weight/plant, NUEY and yield under low nitrogen due to maintained grain number/spike and harvest index. Significant yield losses in the

other genotypes due to nitrogen limitation were attributable to reduced spike number/plant and grain number/spike. Nitrogen rate did not alter 1000-seed weight of any genotype. Interestingly, a linear relationship between NUEY and NUEP was detected at high and low nitrogen; both of these traits were positively correlated with grain number/spike, 1000-seed weight and harvest index under nitrogen-limited conditions. These results suggest that simultaneous improvement of NUEY and NUEP could be achieved through the selection of the three yield components as these components are positively correlated with each other under low nitrogen.

Keywords

Triticum aestivum, nitrogen use efficiency, nitrogen deficiency, carbohydrates

4.1 INTRODUCTION

Nitrogen (N) is globally the highest consumed macronutrient in today's high-yield agricultural production systems, representing a significant cost for growers. The current usage of N fertilizers is nine-fold greater than in the 1960's (*Sutton* et. al., 2013). Application of N is predicted to increase by 40-50% over the next 40 years. An increased input of N fertilizers has been indispensable for continuous increases in crop productivity. However, for most cereal species, only 30-50% of applied N is actually taken up by the plant in the same season (*Dobermann & Cassman*, 2002; *Kitchen* et al. 2008). Nitrogen not absorbed by plants can be released to atmosphere and water bodies, resulting in greenhouse gas emission and eutrophication of fresh water and marine ecosystems (*Vitousek* et al. 1997; *Mueller* et al. 2012; *Zhang* et. al., 2015). Optimization of the rate, timing, and placement of N fertilizers and usage of slow and controlled release fertilizers are effective approaches for the reduction of ammonium emission, denitrification, N leaching and runoff from agricultural systems. In addition to these management-based solutions, genetic improvement of nitrogen use efficiency (NUE) in crops must be of necessity to address the complex environmental issue.

Wheat (*Triticum aestivum* L.) is the most widely grown crop in the world. In 2014, the total area harvested was 222 M ha in wheat, as compared with 183 M ha in maize and 163 M ha in rice (http://faostat3.fao.org/). Protein content is a critical factor determining grain quality and marketability in wheat. To achieve the acceptable amount of protein in grains, a high level of N fertilizers has been supplied for wheat production (*Shewry*, 2006; *Henry* et al., 2016). For these reasons, of the total N produced globally, 18% is applied in wheat cultivation; the largest amount of N applied in crop production (maize, 16% and rice, 16%) (*Ladha* et. al., 2015). Based on the scale of cultivation and the high requirement of N fertilizers, genetic improvement of NUE in

wheat can largely contribute to the suppression of water and air pollution caused by excessive N input as well as the reduction in production costs.

Soft red winter wheat is widely grown over the eastern third of North America. It is generally higher yielding with less grain protein content than hard wheat (*Thomason* et al., 2009). The flour is mainly used for cookies, cakes, crackers and flat bread. In contrast to hard wheat, grain protein content per se is of less importance to end-use quality in soft red winter wheat (*Souza* et al., 2011, 2012). Nitrogen use efficiency for yield (NUEY) and protein (NUEP) are defined as grain dry weight or total grain N divided by fertilizer N supplied to the crop, respectively (*Moll* et al., 1982; *Van Sanford & MacKown*, 1986). It was previously reported that significant genotypic variations for NUEY and NUEP exist among 25 soft red winter wheat genotypes (*Van Sanford & MacKown*, 1986). A recent large-scale survey has also recognized wide diversity for NUEY and NUEP within 281 soft red winter wheat genotypes regionally developed in the Eastern U.S (*Pavuluri* et al., 2014).

Identification of morphological and physiological components that positively and negatively associate with NUEY and NUEP is a necessary step for dissecting the genetic basis of these complex traits. In this project, we evaluated the effect of nitrogen supply on grain yield, grain protein content and their associated traits under three N regimes in four representative soft red winter wheat genotypes previously identified as varying for NUE (*Pavuluri* et al., 2014). This study allowed us to identify key traits which are strongly correlated with NUEY and NUEP, useful diagnostic traits for selection of high NUE accessions in soft red winter wheat.

4.2 MATERIALS AND METHODS

4.2.1 Plant materials and growth condition

Four soft red winter wheat (*Triticum aestivum* L.) accessions (IL07-4415, MD05W10208-11-8, SISSON and OH06-150-57) were analyzed in this study. These accessions were selected based on the genetic diversity survey for NUE within a panel of 281 regionally developed soft red winter wheat genotypes (*Pavuluri* et al., 2014). This study was conducted from October 8, 2014 to May 7, 2015 in a greenhouse located in Blacksburg, Virginia, USA (37º12'N, 80º24'W). Seeds were sterilized in 5% (v/v) sodium hypochlorite and 0.1% (v/v) Tween-20 for 5 min and thoroughly rinsed with deionized water. Sterilized seeds were placed on wet paper towels for 4 d at 25 ºC in the light (50 μmol m^{-2} s^{-1}). Germinated seedlings were then transplanted into soil-containing flats and grown in a greenhouse (natural light, 22 ºC day/ 13 ºC night) until the 3-leaf stage. Subsequently, seedlings were exposed to vernalization treatment at 7 ºC under 8 h light (80 μmol m^{-2} s^{-1}) / 16 h dark cycles for 52 d. Following vernalization, each plant was grown in a pot (2.4 L) containing a mixture of 50% (v/v) Metro Mix 360 and 50% (v/v) sand under greenhouse conditions (natural light; 18 ºC day / 7 ºC night). When plants generated five tillers, the light period and temperature were changed to 16 h day (22 ºC) / 8 h night (13 ºC) using greenhouse heating and supplemental light (1000W metal halide x 4) systems.

A half-strength of Hoagland's minus nitrogen solution (50 mL/plant) was supplied twice a week to all plants until grain filling was complete. As a nitrogen source, ammonium nitrate was applied with 1/2 x Hoagland's solution until the 5-tiller stage (11.4 mg N/plant). After that, three rates of N (high N, 11.4 mg; medium N, 3.8 mg; low N, 1.14 mg) were supplied with 1/2 x Hoagland's solution twice a week till the booting stage. After this stage, the amount of N supplied was doubled for all treatments (high N, 22.8 mg; medium N, 7.6 mg; low N, 2.2 mg).

The total amount of N supplied for each plant was 423 mg for high N, 225 mg for medium N, and 155 mg for low N, respectively.

A total of 240 plants (20 plants x 3 nitrogen treatments x 4 genotypes) were laid out in a completely randomized design for this study. Yield components were measured for 14 of the plants, while the remaining 6 plants (2 plants per biological replicate) were randomly selected for flag leaf analysis. At anthesis, flag leaf blades were harvested at midday, immediately frozen in liquid nitrogen, and stored in -80 ^{o}C until use.

4.2.2 Yield component analysis

Spikes and aboveground tissue were harvested from each plant at maturity and dried at 65 ^{o}C for 3 d. Yield components measured include: 1) grain weight/plant, 2) spike number/plant, 3) grain number/spike, 4) 1000-seed weight, 5) spike length, 6) vegetative biomass weight/plant. Harvest index was calculated as the ratio of grain dry matter to total aboveground dry matter.

4.2.3 Protein content analysis

Protein concentration in grains was estimated using an XDS Rapid Content Analyzer (Foss, Eden Prairie, MN, USA) and adjusted on the basis of protein values obtained by combustion N analysis (FPS 528; Leco Corporation, St. Joseph, MI, USA) (*Pavuluri* et al., 2014). Protein content in flag leaves was quantified using the method of *Tamang* et al. (2014). Total protein was extracted from 50 mg tissue in 800 µL of an extraction buffer containing 50 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 2 m*M* EDTA, 10% (v/v) glycerol, 0.5% (v/v) IGEPAL CA-360 and 1 m*M* phenylmethylsulfonyl fluoride on ice. Protein concentrations were determined by Coomassie

Plus protein assay reagent following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Bovine serum albumin was used to generate a standard curve.

4.2.4 Nitrate, ammonium and total amino acid assays

Frozen tissues (75 mg) were homogenized in 450 µL of 0.83 *M* perchloric acid on ice. After centrifugation, 300 µL of the supernatant was neutralized with 75 µL of 1 *M* bicin (pH 8.3) and 70 µL of 4 *M* KOH. Following centrifugation, the supernatant was used for nitrate, ammonium, and amino acid assays. Nitrate and ammonium concentrations were determined as described by *Alpuerto* et al. (2016). Potassium nitrate and ammonium sulfate were used as the standards. For total amino acids, 80 µL of the extract was mixed with 20 µL of 3 *M* MgO and left in an opened 1.5 mL tube for 16 h at room temperature. Following overnight incubation, 80 µL of the solution was mixed with 50 µL of 0.2 m*M* sodium cyanide resolved in 8 *M* sodium acetate and 50 µL of 168 m*M* ninhydrin resolved in 100% 2-methoxyethanol. The mixture was heated at 100 ^{o}C for 15 min and 1 mL of 50% isopropanol was immediately added to the solution. After cooling, A_{570} was measured with a spectrophotometer. Glycine was used as the standard.

4.2.5 Carbohydrate assays

Total soluble sugars were extracted from 50 mg of frozen tissues as described by *Fukao* et al. (2006). The soluble sugar extract (100 µL) was mixed with 1 mL of 0.14% (w/v) anthrone solution in 100% H_2SO_4 and incubated at 100 ^{o}C for 20 min. After cooling, A_{620} was measured with a spectrophotometer. Glucose was used as the standard. Starch content was assayed using the pellet obtained after soluble sugar extraction. The pellet was re-suspended in 1 mL of water containing 10 units of heat-resistant α-amylase, and incubated at 95 ^{o}C for 30 min. The

suspension was mixed with 25 μL of 1 *M* sodium citrate (pH 4.8) and 5 units of amyloglucosidase. After incubation at 55 ºC for 30 min, the reaction mixture was centrifuged for 30 min at 21,000 g and glucose content in the supernatant was quantified by the anthrone method as described above.

4.2.6 Chlorophyll measurement

Frozen tissue (50 mg) was homogenized in 3 ml of 100% methanol on ice. After centrifugation, the absorbance of the supernatant was quantified at 652.0 and 665.2 nm with a spectrophotometer. Chlorophyll *a* and *b* contents were calculated following the method of *Porra* (2002).

4.2.7 Statistical analyses

Statistical analyses were performed using JMP Pro (ver. 11.0.0) (SAS Institute). One-way ANOVA with Tukey's honest significant (HSD) test was carried out for comparison of means among accessions and nitrogen regimes. Correlation coefficient analysis was conducted to determine R^2 and p-value for each comparison. NUEY and NUEP were calculated as total grain dry weight (g/plant) and total grain N (g/plant) divided by fertilizer N supplied to the plant (g/plant), respectively (*Moll* et al., 1982; *Van Sanford & MacKown*, 1986).

4.3 RESULTS

4.3.1 The effect of N supply on yield components and grain protein content

The influence of nitrogen fertility on yield components, grain protein content, and NUE was evaluated in four soft red winter wheat accessions (Table 1). Grain weight/plant (equivalent to grain yield) was similar among the four genotypes at high N input, but was reduced differentially in response to lower N supply. Sisson exhibited greater grain weight/plant and less yield reduction at low N. By contrast, grain weight/plant of MD05W10208-11-8 was lowest at medium and low N due to significantly greater yield reduction than other accessions. Because NUEY is determined by grain weight/plant divided by the amount of fertilizer N supplied to the plant, genotypic and nitrogen effects on NUEY and grain weight/plant were identical (the results of statistical analysis are identical for these two traits). Grain weight/plant is the product of spike number/plant, grain number/spike, and grain weight. Among them, spike number/plant was reduced under medium and low N input in MD05W10208-11-8, Sisson, and OH06-150-57, whereas this trait was not significantly altered in IL07-4415. On the other hand, low N input reduced grain number/spike in IL07-4415, MD05W10208-11-8, and OH06-150-57, but it did not affect this trait in Sisson. Unlike spike number/plant and grain number/spike, variable N rates did not alter 1000-seed weight in the four genotypes. We also analyzed the effect of nitrogen on spike length. Wide variation for this trait was detected among the four genotypes at high N. Lower N application reduced spike length only in Sisson and OH06-150-57.

Sisson exhibited the greatest harvest index at medium and low N. In the other three genotypes, harvest index was significantly lower at medium N as compared to high N. Yet, no difference was detected under high and medium N regimes in Sisson. Vegetative biomass was

reduced in response to low N supply in all genotypes except for IL07-4415. Of four genotypes, MD05W10208-11-8 and Sisson appeared more sensitive to low N in biomass reduction.

Reduced N application decreased the level of grain protein, with diverse magnitude of reduction among the four genotypes. Sisson and IL07-4415, which showed relatively high grain weight/plant at low N, were more sensitive to N limitation than the other two genotypes in terms of grain protein reduction. Conversely, MD05W10208-11-8, which exhibited lower grain yield and yield components than the other accessions at low N, had the highest grain protein under low N supply. A reduction in N supply from high N to medium N significantly reduced NUEP in all accessions. However, a further reduction in N application rate affected NUEP only in Sisson.

Table 1. The effect of nitrogen application on yield components, grain protein content and nitrogen use efficiency in four soft red winter wheat genotypes.

	Grain weight/plant (g)			NUEY			Grain yield reduction (%)		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{a}10.2_{a}$	$^{a}9.0_{b}$	$^{ab}7.7_{c}$	$^{a}24.2_{c}$	$^{a}40.1_{b}$	$^{ab}49.3_{a}$	-	$^{b}11.9_{b}$	$^{bc}25.1_{a}$
MD05W10208-11-8	$^{a}10.1_{a}$	$^{b}7.6_{b}$	$^{c}\ 6.5_{c}$	$^{a}23.9_{c}$	$^{b}33.9_{b}$	$^{c}\ 41.8_{a}$	-	$^{a}24.7_{b}$	$^{a}\ 35.8_{a}$
SISSON	$^{a}10.3_{a}$	$^{a}9.0_{b}$	$^{a}\ 8.1_{c}$	$^{a}24.4_{c}$	$^{a}39.9_{b}$	$^{a}\ 52.4_{a}$	-	$^{b}13.0_{b}$	$^{c}\ 21.0_{a}$
OH06-150-57	$^{a}10.3_{a}$	$^{a}9.1_{b}$	$^{bc}\ 7.0_{c}$	$^{a}24.3_{b}$	$^{a}40.6_{a}$	$^{bc}\ 45.0_{a}$	-	$^{b}11.0_{b}$	$^{ab}31.9_{a}$
	Spike number/plant			**Grain number/spike**			**1000-seed weight (g)**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{a}\ 5.4_{a}$	$^{a}4.8_{a}$	$^{a}\ 4.6_{a}$	$^{b}\ 56.9_{a}$	$^{b}\ 53.6_{ab}$	$^{b}47.6_{b}$	$^{bc}36.3_{a}$	$^{bc}36.2_{a}$	$^{a}34.4_{a}$
MD05W10208-11-8	$^{a}\ 5.4_{a}$	$^{b}4.1_{b}$	$^{ab}3.9_{b}$	$^{ab}59.0_{a}$	$^{b}\ 55.4_{ab}$	$^{b}50.5_{b}$	$^{c}\ 32.6_{a}$	$^{c}\ 33.9_{a}$	$^{b}33.0_{a}$
SISSON	$^{b}\ 4.4_{a}$	$^{b}3.8_{b}$	$^{b}\ 3.6_{b}$	$^{a}\ 65.3_{a}$	$^{a}\ 62.4_{a}$	$^{a}60.3_{a}$	$^{ab}36.7_{a}$	$^{ab}38.6_{a}$	$^{a}38.4_{a}$
OH06-150-57	$^{ab}4.6_{a}$	$^{b}4.0_{b}$	$^{b}\ 3.7_{b}$	$^{ab}62.0_{a}$	$^{ab}58.7_{a}$	$^{b}52.1_{b}$	$^{a}\ 38.9_{a}$	$^{a}\ 41.8_{a}$	$^{a}38.8_{a}$
	Spike length (cm)			**Harvest Index**			**Vegetative biomass (g)**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{a}11.5_{a}$	$^{a}11.0_{a}$	$^{a}10.9_{a}$	$^{ab}0.49_{a}$	$^{b}0.47_{b}$	$^{b}0.44_{b}$	$^{a}\ 10.6_{a}$	$^{a}10.1_{a}$	$^{a}\ 9.8_{a}$
MD05W10208-11-8	$^{d}9.0\ _{a}$	$^{b}8.9\ _{a}$	$^{c}8.9\ _{a}$	$^{ab}0.49_{a}$	$^{b}0.47_{b}$	$^{b}0.45_{c}$	$^{ab}10.2_{a}$	$^{b}8.5\ _{b}$	$^{b}\ 8.0_{b}$
SISSON	$^{c}9.8\ _{a}$	$^{b}9.2\ _{b}$	$^{c}8.8\ _{b}$	$^{a}\ 0.52_{a}$	$^{a}0.53_{a}$	$^{a}0.49_{b}$	$^{b}\ 9.4\ _{a}$	$^{b}8.4\ _{b}$	$^{b}\ 8.1_{b}$
OH06-150-57	$^{b}10.5_{a}$	$^{a}10.4_{ab}$	$^{b}9.9\ _{b}$	$^{b}\ 0.48_{a}$	$^{b}0.46_{b}$	$^{b}0.44_{c}$	$^{a}\ 11.0_{a}$	$^{a}10.6_{a}$	$^{ab}8.9_{b}$
	Grain protein content (%)			**NUEP**			**Grain protein reduction (%)**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{ab}14.5_{a}$	$^{bc}10.6_{b}$	$^{b}9.2\ _{c}$	$^{ab}3.6_{b}$	$^{a}4.3_{a}$	$^{a}4.5_{a}$	-	$^{a}\ 27.1_{b}$	$^{a}\ 36.3_{a}$
MD05W10208-11-8	$^{a}\ 15.3_{a}$	$^{a}\ 12.2_{b}$	$^{a}10.3_{c}$	$^{ab}3.6_{b}$	$^{a}4.1_{a}$	$^{a}4.4_{a}$	-	$^{b}\ 20.4_{b}$	$^{b}\ 32.3_{a}$
SISSON	$^{a}\ 15.5_{a}$	$^{b}\ 11.1_{b}$	$^{b}9.5\ _{c}$	$^{a}\ 3.8_{c}$	$^{a}4.5_{b}$	$^{a}4.9_{a}$	-	$^{a}\ 28.5_{b}$	$^{a}\ 38.3_{a}$
OH06-150-57	$^{b}\ 13.7_{a}$	$^{c}\ 10.3_{b}$	$^{b}9.4\ _{c}$	$^{b}\ 3.3_{b}$	$^{b}4.3_{a}$	$^{a}4.5_{a}$	-	$^{ab}24.9_{b}$	$^{b}\ 31.8_{a}$

Superscript and subscript letters represent mean comparisons among four accessions and among three nitrogen regimes, respectively. Values not sharing the same letter are significantly different ($P < 0.05$).

4.3.2 The effect of N fertility on correlation coefficient among yield components and grain protein content

Correlation analysis of yield components, grain protein content, and NUE was performed for each N regime separately (Table 2, below diagonal). This analysis allowed us to evaluate whether N fertility linearly affected these components. Correlation values between grain weight/plant and other traits are identical to those between NUEY and other traits because NUEY is determined by grain weight/plant divided by the amount of N supplied to the plant, but the N rate is not taken into account to calculate correlation coefficient at each N input. Grain weight/plant and NUEY were positively correlated with 1000-seed weight and harvest index at all N regimes, whereas yield reduction was negatively correlated with these traits and vegetative biomass. It appears that 1000-seed weight and harvest index are major parameters affecting grain yield and NUEY regardless of N regimes. Grain protein content was inversely associated with 1000-seed weight and grain protein reduction at all N regimes. NUEP was positively related to harvest index at the three N levels. Changes in N fertility affected correlations among yield components, grain protein, and NUE. For example, grain weight/plant and NUEY were positively correlated with most yield components such as spike number/plant, grain number/spike, 1000-seed weight, harvest index and vegetative biomass at low N. However, as nitrogen supply increased, many of these correlations were not significant. Grain weight/plant and NUEY were negatively correlated with grain protein content only at high and medium N, while they were positively associated with NUEP only at high and low N.

Correlation coefficient was also analyzed by combining all datasets collected from three N regimes (Table 2, above diagonal). In this analysis, more traits were significantly correlated with each other because the influence of nitrogen fertility in each trait is taken into account in

determining correlation coefficient. For example, grain weight/plant was positively correlated with all yield components surveyed, most of which were significantly altered by distinct N rates (Table 1). Likewise, positive relationship between grain weigh/plant and grain protein content was observed because N fertility increased both of these values. NUEY was inversely related to most yield components due to the adverse correlation between grain weight/plant and NUEY. However, NUEY was positively associated with 1000-seed weight, consistent with the observation at each N input. Grain protein content was negatively correlated with NUEP because NUEP decreased as N rate increased. A positive association was observed between NUEP and 1000-seed weight, but NUEP was adversely related to spike number/plant and vegetative biomass.

Table 2. Correlation coefficient analysis of yield components, grain protein content and nitrogen use efficiency over all nitrogen regimes (above diagonal) and under each nitrogen regime (below diagonal).

		Grain weight/ plant	NUEY	Grain yield reduction	Spike number/ plant	Grain number/ spike	1000-seed weight	Spike length	Harvest Index	Vegetative biomass	Grain protein content	NUEP	Protein reduction
	H	-											
Grain weight/ plant	M	-	-0.39***	-1.00***	0.43***	0.47***	0.22**	0.27**	0.64***	0.57***	0.59***	-0.34***	-0.19
	L	-											
	H	1.00***	-										
NUEY	M	1.00***	-	-0.33***	-0.33***	-0.23**	0.30***	-0.15	-0.26***	-0.20*	-0.87***	0.77***	0.60***
	L	1.00***	-										
	H	-	-	-									
Grain yield reduction	M	-1.00***	-1.00***	-	-0.29**	-0.37***	-0.49***	-0.24**	-0.55***	-0.57***	-0.69***	0.43***	0.22
	L	-1.00***	-1.00***	-									
	H	-0.02	-0.02	-	-								
Spike number/ plant	M	0.18	0.18	-0.20	-	-0.41***	-0.45***	0.07	-0.05	0.58***	0.42***	-0.36***	-0.17
	L	0.30*	0.30*	-0.32**	-								
	H	0.34*	0.34*	-	-0.74***	-							
Grain number/spike	M	0.14	0.14	-0.13	-0.70***	-	0.14	0.06	0.55***	0.02	0.32***	-0.12	-0.09
	L	0.35**	0.35**	-0.34*	-0.66***	-							
	H	0.36***	0.36***	-	-0.65***	0.26	-						
1000-seed weight	M	0.62***	0.62***	-0.62***	-0.29*	0.05	-	0.25**	0.27***	-0.02	-0.23*	0.22**	0.07
	L	0.43**	0.43**	-0.41**	-0.34*	0.30*	-						
	H	0.00	0.00	-	-0.15	0.02	0.24	-					
Spike length	M	0.34**	0.34**	-0.34*	-0.01	0.01	0.39**	-	-0.07	0.40***	0.07	-0.18	-0.01
	L	0.08	0.08	-0.08	0.08	-0.13	0.20	-					
	H	0.54***	0.54***	-	-0.36**	0.41**	0.41**	-0.12	-				
Harvest Index	M	0.41**	0.41**	-0.40**	-0.34**	0.39**	0.33*	-0.18	-	-0.26***	0.42***	-0.07	-0.18
	L	0.48***	0.48***	-0.47***	-0.28*	0.55***	0.32*	-0.39**	-				
	H	0.05	0.05	-	0.40**	-0.24	-0.24	0.18	-0.81***	-			
Vegetative biomass	M	0.41**	0.41**	-0.42**	0.50***	-0.25	0.18	0.45***	-0.65***	-	0.25**	-0.32***	-0.03
	L	0.69***	0.69***	-0.70***	0.58***	-0.08	0.17	0.39**	-0.30*	-			
	H	-0.54***	-0.54***	-	-0.01	-0.19	-0.35*	-0.27	-0.15	-0.23	-		
Grain protein content	M	-0.56***	-0.56***	0.54***	-0.13	0.01	-0.46**	-0.54***	0.08	-0.52***	-	-0.64***	-0.88***
	L	-0.17	-0.17	0.15	0.05	0.05	-0.43**	-0.41**	0.25	-0.36*	-		
	H	0.67***	0.67***	-	0.11	0.10	0.09	-0.11	0.52***	-0.16	0.27	-	
NUEP	M	0.30	0.30	-0.29	-0.11	0.13	0.24	0.07	0.41**	-0.18	-0.02	-	0.19
	L	0.42**	0.42**	-0.42**	-0.10	0.35*	0.35*	-0.07	0.45**	0.10	0.21	-	
	H	-	-	-	-	-	-	-	-	-	-	-	-
Protein reduction	M	0.43**	0.43**	-0.42**	0.07	0.09	0.26	0.20	0.28	0.08	-0.81***	0.06	-
	L	0.26	0.26	-0.25	-0.13	0.24	0.16	0.01	0.16	0.16	-0.72***	-0.08	-

H, M, and L represent high, medium and low nitrogen conditions, respectively. * P < 0.05; ** P < 0.01; *** P < 0.001.

4.3.3 The effect of N supply on accumulation of N and C compounds in grains

It is anticipated that N fertility changes the composition and abundance of N and C compounds in grains. To evaluate this, the concentrations of protein, total amino acids, ammonium, nitrate, starch and total soluble sugars were quantified in grains of three plants per cultivar and per N rate that were randomly selected from 14 plants used for yield component analysis (Table 3). Among N compounds, protein, total amino acid, and ammonium contents generally declined in response to reduced N application. On the other hand, accumulation of nitrate increased under lower N supply in all genotypes, and grain starch content was slightly elevated in all genotypes except for MD05W10208-11-8. Nitrogen responses of soluble sugar content in grains were genotype-dependent. MD05W10208-11-8, which exhibited the lowest grain yield at low N, contained the highest protein, total amino acids, ammonium, and nitrate, but had the lowest starch and soluble sugars.

Correlation of N and C compounds in grains was analyzed for each N treatment (Table 4, below diagonal). Protein level was positively correlated with amino acid and ammonium contents at all N regimes. However, a positive relationship between amino acid and ammonium contents was detected only at high and medium N. It was also observed that total soluble sugar content was positively correlated with protein and ammonium levels at high N. In contrast, total soluble sugar was adversely related to nitrate at medium N and protein and amino acids at low N. We also analyzed correlations among these traits by combining all N datasets (Table 4, above diagonal). Grain protein, total amino acid, and ammonium contents were positively and strongly correlated with each other, whereas protein and ammonium contents were inversely associated with nitrate level. In addition, a positive relationship between ammonium and total soluble sugar contents was detected.

Table 3. The effect of nitrogen application on the accumulation of nitrogen compounds and carbohydrates in grains of four soft red winter wheat accessions.

	Protein (mg/g)			Total amino acids (μmol/g)			Ammonium (μmol/g)		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{a}146.6_{a}$	$^{b}102.2_{b}$	$^{c}93.2_{c}$	$^{a}30.1_{a}$	$^{c}19.9_{b}$	$^{c}17.9_{c}$	$^{b}839.9_{a}$	$^{c}423.5_{b}$	$^{b}406.7_{b}$
MD05W10208-11-8	$^{a}145.7_{a}$	$^{a}119.8_{b}$	$^{a}116.4_{b}$	$^{a}31.4_{a}$	$^{b}25.1_{c}$	$^{a}26.2_{b}$	$^{b}834.7_{a}$	$^{b}599.2_{c}$	$^{a}655.4_{b}$
SISSON	$^{a}154.4_{a}$	$^{a}116.2_{b}$	$^{b}101.1_{c}$	$^{a}30.5_{a}$	$^{a}27.5_{a}$	$^{b}20.9_{b}$	$^{a}1011.9_{a}$	$^{a}909.8_{b}$	$^{a}637.9_{c}$
OH06-150-57	$^{b}135.2_{a}$	$^{ab}109.2_{b}$	$^{b}101.7_{c}$	$^{b}18.1_{a}$	$^{d}17.4_{a}$	$^{c}16.9_{a}$	$^{c}428.9_{b}$	$^{b}516.2_{b}$	$^{a}670.5_{a}$
	Nitrate (μmol/g)			**Starch (mmol/g)**			**Soluble sugars (μmol/g)**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{a}9.8_{c}$	$^{a}11.3_{b}$	$^{a}13.2_{a}$	$^{a}2.8_{b}$	$^{a}2.8_{b}$	$^{ab}3.2_{a}$	$^{b}227.8_{ab}$	$^{b}218.9_{b}$	$^{a}245.3_{a}$
MD05W10208-11-8	$^{b}7.1_{b}$	$^{ab}10.7_{a}$	$^{ab}12.6_{a}$	$^{a}2.9_{a}$	$^{a}2.9_{a}$	$^{c}2.7_{a}$	$^{bc}209.6_{a}$	$^{b}205.9_{a}$	$^{c}204.8_{a}$
SISSON	$^{b}7.1_{b}$	$^{bc}9.1_{a}$	$^{c}8.6_{a}$	$^{a}2.7_{b}$	$^{a}2.9_{ab}$	$^{bc}3.1_{a}$	$^{a}260.6_{a}$	$^{a}248.8_{a}$	$^{b}226.6_{b}$
OH06-150-57	$^{ab}7.4_{b}$	$^{c}8.3_{b}$	$^{b}11.7_{a}$	$^{a}2.7_{b}$	$^{a}2.9_{b}$	$^{a}3.5_{a}$	$^{c}189.2_{b}$	$^{a}244.7_{a}$	$^{a}249.6_{a}$

Superscript and subscript letters represent mean comparisons among four accessions and among three nitrogen regimes, respectively. Values not sharing the same letter are significantly different ($P < 0.05$).

Table 4. Correlation coefficient analysis of nitrogen compounds and carbohydrate contents in grains under all nitrogen conditions (above diagonal) and under each nitrogen regime (below diagonal).

		Protein	Amino acids	Ammonium	Nitrate	Starch	Total soluble sugars
Protein	H	-	0.72***	0.62***	-0.52**	-0.01	-0.11
	M	-					
	L	-					
Amino acids	H	0.70*	-	0.80***	-0.27	-0.22	-0.01
	M	0.72**	-				
	L	0.75**	-				
Ammonium	H	0.80**	0.88***	-	-0.32*	0.12	0.32*
	M	0.65*	0.82***	-			
	L	0.62*	0.36	-			
Nitrate	H	-0.18	0.18	0.14	-	-0.06	-0.12
	M	-0.26	-0.06	-0.35	-		
	L	0.05	-0.14	-0.47	-		
Starch	H	-0.31	-0.54	-0.32	-0.13	-	0.23
	M	0.22	-0.14	0.34	-0.41	-	
	L	-0.05	-0.34	0.43	-0.03	-	
Total soluble sugars	H	0.79**	0.50	0.69*	-0.19	0.13	-
	M	0.05	-0.03	0.39	-0.61*	0.56	-
	L	-0.73**	-0.64*	-0.26	0.01	0.35	-

H, M, and L represent high, medium and low nitrogen input, respectively. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

4.3.4 Accumulation of N compounds in flag leaves under three N regimes

To evaluate the effect of N supply on the accumulation of N compounds in flag leaves at anthesis, the amount of protein, total amino acids, ammonium, and nitrate were quantified in the four genotypes (Table 5). Significant variation for all of these N compounds was detected among these accessions at high N input. A decline in N application rate reduced the levels of protein, total amino acids, and ammonium in the four genotypes, but the amount of nitrate was elevated in IL07-4415 and Sisson in response to lower N. In Sisson, the level of nitrate was highest under low N supply, whereas the abundance of protein, total amino acid, and ammonium was relatively low as compared to the other three genotypes. The contents of chlorophyll *a* and *b* in flag leaves were almost similar among the four genotypes at high N input. However, the abundance of chlorophyll *a* and *b* was reduced in response to lower N application at distinct rates in these accessions. Under low N input, the amount of chlorophyll *a* and *b* was lowest in Sisson.

Correlation analysis of these traits was performed for each N regime (Table 6, below diagonal). The amount of total amino acids and ammonium was positively correlated at all N regimes. Nitrate level was negatively related to the accumulation of total amino acids and ammonium under all N conditions. No significant correlation was detected between protein and other N-compounds at any N regime. However, protein content was positively and strongly correlated with the accumulation of chlorophyll *a* and *b* but only at low N. Correlation coefficient was also calculated over all N treatments (Table 6, above diagonal). Positive correlation was detected among protein, amino acids, ammonium, and chlorophyll *a* contents. Conversely, nitrate content was negatively correlated with total amino acids, ammonium, and chlorophyll *a* levels.

Table 5. The effect of nitrogen application on the accumulation of nitrogen compounds in flag leaves of four soft red winter wheat accessions.

	Protein (mg/g FW)			**Total amino acids (µmol/g FW)**			**Ammonium (µmol/g FW)**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{ab}20.5_{a}$	$^{a}13.5_{b}$	$^{b}\ 9.0_{c}$	$^{a}43.7_{a}$	$^{a}33.8_{b}$	$^{b}30.0_{c}$	$^{a}5.4_{a}$	$^{a}3.3_{b}$	$^{a}2.5_{c}$
MD05W10208-11-8	$^{b}\ 18.4_{a}$	$^{b}\ 8.6_{c}$	$^{a}12.9_{b}$	$^{a}44.6_{a}$	$^{a}33.7_{b}$	$^{a}32.0_{b}$	$^{a}5.5_{a}$	$^{a}3.2_{b}$	$^{a}2.5_{c}$
SISSON	$^{ab}19.6_{a}$	$^{a}13.3_{b}$	$^{b}\ 9.0_{c}$	$^{b}35.7_{a}$	$^{b}27.2_{b}$	$^{c}23.1_{c}$	$^{c}1.5_{a}$	$^{b}1.6_{a}$	$^{c}1.0_{b}$
OH06-150-57	$^{a}\ 21.1_{a}$	$^{a}14.3_{b}$	$^{a}14.5_{b}$	$^{b}33.5_{a}$	$^{b}26.9_{b}$	$^{c}23.0_{c}$	$^{b}2.4_{a}$	$^{b}1.8_{b}$	$^{b}1.5_{c}$
	Nitrate (µmol/g FW)			**Chlorophyll a (µmol/g FW)**			**Chlorophyll b (µmol/g FW)**		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
IL07-4415	$^{d}12.1_{\ b}$	$^{c}15.7_{a}$	$^{c}16.9_{a}$	$^{a}2.2_{a}$	$^{b}\ 2.0_{\ b}$	$^{bc}1.8_{b}$	$^{b}0.46_{a}$	$^{b}\ 0.42_{a}$	$^{c}0.36_{a}$
MD05W10208-11-8	$^{c}18.0_{ab}$	$^{b}20.4_{a}$	$^{c}16.5_{b}$	$^{a}2.3_{a}$	$^{a}\ 2.2_{ab}$	$^{a}\ 2.1_{b}$	$^{b}0.52_{a}$	$^{ab}0.52_{a}$	$^{b}0.44_{b}$
SISSON	$^{b}31.7_{\ c}$	$^{a}42.2_{b}$	$^{a}61.2_{a}$	$^{a}2.2_{a}$	$^{ab}2.1_{\ a}$	$^{c}\ 1.7_{b}$	$^{b}0.46_{a}$	$^{ab}0.44_{a}$	$^{c}0.33_{b}$
OH06-150-57	$^{a}37.4_{\ a}$	$^{b}23.8_{b}$	$^{b}21.1_{c}$	$^{a}2.3_{a}$	$^{b}\ 1.9_{\ b}$	$^{b}\ 2.0_{b}$	$^{a}0.64_{a}$	$^{a}\ 0.53_{b}$	$^{a}0.53_{b}$

Superscript and subscript letters represent mean comparisons among four accessions and among three nitrogen regimes, respectively. Values not sharing the same letter are significantly different ($P < 0.05$).

Table 6. Correlation coefficient analysis of nitrogen compound and chlorophyll contents in flag leaves over all nitrogen regimes (above diagonal) and under each nitrogen regime (below diagonal).

		Protein	Amino acids	Ammonium	Nitrate	Chlorophyll a	Chlorophyll b
Protein	H	-					
	M	-	0.58***	0.33*	-0.15	0.61***	0.45**
	L	-					
Amino acids	H	-0.04	-				
	M	-0.50	-	0.84***	-0.50**	0.66***	0.19
	L	0.01	-				
Ammonium	H	-0.17	0.83***	-			
	M	-0.49	0.91***	-	-0.66***	0.48**	0.16
	L	0.16	0.87***	-			
Nitrate	H	0.28	-0.80***	-0.82***	-		
	M	0.19	-0.69*	-0.87***	-	-0.33*	-0.13
	L	-0.46	-0.62*	-0.89***	-		
Chlorophyll *a*	H	0.10	0.13	0.26	-0.06	-	
	M	-0.53	0.25	0.13	0.13	-	0.69***
	L	0.71***	0.47	0.56	-0.70*	-	
Chlorophyll *b*	H	0.17	-0.38	-0.20	0.56	0.42	-
	M	-0.25	0.02	0.13	-0.11	0.76***	-
	L	0.82***	-0.07	0.24	-0.58*	0.79***	-

H, M and L represent high, medium and low nitrogen input, respectively. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

4.4 DISCUSSION

This study evaluated the influence of nitrogen fertility on grain yield, grain protein content, NUE and their associated traits in four soft red winter wheat genotypes. Among these accessions, Sisson exhibited the best performance in terms of grain weight/plant, NUEY, and grain yield at low N, which can be attributed to its superior grain number/spike and harvest index under N-limited conditions. Sisson is broadly adapted and was a top yielding cultivar when it was released in 2000 (*Griffey* et al., 2003). In general, modern high-yielding wheat cultivars including Sisson have been developed through the selection at high or optimal N levels. However, a large-scale yield analysis of 225 winter wheat accessions from 11 European countries in four environments revealed that there is a strong linear relationship ($R^2 = 0.74$, $P < 0.001$) between grain yields at high and low N; recently released cultivars yielded more than older ones at both N conditions (*Cormier* et al., 2013). Consistent results have also been observed by other groups in the UK, French, Italian and Mexican winter wheat genotypes (*Ortiz-Monasterio* et al., 1997; *Brancourt-Hulmel* et al., 2003; *Guarda* et al., 2004; *Gaju* et al., 2011). These data suggest that genetic mechanisms underlying grain productivity are similar at high and low N in winter wheat. However, the mechanisms that contribute to grain production mainly under N limitation should not be dismissed as unimportant. Detailed comparative analysis of high and low NUE genotypes at physiological and molecular levels will facilitate the identification of key components that determine grain yield under high N, low N, and both conditions.

The responsiveness of yield components to environmental factors has been evaluated in cereals including wheat (*Sadras* & *Slafer*, 2012; *Slafer* et al., 2014). According to these reports, the responsiveness of spike number/plant is similar to that of grain number/spike. However, their

responsiveness is more sensitive than that of grain weight. Consistently, the current demonstrated that spike number/plant and grain number/spike significantly declined in response to reduced N supply in most accessions, whereas a reduction in N supply did not change 1000-seed weight in any of the genotypes (Table 1). It has been confirmed that responsiveness of yield components to environmental factors is inversely associated with their heritability in wheat and other cereals (*Sadras* & *Slafer*, 2012). Due to its high heritability, grain weight may be a major contributor for genotypic variation in grain yield at variable N rates. Indeed, 1000-seed weight was positively correlated with grain weight/plant (equivalent to grain yield) at all N rates (Table 2). In contrast, correlation of spike number/plant and grain number/spike with grain weight/plant was not significant at higher N levels, presumably due to the lower heritability of these traits. These results suggest that 1000-seed weight (grain weight) can be a key component determining grain yield irrespective of N availability. Further validation of this finding under field conditions is necessary, where there is significantly higher genotype by environmental interaction.

Grain protein content is a critical trait associated with the milling and baking quality of wheat grains. Consistent with other field studies (*Kibite* & *Evans*, 1984; *Oury* & *Godin*, 2007; *Bogard* et al., 2010), grain protein content was inversely associated with grain weight/plant and NUEY at high and medium N (Table 2). Similarly, grain protein content was also adversely correlated with yield components such as spike number/plant, grain number/spike, 1000-seed weight, spike length and vegetative biomass under at least one N regime. These negative relationships have made it difficult to improve grain yield and grain protein content simultaneously (*Bogard* et al., 2010). In fact, grain protein and nitrogen contents decreased with the year of cultivar release in Italian and Spanish winter wheat (*Guarda* et al., 2004; *Acreche* & *Slafer*, 2009). For sustainable wheat production, NUEP is an important trait to reduce N usage

per unit area while maintaining grain protein content. Correlation analysis revealed that NUEP is positively correlated with harvest index regardless of N fertility (Table 2). Likewise, NUEP was positively associated with grain weight/plant, grain number/spike, and 1000-seed weight at low N. Based on these data, it can be anticipated that sink strength of spikes might largely influence NUEP, especially when N availability is limited.

Flag leaves provide the majority of assimilates for grains during post-anthesis and grain filling stages (*Simpson* et al., 1983). It has been reported that increased nitrogen supply results in increased leaf area, leaf nitrogen and chlorophyll contents in flag leaves of wheat (*Evans*, 1983). Conversely, nitrogen limitation reduces the size of leaves due to lower cell numbers and volume together with protein and chlorophyll contents (*Lawlor* et al., 1989). Consistent results were observed in the current study where increased N supply raised protein and chlorophyll contents in flag leaves at anthesis (Table 5). In addition, increasing N supply also resulted in greater accumulation of total amino acids and ammonium, which were positively correlated under all N regimes (Table 6). The effect of N fertility on nitrate accumulation in flag leaves varied among genotypes. Nevertheless, the level of nitrate was inversely and strongly associated with amino acid and ammonium accumulation at all N regimes. It seems that the reduction rates of nitrate and nitrite into ammonium are critical determinants for the concentrations of ammonium and total amino acids in flag leaves, regardless of N fertility. We also observed that protein content was adversely correlated with the levels of chlorophyll *a* and *b* but only at low N. This result suggests that protein and chlorophyll biosynthesis compete with each other when N availability is limited in flag leaves.

Consistent with the observations in flag leaves, limited N supply considerably restricted the accumulation of major N compounds such as protein, total amino acids and ammonium in

grains (Table 3). These N compounds were positively correlated with each other under all N regimes except for the correlation between ammonium and total amino acids at low N (Table 4). In contrast to N compounds, the levels of C compounds such as starch and soluble sugars were stable or only slightly altered at variable N rates (Table 3). It seems that N fertility does not largely affect grain carbohydrate contents in soft red winter wheat. Limitation of N availability reduces chlorophyll, protein and photosynthesis-related enzyme (e.g., Rubisco) contents in flag leaves (*Evans*, 1983; *Lawlor* et al., 1989; *Zhu* et al., 2010), which can result in reduced performance for biosynthesis of both C and N compounds. A large decline in protein content and stabilized carbohydrate level in grains at low N can be attributed to distinct energy costs required for protein, amino acid, soluble carbohydrates and starch synthesis. Indeed, production of protein and amino acids requires more energy and carbon costs than that of soluble carbohydrates and starch (*Vertregt* & *Penning de Vries*, 1987; *Munier-Jolain* & *Salon*, 2005). Reduced accumulation of protein and other N compounds in grain may be an energy-saving strategy to maintain carbohydrate synthesis and minimize yield loss under N-limited conditions. It has also been demonstrated that grain protein content is positively and strongly correlated with total soluble sugar content at high N, whereas these two components are adversely associated at low N (Table 4). This result indicates that trade-off between protein and soluble carbohydrate synthesis is not necessary when a sufficient amount of N is available, but production of these compounds compete with each other under N limitation.

4.5 CONCLUSION

Comparative analysis of yield components and NUE along with N and C components in grains and flag leaves revealed positive and negative relationships among these traits, many of which were considerably altered in response to N fertility. However, some traits with high heritability such as 1000-seed weight and harvest index were consistently associated with grain weight/plant (equivalent to grain yield), NUEY and other yield components at all N regimes. It is widely accepted that there is an inverse relationship between grain yield and grain protein content in wheat and other cereals and we confirmed this under high and medium N regimes. Interestingly, correlation analysis revealed that NUEY is positively related to NUEP at high and low N, suggesting that the two NUE traits could be improved simultaneously in soft red winter wheat. It was also observed that both NUEY and NUEP are positively associated with grain number/spike, 1000-seed weight and harvest index at low N. These yield components likely would be key traits to select for high NUEY and NUEP under N-limited conditions. Analysis of N components in flag leaves and grains demonstrated that relationships among protein, amino acid, ammonium and nitrate contents are conserved in the two tissues. Further analysis (e.g., time-course observation in flag leaves during seed filling) is required to determine how regulation of nitrogen metabolism in flag leaves affect grain yield and grain N content in soft red winter wheat at variable N rates.

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CHAPTER 5

SUMMARY AND CONCLUSION

The climbing global population coupled with changing climate has posed a serious threat in the sustainability of current food production systems. While crop productivity needs to be improved significantly to match the global demand, unfavorable climatic conditions are on the rise, impacting our ability to reach the goal of ensuring a sufficient food supply. In order to address this issue, genetic improvement of abiotic stress tolerance of crops is of primary importance. On the other hand, to improve crop productivity, we have been increasingly adding synthetic nitrogenous fertilizers to agricultural systems. Although the use of such chemicals has significantly increased the quality and quantity of agricultural products, excessive usage has its own caveats that can impact human health and create imbalances in ecosystems. Therefore sustaining economical crop production and protecting the environment requires either a reduction in or more efficient use of fertilizers.

In this study, we characterized physiological and transcriptomic responses to flooding and drought tolerance in soybean and identified key traits associated with nitrogen-use-efficiency in wheat. These two species are commercially important crops widely grown across the globe. Although no nitrogenous fertilizers are used for soybean cultivation (as it is a legume and can fix atmospheric nitrogen in the roots via nitrogen fixing bacterium), it faces devastating flooding and drought events. Thus, to improve our knowledge on soybean flooding and drought tolerance, we undertook the studies of Chapter 2 and 3. On the other hand, wheat cultivation takes place under extensive use of nitrogenous fertilizers. Furthermore, wheat has the highest area coverage for production and consumes the largest share of manufactured nitrogenous fertilizers globally. Therefore, the study in Chapter 4 was formulated to dissect the relationship between nitrogen use

efficiency and agronomic traits, contributing to the breeding of efficient nitrogen-use varieties in wheat.

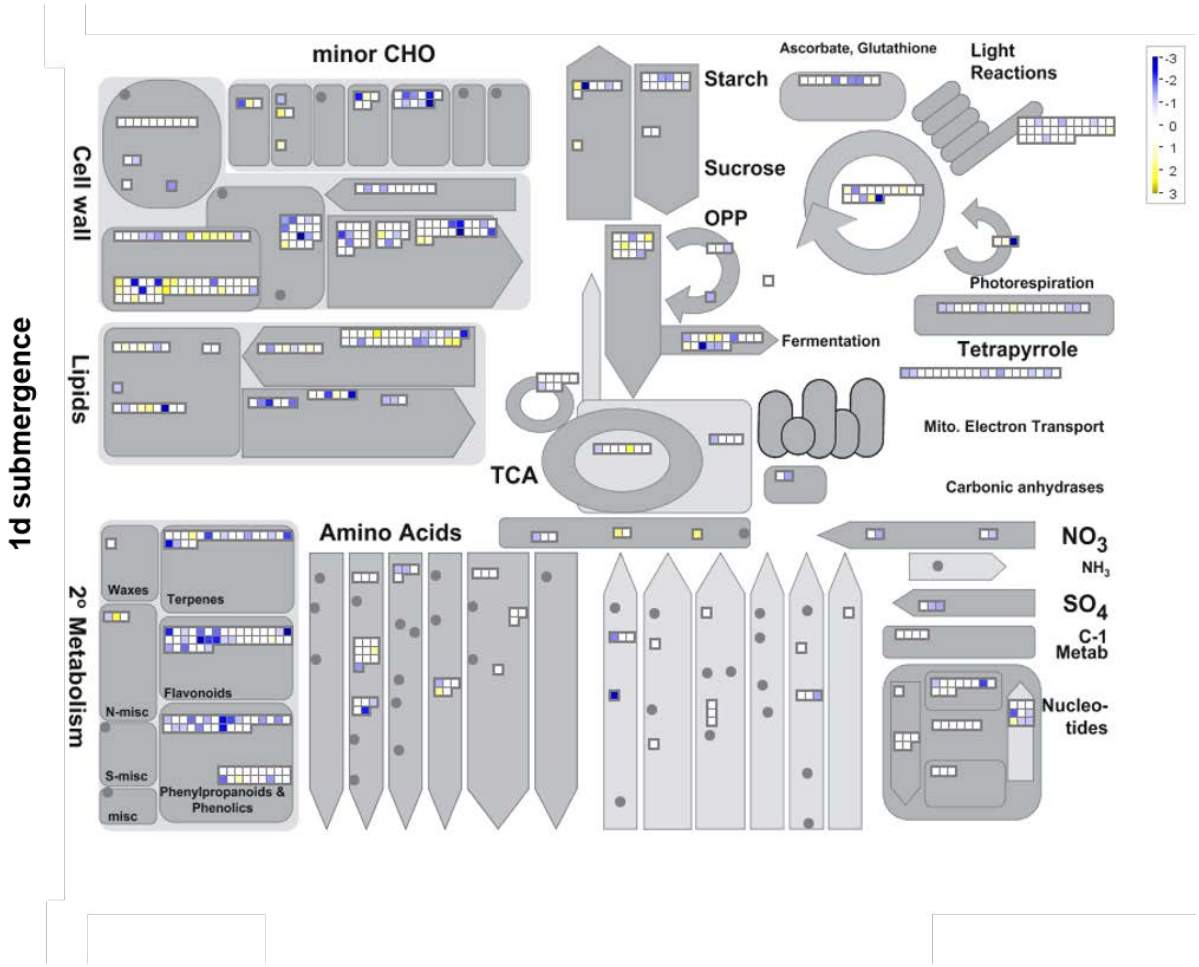
Williams 82 soybean was the cultivar of our choice for Chapter 2 and 3 as it was the cultivar used for genome sequencing. In Chapter 2, we characterized the responses of soybean seedlings during complete submergence and subsequent reoxygenation. In Chapter 3, we integrated drought stress and conducted a combined study of the responses of soybean during complete submergence, gradual drought and recovery from these stresses at the early vegetative stage. Through our comparative physiological and transcriptomic studies, we highlighted stress-specific, organ-specific and overlapped regulation of gene expression and metabolic reconfiguration which enhances the adaptability to the stresses. We also identified nine group-VII ERF transcription factors in soybean genome from our study and characterized their responses under these stresses and recovery from them. This study highlighted the importance of two out of the nine ERF VIIs during submergence and drought. Functional characterization of these genes is underway.

In Chapter 4, we selected four diverse wheat accessions based on the nitrogen-use-efficiency in a study of 281 soft red winter wheat germplasms. Our greenhouse experiment on the genotypes under three nitrogen regimes determined the response to nitrogen fertility on yield and its components. We showed that grain number/spike, 1000-seed weight, and harvest index could be key traits for selection to enhance yield and grain protein content in soft red winter wheat under low nitrogen environments. Time-course observation of gene expression and metabolites associated with nitrogen use efficiency is being carried out in flag leaves of representative soft red winter wheat accessions.

APPENDICES

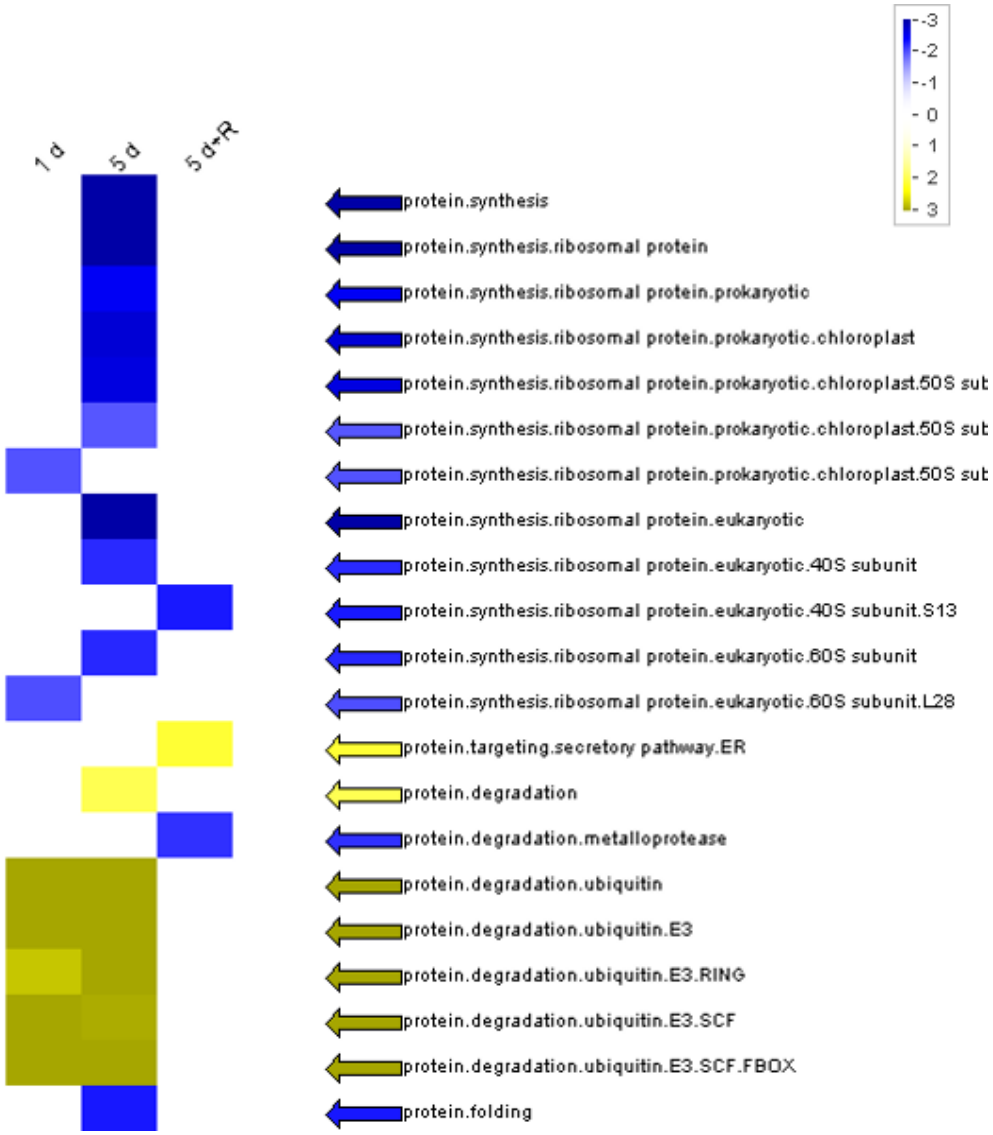
Appendix A: Supplementary Files for Chapter 2

Supplementary Appendix A1



Expression changes of genes encoding enzymes involved in major metabolic pathways in response to 1 d of submergence. Alterations in the abundance of mRNAs with significant adjusted *P*-values ($P < 0.05$) and more than twofold changes in expression (SLR > 1 or SLR< −1) were visualized on the metabolic pathway diagrams. The log2 values of fold changes (1 d submergence versus non-submergence control) are displayed using the color code.

Supplementary Appendix A2



PageMan analysis of pathways associated with protein synthesis, degradation, targeting and folding which were upregulated or downregulated in response to submergence and reoxygenation. Genes with significant adjusted *P*-values ($P < 0.05$) and more than twofold changes in expression (SLR > 1 or SLR < −1) were utilized for the analysis. The log2 values of fold changes are displayed using the color code.

Supplementary Appendix A3

Primer sequences and PCR conditions used for quantitative RT-PCR

Gene	Locus name	Forward primer	Reverse primer	Annealing temp (°C)	Product size (bp)
ERFVII1	Glyma02g01960	TCCCTCCATTCTGCTGACTAAACT	CCGAAGCACTCAATTTAAATTCCTGTG	60	78
ERFVII2	Glyma03g42450	CATCCCCCCATGTGTCTGAAAA	GCCCCATACTTTAGATACAAGAGATGC	60	134
ERFVII3	Glyma07g04950	GGTGAATCTCAGTTTGTGCAGGG	TGGGATTCAATGTCTGCAAGCTC	60	128
ERFVII4	Glyma09g04630	GGTTTGGAGAATGAGCTGCCTG	GTCCAGCATCCACAGGTCATC	60	78
ERFVII5	Glyma10g02080	GCGTTTTAGCAGGGATGGACA	TCAACAAAACCAGATGATAGCCCTAAG	60	150
ERFVII6	Glyma15g16260	TGTCTTGTCCTCTCGCTTTGGT	GCACGACCCAAACGCTAACAC	60	149
ERFVII7	Glyma16g01500	CTTTGGAGGGTGCTGCTGATC	TTGAGCTGGGATTCAATGTCTGC	60	121
ERFVII8	Glyma19g40070	CACGTGGCTTACTCCGTTACG	AGGTGGAGAGGTGTCAGGAAAG	60	147
ERFVII9	Glyma19g45200	GGGAGTAATTCATTTGGGTGGAGTG	TTAGGCTGCTGGTTGGCATTT	60	119
ADH1	Glyma14g27940	CCATGACTGTGTTCTGGGACAG	GCCCTGTCATTCCCAACACT	60	137
ADH3	Glyma06g12780	TGATAGGGCCTTTGCTGCTG	CACACCATTGGACATGCGTTG	60	150
Fbox	Glyma12g05510	AGATAGGGAAATTGTGCAGGT	CTAATGGCAATTGCAGCTCTC	60	93
RPL30	Glyma17g05270	AAAGTGGACCAAGGCATATCGTCG	TCAGGACATTCTCCGCAAGATTCC	60	125

Supplementary Appendix A4

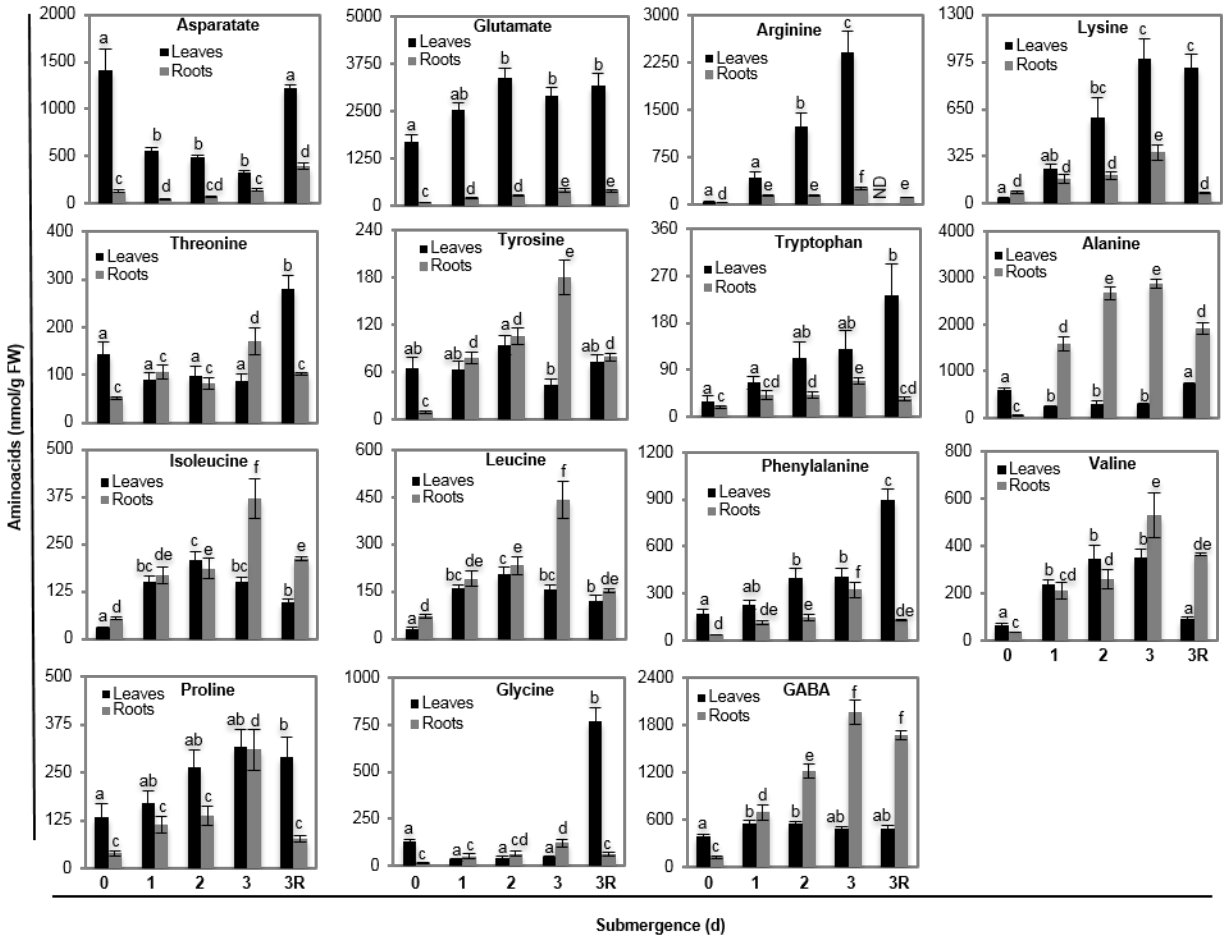
Genes associated with ROS detoxification which were differentially upregulated in response to 1 d of reoxygenation relative to 5 d of submergence.

		1 d reoxy / 5 d sub			
Affy ID	Gene ID	logFC	adj.P.Val	Ath ortholog	Annotation
11771335	Glyma01g36780	1.375373	0.004698	AT5G42180.1	Peroxidase superfamily protein
11780889	Glyma01g34360	1.205302	0.014198	AT2G02380.1	glutathione S-transferase (class zeta) 2
11782083	Glyma01g37550	2.238967	0.000692	AT3G51030.1	thioredoxin H-type 1
11794571	Glyma02g40000	2.428872	0.010271	AT5G05340.1	Peroxidase superfamily protein
11804229	Glyma02g28880	2.801162	0.000271	AT5G06720.1	peroxidase 2
11805514	Glyma02g35780	1.319412	0.041664	AT5G06290.1	2-cysteine peroxiredoxin B
11816130	Glyma03g30180	1.945015	0.006023	AT5G06720.1	peroxidase 2
11817326	Glyma03g33340	2.416631	0.000352	AT5G02790.1	Glutathione S-transferase family protein
11819966	Glyma03g40280	1.137434	0.009787	AT1G08830.1	copper/zinc superoxide dismutase 1
11824225	Glyma03g16600	1.185158	0.034125	AT3G09270.1	glutathione S-transferase TAU 8
11832785	Glyma04g02360	1.420208	0.004441	AT1G20225.1	Thioredoxin superfamily protein
11841623	Glyma04g39930	1.433363	0.005277	AT3G10920.1	manganese superoxide dismutase 1
11848305	Glyma04g17310	1.591804	0.005634	AT3G06730.1	Thioredoxin z
11867917	Glyma05g23090	1.749238	0.011231	AT3G11630.1	Thioredoxin superfamily protein
11889190	Glyma06g05510	2.170089	0.001626	AT4G33040.1	Thioredoxin superfamily protein
11892859	Glyma06g14960	1.207428	0.010934	AT3G10920.1	manganese superoxide dismutase 1
11898682	Glyma06g42850	1.819925	0.003216	AT5G05340.1	Peroxidase superfamily protein
11901801	Glyma07g03660	1.258291	0.026647	AT3G15660.1	glutaredoxin 4
11903866	Glyma07g09240	1.018646	0.014964	AT1G65980.1	thioredoxin-dependent peroxidase 1
11905950	Glyma07g15800	1.013093	0.011341	AT3G09390.1	metallothionein 2A
11918290	Glyma07g19060	1.934129	0.025934	AT5G01600.1	ferretin 1
11922533	Glyma07g39020	1.622382	0.002624	AT4G21960.1	Peroxidase superfamily protein
11926083	Glyma08g07690	1.100274	0.010099	AT5G64210.1	alternative oxidase 2
11930259	Glyma08g18660	3.255126	0.000104	AT1G78380.1	glutathione S-transferase TAU 19
11933691	Glyma08g27720	2.765795	0.005385	AT5G38900.1	Thioredoxin superfamily protein
11945593	Glyma08g18690	1.812732	0.003114	AT1G78380.1	glutathione S-transferase TAU 19
11954225	Glyma09g02590	1.3692	0.032396	AT5G06720.1	peroxidase 2
11954237	Glyma09g02610	1.098331	0.01677	AT5G06730.1	Peroxidase superfamily protein
11954257	Glyma09g02670	1.378489	0.020814	AT5G06720.1	peroxidase 2
11972484	Glyma09g32540	2.875201	0.037087	AT1G65980.1	thioredoxin-dependent peroxidase 1
11974489	Glyma09g38470	1.040393	0.023278	AT5G16400.1	thioredoxin F2
11975625	Glyma09g41440	1.359717	0.02054	AT5G05340.1	Peroxidase superfamily protein
11975632	Glyma09g41450	1.440596	0.019886	AT5G05340.1	Peroxidase superfamily protein
11977628	Glyma10g04570	1.157061	0.011907	AT5G44000.1	Glutathione S-transferase family protein
11996479	Glyma10g33650	1.538898	0.001867	AT1G59670.1	glutathione S-transferase TAU 15
12000235	Glyma10g43730	2.62053	0.003476	AT1G75270.1	dehydroascorbate reductase 2
12010009	Glyma11g31330	1.970418	0.004076	AT1G78380.1	glutathione S-transferase TAU 19

12014348	Glyma11g05300	1.981563	0.008221	AT4G37530.1	Peroxidase superfamily protein
12015339	Glyma11g08520	3.053581	0.000334	AT5G42180.1	Peroxidase superfamily protein
12018981	Glyma11g19840	2.776861	0.005013	AT2G28190.1	copper/zinc superoxide dismutase 2
12019769	Glyma11g25540	1.500376	0.007416	AT3G06730.1	Thioredoxin z
12026532	Glyma12g08650	1.405777	0.012697	AT2G28190.1	copper/zinc superoxide dismutase 2
12031192	Glyma12g32160	1.388556	0.014553	AT1G05260.1	Peroxidase superfamily protein
12035735	Glyma12g07780	1.667093	0.001021	AT1G07890.1	ascorbate peroxidase 1
12044009	Glyma13g03650	1.447278	0.003466	AT5G21105.1	Plant L-ascorbate oxidase
12049008	Glyma13g19830	3.064754	0.000168	AT5G02790.1	Glutathione S-transferase family protein
12059693	Glyma13g04640	1.197651	0.02715	AT2G04700.1	ferredoxin thioredoxin reductase catalytic beta chain family protein
12080934	Glyma14g38210	1.174997	0.011245	AT5G58400.1	Peroxidase superfamily protein
12093703	Glyma15g06650	1.089317	0.013569	AT4G21860.1	methionine sulfoxide reductase B 2
12096271	Glyma15g13510	1.626309	0.002172	AT5G06730.1	Peroxidase superfamily protein
12096304	Glyma15g13560	2.112071	0.001077	AT5G06730.1	Peroxidase superfamily protein
12097760	Glyma15g18310	1.153086	0.006606	AT5G20500.1	Glutaredoxin family protein
12101580	Glyma15g40210	1.033875	0.045289	AT1G78380.1	glutathione S-transferase TAU 19
12112879	Glyma15g40250	1.20633	0.009069	AT1G78380.1	glutathione S-transferase TAU 19
12113599	Glyma15g42660	1.635744	0.002814	AT5G42850.1	Thioredoxin superfamily protein
12116807	Glyma16g07870	1.228736	0.005083	AT5G40370.1	Glutaredoxin family protein
12121998	Glyma16g32490	2.759445	0.001125	AT5G51890.1	Peroxidase superfamily protein
12131818	Glyma17g01720	2.573629	0.000194	AT4G21960.1	Peroxidase superfamily protein
12133289	Glyma17g05150	1.000847	0.019995	AT3G26060.1	Thioredoxin superfamily protein
12141214	Glyma17g34870	2.089375	0.011845	AT3G09390.1	metallothionein 2A
12153067	Glyma17g37280	1.540459	0.002742	AT3G51030.1	thioredoxin H-type 1
12158500	Glyma18g16710	2.330508	0.001016	AT5G39950.1	thioredoxin 2
12162633	Glyma18g44320	1.506365	0.025702	AT5G05340.1	Peroxidase superfamily protein
12164791	Glyma18g50940	1.609226	0.003862	AT5G38900.1	Thioredoxin superfamily protein
12183186	Glyma19g33080	1.917159	0.016364	AT5G06720.1	peroxidase 2
12195475	Glyma19g37500	1.892004	0.000692	AT3G52960.1	Thioredoxin superfamily protein
12201052	Glyma20g12150	1.610058	0.001849	AT5G21105.1	Plant L-ascorbate oxidase
12205358	Glyma20g30910	1.92354	0.000996	AT1G71695.1	Peroxidase superfamily protein
12206156	Glyma20g32850	1.048373	0.030717	AT1G66240.1	homolog of anti-oxidant 1
12209380	Glyma20g01910	1.659429	0.002238	AT1G78370.1	glutathione S-transferase TAU 20
12211027	Glyma20g12510	1.188587	0.022096	AT5G23310.1	Fe superoxide dismutase 3
12212788	Glyma20g23420	1.36382	0.005608	AT3G09270.1	glutathione S-transferase TAU 8
12218204	Glyma20g38440	1.150415	0.029435	AT1G75270.1	dehydroascorbate reductase 2

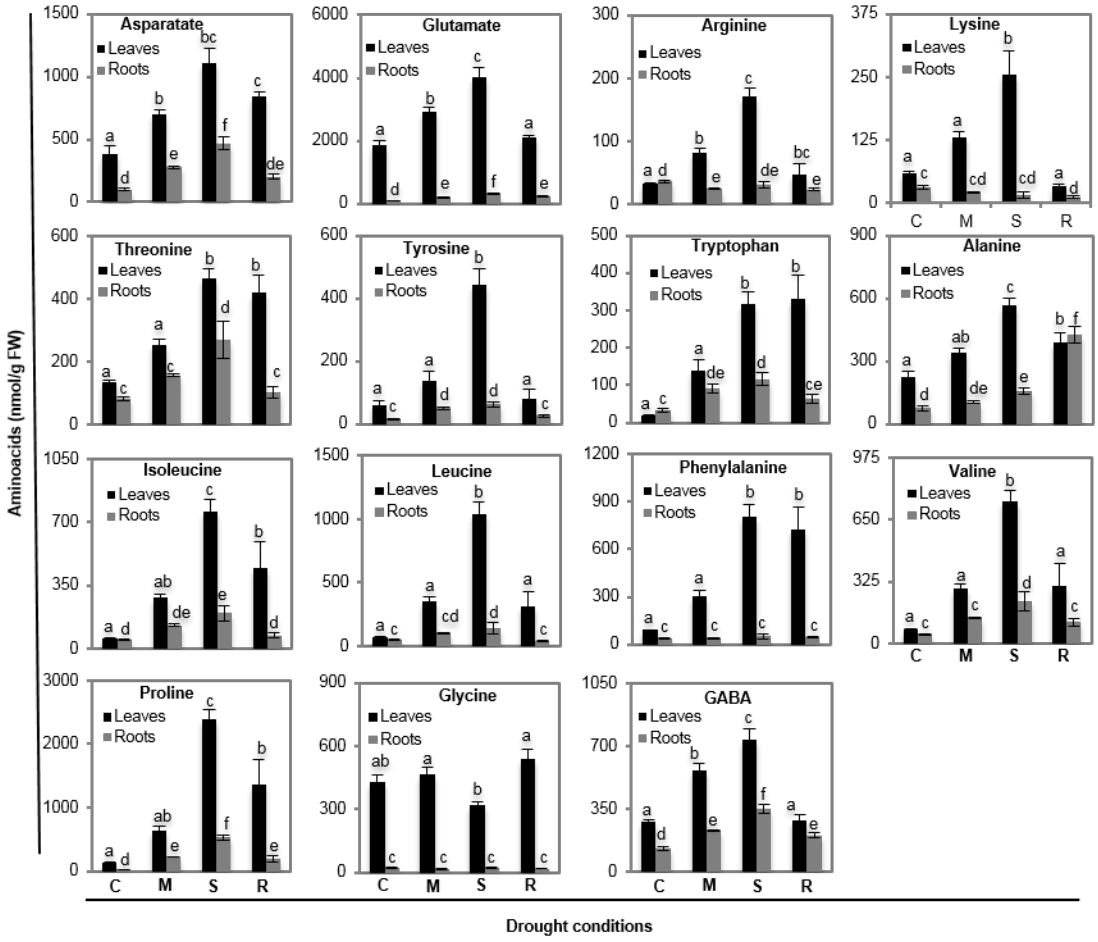
Appendix B: Supplementary Files for Chapter 3

Supplementary Appendix B1a



Individual amino acid responses to submergence and reoxygenation in V1 leaves of soybean. Amino acids contents were analyzed in the V1 leaves exposed to submergence for up to 3 d and 3 d submergence followed by 24 h recovery (3R). Data represent mean ± SE (n = 6). Bars not sharing the same letter are significantly different ($P < 0.05$). (ND= Not determined).

Supplementary Appendix B1b



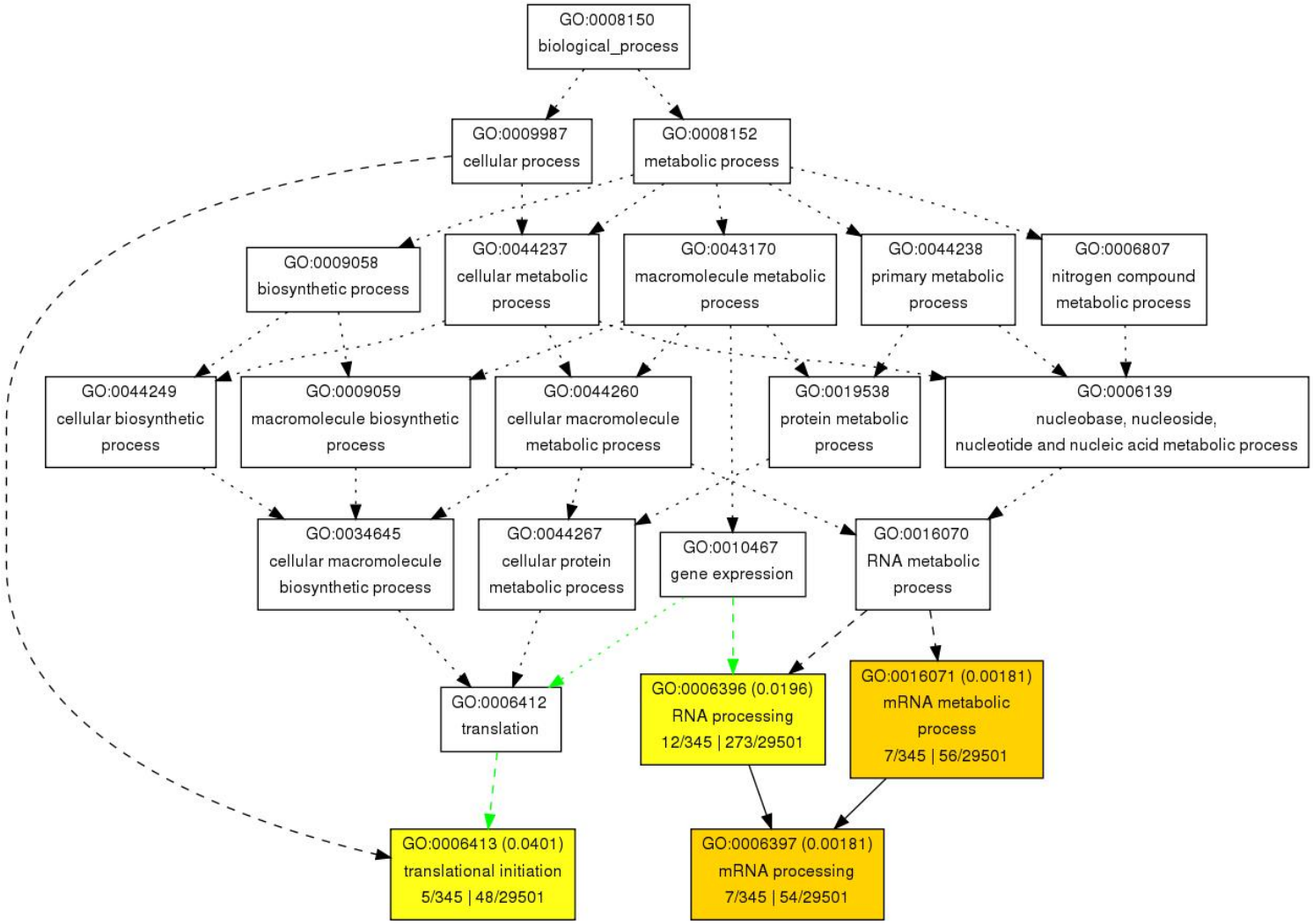
Individual amino acid responses to drought and recovery in V1 leaves and roots of soybean. Amino acids content were analyzed in the V1 leaves and roots exposed to air control (C), moderate drought (M), severe drought (S) and severe drought followed by 24 h recovery(R). Data represent mean ± SE (n = 6). Bars not sharing the same letter are significantly different ($P < 0.05$).

Supplementary Appendix B2: Statistics on RNA-Seq

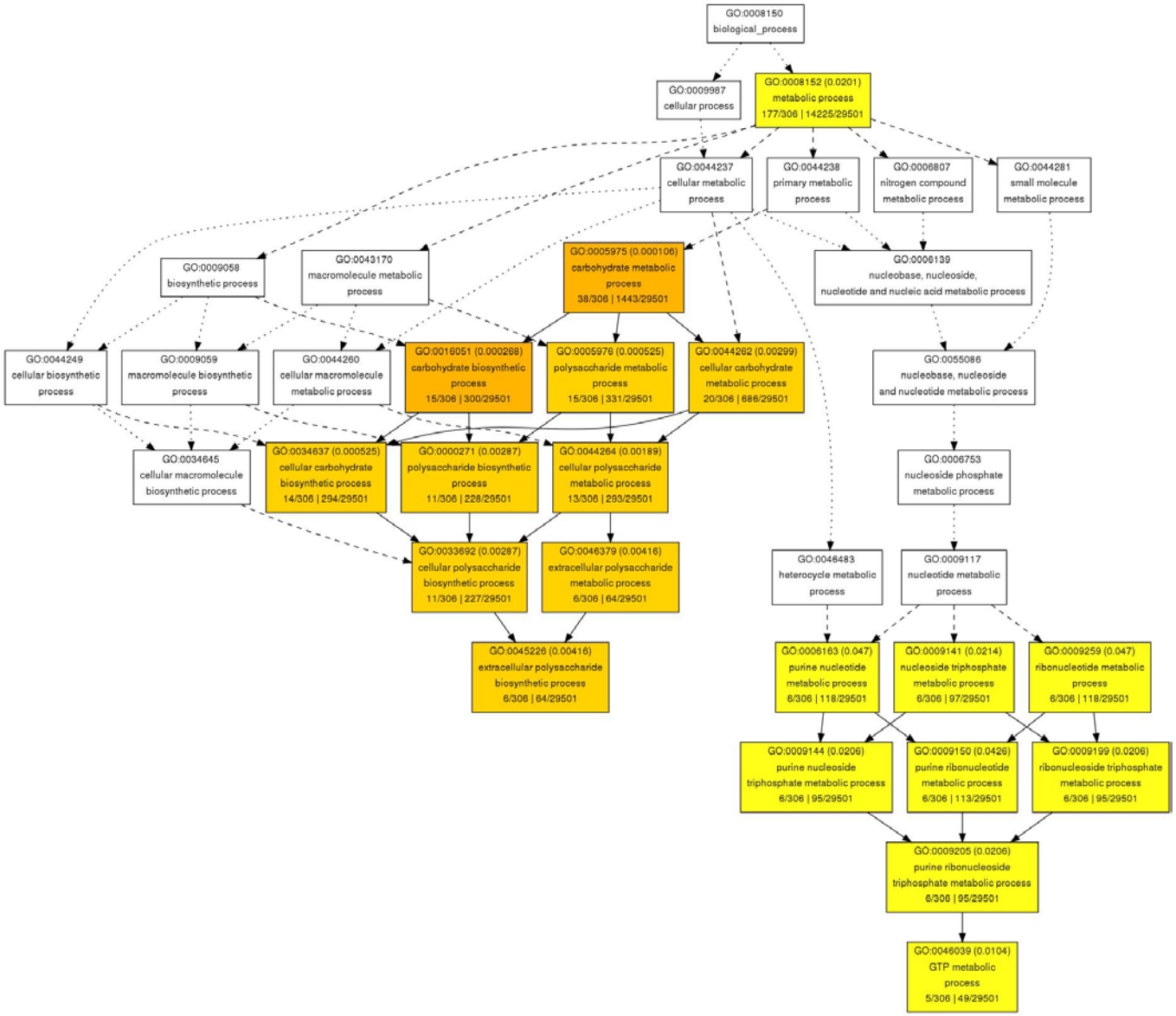
Experimental type	Tissue type	Stress condition	Biological replicate	Total input reads	Uniquely mapped reads	Uniquely mapped reads %	Multimapped reads	Multimapped reads %	Unmapped reads %	Average input read length	Average mapped length
Submergence	V1 leaf	0 d (Control)	1	14150762	9174110	64.83	660855	4.67	30.65	200	192.84
			2	27968457	20709135	74.04	1134648	4.06	21.90	200	194.06
			3	37862565	24285861	64.14	1560288	4.12	31.95	200	191.75
		1 d	1	31331388	23254856	74.22	661036	2.11	23.71	200	193.54
			2	32891007	21311488	64.79	749630	2.28	33.30	200	191.44
			3	34423868	23114549	67.15	841680	2.45	30.53	200	191.62
		2 d	1	22928711	15959654	69.61	421618	1.84	28.61	202	194.40
			2	26706851	20446217	76.56	478971	1.79	21.66	202	195.36
			3	28777916	21129856	73.42	562051	1.95	24.68	202	194.24
		3 d	1	27244701	22180228	81.41	533894	1.96	16.64	202	197.92
			2	29214572	21409963	73.29	617759	2.11	24.64	202	194.95
			3	25980690	20963219	80.69	502439	1.93	17.41	202	197.75
		3 d + 1 d recovery	1	30510074	22561310	73.95	824865	2.70	23.39	202	195.05
			2	29973689	22080208	73.67	816644	2.72	23.66	202	195.13
			3	32164668	23648265	73.52	872398	2.71	23.88	202	194.96
	V1 root	0 d (Control)	1	33944011	21521889	63.40	521620	1.54	35.12	202	192.97
			2	27645772	20200089	73.07	414181	1.50	25.44	202	195.07
			3	31444676	19457824	61.88	512696	1.63	36.50	202	192.58
		1 d	1	31846906	23291936	73.14	604379	1.90	24.97	202	194.36
			2	30344920	22882110	75.41	527560	1.74	22.89	202	195.85
			3	31632868	22878436	72.32	544859	1.72	25.96	202	194.33
		2 d	1	33216308	22943928	69.07	577611	1.74	29.31	202	194.69
			2	26044950	20435170	78.46	431951	1.66	19.91	202	196.95
			3	20804402	15880101	76.33	347883	1.67	22.10	202	195.69
		3 d	1	35892010	25821038	71.94	865189	2.41	25.65	202	194.68
			2	28887920	22802119	78.93	675551	2.34	18.76	202	196.97
			3	29029277	22560762	77.72	756224	2.61	19.71	202	196.55
		3 d + 1 d recovery	1	33743726	21156992	62.70	629251	1.86	35.49	202	192.95
			2	33709354	24725191	73.35	607874	1.80	24.84	202	194.92
			3	90465689	53657492	59.31	1871535	2.07	38.62	202	190.41

Experimental type	Tissue type	Stress condition	Biological replicate	Total input reads	Uniquely mapped reads	Uniquely mapped reads %	Multimapped reads	Multimapped reads %	Unmapped reads %	Average input read length	Average mapped length
Drought	**V1 leaf**	0 d (Control)	1	32469979	23846811	73.44	736001	2.27	24.35	202	194.49
			2	32823842	24073174	73.34	727932	2.22	24.48	202	194.28
			3	34596946	26708698	77.20	793707	2.29	20.53	202	195.07
		5 d	1	36216581	23340946	64.45	552382	1.53	34.25	202	192.57
			2	32282314	24557822	76.07	516591	1.60	22.37	202	195.30
			3	32201028	22905488	71.13	574803	1.79	27.15	202	193.40
		6 d	1	41277649	27679743	67.06	590440	1.43	31.59	202	193.08
			2	32643394	25461970	78.00	441409	1.35	20.70	202	195.69
			3	29474820	22412079	76.04	424845	1.44	22.58	202	194.98
		6 d + 1 d recovery	1	28057264	20532439	73.18	740399	2.64	24.30	202	194.98
			2	29100620	19577030	67.27	832602	2.86	29.93	202	193.32
			3	15086201	10632226	70.48	339662	2.25	27.29	202	194.71
	V1 root	0 d (Control)	1	22737169	14788588	65.04	350625	1.54	33.42	210	199.40
			2	22349298	17310496	77.45	293098	1.31	21.24	210	202.99
			3	20516376	14659602	71.45	286599	1.40	27.19	210	201.57
		5 d	1	23253954	16697176	71.80	329082	1.42	26.78	210	200.78
			2	26416044	18834135	71.30	388175	1.47	27.24	210	200.38
			3	21707388	18031068	83.06	291405	1.34	15.60	210	205.53
		6 d	1	32564219	24978540	76.71	554802	1.70	21.60	210	203.98
			2	25141833	20575248	81.84	375202	1.49	16.68	210	205.03
			3	26465079	22071980	83.40	378938	1.43	15.17	210	205.21
		6 d + 1 d recovery	1	9997336	8279298	82.82	118019	1.18	15.99	210	205.19
			2	26393599	19785665	74.96	433239	1.64	23.40	210	203.03
			3	16836866	13747307	81.65	216447	1.29	17.09	210	204.59

Supplementary Appendix B3A: Hierarchy of biological processes-associated GO terms under Group A1



Supplementary Appendix B3B: Hierarchy of biological processes-associated GO terms under Group A2



Supplementary Appendix B3C: Hierarchy of biological processes-associated GO terms under Group E1

Supplementary Appendix B4: Genes under specialized GO child terms in the GO hierarchy of Group A1

GO:0006397 (mRNA processing)	**Annotated function**
Glyma.01G031400	Splicing factor PWI domain-containing protein / RNA recognition motif (RRM)-containing protein
Glyma.11G136000	Splicing factor PWI domain-containing protein
Glyma.16G077700	SKIP | chromatin protein family
Glyma.17G001900	Nucleic acid binding; RNA binding
Glyma.12G059700	Splicing factor PWI domain-containing protein
Glyma.01G008600	SKIP | chromatin protein family
Glyma.08G153100	RNA binding (RRM/RBD/RNP motifs) family protein
GO:0006413 (Translation initiation)	
Glyma.03G206400	Eukaryotic translation initiation factor 3C
Glyma.05G001200	Translation initiation factor IF2/IF5
Glyma.02G090800	Translation initiation factor IF2/IF5
Glyma.16G153800	Novel cap-binding protein
Glyma.12G135100	Translation initiation factor SUI1 family protein

Supplementary Appendix B5: Biological process associated enriched GO terms

A. UPREGULATED GO terms during submergence in both tissues (Group C1)

GO term	Biological Process	FDR
GO:0006464	Cellular protein modification process	0.00078
GO:0016192	Vesicle-mediated transport	0.012999
GO:0032312	Regulation of ARF GTPase activity	0.031996
GO:0006793	Phosphorus metabolic process	0.0016
GO:0006352	DNA-templated transcription, initiation	0.043003
GO:0043412	Macromolecule modification	0.0016
GO:0006796	Phosphate-containing compound metabolic process	0.0016
GO:0006367	Transcription initiation from RNA polymerase II promoter	0.036
GO:0016070	RNA metabolic process	0.0082
GO:0044260	Cellular macromolecule metabolic process	0.024998

B. DOWNREGULATED GO terms during submergence in both tissues (Group C2)

GO term	Biological Process	FDR
GO:0008152	Metabolic process	0.031003

C. UPREGULATED GO terms during drought in both tissues (Group C3)

GO term	Biological Process	FDR
GO:0006457	Protein folding	0.0027
GO:0006355	Regulation of transcription, DNA-templated	0.0027

D. UPREGULATED GO terms during submergence in leaves (Group D1)

GO term	Biological Process	FDR
GO:0000003	Reproduction	0.029
GO:0008152	Metabolic process	0.00097
GO:0009856	Pollination	0.029
GO:0022414	Reproductive process	0.029
GO:0043687	Post-translational protein modification	6.50E-06
GO:0051704	Multi-organism process	0.044999
GO:0044238	Primary metabolic process	0.00033
GO:0008037	Cell recognition	0.029
GO:0006796	Phosphate-containing compound metabolic process	0.00017
GO:0043412	Macromolecule modification	7.70E-05
GO:0006793	Phosphorus metabolic process	0.00017
GO:0019538	Protein metabolic process	0.029

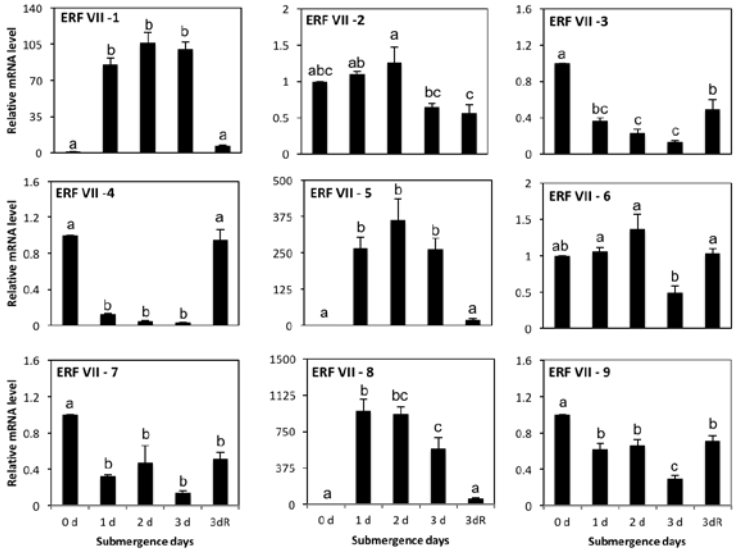
E. UPREGULATED GO terms during submergence in roots (Group D2)

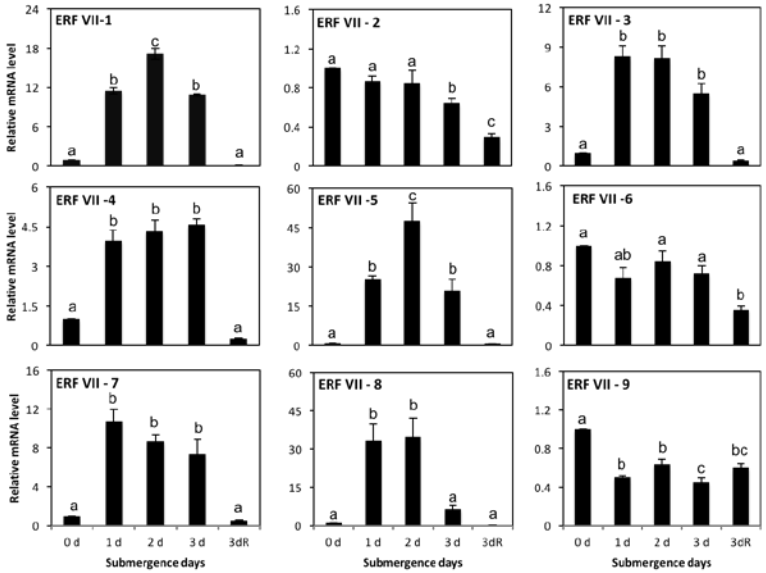
GO term	Biological Process	FDR
GO:0044262	Cellular carbohydrate metabolic process	4E-05
GO:0065007	Biological regulation	0.016998
GO:0044282	Small molecule catabolic process	7.9E-05
GO:0050794	Regulation of cellular process	4E-05
GO:0006091	Generation of precursor metabolites and energy	0.028003
GO:0016137	Glycoside metabolic process	0.0045
GO:0032774	RNA biosynthetic process	0.00033
GO:0046164	Alcohol catabolic process	4.9E-05
GO:0044265	Cellular macromolecule catabolic process	0.0046

F. UPREGULATED GO terms during drought in roots (Group D4)

GO term	Biological Process	FDR
GO:0043648	Dicarboxylic acid metabolic process	0.0031
GO:0016053	Organic acid biosynthetic process	0.024998
GO:0009073	Aromatic amino acid family biosynthetic process	0.0045
GO:0009072	Aromatic amino acid family metabolic process	0.0084
GO:0046394	Carboxylic acid biosynthetic process	0.024998
GO:0008652	Cellular amino acid biosynthetic process	0.0045
GO:0009309	Amine biosynthetic process	0.006001
GO:0006725	Cellular aromatic compound metabolic process	0.012999
GO:0055114	Oxidation-reduction process	0.0051
GO:0019438	Aromatic compound biosynthetic process	0.029
GO:0046417	Chorismate metabolic process	0.0022
GO:0044271	Cellular nitrogen compound biosynthetic process	0.031996

Supplementary Appendix B6a: qPCR assay of nine soybean ERF-VII genes in leaves and roots under submergence and recovery

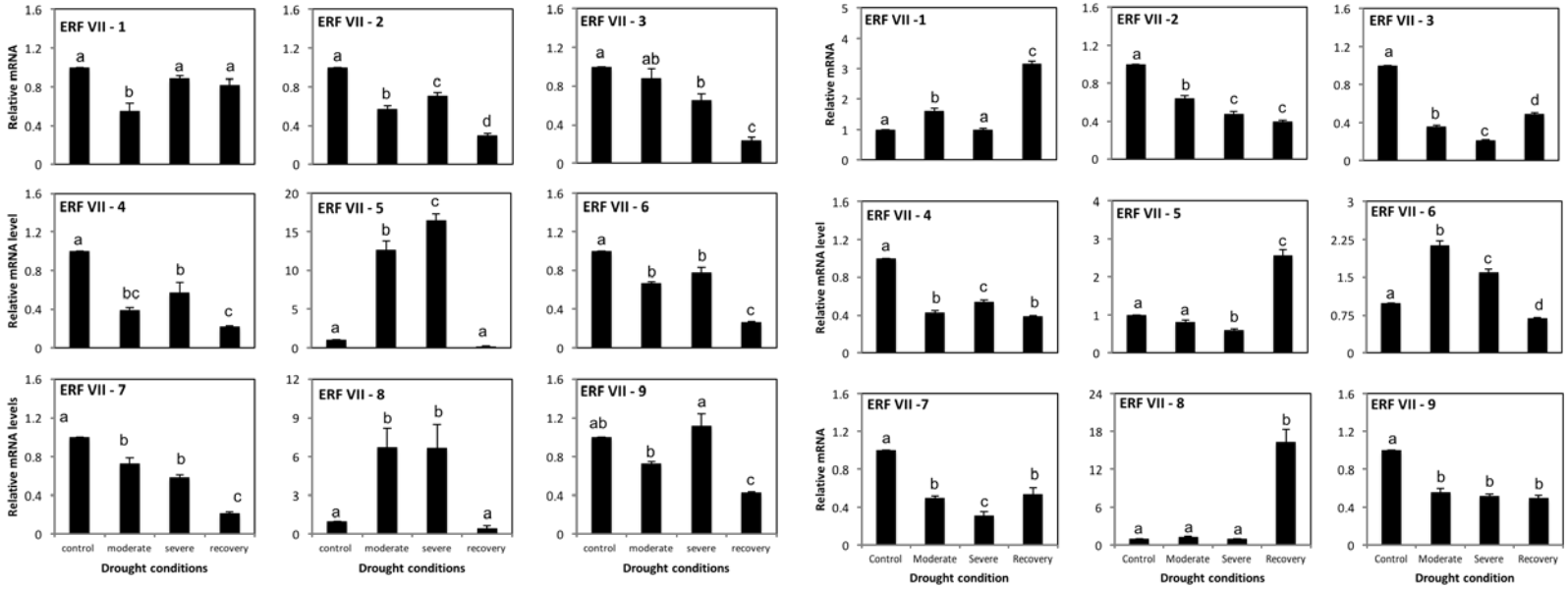




Leaves **Roots**

The effect of submergence and recovery on mRNA accumulation of group VII ERFs in VI leaves (left panel) and roots (right panel) of soybean. Relative mRNA levels of group VII ERF genes were monitored up to 3 d of submergence and during 24 h recovery from 3 d submergence. Data represent mean ± SE ($n = 3$). Bars not sharing the same letter are significantly different ($P < 0.05$).

Supplementary Appendix B6b: qPCR assay of nine soybean ERF-VII genes in leaves under drought and recovery



Leaves **Roots**

The effect of submergence and recovery on mRNA accumulation of group VII ERFs in VI leaves (left panel) and roots (right panel) of soybean. Relative mRNA levels of group VII ERF genes were monitored up to 3 d of submergence and during 24 h recovery from 3 d submergence. Data represent mean ± SE ($n = 3$). Bars not sharing the same letter are significantly different ($P < 0.05$).

Supplementary Appendix B7a: List of 73 differentially expressed AP2/ERF TFs

Gene_ID	Cluster No	Gene_ID	Cluster No	Gene_ID	Cluster No
Glyma.18G159900	1	Glyma.14G089200	15	Glyma.12G073300	29
Glyma.15G077000	3	Glyma.05G108600	15	Glyma.19G213100	29
Glyma.11G053600	3	Glyma.04G084000	15	Glyma.18G018200	29
Glyma.10G016500	3	Glyma.14G056200	16	Glyma.13G236600	29
Glyma.08G261500	3	Glyma.15G152000	17	Glyma.08G348300	29
Glyma.08G145300	3	Glyma.19G138000	21	Glyma.01G081100	29
Glyma.06G111300	3	Glyma.15G044400	21	Glyma.16G012600	30
Glyma.05G200100	3	Glyma.13G329700	21	Glyma.10G118900	30
Glyma.05G186700	3	Glyma.12G117000	21	Glyma.09G041500	30
Glyma.02G016100	3	Glyma.06G290000	21	Glyma.08G320700	30
Glyma.02G087400	4	Glyma.06G042100	21	Glyma.07G044300	30
Glyma.20G195900	5	Glyma.01G188400	22	Glyma.14G050100	32
Glyma.10G186800	5	Glyma.12G162700	22	Glyma.13G236500	32
Glyma.17G170300	7	Glyma.08G137600	22	Glyma.05G091200	36
Glyma.20G203700	9	Glyma.04G103900	23	Glyma.20G070100	36
Glyma.14G205600	9	Glyma.03G177500	25	Glyma.20G070000	36
Glyma.14G171500	10	Glyma.01G195900	25	Glyma.17G062600	37
Glyma.14G084700	10	Glyma.10G274600	25	Glyma.01G022500	37
Glyma.02G261700	10	Glyma.07G078600	25	Glyma.15G077100	37
Glyma.12G203100	11	Glyma.04G062900	25	Glyma.03G263700	37
Glyma.17G145300	12	Glyma.01G188600	25	Glyma.13G096900	38
Glyma.19G178200	13	Glyma.20G115300	26	Glyma.18G281400	39
Glyma.06G085700	13	Glyma.12G226600	27	Glyma.10G119100	39
Glyma.05G179900	13	Glyma.09G242600	27		
Glyma.U022100	14	Glyma.02G066200	27		

Supplementary Appendix B7b: List of commonly regulated AP2/ERFs by both stresses in both tissues

Upregulated	**Downregulated**
Glyma.12G073300	Glyma.06G085700
Glyma.02G016100	Glyma.19G178200
Glyma.12G117000	Glyma.18G159900
Glyma.06G042100	Glyma.13G329700
Glyma.14G205600	Glyma.01G188600
Glyma.13G236600	Glyma.05G179900
Glyma.10G016500	
Glyma.14G056200	
Glyma.20G070100	

Supplementary Appendix B8a: List of 91 differentially expressed MAP kinases

Gene_ID	Cluster No	Gene_ID	Cluster No	Gene_ID	Cluster No
Glyma.12G097200	3	Glyma.08G309500	14	Glyma.19G056300	29
Glyma.01G161600	3	Glyma.09G275900	14	Glyma.04G020100	29
Glyma.16G022600	4	Glyma.08G356500	14	Glyma.14G182700	29
Glyma.01G149000	5	Glyma.12G073700	15	Glyma.03G245500	29
Glyma.19G242900	5	Glyma.05G036600	16	Glyma.13G263600	30
Glyma.06G177700	6	Glyma.08G223400	17	Glyma.U021800	30
Glyma.14G028100	7	Glyma.17G105100	17	Glyma.12G073000	30
Glyma.17G033700	7	Glyma.14G016400	17	Glyma.16G032900	30
Glyma.11G007100	7	Glyma.16G088700	18	Glyma.13G106900	30
Glyma.14G203700	7	Glyma.15G074900	21	Glyma.17G052400	30
Glyma.14G080100	8	Glyma.15G202000	21	Glyma.06G036400	30
Glyma.08G126000	9	Glyma.10G248400	21	Glyma.13G151100	30
Glyma.03G036000	9	Glyma.10G232800	22	Glyma.05G167600	30
Glyma.13G290400	10	Glyma.05G002600	22	Glyma.07G003200	32
Glyma.20G145800	10	Glyma.08G078200	23	Glyma.17G090600	32
Glyma.12G128700	11	Glyma.06G153200	23	Glyma.07G197200	32
Glyma.01G236100	11	Glyma.19G191600	23	Glyma.18G215100	33
Glyma.16G074300	12	Glyma.03G191000	23	Glyma.07G053500	35
Glyma.10G066000	12	Glyma.15G213400	23	Glyma.09G212900	36
Glyma.20G031300	12	Glyma.08G026500	23	Glyma.07G066800	36
Glyma.11G082200	12	Glyma.01G132300	23	Glyma.02G165800	36
Glyma.13G179000	12	Glyma.06G151000	23	Glyma.18G054100	36
Glyma.07G101600	12	Glyma.02G138800	24	Glyma.18G106800	37
Glyma.05G245300	12	Glyma.10G284400	24	Glyma.05G220200	37
Glyma.08G052700	12	Glyma.05G123200	25	Glyma.16G069200	37
Glyma.07G255400	13	Glyma.19G009500	26	Glyma.18G174200	37
Glyma.03G237600	13	Glyma.03G036500	27	Glyma.18G236800	39
Glyma.01G220700	13	Glyma.15G240700	28	Glyma.01G132000	39
Glyma.15G261100	13	Glyma.07G239600	28	Glyma.09G276300	39
Glyma.06G029700	14	Glyma.02G235700	28		
Glyma.02G286800	14	Glyma.05G191700	29		

Supplementary Appendix B8b: List of commonly regulated MAP kinases by both stresses in both tissues

Upregulated	**Downregulated**
Glyma.11G082200	Glyma.14G028100
Glyma.16G032900	Glyma.02G235700
Glyma.16G022600	Glyma.17G033700
Glyma.05G123200	Glyma.14G203700
	Glyma.01G132000