HIGH THROUGHPUT PROFILING OF TRANSCRIPTION FACTORS INVOLVED IN SOYBEAN ROOT GROWTH UNDER WATER DEFICIT

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Henry Nguyen

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ABSTRACT

Drought is the major abiotic stress factor limiting crop productivity worldwide. Plant root and shoot systems respond to environmental changes by altering the expression of complex gene networks through sensing environmental stresses and modifying signaling and metabolic pathways. Previous work (Yamaguchi et al., 2009) showed that the soybean primary root adapts to low water potential (-1.6 MPa) by maintaining longitudinal expansion in the apical 4 mm (region 1), whereas in the adjacent 4 mm (region 2), longitudinal expansion reaches a maximum in well-watered roots but is progressively inhibited at low water potential.

To identify the key transcription factors (TFs) that determine these responses to low water potential, we have conducted high-throughput profiling of root-related TF expression in regions 1 and 2 of water-stressed and well-watered roots using quantitative real-time PCR. 186 root- and stress-related TFs were selected to identify their specific expression patterns in root regions 1 and 2 of well-watered and water-stressed soybean seedlings at four time points (5h, 12h, 24h, and 48h) after transplanting. Several stress-specific and root-region-specific transcripts were identified which may contribute to root responses to water deficits. Among these were zinc-finger protein, MYB-related protein, GmNAC3, GmNAC4, and bZIP transcription factors. These TFs were differentially expressed in distinct root regions, and therefore they can be targeted for functional characterization and further genetic engineering for enhanced drought resistance in soybean.

CHAPTER 1: INTRODUCTION

Due to the exponential rate of human population increase, agricultural production is one of the most critical issues today. Our ability to feed this growing population rests on our ability to produce adequate crop yields in less than ideal environments. According to the United States Geological Survey, over 35% of the world's land is arid or semi-arid and inadequate for agricultural usage. Several abiotic events, including drought, salinity, cold, and heat stresses have major impacts on crop productivity. Among them, drought is the major abiotic stress, causing significant reduction of crop yields worldwide (Boyer, 1982).

Drought can be defined as an extended period of abnormally dry weather that causes water shortages and crop damage or as a general term implying a deficiency in precipitation of sufficient magnitude to interfere with some economic phase (Si-tech encyclopedia). According to the National Drought Mitigation Center, there are four disciplinary perspectives on drought: meteorological, hydrological, agricultural, and socioeconomic. Meteorological drought is usually defined by comparing the degree of dryness and the duration of the dry period to some "normal" or average amount or time period. Hydrological drought is associated with the effects of periods of precipitation (including snowfall) or shortfalls in surface or subsurface water supply (i.e., stream flow, reservoir and lake levels, or ground water). Agricultural drought links various characteristics of meteorological or hydrological drought to their impacts on agriculture,

focusing on precipitation shortages, differences between actual and potential evapo-transpiration, soil-water deficits, reduced groundwater or reservoir levels, etc. Socioeconomic definitions of drought associate the supply and demand of some economic good with elements of meteorological, hydrological, and agricultural drought.

Water Deficit Development in Plants

Water deficit occurs in plants when the water demand for transpiration and soil water evaporation exceeds the amount of water available in the soil. Water flow through the plant from the soil to the atmosphere is driven by the difference between the water potential in the atmosphere (usually low) and the higher water potential of the soil. During transpiration, leaf water potential is reduced because of water loss, which will then cause water to flow from the soil. Therefore, in order to extract water during water-stress conditions, the leaf water potential must be further reduced to create a difference with soil water potential. However, further reduction in leaf water potential will also lead to reduction in turgor potential, affecting stomatal function, CO_2 fixation, and photosynthetic activity (Mahajan, 2005). At the whole-plant level, water deficit also affects plant growth, development, and reproduction processes. Therefore, the maintenance of turgor potential and transpiration rate during water stress is critical to the plant's survival.

Turgor maintenance can be achieved by the reduction in osmotic potential through osmotic adjustment or by the maintenance of leaf water potential through the extraction of more water from the drying soil. Extracting more water from the soil-plant

continuum by developing better plant root systems has received little attention in terms of physiological and molecular aspects.

Drought Resistance Mechanisms

In response to drought, plants undergo different drought-resistance mechanisms, such as drought escape, dehydration postponement, and dehydration tolerance (Turner et al., 2001). Drought escape allows the plant to complete its life cycle during the period of sufficient water supply before the onset of drought. Normally, the life cycle of the plant is shorter and it is able to set some seeds instead of experiencing complete crop failure.

Plants can postpone dehydration by maintaining turgor, which can be achieved by water-uptake maintenance, water-loss reduction, or osmotic adjustment (Turner et al., 2001). Plants can maintain water uptake and reduce water loss through several strategies, such as: a) the development of better root systems that can reach the water available in deeper soil; b) the increase of root/shoot ratio; c) the decrease of shoot growth, which will reduce water losses from the shoot; and d) the increase of efficient water transport via xylem; or the control of shoot water loss via stomatal closure or low cuticular conductance (Verslues et al., 2006). All these strategies are important in the maintenance of equilibrium between the water-loss rate and water uptake. In crop plants, root growth is an important trait because of its essential role in water uptake.

Dehydration tolerance is defined as the ability of cells to withstand suboptimal water levels through the development of mechanisms which permit metabolism to occur at low leaf water status (Turner et al., 2001). Plants develop dehydration-tolerance

mechanisms in two ways: a) by the synthesis of protective proteins (e.g., dehydrins or Late Embryogenesis Abundant [LEA] proteins), which act as molecular chaperones to protect proteins and other molecules from denaturation and aggregation; or b) by developing the scavenging mechanisms of reactive-oxidative species (ROS) formed during water-stress conditions (Verslues et al., 2006).

Root Growth and Water Deficit

The root system is an integral part of the plant, extracting water from the soil in order to meet the evaporation and transpiration demands of the leaves. Under drought conditions, root length is one of the primary traits that allows crop resistance to limited water conditions (Sponchiado et al., 1989). Under severe water-deficit conditions, some roots can continue elongation at substantial rates even when shoot growth is completely inhibited (Sharp and Davies, 1989; Spollen et al., 1993).

Previous studies in maize seedlings showed that under water-stress conditions (water potential of -1.6 MPa), the elongation rate of the first root segment (0-3 mm behind the root tip) was well maintained, while the elongation rate of the adjacent segment (3-9 mm) progressively decreased compared to well-watered conditions (Sharp et al., 1988; Liang et al., 1997). The maintenance of root growth under severe water stress involves the accumulation of endogenous abscisic acid (ABA) to prevent excess production of ethylene (Spollen et al., 2000). Osmotic adjustment and enhanced cell-wall extensibility have also been shown to contribute to the maintenance of root growth under water deficit (Sharp et al., 2004). The progressive inhibition of root growth at the basal

region (3-9 mm) was found to be associated with reduction in acid-induced extensibility of cell walls in water-stressed roots. Other reported reasons for the reduction in root growth in the basal region under water-stress conditions were the accumulation of phenolic substances and the increased deposition of lignin in the cell wall, which resulted in wall stiffening and progressive inhibition of cell-wall extensibility (Fan et al., 2006).

Root growth and development consists of two important processes: the continuous production of new cells by cell division in the root apical meristem and cell elongation in the root elongation zone. The effect of water stress on cell division was reported on pea root tips. Mitotic activity was shown to rapidly decrease, which was correlated with a large number of arrested root-tip meristem cells from dehydrated seedlings in the G2 phase (Bracale et al., 1997). In water-stressed maize, Sacks et al., (1997) reported a 40% reduction in the rate of cell division in cortical cells of the primary root, based on the measurement of cell production rate. The decrease in mitotic activity of cycling cells under water deficit has been shown to relate to the decrease in expression of cyclin-dependent kinase proteins (CDKs) (Schupple et al., 1998). Maize *ZmCdc2* transcript was shown to be down-regulated under water stress, resulting in the decrease of mitotic activity (Setter and Flanningan, 2001).

Cell expansion consists of two components: cell wall extensibility and cellular turgor. These two processes are coordinately regulated at the whole-plant level and are dependent on the availability of water. Transcriptome and proteomic studies have focused on the gene and on the protein-expression profiles related to root-elongation regulation. Two α-expansin (*Exp1* and *Exp5*) and two β-expansin (*ExpB2* and *ExpB8*) genes encoding

cell-wall-loosening proteins were shown to be specifically expressed in the growing region of well-watered maize primary roots, while three genes, *Exp1*, *Exp5*, and *ExpB8,* rapidly accumulated in the apical region of water-stressed roots (Wu et al., 2001). This result correlated with the maintenance of elongation rate in the apical region of water-stressed roots (Wu et al., 2001; Sharp et al., 2004). Transgenic tobacco plants over-expressing soybean root-specific expansin *GmEXP1,* which is highly expressed in the elongation zone of soybean roots, displayed root-growth acceleration (Lee et al., 2003). The increase in transcript level of expansin gene *CpExp1* in the resurrection plant *Craterostigma plantagineum* was shown to correlate with an increase of cell wall extensibility in response to dehydration (Jones and McQueen-Mason, 2004). Transcript profiling has been conducted to further identify promising candidate genes which might be involved in the maintenance of maize root growth under water-stress conditions (Spollen et al., 2008).

Drought-responsive Transcription Factors

In response to environmental factors (including drought), plants first receive external stimuli via signal receptors or sensors, then initiate signaling cascades which in turn will affect the expression of stress-responsive genes, triggering appropriate responses. Signaling pathways consist of: a) signaling molecules (reactive oxygen species (ROS), NO, Ca^{2+}, etc.), and b) protein-protein interactions involving MAPKinases (Mitogen-Activated Protein Kinases), CDPKs (Calcium-dependent protein kinases), and

Phospholipases, which in turn regulate the expression of specific transcription factors (Bartels and Sunkar, 2005).

Transcription factors (TFs) are master-control proteins in all living cells. Transcription factors play critical roles in all aspects of a higher plant's life cycle. They often exhibit sequence-specific DNA binding and are capable of activating or repressing transcription of multiple target genes. In this way, they control or influence many biological processes, including cell-cycle progression, metabolism, growth and development, and responses to the environment (Czechowski et al., 2004).

The plant hormone ABA is known to accumulate in response to water stress. Two possible primary roles of ABA in drought response are to maintain water balance through stomatal closure and to induce gene-expression encoding for proteins responsible for cellular dehydration tolerance (Zhu, 2002). However, there are several drought-responsive genes that do not require ABA induction for their expression (Shinozaki, 1997). Therefore, the regulatory systems for gene expression in response to drought are ABA-independent and ABA-dependent (Shinozaki, 1997; Zhu, 2002; Valliyodan and Nguyen, 2006). Both *cis*-acting elements and transcription factors involved in these pathways have been well studied (Shinozaki et al., 2006; Zhu, 2002; Bartels, 2005; Valliyodan and Nguyen, 2006). There are two major *cis*-elements present in the promoter region of stress-inducible genes under drought stress. One is the DRE/CRT (dehydration-responsive element/C-repeat) which consists of a 9-base pair direct repeat (TACCGACAT) involved in the ABA-independent pathway. The other is the ABA-

responsive element (ABRE), the *cis*-acting element involved in ABA-dependent regulatory pathway responses to drought.

Transcription factors (TFs) regulate gene expression by binding with *cis*-acting elements in the promoter or enhancer regions of target genes. Most drought-responsive TFs belong to gene families such as AP2/ERF-Type, bZIP (basic region leucine zipper), HD-ZIP (Homeodomain-leucine zipper), HB-Zip (homeobox TF), b-HLH (basic helix-loop-helix), MYB (Myoblastoma), NAC (NAM, ATAF1and 2, CUC2), WRKY, and Zinc-finger proteins.

AP2/EREBP-type Transcription Factors

The APETALA2/Ethylene-Responsive Element Binding Protein (AP2/EREBP)-type transcription factor family is unique to plants. In Arabidopsis, this family has 147 members divided into three subgroups: DREB (DRE-binding proteins), EREBP (Ethylene-Responsive Element Binding Proteins), and RAV (Qu and Zhu, 2006). AP2/EREBP transcription factors function in many developmental stages of the plant and respond to biotic and environmental stresses (Riechmann et al., 1998). DREB genes govern the expression of several stress-inducible genes and have been extensively studied (Bartels et al., 2005; Shinozaki et al., 2006).

By using yeast one-hybrid screening, two Arabidopsis DRE-binding proteins, DREB1A and DREB2A, were found to interact with the DRE motif in the 71-bp promoter region of the *rd29A* gene, whose expression was induced by cold, drought, and ABA treatment (Liu et al., 1998). The expression of DREB1A and its two homologs

DREB1B/CBF1 (C-Repeat Binding Factor) and DREB1C were significantly induced by freezing, while the expression of DREB2A and its homolog DREB2B were induced by dehydration. Over-expression of DREB1A in Arabidopsis transgenic plants exhibited freezing and drought tolerance with dwarf phenotypes with increased levels of rd29A transcripts (Liu et al., 1998). Transgenic wheat over-expressing AtDREB1A showed substantial resistance to water stress in greenhouse conditions (Pellegrineschi et al., 2004). Unlike the other three identified CBF/DREB1 homologs that were induced in freezing stress (DREB1A, DREB1B, and DREB1C), CBF4 (C-repeat binding factor) was also induced by drought (Haake et al., 2002). Transgenic Arabidopsis plants over-expressing CBF4 displayed increased drought and freezing tolerance with the up-regulation of stress-induced genes.

Though DREB2A and its homologs were induced by dehydration, transgenic Arabidopsis plants over-expressing DREB2A did not show growth retardation or improved stress tolerance, and had no significant effect on the rd29A transcript level under unstressed conditions. This suggests that that a post-translational modification of DREB2A is needed for its function (Liu et al., 1998). It was demonstrated that the intact AtDREB2A is needed to perform a modification to release the negative regulatory domain in the central region, resulting in the constitutively active form, AtDREB2A CA (Constitutive Active) for its expression (Sakuma et al., 2006a). Transgenic plants over-expressing AtDREB2A CA showed dual function in both water and heat stress tolerance with the up-regulation of many drought-responsive and heat-shock-responsive genes (Sakuma et al., 2006a and 2006b). A *Pennisetum glaucum* DREB2A homolog,

PgDREB2A, was reported to encode a phospho-protein, and its phosphorylation occurred at threonine residues, confirming a post-translational modification by phosphorylation of DREB2A (Agarwal et al., 2007).

DREB transcription factors have been reported to be isolated from several other crops. *Triticum aestivum* TaDREB1 was induced in different wheat varieties not only by drought, but also by other stresses, including salinity and cold (Shen et al., 2003). Five DREB homologs isolated from rice are OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D, and OsDREB2A. Among these, only OsDREB2A expression was induced by drought and high salinity, whereas expression of OsDREB1A and OsDREB1B was induced by cold treatment. Transgenic Arabidopsis plants over-expressing OsDREB1A exhibited improved tolerance to drought, cold, and salinity stresses (Dubouzet et al., 2003).

A DREB transcription factor, PhDB1, was also identified in the moss *Physcomitrella patens* (Liu et al., 2007), and was shown to be induced by various abiotic stresses and phytohormones. Expression of PhDB1 in transgenic tobacco plants enhanced tolerance to drought, salt, and cold stresses (Liu et al., 2007). Three DREB homologs have been identified in soybean: GmDREBa, GmDREBb, and GmDREBc. Under salt and drought treatments, GmDREBa and GmDREBb transcripts were increased in soybean leaves; however, in soybean roots, only GmDREBc showed a significant increase under salt, drought, and exogenous ABA treatments (Li et al., 2005).

The expression of wheat WDREB2 was demonstrated to be induced by cold, drought, salinity, and exogenous ABA treatment (Egawa et al., 2006). ZmDREB2A in

maize seedlings was also shown to be induced by major abiotic stresses such as drought, cold, salinity, and heat. ZmDREB2A transcript has two forms, but only the functional form was significantly expressed during stress treatments (Qin et al., 2007). The authors found that transgenic plants over-expressing ZmDREB2A showed drought tolerance as a result of enhanced expression of several stress-inducible genes including LEA, heat shock, and detoxification-related genes (Qin et al., 2007).

Other AP2 TFs, which are not part of the DREB group, have been found to be induced by drought. Transgenic Arabidopsis plants over expressing SHN, which belongs to the AP2/EREBP transcription factor family, displayed significant drought tolerance by reducing stomatal density. *shn* mutants showed an approximate 6-fold increase in cuticular wax level when compared to wild-type leaves. Over-expression of SHN resulted in enhanced cuticle permeability, and in altered trichome number, leaf and epidermal cell structure, branching, and stomatal index (Aharoni et al., 2004). CARAV1, an AP2 transcription factor isolated from pepper, was induced not only by environmental stresses, but also by pathogen infection and abiotic elicitors. CARAV1, which contains two DNA binding domains (AP2 and B3), showed interaction with two recognition motifs (CAACA and CACCTG) when subjected to yeast one-hybrid screening. Ectopic expression of CARAV1 in transgenic Arabidopsis plants resulted in enhanced tolerance to dehydration, salinity treatments, and some pathogen infections (Sohn et al., 2006). Another AP2/ERF-like transcription factor, HARDY, was recently reported in Arabidopsis. Rice plants over-expressing the Arabidopsis HARDY (HRD) TF increased drought tolerance and improved water-use efficiency as a result of enhanced

photosynthetic assimilation and reduced transpiration. (Karaba et al., 2007). A gain-of function Arabidopsis mutant, *hrd*-D, also displayed enhanced root branching and exhibited drought and salt tolerance concurrent with an increase in expression of many stress-inducible genes (Karaba et al., 2007).

bZIP Transcription Factor Family

Basic region leucine zipper (bZIP) transcription factors have a DNA-binding domain rich in basic amino acids and a leucine zipper dimerization motif that interacts with other transcription factors. The Arabidopsis genome contains 75 bZIP transcription factors classified into ten groups. Plant bZIP transcription factors function in pathogen defense, light and stress signaling, seed maturation, and flower development (Jakoby et al., 2002).

Several stress-inducible bZIP transcription factors, including ABRE- binding proteins (AREB) and ABRE-binding factors (ABFs), were isolated from Arabidopsis. ABFs were reported to be involved in ABA-signaling (Shinozaki et al., 2006). AREB1/ABF2, AREB2/ABF4, and ABF3 were up-regulated by drought and salinity stresses and by ABA treatment in vegetative tissues (Uno et al., 2000; Choi et al., 2000). The expression of AREB1 and AREB2 was required for post-transcriptional modification by phosphorylation via the ABA-dependent activation of a protein kinase (Uno et al., 2000). Transgenic Arabidopsis plants constitutively over-expressing ABF3 and ABF4 exhibited reduced transpiration, ABA hypersensitivity, and improved drought tolerance (Kang et al., 2002). CAbZIP1, a bZIP transcription factor isolated from pepper, was

reported to be induced by both biotic and abiotic stresses. CAbZIP1 is specifically expressed in root and flower tissues. Constitutive expression of CAbZIP2 in transgenic Arabidopsis plants resulted in a dwarf phenotype, in enhanced resistance to pathogens, and in increased tolerance to drought, salt, and methyl viologen-oxidative stresses during different growth stages (Lee et al., 2006). A water-stress-responsive bZIP transcription factor was reported in tepary bean (*Phaseolus acutifolius*) and common bean (*P. vulgaris*). This bZIP transcription factor was expressed specifically in roots, particularly in epidermal and vascular cells (Rodriguez-Uribe et al., 2006). Recently, the stress-induced expression of two bZIP transcription factors, TRAB1 (Transcription factor Responsible for ABA regulation) and ABF3 (an Arabidopsis ABA-response element binding protein), was reported in maize seedling root tips (Spollen et al., 2008).

NAC Transcription Factor Family

NAC (NAM, ATAF1 and 2, and CUC2) TFs are abundant and specific to plants. Approximately 109 genes were reported in Arabidopsis (Riechmann et al., 2000). NAC TFs play roles in embryonic, floral, and vegetative development; lateral root formation; auxin signaling; defense; and abiotic stress responses (Olsen et al., 2005).

Three drought-inducible Arabidopsis NAC transcription factors that have recently been investigated are ANAC091, ANAC055, and ANAC072. A yeast one-hybrid assay revealed that these TFS interacted with promoter regions containing the CATGTG motif of the *ERD1* gene (Early Responsive to Dehydration stress 1) (Tran et al., 2004). Fujita et al. (2004) found that transgenic plants over-expressing each one of these transcription

factors displayed increased drought tolerance as a result of the increased expression of some stress-inducible genes. The NAC072 protein RD26 was further shown to be induced not only by drought, but also by ABA and salinity treatments. RD26 was reported to be a transcription activator involved in the ABA-dependent signaling pathway in response to abiotic stresses (Fujita et al., 2004).

Another Arabidopsis stress-inducible NAC transcription factor, ATAF1, was demonstrated to negatively regulate the expression of several stress-inducible genes under drought stress in Arabidopsis. ATAF1 expression was strongly induced under dehydration and ABA treatment. Arabidopsis atf1 knockout mutant plants exhibited enhanced transcript levels of the stress-induced genes COR47, ERD10, KIN1, RD22, and RD29 under drought stress (Lu et al., 2007). One drought-inducible member of the NAC transcription factor family, OsNAC6, was shown to be a transcriptional activator in rice. Transgenic rice over-expressing OsNAC6 demonstrated increased tolerance to dehydration and high-salinity stress (Nakashima et al., 2007). The expression of OsNAC6 was also induced by wounding, cold, salt, ABA, and jasmonic acid (JA) treatment (Ohnishi et al., 2005).

MYB Transcription Factor Subfamily

The MYB subfamily is the largest transcription factor subfamily in plants, with 190 genes identified in the Arabidopsis genome (Riechmann et al., 2000). This subfamily is further classified into three families: R2R3, R1R2R3, and MYB-related families (Qu and Zhu, 2006). An Arabidopsis MYB (AtMYB2) transcription factor was demonstrated

to function as a transcription activator, interacting with the MYB recognition site in the 67-bp promoter region of the ABA-mediated and dehydration-responsive gene *rd22* (Abe et al., 1997). Arabidopsis transgenic plants over-expressing AtMYB2 exhibited ABA hypersensitivity, along with the up-regulation of several ABA-inducible genes (Abe et al., 2003).

Another MYB transcription factor, AtMYB60, was reported to be involved in stomatal movement and drought stress in Arabidopsis. AtMYB60, a R2R3-MYB gene, is expressed specifically in guard cells and is induced by drought stress. AtMYB60 null mutant plants exhibited a constitutive reduction in stomatal opening and a decrease in wilting under water-stress conditions (Cominelli et al., 2005). The CpMYB10 TF from *Craterostigma plantagineum* was shown to be rapidly induced by dehydration and ABA treatments in leaves and roots. Arabidopsis transgenic plants over-expressing CpMYB10 displayed tolerance to desiccation and salt stresses with glucose-insensitive and ABA-hypersensitive phenotypes, suggesting that CpMYB10 might function in both ABA and glucose signaling pathways in response to abiotic stress (Villalobos et al., 2004).

Among 156 GmMYB genes identified in soybean, 43 were shown to be induced by treatments with ABA, salt, drought, and/or cold stress (Liao et al., 2008). In comparison with wild-type plants, the transgenic Arabidopsis plants over-expressing GmMYB76, GmMYB177, and GmMYB92 displayed decreased sensitivity to ABA treatment at the germination stage. These three MYB transcription factors may play different roles in tolerance mechanisms to stress treatment via the regulation of different subsets of stress-responsive genes (Liao et al., 2008)

bHLH Transcription Factor Family

The bHLH (basic helix-loop-helix) family is the second-largest transcription factor family, with 139 genes in Arabidopsis (Riechmann et al., 2000). The bHLH transcription factor contains a DNA-binding domain with a basic region and the helix-loop-helix (HLH) region which is responsible for dimerization with other proteins. bHLH transcription factors function in regulating cell proliferation and cellular differentiation pathways, pigmentation in tissues, and light-signaling pathways (Heim et al., 2003).

An Arabidopsis bHLH transcription factor, AtMYC2, was shown to function as a transcription activator in the ABA-dependent signaling pathway under drought stress. Similar to AtMYB2, AtMYC2 showed interaction with the MYC recognition site in the promoter region of the rd22 gene (Abe et al., 1997). Transgenic Arabidopsis plants over-expressing AtMYC2 exhibited ABA hypersensitivity with increased expression of ABA-inducible genes such as rd22 and AtADH1. Knockout mutants of AtMYC2 resulted in ABA insensitivity with reduced expression of rd22 and AtADH1 (Abe et al., 2003). Another bHLH transcription factor identified in Arabidopsis, AtAIB, was reported to positively regulate genes involved in the ABA-dependent signaling pathway in response to drought. The expression of *AtAIB* was induced by ABA and PEG (Polyethylene Glycol). Knockdown of *AtAIB* resulted in decreased ABA sensitivity. Transgenic plants over-expressing *AtAIB* exhibited an ABA-hypersensitive phenotype and an increased tolerance to drought (Li et al., 2007).

Zinc-finger Proteins

Two Arabidopsis ZPT-2-related proteins (AZF2 and STZ), consisting of two Cys-2/His-2 type zinc-finger motifs, have been demonstrated to repress transcription of other transcription factors under abiotic stress conditions. The expression of AZF2 and STZ were shown to be significantly induced in leaves but not in roots under dehydration, cold, high salinity, and ABA treatment. Transgenic Arabidopsis plants over-expressing STZ displayed increased drought tolerance (Sakamoto et al., 2004).

A petunia Cys2/Hus2-type zinc-finger protein, ZPT2-3, was also reported to be induced by drought and cold treatments. The expression of ZPT2-3 was also mediated by jasmonic acid-dependent and ethylene-independent pathways in response to mechanical wounding. Transgenic petunia plants over-expressing ZPT2-3 exhibited improvement in dehydration tolerance (Sugano et al., 2003).

Tran et al. (2007) reported another stress-inducible zinc-finger homeodomain, ZFHD1, which specifically interacted with ZFHD recognition sites in the 62-bp promoter region of ERD1. The expression of ZFHD1 was not only induced by drought, but also by high salinity and ABA treatments. The DNA-binding domain resides in the C-terminal homeodomain, while the activation domain was localized in the N-terminal zinc-finger domain. Transgenic plants over-expressing ZFHD1 displayed improved drought tolerance and smaller phenotypes. Also, ZFHD1 was shown to interact with NAC proteins in yeast two-hybrid screening. Over-expression of both ZFHD1 and NAC genes

resulted in transgenic plants with nearly normal phenotypes and increased levels of the ERD1 transcript.

WRKY Transcription Factor Family

WRKY transcription factors contain one or two conserved WRKY domains, about 60 amino acid residues with the WRKYGQK sequence followed by a C2H2 or C2HC zinc-finger motif (Wu et al., 2005). There are an estimated 72 WRKY transcription factors in the Arabidopsis genome (Riechmann et al., 2000) and 102 WRKY transcription factors in the rice genome (Wu et al., 2005). The WRKY proteins play roles in the regulation of plant development and plant responses to biotic and abiotic stresses. A barley WRKY gene, Hv-WRKY38, was reported to be involved in cold and dehydration responses. The expression of Hv-WRKY38 was continuously induced during dehydration and freezing treatments (Marè et al., 2004).

64 *GmWRKY* genes were identified in soybean and found to be differentially expressed under abiotic stresses. Three *GmWRKY* genes, including *GmWRKY13*, *GmWRKY21,* and *GmWRKY54,* may possibly play differential roles in abiotic stress tolerance (Zhou et al., 2008). While Arabidopsis transgenic plants over-expressing *GmWRKY21* exhibited tolerance to cold stress, transgenic plants over-expressing *GmWRKY54* displayed salt and drought tolerance, possibly through the regulation of DREB2A and STZ/Zat10. Transgenic Arabidopsis plants over-expressing *GmWRKY13* showed enhanced sensitivity to salt and mannitol stresses and increased lateral root number, but they showed a decrease in ABA sensitivity compared to wild-type plants.

Kinematic Analysis of Soybean Root Growth under Water Deficit

The spatial distribution of root growth under low water potential (-1.6 MPa) was first studied in maize to reveal the differential response of distinct regions (Sharp et al., 1988). In the apical region (0-3 mm) of maize primary roots, the elongation rate was similar in both well-watered and water-stress conditions. In the basal region (3-7 mm), elongation rate reached a maximum in well-watered roots, but in water-stressed roots, the rate decreased and ceased at 7mm from the root apex (Liang et al., 1997). Taking advantage of the kinematic analysis approach, the spatial distribution of elongation rate of root growth was also studied in other plants, including soybean (Yamaguchi et al., 2009). In this study, soybean seedlings were transplanted into well-watered and severe water-stressed media (-1.6 MPa). The primary root growth of soybean seedlings measured at 48 hours after transplanting showed distinct growth responses in different regions of the elongation zone. Soybean primary roots adapted to low water potential (-1.6 MPa) by maintaining longitudinal expansion in the apical 4 mm (region 1), but by progressively inhibiting longitudinal expansion in the adjacent 3 mm (region 2). These results provide a developmental framework to elucidate the molecular mechanisms of root responses to water-deficit conditions. Plant root and shoot systems respond to environmental changes by altering the expression of complex gene networks through sensing environmental stresses which result in modification of signaling and metabolic pathways. These transcriptional changes regulate gene expression, ultimately leading to

stress tolerance. The molecular responses of key regulatory pathways involved in root growth and development under water-deficit conditions are not completely understood.

Transcription Factor Profiling By Using High-Throughput Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was shown to be 100 times more sensitive than microarray technology in gene expression profiling (Czechowski et al., 2004), making it more suitable for the investigation of transcription factor expression. (Czechowski et al., 2004). The high-throughput quantitative real-time RT-PCR method was recently used to analyze transcription factor expression in Arabidopsis, rice, and Medicago. Among 1,465 Arabidopsis transcription factors, 35 were shown to be root-specific and 52 were shown to be shoot-specific (Czechowski et al., 2004). Over 1,000 *Medicago truncatula* transcription factor primer pairs were examined for efficiency and gene specificity and were later utilized for the identification of organ-specific TFs which may play important roles in organ development (i.e., leaves, stem, roots, flowers, pods, and nodules) (Kakar et al., 2008). Approximately 2,500 TF primer pairs and synthesized cDNA from shoot and root tissues of two rice cultivars from two treatments (control and salt stress) were used to examine the accuracy, precision, reaction, and primer specificity of the qRT-PCR method. The authors proposed an optimized protocol for qRT-PCR in large-scale gene expression profiling and revealed several reference genes for qRT-PCR in rice *Oryza sativa L.* (Caldana et al., 2007). The confirmation of the method's specificity, precision, accuracy, and sensitivity in transcription factor profiling

experiments enabled the use of the high-throughput qRT-PCR method in this study to investigate the stress-responsive transcription factors involved in soybean root growth under water-deficit conditions.

Hypothesis

Root growth of a soybean (*Glycine max* L.) cultivar, Magellan, exhibited superior response to water-stress conditions. Root-elongation rates remained constant 36 hours after transplanting to severe water-stressed conditions, when root water potential reached -1.6 MPa (Yamaguchi et al., 2009). The elongation rates of different regions of soybean root tips were measured at 48 hours after transplanting. The relative elongation rate of the apical 4 mm (region 1) of water-stressed roots was completely maintained at the well-watered level under severe water stress. The basal 4-7 mm (region 2) of water-stressed roots showed a maximum elongation rate in well-watered conditions; however, water-stressed roots displayed a decelerated relative elongation rate. At 7mm from the apex onward (region 3), elongation stopped in water-stressed roots, while elongation continued in well-watered roots. Well-watered roots showed a progressively decreasing rate of elongation in region 3, which ceased at 15 mm (Figure 1) (Yamaguchi et al., 2009).

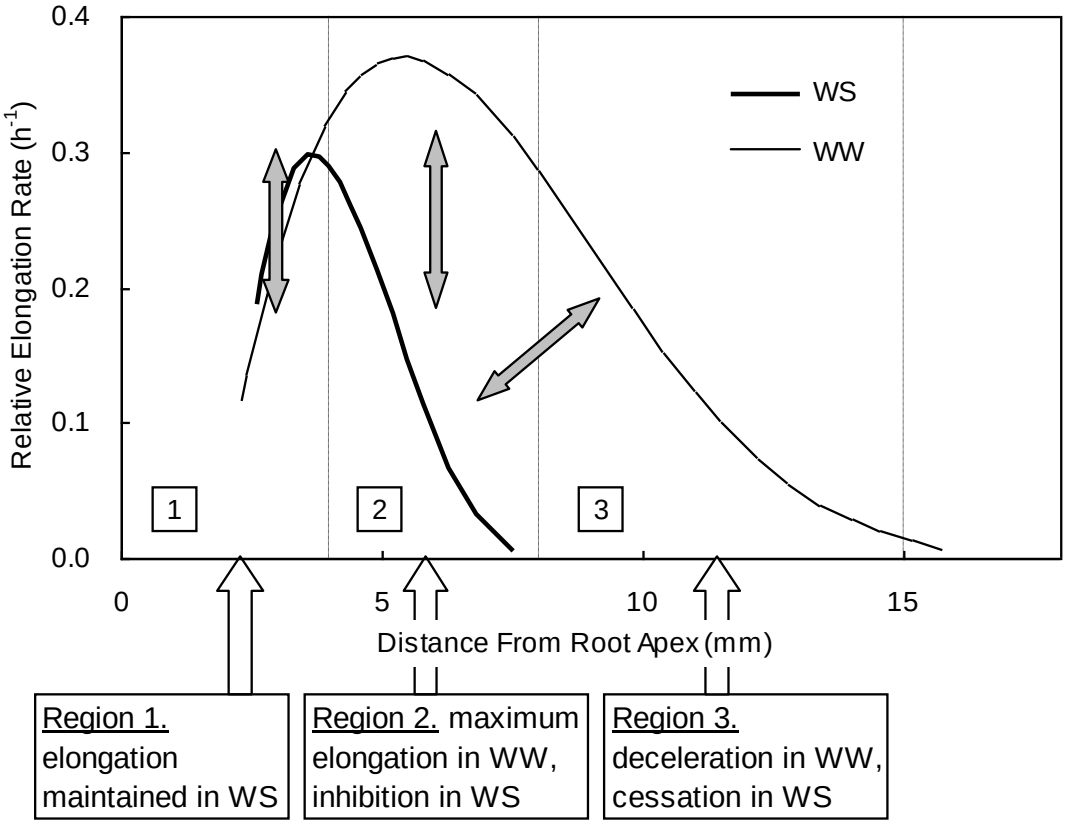
A hypothesis was developed that might help to elucidate the genetic regulations pertaining to different responses of distinct root regions of soybean seedling when subjected to water stress. From the available literature summarized above, it can be concluded that transcription factors play a significant role in plant stress response. It is hence highly probable that region-specific transcriptional regulatory mechanisms underlie

the maintenance and decrease of the elongation rate in the apical and basal growth zones, respectively, of soybean seedling roots under severe water-stress conditions.

Objectives

The major objective of this study was to profile 186 soybean root-related and stress-responsive transcription factors using quantitative real-time PCR to identify soybean root region-related and drought-responsive transcription factors under water-stress conditions. The second objective was to analyze the expression pattern of several stress-related, root region-specific transcription factors at different time points (5, 12, 24, and 48 hours) after transplanting to water-stress conditions, revealing their possible involvements in early, middle, and late events of the adaptation process of soybean root growth under low soil water potential. Later, more detailed hypotheses may be developed regarding TFs which may be involved in genetic regulations underlying the physiological mechanisms that contribute to root growth maintenance during water stress, e.g., ABA accumulation, osmotic adjustment, or enhanced cell-wall loosening.

Figure 1. Spatial distribution of relative elongation rate within the primary root of well-watered (WW) and water-stressed (WS) soybean seedlings at 48 hours after transplanting (Yamaguchi et al., 2009). For transcript profiling analysis, root growth regions 1-2 and 1-3 were collected from water-stressed and well-watered roots, respectively. Arrows indicate the regions selected for comparisons by transcript profiling (see chapter 2, page 31-32).



CHAPTER 2: MATERIALS AND METHODS

Soybean Seedling Assay and Root Tissue Collection

The soybean seedling assay developed in Dr Robert Sharp's laboratory at the University of Missouri was used for these experiments. Soybean (*Glycine max* L.) cv. Magellan seeds were sterilized with 20% bleach for 2 minutes and rinsed with running tap water for about 20 minutes. Seeds were then placed on germination paper saturated with 10 mM $CaCl_2$ plus 10 mM $Ca(NO_3)_2$ solution and germinated in the dark at 29°C and nearly 100% relative humidity. A mixture of vermiculite and Turface at a 1:1 volumetric ratio was used as the culture medium. Plastic tubes covered with fiberglass mesh at the bottom and plastic boxes used as growth containers were filled with either well-watered (pre-soaked with 10 mM $CaCl_2$ and 10 mM $Ca(NO_3)_2$ solution) or water-stressed medium. The water-stressed medium was pre-mixed with 10 mM $CaCl_2$ and 10 mM $Ca(NO_3)_2$ solutions to achieve a water potential of -1.6 MPa, which was measured with isopiestic thermocouple psychrometers (Boyer and Knipling, 1965). The plastic tubes and boxes containing culture medium were then placed in the "humid room", where the parameters were set for seedling growth (29^0C and 100% RH, no light), for one day before transplanting.

Uniform seedlings with a root length of 11-25 mm were transplanted to boxes and tubes containing either well-watered or water-stressed medium. At 5h, 12h, 24h, and 48h after transplanting, three biological replicates of soybean seedlings were harvested. The

5h, 12h, and 24h time points were chosen to permit the identification of early-responsive transcription factors after the initiation of water-stress treatment and before root water potential reach -1.6 MPa (equal to the water potential of medium) (Sharp et al., 2004). The root water potentials at 5h, 12h, and 24h after transplanting were -0.4 , -1.1, and -1.4 MPa, respectively (Yamaguchi, personal communication). The 48h time point was selected to identify transcription factors that might be responsible for the changes associated with the consistence of the relative elongation rate of soybean seedling roots in response to water-stress conditions. The primary roots were divided into 3 regions based on the previously-characterized expansion profiles: region 1 is from 0-4 mm (including the root cap), region 2 is from 4-7 mm, and region 3 is from 7-15 mm. Regions 1-2 and regions 1-3 were collected in water-stressed roots and well-watered roots, respectively These root regions were frozen immediately in liquid nitrogen and then stored at -80^{o}C. Comparison of gene expression between region 2 of water-stressed roots and region 3 of well-watered roots will distinguish the responses induced by water stress from responses resulting from changes in normal growth deceleration (Zhu et al., 2007; Yamaguchi et al., 2009).

RNA Isolation

Total RNA from regions 1 and 2 at all time points (5, 12, 24, and 48 hours) of well-watered and water-stressed roots, and from region 3 of well-watered roots at 24h and 48h (54 samples, total) were extracted using TRIzol reagent. Root tissues were ground in liquid N_2 using a chilled mortar and pestle, and the powdered samples were transferred to 15 ml centrifuge tubes. 4 ml of TRIZOL reagent (INVITROGEN) was

added to each sample tube, which was then incubated at room temperature for 10 minutes with intermittent vortexing. After incubation, 0.8ml of chloroform was added to each sample, which was then mixed well and incubated at room temperature for 15 minutes. After the incubation, sample tubes were centrifuged at 5,000 rpm at 4^0C for 40 minutes. The top aqueous phase containing RNA was transferred to fresh 15 ml tubes. Equal volumes of isopropanol (2.3 ml) were added to each tube and the tubes were incubated for 60 minutes at room temperature for the RNA precipitation. Next, the tubes were centrifuged at 5,000 rpm at 4^0C for 40 minutes and the supernatant in each tube was decanted, keeping the pellet containing RNA. 1 ml of 75% ethanol was added to each tube to wash the pellet. The liquid in each tube was decanted, and the traces of EtOH were carefully removed with clean Kimwipes. The pellet in each tube was dissolved with 400 µl of RNase-free water and incubated on ice for 1-2h, pipetting occasionally and tapping gently to ensure that the pellet dissolved completely. After the RNA was totally dissolved, the tubes were centrifuged at 1,000 rpm at 4^0C for 1 minute and each RNA sample was transferred into a new 1.5 ml tube. To remove any remaining un-dissolved particles, the tubes were centrifuged again at 13,000 rpm at 4^0C for 10 minutes. Later, the clear supernatant was transferred into a new 1.5 ml tube. To obtain high-quality RNA, 1/10 volume of sodium acetate (0.8M sodium citrate and 1.2M NaCl) and an equal volume of ethanol were added to each sample. The tubes were gently inverted by hand several times and incubated at -80^0C overnight.

After the overnight incubation, the tubes were kept on ice for 1-2 hours. Then the sample tubes were centrifuged at 13,000 rpm at 4^0C for 20 minutes. After decanting the

supernatant, the pellet was kept at the bottom of the tube and the pellet was then washed with 75% EtOH. After removing the EtOH supernatant, the pellets were left to dry at room temperature for 10 minutes. The ethanol-free pellets were then re-suspended in 50-200 µl of RNase-free water, depending on the pellet size. The samples were kept on ice for 1-2 hours so the RNA could dissolve completely. Total RNA concentration was quantified using a NanoDrop ND-1000UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA concentration was determined by absorbance at 260 nm. All RNA samples were kept at -80^{0}C until use.

DNase Treatment and cDNA Synthesis

Total RNA samples were treated with Turbo DNase (Ambion) to remove contaminating genomic DNA. In a 100 µl reaction, 20 µg of total RNA from each sample was treated with 2 µl of TURBO DNase (Ambion) and 10 µl of TURBO DNase buffer (according to the manufacturer's instructions) to remove any residual DNA, mixing/tapping gently by hand. To activate the DNase, the samples were incubated at 37^{0}C for 30 minutes. After the reaction, 10 µl of DNase inactivation reagent was added, vortexed, and incubated at room temperature for 5 minutes. Sample tubes were then gently tapped by hand and centrifuged at 10,000 rpm at 4^{0}C for 5 minutes. The supernatant containing RNA was transferred into a fresh 1.5 ml tube. Total RNA concentration was quantified using a NanoDrop ND-1000UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All stock RNA samples were kept at -80^{0}C until cDNA synthesis.

First-strand cDNA synthesis was conducted following the manufacturer's protocol (Invitrogen, Carlsbad, CA). 1 µg of DNase-treated RNA was mixed with 1 µl of random hexamer oligonucleotides and 1 µl of 10 mM dNTP mix to make a total volume of 10 µl and was then incubated at 65^0C for 5 minutes. A reaction mixture for cDNA synthesis comprising of 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M dithiothreitol, 1 µl of RNase inhibitor, and 1µl of reverse transcriptase (200U/µl) was added to each RNA/primer mixture and incubated at 25^0C for 10 minutes and then at 50^0C for 50 minutes. The cDNA synthesis reaction was terminated at 85^0C for 5 minutes. All stock cDNA samples were kept at -20^0C for further use and for RT-PCR reactions.

Selection of Transcription Factor Primer Sets for qRT-PCR

The soybean genome was recently sequenced by the Department of Energy-Joint Genome Institute (DOE-JGI) and is publicly available. Mining of this sequence identified 5,683 soybean genes as putative regulatory genes which included transcription factors. qRT-PCR primers have been developed to allow for sensitive measurement of the expression of 1,400 different soybean transcription factors (25% of total soybean TF genes) (unpublished data). All the primers were designed using the modified program Primegene (Dong Xu et al., unpublished). These primer sets have been used to profile gene expression in various soybean tissues including developing seeds and also under various biotic and abiotic stress conditions using qRT-PCR. The Nguyen lab has extensively researched on screening various soybean tissues such as leaf, root, and stem at vegetative stages under water deficit conditions (Valliyodan et al., unpublished data).

This screening for drought related transcription factors helped to select a subset of 186 root related and stress related TFs plus 6 housekeeping gene controls for this study.

Real-time PCR Conditions and Data Analysis

Polymerase chain reactions were performed in an optical 384-well plate with an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using SYBR® Green detection dye. Each well contained 5 µl 2× SYBR® Green Master Mix reagent (Applied Biosystems), 1.0 ng cDNA, and 200 nM of each gene-specific primer in a final volume of 10 µl. Master mixes were prepared with cDNA and 2× SYBR® Green reagent prior to dispensing into individual wells. Biomeck liquid handing system (Biomeck® FX, Beckman Coulter, Inc.) was used for pipetting, which helps to reduce pipetting errors and to ensure that each reaction contained an equal amount of cDNA and the reaction mix. The reaction plates were spun down after the pipetting step and were sealed using an optical seal (ABI). The following standard thermal profile was used for all PCR reactions: 50^0C for 2 min, 95^0C for 10 min, 40 cycles of 95^0C for 15 sec, and 60^0C for 1 min. SYBR® Green fluorescence was measured continuously through 40 reactions. Melting curves after 40 cycles were generated by heating samples to 95^0C for 15 seconds, then cooling down to 60^0C for 15 seconds and heating samples to 95^0C for 15 seconds.

Data were analyzed using the SDS 2.0 software (Applied Biosystems). To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔR_n) versus cycle number, baseline data were collected between cycles 3 and 15. All amplification plots were analyzed with an R_n threshold of 0.1 to obtain the C_T (threshold

cycle) values. In order to compare data from different PCR runs or cDNA samples, C_T values for all TF genes were normalized to the C_T value of the endogenous control *GmUBI.* This gene exhibited the highest inter-sample stability among the five house-keeping genes run for each sample. The average C_T value for *Glycine max UBI* was 22.76 ± SD 1.31) for all 56 plates/templates measured in this series of experiments. For those genes presenting low quantity in the sample which in turn lead to the undetermined C_T values, C_T values were assumed to be 40 for the quantification.

Relative quantification is the method to quantify the difference in expression level of a target gene among different samples. In this study, we refer to the relative quantification as a fold ratio. In each sample, the expression level of the target genes was normalized to that of the housekeeping genes. To normalize the data in this study, the housekeeping gene *Glycine max* UBI C_T value was subtracted from the C_T value of the target transcription factor gene of interest (Tran et al., 2009; Adrich et al., 2009, unpublished).

$$\Delta C_{T\ target/stress} = C_{T\ target/stress} - C_{T\ housekeeping/stress}$$

$$\Delta C_{T\ target/control} = C_{T\ target/control} - C_{T\ housekeeping/control}$$

The means of the ΔC_T values of the three biological replicates of each experimental condition (well-watered and water-stressed) at different time points were calculated as Avg. $\Delta C_{T\ target/stress}$ and Avg. $\Delta C_{T\ target/control}$, respectively. The fold ratio of gene expression between water-stressed roots and well-watered roots was calculated using the following formula:

$$\text{Relative Expression (Fold Ratio)} = 2^{-\Delta\Delta C_T}$$

$$\Delta\Delta C_T = \text{Avg. } \Delta C_{T\ \text{target/stress}} - \text{Avg. } \Delta C_{T\ \text{target/control}}$$

Avg. $\Delta C_{T\ \text{target/stress}}$ and Avg. $\Delta C_{T\ \text{target/control}}$ were averages of three biological replicates of the two samples being compared. The efficiency for all RT-PCR reactions in this relative expression is around 100%.

Three pair-wise comparisons of gene expression were conducted in this study to compare the relative expression level of each transcription factor from water-stressed and well-watered tissues in the different root tip regions (Spollen et al., 2008). The R1 comparison was chosen to compare the gene expression level in region 1 (0-4mm) between water-stressed roots and well-watered roots, which have a similar elongation rate (Yamaguchi et al., 2009). Therefore, differentially expressed transcription factors in R1 could have roles in soybean root response to water-stress conditions, especially in the maintenance of the elongation rate under water deficit. The R2 comparison was made between gene expression of region 2 between water-stressed and well-watered roots. Because the elongation rate in region 2 of water-stressed roots is decreased and ceases at 7 mm, while the rate of well watered roots reaches its maximum, more transcription factors are expected to be differentially expressed in the R2 comparison.

Like region 2 of water-stressed roots, the elongation rate of region 3 of well-watered roots was also decreased (Figure 1). Therefore, the genes differentially expressed in region 2 of water-stressed roots might be involved in either tissue development or stress response. A distinction of differentially expressed genes in region 2 was made to classify transcription factors involved in response to water stress and those related to

tissue maturation (Spollen et al., 2008). Thus, a R2R3 comparison was conducted to compare the gene expression between region 2 of water-stressed roots and region 3 of well-watered roots. Differentially expressed transcription factors present in both R2 and R2R3 comparison are likely to be stress-inducible genes and may play roles in the inhibition of the elongation rate in response to water deficit. On the other hand, differentially expressed transcription factors which are present in the R2 comparison but not in the R2R3 comparison are likely related to root maturation processes such as cell-wall thickening, vascular differentiation, etc. (Zhu et al., 2007; Spollen et al., 2008).

Statistical Analysis

The R programming environment, including the parametric *t*-test package in Real-Time StatMiner software (ABI), was used to process and statistically analyze real-time PCR data. Two-sided Student's (parametric) *t*-test for pair-wise comparisons of difference in gene expression level of each target TF was conducted to identify TFs differentially expressed in a statistical manner. A P-value threshold of 0.05 was selected as the cutoff for statistical significance of the data. In this study, ΔC_T values of each transcription factor gene in three biological replicates of water-stressed and well-watered treatments were subjected to a *t*-test. Averages of $\Delta\Delta C_T$ were calculated from three biological replicates in each treatment (water-stressed and well-watered), which generated the estimation of $\Delta\Delta C_T$ calculated from the formula above. The SAS procedure TTEST program was also used to analyze the data. In the program, all P-values were derived from testing the null hypothesis that $\Delta\Delta C_T$ are equal to 0, which means that there

is no difference between means of ΔC_T between water-stressed and well-watered roots. Therefore, a small P-value for each gene in each comparison (<0.05) indicates that the $\Delta\Delta C_T$ is significantly different from 0, demonstrating a significant effect. Thus, the expression of that TF gene is significantly different between the water-stressed and well-watered tissues.

CHAPTER 3: RESULTS AND DISCUSSION

The kinematic analysis of soybean root growth under water deficit was conducted at 48 hours after transplanting, when the seedlings were already acclimated to severe water-stress conditions. At that time point, the relative elongation rate of soybean roots had a steady, consistent rate of increase and the root water potential reached -1.6 MPa (equal to the water potential of the medium) (Yamaguchi et al., 2009). Since transcription factors act one step above stress-responsive genes so their expression can result in appropriate response to drought tolerance, more transcription factors are expected to be differentially expressed before the 48-hour time point. Therefore, in this study, we also profiled TFs before root growth had adapted to water-stress conditions. Profiles were taken at the 5h, 12h, and 24h time points, in addition to the 48 h time point. Transcript profiling of the 186 selected transcription factors at these time points will help identify early stress-responsive transcription factors that might be involved in soybean root growth after the initiation of the water-stress treatment.

After transplanting to the severely water-stressed medium at -1.6 MPa, soybean primary roots of pre-germinated seedlings reached a water potential of -1.6 MPa in approximately 36 hours. Therefore, TFs differentially expressed at 5 hours after transplanting might be involved in the response to mild water-stress conditions. TFs expressed at 12h and 24h after transplanting, on the other hand, might be involved in soybean-root response to more severe water-stress conditions.

Profiling of Soybean Transcription Factor Expression under Water Deficit

The pair-wise comparisons of gene expression between well-watered and water-stressed soybean roots were conducted for four time points (5h, 12h, 24h and 48h after transplanting) and in different root-tip regions. Our primary focus was the comparison of soybean TF expression at 5h and 48 hours in the different root regions, which correlate with the early and late events of adaptation of root tips to the imposition of water-stress conditions. Three separate comparisons were made: R1 (which compared region 1), R2 (which compared region 2), and R2R3 (which compared region 2 of water-stressed roots to region 3 of well-watered roots) (see chapter 2, page 31-32).

The qRT-PCR analysis of TFs in soybean root regions at various time points under water-deficit conditions revealed ranges of differential expression patterns of several transcription factors (TFs). The 24hR1 comparison exhibited the maximum number of differentially expressed TFs (62), followed by 5hR2 (60), 24hR2 (45), 12hR1 (25), 5hR1 (22), 12hR2 (20), 48hR2 (14), and 48hR1 (5) (Table 1). More TFs were differentially expressed at 5h, 12h, and 24h than at 48 hours after transplanting, indicating that more TFs play roles in the early events of the adaptation process of soybean root response to water stress. At 48 hours after transplanting, the roots displayed an adaptation response to water-stressed conditions through several physiological mechanisms, including ABA accumulation, ABA-dependent and –independent pathways, and osmotic adjustment. The adaptation occurred because molecular regulation mechanisms that control these biological processes were triggered. Ultimately, the

appropriate responses were triggered because the stress-responsive target genes were expressed, which activated the downstream molecular regulation. These gene expressions are controlled by the master proteins, transcription factors (TFs). TFs can activate or repress the expression of target genes by binding gene sequences (promoter or enhancer regions) or by interacting with other TFs. Therefore, more TFs are expected to express before 48 hours, when the soybean root adaptation response to water-stress conditions is already established. As expected, our results showed a larger number of differentially expressed TFs at 5 hours than at 10 hours, and a larger number of differentially expressed TFs in Region 2 than in Region 1 (60 at 5hR2, 14 at 48hR2, 22 at 5hR1, and 5 at 48hR1) (Table 1). However, 12hR1 (25) and 24hR1 (62) showed comparatively more differentially expressed TFs than 12hR2 (20) and 24hR2 (45) (Table 1). It is possible that more TFs are needed to be differentially expressed during those middle events of the adaptation process of soybean root growth to the water-stressed condition.

In different regions, at each time point, differentially expressed TFs were classified into either up-regulated or down-regulated groups. Of the total of 22 differentially expressed TFs in 5hR1, 4 were up-regulated and 18 were down-regulated (Table 1). In 5hR2, only 3 of the total 60 transcripts were up-regulated, while 57 transcripts were down-regulated (Table 1). The TFs found in these comparisons at 5 hours after transplanting might be involved in the response to mild stress (-0.4 MPa). Comparisons between 5hR1 and 5hR2 showed that only one transcription factor (belonging to the MYB family) was up-regulated in both soybean root regions (Figure 2).

Relative expression quantification showed that MYB transcription factor S4896043 was up-regulated approximately 17 fold in 5hR1, and approximately 3.6 fold in 5hR2 (Table 2, Table 3). There were ten differentially down-regulated TFs in both 5hR1 and 5hR2: *Glycine max* NAC1 (AY974349), three Zinc-finger proteins (S4913507, S5045942, and S5100831), DNA-binding protein (S4912250), one AP2-like (TC205929), bZIP (TC216155), one HB-homeobox (S5075763), WRKY (TC225723), and one TC235019 belonging to another TF family.

Among 25 differentially expressed TFs in 12hR1, six were up-regulated and 19 were down-regulated. In the 12hR2 comparison, the number of up-regulated and down-regulated TFs was 1 and 19, respectively (Table 1). 12hR1 and 12hR2 had one up-regulated gene in common, putative TF TC220047, which was up-regulated at very high levels under water deficit in both comparisons (Table 4, Table 5). The transcript amount of this gene was so small in well-watered roots that it was impossible to determine C_T values in all three replicates. 12hR1 and 12hR2 shared two common down-regulated TFs, belong to the MYB (S5046001) and the AP2-like (TC206902) families (Figure 3).

Of the 62 differentially expressed TFs in 24hR1, 17 were up-regulated and 45 were down-regulated. Among 46 differentially expressed TFs in 24hR2, one gene was up-regulated in both 24hR2 and 24hR2R3, (Table 1) (Figure 4). This TF WRKY domain TC223128 displayed very high fold difference of expression in both 24hR2 and 24hR2R3; in fact, its transcripts were undetectable in well-watered roots but detectable in region 2 of water-stressed roots (Table 7, Table 8). Comparison between 24hR1 and

24hR2 showed 14 down-regulated transcription factors in common (Table 6, Table 7). In the 24hR2 comparison, three of the 20 TFs showed down-regulation; their transcripts were undetectable in water-stressed roots, but were detectable in well-watered roots. These three transcription factors are: DNA-binding protein (S4872717), Auxin Response Factor (ARF) (S4981647), and MYB domain TF (TC232363). Among 17 differentially expressed TFs common in both 24hR1 and 24hR2, three TFs showed higher levels of up-regulation in 24hR1, but showed down-regulation in 24hR2. These three TFs are AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family (S4861942), EIN3+EIN3-like (EIL) transcription factor (S5035170) and one RING zinc finger protein (S4904949). The RING zinc finger protein was not expressed in soybean well-watered root region 1 but was detected in well-watered soybean root region 2.

All of the five differentially expressed TFs in 48hR1 were up-regulated (Table 1). Among those, one MYB transcription factor was up-regulated 64 fold. Two TFs were not detected under well-watered conditions in root region 1, but were detected in water-stressed root region 1 at 48 hours after transplanting. These are members of the unidentified TF family (S4953170) and the chromatin-remodeling complex subunit (S490388) (Table 9). In the case of 48hR2, 10 TFs were up-regulated and 4 were down-regulated (Table 1). 48hR1 and 48hR2 showed a higher percentage of up-regulated transcription factors (100% and 71.43%, respectively) compared to the other time points (5, 12 and 24 hours). Comparisons of 48hR2 and 48hR2R3 showed one up-regulated and two down-regulated TFs in common (Figure 5). These results suggest their possible

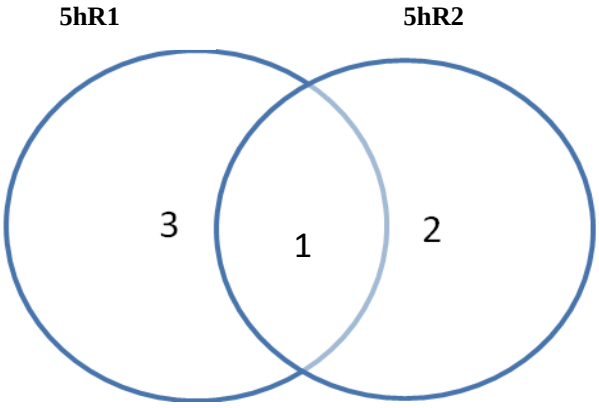
involvement in the stress response of soybean root at 48 hours after transplanting to water-stress conditions. *Glycine max* NAC4 (AY974352) showed up-regulation in three comparisons: 48hR1, 48hR2, and 48hR2R3. Another transcription factor belonging to another transcription factor family (S5002246) showed down-regulation in 48hR2 and 48hR2R3 at a high level (Table 9). Four TFs were up-regulated in both 48hR1 and 48hR2 (Figure 5). Among them, one TF, S4953170, belonging to another TF family, showed a very high fold change in both comparisons because its transcripts were undetectable in water-stressed root regions but detectable in well watered roots at 48 hours after transplanting. However, this TF was not differentially expressed in 48hR2R3, suggesting that this TF may play important roles in the maintenance of the elongation rate of root region 1, but not in the decrease of the elongation rate in root region 2 under water-deficit conditions. In addition, the differential expression of transcription factor S4953170 in water-stressed root region 2 after 48 hours of transplanting may suggest its involvement in cell maturation processes. Another TF differentially expressed in both 48hR1 and 48hR2, but not in 48hR2R3, was MYB domain transcription factor S4896043, suggesting its possible involvement in the maintenance of the elongation rate of water-stressed soybean root.

Very few TFs were present in all three comparisons (R1, R2, and R2R3) at 12 and 48 hours after transplanting. This supports our hypothesis that response to water-stress depends largely on root region-specific responses, and not on global expression of specific stress-inducible genes.

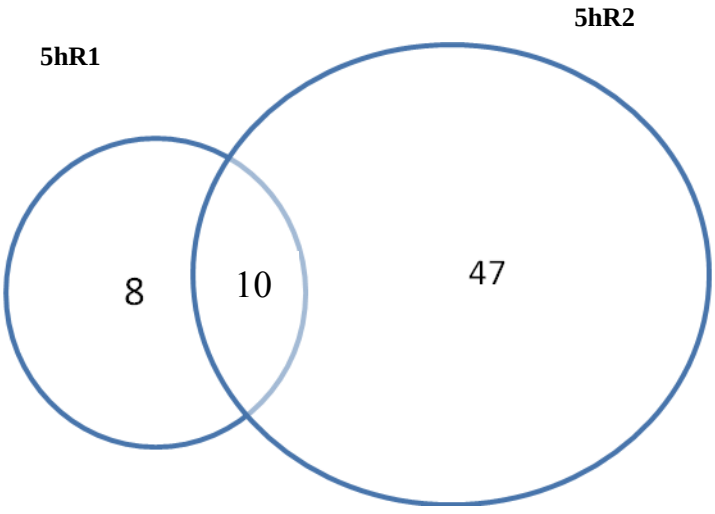
Comparisons were made between root regions at 5h and 48h after transplanting to find any common TFs differentially expressed in both early-event and late-event adaptation responses of soybean seedling roots to water-stress conditions. Two transcription factors showed up-regulation in both 5hR1 and 48hR1 (Figure 6). Glycine max NAC4 AY974352 showed a similar fold ratio of changes in relative expression quantification, with a 4-fold increase in 5hR1 and a 7-fold increase in 48hR1. MYB transcription factor showed a much higher fold ratio in 48hR1 (64-fold increase) than in 5hR1 (17-fold increase). Among 8 differentially expressed transcription factors common to both 5hR2 and 48hR2 (Figure 7), three were up-regulated and three were down-regulated in both comparisons.

Two TFs (DNA-binding protein S4911726 and zinc-finger protein S51008831) were down-regulated in 5hR2 but up-regulated in 48hR2. Only one of the eight differentially expressed transcription factors, HB-Homeobox transcription factor S5075763, was also found in comparison 48hR2R3. This transcription factor might be related to the response of soybean root region 2 to water-stress conditions. The remaining seven TFs might be related to cell maturation rather than the stress-response process. Given the fact that, among 186 TFs, only two differentially expressed TFs were present in both5hR1 and 48hR1, and only one was present in 5hR2, 48hR2, and 48hR2R3, we can conclude that stress responses of soybean root regions are time specific and region specific.

Figure 2: Differentially expressed transcription factors in soybean roots (up-regulated and down-regulated) after 5 hours of water-stress conditions. 5hR1 and 5hR2 refer to the comparison between well-watered and water-stressed conditions in region 1 (0-4 mm) and region 2 (4-7 mm), respectively.

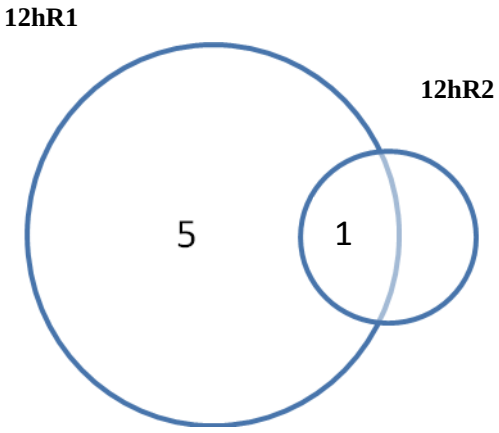


UP-REGULATED

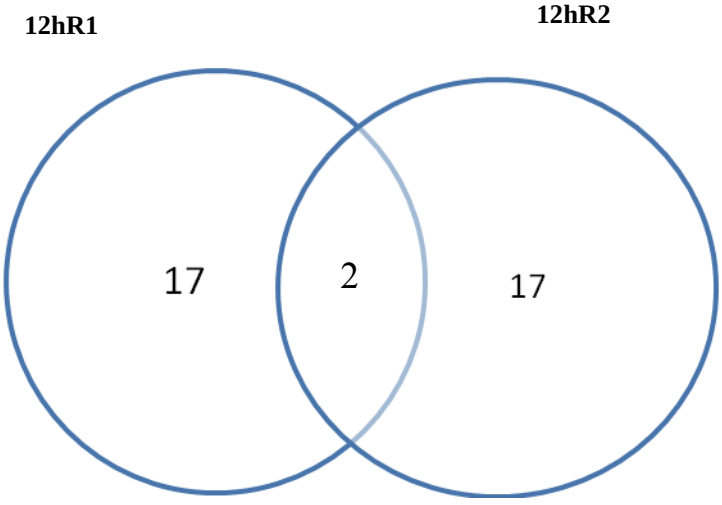


DOWN-REGULATED

Figure 3: Differentially expressed transcription factors in soybean roots (up-regulated and down-regulated) after 12 hours of water-stress conditions. 12hR1 and 12hR2 refer to the comparison between well-watered and water-stressed conditions in region 1 (0-4 mm) and region 2 (4-7 mm), respectively.

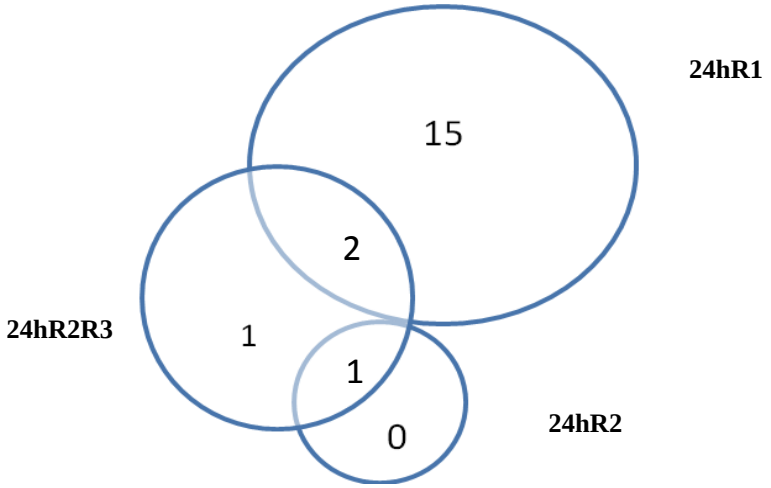


UP-REGULATED

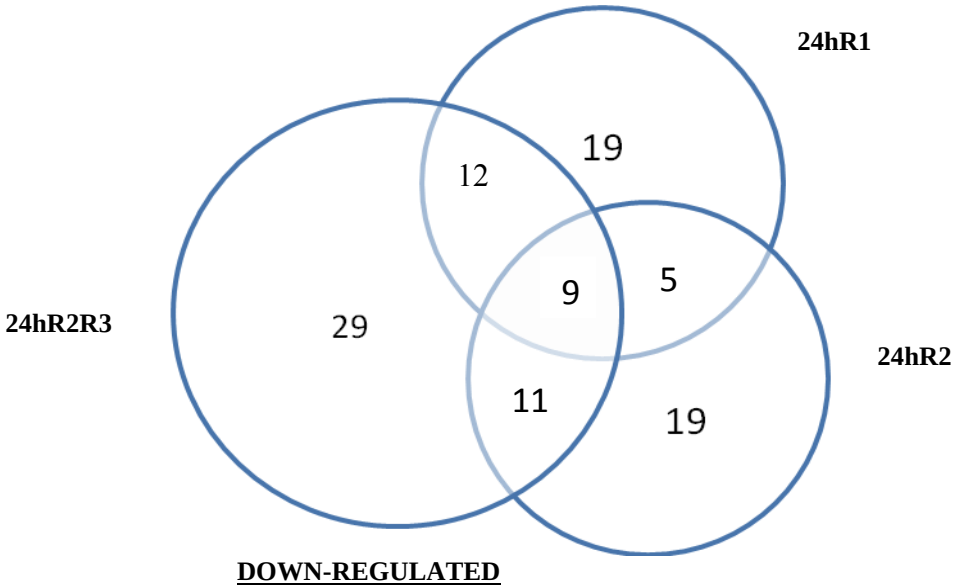


DOWN-REGULATED

Figure 4: Differentially expressed transcription factors in soybean roots (up-regulated and down-regulated) after 24 hours of water-stress conditions. 24hR1 and 24hR2 refer to the comparison between well-watered and water-stressed conditions in region 1 (0-4 mm) and region 2 (4-7 mm), respectively. 24hR2R3 refers to the comparison of region 2 (4-7 mm) of water-stressed roots with region 3 (7-15 mm) of well-watered roots.

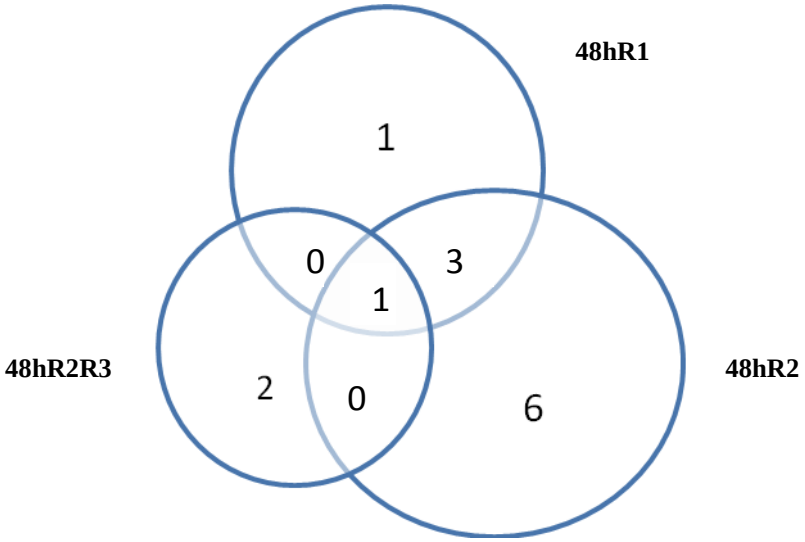


UP-REGULATED

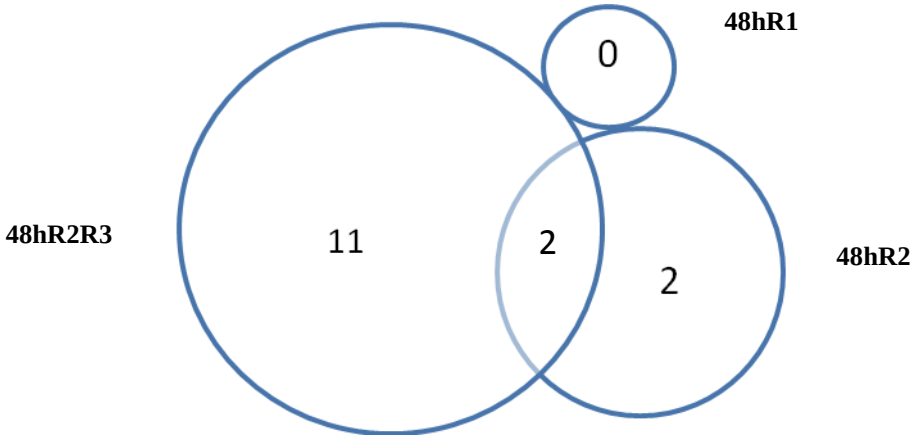


DOWN-REGULATED

Figure 5: Differentially expressed transcription factors in soybean roots (up-regulated and down-regulated) after 48 hours of water-stress conditions. 48hR1 and 48hR2 refer to the comparison between well-watered and water-stressed conditions in region 1 (0-4 mm) and region 2 (4-7 mm), respectively. 48hR2R3 refers to the comparison of region 2 (4-7 mm) of water-stressed root with region 3 (7-15 mm) of well-watered root.

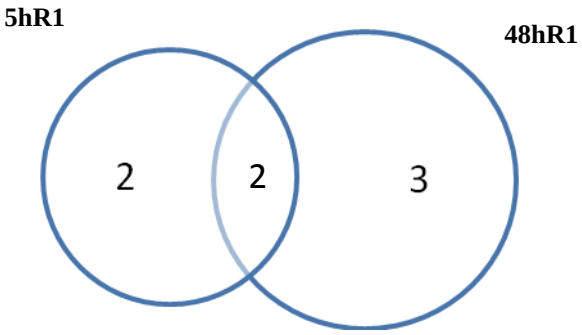


UP-REGULATED

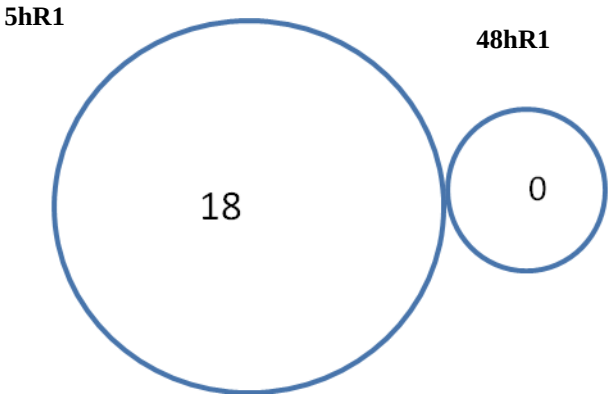


DOWN-REGULATED

Figure 6: Differentially expressed transcription factors in soybean roots (up-regulated and down-regulated) at Region 1 after 5 hours and 48 hours of water-stress condition. 5hR1 and 48hR1 refer to the comparison between well-watered and water-stress conditions in region 1 (0-4 mm) at 5 hours and 48 hours, respectively.

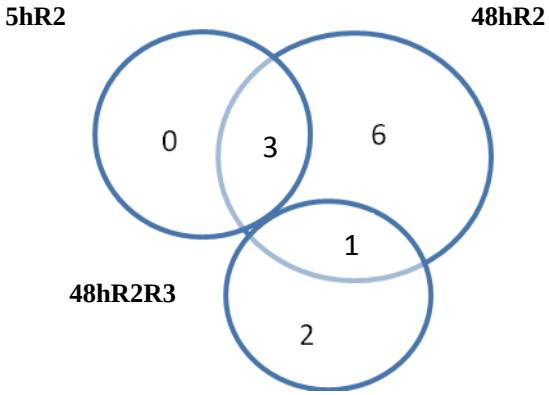


UP-REGULATED

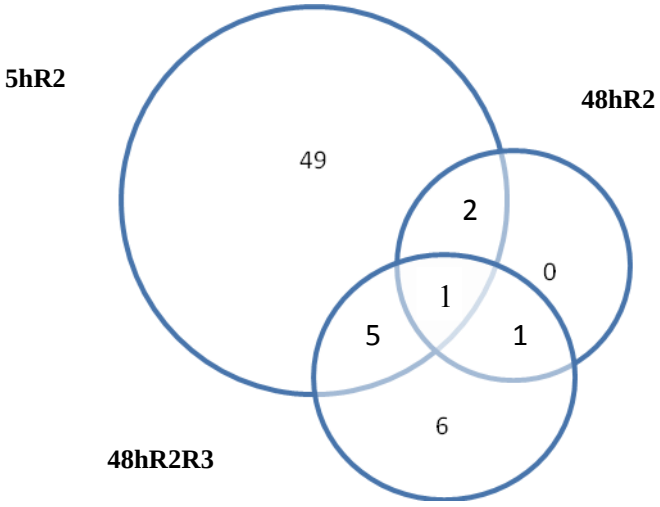


DOWN-REGULATED

Figure 7: Differentially expressed transcription factors in soybean roots (up-regulated and down-regulated) at Region 2 after 5 hours and 48 hours of water-stress conditions. 5hR2 and 48hR2 refer to the comparison between well-watered and water-stress conditions in region 2 (4-7 mm) at 5 hours and 48 hours, respectively. 48hR2R3 refers to the comparison between region 2 (4-7 mm) of water-stressed roots and region 3 (7-15 mm) of well-watered roots.



UP-REGULATED



DOWN-REGULATED

Table 1: The number of differentially expressed transcription factors in each comparison at four time points (5h, 12h, 24h, and 48h) after transplanting to water-stress conditions. R1 refers to a comparison of water-stressed soybean roots to well-watered soybean roots in region 1 (0-4 mm). R2 refers to a comparison of water-stressed soybean roots to well-watered soybean roots in region 2 (4-7 mm). R2R3 refers to a comparison between region 2 (4-7 mm) of water-stressed roots and region 3 (7-15 mm) of well-watered roots.

Comparison	Up-regulated	Down-regulated	Total
5hR1	4	18	22
5hR2	3	57	60
12hR1	6	19	25
12hR2	1	19	20
24hR1	17	45	62
24hR2	1	44	45
24hR2R3	4	61	65
48hR1	5	0	5
48hR2	10	4	14
48hR2R3	3	13	16

Table 2: Differentially expressed transcription factors in the 5hR1 comparison between water-stressed roots and well-watered roots in region 1 (0-4 mm) after 5 hours of water-stress conditions.

Fold Ratio (S-C): Relative quantification of gene expression between stress and control.

Gene ID	Gene Name Function	Fold Ratio (S-C)
AY974349	Glycine max NAC1	-2.23
AY974352	Glycine max NAC4	3.95
DQ054363	Glycine max DREB2 gene	-1.99
S18531023	Zinc-finger protein	-1.90
S21566080	Zinc-finger protein	-1.74
S23062231	Zinc-finger protein	-3.01
S4896043	MYB domain transcription factor	17.78
S4912250	DNA-binding protein	-7.80
S4913507	Zinc-finger protein	-2.44
S5045942	Zinc-finger protein	-2.78
S5075763	HB,Homeobox transcription factor	-6.51
S5076266	bZIP transcription factor	-1.90
S5100831	Zinc-finger protein	-1.82
S5103646	Agamous like	-2.15
S5142323	Other transcription factor families	-1.90
S5146255	Putative transcription factor	2.14
TC205929	AP2 transcription factor like	-2.29
TC206902	AP2 transcription factor like	-11.31
TC209970	bZIP transcription factor	1.85
TC216155	bZIP transcription factor	-2.89
TC225723	WRKY domain transcription factor	-4.34
TC235019	Other transcription factor families	-1.60

Note: A negative (-) number indicates down-regulation; a positive number indicates up-regulation.

Table 3: Differentially expressed transcription factors in the 5hR2 comparison between water-stressed roots and well-watered roots in region 2 (4-7 mm) after 5 hours of water-stress conditions.

Fold Ratio (S-C): Relative quantification of gene expression between stress and control.

Gene ID	Gene Name Function	Fold Ratio (S-C)
AY974349	Glycine max NAC1	-2.69
AY974351	Glycine max NAC3	6.93
S15850391	Other transcription factor families	-4.99
S15940089	Zinc-finger protein	-3.20
S21538405	Zinc-finger protein	-3.64
S21538802	Other transcription factor families	-3.97
S21540786	General Transcription	-2.76
S21540792	Zinc-finger protein	-2.98
S21565183	bHLH,Basic Helix-Loop-Helix	-2.93
S22951976	Aux/IAA	-3.08
S22952905	Putative transcription factor	-4.85
S22953062	WRKY domain transcription factor	-4.31
S23061205	Leucine zipper transcription factor	-3.13
S23061947	Trihelix, Triple-Helix transcription factor	-2.59
S23064130	General Transcription	-3.85
S23064915	CCAAT box binding factor	-3.36
S23068684	bZIP transcription factor	-4.34
S23069233	Putative transcription factor	-4.12
S23070183	DNA-binding protein	-3.19
S23070418	C2H2 zinc finger	-2.48
S23071068	TCP transcription factor	-3.63
S23071935	Other transcription factor families	-3.42
S4875903	WRKY domain transcription factor	-2.33
S4876683	ARF, Auxin Response Factor	-3.33
S4877491	MYB domain transcription factor	-3.04
S4882183	DNA- binding protein	-3.52
S4884795	putative transcription factor	-2.50
S4896043	MYB domain transcription factor	3.65
S4898613	Zinc-finger protein	-4.62
S4901877	Other transcription factor families	-4.49
S4908810	C2H2 zinc finger	-3.43
S4911235	Other transcription factor families	-4.70
S4912250	DNA-binding protein	-14.45
S4913507	Zinc-finger protein	-3.79
S4932151	DNA-binding protein	-4.91
S4932942	CHP-rich	-3.06
S4948369	Zinc-finger protein	-2.45
S4950242	DNA-binding protein	-3.41
S4953170	Other transcription factor families	264.88
S4976159	AT-rich interaction domain containing transcription factor	-2.75
S4980774	Chromatin remodeling complex subunit	-2.36
S5045942	Zinc-finger protein	-3.55

S5046001	MYB domain transcription factor	-2.74
S5075763	HB,Homeobox transcription factor	-11.60
S5088770	Other transcription factor families	-4.04
S5100831	Zinc-finger protein	-2.98
S5126262	MYB domain transcription factor	-2.65
S5130128	DNA-binding protein	-2.92
S5146158	bZIP transcription factor	-3.14
S6675518	Putative transcription factor	-3.24
TC205627	bZIP transcription factor	-5.93
TC206902	AP2 transcription factor like	-6.42
TC211951	MYB domain transcription factor	-4.43
TC214232	Cyclic-AMP-dependent transcription factor	-3.54
TC214990	MYB domain transcription factor	-3.29
TC216155	bZIP transcription factor	-7.21
TC225047	Other transcription factor families	-3.19
TC225723	WRKY domain transcription factor	-4.51
TC232307	Putative transcription factor	-2.48
TC235019	Other transcription factor families	-2.79

Note: A negative (-) number indicates down-regulation; a positive number indicates up-regulation.

Table 4: Differentially expressed transcription factors in the 12hR1 comparison between water-stressed roots and well-watered roots in region 1 (0-4 mm) after 12 hours of water-stress conditions.

Fold Ratio (S-C): Relative quantification of gene expression between stress and control.

Gene ID	Gene Name Function	Fold Ratio (S-C)
AY974352	Glycine max NAC4	7.04
DQ055134	Glycine max C2H2	-6.61
S23061550	bHLH,Basic Helix-Loop-Helix	4.60
S23064932	MYB domain transcription factor	-25.07
S4861946	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	-184.04
S4863935	CCAAT box binding factor	-12.16
S4864621	Other transcription factor families	-16.48
S4867907	Putative transcription factor	-171.41
S4870629	MYB domain transcription factor	5.54
S4879817	Zinc-finger protein	-10.64
S4884782	RING zinc-finger protein	-7.72
S4891674	MADS box transcription factor	-45.30
S4900633	Other transcription factor families	-8.11
S4904584	WRKY domain transcription factor	-5.12
S4904949	RING zinc-finger protein	-13.15
S4907367	MADS box transcription factor	-10.60
S4909265	Putative transcription factor	-8.35
S4917467	Zinc-finger protein	6.37
S4981647	ARF, Auxin Response Factor	-9.61
S5002246	Other transcription factor families	-168.75
S5035170	EIN3+EIN3-like(EIL) transcription factor	-15.47
S5046001	MYB domain transcription factor	-5.88
S5146255	Putative transcription factor	5.23
TC206902	AP2 transcription factor like	-8.50
TC220047	Putative transcription factor	5814248.62 (*)

Note: A negative (-) number indicates down-regulation; a positive number indicates up-regulation.

(*): Transcripts were undetectable in well-watered roots but detectable in water-stressed roots.

Table 5: Differentially expressed transcription factors in the 12hR2 comparison between water-stressed roots and well-watered roots in region 2 (4-7 mm) after 12 hours of water-stress conditions.

Fold Ratio (S-C): Relative quantification of gene expression between stress and control.

Gene ID	Gene Name Function	Fold Ratio (S-C)
S15849836	DNA-binding protein	-5.63
S18531023	Zinc-finger protein	-4.34
S22953062	WRKY domain transcription factor	-3.33
S23061682	Alfin-like	-3.13
S23068684	bZIP transcription factor	-6.02
S23070183	DNA-binding protein	-3.34
S4885901	Putative transcription factor	-4.79
S4898613	Zinc-finger protein	-3.75
S4901877	Other transcription factor families	-5.00
S4912250	DNA-binding protein	-9.07
S4950242	DNA-binding protein	-5.69
S4953170	Other transcription factor families	-3.78
S5046001	MYB domain transcription factor	-3.34
S5088770	Other transcription factor families	-2.88
S5146871	Aux/IAA	-5.07
TC205627	bZIP transcription factor	-7.69
TC206902	AP2 transcription factor like	-6.10
TC216155	bZIP transcription factor	-7.34
TC220047	Putative transcription factor	248785.46 (*)
TC225723	WRKY domain transcription factor	-4.53

Note: A negative (-) number indicates down-regulation; a positive number indicates up-regulation.

(*): Transcripts were undetectable in well-watered roots but detectable in water-stressed roots

Table 6: Differentially expressed transcription factors in the 24hR1 comparison between water-stressed roots and well-watered roots in region 1 (0-4 mm) after 24 hours of water-stress conditions.

Fold Ratio (S-C): Relative quantification of gene expression between stress and control.

Gene ID	Gene Name Function	Fold Ratio (S-C)
AY974351	Glycine max NAC3	4.25
AY974352	Glycine max NAC4	5.85
S15849836	DNA-binding protein	-2.24
S15850208	Hunchback protein like	-2.35
S15850391	Other transcription factor families	-2.38
S21537216	MYB domain transcription factor	-1.82
S21537821	SET-domain transcriptional regulator family	-1.92
S21538617	MADS box transcription factor	-2.20
S21538802	Other transcription factor families	-2.12
S21539727	Homeodomain transcription factor	-2.54
S21540786	General Transcription	-1.97
S21565183	bHLH,Basic Helix-Loop-Helix	-2.08
S22952905	Putative transcription factor	-2.08
S22953062	WRKY domain transcription factor	-2.66
S23061430	LUG	-2.47
S23061682	Alfin-like	-2.31
S23061947	Trihelix, Triple-Helix transcription factor	-2.48
S23062231	Zinc-finger protein	-2.02
S23066857	Bromodomain proteins	-2.70
S23067564	MYB domain transcription factor	-2.08
S23068300	myb-related protein	-1.98
S23068684	bZIP transcription factor	-2.55
S23070418	C2H2 zinc finger	-2.48
S23070894	SBP,Squamosa promoter binding protein	-1.85
S4861946	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	736.78 (*)
S4864621	Other transcription factor families	12269.38(*)
S4867907	Putative transcription factor	474.47 (*)
S4875857	Zinc-finger protein	-2.14
S4892093	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	23.84
S4896043	MYB domain transcription factor	12.57
S4901877	Other transcription factor families	-3.22
S4904547	Other transcription factor families	2.52
S4904949	RING zinc finger protein	1748.48 (*)
S4907367	MADS box transcription factor	1422.98 (*)
S4910460	MYB domain transcription factor	-1.94
S4910851	EIN3+EIN3-like(EIL) transcription factor	333.25 (*)
S4913507	Zinc-finger protein	-2.17
S4917467	Zinc-finger protein	2.51
S4917546	MYB domain transcription factor	-1.80
S4925034	Other transcription factor families	-2.35
S4932151	DNA-binding protein	-3.12

S4981395	Other transcription factor families	972.60 (*)
S5026438	General Transcription	-2.04
S5035170	EIN3+EIN3-like(EIL) transcription factor	20.79
S5050636	NAC domain transcription factor	1502.59 (*)
S5126262	MYB domain transcription factor	-1.91
S5129107	Other transcription factor families	-2.01
S5142323	Other transcription factor families	-1.95
S5146307	Putative transcription factor	-2.75
S5146871	Aux/IAA	-3.11
S6675518	Putative transcription factor	-2.74
TC205125	Homeodomain transcription factor	11637.93 (*)
TC205929	AP2 transcription factor like	-2.62
TC206902	AP2 transcription factor like	-2.69
TC208789	MADS box transcription factor	-1.98
TC214232	Cyclic-AMP-dependent transcription factor	-2.58
TC214990	MYB domain transcription factor	-2.19
TC215913	MYB domain transcription factor	-2.16
TC216155	bZIP transcription factor	-3.77
TC220047	Putative transcription factor	1968027.11 (*)
TC220458	bZIP transcription factor	-2.79
Z46956	Glycine max HSTF5	-1.81

Note: A negative (-) number indicates down-regulation; a positive number indicates up-regulation.

(*): Transcripts were undetectable in well-watered roots but detectable in water-stressed roots.

Table 7: Differentially expressed transcription factors in the 24hR2 comparison between water-stressed roots and well-watered roots in region 2 (4-7 mm) after 24 hours of water-stress conditions.

Fold Ratio (S-C): Relative quantification of gene expression between stress and control.

Gene ID	Gene Name Function	Fold Ratio (S-C)
DQ054363	Glycine max DREB2 gene	-7.48
S15850391	Other transcription factor families	-6.27
S15940089	Zinc-finger protein	-7.97
S21539619	Other transcription factor families	-10.19
S22952905	Putative transcription factor	-11.98
S22953062	WRKY domain transcription factor	-11.10
S23061205	Leucine zipper transcription factor	-8.55
S23061682	Alfin-like	-11.39
S23063489	C3H zinc finger	-7.30
S23064915	CCAAT box binding factor	-10.59
S23068684	bZIP transcription factor	-19.00
S23070183	DNA-binding protein	-9.37
S23071068	TCP transcription factor	-8.11
S4872717	DNA-binding protein	-692.50 (**)
S4876683	ARF, Auxin Response Factor	-8.42
S4882183	DNA-binding protein	-14.14
S4885901	Putative transcription factor	-9.53
S4888307	ARR	-265.27 (**)
S4891443	bZIP transcription factor	-7.02
S4892093	AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	-53.48
S4897794	bHLH,Basic Helix-Loop-Helix	-982.71 (**)
S4901877	Other transcription factor families	-9.83
S4904949	RING zinc finger protein	-49.62
S4909265	Putative transcription factor	-14.21
S4910460	MYB domain transcription factor	-8.36
S4912250	DNA-binding protein	-21.97
S4932942	CHP-rich	-11.02
S4980388	Chromatin remodeling complex subunit	-160.28
S4981647	ARF, Auxin Response Factor	-461.20 (**)
S4981738	Zinc-finger protein	-219.06 (**)
S5026438	General Transcription	-7.36
S5035170	EIN3+EIN3-like(EIL) transcription factor	-70.37
S5146158	bZIP transcription factor	-10.98
S5146871	Aux/IAA	-24.16
S6675518	Putative transcription factor	-6.04
TC205627	bZIP transcription factor	-15.64
TC206902	AP2 transcription factor like	-29.48
TC211951	MYB domain transcription factor	-11.38
TC214232	Cyclic-AMP-dependent transcription factor	-7.11
TC214990	MYB domain transcription factor	-8.22
TC216155	bZIP transcription factor	-13.10
TC221650	bZIP transcription factor	-11.28

TC223128	WRKY domain transcription factor	91.80 (*)
TC232307	Putative transcription factor	-8.21
TC232363	MYB domain transcription factor	-39.88 (**)

Note: A negative (-) number indicates down-regulation; a positive number indicates up-regulation.

(*): Transcripts were undetectable in well-watered roots but detectable in water-stressed roots.

(**): Transcripts were detectable in well-watered roots but undetectable in water-stressed roots.

Table 8: Differentially expressed transcription factors in the 24hR2 comparison between water-stressed roots and well-watered roots in region 2 (4-7 mm) after 24 hours of water-stress conditions.

Fold Ratio (S-C): Relative quantification of gene expression between stress and control.

Gene ID	Gene Name Function	Fold Ratio (S-C)
AY974351	Glycine max NAC3	3.48
AY974352	Glycine max NAC4	4.68
DQ054363	Glycine max DREB2 gene	-2.86
DQ055134	Glycine max C2H2	-3.25
S15850208	Hunchback protein like	-1.83
S18531023	Zinc-finger protein	-8.16
S21538802	Other transcription factor families	-3.30
S21539162	Other transcription factor families	-2.28
S21540786	General Transcription	-2.11
S21540792	Zinc-finger protein	-3.19
S21567785	WRKY domain transcription factor	-2.07
S22951976	Aux/IAA	-1.98
S22952905	Putative transcription factor	-4.06
S22953062	WRKY domain transcription factor	-3.06
S23061682	Alfin-like	-3.37
S23062231	Zinc-finger protein	-2.10
S23063261	myb-related protein	-3.38
S23064915	CCAAT box binding factor	-2.46
S23065007	Other transcription factor families	-2.38
S23066857	Bromodomain proteins	-2.01
S23068300	myb-related protein	-2.83
S23068684	bZIP transcription factor	-2.82
S23070183	DNA-binding protein	-2.77
S23070876	General Transcription	-2.63
S23070894	SBP,Squamosa promoter binding protein	-2.28
S23071068	TCP transcription factor	-2.67
S23071477	bHLH,Basic Helix-Loop-Helix	-2.90
S23071935	Other transcription factor families	-2.33
S4869132	TUB transcription factor	-78.13 (**)
S4872717	DNA-binding protein	-84.16 (**)
S4876683	ARF, Auxin Response Factor	-2.70
S4877094	Zinc-finger protein	-3.34
S4898613	Zinc-finger protein	-2.97
S4901375	EIN3+EIN3-like(EIL) transcription factor	-4.92
S4910460	MYB domain transcription factor	-3.91
S4911235	Other transcription factor families	-3.68
S4912250	DNA-binding protein	-36.97
S4925034	Other transcription factor families	-2.83
S4930680	DNA-binding protein	-15.00
S4932151	DNA-binding protein	-2.25
S4932942	CHP-rich	-2.31
S4950242	DNA-binding protein	-2.34

S4953170	Other transcription factor families	2.23
S4967941	MADS box transcription factor	-2.75
S4980388	Chromatin remodeling complex subunit	-381.41
S4981647	ARF, Auxin Response Factor	-280.49 (**)
S5011331	Other transcription factor families	-2.20
S5019221	Putative transcription factor	-11.35
S5045942	Zinc-finger protein	-4.63
S5046001	MYB domain transcription factor	-4.10
S5088770	Other transcription factor families	-3.61
S5100831	Zinc-finger protein	-4.40
S5103646	Agamous like	-2.13
S5126262	MYB domain transcription factor	-2.21
S5142323	Other transcription factor families	-4.09
S5146871	Aux/IAA	-6.50
S6675518	Putative transcription factor	-2.79
TC206511	Other transcription factor families	-2.68
TC206902	AP2 transcription factor like	-9.17
TC208789	MADS box transcription factor	-2.76
TC214990	MYB domain transcription factor	-3.10
TC223128	WRKY domain transcription factor	65.93 (*)
TC225047	Other transcription factor families	-2.11
TC225723	WRKY domain transcription factor	-4.62
TC232363	MYB domain transcription factor	-37.54 (**)

Note: A negative (-) number indicates down-regulation; a positive number indicates up-regulation.

(*): Transcripts were undetectable in well-watered roots but detectable in water-stressed roots.

(**): Transcripts were detectable in well-watered roots but undetectable in water-stressed roots.

Table 9: Differentially expressed transcription factors in the 48hR1, 48hR2, and 48hR2R3 comparisons. 48hR1 refers to the region 1 (0-4mm) comparison of water-stressed to well-watered soybean roots. 48hR2 refers to the region 2 (4-7mm) comparison of water-stressed to well-watered soybean roots. 48hR2R3 refers to the comparison of region 2 of water-stressed roots to region 3 (7-15mm) of well-watered roots at 48 hours after transplanting.

Fold Ratio (S-C): Relative quantification of gene expression between stress and control.

Gene ID	**Gene Name Function**	**Fold Ratio (S-C)**		
		48hR1	**48hR2**	**48hR2R3**
AY974351	Glycine max NAC3		13.99	
AY974352	Glycine max NAC4	4.48	12.67	5.49
S21566080	Zinc-finger protein			-2.89
S22953062	WRKY domain transcription factor			-3.38
S23063261	myb-related protein			-4.39
S23068684	bZIP transcription factor		-3.00	
S23071477	bHLH, Basic Helix-Loop-Helix			-4.39
S4884782	RING zinc-finger protein			-68.11
S4896043	MYB domain transcription factor	64.97	9.33	
S4901375	EIN3+EIN3-like(EIL) transcription factor			-262.29
S4911726	Putative transcription factor		12.24	
S4912250	DNA-binding protein		4.40	
S4917467	Zinc-finger protein	3.90	5.27	
S4953170	Other transcription factor families	32369.21 (*)	27773.58 (*)	
S4980388	Chromatin-remodeling complex subunit	8440.13 (*)		
S5002246	Other transcription factor families		-202.02 (**)	-180.54 (**)
S5046001	MYB domain transcription factor			-5.60
S5075763	HB, Homeobox transcription factor		-5.74	-4.39
S5100831	Zinc-finger protein		3.14	
S5129107	Other transcription factor families		2.94	
TC205627	bZIP transcription factor			-3.60
TC205929	AP2 transcription factor like			10.46
TC206902	AP2 transcription factor like			-7.93
TC209970	bZIP transcription factor		7.85	
TC216155	bZIP transcription factor		-3.11	
TC223128	WRKY domain transcription factor			89.60 (*)
TC225723	WRKY domain transcription factor			-5.56
TC232363	MYB domain transcription factor			-50.81 (**)

Note: A negative (-) number indicates down-regulation; a positive number indicates up-regulation.

(*): Transcripts were undetectable in well-watered roots but detectable in water-stressed roots.

(**): Transcripts were detectable in well-watered roots but undetectable in water-stressed roots.

Role of Selected Differentially Expressed Transcription Factors

Stress-Inducible, Root Region-Related NAC Transcription Factors

NAM (NO APICAL MERISTEM), ATAF1,2, and CUC2 (CUP-SHAPED COTYLEDON2) (NAC) proteins belong to one of the largest plant-specific transcription factor families and play important roles in embryonic, floral, and vegetative development; lateral root formation; auxin signaling; and plant response to abiotic stress (Olsen et al., 2005). Six *Glycine max NAC* proteins were initially isolated, cloned, and characterized by Meng et al. (2007), including GmNAC1 to GmNAC6. Tran et al. (2009) reported the expression analysis of 31 *GmNAC* genes. The GmNAC002, 003, 004, 010, 012, 013, 015 and 028 genes were shown to be drought-inducible when 14-day-old soybean plants were subjected to 10 hours of dehydration treatment.

In this study, the relative gene expression of *Glycine max NAC1* was down-regulated approximately two fold in both region 1 (0-4 mm) and region 2 (4-7 mm) of water-stressed roots at 5 hours after transplanting. *GmNAC1* was shown to be predominantly expressed in the roots of seedlings, floral buds, and flowers (Meng et al., 2007). Phylogenetic analysis of *GmNAC* genes demonstrated that *GmNAC1* belongs to the NAP (NAC-like, activated by APETALA 3/PISTILLATA) group. *GmNAC1* showed a highly similar sequence with Arabidopsis ANAC029. ANAC029 was reported to be directly activated by a heterodimer of the APETALA 3 and PISTILLATA proteins belonging to the MADS box TF family. ANAC029 transcripts are expressed in leaves, flowers, the primary root apical meristem, and the quiescent center, and they are involved

in multi-cellular organismal development, multi-dimension cell growth, flower development, and leaf senescence. Therefore, the down-regulation of the expression of this NAC gene after 5 hours of transplanting to severe water-stress conditions may affect root growth, possibly by affecting cell division and cell cycles in the primary root apical meristem and quiescent center.

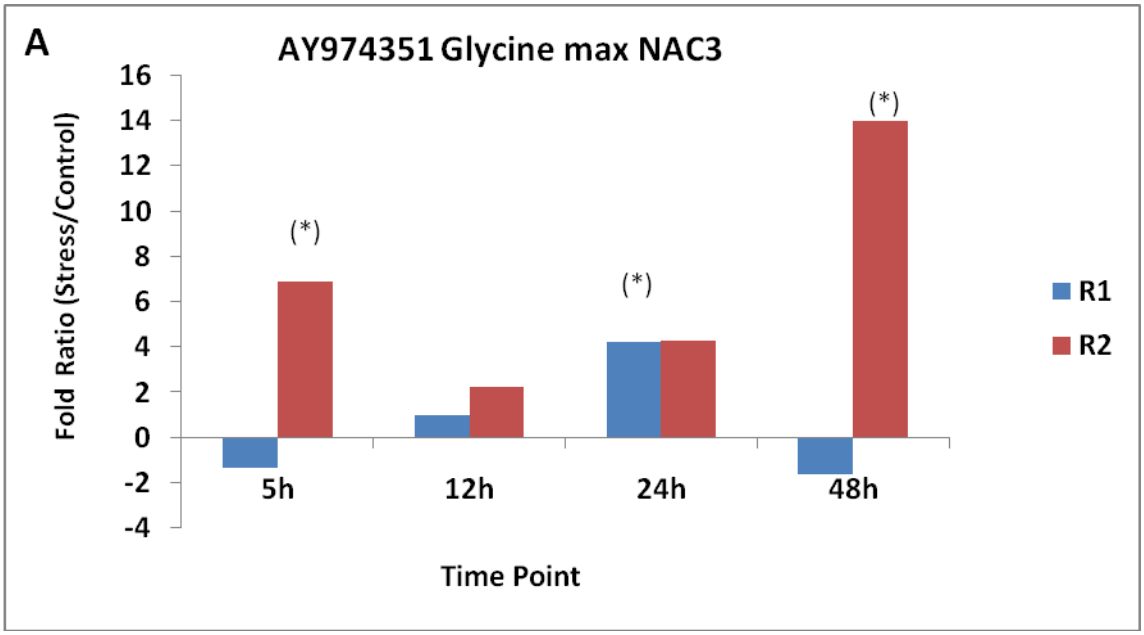
Relative gene expression showed that *GmNAC3* (AY974351) was up-regulated 4-fold in soybean water-stressed region 1 (0-4 mm) 24 hours after transplanting, but not at any other time points (Figure 7A). Thus, *GmNAC3* expression may be induced as a response to a water potential of -1.4 MPa, and may be involved in middle events (24 hours) of the adaptation response of root systems to water stress, especially in root-elongation maintenance in region 1 (0-4mm) under water-stress conditions at a later time point (48 hours). Expression level of *GmNAC3* also increased in region 2 of soybean water-stressed roots after 5 hour and 48 hour of transplanting (Figure 8A), with up-regulation of 7 fold and 14 fold, respectively. However, this differential expression was not identified in comparison 48hR2R3, which means that the expression of *GmNAC3* in region 2 of soybean water-stressed roots might primarily be due to cell maturation processes.

Although *GmNAC3* and *GmNAC4* sequences are very similar, *GmNAC3* expression level is higher in leaves, floral buds, and pods, and is weaker in seedling roots; whereas *GmNAC4* expression level was similar in all tested tissues (Meng et al., 2007). In our study, compared to *GmNAC3*, *GmNAC4* was up-regulated in water-stressed root

region 1 at all four tested time points, with relative expression fold change ranging from 4-7 folds (Figure 8B). The expression of *GmNAC4* in region 1 (0-4 mm) at all tested time points suggests that it may have a significant contribution towards root-elongation maintenance during water-stress conditions. However, the expression level of *GmNAC4* was found to increase by 13 fold in the 48 hour water-stressed region 2 (4-7mm). Also, *GmNAC4* showed differential expression in comparison 48hR2R3, suggesting that *GmNAC4* was involved mainly in the decrease of the elongation rate in root region 2 after 48 hours of transplanting to water-stress conditions, and not in cell maturation.

It is reported that *GmNAC3* and *GmNAC4* transcripts were induced by treatments with exogenous ABA and dehydration (Tran et al., 2009). Accumulation of endogenous ABA plays an important role in the maintenance of maize root elongation by preventing excessive ethylene production (Spollen et al., 2000). In this study, *GmNAC3* may have some function in the response of roots to water stress at a water potential of -1.4 MPa and be involved in middle events (at 24 hours) of the adaptation process through an ABA-dependent pathway. Likewise, *GmNAC4* may play important roles in both the maintenance of the root-elongation rate in region 1 and the decrease of the root-elongation rate of region 2 of soybean roots in response to water-stress conditions.

Figure 8: Relative expression of NAC transcription factors of soybean roots at 5, 12, 24, and 48 hours of low water potential. A) *Glycine max* NAC3 (AY974351). B) *Glycine max* NAC4 (AY974352). R1 stands for Region 1 (0-4mm); R2 stands for Region 2 (4-7mm). (*): Statistical significance (P value <0.05).



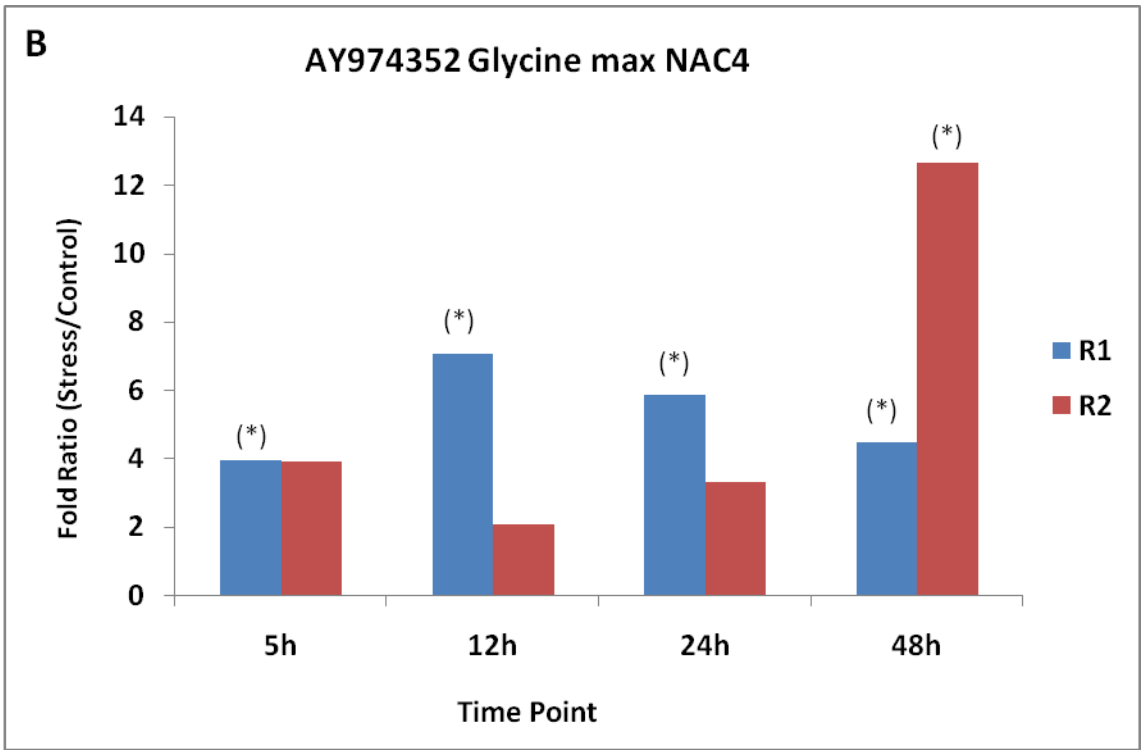
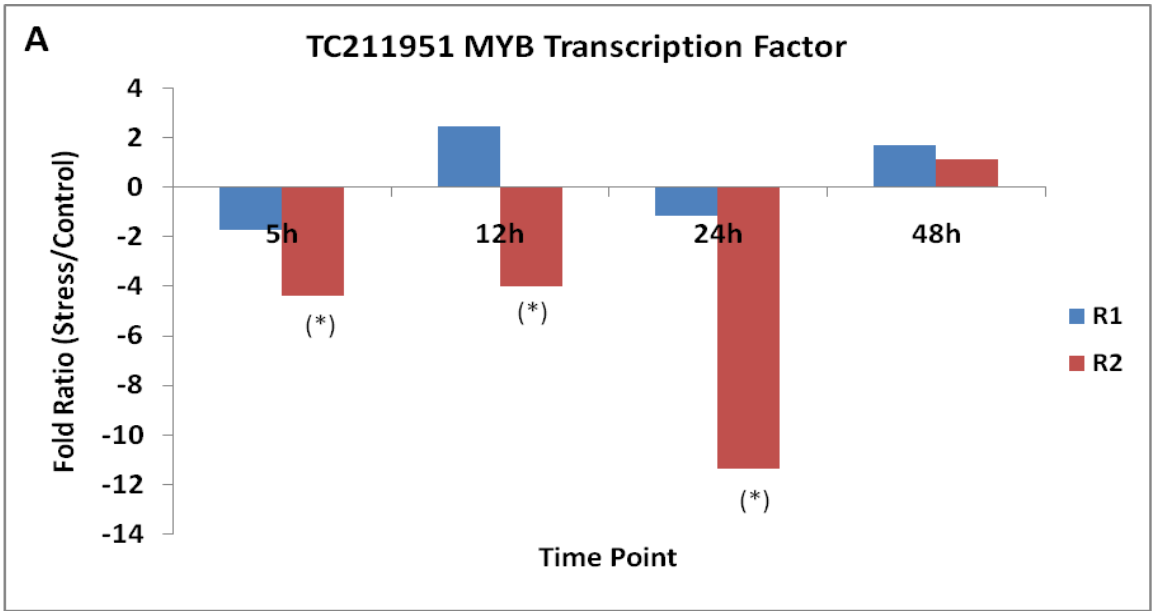
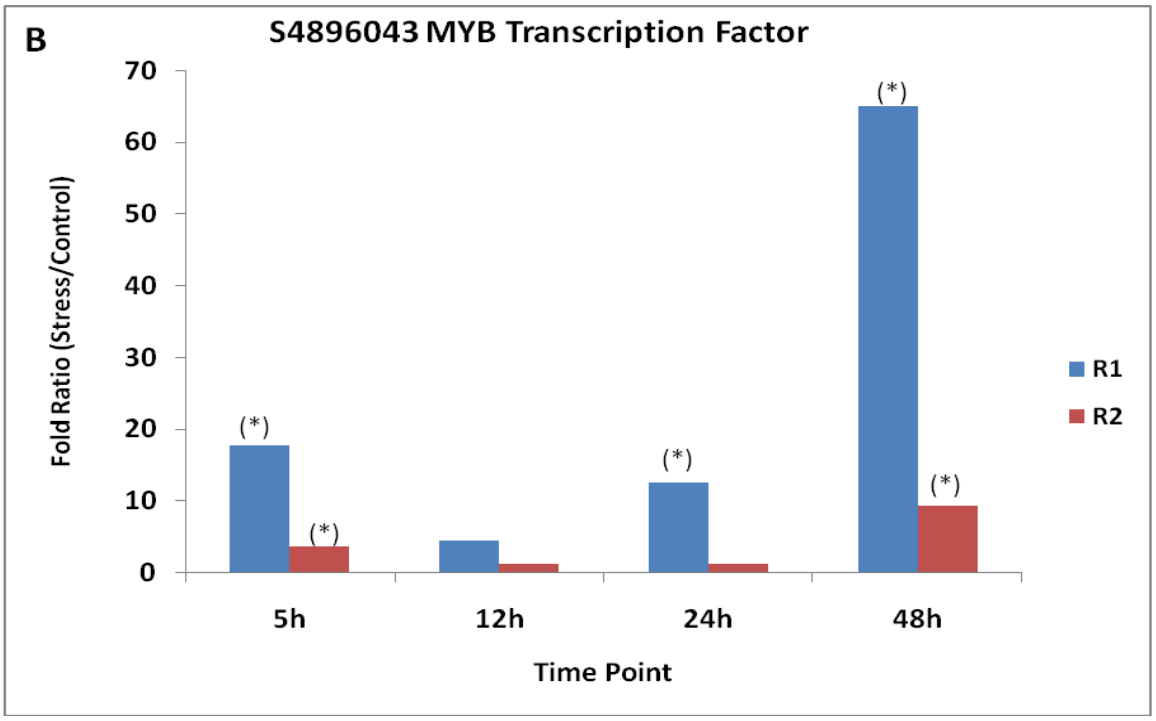


Figure 9: Relative expression of MYB transcription factors of soybean roots at 5, 12, 24, and 48 hours of low water potential. A) TC211951. B) S4896043. R1 stands for Region 1 (0-4mm); R2 stands for Region 2 (4-7mm). (*): Statistical significance (P value <0.05).





Stress-Inducible, Region-Specific MYB Transcription Factors

MYB (Myoblastoma) TFs are spread widely among animals, fungi, and plants, and are characterized by the conserved MYB DNA-binding domain. This domain, which generally consists of up to three repeats in which each repeat can form a three-dimensional helix-turn-helix structure of about 50-53 amino acids, is characterized by a tryptophan cluster formed by three regularly spaced tryptophan residues (Martin, 1997; Stracke, 2001). MYB TFs can be classified into three subfamilies depending on the number of repeats in the MYB domain: MYB1R factors (one repeat), R2R3-type MYB (two repeats), and MYB3R (three repeats) (Stracke et al., 2001). R2R3 MYB proteins, the largest MYB gene family in plants, are involved in the regulation of many secondary metabolism pathways (e.g., phenylpropanoid metabolism and tryptophan biosynthesis). They also play role in many plant-specific processes such as development and determination of cell fate and identity and response to both biotic (pathogen, fungi) and abiotic stresses (drought, low oxygen, cold, osmotic, and salt stress) (Stracke et al., 2001; Lippold et al., 2009).

One MYB transcription factor, TC211951, showed significant down-regulation in water-stressed root region 2 at 5h, 12h, and 24 hours, but not at 48 hours after transplanting (Figure 9A). However, the differential expression of this MYB TF was not identified in comparison 24hR2R3. Thus, the decrease of the expression level of this MYB TF may be due to its involvement in cell maturation processes. The nucleotide sequence of TC211915 shares 98% identity with *GmMYB160* (E value=0), with 77% query coverage (BLASTX NCBI). GmMYB160 protein shares 62% identity with

Arabidopsis *AtMYB61* (E value=5e-73). The *AtMYB61* gene, encoding a protein belonging to the Arabidopsis R2R3-MYB transcription factor family, expresses specifically in guard cells, and it is therefore involved in the control of stomatal aperture via the ABA-independent pathway (Liang et al., 2005).

Relative expression quantification of another MYB TF, S4896043, demonstrated an increased level in gene expression in region 1 of water-stressed roots at 5h, 24h, and 48 hours after transplanting, with increases of 17, 12, and 64 fold, respectively (Figure 9B). Although its expression level was up-regulated in region 2 (4-7 mm) at 5h and 48 hours after transplanting, no differential expression was found in the 48hR2R3 comparison. Therefore, the MYB TF S4896043 might have roles in cellular process related to development (such as cell maturation) in region 2 (4-7 mm), but it might also have a significant role in the maintenance of the elongation rate in region 1 (0-4 mm) of water-stressed roots, especially in the later events of the adaptation process to water-stress conditions. The nucleotide sequence of S4896043 shares 99.4% identity with the Glyma03g38410 gene, which in turn shares 97% identity with *GmMYB64* (E value=1e-101). Interestingly, like *GmMYB160* mentioned above, the amino acid sequence of the Glyma03g38410 protein has 43% identity with the AtMYB61 protein (E value=2e-78). Taken together, MYB transcription factors S4896043 and TC211915 are possibly homologues, and they may be involved in different mechanisms in response to low water potentials of soybean seedling roots.

Stress-Inducible, Region 2-Related CCAAT Box Binding Transcription Factor

The CCAAT-box binding proteins are transcription factors (TF) that bind with the CAAT-box motif of the promoters of a variety of genes, playing distinct roles in gene expression and DNA replication. The expression level of one CCAAT-box binding TF (S23064915) was down-regulated in water-stressed region 2 at 5h, 12h, and 24h after transplanting with decreases of 3, 3 (P=0.05), and 10 fold, respectively (Figure 10A). S23064915 was also significantly down-regulated in the comparison 24hR2R3. These results suggest that this TF (S23064915) may be involved in the responses of roots to water-stress conditions, especially the decrease of the elongation rate in region 2 (4-7 mm), as an early response at 24 hours after transplanting.

The soybean CCAAT-box TF S23064915 nucleotide sequence shares 100% similarity with the *Glycine max* gene identified as Glyma10g33550 (E value=0) (PHYTOZOME BLAST at phytozome.net). Phylogenetic analysis of this *Glycine max* gene in Arabidopsis showed that its amino acid sequence shares 72% similarity with Arabidopsis *NF-YB8* (AT2G37060) (NUCLEAR FACTOR Y SUBUNIT B8), which is a putative Arabidopsis CCAAT-box transcription factor. Nuclear factor Y, a heterotrimeric complex, consists of three distinct subunits: NF-YA, NF-YB, and NF-YC. The assembly of the three subunits is necessary for the CCAAT-box-binding activity of each NF-Y complex. In animals and yeast, the three subunits of NF-Y are encoded by single-copy genes; however, plants posess an extensive gene family for each of the three different subunits. In *Triticum aestivum*, 10, 11, and 14 genes encode the NF-Y subunits A, B, and

C, respectively, which are involved in many specific tasks during plant development, in response to environmental stress factors and phytohormones (Stephenson et al., 2007). *Arabidopsis thaliana NFYA5* was reported to be induced by drought stress in an ABA-dependent manner at both the transcriptional and posttranscriptional levels. Transgenic *Arabidopsis* plants over-expressing *NFYA5* showed reduction in leaf water loss and were more resistant to drought treatment than the wild type (Li et al., 2008).

Transgenic Arabidopsis plants over-expressing *AtNF-YB1* displayed the maintenance of higher water potential and photosynthetic rates, and less severe wilting, than wild-type plants at the early reproductive stage under severe drought treatment (water withholding for 8 days) (Nelson et al., 2007). Maize transgenic lines over-expressing orthologous *ZmNF-YB2* demonstrated an enhancement of drought tolerance and yield improvement under drought stress in field trials. While At*NFYA5* was induced by water stress via the ABA-dependent pathway, the involvement of *AtNF-YB1* in enhancement of drought tolerance was also shown to be independent of the ABA pathway (Nelson et al., 2007). Thus, the CCAAT-box binding TF (S23064915) may be involved in the early events of the adaptation process in response to water stress in root region 2 via the ABA-independent pathway.

Stress-Inducible AP2-like Transcription Factor

One AP2-like transcription factor (TC206902) was down-regulated in both region 1 and region 2 of soybean water-stressed roots at 5, 12, and 24 hours, but not at 48 hours after transplanting (Figure 10B). The relative expression level of the AP2-like TF in

region 1 of water-stressed roots showed a higher fold ratio in 12hR1 and in 24hR1 than in 5hR1. Likewise, it was down-regulated at the same fold ratio for 5hR2 and 12hR2, but had a much higher fold ratio in 24hR2. The differential expression of this gene was also found in the 24hR2R3 comparison, suggesting that the AP2-like TF TC206902 may be involved in the response of root growth to water-stress conditions. Because the AP2-like TF TC206902 was differentially expressed in both root regions at early hours of transplanting, it could possibly play roles in both the maintenance of the root elongation rate in region 1 and in the reduction in the elongation rate in root region 2 during early stages of the adaptation process.

The AP2-like TF TC206902 nucleotide sequences shares 97.3% identity with the *Glycine max* gene Glyma10g33810 (PHYTOZOME BLAST). The amino acid sequence of this *Glycine max* protein shares 40% identity with the Arabidopsis gene AT5G61590 (E value=3e-30) and 39% similarity with AtERF5 (E value=2e-29). Both of these genes encode proteins belonging to the ERF (ethylene-response factor) subfamily B-3 of the ERF/AP2 transcription factor family. ERF proteins, one of plant-specific transcription factor families, contain a highly conserved DNA-binding domain (known as the ERF domain). This domain consists of 58 or 59 amino acids which interacts with GCC box *cis*-acting element in target genes (Fujimoto et al., 2000). AtERF5, which consists of a putative site for MAP kinase-mediated phosphorylation, and which acts as a transcription activator of GCC box genes, was reported to be induced by cold stress in but not by exogenous ABA treatment (Fujimoto et al., 2000).

Stress-Inducible, Region 2-Related DNA-Binding Proteins

The expression patterns of the two selected DNA-binding proteins suggest their possible involvement in the early events of the water-stress adaptation processes in region 2 of soybean roots. One of the DNA-binding proteins, S23070183, was significantly down-regulated in region 2 of water-stressed soybean roots at 5, 12, and 24 hours after transplanting (See Figure 11A). The differential expression of the DNA-binding protein S23070183 was also detected in comparison 24hR2R3. Therefore, the down-regulation of the S23070183 DNA-binding protein in soybean root region 2 under water deficit may be related to stress responses and not to the root maturation process. The nucleotide sequence of gene S23070183 shares 99% identity with the *GmWRKY4* gene, with 95% sequence coverage; this confirms the designation of this TF as WRKY.

Another DNA-binding protein, S4912250, displayed a similar gene expression pattern to S23070183; it was also down-regulated in 5hR2, 12hR2, and 24hR2, and it was differentially expressed in the 24hR2R3 comparison, as well (Figure 11B). S4912250 was shown to be differently expressed in 24hR2R3 comparison as well. The up-regulation of S4912250 found in the 48hR2 comparison, but not in the 48hR2R3 comparison, may be due to its possible involvement in the root maturation processes in region 2. The nucleotide sequence of S4912250 shares 84% sequence coverage at 96% identity with GmWRKY57. Its amino acid sequence also shares 38% similarity with the AtWRKY70 protein (E value=8e-32). In this study, two soybean WRKY transcription factors sharing high similarity with GmWRKY4 and GmWRKY57 were differentially

expressed in soybean root region 2 in responses to low water potential. However, *GmWRKY4* and *GmWRKY57* were reported not to be induced by cold, salt, and dehydration treatment subjected to 14-day-old soybean seedlings (Zhou et al., 2008). It is also found that the members of WRKY family in Medicago truncatula (Mt) plants have roles in orchestrating secondary metabolic responses to biotic stress conditions (Naoumkina et al., 2008). Increase in soluble and wall bound phenolics and lignin compounds in tobacco plants over expressed with MtWRKY gene support the regulatory role of WRKY genes in secondary metabolic pathways under stress conditions.

Region-Specific, Stress-Inducible Homeodomain Lecine Zipper Transcription Factors

Homeodomain Leucine zipper (HDZip) transcription factors are unique to plants and characterized by a DNA-binding homeodomain which includes a 61-amino acid sequence and an adjacent putative leucine zipper motif (Schena and Davis, 1992). The Arabidopsis genome consists of 47 HDZip genes which are classified into four groups (from I to IV) based on sequence similarity criteria and supported by the intron/exon patterns. The HDZip TFs participate in several plant developmental processes, including vascular tissue, trichome development and mediation of external signals in plant growth regulation (Henriksson et al., 2005).

In this study, two HDZip genes were differentially expressed in soybean roots under severe water stress in different ways. The first one, putative transcription factor S5146255, displayed an increase of expression level in region 1 (Figure 12A). This result indicates that this HD TF may play an important role in root-growth maintenance in

region 1 under water stress. The annotation of this TF is *Glycine max* homeodomain-leucine zipper protein 57 (Hdl57) (NCBI BLAST). Tang et al., (2001) reported that GmHdl57 interacts specifically with promoters of VSP genes (Vegetative Storage Proteins), which are regulated during plant development and induced by wounding, water deficit, light, phosphate, carbon, nitrogen, and also by jasmonic acid and auxin. *Craterostigma plantagineum CpHB-7*, sharing 43% identity with *GmHdl57* (at E value= 9e-42), acts as a negative regulator of many ABA-responsive genes in response to exogenous ABA, cold, and drought treatments (Deng et al., 2006).

The amino acid sequence of GmHdl57 shares 63% and 68% similarities with Arabidopsis thaliana ATHB1 (E value=2e-35) and ATHB6 (E value=9e-32), respectively. Both ATHB1 and ATHB6 belong to the group HDZip I, which are widely spread and function as transcriptional activators, and which are induced by exogenous ABA treatment and water-deficit conditions (Söderman et al., 1999; Henriksson et al., 2005). Tobacco transgenic plants over-expressing the fused protein containing the DNA-binding domain and transactivating domain of *Arabidopsis thaliana* ATHB1 displayed the de-etiolated phenotypes in the dark. The defect in the development of palisade parenchyma under light treatment indicates its possible function in leaf development and response to light treatment (Aoyama et al., 1995). Arabidopsis gene *ATHB6* is highly expressed in guard cells and cells surrounding the stomatal pore, in leaf primordia, in developing cotyledons, in leaves, in primary roots, and in lateral roots, suggesting its function in cell division and differentiation (Söderman et al., 1999). Based on these findings, this homeodomain transcription factor may have roles in the maintenance of

root growth and may be involved in root cell division or differentiation under water-deficit conditions via the ABA-dependent pathway.

In contrast, the homeobox TF S5075763 was not only down-regulated in region 1 of 5-hour water-stressed roots, but also in root region 2 at 5 and 48 hours after transplanting (Figure 12B). The differential expression of S5075763 at 5 hours after transplanting in both regions may be a result of a response to mild water stress (root water potential of -0.4 MPa) at an early stage of the adaptation process. The HB TF S5075763 was also found to be down-regulated in the 48R2R3 comparison, which suggests that its differential expression in region 2 at 48 hours after transplanting is possibly related to stress response, but not root-maturation processes. The nucleotide sequence of this HB-TF shares 99.7% similarity with the *Glycine max* gene Glyma0902750 (PHYTOZOME BLAST). The amino acid sequence of this *Glycine max* gene has 80% identity (678/ 846 amino acids at E value=0) with the Arabidopsis *PHABULOSA* (PHB) gene belonging to the HD-ZIP III class of transcription factors. The PHB gene was reported to be present in the early stage of embryogenesis, but the domain expression is confined to the adaxial domain of the cotyledons and the central SAM (shoot apical meristem). PHB functions as the receptor for an adaxializing signal in determining radial patterning in shoots (McConnell et al., 2001).

Stress-Responsive, Region 1- Specific Zinc-Finger Proteins

The expression level of Zinc-finger protein S4917467 was significantly increased in region 1 of water-stressed roots compared to well watered roots at 12, 24 and 48 hours after transplanting (Figure 13A). This zinc-finger protein may possibly play an important

role in the maintenance of root growth under severe water-deficit conditions. The differential expression of this TF was found in 48hR2, but not in 48hR2R3. This result suggests that the up-regulation of zinc-finger protein S4917467 in region 2 of water-stressed roots may be related to root maturation processes, but not to stress response. No soybean genes or annotations in the genome database (PHYTOZOME and NCBI) have been found to share similarity in nucleotide sequence and amino acid sequence with this zinc-finger protein EST.

Another zinc-finger protein, S4879817, displayed differential expression in region 1, but not in region 2, of water-stressed roots (Figure 13B). It was down-regulated in 12hR1 but up-regulated in 48hR1, with a relative increase of 16 fold (P=0.075). The differential expression of this zinc-finger protein in region 1 at both 12 and 48 hours of severe water stress suggests its potential involvement in the maintenance of the elongation rate of root region 1. The nucleotide sequence of S4879817 shares 89% similarity with the *Glycine max* gene AK245208. The amino acid sequence of this TF shares 56% similarity (E value=1e-29) with Arabidopsis RHC1A (RING H2-type finger C1A) protein (BLAST NCBI). RING zinc-finger proteins consist of two types: RING-HC (C3HC4) and RING-H2 (C3H2C3), which function in plant development, defense, and response to abiotic stress by participating in gene regulation via the interaction with other regulatory proteins (Kam et al., 2007). Transgenic Arabidopsis plants over-expressing a RING-H2 zinc-finger gene named XERICO displayed hypersensitivity to salt and osmotic stress and to ABA treatments during germination and early seedling growth. XERICO also showed interaction with an E2ubiquitin-conjugating enzyme (AtUBC8)

and an ASKA-1 interacting F-box protein (AtTLP9) which was involved in the ABA signaling pathway, suggesting that XERICO could possibly play a role in ABA homeostasis through the ubiquitin/proteasome pathway (Ko et al., 2006). Among four *Triticum aestivum* genes (*TaRZF8*, *TaRZF38*, *TaRZF59,* and *TaRZF70*) encoding RING-H2 zinc-finger proteins which differentially expressed under drought treatment, only the *TaRZF70* gene was shown to be up-regulated in leaves and down-regulated in roots of bread wheat plants under both drought and exogenous ABA treatment (Kam et al., 2007). As the similar results in these studies show, zinc-finger proteins have specific roles in root-elongation maintenance in response to low water potential via the ABA-dependent pathway.

Stress-Responsive, Region-Specific bZIP Transcription Factors

Several bZIP TFs were differentially expressed in soybean water-stressed roots. The bZIP TF, TC216155, was down-regulated in region 1 of water-stressed roots at 5 and 24 hours after transplanting, when compared to well-watered roots (Figure 14A). This result suggests the possible relation of TC216155 to the maintenance of root growth at early stages of the water stress adaptation processes. This TF also exhibited differential expression in region 2 of water-stressed roots at all time points. The nucleotide sequence of this bZIP TF shares 98% similarity with *GmbZIP111* (E value=0), whose protein sequence shares 53% identity with the amino acid sequence of *ATbZIP11* (E value at 8E-28) (NCBI BLAST). The expression of the *ATbZIP11* gene is induced by light treatment and sucrose (Rook et al., 1998, Wiese et al., 2005). The fact that the target genes of *AtbZIP11* include *ASPARAGINE SYNTHETASE 1* and *PROLINE DEHYDROGENASE2*

suggests its involvement in amino acid metabolism (Hanson et al, 2008). ProDH controls proline level by the oxidation of L-proline to P5C, which is then converted to L-glutamic acid by P5C dehydrogenase. Proline concentration was shown to be increased in the maize primary root tip under low water potential via an ABA-dependent pathway (Ober and Sharp, 1994). The increase of proline accumulation in the 3mm apical region of water-stressed maize roots in turn contributes to the osmotic adjustment, which plays an important role in root-growth maintenance (Sharp et al., 2004). These results suggest that the down-regulation of the bZIP TF TC216155 in soybean water-stressed roots may lead to the decrease in expression level of ProDH, which in turn may contribute to increased proline accumulation, leading to root-growth maintenance in water-stress conditions.

Another bZIP transcription factor that was down-regulated in region 2 of water-stressed roots at 5, 12, 24, and 48 hours after transplanting was S23068684 (Figure 14B). Interestingly, the differential expression of this bZIP was also identified in region 1, but only in the 24 hour comparison, where it was down-regulated 2.5 fold. The bZIP transcription factor S23068684 represents *GmbZIP110,* with 100% similarity (NCBI BLAST). However, Liao et al., (2008) reported that the expression of *GmbZIP110* was not induced by dehydration or by 100 μM ABA treatment in 14-day-old soybean plants. GmbZIP110 shares 49% identity with *AtbZIP44,* which belongs to the same bZIP group S as *AtbZIP11* (mentioned above). However, *AtbZIP44* roles in plant growth and development have not yet been investigated (Jakoby et al., 2002).

Spollen et al. (2008) recently reported two bZIP TFs differently expressed in maize root tips under severe water-stress conditions. Rice TRAB1 (Transcription factor

Responsible for ABA regulation) and ABF3 were up-regulated in the 3-7 mm region and in the 0-3 mm region of water-stressed maize root tips, respectively (Spollen et al., 2008). Nevertheless, those two TFs were not found to be differentially expressed in soybean seedlings in this study. In summary, two bZIP TFs found in this study might be involved in the regulation of proline accumulation, which in turn contributes to osmotic adjustment in the root-system response to water stress via the ABA-dependent or the ABA-independent pathways.

Possible Hypotheses about TFs Involving Root-Growth Responses to Water Deficit

Physiological mechanisms underlying the root response to water deficit have been well studied in maize using seedling systems. Mechanisms that contributed to root-growth maintenance in the apical region include osmotic adjustment, enhanced cell-wall loosening, membrane hyperpolarization, and an increase in ABA accumulation (Sharp et al., 2004). However, the molecular regulations that control these physiological mechanisms have not been well studied. The identification of differentially expressed transcripts, especially transcription factors in different regions of water-stressed roots, might help to select candidate genes responsible for genetic control of the distinct growth responses. Using high-throughput real-time RT-PCR to profile 186 soybean transcription factors in different regions of well-watered and water-stressed roots, we revealed several candidate TFs, including bZIP, AP2, zinc-finger proteins, NAC genes, and homeodomain TFs. In this research, several possible genetic mechanisms related to those promising TFs were discovered, with the hope that further study can help to elucidate the physiological mechanisms underlying distinct root-growth responses to water deficit.

The TFs that might be involved in the maintenance of root growth under water-stress conditions are: Glycine max NAC4, MYB TF S4896043, GmHdl57, and Zinc-finger proteins S4917467 and S4879817. All of these transcription factors were shown to be up-regulated in region 1 of water-stressed roots, suggesting their potential involvement with root-growth maintenance during water deficit. Several hypotheses about the relationship of these TFs to root-growth maintenance were generated based on literature review. Further study of these hypotheses might give some understanding about genetic mechanisms underlying root-growth response to water deficit.

The study showed that the GmNAC4 transcript level was increased in region 1 of water-stressed roots at all four tested time points. GmNAC4 was also reported to be induced by exogenous ABA treatment (Tran et al., 2009). The increase in ABA accumulation in water-stressed roots will trigger the expression of several ABA-dependent genes, including GmNAC4. The increased expression of GmNAC4 will in turn contribute to the induction of several stress-responsive genes, resulting in appropriate responses which ultimately confer the maintenance of the elongation rate in region 1. The stress-responsive genes which act down-stream of GmNAC4 have not been well studied. Further investigation of these genes may give more understanding about the possible function of GmNAC4 in specific genetic and physiological mechanisms underlying root-growth maintenance under water deficit.

The MYB TF S4896043 was significantly up-regulated (with a very high fold ratio) in region 1 of water-stressed roots, suggesting its possible involvement in root-growth maintenance. The Arabidopsis homologue of this MYB TF, AtMYB61, was

reported to be involved in the control of the stomatal aperture via the ABA-independent pathway; however, the function of AtMYB61 in roots has not yet been investigated. Therefore, we hypothesize that the increase in MYB TF S4896043 expression level in water-stressed roots is independent of ABA and plays important roles in the maintenance of the elongation rate of the apical region.

The expression pattern of GmHdl57 suggests that it may act as a negative regulator or transcription activator and is induced by water stressed condition via ABA dependent pathway (perhaps related to cell division and differentiation in primary root tips), and that contributes to the root growth maintenance under water deficit. The extent of GmHdl57's role in primary roots is unknown and has not been adequately studied. The NAC protein's function as transcription activators along with the ZFHD (zinc finger homeodomain) proteins and the increased drought tolerance through the overexpression of these genes (Yamaguchi-Shinozaki and Shinozaki, 2006) suggests detailed investigation of these TFs in soybean.

The drought specific expression of zinc finger proteins S4917467 and S4879817 lead to test the hypothesis focusing on the role of these TFs in root growth maintenance during water deficits. Under water-stress conditions, these zinc-finger proteins were up-regulated, possibly triggering ABA homeostasis through ubiquitin/proteasome pathway, leading to the ABA accumulation in root tip, ultimately causing the maintenance of root growth under water deficit.

The bZIP transcription factor TC216155 is very similar to Arabidopsis ATbZIP11, which activates the expression of PROLINE DEHYDROGENASE2

(ProDH). ProDH participates in the oxidation of proline; therefore, the activation of the ProDH gene can lead to a decrease in proline concentration. Proline concentration was reported to be increased in the apical region of maize root tip via the ABA-dependent pathway and it was partially responsible for the maintenance of root growth in this region. In this research, bZIP transcription factor TC216155 was significantly down-regulated in region 1 of soybean primary root tips at 5 and 24 hours after transplanting. The down-regulation of this gene should result in the repression of ProDH, which might play a role in the increase of proline accumulation in region 1. In region 2, bZIP transcription factor TC216155 was down-regulated in all of the four tested time points. The down-regulation of this gene in region 2 might result in an increase in proline accumulation in region 2 of water-stressed roots. Proline accumulation can contribute to osmotic adjustment, which is one of the mechanisms that helps to maintain root growth under water-stress conditions. Therefore, the enhanced accumulation of proline as a result of the decreased expression of bZIP TC216155 can lead to two possibilities. The first one is that the proline increase in region 2 is not enough to maintain root growth. The second one is that proline concentration in region 2 might be transported to region 1, which helps to increase proline accumulation in this region. Results of Verslues and Sharp (1999) suggested that the transport rate of proline to the maize primary root tip increased during water stress conditions. Either way, it is clear that the down-regulation of bZIP TC216155 in water-stressed roots may play an important role in proline accumulation, contributing to the distinct responses of soybean roots to water-stress conditions.

These results show that the genetic controls underlying physiological responses of root growth under water-stress conditions are complex. They do not depend on the global expression of particular genes; rather, they rely on temporally and regionally distinct expression of many genes that are responsible for various mechanisms. Understanding regulatory mechanisms related to specific genes can help to select candidate genes, which may ultimately lead to improved drought tolerance through genetic and metabolic engineering.

Figure 10: Relative expression of CAAT-box and AP2-like transcription factors in soybean roots at 5, 12, 24, and 48 hours of low water potential. A) CCAAT-box transcription factor (S23064915). B) AP2-like transcription factor (TC206902). R1 stands for Region 1 (0-4mm); R2 stands for Region 2 (4-7mm). (*): Statistical significance P value <0.05.

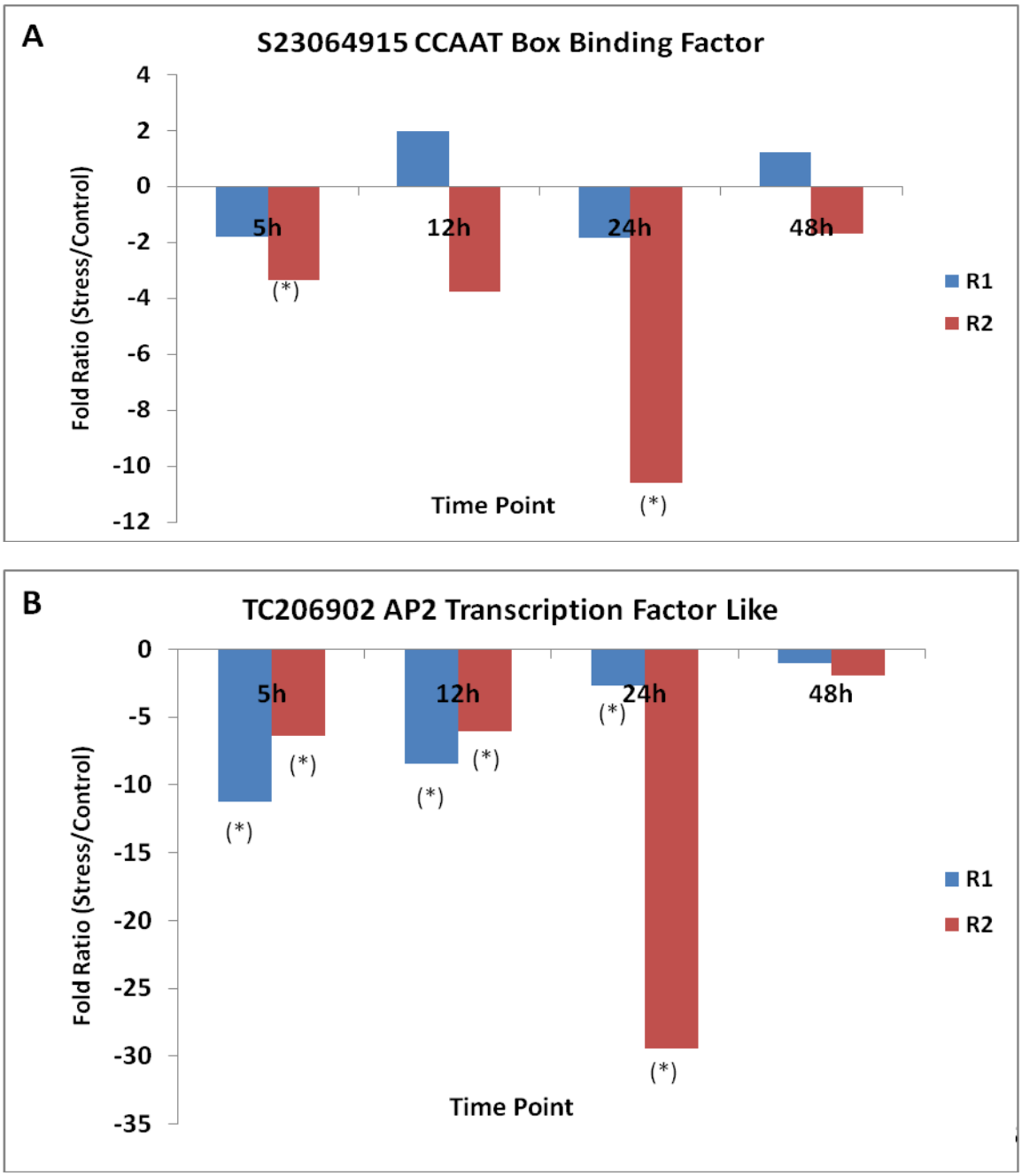
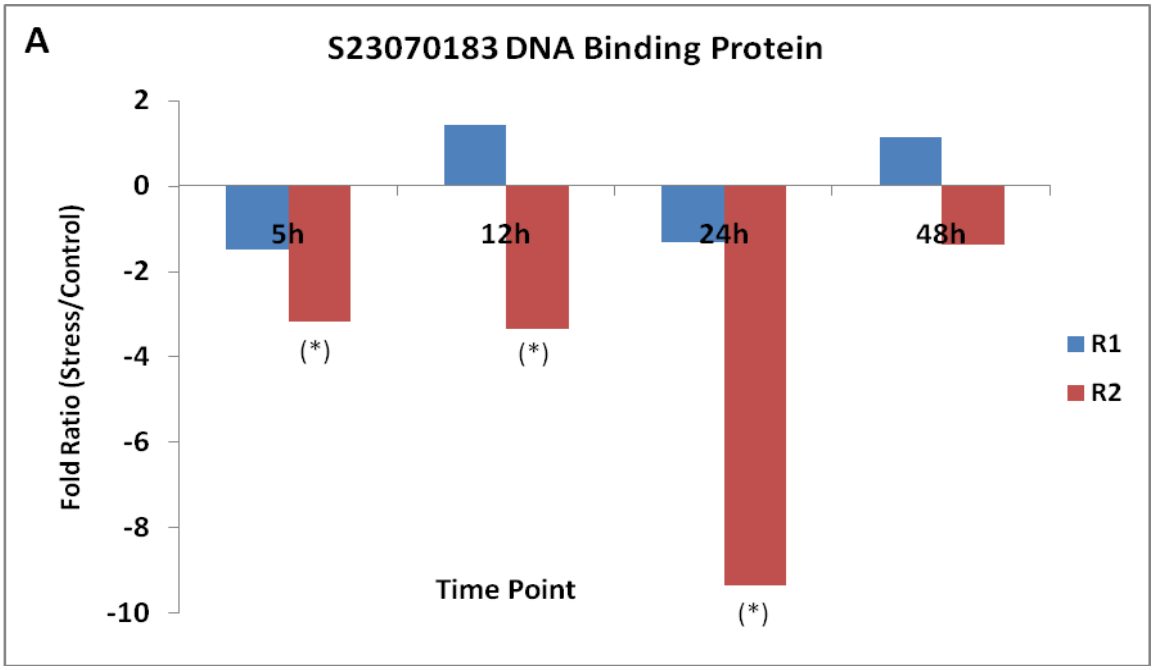


Figure 11: Relative expression of DNA-binding proteins of soybean roots at 5, 12, 24, and 48 hours of low water potential. A) S23070183. B) S4912250. R1 stands for Region 1 (0-4mm); R2 stands for Region 2 (4-7mm). (*): Statistical significance P value <0.05.



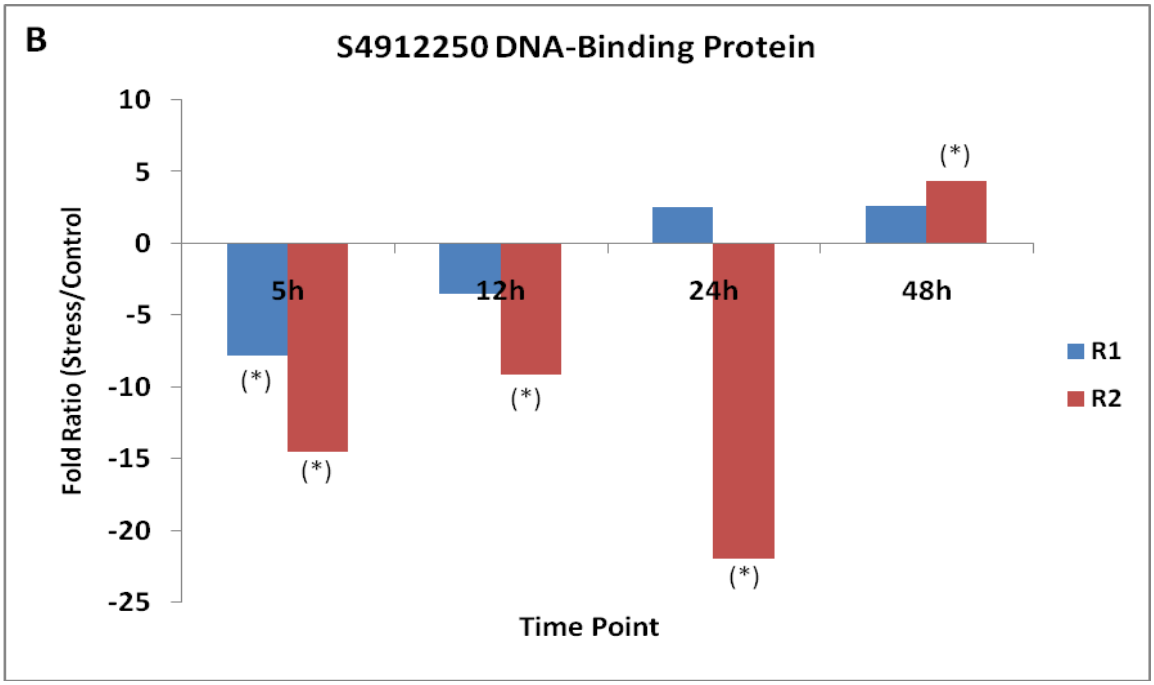
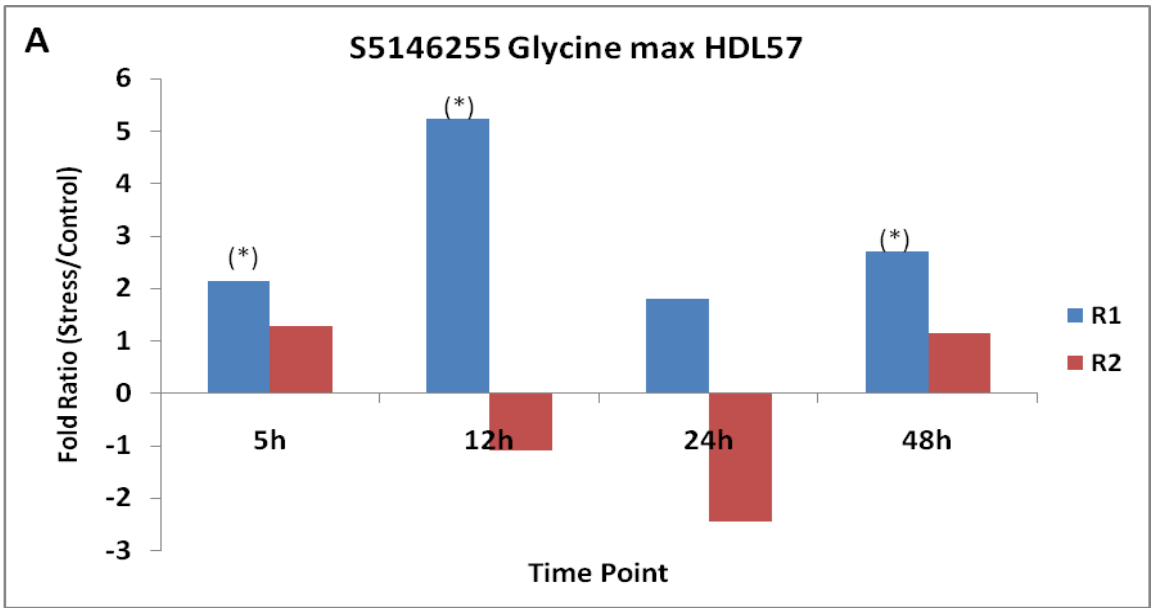


Figure 12: Relative expression of homeodomain leucine zipper transcription factors of soybean roots at 5, 12, 24, and 48 hours of low water potential. A) S5146255. B) S5075763. R1 stands for Region 1 (0-4mm); R2 stands for Region 2 (4-7mm). (*): Statistical Significance P value <0.05.



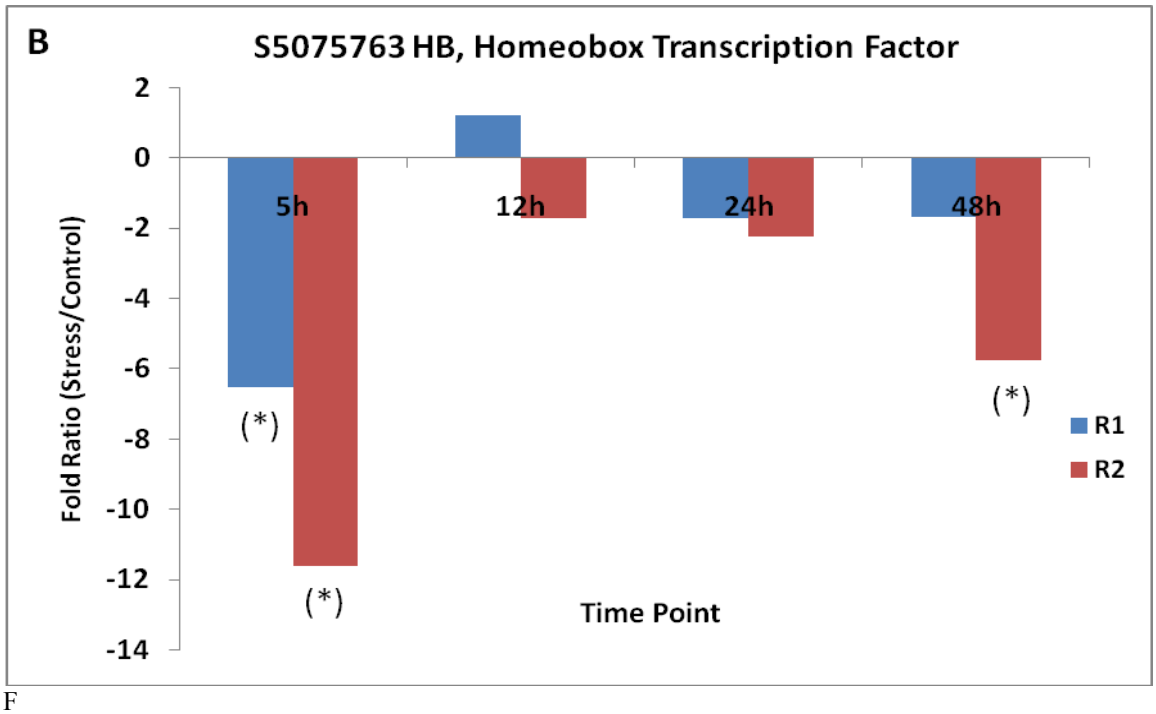


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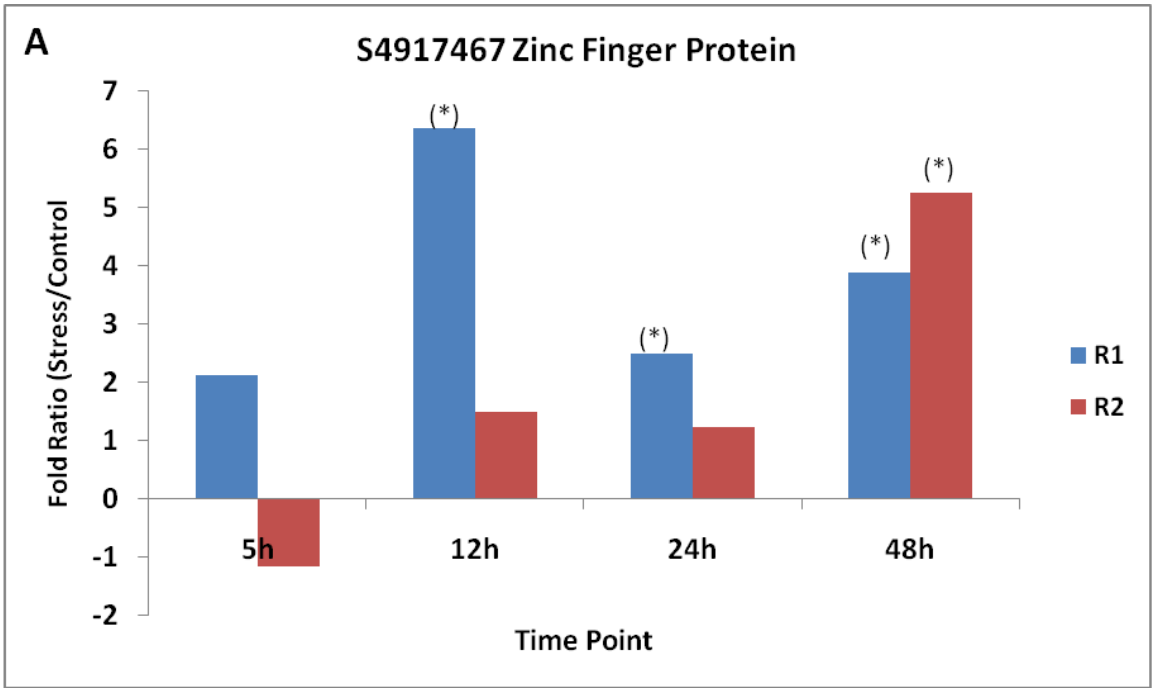
Figure 13: Relative expression of zinc-finger proteins of soybean roots at 5, 12, 24, and 48 hours of low water potential. A) S4917467. B) S4879817. R1 stands for Region 1 (0-4mm); R2 stands for Region 2 (4-7mm). (*): Statistical Significance P value <0.05.



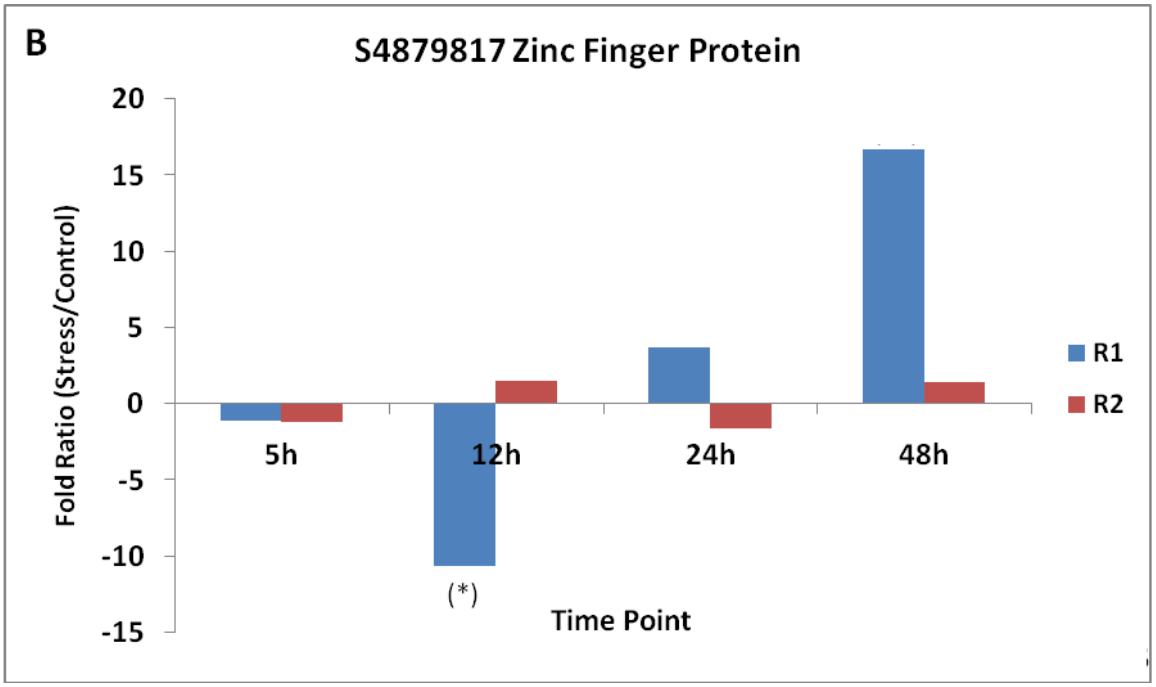
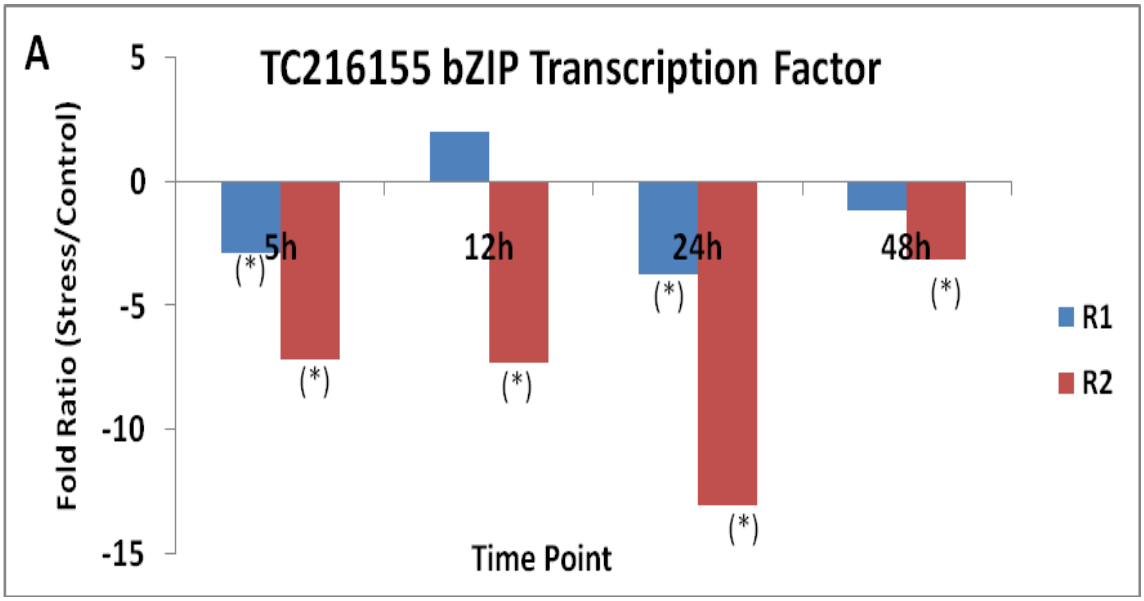


Figure 14: Relative expression of bZIP transcription factors of soybean roots at 5, 12, 24, and 48 hours of low water potential. A) TC216155. B) S23068684. C) TC205627. R1 stands for Region 1 (0-4mm); R2 stands for Region 2 (4-7mm). (*): Statistical Significance P value <0.05.



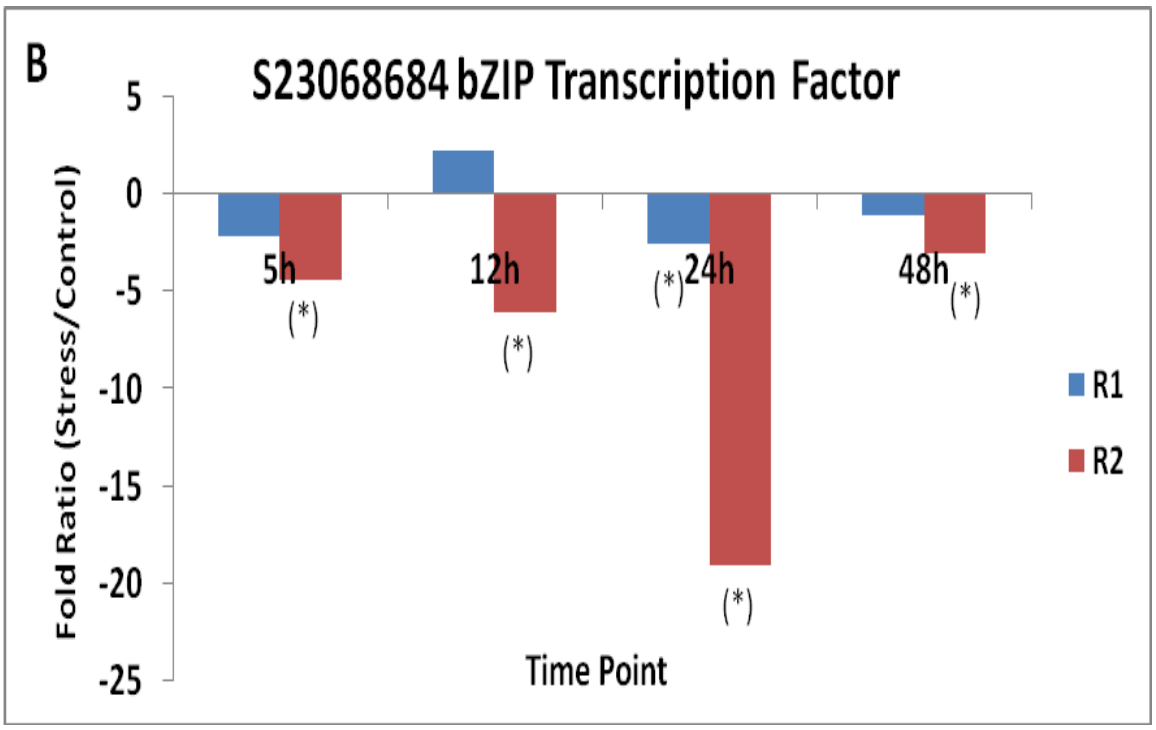


Table 10: Selected soybean stress-induced, region-specific transcription factors.
Relative quantification of gene expression in water-stress conditions compared to well-watered conditions in two soybean root regions: Region 1- R1 (0-4mm) and Region 2- R2 (4-7mm) at 5, 12, 24, and 48 hours after stress induction.

EST ID	Gene Name/ Function	Relative Expression (Fold Ratio between Stress/Control)								Accession No
		5hR1	12hR1	24hR1	48hR1	5hR2	12hR2	24hR2	48hR2	
AY974351	Glycine max NAC3	-1.33	1.02	4.25	-1.61	6.93	2.24	4.31	13.99	DQ028771
AY974352	Glycine max NAC4	3.95	7.04	5.85	4.48	3.89	2.08	3.32	12.67	DQ028772
S23064915	CCAAT-box binding factor	-1.81	1.95	-1.84	1.21	-3.36	-3.77	-10.59	-1.69	Glyma10g33550
S23068684	bZIP TF	-2.16	2.29	-2.55	-1.02	-4.34	-6.02	-19.00	-3.00	DQ787051
S23070183	DNA-binding protein	-1.50	1.43	-1.31	1.15	-3.19	-3.34	-9.37	-1.38	EU375355
S4879817	Zinc-finger protein	-1.08	-10.64	3.72	16.70	-1.19	1.55	-1.62	1.46	AK245208
S4896043	MYB domain transcription factor	17.78	4.57	12.57	64.97	3.65	1.32	1.31	9.33	Glyma03g38410
S4912250	DNA- binding protein	-7.80	-3.51	2.58	2.61	-14.45	-9.07	-21.97	4.40	EU375353
S4917467	Zinc-finger protein	2.13	6.37	2.51	3.90	-1.16	1.49	1.24	5.27	
TC211951	MYB domain transcription factor	-1.74	2.45	-1.16	1.67	-4.43	-4.03	-11.38	1.10	DQ822966
S5075763	HB, Homeobox transcription factor	-6.51	1.21	-1.71	-1.68	-11.60	-1.71	-2.24	-5.74	Glyma0902750
S5146255	Glycine max Hdl57	2.14	5.23	1.80	2.70	1.26	-1.10	-2.46	1.14	AF184278
TC206902	AP2 transcription factor like	-11.31	-8.50	-2.69	-1.06	-6.42	-6.10	-29.48	-2.00	Glyma10g33810
TC216155	bZIP transcription factor	-2.89	2.05	-3.77	-1.19	-7.21	-7.34	-13.10	-3.12	DQ787052

CHAPTER 4: CONCLUSION AND FUTURE PERSPECTIVES

Root growth is one of the crucial traits that allow crop plants to extract more water from the soil during drought conditions. Yamaguchi et al. (2009) reported the different response of distinct regions in the elongation zone of the soybean primary root under severe water deficit (a water potential of -1.6 MPa). In this study, we investigated the expression profiling of 186 soybean transcription factors (TFs) in different root regions of soybean primary roots at 5, 12, 24, and 48 hours after transplanting to a low-water-potential medium.

The results showed that the number of differentially expressed TFs at 5h, 12h, and 24h in both region 1 and region 2 is larger than it is at 48 hours after transplanting, suggesting that more TFs play roles in early events of the adaptation process of soybean root response to low water potential. The results also suggest that more TFs were differentially expressed in response to the low water potential of -0.4 MPa (5hrs), -1.1 MPa (12hrs), and -1.4 MPa (24hrs) than in response to -1.6 MPa (48hrs). The results also support the hypothesis that more TFs are differentially expressed before 48 hours, and that they are ultimately responsible for the expression of stress-responsive target genes. The induction of target genes may eventually trigger appropriate responses in the root adaptation process to water deficit through physiological mechanisms, such as osmotic adjustment, ABA accumulation, and enhanced cell-wall loosening. At the earliest and

latest events of the adaptation process at 5 and 48hours after transplanting there were more differentially expressed TFs in region 2 than in region 1. These results confirm the expectation that more TFs are required to be differentially expressed within the region 2, which has the reduction in root-elongation rate and has more metabolic machinery involving the root-development process. However, region 1 of water-stressed roots at 12h and 24 hours after transplanting showed slightly higher numbers of differentially expressed TFs than for the same time points in region 2. These results suggest that more TFs are needed to be differentially expressed during those middle dynamic events of the adaptation process of soybean root growth to water-stress conditions.

Transcription factors differentially expressed within region 1 may play a role in the maintenance of root elongation in response to severe water deficit. These TFs that we identified are zinc-finger protein S4879817, AP2-like TF TC206902, zinc-finger protein S4917467, MYB TF S4896043, *GmHdl57,* and *GmNAC4*. Meanwhile, differentially expressed TFs within region 2 may function only in the stress response of the primary root, or they may possibly play major roles in both stress-response and cell-maturation processes of root growth. These TFs that we identified are: bZIP S23068684, bZIP TF TC216155, DNA-binding protein S23070183, S4912250, CCAAT-box binding factor S23064915, homeobox TF S5075763, and *GmNAC4*. Several hypotheses can be developed from these results towards studies focused on deeper understanding of molecular regulatory mechanisms under water deficit conditions and there by selecting candidate genes/TFs for metabolic engineering for drought tolerance in crop species. For example, the expression pattern of certain TFs suggests the specific regulatory role of

root region specific and stress inducible TFs in metabolic pathways such as proline, ABA, phenolics and lignin (e.g. specific members of AP2, MYB, WRKY, bZIP and NAC families). These pathways have major physiological roles in drought resistance in plants.

Our results provide molecular details regarding the role of specific transcription factors that may be involved in root growth maintenance under water deficit. The transcription factors identified through this study and the related interacting partners may be associated with osmotic adjustment or to ABA accumulation and signaling, or perhaps to other processes that have not yet been revealed. This study has identified several potential TF candidates which are root-region specific and stress related, and will help dissect the molecular mechanism and gene-expression networks related to water-stress adaptation in crop plants in future investigations. In addition, the functional analysis of these root-region-specific and stress-related TFs will assist with the confirmation and selection of specific targets for soybean crop improvement under water-deficit conditions through genetic engineering approaches.

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