

FUNCTIONAL ANALYSIS OF DROUGHT RESPONSIVE  
SOYBEAN GmNAC003 AND GmNAC004 TRANSCRIPTION FACTORS  
IN LATERAL ROOT DEVELOPMENT IN ARABIDOPSIS

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Doctor of Philosophy

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by  
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FUNCTIONAL ANALYSIS OF DROUGHT RESPONSIVE  
SOYBEAN GmNAC003 AND GmNAC004 TRANSCRIPTION FACTORS  
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## LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	Abscisic acid
ABI	ABA insensitive
ARF	Auxin response factor
ATAF	Arabidopsis transcription activation factor
CUC	Cup-shape cotyledon
ERD	Early response to dehydration
GUS	Beta Glucuronidase
KRP	Kip-related protein
LBD	Lateral organ boundary domain
LR	Lateral root
LSD	Least significant difference
NAC	NAM, ATAF, and CUC; all belong to NAC transcription family
NAM	Non apical meristem
NPA	Naphthylphthalamic acid
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
QC	Quiescent center
RAM	Root apical meristem
SAM	Shoot apical meristem
SEM	Standard error of the mean
TF	Transcription factor
XTH	Xyloglucan endotransglycosylase/hydrolase

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ABSTRACT

NAC (CUC, ATAF and NAM) transcription factors have been found to promote lateral root (LR) numbers in Arabidopsis through the auxin signaling pathway. In soybean, a majority of genotypes increased LR number in response to water deficit. With the hypothesis that drought inducible soybean NAC transcription factors may be involved in the regulation of this enhanced LR number, two soybean *GmNAC003* and *GmNAC004* genes were included in the study.

*GmNAC003* and *GmNAC004* showed a high expression level in roots, leaves and flowers of soybean cultivar Williams 82. They were strongly induced by drought stress and moderately induced by abscisic acid (ABA). *GmNAC004* but not *GmNAC003* showed a moderate response to treatment of 2,4-dichlorophenoxyacetic acid (2,4-D). *Arabidopsis* transgenic *GmNAC003* plants did not show any response, while the transgenic *GmNAC004* plants showed an increase in LR number and length under non-stress conditions. The *GmNAC004* plants also maintained a higher LR number and length than the wild-type (WT) under mild water stress conditions. Treatment with ABA suppressed LR number more in the wild type than in the *GmNAC004* transgenic plants suggesting

that GmNAC004 counteracts ABA-induced inhibition of LR number. Treatment with 2,4-D increased LR in both GmNAC004 transgenic and WT plants but the promotion was higher in the transgenic plants. Conversely, treatment with Naphthylphthalamic acid inhibited LR number and resulted in no difference in the trait between the transgenic GmNAC004 and WT plants. These results suggest that GmNAC004 suppresses ABA while promoting auxin signaling to increase LR number in *Arabidopsis*.

## **CHAPTER 1: INTRODUCTION**

The root system is vital to plant growth and development. It provides minerals and water for plant growth, mechanical support, and anchors the plant to the soil. Root systems generate various chemical signals to regulate shoot growth and development (Schachtman and Goodger 2008), and in return the roots take energy from the shoot system for many biological processes. Genetic factors provide certain limits on root growth, but roots also exhibit plasticity in growth and development in response to environment stimuli (e.g., water, nutrient, and biotic stresses). This plasticity allows plants to adapt to changes in environmental conditions. The plant can optimize its root architecture through the regulation of growth and development in order to acquire optimal water and nutrients and to avoid negative effects of the environment, such as toxicity or other growth-limiting constraints. Understanding the molecular mechanisms regulating root architecture in response to environmental conditions will allow genetic engineering for better root growth, to enhance crop production in areas prone to water and nutrient stresses.



## LATERAL ROOT DEVELOPMENT

The plant root is comprised of three regions involved in root growth: the root apex, consisting of the root apical meristem (RAM); the root-elongation zone, where active cell division and cell expansion occur; and the maturation zone. The RAMs are conserved in both primary roots and lateral roots (LRs), with a cell set to initiate various tissue cell types around the quiescent center (QC). The QC directs the surrounding cells to produce the initials of ground tissue and stele above it (proximal initials) and the initials of root cap below it (distal initials). Active cell division and differentiation produce most of the vascular and ground tissues; those differentiated cells will later go through a rapid cell expansion to maturation. In *Arabidopsis*, at a certain distance from the RAM, the first order of lateral roots can be initiated from the primary root (Peret *et al.* 2009a).

The formation of lateral roots in *Arabidopsis* goes through several major stages (reviewed by Peret *et al.* 2009a). The first stage is lateral root pre-initiation that occurs at the very end of the root tip in the basal meristem, which is adjacent to the root apical meristem (a transition region between the meristem and the elongation zone) (De Smet *et al.* 2007). Certain xylem pericycle cells receive signals to be the lateral root primordia founder cells that are ready to divide and advance to the G2 stage of mitosis, while other pericycle cells remain at G1 (Beeckman *et al.* 2001; De Smet *et al.* 2007). The signal for this process of cell priming is dependent on auxin accumulation, which occurs at approximately 15-hour intervals at the basal meristem. The accumulation is

associated with the formation of executive lateral roots at a later stage (De Smet *et al.* 2007). The second stage is the formation of a dome-shaped lateral root primordium from the division of one or a pair of pericycle cells adjacent to the xylem pole. In the third stage, the division of the pericycle cells produces the full shape of the lateral root. During the final stage, lateral root stem cells are activated and start their cell division, and the lateral root breaks through the outer root layers and emerges from the main root. Although these steps proceed in a coordinated manner, it has been shown that distinct molecular mechanisms regulate lateral root initiation and emergence (Swarup *et al.* 2008; Peret *et al.* 2009b; Hodge *et al.* 2009), which is discussed in detail below.

### **Hormonal regulation and genetics of lateral root development**

Molecular analysis has revealed a coordinated network involving hormonal interactions in the regulation of lateral root development. The phytohormone auxin is the primary hormone acting in the regulatory process. Other hormones may interact with auxin in a responsive manner, which may require signals from growth and environmental stimuli (Peret *et al.* 2009a; Peret *et al.* 2009b; Hodge *et al.* 2009; Malamy 2005). The lateral root founder cells are generated every 15 hours; however, those cells must go through lateral root initiation and emergence processes, which require various regulatory factors, to eventually develop into lateral roots.

## Auxin

Auxin is synthesized in both young parts of the shoot and in the roots. Shoot-generated auxin is transported rootward via phloem to the root tips, a major auxin sink. From the root tips, auxin is transported horizontally and shootward through the lateral root cap, root epidermis, and other root cells via the coordination of polar auxin transporters.

There are several known auxin transporter families (Overvoorde *et al.* 2010): auxin influx carriers (AUX1, auxin resistant and LIKE-UAX1) and auxin efflux carriers (PIN-FORMED (PIN) and P-GLYCOPROTEIN ABC transporter family).

The auxin signal is required at early events of pericycle cell priming (see above), which happens at the basal meristem and determines the founder cells and spatial distribution of lateral roots along the root axes. At the basal root region, there is a specific accumulation of auxin every 15 hours at the xylem pole pericycle cells, and these cells later become founder cells for lateral root initiation in the maturation region (De Smet *et al.* 2007). The accumulation of auxin causes the cytoplasm of these cells to thicken, restructures cell organelles, and activates various cell cycle proteins to direct cell division (Himanen *et al.* 2004). In Arabidopsis, accumulation of auxin by AUX1 and PIN, local auxin transporters, causes degradation of IAA14, a negative regulator of auxin-regulated transcription. Degradation of IAA14 results in the repression release of auxin response factors ARF7/19 that allows them to bind to the promoters and activate the expression of lateral organ boundary domains *LBD16/LBD29*, which in turns activates cell cycle and cell patterning (Peret *et al.* 2009a&b). Accumulation of auxin also causes

the degradation of IAA3, resulting in the activation of cell wall remodeling enzymes that facilitate cell separation and cell shape modification (Swarup *et al.* 2008). The modification of cell walls in the outer cell layers facilitates the emergence of the lateral root (Peret *et al.* 2009a).

Genetic analysis shows that modification of auxin biosynthesis, transport, and signaling affects lateral root development. Increased auxin levels by either over-expression of auxin biosynthesis or mutation of the negative regulators increased lateral root number (Overvoorde *et al.* 2010). For example, mutants in auxin influx and efflux transporters such as *aux-lax* and *pin* showed abnormal lateral root formation. Mutation in *Aux1* resulted in decreased lateral root primordia (Laskowski *et al.* 2008). In accordance with this, the *pin2* mutant that blocked the efflux auxin transport resulted in the accumulation of auxin at the lateral root primordial tip stimulating lateral root emergence (Swarup *et al.* 2008).

## **Cytokinin**

Cytokinin levels in roots are negatively associated with lateral root development, as shown in the application of cytokinin, in the reduction or over-expression of cytokinin biosynthetic and signaling enzymes (Werner *et al.* 2003; Laplaze *et al.* 2007), and in mutants that are defective in cytokinin biosynthetic and signaling factors (Riefler *et al.* 2006). In *Arabidopsis*, the action of cytokinin is limited to the initiation events that occur at the xylem pericycle cells, rather than at the primordial formation stage (Laplaze *et al.* 2007). In LR development, cytokinin has an antagonistic role to auxin. Cytokinin

suppresses a number of *PIN* genes in the LR founder cells, which results in alteration of the auxin gradient essential for primordial patterning. Down-regulation of cytokinin showed an increased LR number. In the presence of cytokinin, apical dominance is favored and represses lateral root development (Lloret and Casero 2002).

## **Ethylene**

Ethylene can have either an inhibitory or stimulatory effect on lateral root development, depending on its concentrations in the tissues. Treatments with low concentrations of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) increase the number of lateral roots, but higher doses of ACC inhibit the initiation of new lateral root primordia and promote the emergence of existing lateral root primordia (Ivanchenko *et al.* 2008). XBAT32, a RING E3 ligase, regulates lateral root production through its role in ethylene biosynthesis (Prasad *et al.* 2010). The *xbat32* mutants showed elevated ethylene production, which resulted in inhibition of lateral root number in Arabidopsis.

## **Abscisic acid (ABA)**

ABA plays various roles in the regulation of plant growth, including seed maturation and dormancy (Koornneef *et al.* 2002), leaf senescence (Lim *et al.* 2007), stomatal aperture regulation (Hose *et al.* 2002), and maintenance of root growth under low water potentials (Sharp and LeNoble 2002; Sharp 2002). In plant development, ABA is required to maintain the meristem structure and function. For example, ABI8, a protein mediating ABA and sugar response, is essential for normal root meristem activity. The *abi8* mutants were still able to initiate lateral roots, but root meristems lost their

capacity to divide (Brocard-Gifford *et al.* 2004). Under water stress, ABA is necessary to maintain root growth and development. For example, *abi1-1* and *aba-1* mutants in ABA signaling showed greatly reduced capacity to produce short root and hairless roots, which are critical to the production of a new functional root system upon rehydration (Vartanian *et al.* 1994). In maize, ABA accumulation suppresses excess ethylene production to maintain root growth in plants under water deficit stress (Sharp and LeNoble 2002; Sharp 2002).

ABA is also an inhibitor of lateral root development (De Smet *et al.* 2006), with an antagonistic effect to auxin on lateral root primordial formation and emergence, under both normal and osmotic stress conditions. Exogenous ABA treatments suppress LR numbers in wild-type *Arabidopsis* (Razem *et al.* 2006), while the ABA-deficient mutants *aba2-1* and *aba3-1* showed larger root systems under osmotic stress (using mannitol) than the wild-type plants (Deak and Malamy 2005). Another ABA-signaling mutant, *Ird2*, also had more lateral roots under osmotic stress than the wild type (Deak and Malamy 2005).

Although the molecular connection of ABA in LR development is not well understood, some studies have shown that ABA inhibits LR development in both initiation and post-initiation of LR development. The antagonism between ABA and auxin signaling in the regulation of lateral root development has been shown as early as the lateral root initiation stage. ABA was found to promote expression of the cell cycle regulation genes, KRPs, which encode the cell cycle inhibitors. In contrast, in the presence of auxin,

expression of KRPs was repressed (Himanen *et al.* 2002; De Veylder *et al.* 2001). In addition, transcript profiling of the lateral root-less mutant revealed that some of the lateral root-initiation genes, such as *AIR12* and *IAA19*, were down-regulated under ABA treatment (Hoth *et al.* 2002). ABA inhibition of LR development was even more evident at the post-initiation stage. *Arabidopsis* cv. Columbia did not produce any LRs after 9 days of treatment with ABA, but when the seedlings were transferred to normal conditions, they were able to produce many LRs (De Smet *et al.* 2003).

One possible crosstalk between ABA and auxin-signaling pathways to control lateral root development is found through *ABI3* (De Smet *et al.* 2006). *ABI3* is a positive regulator of the ABA-signaling pathway and shares a DNA-binding motif with auxin response factors (ARFs) (Ulmasov *et al.* 1997). Two of the ARF members, ARF7 and ARF19, directly activate the expression of key genes in lateral root development: LBD16 and LBD29 (Okushima *et al.* 2007). Brady *et al.* (2003) showed that *ABI3* was induced by auxin in lateral root primordia and that the *abi3* mutant reduced the initiation of lateral roots in auxin-treated *Arabidopsis*, indicating that ABA signaling is involved in auxin regulation of lateral root development.

## **ROOT GROWTH IN RESPONSE TO ABIOTIC STRESSES**

Root architecture in dicot plants is defined by tap root length and by lateral root length, density and distribution. The tap root is produced during embryogenesis, while lateral roots are produced throughout the life of the plant. Phenotypic variation in root

architecture in plants is controlled by genetic (G) factors, environmental factors (E), and the interaction between G and E (Malamy 2005). Optimum root architecture (root length, lateral root density, and distribution) is the result of coordinated action between the genetic limit and the environmental factors (Malamy 2005). Environmental conditions include the nutrient composition of the soil; soil physical properties such as particle size and compactness, soil temperature, and porosity; soil water content; and the living organisms around the plant. The plant that is able to change its root morphology and structure exhibits plasticity in root architecture to optimize root growth and development to maximize acquisition of resources.

### **Root growth in response to phosphorous and nitrogen availability**

Root development and growth exhibits a high plasticity in response to nutrient conditions. In barley, localized promotion of lateral root number and length was found in root regions rich in phosphorus or nitrogen, but no promotion was found in potassium-rich regions (Drew 1975). This root architecture plasticity demonstrates a highly effective utilization of environmental resources to survive and reproduce in response to stresses. In Arabidopsis, genotypes with the highest root/shoot mass had high phosphorus uptake efficiency. These genotypes can increase their root length and root hairs to promote survival in phosphorous-deficient soils (Narang *et al.* 2000). In common bean, response to phosphorus deficiency results in a decrease in the primary root length and an increase in lateral root number and length in the top soil, where absorbable phosphorus is more available (Liao *et al.* 2001). Auxin has been found to be a



major player in this regulation of root development. For example, low phosphate in the media induces changes in cell cycle gene expression by increasing auxin sensitivity in the *Arabidopsis* root system (Pérez Torres *et al.* 2009).

Response to nitrogen in *Arabidopsis* depends on whether the source is ammonium or nitrate. Nitrate has a localized effect to stimulate the elongation of lateral roots and is regulated by a putative MADS-box transcription factor, ANR1 (Zhang and Forde 1998). A high nitrate concentration has a systemic inhibitory effect on LR number in distal regions, specifically on the emergence of the lateral root primordium (Zhang *et al.* 1999). Ammonium application, however, did not alter LR length, suggesting that nitrate acts as a signaling effect rather than a nutrient effect (Zhang *et al.* 1999). This inhibitory effect on LR development through local nitrate application requires ABA signaling; several ABA mutants (ABA-insensitive mutants *abi4-1*, *abi4-2*, and *abi5-1*; and ABA-biosynthesis mutants *aba1-1*, *aba2-3*, *aba2-4*, and *aba3-1*) showed significant reduction in inhibitory response (Signora *et al.* 2001).

## **Root growth in response to water deficit condition**

Water deficit has the most dramatic effect on growth and productivity of plants (Boyer 1982). Among plant tissues, shoot growth is more sensitive to drought than the root, which maintains some growth even at levels where shoot growth is completely inhibited (Sharp *et al.* 2004; Yamaguchi and Sharp 2010). Maintenance of root growth under drought is considered to be a mechanism to maintain tissue water content through greater extraction of water from the soil (Turner *et al.* 2001). In the plant root system,

root hairs may cover up to 70% of the root surface area and they are located between the root tip and the lateral root formation region (Wang *et al.* 2006). The root hair zone is the most permeable zone, with high radial and axial conductivities. Thus it plays a major role in water and nutrient uptake (Segal *et al.* 2008). Deep tap root growth may theoretically reach deeper soil profiles where water is more available, while optimized localization of LR density and length may allow more effective water and nutrient extraction from soil under water deficit conditions.

Plants adapted to drought conditions may have a constitutively deep root system that is pre-developed before the occurrence of drought. It has been shown in rice that many drought-resistant genotypes have deeper and more branched root systems compared to drought-sensitive genotypes in a hydroponic screening system (Price *et al.* 1997). Deep root systems might provide an advantage to plants during long-term water scarcity. A crop modeling analysis of maize productivity over 20 years at Columbia, MO showed that deep rooting in maize increased yield and transpiration efficiency (Sinclair and Muchow 2001). However, in soybean, a crop modeling analysis over the span of two years has shown that rooting depth does not improve yield in either dry or wet locations among 2655 tested locations (Sinclair *et al.* 2010). The soybean model used five plant traits: rooting depth extension, rate of leaf area development, decreased stomata conductance at high soil water content, reduced maximum transpiration rate, and drought-tolerant nitrogen fixation. Based on the model, it is suggested that reduced water loss by early responsive stomata and maintenance of nitrogen fixation are most critical for soybean productivity in drought-prone areas. The model did not, however,

clearly define the root depth and root density, which may limit the interpretation and the application of the model. This is because root hairs are located between the root tip and the lateral root formation region, higher LR density (or higher density of root tips) is greatly important for water and nutrient absorption (Wang *et al.* 2006).

Genetic modification has produced a number of transgenic plants with a larger root system by overexpressing candidate genes for drought tolerance. Having larger root systems may potentially enhance water extraction, which in turn should improve drought tolerance in plants (Turner *et al.* 2001; Manavalan *et al.* 2009). Tobacco plants overexpressing a gene encoding mothbean [ $\delta$ ]-pyrroline-5-carboxylate synthetase, an enzyme that functions in proline biosynthesis, showed longer roots in both well-watered and water-deficit conditions (Kishor *et al.* 1995). The authors claimed that the transgenic plants had better tolerance to drought and salinity; however, further tests are needed to confirm their conclusions (Blum *et al.* 1996). Recently, a HDSTART transcription factor gene identified in *Arabidopsis* showed enhanced root development (root length and lateral root number) in both *Arabidopsis* and tobacco (Yu *et al.* 2008). The transgenic plants showed improved drought tolerance by (i) reducing water loss which resulted from reduced stomatal density, and (ii) increased oxidative stress tolerance. Unfortunately, the role of the larger root system in the increased tolerance has not yet been investigated.

The developmental plasticity of the roots is an advantage that allows plants to adapt to a water-deficit environment. For example, in common bean, the two drought-tolerant

genotypes had a higher root density in deeper root profiles in drought conditions, while drought-sensitive genotypes did not (Sponchiado *et al.* 1989). Under non-drought conditions, both tolerant and sensitive genotypes showed comparable yields, but under drought stress, the drought-tolerant genotypes had a higher yield than the drought-sensitive genotypes.

In response to drought stress, plants may prioritize growth and development in certain parts that are most beneficial to the plant in order to maintain growth and reproduction. The distribution of carbon assimilates could be more directed to support root growth, which in turn, would help the plant extract more water under drought stress. It has been shown that root depth is correlated to water uptake from soil (Allmaras *et al.* 1975). The depth of the root system, the root biomass, the root elongation rate, and the lateral root numbers in many plants have been reported to be affected by water-deficit stresses (van der Weele *et al.* 2000; Deak and Malamy 2005, Manavalan *et al.* 2009). A common response to water deficit stress in plants is that shoot growth rate is reduced while root growth is less affected, or stimulated in many cases (Read and Bartlett 1972; Sharp *et al.* 1998; He 2008). Localized stimulation of root growth has been observed in response to nutrient and water deficiency while a systemic inhibition of growth was found in the shoot and in the root region where the absorbable sources are less available (Drew 1975; Zhang *et al.* 1999; Huck *et al.* 1983; Hoogenboom *et al.* 1987).

## **ROOT GROWTH IN RESPONSE TO WATER-DEFICIT CONDITIONS IN SOYBEAN**

In soybean, the roots are distributed in the top soil when water is sufficient, but under water stress, extensive root growth and development occurs deeper in the soil profile (Allmaras *et al.* 1975; Hoogenboom *et al.* 1987; Huck *et al.* 1983; He 2008). Early establishment of the root system (seedling vigor) could be one of the important traits in the selection of soybean genotypes for improvement of soybean production in drought-prone areas (Manavalan *et al.* 2009). Not surprisingly, under water-deficit conditions, root growth rate is relatively less inhibited while shoot growth is significantly inhibited (Read and Bartlett 1972; He 2008).

There are three contiguous regions of root growth: cell division, elongation, and maturation. Root elongation is the result of cell division and cell elongation. Extensive research has been done in maize and soybean for the physiological mechanisms of the maintenance of root elongation in response to low water potential conditions. Under drought stress, cell expansion is more severely affected, while the rate of cell division is much less affected (Hsiao 1973). Root-elongation kinematics under water deficiency in maize and soybean showed that the elongation of the root tip region (3 mm in maize and 4 mm in soybean) was not affected at a water-stress level of -1.6 MPa in comparison with the control (-0.03 MPa), while the adjacent regions were significantly affected (Sharp *et al.* 1988; Yamaguchi *et al.* 2010). Cell expansion is the result of internal cell pressure and cell wall extensibility. Internal pressure is produced by the

synthesis and accumulation of low molecular weight compatible organic solutes in the cytoplasm. Under -1.6 MPa water stress, turgor pressure was reduced in the maize primary root, compared to the control, and no longitudinal variation in turgor pressure was observed (Spollen and Sharp 1991). The region that maintained its elongation rate showed reduced radial expansion, but its cells were longer than in the control condition (Fraser *et al.* 1990). Furthermore, there is involvement of proton flux (Fan and Neumann 2004) and expression of several cell wall loosening enzymes (expansins and xyloglucan endotransglycosylase/hydrolases (XTH)) that correlate with the elongation zone (Wu *et al.* 1994; Wu *et al.* 1996). The maintenance of root elongation at the root tip under drought condition in maize, therefore, is likely to be regulated by both cell wall loosening and the direction of cell elongation. Progress in molecular mechanisms regulating the maintenance of root length under water stress showed a complex response. The molecular mechanisms may include the accumulation of reactive oxygen species that regulate cell wall loosening properties and the spatial distribution of phenylpropanoid compounds in root zones, as predicted from maize and soybean transcript and protein profiling (Yamaguchi and Sharp 2010).

Lateral roots play major roles in the acquisition of water and nutrients and are highly flexible in response to environmental conditions (Malamy 2005). In soybean, lateral root number was stimulated under drought, and the levels of responses were positively correlated with increasing drought stress (Read and Bartlett 1972). Recently, He (2008) reported a similar response when soybeans were exposed to mild water stress (-0.25 MPa). An increase in lateral root number of up to 30% was seen in eight of the eleven

soybean genotypes tested. Significant increase in the total LR length was also seen in ten of the eleven genotypes, suggesting a common response in soybean. The response of those genotypes was relatively consistent in experiments conducted in a growth chamber at early seedling stage and in 3-week soil drying treatments. It is noted that the primary root lengths were similar between the mild stress and the control treatment. In response to the mild water-deficit condition, six of the eleven genotypes showed reduced root biomass, while all of the genotypes showed reduced shoot growth (He 2008). The data suggest that soybean plants can optimize resources to prioritize lateral root development to adapt to stress. The study provides excellent information for further genetic analysis towards an understanding of the regulation of LR development in soybean under water deficit conditions.

A greater understanding of molecular mechanisms controlling root growth and development has gained been achieved (Peret *et al.* 2009a&b; Hodge *et al.* 2009); however, the molecular knowledge of how plants regulate their structure in response to water stress is poorly understood. Malamy (2005) suggested that root growth and development was strongly regulated by environmental stimuli as extrinsic regulation that incorporates the intrinsic root growth regulation pathways. Profiling of transcripts, proteins, and metabolites, together with functional genomics, may eventually allow the identification of the genes and gene networks regulating root growth and maintenance in response to environmental stress. This information can then be used for genetic engineering to develop crops with improved productivity for water-deficit cultivation zones.

## NAC TRANSCRIPTION FACTORS

### NAC transcription factors in adaptation to environmental stress

The NAC (NAM-no apical meristem, ATAF- Arabidopsis transcription activation factor, and CUC-cup-shaped cotyledon) transcription-factor family is a large plant-specific transcription factor family that has more than 100 members in *Arabidopsis* and rice (Olsen *et al.* 2005). NAC proteins have a conserved NAC domain, which is located at the amino terminal, and a diverse carboxyl terminal (Ooka *et al.* 2003). The NAC domain consists of 5 sub-domains, A, B, C, D, and E, which are important for DNA binding activity, while the carboxyl terminal is important for transcriptional activation (Duval *et al.* 2002).

NAC transcription factors are involved in diverse processes including development, defense, and abiotic stress responses. Many mutants in the *nam* (no apical meristem) in *petunia* do not have the shoot apical meristem and die at the seedling stage (Souer *et al.* 1996), which is similar to the *cuc* (cup-shaped cotyledon) in *Arabidopsis* (Aida *et al.* 1997). CUC regulates embryonic shoot apical meristem (SAM) formation through both the STM (shoot meristemless) pathway and the STM-independent pathway (Hibara *et al.* 2003). Several NAC proteins have been shown to be involved in plant defense to biotic pathogen attacks (Olsen *et al.* 2005; Nakashima *et al.* 2007; Jensen *et al.* 2008).

In abiotic stress signaling, NAC transcription factors are involved in both the ABA-dependent and the ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki



2007). Over-expression of several NAC proteins showed improved drought tolerance in both rice and *Arabidopsis*. In rice, *SNAC1* (Hu *et al.* 2006) and *OsNAC6* (Nakashima *et al.* 2007) are positive regulators of abiotic signaling, and their expression induces various abiotic stress-inducible genes. Over-expression of *SNAC1* and *OsNAC6* improve rice tolerance to drought and salinity. *SNAC1* is expressed differentially in guard cells, and its expression promotes stomatal closure, reduces water loss, and improves spikelet fertility in transgenic rice (Hu *et al.* 2006). In *Arabidopsis*, over-expression of *ANAC019*, *ANAC055*, and *ANAC072* induces expression of many stress-related genes, including a gene encoding a glyoxalase I family protein (Tran *et al.* 2004) that is known to function in detoxification (Thornalley 2003). The survival rate is significantly higher in these transgenic plants; however, the physiological mechanisms of the increased tolerance have not yet been dissected. It is important to note that not all drought- and salinity-inducible NAC genes are positive regulators of stress adaptation. A recent study showed a drought-inducible *Arabidopsis* NAC transcription factor, *ATAF1*, acting as a negative regulator in drought signaling. The *ataf1* mutant showed up-regulation of drought-inducible genes and improved recovery rate after drought stress (Lu *et al.* 2007).

## **NAC transcription factors and lateral root development**

Auxin plays a major role in the regulation of lateral root development in *Arabidopsis*, and NAC is required for the regulation of lateral roots by auxin. The *Arabidopsis* transcription factor *NAC1* acts downstream of *TIR1*, an auxin receptor, to regulate several proteins in the auxin signaling pathway. Over-expression of *NAC1* increased

lateral root number, while down-regulation of NAC1 gene expression by antisense RNA reduced lateral root development (Xie *et al.* 2000) suggesting that NAC must be present for the auxin-induced lateral root development. AtNAC2, another Arabidopsis NAC transcription factor, showed stimulation of lateral root number in transgenic plants (He *et al.* 2005). AtNAC2 was induced by dehydration, ABA, and salt stresses. The protein promotes lateral root development in *Arabidopsis* through auxin and ethylene signaling (He *et al.* 2005).

Soybean contains a large family of NAC transcription factors, with an estimated 226 members (Wang *et al.* 2010; Soydb: <http://casp.rnet.missouri.edu/soydb/>). Many of these genes may be presented in duplication blocks in the soybean genome (Schmutz *et al.* 2010). Genome-wide transcriptome analysis estimates that about 25-30% of NAC genes from Arabidopsis and rice may function in stress response (Tran *et al.* 2010). In our previous report, expression profiling of 31 soybean NAC genes identified 9 dehydration-inducible NAC genes (Tran *et al.* 2009). Eight of the nine transcription factors showed the ability to activate transcription in a yeast assays. Among the nine genes, only two genes, *GmNAC003* and *GmNAC004*, showed differential expression in response to ABA treatment. These two transcription factors may be important regulators for the adaptation of soybean to drought stress in relation to the ABA-signaling pathway. They were expressed strongly in the root, suggesting that they may be involved in the regulation of growth and development of the root system in response to the water-deficit conditions.

## **HYPOTHESIS AND OBJECTIVE**

As discussed earlier, there is increasing evidence that NAC transcription factors are important for stress tolerance in plants. In root development, over-expression of *Arabidopsis* NAC1 and AtNAC2 transcription factors showed enhanced lateral root numbers in transgenic *Arabidopsis* and the action involved the auxin-signaling pathway (Xie *et al.* 2000; He *et al.* 2005). In soybean, lateral root number increased in response to mild water stress. Taken together, it is possible that soybean drought-inducible GmNAC transcription factors may play a role in enhanced lateral root number in response to drought stress. The hypothesis of this study is: Drought-inducible GmNAC transcription factors may regulate lateral root number in soybean in response to drought stress.

The specific objective of this study is to characterize the roles of drought-inducible *GmNAC003* and *GmNAC004* genes in regulation of lateral root number using the *Arabidopsis* model.

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## **CHAPTER 2: TRANSCRIPT EXPRESSION OF *GmNAC003* AND *GmNAC004***

### **INTRODUCTION**

Increasing evidence shows that NAC transcription factors function in multiple biological processes of plant development and adaptation to environmental stresses. They possess development- and tissue-specific responses, as well as interaction with the regulatory system, to control gene expression. For examples, in Arabidopsis, two NAC transcription factor genes, *NST1* and *NST2*, were strongly expressed in the anthers and had specific roles in the regulation of the pollen grain dehiscence process (Yang *et al.* 2007). In rice, *SNAC1* was expressed differentially in guard cells under dehydration stress to control stomatal closure. Under water deficit conditions, optimizing root growth in order to effectively extract water is an essential factor in maintaining growth and productivity. This chapter discusses the expression patterns of two transcription factor genes, *GmNAC003* and *GmNAC004*, in various developmental- and stress-specific responses, with focus on root expression in order to predict possible functions that regulate of soybean root growth under water deficit conditions.

If *GmNAC003* and *GmNAC004* are responsible for enhanced lateral root (LR) number in soybean under water-deficit conditions (Read and Bartlett 1972; He 2008), they are likely to be drought responsive, or undergo protein conformation modification under stress, and they might be involved in ABA and auxin signaling. To predict these functions in soybean, a series of experiments were conducted to investigate the transcript expression patterns of these two genes in plant development as well as in the response to environmental conditions. Different levels of stress were used to generate both fast responses and slow responses to mimic real field drought conditions on the soybean cultivar Williams 82, which showed a significantly enhanced LR number in response to mild water stress (He 2008).

It has been shown that *GmNAC003* and *GmNAC004* are expressed strongly in roots, leaves and flowers. They are strongly induced by drought and NaCl, particularly in roots and leaves, and they are responsive to ABA but not to cold treatment, which is similar to several other *NAC* genes in rice and *Arabidopsis* (Shinozaki and Yamaguchi-Shinozaki 2007). The response of *GmNAC004* to treatment of 2,4-Dichlorophenoxy acetic acid (2,4-D), a synthetic auxin, indicates that this gene may be involved in auxin signaling and may function in the regulation of development. Because these two genes are strongly expressed in roots, and because it has been reported that *NAC* proteins regulate LR development (Xie *et al.* 2000; He *et al.* 2005), the data suggest that *GmNAC003* and *GmNAC004* may be involved in the regulation of the enhanced LR number under water-deficit conditions in soybean.

## MATERIALS AND METHODS

### Plant materials and stress treatment

**Dehydration, cold, NaCl, ABA stresses.** Soybean cv. Williams 82 was grown in greenhouse conditions (28/20 °C day/night temperature, photoperiod of 14/10 h, 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity, and 60% relative humidity) with a density of 4-5 plants per 1.5-gallon pot, containing a mixture of sand and turface in a 1:1 ratio. The plants were watered every two days during the course of the experiment. When the plants reached the V1 growth stage (first fully open trifoliolate leaf, approximately 14 days from sowing), they were carefully harvested to avoid any mechanical damage. The harvested plants were then transferred to the stress conditions described below. For cold treatment, the plants were placed with their roots submerged in cold water (4°C). For hormonal and NaCl treatments, soybean roots were placed in hormonal solutions containing either 100  $\mu\text{M}$  ABA; 10  $\mu\text{M}$  2,4-D; or 250 mM NaCl. A water treatment was used as the control because all chemicals and cold stresses were imposed in solution. The hormonal solutions were prepared as described by Tran *et al.* (2009) and Bolle (2009). For dehydration treatment, harvested plants were placed on tissue papers in growth chamber conditions (75% relative humidity, day/night temperatures of 22/18°C and a photoperiod of 16/8 hours). After designated time points of 1, 2, 5, 10, and 24 hours under various stress treatments, whole plant tissue was collected and immediately frozen in liquid nitrogen. Plants were then kept at -80°C until RNA isolation. Three individual plants were sampled randomly for each treatment.

**Drought stress.** For the drought treatment, soybean cv. Williams 82 was grown under greenhouse conditions as described above. Drought treatments were started when the plants reached the V1 growth stage by withholding water until the desired stem water potentials were reached. There were three drought treatments with stem water potentials of -0.5 MPa (day 18), -1.0 MPa (~day 22), and -1.5 MPa (~day 34). The control treatments (V2, V3, and V6) each had a stem water potential of ~-0.25 MPa (Table 4). The control plants in well-watered conditions grew at an approximate rate of 3 days per leaf from the date when the drought treatment started. On the other hand, the plants in drought treatments grew slowly and ceased further growth at V2. There were temporal and developmental controls for each treatment. The developmental control at the V2 stage under well-watered conditions was used for all comparisons for developmental reference. To obtain the desired stem water potentials, the stems were cut at the middle of the hypocotyl and were immediately measured for water potential using a pressure chamber (PMS Instrument Co. Albany, CA, USA) at around 4 AM. Pre-dawn tissue collection was selected for stress measurement because at this time, the transpiration is at the minimum and the stem water potential is closest to soil water potential. There were three separate plants as three replications for each treatment.

**Tissue specific expression.** For tissue specific expression of *GmNAC003* and *GmNAC004*, tissues were taken from the drought stress experiment above. For reproductive tissue, flowers at the R2 (full flowering) and seeds at the R4 (full pod) growth stage were collected and immediately frozen in liquid nitrogen. Plants were then kept at -80°C until RNA isolation. There were three biological repeats for the experiment.

## **RNA isolation and cDNA synthesis**

The frozen tissues were ground in liquid nitrogen using mortars and pestles. Trizol<sup>®</sup> (Invitrogen Carlsbad, CA, USA) was used to extract RNA, as described by the manufacturer's protocol. After phase separation by chloroform, RNA was precipitated with high salt concentration and isopropyl alcohol. The RNA was dissolved in TE buffer and then re-precipitated with an equal volume of isopropyl alcohol and 1/10 volume of 3 M sodium acetate. After washing with 75% ethanol, the RNA pellet was dissolved in water. RNA integrity was verified by electrophoresis using 1% agarose, which showed two distinct bands of ribosomal RNA. RNA quality was also determined by the Nanodrop model ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), which showed an optical density ratio (260 nm/280 nm) of 1.9-2.0, indicating good quality. The RNA was purified from DNA contamination using the Turbo DNA-free DNaseI (Ambion, Austin, TX, USA) according to the manufacturer's instructions. 1 µg of total RNA was used to synthesize cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, U.S.A) in a reaction volume of 20 µL according to the manufacturer-supplied protocol. The primer mix of oligodT and random hexa primers was used for the reverse transcriptase PCR. The cDNA was then diluted to obtain a final volume of 200 µL with milli-Q water and was then ready for quantitative real-time PCR reactions.



## Quantitative real-time PCR (qRT-PCR)

qRT-PCR primers for *GmNAC003* and *GmNAC004* were designed using the Primer 3 software (Rozen and Helen 2000) with the online version available at <http://frodo.wi.mit.edu/primer3/>. The PCR amplicons were set to have lengths between 60 to 160 bp (Table 2). The primers were designed to have a  $T_m$  of 60°C, 30-60% GC content. Self complimentary and GC clamps settings were used as the default. The primers were checked for binding specificity by blasting each primer sequence against the soybean genome sequence information at Phytozome (<http://www.phytozome.net/search.php?show=blast>) using the BLASTN algorithm with a low score setting to allow a high mismatch alignment for easier manual selection of primers. The best primer pairs were used to run the qRT-PCR. Nine internal controls (Table 1) were included for the qRT-PCR reactions and the best 4 or 5 genes were selected to calculate the normalization factors using GeNorm (Vandesompele *et al* 2002).

Quantitative real-time PCR was performed on the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using 384-well plates. The 10  $\mu$ L reaction volume consisted of 5  $\mu$ L 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 3  $\mu$ L cDNA, and primers, with a final concentration of 0.4  $\mu$ M for each primer.

The reactions were set with a thermal profile of 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Dissociation curves were

obtained from an extra thermal melting profile of 95°C for 15 seconds, followed by a constant increase of the temperature from 60°C to 95°C for 1 minute. The data were analyzed with the SDS 2.3 software package and a common signal threshold was set to 0.1. Ct values and clipped data were obtained for further analysis of PCR efficiency and relative expression levels for each gene from every tissue sample.

Relative RNA abundance of each gene was calculated as  $GOI/N$ , where GOI is the expression quantity the gene of interest, N is the normalization factor. N was calculated by GeNorm as the geometric mean of the selected internal genes. For example, in case 4 internal genes are selected, N is calculated as:  $N = \sqrt[4]{\dots}$ .

PCR efficiency was obtained using the assumption-free PCR efficiency calculated by LinRegPCR (Ruijter *et al.* 2009).

**Table 1. Reference genes and their primers used for qRT-PCR analysis.**

<b>Gene symbol</b>	<b>Function</b>	<b>NCBI Accession/ Plant GI</b>	<b>Forward primer [5'-3']</b>	<b>Reverse primer [5'-3']</b>	<b>Amp. length</b>	<b>Reference</b>
<i>ACT11</i>	Cytoskeletal structural protein	BW652479	ATCTTGACTGAGCGTGGT TATTCC	GCTGGTCCTGGCTGTCTC C	126	Hu <i>et al</i> 2009
<i>TIP41</i>	TOR (Target of Rapamycin) signaling element	EV263725	AGGATGAACTCGCTGATA ATGG	CAGAAACGCAACAGAAG AAACC	88	Hu <i>et al</i> 2009
<i>TUB4</i>	Structural constituent of cytoskeleton	EV263740	GGCGTCCACATTCATTGG A	CCGGTGTACCAATGCAA GAA	111	Hu <i>et al</i> 2009
<i>UKN1</i>	Unknown	BU578186	TGGTGCTGCCGCTATTTA CTG	GGTGAAGGAACTGCTA ACAATC	74	Hu <i>et al</i> 2009
<i>UKN2</i>	Unknown	BE330043	GCCTCTGGATACCTGCTC AAG	ACCTCCTCCTCAAACCTCT CTG	79	Hu <i>et al</i> 2009
<i>CYP2</i>	Cyclophilin	TC224926	CGGGACCAGTGTGCTTCT TCA	CCCCTCCACTACAAAGGC TCG	154	Jian <i>et al</i> 2008
<i>IDE</i>	Insulin degrading enzyme	AW310136	ATGAATGACGGTCCCAT GTA	GGCATTAAAGGCAGCTCA CTCT	114	Libault <i>et al</i> 2008
<i>SUB1-2</i>	Ubiquitin	D26092	AGCTATTCGCAGTTCCCA AAT	CAGAGACGAACCTTGAG GAGA	84	Libault <i>et al</i> 2008
<i>CDPK</i>	CDPK-related protein kinase	AW396185	TAAAGAGCACCATGCCTA TCC	TGGTTATGTGAGCAGAT GCAA	97	Libault <i>et al</i> 2008

**Table 2. Primers used in quantitative RT-PCR for *GmNAC003* and *GmNAC004*.**

Primer name	Primer sequence (5'-3')	Amplicon length
GmNAC003-qRT-PCR-F	CCCTGCCACGAGTGAAC	66 bp
GmNAC003-qRT-PCR-R	TTCGGAGACCCGAATTTCT	
GmNAC004-qRT-PCR-F	CGTCAGTCCGCAAAAGAT	62 bp
GmNAC004-qRT-PCR-R	GACCCGTTGGTTTCTCAC	

## **Reporter gene construction, GUS histochemical staining and microscopy**

The promoter regions of *GmNAC003* and *GmNAC004* were amplified from soybean cv. Williams 82 genomic DNA using the primers listed in Table 3. The fragments were digested with *Bam*HI and *Xma*I and ligated to the pZY-GUS vector, which was predigested with the same enzymes to produce pZY-PNAC003-GUS and pZY-PNAC004-GUS. The gene constructs were verified by sequencing. These plasmids were then transformed into *Agrobacterium tumefaciens* strain C58 for Arabidopsis transformation. Arabidopsis transformation was conducted two times as previously described (Clough and Bent 1998). Transgenic Arabidopsis plants from generations T1 and T2 were screened in ¼ MS (Murashige and Skoog Basal Salt Mixture, and Murashige and Skoog Vitamin) medium containing 25 mg/L glufosinate.

GUS histochemical staining for GmNAC promoter activity in roots, stem, leaves, flowers, and young seeds was performed as per Jefferson *et al.* (1987) from at least 5 transgenic events. All samples for GUS assays were from the second transgenic generation (T2

generation). For root, stem, and leaf expression, 3- and 7-day-old agar-grown seedlings were collected. Flowers and young pods were collected from 40-day-old plants at the flowering and pod development stages. For stressed tissues, the 4-day-old seedlings were transferred from a nursery plate to agar plates containing either 20% polyethylene glycol (PEG MW8000), 10  $\mu$ M abscisic acid (ABA), or control (1/4 MS basal media) for three days. The harvested tissues were then transferred to the staining solutions ( $\beta$ -Glucuronidase Reporter Gene Staining Kit, Sigma Aldrich, St. Louis, MO, USA, Cat number: GUSS-1KT). After an incubation period of 12 hours in the dark, the solutions were removed and the samples were cleared of chlorophyll with repeated washings of 70% ethanol. The samples were mounted on microscope glass slides and kept from dehydrating by adding a drop of 50% glycerol. Photographs were taken using a Leica EZ4 D stereo-microscope (Leica Microsystems, Switzerland) equipped with a Leica EC3 digital camera and LAS EZ (Leica Application Suite Educational Zoom) software for image acquisition and analysis.

**Table 3. Primers for cloning of *GmNAC003* and *GmNAC004* promoters**

<b>Primer name</b>	<b>Primer sequence (5'-3')</b>	<b>Restriction site</b>
<b>GmNAC003-ProF</b>	ATTGGATCCCAAAAGGAGCAAAACCTCA	<i>BamHI</i>
<b>GmNAC003-ProR</b>	CCCGGGTAAAAAGTTATAAAACAAATTTTTCTTT CTC	<i>XmaI</i>
<b>GmNAC004-ProF</b>	GGATCCATGTGTCCAACGGTGGAAG	<i>BamHI</i>
<b>GmNAC004-ProR</b>	CCCGGGTTTTTCAAAAACACAATTCGATC	<i>XmaI</i>

## **Data analysis**

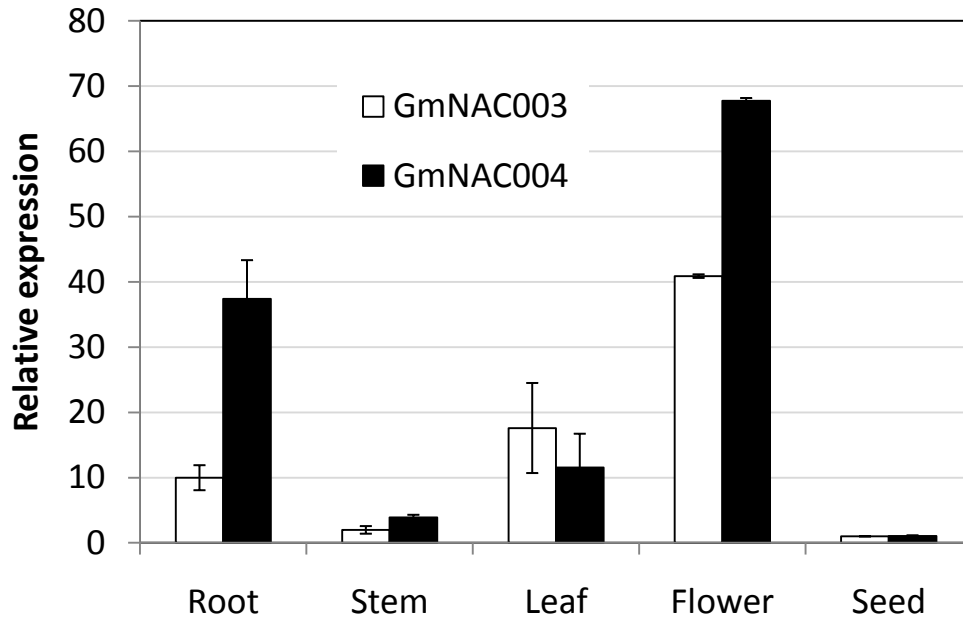
Relative gene expression differences between the stress and control conditions was estimated using the one-tailed Student's t-test (Microsoft Excel 2007). Fold change was calculated as the relative expression levels of the gene under stressed conditions divided by the relative expression of the gene under controlled conditions. Fold change was only calculated when there was a significant difference between the means of the stress and control conditions. In the situation where multiple controls were present, the treatment with the lowest expression was transformed to a value of 1 for convenient comparisons.

## **RESULTS**

### **Expression of *GmNAC003* and *GmNAC004* is strong in roots, leaves and flowers**

Activation or repression of gene expression in a tissue-specific manner is an important role of transcription factors (TF) in the regulation of plant development. NAC TFs have been shown to involve many processes of plant growth and development (Xie *et al.* 2000; He *et al.* 2005; Hu *et al.* 2006). Root development is one of the key traits governing adaptation to water stress: deep and branching root systems help to extract more water under drought conditions. Organ specific or differential expression of genes

will provide clues about their potential functions in the regulation of development. Using this research approach, the expression patterns of *GmNAC003* and *GmNAC004* were analyzed in different plant tissues (root, stem, leaf, flower, and young seed) using qRT-PCR.



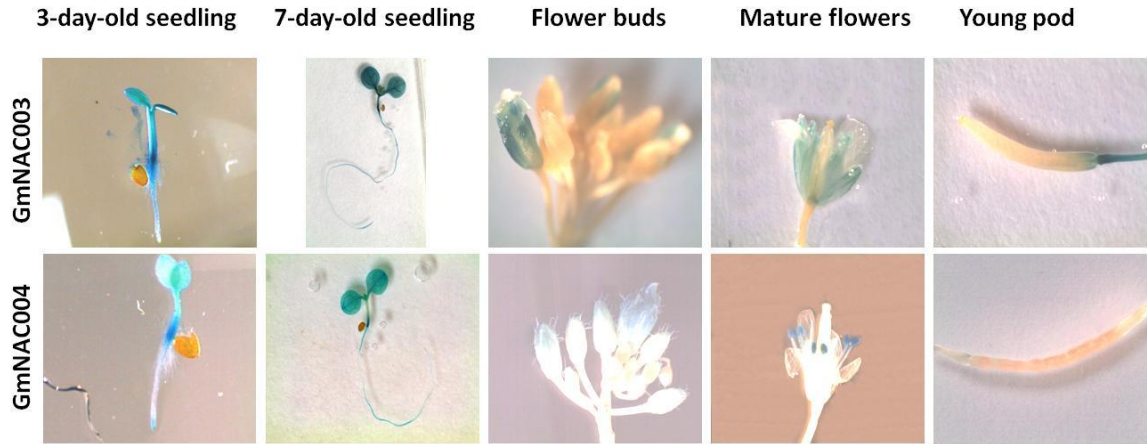
**Figure 1. Relative expression of *GmNAC003* and *GmNAC004* genes in soybean cv. Williams 82 grown under greenhouse conditions. The root, stem, and leaf tissues were collected from the soybean plants at the V3 growth stage, the flowers at the R2 stage, and the seeds at the R4 stage. Three biological replicates were used and the data were normalized to the 4 best reference genes (Appendix Figure A.3). Mean relative expression levels were normalized to a value of 1 for the sample having the lowest expression (seeds). Error bars are standard errors of the means (SEMs).**

Figure 1 shows similar patterns of gene expression of *GmNAC003* and *GmNAC004* in all tissues tested, with a high abundance in soybean roots, leaves, and flowers, but low

expression in stems and seeds. *GmNAC004*, however, showed a stronger relative expression in roots and flowers. The data showed a relative divergence from the work of Meng *et al.* (2006), in which they did a semi-quantitative PCR analysis for the soybean cultivar Bogao. They found that *GmNAC003* and *GmNAC004* were expressed strongly in leaves and seeds but showed low expression in roots and flowers.

To further examine the expression of *GmNAC003* and *GmNAC004*, the GUS expression driven by *GmNAC003* and *GmNAC004* promoters was analyzed using the Arabidopsis model. Overall, the GUS expression was consistent with the qRT-PCR data from the transcript profiling of the two genes in soybean: GUS was expressed in Arabidopsis roots and flowers, but almost absent in pods (Figure 2). The genes were expressed more in open flowers and less in young flower buds. In the matured flowers, *GmNAC003* expressed more in the petals, while *GmNAC004* mainly expressed in the anthers. Given that the rice gene *SNAC1* showed differential guard cell expression that improved drought tolerance by reduced water loss (Hu *et al.* 2006), it would be interesting to see the expression of *GmNAC003* and *GmNAC004* in stomata. The GUS expression was visualized at a higher resolution to see if the two genes expressed in guard cells, but no differential expression was observed (data not shown).





**Figure 2. Expression of GUS protein driven by *GmNAC003* and *GmNAC004* promoters in Arabidopsis.** Seedlings were sampled from 3- and 7-day-old plants grown in ¼-MS nutrient agar plates. Reproductive tissues were collected from plants in growth chamber conditions for 40 days. Samples were stained with GUS for 12 hours and viewed using an Olympus stereo microscope. This picture is a representative of several transgenic events with same expression patterns.

## **Response to stress and hormone treatments of *GmNAC003* and *GmNAC004***

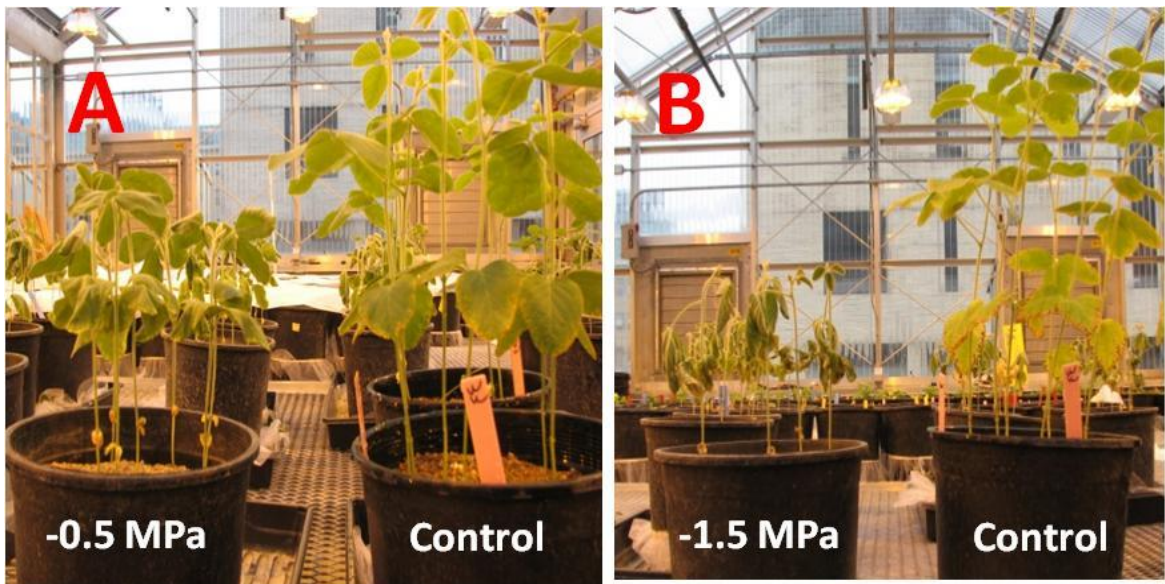
Response to environmental stresses has been shown in a number of NAC genes in Arabidopsis and rice (Tran *et al.* 2004; Hu *et al.* 2006). In abiotic stress signaling, common molecular responses have been seen with both dehydration and NaCl, but these are distinct from cold responses (Shinozaki and Yamaguchi-Shinozaki 2007; Yamaguchi-Shinozaki and Shinozaki 2006). ABA has been associated with osmotic stress, but not with cold stress. Molecular and biochemical analyses have suggested a model that consists of ABA-dependent and ABA-independent signaling pathways to regulate gene expression in response to abiotic stresses. With an interest in drought adaptation

studies, a set of tissues were collected from various environmental stress conditions, including drought, cold, salinity, and ABA treatments, to understand environmental and hormonal signals that regulate the expression of *GmNAC003* and *GmNAC004* as well as their possible roles in stress adaptation. These genes were also studied for their response to 2,4-D treatment in order to investigate their possible interaction with auxin in the regulation of lateral root development.

### ***GmNAC003* and *GmNAC004* are induced by drought**

In nature, drought stress often occurs gradually and the plants may initiate molecular responses according to the levels of stress severity. A greenhouse experiment was conducted to mimic this slow drying by withholding water. Tissues were collected at three drought levels of -0.5, -1.0, and -1.5 MPa stem water potentials that represented mild, intermediate, and severe stress, respectively. It took approximately 20 days to reach the stem water potential of -1.5 MPa, a severe stress that is closely equivalent to the non-recoverable wilting point in field conditions. The slow and gradual stress process helped avoid the unexpected effects of fast and severe dehydration stress and allowed a field-like drought response. As shown in Figure 3 and Table 4, the control soybean plants grew at a rate of about 3 days per new leaf, and the developmental stages of the controls were different from the stressed plants, which almost ceased their growth when stress started and had only 2 or 3 trifoliolate leaves (Figure 3). To avoid this difference in development, two controls were required: a developmental control having the same growth stage as the stressed treatments and a temporal control

with a similar duration of growth. The control V2 is considered a developmental control because the stressed plants did not produce any more leaves. For the temporal comparison, V2 is the control for -0.5 MPa, and similarly, V3 for -1.0 MPa and V6 for -1.5 MPa (Table 4). A gene that responds to stress should have changes in expression relative to both developmental and temporal controls.

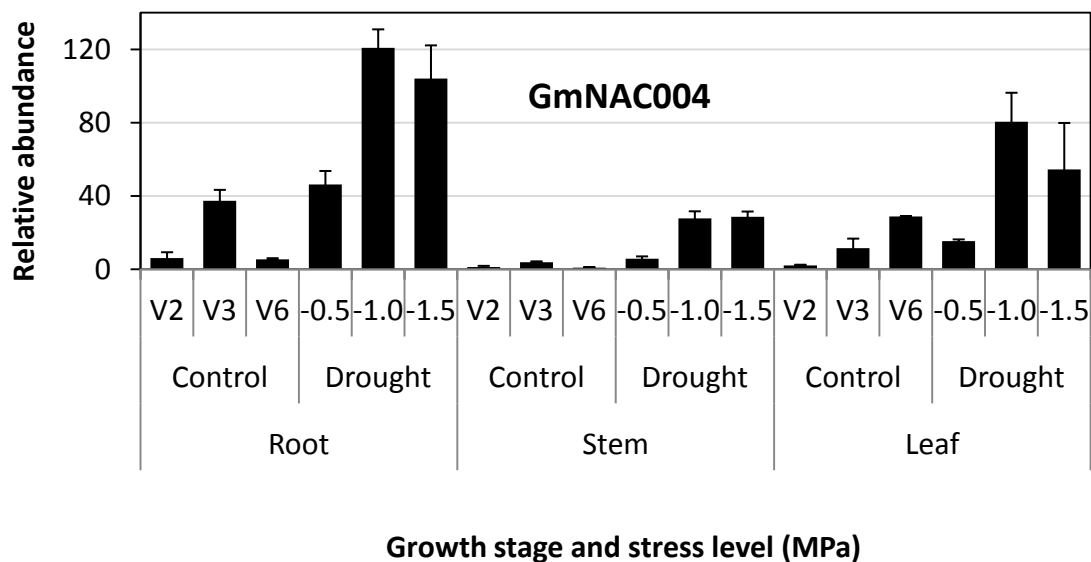
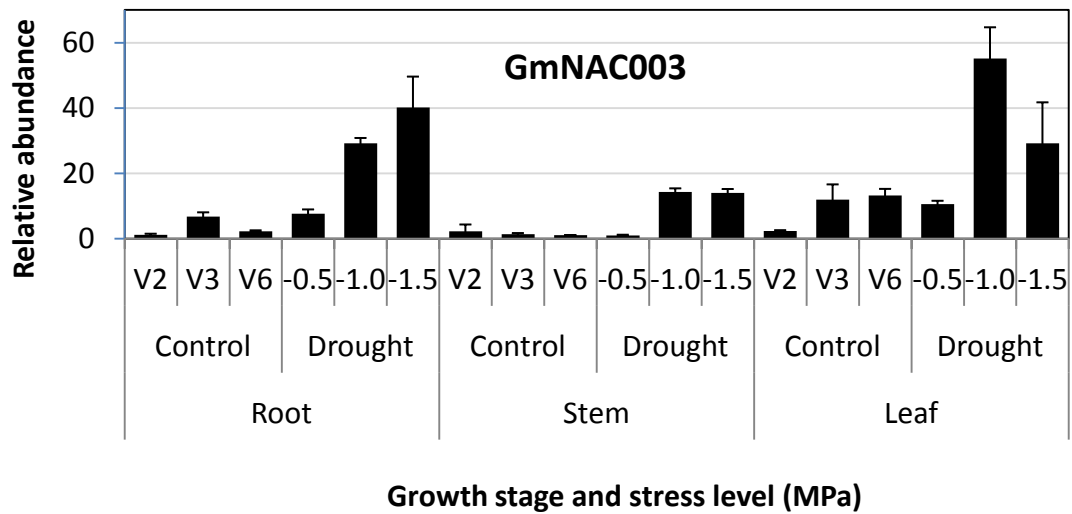


**Figure 3. Soybean drought treatment for the transcript expression profiling of *GmNAC003* and *GmNAC004*. Soybean cv. Williams 82 was grown in greenhouse conditions and drought stress treatment at V1 growth stage. (A) Drought stress at -0.5 MPa and the corresponding control. (B) Drought stress at -1.5 MPa and the corresponding control. Note that plant growth under drought was significantly inhibited. After a long drought stress period, only 0.5-1 leaves were developed, while the control had a growth rate of about three days per new leaf.**

Expression of *GmNAC003* and *GmNAC004* was strongly induced by drought stress (Figure 4). Both genes responded relatively early at mild drought stress (-0.5 MPa), with strong expression in root and leaf, but not in stem. At the more severe stress levels, stronger expression was seen in both genes in the roots and leaves of the stressed plants. This differential expression in response to drought stress in roots and leaves indicates the roles of the two genes in the regulation of development in response to drought.

**Table 4. Soybeans cv. Williams 82 tissue collection chart for the investigation of the inducibility of *GmNAC003* and *GmNAC004* under drought stress. For drought treatments, the plants were not supplied any water after day 14 when they reached the V2 growth stage. Droughted tissues were collected when the stem water potential reached -0.5, -1.0, and -1.5 MPa. Corresponding controls reached the V2, V3, and V6 growth stages, respectively; while the stressed plants could not produce any more leaves (Figure 3).**

Days from sowing	Control plants		Droughted plants	
	Growth stage	Stem water potential (MPa)	Growth stage	Stem water potential (MPa)
18	V2	-0.25	V2	-0.50
22	V3	-0.25	V2	-1.00
34	V6	-0.25	V2	-1.50



**Figure 4.** Transcript abundance of *GmNAC003* and *GmNAC004* in response to drought stress. Drought stress started when the plants reached the V1 growth stage (first trifoliolate leaf). Transcript abundance was quantified using qRT-PCR and data were normalized to the four best internal control genes (*ACT*, *IDE*, *UNK1*, and *UNK2*; Table 1 and appendix Figure A.2) based on the M stability of geNorm. Mean relative expression levels were normalized to a value of 1 for the samples having lowest expression. Data were from three biological repeats. Error bars are SEMs.

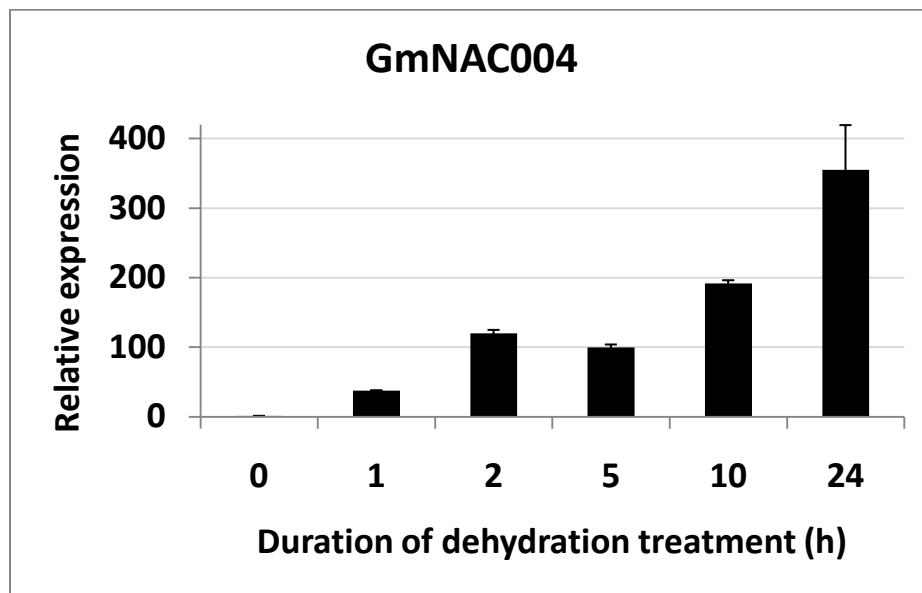
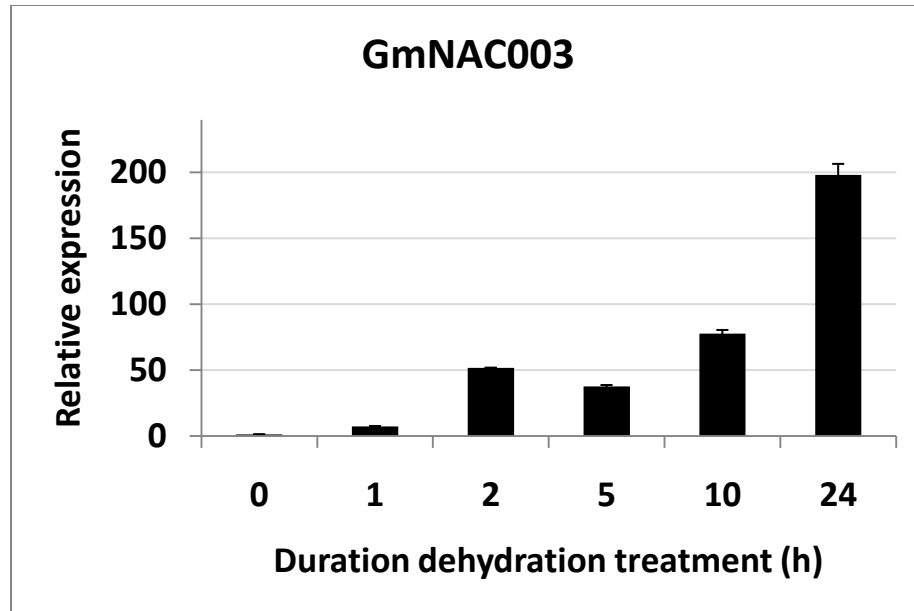
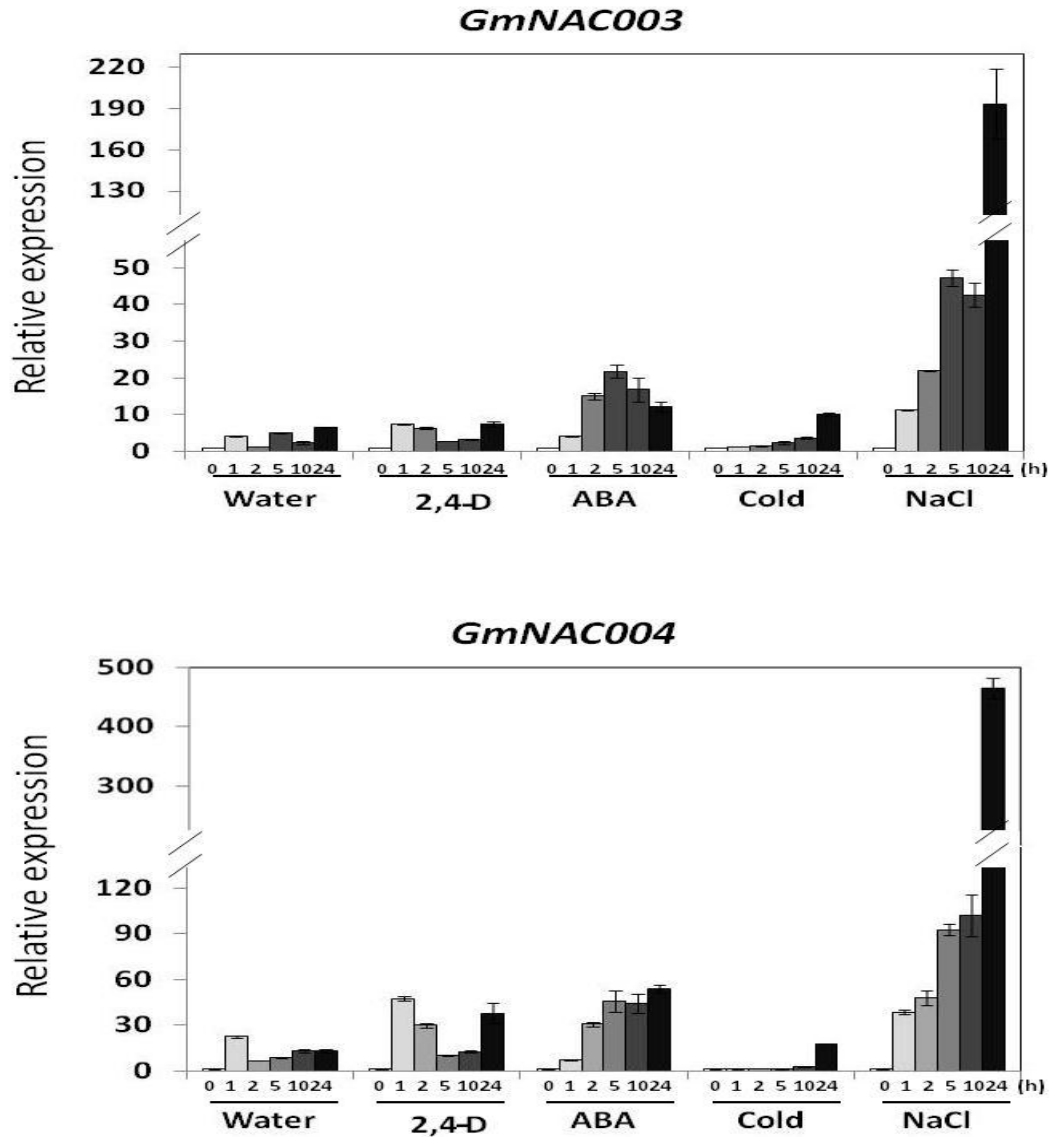


Figure 5. Dehydration inducibility of soybean *GmNAC003* and *GmNAC004* genes. The tissues were from the V1 growth stage (first fully open trifoliolate) of soybean cv. Williams 82 grown in greenhouse conditions and dehydrated for 0h, 1h, 2h, 5h, 10h and 24h in growth chamber conditions. Data were normalized to the five best internal reference genes (*ACT*, *CYP2*, *IDE*, *UNK1* and *UNK2*; Table 1 and Appendix Figure A.3). Mean relative expression levels were normalized to a value of 1 for the samples having lowest expression (0 h). Error bars are SEMs (n=3 plants).

The data from the drought experiment demonstrated that *GmNAC003* and *GmNAC004* show early responses to drought, even at a mild stress level of -0.5 MPa (about 4 days after removal of water supply). To further understand the sensitivity of the response, a series of whole-plant tissues were collected from dehydration treatments by placing the young soybean plants at V1 growth stage on tissue papers for 0h, 1h, 2h, 5h, 10h and 24h. The severity of the stress was measured using a pressure chamber for stem water potentials and recorded as followed: -0.25 MPa for control, -0.83 MPa for 1 hour, and -2.8 MPa for 5 hour treatment. Water potential of the 10h and 24h dehydrated plants was too low to be measured using the pressure chamber. The data in Figure 5 showed that the expression of the two genes starts as early as 1 hour from the onset of dehydration. At 2 hours of stress, the expression was almost 50-fold for *GmNAC003* and 100-fold for *GmNA004* with similar patterns of response for both genes. These data confirm the drought response of the two genes and also verify that the drought experiment shown in Table 4 was properly conducted with adequate and proper controls.



**Figure 6. Inducibility of *GmNAC003* and *GmNAC004* by different stress and hormonal treatments.** Expression of dehydration-inducible *GmNAC* genes was studied by qRT-PCR in 14-day-old soybean plants that had been transferred to hydroponic growth at 28°C in water (Water) for control, 20 nM 2,4-D (2,4-D), 100 μM ABA (ABA), 4 °C (Cold), and 250 mM NaCl (NaCl) for the indicated durations. Data were normalized to the five best internal reference genes (*ACT*, *CYP2*, *IDE*, *UNK1*, and *UNK2*; Table 1 and the Appendix Figure A.3) and transformed to a value of 1 for the unstressed plant (0 h). The error bars are standard errors of the means (n=3 plants).



## ***GmNAC003* and *GmNAC004* are responsive to NaCl and ABA, but not to cold treatment**

To regulate plant growth and development and the adaptation of plants to stress conditions, the action of proteins is often associated with hormones and changes in environmental conditions. NAC genes are well known for their expression dependence on ABA in regulation of responses to osmotic stress (Shinozaki and Yamaguchi-Shinozaki 2007; Hu *et al.* 2006; Tran *et al.* 2004). In this study, qRT-PCR was employed to quantify the expression of the two genes in response to NaCl, cold and ABA treatment, to help predict the roles of GmNAC003 and GmNAC004 proteins in abiotic stress adaptation.

Figure 6 shows that both genes were highly expressed under the NaCl treatment, with a similar trend seen in response to dehydration (Figure 5). ABA treatment resulted in a moderate response of gene expression. In contrast, cold stress (in water) had no effect on the expression of the genes compared to the water control treatment. Among the three stresses, NaCl and drought are known to induce ABA accumulation (Jakab *et al.* 2005; Jia *et al.* 2002), but cold stress has no effect on the accumulation of ABA (Lee *et al.* 2005). The response to NaCl occurred as early as the first hour of treatment, and it occurred earlier than in the ABA treatment, suggesting that the genes do not require ABA to respond to NaCl or dehydration. Taken together, the data suggests that the two genes function in both the ABA-dependent and ABA-independent pathways.

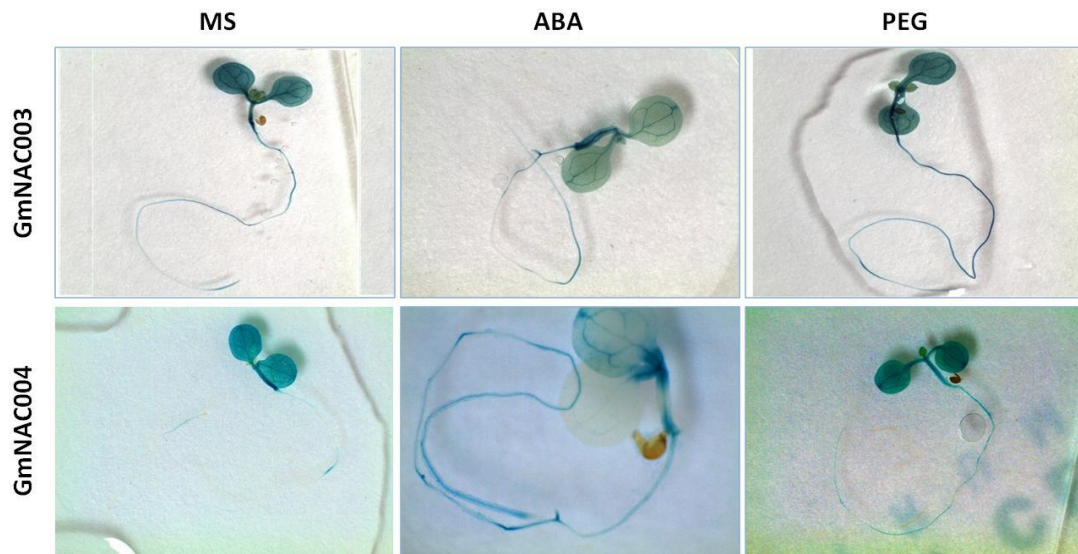
### ***GmNAC004* is auxin inducible**

For the regulation of development, including LR development, NACs have been shown to associate with auxin signaling (Xie et al. 2000, He et al. 2005). For example, auxin stimulates the expression of NAC1 but also induces the expression of miRNA164, which targets degradation of NAC1 (Guo et al. 2005). To predict the involvement in the development of LRs, *GmNAC003* and *GmNAC004* expression in response to 2,4-D was investigated. The expression of *GmNAC003* in response to 2,4-D was negligible, while there was a moderate expression of *GmNAC004*. The response of *GmNAC004* was rapid but transient for the first few hours, and then expression was regained after a longer time of treatment (Figure 6). The data indicate that *GmNAC004* may be involved in the auxin-signaling pathway.

### ***GmNAC003* and *GmNAC004* are not differentially expressed in root primordia under stress**

As described above, *GmNAC003* and *GmNAC004* are induced by drought and ABA and are strongly expressed in the roots of soybean. To further predict whether the genes are specific for root development in response to ABA and drought stress, investigations were conducted on *GmNAC003*- and *GmNAC004*-driven GUS expression in Arabidopsis transgenic plants in the control, ABA and PEG treatments (Figure 7). In this experiment, PEG was used to produce lower water potential and ABA was used to investigate if ABA altered location of *GmNAC003* expression. The data show that, under control conditions, there was a difference in expression patterns of the two genes at the

seedling stage (Figures 2 and 7). *GmNAC003* appeared to express ubiquitously in the entire seedling, whereas *GmNAC004* showed low expression in the middle section of the root. However, neither of the genes showed any differential expression in the LR primordial region. Observations of GUS expression under ABA and PEG treatments did not detect any differential response in the LR formation region (Figure 7). The data clearly showed that these two genes are osmotic-stress inducible in various tissues but are not restricted to LR tissues.



**Figure 7. GUS visualization for expression patterns of *GmNAC003* and *GmNAC004* promoters: GUS fusions in control (MS), 10  $\mu$ M ABA (ABA) and 20% polyethylene glycol (PEG). The plants were grown for 4 days in a nursery plate and then transferred to ABA and PEG plates for three additional days. Consistent expression patterns were seen in five transgenic events from each transgenic construct.**

## DISCUSSION

Enhanced LR number and length in certain root regions is beneficial for plants to survive and reproduce under drought conditions. Genetic variation in LR development in response to environmental stresses has been identified in plants. In soybean, enhanced LR numbers and increased LR development in lower soil profiles in response to stress have been reported in many genotypes (Read and Bartlett *et al* 1972; Huck *et al.* 1983; Hoogenboom *et al.* 1987; He 2008). This plasticity of LR number and length must be regulated by changes in gene expression and activation of responsive proteins that control growth and optimize development for adaptation to environmental conditions. To date, information on the molecular regulation of root architecture that maintains root elongation and LR development under water deficit is still poorly understood. Comparing transcript profiling of different root growth regions between droughted and non-droughted roots or between drought-tolerant and drought-sensitive plants will detect candidate genes for controlling root architecture in response to drought stress. This research data provides some additional clues to the molecular regulation of drought adaptation in plants. The two genes that were studied, *GmNAC003* and *GmNAC004*, were expressed strongly in soybean roots, leaves, and flowers in response to drought stress, suggesting that they may be involved in drought adaptation and that their function might not be specific to root tissue. Previous studies have reported that drought-inducible NAC TFs improved drought tolerance in plants. For example, Hu *et al.* (2006) found that a drought-inducible *SNAC1* gene expressed differentially in the guard

cells of rice. Transgenic rice plants that overexpressed *SNAC1* could maintain leaf turgor, reduce water loss, and maintain better spikelet fertility under drought stress. Hu *et al.* (2008) also studied another dehydration-inducible rice gene, *SNAC2*, which showed up-regulation of a large number of genes that encode proteins with predicted drought tolerance mechanisms, such as detoxification and redox homeostasis. Transgenic rice overexpressing *SNAC2* showed significant improvement in the survival rate of plants subjected to salinity treatment. In Arabidopsis, overexpression of the dehydration-inducible genes *ANAC019*, *ANAC055*, *ANAC072* (Tran *et al.* 2004) improved drought and salinity tolerance. Microarray data showed that drought tolerance may be involved in detoxification, but the drought tolerance mechanisms of these transgenic plants have not been investigated. Two other NAC genes in Arabidopsis, *NAC1* and *AtNAC2* TFs, have been shown to be involved in the regulation of the number of LRs, acting downstream of auxin signaling (Xie *et al.* 2000, He *et al.* 2005). *AtNAC2* was up-regulated by ABA and salinity treatments, suggesting the interaction of root development and osmotic stress. The involvement of NAC transcription factors in drought tolerance and LR development provides evidence that *GmNAC003* and *GmNAC004* could play a role in regulation of LR development in soybean under mild water-deficit conditions.

Auxin has a central role in plant organelle development, including regulation of shoot apical meristem and LR development. Both Arabidopsis *NAC1* and *AtNAC2* transcription factors are involved in LR development through auxin signaling (Xie *et al.* 2000, He *et al.* 2005), although their direct interaction in the pathway is not known. *CUC1* and *CUC2*, two other NAC proteins from Arabidopsis, were shown to be regulated by auxin

signaling in order to maintain normal cotyledon development (Furutani *et al.* 2004). Furthermore, Willemsen *et al.* (2008) reported that two NAC domain transcription factors, FEZ and SOMBRERO, regulated the orientation of the cell division plane in Arabidopsis root stem cells. FEZ is expressed in root cap stem cells and functions to promote periclinal root cap-forming cell divisions, while SOMBRERO represses the activity of FEZ. Since the LR apical meristem resembles the main root apical meristem (reviewed by Hodge *et al.* 2009), it is likely that the functions of FEZ and SOMBRERO are conserved in the LR meristem to regulate LR emergence and growth. The results of the present study demonstrate that *GmNAC004* is auxin-inducible when treated with 2,4-D. In contrast, *GmNAC003* did not respond to this treatment. The involvement of this TF in auxin signaling suggests that the *GmNAC004* protein may potentially interact with auxin in the regulation of root architecture.

In contrast to auxin, ABA has been found to inhibit lateral root development via multiple processes from primordia development through emergence. It is thought that there is an antagonistic action between ABA and auxin (De Smet *et al.* 2006). From our recent study (Tran *et al.* 2009), among nine dehydration-inducible GmNAC genes, only *GmNAC003* and *GmNAC004* were ABA responsive. The data from Figure 6 show that both genes responded more slowly to ABA treatment than they did to NaCl stress, which indicates that they belong to both the ABA-dependent and the ABA-independent pathways in osmotic signaling. This slow response to ABA implies that they may not be the direct target of ABA, but that they may be involved with other indirect factors to activate expression. The involvement of *GmNAC004* in both auxin and ABA-regulated

response suggests that the gene may have a role in crosstalk between the two signaling pathways.

The expression of both *GmNAC003* and *GmNAC004* is ubiquitous in many plant organs, suggesting diverse roles in the regulation of gene expression. Histochemical assays showed that the *GmNAC004* promoter was highly active in the anthers of transgenic *Arabidopsis* plants, where desiccation may occur. This desiccation assists with the pollen grain dehiscence process. Yang *et al.* (2007) reported that two *Arabidopsis* NAC TF proteins, NST1 and NST2, were involved in these processes. NST1 and NST2 functioned in the secondary thickening of the anther endothecium and caused dissimilar forces in the cell wall when shrinkage due to desiccation occurred. This in turn facilitated pollen grain dehiscence. Data has shown that *GmNAC003* and *GmNAC004* were strongly expressed in soybean roots and leaves. Strong expression in the root may be an indication that the genes are involved in the intrinsic pathways of the regulation of root development (Malamy 2005). Since the expression of the genes was not specific to the LR emerging site, their function might not be specific to LR development in soybean.

In conclusion, the observations in the present study indicate that the genes *GmNAC003* and *GmNAC004* play a potential role in the regulation of root architecture in response to drought. First, both genes expressed strongly in roots, and their expression was significantly induced in roots in response to drought stress. Second, one of the genes, *GmNAC004*, showed induction by the application of 2,4-D, suggesting its interaction with auxin signaling. This evidence, combined with the known roles of NAC TFs in the

regulation of root development in the literature, suggests that the two genes are potential candidates for further characterization for their roles in LR development, which will be discussed in Chapter 3.

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## **CHAPTER 3: GmNAC004 STIMULATES LATERAL ROOT DEVELOPMENT IN TRANSGENIC ARABIDOPSIS**

### **INTRODUCTION**

Plants adapt to environmental stress through a number of mechanisms, including the regulation of root growth and development to optimize water and nutrient extraction. In soybean, water deficit inhibits shoot dry weight (Read and Bartlett 1972; Yamaguchi and Sharp 2010), but promotes an increase in lateral root number and total root length, with more root length density at deeper soil profiles (Huck *et al.* 1983; Hoogenboom *et al.* 1987). Recently, He (2008) reported similar observations and found that there was genetic variation in soybean response to mild water stress: eight of the eleven studied genotypes showed increased number and length of lateral roots, but slightly decreased primary root length. Because enhanced lateral root growth and development is a responsive action, this regulation of root growth must be regulated by responsive genes/proteins in the plant root system. The characterization and identification of these genes will facilitate the understanding of root growth in response to water deficit and

may help improve future crop productivity during drought conditions through genetic manipulation.

Several plant hormones play roles in the regulation of root growth in response to environmental changes. ABA, a plant hormone accumulated under water stress and helped maintain root growth by acting at least in part through the inhibition of excess ethylene production (Sharp 2002; Sharp and LeNoble 2002). Ethylene has been shown to induce adventitious root development in response to low-oxygen stress in maize (Drew *et al.* 1979). Recently, Ivanchenko (2008) reported that lateral root number in *Arabidopsis* was promoted by low concentrations of ethylene, but that it was inhibited by higher concentrations of the hormone. Auxin, which plays a critical role in all processes of lateral root development, has also been shown to act competitively with ABA in the regulation of lateral root development (Peret *et al.* 2009a; Peret *et al.* 2009b). ABA interferes with auxin signaling, and crosstalk between ABA and auxin may occur at multiple steps from lateral root initiation to root emergence. One possible interaction between these two signaling pathways is through ABI3, a positive regulator in ABA signaling. This gene is induced by auxin and can activate the expression of both ABA- and auxin-responsive genes (Nag *et al.* 2005). Another interaction between two pathways is with the AtMYB96 transcription factor gene, which is induced by both ABA and auxin and has been shown to suppress auxin action in the activation of the lateral root meristem (Seo *et al.* 2009). In addition, the ABA-responsive mutants *abi3*, *lrd2*, *aba2-1* and *aba3-1* showed a reduced response to polar auxin transport inhibitors,

suggesting that these genes may be involved in the ABA-auxin interaction (De Smet *et al.* 2006).

NAC (NAM: no apical meristem, ATAF, and CUC: cuc-shaped cotyledon) transcription factors play various roles in growth, development and adaptation to environmental stress in plants. Treatments with salinity and drought induced NAC gene expression in various plant species (Tran *et al.* 2004; Hu *et al.* 2006; Tran *et al.* 2009). The previous chapter identified two soybean NAC genes, *GmNAC003* and *GmNAC004*, that are highly responsive to drought stress and moderately induced by ABA. One of the genes, *GmNAC004*, showed an increased expression under 2,4-D treatment, suggesting its involvement in auxin signaling. In Arabidopsis, NAC1 and AtNAC2 have been shown to stimulate lateral root development downstream of the auxin signaling pathway (Xie *et al.* 2000, He *et al.* 2005). Therefore, it is possible that soybean drought-inducible *GmNAC003* and *GmNAC004* genes may be a part of the signaling network regulating the enhancement of LR number in soybean in response to water-deficit conditions (He 2008). The objective of this study was to investigate the roles of *GmNAC003* and *GmNAC004* in the regulation of lateral root development using transgenic Arabidopsis plants overexpressing the two genes.

## **MATERIALS AND METHODS**

### **Plasmid constructs and transformation**

The open reading frames of soybean *GmNAC003* and *GmNAC004* genes (Appendix Figure A.1) were amplified by KOD hot start polymerase enzyme (EMD Chemicals, Gibbstown, NJ, USA), using the primers listed in Table 5 to produce blunt-end PCR fragments. These PCR-generated blunt-end fragments were transformed into the *EcoRV*-digested Bluescript vector (pKS vector, Stratagene, La Jolla, CA) to produce pKS-*GmNAC003* and pKS-*GmNAC004*. The positive colonies were verified by sequencing the plasmid DNA using T3 and T7 primers. The DNA fragments were then excised from the pKS plasmid using *BglII* and *EcoRV* sites and ligated into a pGreen plasmid that had been pre-inserted with the CaM35S promoter to produce pGreen-p35S-*GmNAC003* and pGreen-p35S-*GmNAC004* (Table 6). The two final vectors containing the soybean NAC genes were verified by sequencing and then transformed into *Agrobacterium tumefaciens* C58, which had been transformed with helper vector pSoup, the vector that facilitates plant transformation for pGreen. Ampicillin (100mG/L) was used to screen the transformed bacteria colonies of pKS and kanamycin (50 mG/L) was used to select pGreen transformants.

### **Arabidopsis transgenic plant transformation and regeneration**

The gene cassettes were introduced to *Arabidopsis thaliana* cv. Columbia using the floral dip transformation method (Clough and Bent 1998). Transgenic plants were

screened for survival in ¼ MS (a combination of Murashige [Sigma cat: M5524] and Skoog Vitamin Powder [Sigma cat: M7150] agar medium containing 25 mg/L hygromycin) for the T1, T2 and T3 generations. To reduce unstable gene expression and possible interference to the host genome, only transgenic events having a single insertion locus were kept for the generation of homologous transgenic plants. Determination of locus numbers was based on the segregation ratio of hygromycin resistance at the T2 generation. Plants of homozygous genotypes of at least five independent events having transgene expression were selected for morphology studies. Two events with visually normal plant growth were selected for phenotypic assays for root growth and morphology.

**Table 5. Primers used to clone the full length of soybean *GmNAC003* and *GmNAC004* genes.**

Primer name	Sequence (5'-3')
GmNAC003- <i>Bgl</i> II-Forward	GTTAGATCTATGGGAGTTCAGAGAGAGACCCTCTTG
GmNAC003- <i>Eco</i> RV-Reverse	TAAGATATCTCAGTCCCTAAACCCGAACTCAAC
GmNAC004- <i>Bgl</i> II-Forward	AATAGATCTATGGGAGTTCAGAGGAAGACCCTCTTTCC
GmNAC004- <i>Eco</i> RV-Reverse	TAAGATATCTCAATTCCTGAACCCGAAACCCACCG

Transgenic plants were screened for transgene insertion and for expression of the overexpressed genes: *GmNAC003* and *GmNAC004*. DNA was isolated from both wild-type (control) and transgenic plants using the CTAB method (Saghai-Marooft *et al.* 1984). DNA quantity and quality were measured using a Nanodrop model ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Transgene insertions were verified using PCR and electrophoresis for DNA sequence specificity. For

the transgene expression in the Arabidopsis transgenic plants, tissues from the wild type (control) and from all the events of Arabidopsis transgenic plants overexpressing soybean *GmNAC003* and *GmNAC004* were collected from 20-day-old seedlings grown on ¼ MS nutrient agar plates. Total RNA isolation, complimentary DNA synthesis, and quantitative real-time PCR were conducted as described in Chapter 2. The Arabidopsis ubiquitin gene was used as the reference gene for relative expression quantification.

Arabidopsis wild-type and transgenic plants were maintained and regenerated in a greenhouse with day/night temperatures of 22°C/18°C. During the summer season, the plants were maintained in growth chambers with the following conditions: 16-hour day length, light density of ~200 uE m<sup>-2</sup> sec<sup>-1</sup>, day/night temperatures of 22°C/18°C respectively, and a constant relative humidity of 70%. Phenotypic assays for root growth and seed germination were conducted in growth-chamber conditions of 75% relative humidity, day/night temperatures of 22/18°C and a photoperiod of 16/8 hours.

**Table 6. pGreen plasmids containing *GmNAC003* and *GmNAC004* constructs for Arabidopsis transformation. Both genes are overexpressed using the CaMV35S (35S) promoter.**

<b>Gene constructs</b>	<b>Final plant transformation pGreen vector containing the soybean genes</b>	<b>Transformation helper vector</b>
<i>GmNAC003</i>	pGreen-35S- <i>GmNAC003</i>	pSoup
<i>GmNAC004</i>	pGreen-35S- <i>GmNAC004</i>	pSoup



## **Arabidopsis root growth assays under low water potential and hormonal treatments**

Arabidopsis transgenic *GmNAC003* and *GmNAC004* and wild-type plants were subjected to root-growth assays in response to water stress and hormonal treatments on agar plates. The agar plates contained basal nutrient medium plus 1.2% Difco bacto agar. The basal nutrient medium comprised: ¼ MS strength (Murashige and Skoog Basal Salt Mixture, and Murashige and Skoog Vitamin), 0.5 g/L monohydrate 2-N-morpholino ethanesulfonic acid (MES), adjusted to pH 5.72. In this experimental series, no sucrose was added to avoid any interference of sugar on growth and molecular signaling (Verslues *et al.* 2006).

### **PEG treatment**

To lower the water potential of the medium, polyethylene glycol (PEG) with a molecular weight of 8000 (Catalog # P5413, Sigma Inc, Saint Louis, MO, USA) was used. The role of PEG is to lower the osmotic potential, which facilitates water deficit in the medium; therefore, the term “water stress” as used in this study refers to low water potential stress.

The diffusion of PEG into the nutrient agar plates was conducted as described in the study by van der Weele *et al.* (2000). The assays were performed on 245 x 245 mm bioassay plates (cat: 431272, Corning, NY, USA). 160 mL of basal nutrient medium solutions containing 0%, 10%, 15%, and 20% PEG were poured into plates containing an

equal amount of solidified nutrient agar media. The plates were gently shaken for 16 hours to facilitate PEG diffusion and equilibrate the water potentials. The PEG solutions were then completely removed by a vacuum generator and the plates were ready for seedling transplanting.

*Arabidopsis* seeds were surface-sterilized with 70% ethanol for 2 minutes, 10% household bleach for 2 minutes, and rinsed four times with distilled water. The seeds were then stratified by incubating them for four days at 4°C in darkness before transferring them to a nursery plate. After plating (day zero), the seeds were treated with red light for 1 hour and kept in the dark for 24 hours at 22°C to promote equal germination. The plates were then placed in a growth chamber in a vertical position to allow downward root growth. At day 4, seedlings with a primary root length between 9 and 11 mm were transferred to bioassay plates with designated PEG concentrations as described above (0%, 10%, 15%, and 20%). During transplantation, seedlings were exposed to laboratory fluorescent lighting for about 10 minutes in laminar flow bench conditions. The plates were sealed by two layers of micropore tape (Micropore, 3M Company, St Paul, MN) and placed vertically in a growth chamber (Conviron model A1000TC). The positions of plates were changed daily to avoid variation in lighting condition in growth chamber conditions. At day 12, the plates were photographed and measured for LR number, LR length and tap root length. Lateral root number was counted visually using a magnifying glass. Total root length was measured using the WinRHIZO program (Regent Instruments Inc.). Primary root length was measured by a ruler from the root/shoot junction to the root tip. Total LR number was calculated by

subtracting the primary root length from the total root length. The experiment was repeated three times for consistent results. Each biological replicate had six replications of five seedlings per replication. At the end of the experiment, water potentials from agar plates were measured using isopiestic thermocouple psychrometry (Boyer and Knipling 1965).

### **Hormonal treatment**

To investigate the response of GmNAC004 transgenic plants to hormonal treatments, seedlings were prepared as described above for PEG treatment. At day 4 (4 days after sowing), they were transferred to bioassay agar plates containing hormonal treatments. Each hormonal treatment contained the basal nutrient medium, with added ABA; 2,4-D; NPA; ABA + 2,4-D; or ABA + NPA, and control solute with concentrations as listed in Table 7. The plants were grown in the same conditions as for the PEG treatments described earlier. The experiment was repeated two times for consistent results. Each biological replicate had six replications of four plants. Lateral root number and length was measured at day 12, as described earlier for the PEG treatments.

**Table 7. Chemicals used for the hormonal treatments for *Arabidopsis* wild type and for transgenic GmNAC004 plants.**

<b>Chemical</b>	<b>Concentration</b>	<b>Purpose</b>
2,4-dichlorophenoxyacetic acid (2,4-D)	20 nM	Auxin treatment
Abscisic acid (ABA)	5 $\mu$ M	ABA treatment
Naphthylphthalamic acid (NPA)	2 $\mu$ M	Auxin transport inhibitor

## **Germination assay**

Sterilized Arabidopsis seeds were sown at low density (70-100 seeds per plate) on agar plates containing basal nutrient medium, either with 1  $\mu\text{M}$  ABA (ABA treatment) or without ABA (control treatment). The plates were incubated in darkness at 4°C for stratification for 4 days and were then placed in a growth chamber (16-hour day length, light density of  $\sim 100 \text{ uE m}^{-2} \text{ sec}^{-1}$ , day/night temperatures of 22°C/18°C respectively, and a constant relative humidity of 70%). Seeds with visible roots were counted after 5 days under normal growth conditions (Bolle 2009). The germination rate was then calculated from the total number of seeds sown, normalized to the germination rate of the control plates. A completely randomized design with 8 replications of about 50-100 seeds was used for the experiment.

## **Experimental design and data analysis**

For root growth assays under low water potential stress, a randomized block design with six replications was used. For seed germination assays, a completely randomized design with 8 replications was used. The differences among the means were estimated using ANOVA and Duncan's multiple range tests, calculated with GENSTAT statistical software (VSN International Ltd, UK). Details of ANOVA data were presented in the Appendix section of this Dissertation.

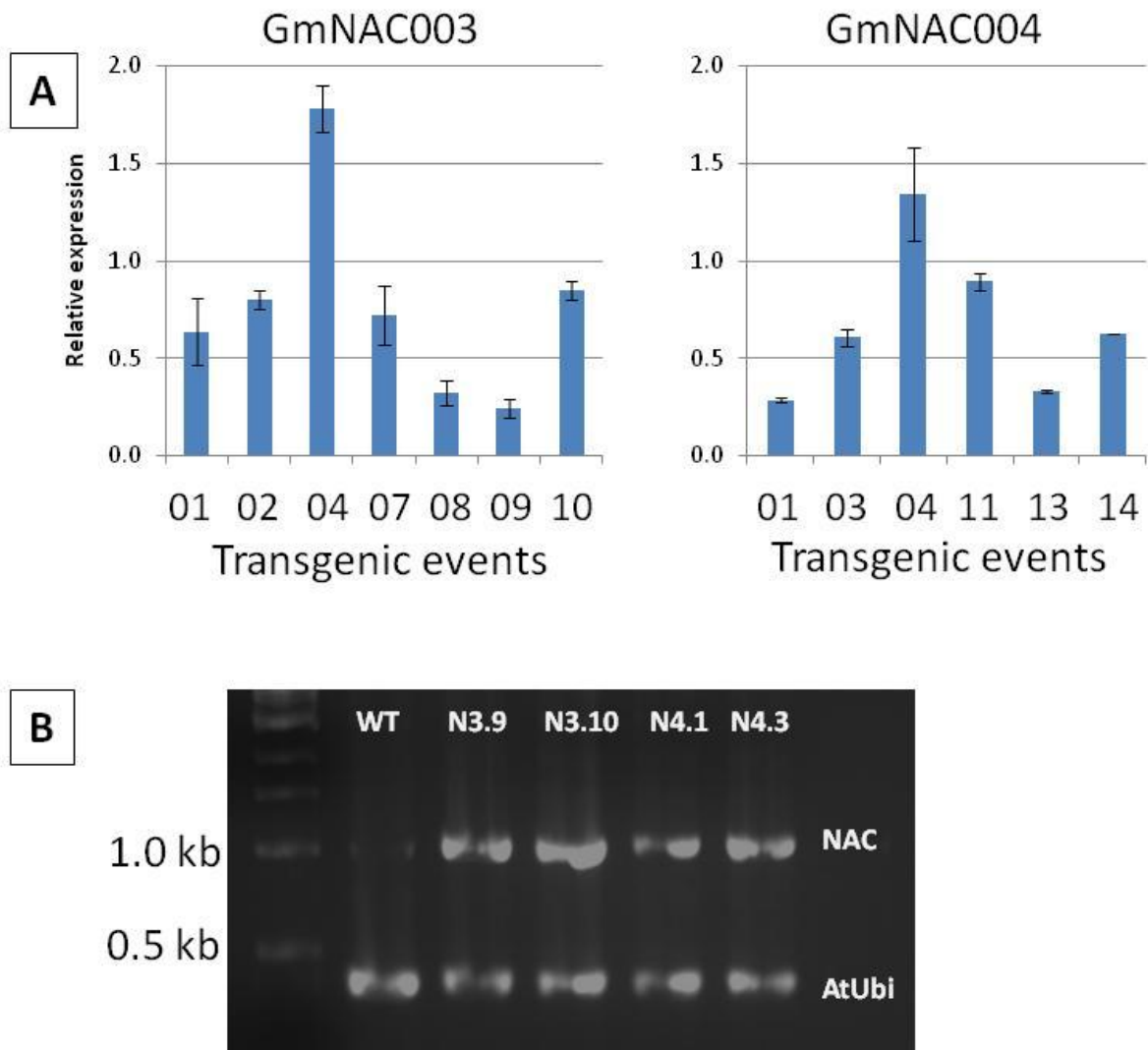
For root growth in response to hormonal treatments, six replications in two bioassay plates (three replication in one plate) were used and the pairwise Student's t-test was

used to compare the means of wild-type and transgenic GmNAC004 plants. The standard error of the mean (SEM) was calculated using Microsoft Excel 2007.

## **RESULTS**

### **Development of Arabidopsis transgenic plants overexpressing soybean *GmNAC003* and *GmNAC004* genes**

Soybean *GmNAC003* and *GmNAC004* driven by CaMV35S promoter were delivered to *Arabidopsis thaliana* cv. Columbia. At least 10 hygromycin-resistant events were selected for transgenic analysis. At the second generation (T<sub>2</sub>), seven *GmNAC003* and six *GmNAC004* transgenic events having a single transgene insertion were selected based on a 3:1 segregation ratio. Transgenic plants over-expressing transcription factors may show altered growth and development. Altered phenotypes such as reduced growth rate and plant size are frequently associated with transgenic plants and may obscure the true growth and development functions of the gene during phenotypic assays. Therefore, among all of the transgenic events expressing the transgenes (Figure 8A), two homozygous events from each gene construct (*GmNAC003* events 9 and 10; and *GmNAC004* events 1 and 3) that showed growth equivalent to the wild type (Figure 9) and the presence of the transgenes by PCR (Figure 8B) were selected for further root growth assays.



**Figure 8. Verification of transgenic Arabidopsis plants overexpressing soybean *GmNAC003* and *GmNAC004*.**

**(A)** Expression of the transgenes in transgenic plants by qRT-PCR using Arabidopsis ubiquitin as the reference gene.

**(B)** Insertion verification by PCR from genomic DNA for selected events that were used in further characterizations. The upper band (NAC) shows transgene PCR product (*GmNAC003*: N3.9 and N3.10; *GmNAC004*: N4.1 and N4.3) and the lower band corresponds to the control gene Arabidopsis ubiquitin (AtUbi).

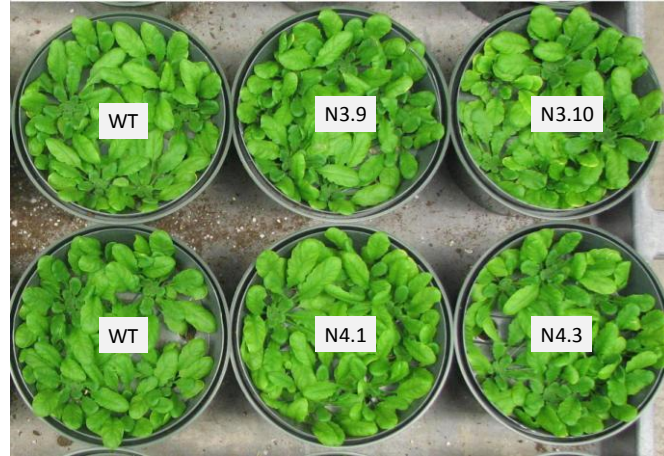


Figure 9. Growth of the transgenic GmNAC003 (events N3.9 and N3.10) and GmNAC004 (events N4.1 and N4.3) and the wild-type (WT). The picture was taken at 4 weeks of growth in growth chamber conditions.

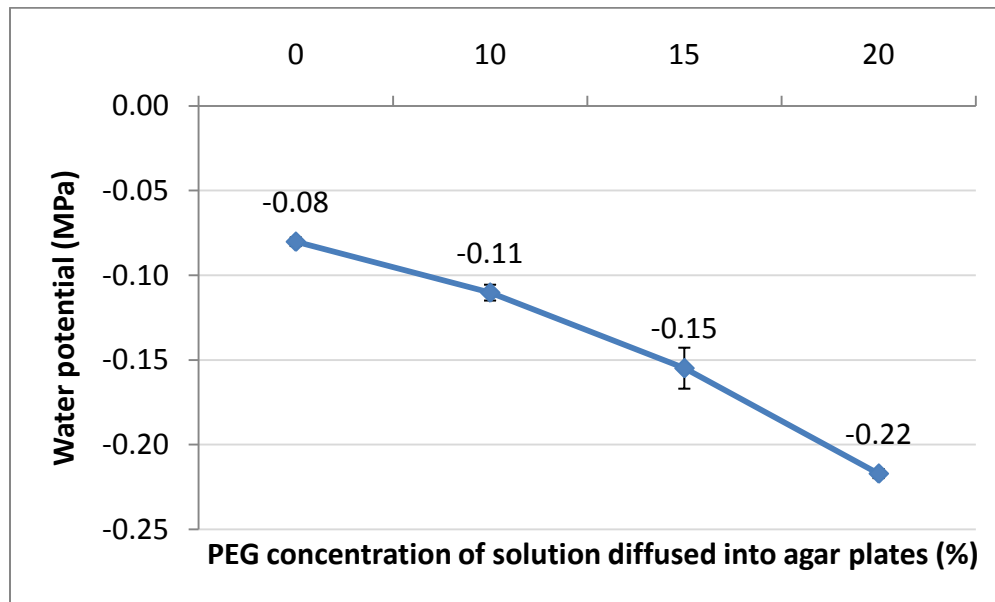


Figure 10. Water potentials of PEG-diffused agar plates of the first biological replicate. Water potentials were measured at the end of the experiment, 8 days from the date of stress exposure. Data are from 6 replications. Error bars are the standard errors of the means (SEMs).

## **GmNAC004 stimulates lateral root number and length in Arabidopsis at high water potential conditions**

Since the Arabidopsis NAC1 and AtNAC2 transcription factors have a positive role in the regulation of lateral root numbers in Arabidopsis (Xie *et al.* 2000; He *et al.* 2005), it is a reasonable hypothesis that, if soybean NAC genes have similar functions, they would show similar effects in the Arabidopsis plants. To generate mild water-deficit conditions, PEG was diffused into the agar medium, which resulted in a lower water potential of the medium (Figure 10). This agar system was used to investigate the roles of GmNAC003 and GmNAC004 in the regulation of LR number because it has been established for Arabidopsis root growth assays (van der Weele *et al.* 2000). The water potentials of PEG-diffused agar plates were reproducible and constant throughout the 5 days in growth chamber conditions (van der Weele *et al.* 2000), allowing accurate measurement of stress level at the end of experiment. The range of water deficit (from -0.08 to -0.22 MPa) in this study was comparable to the stress level in which He (2008) found the promotion of lateral root number and length in soybean. Therefore, this water deficit range was considered suitable for the investigation of the roles of *GmNAC003* and *GmNAC004* genes in the regulation of enhanced lateral root growth and development under water stress.

Figures 11 and 12 show that there is no promotion of lateral root number in Arabidopsis under mild water-stress conditions. Adding 10% PEG to the agar plates reduced the water potential from -0.08 to -0.11 MPa (Figure 10), which was roughly equivalent to



the well-watered condition (-0.1 MPa) of the study by van der Weele *et al.* (2000). This change in water potential caused a slight reduction in lateral root number (Figure 12). When the water potential was reduced to -0.15 MPa, suppression of lateral root number was significant in both wild type and transgenic plants. At the water potential of -0.22 MPa, lateral root number was significantly inhibited. This trend is consistent with the finding of Deak and Malamy (2005) and van der Weele *et al.* (2000), where they found that small changes in water potentials could suppress LR formation.

Transgenic *Arabidopsis* plants overexpressing *GmNAC004* showed an increase in LR number (Figures 11 and 12), while *GmNAC003* overexpression showed no effect on this trait (Figure 12). Specifically, the promotion of LR number in the transgenic *GmNAC004* plants is observed only under non-stress conditions (0%PEG). Under mild water-deficit condition, a significant reduction in LR number was observed in both the transgenic and the WT plants. Nevertheless, the *GmNAC004* transgenic plants still had more lateral root than the WT. Furthermore, lowering the water potential to -0.22 MPa strongly suppressed the LR number of both transgenic and wild type plant. These data demonstrated that *GmNAC004* increases lateral root number under non-stress conditions and maintained higher of LR number under mild water stress compared to the control, but it did not promote lateral root number under low water potentials.

Since total LR length is partially product of LR density and in soybean LR length was increased under mild water stress conditions, response of LR length was also investigated. Similar to the effect on LR number, *GmNAC004* also increased LR length,

with a higher magnitude (Figure 13) under the control condition and had higher total LR length under mild water stress compared to the WT. The higher stimulation of LR length than LR number in GmNAC004 plants perhaps was due to the earlier emergence of LR (by visual observation).

Although there was variation in LR number and length among biological repeats, the trend of response was consistent over three biological replicates of the study. On average, transgenic GmNAC004 had a LR number of approximately 32% and LR length of 65% higher than the WT plants under control conditions. At severe stress (-0.22MPa), LR number was strongly suppressed, but the transgenic plants still had about 25% higher LR number than the WT plants. For LR length, on average under control condition, the GmNAC004 transgenic plants had about 65% longer length than the WT. LR length was decreased under mild water deficits but LR length was still significantly higher in transgenic plants. At severe stress (-0.22MPa), there was a significant reduction in LR length, nevertheless, GmNAC004 transgenic plants still had a slightly higher LR length compared to the WT. Since both LR number and length of GmNAC004 plants were higher than the WT in at least three conditions of 0, 10 and 15% PEG, an analysis for genotype (G) x treatment (T) interaction on LR number and length was investigated for the three PEG concentrations. All three biological replicates showed no GxT interaction (Appendix Tables A.2 and A.4) suggesting that GmNAC004 functioned to increase LR number and length in a range of treatments from 0-15% PEG, but did not promote LR number or length under mild water stress.

Figures 12 and 13 show that GmNAC003 did not have any promotion on lateral root number and total LR length in transgenic Arabidopsis, compared to the WT. Therefore, GmNAC003 was excluded from further functional characterization in Arabidopsis involving lateral root number regulation. In several cases, GmNAC003 transgenic plants are included as a reference for the investigation of GmNAC004 functions.

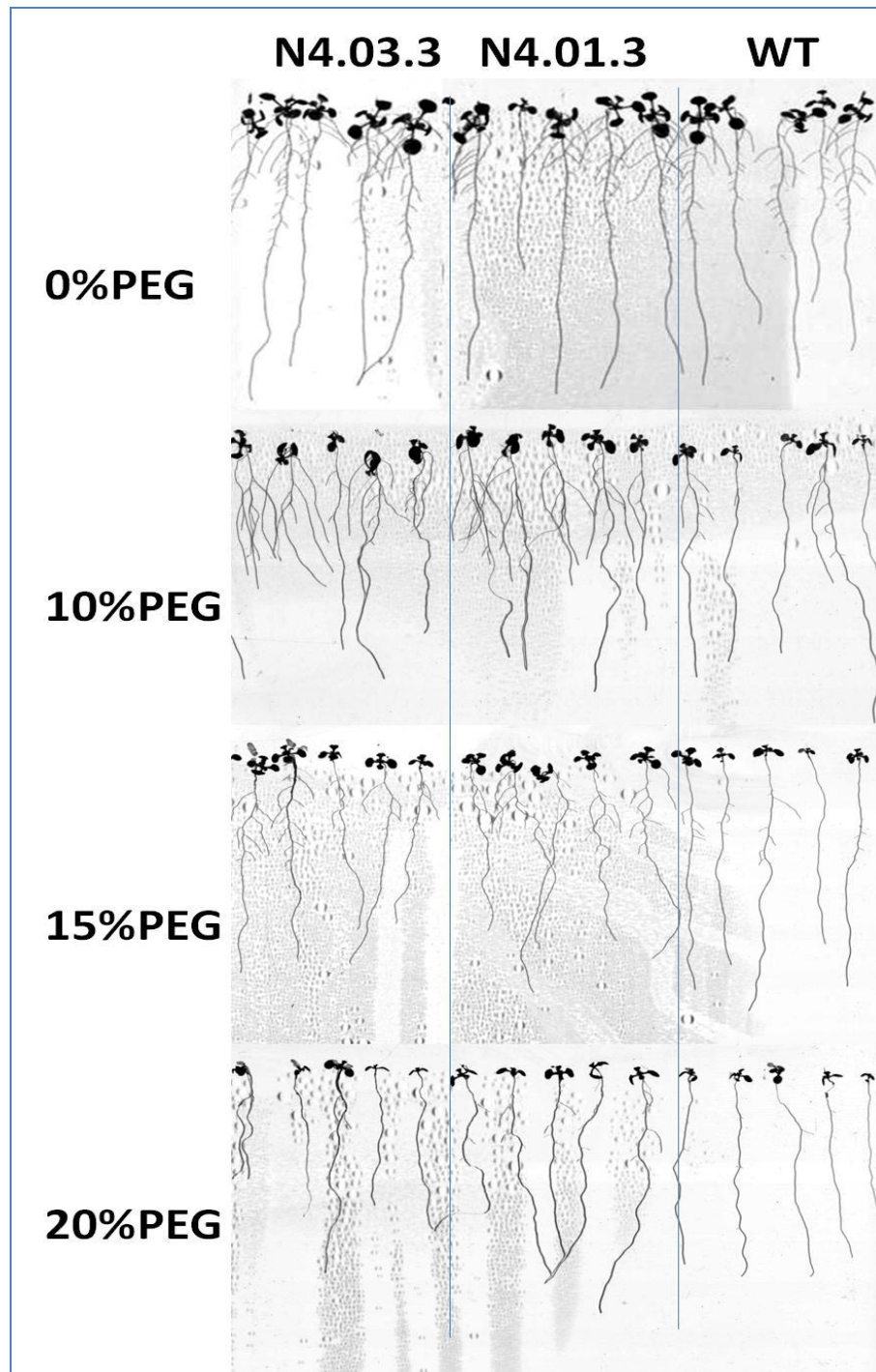
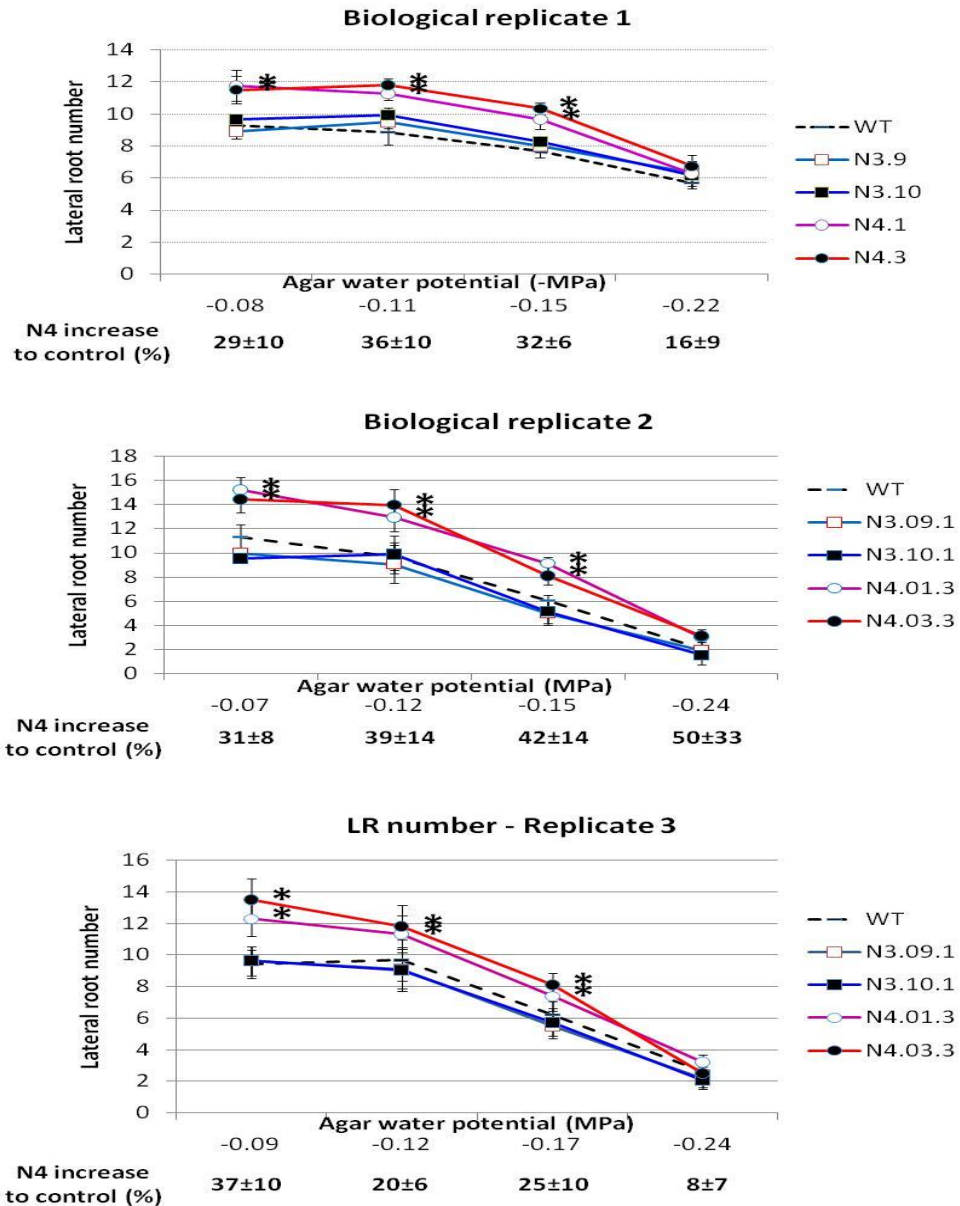
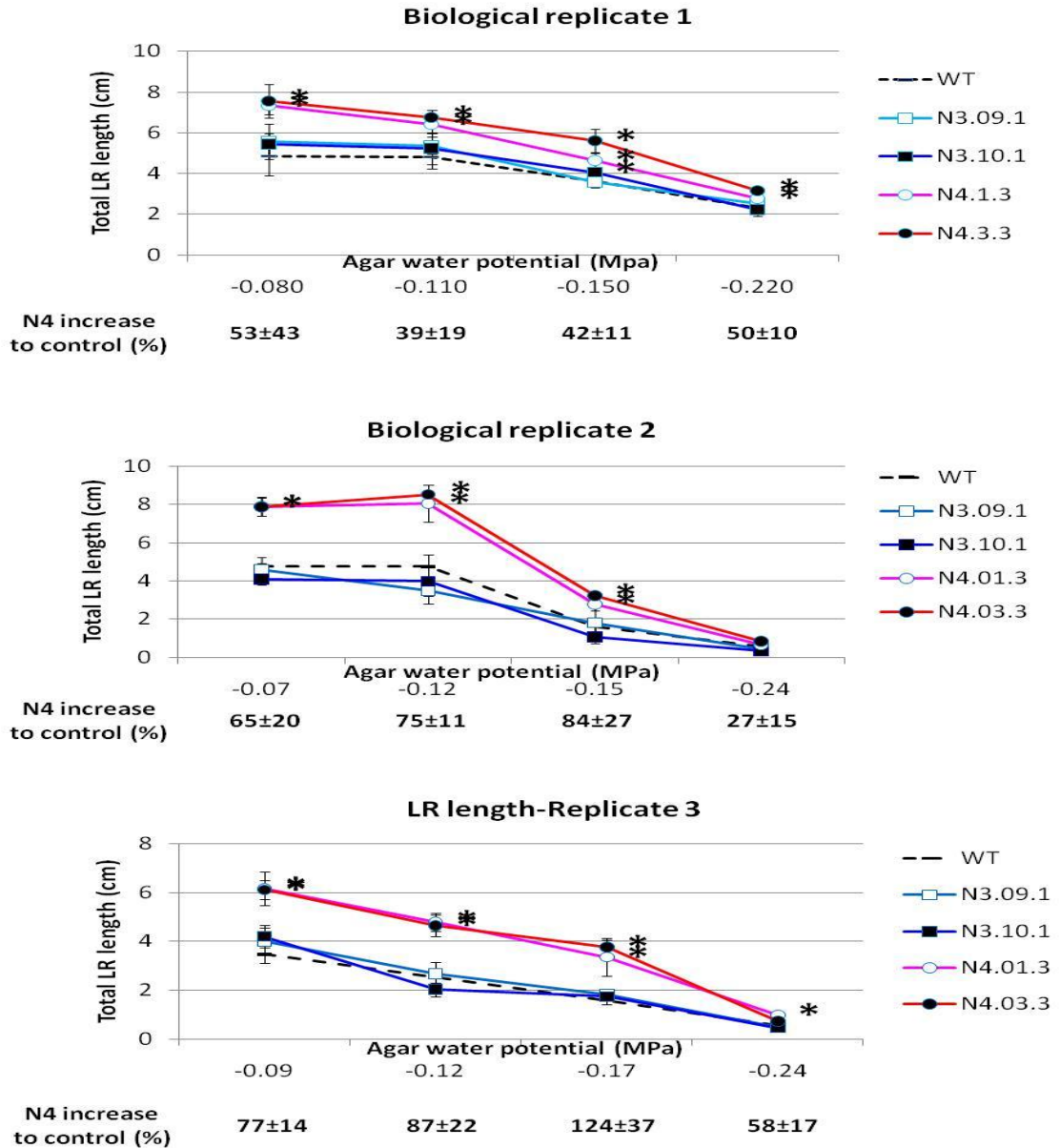


Figure 11. Representative of root growth of two GmNAC004 transgenic events and wild-type lines in response to water deficit conditions. Transgenic lines GmNAC004 (events N-4.1 and N-4.3) and the wild type (WT) were grown in nutrient agar plates diffused with different concentrations of PEG. The pictures were taken for the plants at 12 days old (8 days after stress exposure).



**Figure 12. Lateral root numbers of the Arabidopsis transgenic plants overexpressing soybean *GmNAC003* and *GmNAC004* and of the wild type (WT) in response to mild water stress.**

Transgenic lines *GmNAC003* (events N-3.9 and N-3.10), *GmNAC004* (events N-4.1 and N-4.3), and the wild type (WT) were grown in nutrient agar plates diffused with different concentrations of PEG. Lateral root numbers were measured on the 12<sup>th</sup> day after sowing (8 days of stress exposure). (\*) denotes significant difference at 95% confidence level using Duncan's multiple range test (Appendix Tables A.1 & A.2).



**Figure 13.** Total lateral root length (cm/plant) of *Arabidopsis* transgenic plants overexpressing soybean *GmNAC003* and *GmNAC004* and of wild type (WT) in response to mild water stress. Transgenic lines *GmNAC003* (events N-3.9 and N-3.10), *GmNAC004* (events N-4.1 and N-4.3), and the wild type (WT) were grown in nutrient agar plates diffused with different concentrations of PEG. Lateral root length was measured on the 12<sup>th</sup> day after sowing (8 days of stress exposure). (\*) denotes significant difference at 95% confidence level using Duncan's multiple range test (Appendix Tables A.4 & A.5).

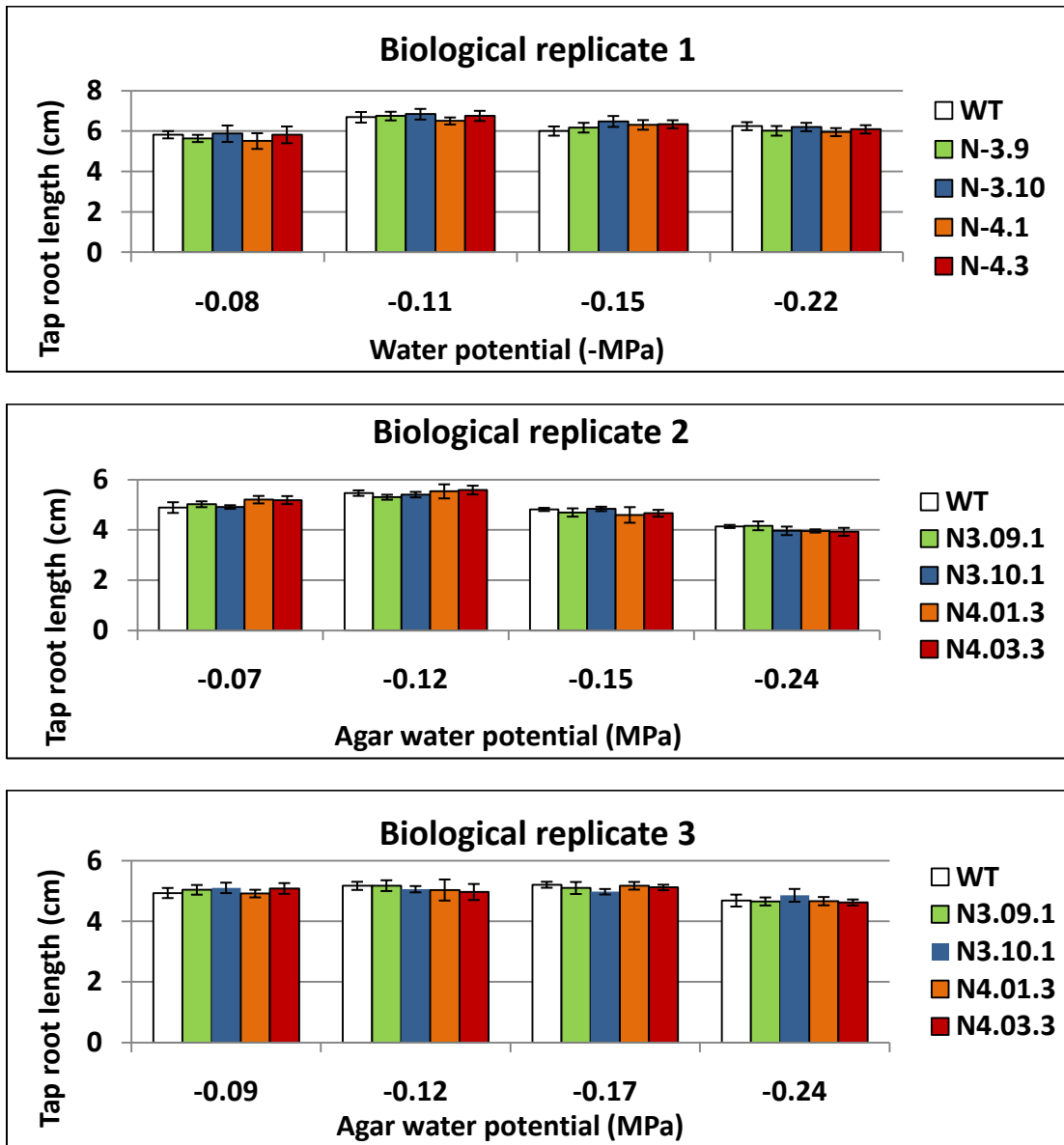


Figure 14. Primary root length of *Arabidopsis* transgenic plants overexpressing soybean GmNAC003 and GmNAC004 and of the wild type (WT) in response to mild water stress. Transgenic lines GmNAC003 (events N-3.9 and N-3.10), GmNAC004 (events N-4.1 and N-4.3), and the wild type (WT) were grown in nutrient agar plates diffused with different concentrations of PEG. Root length was measured on the 12th day after sowing (8 days of stress exposure). (\*) denotes significant difference at 95% confidence level using Duncan's multiple range test (Appendix Tables A.3 & A.4).

## **GmNAC003 and GmNAC004 transcription factors do not promote primary root length in Arabidopsis.**

Figure 14 shows that, in response to mild water-stress treatments, primary root length was slightly promoted in all genotypes at a water potential of -0.11 MPa (10% PEG). At higher stress levels, primary root length remained comparable to non-water-stressed plants, with no reduction in primary root length. The trends of response in this study are in agreement with the study by van der Weele *et al.* (2000), who found that in Arabidopsis, primary root length was slightly stimulated under mild water stress. They also reported that the primary root length was reduced at a water potential of -0.5 MPa and was significantly inhibited at higher stress levels. Figure 14 also shows that transgenic plants overexpressing *GmNAC003* and *GmNAC004* did not show significant changes in root length compared to the wild type in all of the experimental water potentials.

## **GmNAC004 counteracts the ABA-induced inhibition of seed germination**

The *GmNAC004* gene is induced by both ABA and drought; therefore, the action of GmNAC004 may be involved in the ABA-signaling pathway in the regulation of LR number. ABA is a stress-related hormone that has been suggested to inhibit lateral root development (De Smet *et al.* 2006). It is also known to be a dormancy hormone that suppresses seed germination. To illuminate the ABA-signaling network, which might be



involved in the function of GmNAC004, a classical test of seed germination in response to ABA treatment was conducted using both transgenic and wild-type lines. Because two transgenic events from each transgenic construct were shown to perform equally, only one event from each gene was selected for this and further studies. Under the 1  $\mu$ M ABA treatment, as shown in Figure 15, seed germination of the WT was inhibited by nearly 15%. Transgenic line GmNAC003 showed significantly higher inhibition, suggesting that GmNAC003 is a positive regulator of ABA signaling. In contrast, GmNAC004 transgenic plants showed less inhibition in the presence of ABA. Clearly, this data suggests that GmNAC004 counteracts ABA signaling, at least in seed germination.

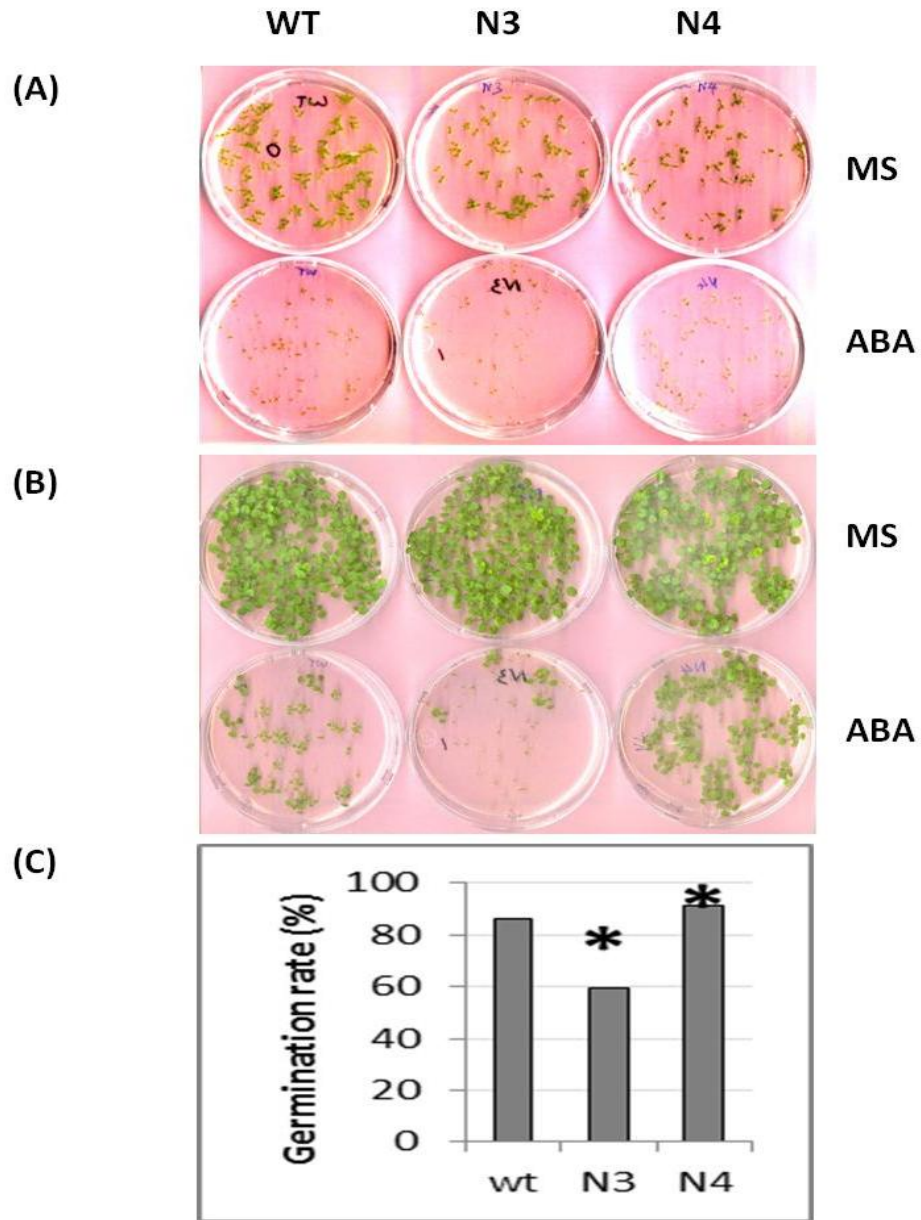


Figure 15. Germination of GmNAC003 (N3) and GmNAC004 (N4) transgenic seeds and of the wild-type (WT) seeds on agar plates without ABA (MS) or with  $1\mu\text{M}$  ABA (ABA). Pictures were taken at (A) 7 days and (B) 14 days after exposure to white light plating. (C) Quantification of germination rates at 5 days after white light exposure. (\*) denotes significant difference at 95% of confidence level using Duncan's multiple range test (Appendix Tables A.7 & A.8).

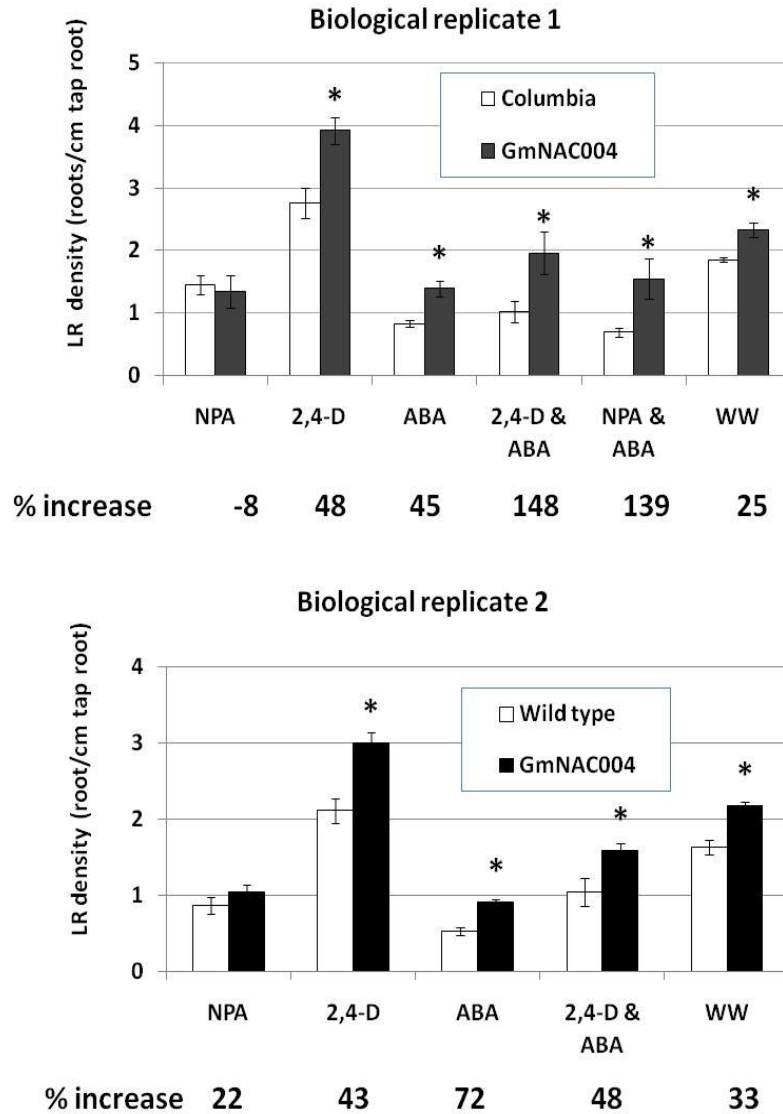
## **GmNAC004 interacts with ABA and auxin signaling to regulate lateral root number**

It is evident that ABA has a negative role in LR development in Arabidopsis under both normal and stress conditions. Several mutants with reduced endogenous ABA show some stimulation of lateral roots. For example, the ABA biosynthesis mutant *nced3*, showed an increase in LR number in peanut (Guo *et al.* 2009). In Arabidopsis, ABA-deficient mutants *aba2-1* and *aba3-1* and ABA-response-defect mutant *lrd2* showed an increase in lateral root system size and number under both normal and osmotic-stress conditions (Deak and Malamy 2005). Previous studies of the suppression of ABA on LR number, together with the current experimental result of ABA inhibition of seed germination (Figure 15), suggest that GmNAC004 may suppress the ABA-induced inhibition of LR development.

To investigate the involvement of GmNAC004 in the ABA-signaling processes that regulate LR development, two approaches were taken for this experiment. First, fluridone, an inhibitor of ABA biosynthesis, was applied to reduce endogenous ABA (Bolte 2009). Unfortunately, this chemical severely damaged the seedlings under light grown condition, and the treated plants did not grow or died within a week of treatment with low concentration (100 nM). The second approach was the application of exogenous ABA. Endogenous ABA can suppress LR number under normal (non-stress) conditions (Deak and Malamy 2005), therefore, it is possible to investigate the

involvement of ABA and GmNAC004 in the regulation of lateral root under normal conditions. Figure 16 shows that in all ABA treatments, either with or without other substances, the WT always showed a higher level of suppression when compared to the transgenic plants. Under treatments of ABA, ABA and NPA, and ABA and 2,4-D, the suppression of the wild-type roots ranged from 45-63%, while the suppression of transgenic GmNAC004 roots ranged from 16-40%. Although there was some variation in the magnitude of the response between two replicates, biological replicate 2 showed a similar response to replicate 1. This suggests that GmNAC004 partially represses ABA-induced inhibition of LR number.

Auxin is known to stimulate LR development. The involvement of GmNAC004 in auxin signaling in LR development was demonstrated with the use of NPA, which is known to inhibit polar auxin transport from the shoot to the root and to inhibit lateral root formation (Casimiro *et al.* 2001). In NPA-treated plants, lateral root number and density were significantly reduced, with no difference between the wild-type and the transgenic plants, while in the control treatment (WW), GmNAC004 showed the expected increase in LR number (Figure 16). In contrast, plants treated with 2,4-D showed a greater promotion of lateral root number in the transgenic plants. These results clearly show that GmNAC004 stimulation of LR development requires auxin, and that GmNAC004 stimulates LR development in the auxin-dependent signaling pathway.



**Figure 16. Effect of hormonal treatments on lateral root density of transgenic *Arabidopsis* plants overexpressing soybean GmNAC004. The wild-type (WT) and GmNAC004 transgenic plants were exposed to 5  $\mu$ M ABA, 20 nM 2,4-D, 2  $\mu$ M NPA, and their combinations for 7 days, starting with 4-day-old seedlings. Well-watered (WW) is the control condition. SEMs are the standard errors of the means. \* denotes significant difference between the WT and transgenic plants from Student's t-test analysis.**

## DISCUSSION

### **Roles of GmNAC004 in the regulation of LR number and length in Arabidopsis**

Arabidopsis has been successfully used as a model in the characterization of foreign genes for drought adaptation, and from this model plant conserved functions of many genes have been found in different plant systems. For example,  $\beta$ -subunit of farnesyltransferase (ERA1) is a negative regulator of ABA signaling in the regulation of stomatal closure. Down-regulation of this gene showed an increase in stomatal closure which resulted in drought tolerance in both Arabidopsis and canola (Wang *et al.* 2005). In addition, because Arabidopsis NAC1 and AtNAC2 showed a promotion of LR number (Xie *et al.* 2000; He *et al.* 2005), it is feasible to use this model system to investigate functions of soybean GmNAC003 and GmNAC004 transcription factors for their roles in the regulation of lateral root number. The data from Figure 12 clearly shows that GmNAC004 significantly promoted LR number under non-stress conditions. However, under low water potential conditions, there was no promotion of LR number in the GmNAC004 transgenic plants. The increased LR number in transgenic GmNAC004 plants was at least partially responsible for the increased in total LR length, which was higher compared to the WT under both control and mild water stress conditions. In contrast, although having a relative conserved DNA binding domain, transcription activation activity and transcript expression pattern with GmNAC004, GmNAC003 did not promote lateral root number.

The inducibility of the *GmNAC004* gene in response to ABA and 2,4-D treatments (Figure 6) suggests that LR stimulation by *GmNAC004* may be involved in the ABA- and auxin-signaling pathways. Auxin and ABA have antagonistic actions: auxin stimulates LR development, while ABA suppresses it (De Smet *et al.* 2006). The suppression of ABA inhibition of seed germination by *GmNAC004* (Figure 15) suggests that *GmNAC004* partially inhibits ABA signaling in LR development. This was shown when all the ABA treatments (ABA, ABA&2,4-D, ABA&NPA) dramatically inhibited LR numbers in both the transgenic and the WT lines, but the inhibition was not as strong in the transgenic plants overexpressing *GmNAC004* (Figure 16). Previous studies showed that the inhibition of ABA on LR development may occur in most of the stages from LR initiation to LR emergence from the main root (De Smet *et al.* 2006), but the major inhibition of ABA may be at the post-initiation stage. *Arabidopsis thaliana* cv. Columbia did not produce any LR after 9 days of treatment with 0.5  $\mu$ M ABA, but it was able to produce many LR when the seedlings were transferred to normal conditions (De Smet *et al.* 2003), suggesting that the LR primordia could develop but that the emergence was arrested under ABA treatment.

Figure 16 also shows that the role of *GmNAC004* in the regulation of LR was in connection with auxin: 2,4-D treatment had a greater promotion in LR number in the transgenic plants, compared to the WT. In addition, *GmNAC004* required auxin for its action in the regulation of LR because there was no promotion when the auxin gradient was blocked (by NPA treatment). The data from this study indicates that the *GmNAC004* may enhance auxin but suppress ABA signaling to regulate LR development in

Arabidopsis where auxin and ABA have been shown to have antagonistic roles throughout the process from LR pre-initiation to emergence (De Smet *et al.* 2006). These results show an additional crosstalk between the auxin and ABA signaling pathways in the regulation of LR development. The specificity of the interactions, however, will require further investigation for specific stages of LR development.

### **Possible role of GmNAC004 in regulation of lateral root number in soybean**

Using the Arabidopsis model, the data presented in this study show that the drought-inducible soybean *GmNAC004* transcription factor gene has a positive role in the regulation of LR development. The gene appears to stimulate auxin but repress ABA in the regulation of lateral root number. In addition, *GmNAC004* is inducible under drought, ABA, and auxin; therefore, it is possible that this gene has a conserved role in promoting lateral root number in soybean under water deficit conditions. This is because the role of Arabidopsis NAC genes in LR development was reported (Xie *et al.* 2000; He *et al.* 2005) and because various genes have conserved functions in root development across Arabidopsis and crop plants. For example, an Arabidopsis HDSTART transcription factor showed increased root growth in both transgenic Arabidopsis and canola (Yu *et al.* 2008). Similarly, the Arabidopsis HARDY protein showed improved drought tolerance in both transgenic Arabidopsis and rice because of a greater root system along with other drought tolerance traits (Karaba *et al.* 2007). The root system is known to help plants maintain high tissue water content as a drought avoidance



mechanism (Turner *et al.* 2001, Manavalan *et al.* 2009). Although the role of GmNAC004 needs to be verified in soybean, GmNAC004 is certainly a strong candidate for regulating the development of lateral roots. Once the role of this gene in soybean is proven, it can potentially be used for genetic engineering for a higher lateral root number, which in turn, may partially facilitate increased lateral root length, at least under normal growth conditions. Plants with early development of a large and extensive root system may benefit adaptation to drought during later growth stages, where water deficit may occur.

GmNAC004, however, does not show promotion of LR in response to water-deficit conditions in Arabidopsis. Therefore, it is unclear whether the gene is responsible for the enhanced LR number of soybean in response to mild water-deficit. One possible limitation can be that the Arabidopsis model represses LR number under osmotic stress which has been reported previously. In response to osmotic stresses induced by PEG, mannitol, or nitrogen salt, lateral root number was inhibited in Arabidopsis ecotypes Landsberg erecta (Ler), Columbia (Col), and Wassilewskija (Ws) (van der Weele *et al.* 2000; Deak and Malamy 2005; Gerald *et al.* 2006; Voothuluru, unpublished). Therefore, there may be limitations in the Arabidopsis system that does not facilitate GmNAC004 to express the enhanced LR number under low water potential conditions. It is not possible to conclude from this study that GmNAC004 does not have a role in stimulation of soybean LR number and length under drought stress.

Soybean, in contrast to Arabidopsis, shows stimulation of LR development under water-deficit conditions. He (2008) reported that, in response to mild water deficit (-0.25MPa media water potential), LR number increased in 8 of 11 studied genotypes, but that there was a reduction in tap root length, and reduction or no change in root biomass. In contrast to root growth, shoot biomass was significantly reduced under mild water-deficit conditions. More importantly, the increase in LR number and length occurred at lower soil profiles. The study also shows that the majority of the soybeans show the same trend in their response, suggesting that soybean plants prioritize root growth and development when adapting to water deficit conditions. LR number promotion was also observed in soybeans growing under drought conditions (Read and Bartlett 1972). Therefore, in soybean, there is a molecular network system that supports gene action to promote LR number under mild water deficit. Using transgenic soybeans, thus, might be useful to investigate the function of GmNAC004 in the enhanced lateral root number, which in turn is partially responsible for the enhanced root density in response to water deficit stress.

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## CHAPTER 4. SYNTHESIS AND CONCLUSION

Drought adaptation in plants involves three major mechanisms: drought escape, drought avoidance, and drought tolerance (Turner *et al.* 2001). Plants with a drought-escape mechanism are able to grow faster and complete their life cycles before the onset of drought. Plants may also endure drought by maintaining a high tissue water content through enhanced water uptake or reduced water loss (drought avoidance), or by tolerating low water content in their tissues with mechanisms that protect cellular organelles from chemical and mechanical damages (drought tolerance). Under mild water-stress conditions, the water supply is below the optimum level; therefore, to maintain productivity, enhanced water uptake by a restructured root system may be favored in natural selection for soybean. There is substantial evidence in soybean that lateral roots increase in both number and total length under mild water stress conditions, particularly at lower soil profiles (Read and Bartlett 1972; Huck *et al.* 1983; Hoogenboom *et al.* 1987; He 2008).

The present study shows that drought-inducible soybean genes *GmNAC003* and *GmNAC004* have several properties which show that they may be potentially involved in enhanced lateral root number in response to water deficit in soybean, as discussed in

Chapter 2. First, they belong to NAC transcription factor family, which is known to affect plant adaptation to drought and other abiotic stresses. Second, several NAC members have been identified for their role in the regulation of lateral root (LR) number. Specifically, Arabidopsis *NAC1* is required for and stimulates the development of LR; the Arabidopsis plant has a significant reduction in lateral root number when this gene is silenced (Xie *et al.* 2000). Another gene, Arabidopsis *AtNAC2*, has been shown to promote LR number (He *et al.* 2005). Both Arabidopsis *NAC1* and *AtNAC2* are involved in auxin signaling and function downstream of the auxin-signaling pathway, which regulates many processes of lateral root development (Peret *et al.* 2009a&b). Third, soybean *GmNAC003* and *GmNAC004* genes are up-regulated in response to drought stress (Chapter 2), in conditions where LR number in soybean is stimulated (He 2008, Read and Bartlett 1972). Finally, one of the genes, *GmNAC004*, showed increased expression in response to the treatment of 2,4-D, a synthetic auxin (Figure 6). This suggests that *GmNAC004* may interact or act downstream of auxin signaling in the regulation of LR number in soybean, and that it may be responsible for enhanced lateral root number in response to the mild water deficit.

Soybean *GmNAC003* and *GmNAC004* were studied for their function in lateral root development using the Arabidopsis model. Transgenic Arabidopsis plants overexpressing these genes were grown in nutrient agar plates diffused with different amounts of polyethylene glycol (PEG) which produce a range of water potentials from -0.08 to -0.22 MPa. While no difference of response was observed for *GmNAC003* transgenic plants, the transgenic *GmNAC004* plants had a higher lateral root number in

the control and it can maintain higher LR number under mild water potential treatments, compared to the WT. The higher lateral root number was associated with total LR length which had a similar pattern of response as LR number. Since *Arabidopsis* NAC1 and AtNAC2 (Xie *et al.* 2000; He *et al.* 2005) also showed promotion of lateral root number, these results provide additional support that NAC proteins are important for the stimulation of lateral root number.

Because *GmNAC004* is induced by two hormones that regulate lateral root development, ABA and auxin (De Smet *et al.* 2006), the focus of this study was on the interaction of *GmNAC004* with ABA and auxin. ABA accumulates in plant tissues in response to drought stress (López-Carbonell 2005), and the role of ABA in plant adaptation has been shown in stomatal regulation (Hose *et al.* 2002) and in drought tolerance (Thompson *et al.* 2007). ABA accumulation under drought-stress is also necessary for the maintenance of root growth in maize (Sharp and LeNoble 2002; Sharp 2002). In contrast with ABA's maintenance of root elongation in maize, LR development is negatively associated with ABA accumulation in *Arabidopsis*. Under both well-watered and water-stress (using mannitol) conditions, *Arabidopsis* ABA-deficient mutants *aba2-1* and *aba3-1*, and an ABA signaling mutant *lrd2*, showed a higher number of LR than in the WT (Deak and Malamy 2005). A similar finding was observed in a peanut mutant in ABA biosynthesis enzyme 9-cis-epoxycarotenoid dioxygenase, *nced3*, which showed increased LR numbers under the control condition (Guo *et al.* 2009). The inhibition of LR number by ABA may happen at various stages of LR formation, from initiation to emergence, where ABA appears to be antagonistic to the auxin action (De Smet *et al.*

2006). The evidence that the GmNAC004 protein suppressed the ABA-induced inhibition of seed germination (Figure 15) suggests that GmNAC004 may counteract the ABA-mediated inhibition of LR development. The suppression of ABA-induced inhibition of LR number by GmNAC004 is evident in this study. In biological replicate 1, for example, all treatments with ABA (ABA alone or in combination with other chemicals) LR number of transgenic GmNAC004 was higher than in the wild type (67-123% increase), while under control conditions, there is only a 26% increase (Figure 16). For auxin, GmNAC004 may act downstream of auxin signaling because the gene is up-regulated by 2,4-D treatment (Figure 6). The present study shows that auxin is necessary for the development of enhanced LR number in the GmNAC004 transgenic plants (Figure 16). The data suggest that GmNAC004 interacts with the auxin and ABA-signaling pathways to regulate LR development.

Because soybean *GmNAC004* is drought responsive in soybean roots, it is reasonable to hypothesize that it may have a role in the enhanced LR number, which in turn also helps increase the total LR length in soybean in response to water deficit. Figure 12 clearly shows that GmNAC004 transgenic plants exhibited an increase of lateral root number over the wild-type line under control conditions and under two of the PEG treatments (-0.11 and -0.15 MPa agar water potentials), but there was no promotion of LR under mild water deficit in the transgenic plants. Thus, the results of this study do not support the role of GmNAC004 in the enhanced LR number under the water deficit in Arabidopsis. There are two possibilities: (i) the soybean GmNAC004 protein does not have a function in increased lateral root development in soybean under water stress; or (ii) it has a



function, but it cannot express the increased lateral root phenotype under stress in the Arabidopsis model. The first possibility is supported by a similar study in Arabidopsis in response to salinity stress (He *et al.* 2005). In Arabidopsis, NaCl stress increases lateral root numbers. Overexpression of *AtNAC2*, a salinity responsive gene, stimulated lateral root number under control conditions. The *nac2* mutant, however, did not show any change in response; that is, the mutant still showed increased lateral root number in response to salinity stress, similar to the wild-type. This suggests that *AtNAC2*, although a promoter of lateral root number under normal growth, is not the protein that regulates the enhanced lateral root number in Arabidopsis in response to salinity stress. If *GmNAC004* does not have a role in the enhanced LR in soybean under water deficit condition, there could be a different molecular mechanism to regulate the LR promotion in soybean.

The second possibility is that Arabidopsis does not support a gene to express enhanced lateral root number under water-deficit stress. Lateral root development in Arabidopsis is significantly inhibited in response to low water potential conditions induced by mannitol (Deak and Malamy 2005) and by PEG (van der Weele *et al.* 2000; Voothuluru, unpublished). Thus, Arabidopsis may have a mechanism to suppress LR number in response to water-deficit stress, so that *GmNAC004* is not able to express the trait. Therefore, to understand whether *GmNAC004* has a role in the stimulation of LR number in response to drought, the gene function should be investigated in soybean. The other possibility is the regulation of *GmNAC004* expression that allows the gene function. For example, if the expression of the gene is too low, it cannot express the

trait under normal condition in soybean. On the other hand, under mild water stress, higher expression may allow the gene function to express to enhance LR number.

In conclusion, although the data cannot rule out other factors (including other soybean transcription factors) that may act to enhance LR number in response to water deficit in soybean, this study showed that the soybean transcription factor *GmNAC004* has a role in the stimulation of LR number and total LR length under non-stress condition.

*GmNAC004* is strongly induced by water stress, and is strongly expressed in roots, thus suggesting its role in enhanced LR number under stress conditions in soybean. However, the present data using the Arabidopsis model does not support the role of *GmNAC004* gene in the stimulation of LR development under water deficit. Therefore, its role in root system architecture in soybean, specifically in the stimulation of LR under mild water deficit requires further investigation. Once the molecular mechanisms regulating root plasticity are identified, it will benefit strategies for genetic improvement of drought adaptation and productivity in soybean.

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## APPENDIX

>GmNAC003 coding sequence

```
ATGGGAGTTCCAGAGAGAGACCCTCTTGACAATTGAGTTTGCCTCCTGGGTTTAGGTTTTACCCACAGATGAGGA
GCTTTTGGTTACAGTACCTTTGCCGCAAGGTTGCTGGCCATCATTCTCTTCCAATCATTGCTGAAAGTTGATTTGTAC
AAGTTTGATCCATGGGTTCTTCCAGGTAAGGCAGTGTGGAGAGAAGGAGTGGTACTTTTTAGCCCAAGAGACA
GGAAGTACCCGAATGGTTCACGACCAACAGAGTCGCGGGTCTGGGTATTGGAAAGCAACTGGAACAGACAAGA
TCATCACCCTGAAGGTAGAAAAGTTGGCATAAAAAAAGCACTTGTCTTCTACATTGGCAAAGCACCCAAAGGCTCC
AAAACCAATTGGATCATGCACGAGTATCGCCTTCTCGATTCTCCCGCAAACACAACCTCGGAACCGCAAAGCTTGAT
GATTGGGTTCTATGTCGTATCTATAAGAAGAACTCAAGTTCACAAAAGGTTGAGGCTAATTTTTGGCTATGGAATG
CAGCAATGGGTCATCACCTTCTTATCGTCCACGTGGATGACATGCTGGGATCGTTGCCGGAGATAAATGACCGGT
GCTTCACCTGCCACGAGTGAACCTCACTCAGAACAATGCACCAGCAGGATGAGAAAATCGGGTCTCCGAACATGGG
ATCCGGGTTTTCTCGGATTGGGTTAACTCGACCGATCTCGATTGATTCCGAATTCGAGTCGGGTTGCCAAACCCA
AAGAATGGTGAATTATGATTGCAATGACTTTTTGTTCTTCTGCGCCCTTGGGCCATGTGGACTACATGGTGGGA
TGCACCTTTGGAGGAGGAGGTTCAAAGTGGTGTGAGAACCCGACGGGTCGACGGGCCGGGGCATTTCACCGAA
TCCAGATACCCGATTGTTACCGGGCTCAGGTGACCATTGCGGTTTGGGTTATTATGGGTCAGCAAGTTGAGTTCG
GGTTTAGGGACTGA
```

>GmNAC003 protein

```
MGVPERDPLAQLSLPPGFRFYPTDEELLVQYLCRKVAGHHFSLPIIAEVDLYKFDPWVLPKAVFGEKEWYFFSPRDRKYP
NGSRPNRVAGSGYWKATGTDKIITTEGRKVGIKKALVFYIGKAPKGSKTNWIMHEYRLDSSRKHNLTAKLDDWVLCRI
YKKNSSSQKVEANFLAMECSNGSSPSSSHVDDMLGSLPEINDRCFTLPRVNSLRMHQOQDEKFGSPNMGSGFFSDWV
NSTDLDSISEFESGCQTRMVNYDCNDFVPSLPLGHVDYMDAPLEEEVQSGVRRRVDGPGHFQPNPDRLLPGS
GDPFGFGFIMGQQVEFGFRD
```

>GmNAC004 coding sequence

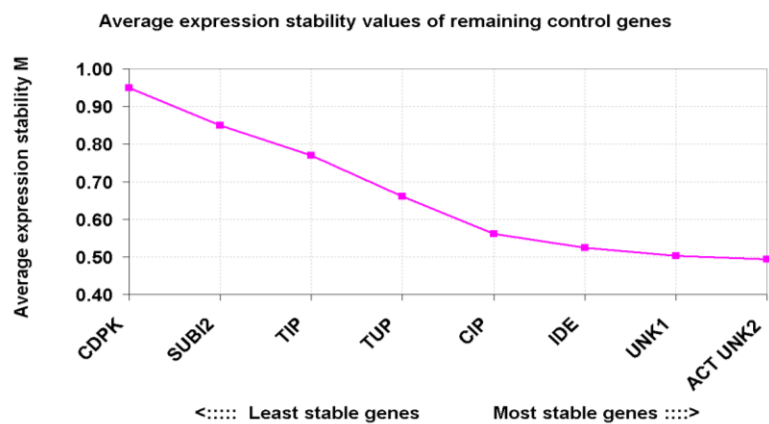
```
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GCTCCTCGTTACAGTATCTATGCCGCAAGGTCGCTGGCCACCATTTCTCTTCCAATCATTGCTGAAATTGATTTGTAC
AAGTTGACCCATGGGTTCTTCCAAGTAAGGCGATTTTCGGTGAGAAAGAGTGGTACTTTTTAGCCCTAGAGACAG
GAAATACCCTAACGGGTCCCGACCCAACAGAGTAGCCGGGTGGGTTATTGGAAAGCCACCGGAACCGACAAGATC
ATCACCCGAAGGTAGAAAAGTTGGCATAAAAAAAGCCCTCGTTTTCTACATTGGCAAAGCCCCAAAGGCACCAA
AACCAATTGGATCATGCACGAGTATCGCCTCCTCGACTCTCCCGAAAAAACACCGGCACCAAGCTTGATGACTGGG
TTCTGTGTCGTATCTACAAGAAGAACTCGAGTGACAGAAAGGCGGTGCAAAACGGCGTCTCCGAGCAACGAGCA
CACTCAATACAGCAACGGTTCCTCTTCTTCTTCTCGTACAACCTCGACGACGTTCTGGAATCGCTGCCAGCGATTGA
TGAACGGTGTTCGGATGCCACGTGTCAACACGCTGCAGCAACAGCAGCAGCAGGAGAAGGTCAATGTTGAGAAC
TTGGGTGAAGGGGGTACTGGATTGGACCAACCCTTCGGTCTGAATTCGGTCTGATTTCGTATCGGGGAATAA
TAATCATAATCAATTGGTGCAGGACCAGACGCAAGGCATGGTGAACATAACGCGTGAATGACCTCTATGTCCTA
CGTTATGCCATGTGGGCACGTCAGTTCGCAAAAGATGGAGGAAGAGGTGCAAAGCGGCGTGAGAAACCAACGGG
TCCAGAACAAATTCCTGGTTCTTCAAGATGATTTACACAGGGGTTTCAAGATTCGGTTGACACGCTCTGGTTTAAGT
ACCCGGTTCAACCGGTGGGGTTCGGGTTCAAGGAATTGA
```

>GmNAC004 protein

```
MGVPEEDPLSQLSLPPGFRFYPTDEELLVQYLCRKVAGHHFSLPIIAEIDLYKFDPWVLPKAVFGEKEWYFFSPRDRKYPN
GSRPNRVAGSGYWKATGTDKIITTEGRKVGIKKALVFYIGKAPKGTKTNWIMHEYRLDSSRKNTGKLDWVLCRIYKK
NSSAQKAVQNGVPSNEHTQYSNGSSSSSSQLDDVLESPLAIDERCFFMPRVNTLQQQQHEEKVNVQNLGEGGLLDW
TNPSVLNSVDFVSGNNHNHNLVQDQTQGMVNYNACNDLYVPTLCHVGTSPVQKMEEEVQSGVRNQRVQNNVSWFL
QNDFTQGFQNSVDTSGFKYPVQPVGFGFRN
```

**Figure A.1. DNA sequences of *GmNAC003* and *GmNAC004* and their predicted proteins. *GmNAC004* has one 1 base different from the sequence cloned by Meng *et al.* (2005), AY974352 at the position 847 (G to A) that causes a change in amino acid in position 283 (Adenine-A to Threonine-T), which is underlined.**

(A)



(B)

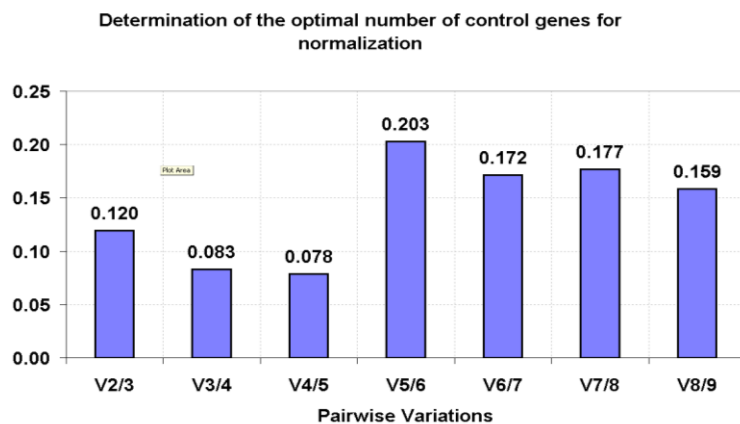
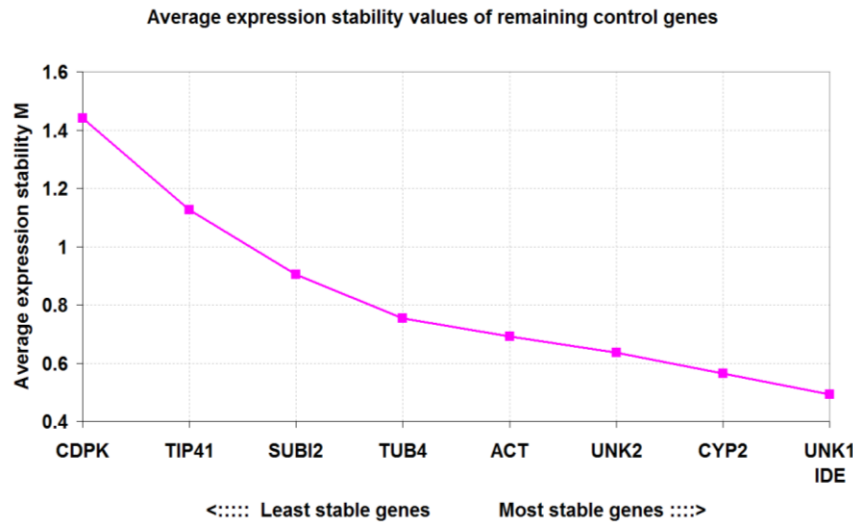


Figure A.2. Determination of reference genes for qRT-PCR for the quantification of gene expression in response to drought in soybean. The expression data was collected from 18 samples: 9 drought samples (3 drought treatments of -0.50, -1.00 and -1.50 MPa for 3 tissues of root, stem and leaf) and 9 samples from control condition (3 growth stages of V2, V3, and V6 for 3 tissues of root, stem and leaf). Data was analyzed by GeNorm (Vandesompele *et al.* 2002).

(A) Average expression stability M of nine reference genes (Table 1) over the tissues tested. The gene with lower average expression stability (M index) has higher expression stability. (B) pairwise variation V between two sequential normalization factors containing an increasing number of genes. The pairwise variations (V) define whether the addition of a reference gene has a significant effect and should preferably be included for calculation of a reliable normalization factor. Based on this data, the optimum number of the reference genes selected to calculate the normalization factor would be either 4 or 5. In this experiment, 4 reference genes: ACT, IDE, UNK1 and UNK2, were selected.

(A)



(B)

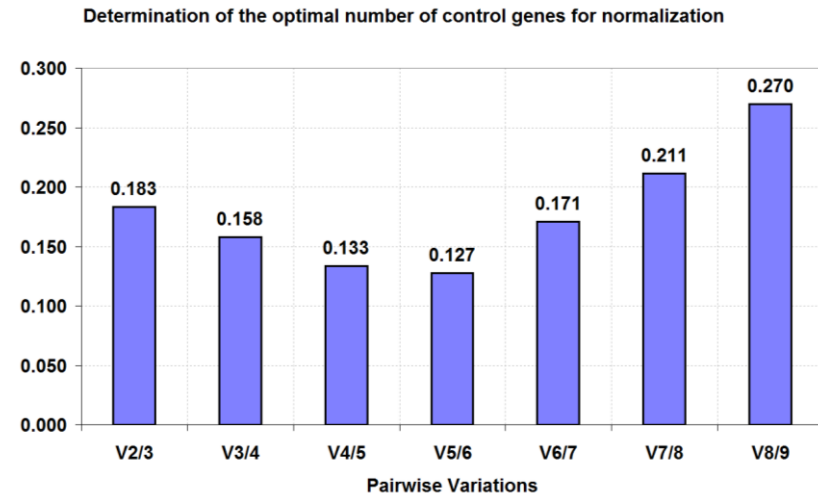


Figure A.3. Determination of reference for qRT-PCR for the calculation of normalization factor for the quantification of gene expression in response to abiotic stress and hormonal treatments. The data comes from 53 samples of the abiotic stress treatments (7 treatments including water, cold (4 oC), 250 mM NaCl, 100uM ABA, 10uM 2,4-D, and dehydration over 5 time points of 1, 2, 5, 10, and 24 hours. Whole plant tissue was sampled from most of stresses and time points. In several cases, root and shoot were sampled separately for ABA and dehydration treatments). (A) Average expression stability M of nine reference genes (Table 1) over the tissues tested. (B) pairwise variation V between two sequential normalization factors containing an increasing number of genes. Data was analyzed as described in Appendix Figure A.2. In this experiment, 5 reference genes: *ACT*, *IDE*, *CYP2*, *UNK1* and *UNK2*, were selected.



**Table A.1. Means and coefficients of variation for lateral root number of Arabidopsis transgenic GmNAC003 (events N3.9.1 and N3.10.1), GmNAC004 (events N4.1.3 and N4.3.3), and the wild type (WT) in response to various treatments of PEG.**

**Biological replicate 1**

<b>Genotypes<sup>*</sup></b>	<b>0% PEG</b>	<b>10% PEG</b>	<b>15% PEG</b>	<b>20% PEG</b>
WT	9.28 <sup>a</sup>	8.87 <sup>a</sup>	7.69 <sup>a</sup>	5.68 <sup>a</sup>
N3.9.1	8.90 <sup>a</sup>	9.48 <sup>a</sup>	8.00 <sup>a</sup>	6.35 <sup>a</sup>
N3.10.1	9.67 <sup>a</sup>	9.92 <sup>ab</sup>	8.26 <sup>a</sup>	6.17 <sup>a</sup>
N4.1.3	11.77 <sup>b</sup>	11.26 <sup>bc</sup>	9.67 <sup>b</sup>	6.27 <sup>a</sup>
N4.3.3	11.50 <sup>b</sup>	11.79 <sup>c</sup>	10.33 <sup>b</sup>	6.74 <sup>a</sup>
Grand mean	10.22	10.26	8.97	6.24
CV%	13.7	13.1	13.2	25.8

**Biological replicate 2**

<b>Genotypes<sup>*</sup></b>	<b>0% PEG</b>	<b>10% PEG</b>	<b>15% PEG</b>	<b>20% PEG</b>
WT	11.33 <sup>a</sup>	9.70 <sup>a</sup>	6.07 <sup>a</sup>	2.05 <sup>a</sup>
N3.9.1	9.99 <sup>a</sup>	9.06 <sup>a</sup>	5.01 <sup>a</sup>	1.93 <sup>a</sup>
N3.10.1	9.56 <sup>a</sup>	9.88 <sup>a</sup>	5.14 <sup>a</sup>	1.44 <sup>a</sup>
N4.1.3	15.25 <sup>b</sup>	12.94 <sup>b</sup>	9.14 <sup>b</sup>	3.01 <sup>a</sup>
N4.3.3	14.43 <sup>b</sup>	13.98 <sup>b</sup>	8.13 <sup>b</sup>	3.14 <sup>a</sup>
Grand mean	12.11	11.12	6.70	2.32
CV%	12.5	19.8	23.0	28.8

**Biological replicate 3**

<b>Genotypes<sup>*</sup></b>	<b>0% PEG</b>	<b>10% PEG</b>	<b>15% PEG</b>	<b>20% PEG</b>
WT	9.43 <sup>a</sup>	9.68 <sup>a</sup>	6.20 <sup>a</sup>	2.65 <sup>a</sup>
N3.9.1	9.62 <sup>a</sup>	9.13 <sup>a</sup>	5.51 <sup>a</sup>	2.19 <sup>a</sup>
N3.10.1	9.63 <sup>a</sup>	9.05 <sup>a</sup>	5.74 <sup>a</sup>	2.08 <sup>a</sup>
N4.1.3	12.29 <sup>b</sup>	11.34 <sup>b</sup>	7.39 <sup>b</sup>	3.23 <sup>a</sup>
N4.3.3	13.51 <sup>b</sup>	11.83 <sup>b</sup>	8.13 <sup>b</sup>	2.50 <sup>a</sup>
Grand mean	10.90	10.21	6.59	2.53
CV%	18.9	25.9	25.9	36.0

\* Similar letters are not significantly different at the 0.05 level of probability according to Duncan Multiple Range Test.

**Table A.2. ANOVA tables of lateral root (LR) number in response to PEG treatments.**

**Biological replicate 1**

A. Variate: LR number under 0% PEG treatment

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	27.034	5.407	2.76	
Genotype	4	41.739	10.435	5.33	0.004
Error	20	39.141	1.957		
Total	29	107.914			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

B. Variate: LR number under 10% PEG treatment

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	5.681	1.136	0.63	
Genotype	4	36.030	9.008	4.99	0.006
Error	20	36.118	1.806		
Total	29	77.83			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

C. Variate: LR number under 15% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	2.268	0.454	0.33	
Genotype	4	31.581	7.895	5.82	0.003
Error	20	27.118	1.356		
Total	29	60.967			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

D. Variate: LR number under 20% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	6.474	1.295	0.5	
Genotype	4	3.478	0.870	0.34	0.851
Error	20	51.833	2.592		
Total	29	61.785			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

E. Genotype x PEG treatment interaction on LR number

Source of variation	df	SS	MS	F	P
Replication	5	3.280	0.656	0.34	
Genotype (G)	4	105.600	26.400	13.78	<.001
Treatment (PEG 0, 10, 15%) (T)	2	42.268	21.134	11.03	<.001
GxT	8	3.750	0.469	0.24	0.981
Error	70	134.080	1.915		
Total	89	288.978			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

## Biological replicate 2

### A. Variate: LR number under 0% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	57.501	11.500	3.82	
Genotype	4	161.310	40.327	13.40	<.001
Error	20	60.168	3.008		
Total	29	278.979			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

### B. Variate: LR number under 10% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	121.519	24.304	5.24	5
Genotype	4	115.695	28.924	6.23	0.002
Error	20	92.800	4.640		
Total	29	330.014			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

C. Variate: LR number under 15% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	59.134	11.827	6.87	
Genotype	4	81.959	20.490	11.90	<.001
Error	20	34.432	1.722		
Total	29	175.525			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

D. Variate: LR number under 20% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	10.530	2.106	0.78	
Genotype	4	12.907	3.227	1.19	0.346
Error	20	54.274	2.714		
Total	29	77.711			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

E. Genotype x PEG treatment interaction on LR number

Source of variation	df	SS	MS	F	P
Replication	5	79.613	15.923	3.22	
Genotype (G)	4	339.021	84.755	17.15	<.001
Treatment (PEG 0, 10, 15%) (T)	2	498.225	249.113	50.41	<.001
GxT	8	19.944	2.493	0.50	0.849
Error	70	345.941	4.942	70	345.941
Total	89	1282.743			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

### Biological replicate 3

#### A. Variate: LR number under 0% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	106.357	21.271	7.97	
Genotype	4	84.917	21.229	7.96	<.001
Error	20	2.1705	0.1085		
Total	29	244.620			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

#### B. Variate: LR number under 10% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	174.905	34.981	18.61	
Genotype	4	40.205	10.051	5.35	0.004
Error	20	37.590	1.879		
Total	29	252.700			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

C. Variate: LR number under 15% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	73.0947	14.6189	19.37	
Genotype	4	30.4256	7.6064	10.08	<.001
Error	20	15.0954	0.7548		
Total	29	118.6157			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

D. Variate: LR number under 20% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	21.2475	4.2495	5.35	
Genotype	4	4.9398	1.2349	1.55	0.225
Error	20	15.8998	0.7950		
Total	29	42.0871			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

A. Genotype x PEG treatment interaction on LR number

Source of variation	df	SS	MS	F	P
Replication	5	53.510	10.702	1.84	
Genotype (G)	4	145.367	36.342	6.25	<.001
Treatment (PEG 0, 10, 15%) (T)	2	320.181	160.090	27.54	<.001
GxT	8	10.181	1.273	0.22	0.986
Error	70	406.877	5.813	70	406.877
Total	89	936.116	89		

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability



**Table A.3. Means and coefficients of variation for primary root length (cm) of Arabidopsis transgenic GmNAC003 (events N3.9.1 and N3.10.1), GmNAC004 (events N4.1.3 and N4.3.3), and the wild type (WT) lines in response to various treatments of PEG.**

**Biological replicate 1**

<b>Genotypes<sup>*</sup></b>	<b>0% PEG</b>	<b>10% PEG</b>	<b>15% PEG</b>	<b>20% PEG</b>
WT	5.82 <sup>a</sup>	6.69 <sup>a</sup>	6.00 <sup>a</sup>	6.25 <sup>a</sup>
N3.9.1	5.64 <sup>a</sup>	6.75 <sup>a</sup>	6.17 <sup>a</sup>	6.02 <sup>a</sup>
N3.10.1	5.87 <sup>a</sup>	6.84 <sup>a</sup>	6.48 <sup>a</sup>	6.20 <sup>a</sup>
N4.1.3	5.51 <sup>a</sup>	6.50 <sup>a</sup>	6.31 <sup>a</sup>	5.95 <sup>a</sup>
N4.3.3	5.82 <sup>a</sup>	6.76 <sup>a</sup>	6.34 <sup>a</sup>	6.09 <sup>a</sup>
Grand mean	5.74	67.10	6.27	6.10
CV%	9.6	4.5	9.9	8.1

**Biological replicate 2**

<b>Genotypes<sup>*</sup></b>	<b>0% PEG</b>	<b>10% PEG</b>	<b>15% PEG</b>	<b>20% PEG</b>
WT	4.89 <sup>a</sup>	5.47 <sup>a</sup>	4.81 <sup>a</sup>	4.14 <sup>a</sup>
N3.9.1	5.02 <sup>a</sup>	5.30 <sup>a</sup>	4.69 <sup>a</sup>	4.16 <sup>a</sup>
N3.10.1	4.91 <sup>a</sup>	5.41 <sup>a</sup>	4.84 <sup>a</sup>	3.96 <sup>a</sup>
N4.1.3	5.20 <sup>a</sup>	5.54 <sup>a</sup>	4.60 <sup>a</sup>	3.96 <sup>a</sup>
N4.3.3	5.19 <sup>a</sup>	5.59 <sup>a</sup>	4.66 <sup>a</sup>	3.92 <sup>a</sup>
Grand mean	5.04	5.46	4.71	4.03
CV%	4.3	4.5	5.0	3.6

**Biological replicate 3**

<b>Genotypes<sup>*</sup></b>	<b>0% PEG</b>	<b>10% PEG</b>	<b>15% PEG</b>	<b>20% PEG</b>
WT	4.94 <sup>a</sup>	5.17 <sup>a</sup>	5.21 <sup>a</sup>	4.69 <sup>a</sup>
N3.9.1	5.04 <sup>a</sup>	5.18 <sup>a</sup>	5.10 <sup>a</sup>	4.66 <sup>a</sup>
N3.10.1	5.11 <sup>a</sup>	5.06 <sup>a</sup>	4.98 <sup>a</sup>	4.86 <sup>a</sup>
N4.1.3	4.92 <sup>a</sup>	5.03 <sup>a</sup>	5.17 <sup>a</sup>	4.67 <sup>a</sup>
N4.3.3	5.09 <sup>a</sup>	4.97 <sup>a</sup>	5.12 <sup>a</sup>	4.62 <sup>a</sup>
Grand mean	5.01	5.07	5.12	4.70
CV%	7.3	9.2	3.5	6.8

\* Similar letters are not significantly different at the 0.05 level of probability according to Duncan Multiple Range Test.

**Table A.4. ANOVA tables of primary root length in response to PEG treatments**

**Biological replicate 1**

A. Variate: primary root length under 0% PEG

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	1053.25	210.65	6.9	
Genotype	4	56.16	14.04	0.46	0.764
Error	20	610.26	30.51		
<b>Total</b>	<b>29</b>	<b>1719.67</b>			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

B. Variate: primary root length under 10% PEG treatment

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	658.699	131.74	14.75	
Genotype	4	38.214	9.553	1.07	0.398
Error	20	178.684	8.934		
<b>Total</b>	<b>29</b>	<b>875.597</b>			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

C. Variate: primary root length under 15% PEG treatment

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	65.37	13.07	0.34	
Genotype	4	78.44	19.61	0.51	0.729
Error	20	769.9	38.5		
Total	29	913.71			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

D. Variate: primary root length under 20% PEG treatment

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	171.62	34.32	1.4	
Genotype	4	36.64	9.16	0.37	0.825
Error	20	491.55	24.58		
Total	29	699.81			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

## Biological replicate 2

### A. Variate: primary root length under 0% PEG

Source of variation	df	SS	MS	F	P
Replication	5	1.1809	0.2362	2.18	
Genotype	4	0.5317	0.1329	1.22	0.332
Error	20	2.1705	0.1085		
<b>Total</b>	<b>29</b>	<b>3.8831</b>			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

### B. Variate: primary root length under 10% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	1.4765	0.2953	2.12	
Genotype	4	0.2953	0.0738	0.53	0.716
Error	20	2.7895	0.1395		
<b>Total</b>	<b>29</b>	<b>4.5612</b>			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

C. Variate: primary root length under 15% PEG treatment

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	1.3831	0.2766	1.73	
Genotype	4	0.2853	0.0713	0.45	0.774
Error	20	3.1997	0.1600		
Total	29	4.8680			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

D. Variate: primary root length under 20% PEG treatment

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	0.5386	0.1077	0.96	
Genotype	4	0.3020	0.0755	0.67	0.620
Error	20	2.2494	0.1125		
Total	29	3.0900			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

### Biological replicate 3

#### A. Variate: primary root length under 0% PEG

Source of variation	df	SS	MS	F	P
Replication	5	3.31618	0.66324	21.37	
Genotype	4	0.18389	0.04597	1.48	0.245
Error	20	0.62081	0.03104		
<b>Total</b>	<b>29</b>	<b>4.12088</b>			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

#### B. Variate: primary root length under 10% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	5.4109	1.0822	9.99	
Genotype	4	0.2283	0.0571	0.53	0.717
Error	20	2.1654	0.1083		
<b>Total</b>	<b>29</b>	<b>7.8046</b>			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

C. Variate: primary root length under 15% PEG treatment

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	0.80817	0.16163	2.00	
Genotype	4	0.19362	0.04840	0.60	0.669
Error	20	1.61995	0.08100		
Total	29	2.62174			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

D. Variate: primary root length under 20% PEG treatment

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	2.53220	0.50644	7.38	
Genotype	4	0.20400	0.05100	0.74	0.574
Error	20	1.37245	0.06862		
Total	29	4.10864			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability



**Table A.5. Means and coefficients of variation for total lateral root length (cm) of Arabidopsis transgenic GmNAC003 (events N3.9.1 and N3.10.1), GmNAC004 (events N4.1.3 and N4.3.3), and the wild type (WT) lines in response to various treatments of PEG.**

**Biological replicate 1**

<b>Genotypes<sup>*</sup></b>	<b>0% PEG</b>	<b>10% PEG</b>	<b>15% PEG</b>	<b>20% PEG</b>
WT	4.9 <sup>a</sup>	4.8 <sup>a</sup>	3.6 <sup>a</sup>	2.3 <sup>a</sup>
N3.9.1	5.6 <sup>ab</sup>	5.4 <sup>a</sup>	3.6 <sup>ab</sup>	2.5 <sup>abc</sup>
N3.10.1	5.4 <sup>a</sup>	5.2 <sup>a</sup>	4.1 <sup>bc</sup>	2.2 <sup>ab</sup>
N4.1.3	7.3 <sup>bc</sup>	6.4 <sup>b</sup>	4.6 <sup>c</sup>	2.8 <sup>bc</sup>
N4.3.3	7.6 <sup>c</sup>	6.7 <sup>a</sup>	5.6 <sup>d</sup>	3.2 <sup>c</sup>
Grand mean	6.20	5.71	4.33	2.61
CV%	8.6	9.2	6.1	13.4

**Biological replicate 2**

<b>Genotypes<sup>*</sup></b>	<b>0% PEG</b>	<b>10% PEG</b>	<b>15% PEG</b>	<b>20% PEG</b>
WT	4.77 <sup>a</sup>	4.75 <sup>a</sup>	1.63 <sup>ab</sup>	0.60 <sup>abc</sup>
N3.9.1	4.58 <sup>a</sup>	3.50 <sup>a</sup>	1.82 <sup>b</sup>	0.46 <sup>ab</sup>
N3.10.1	4.10 <sup>a</sup>	3.99 <sup>a</sup>	1.07 <sup>a</sup>	0.35 <sup>a</sup>
N4.1.3	7.90 <sup>b</sup>	8.07 <sup>b</sup>	2.78 <sup>c</sup>	0.66 <sup>bc</sup>
N4.3.3	7.87 <sup>b</sup>	8.54 <sup>b</sup>	3.24 <sup>c</sup>	0.86 <sup>c</sup>
Grand mean	5.85	5.77	2.10	0.58
CV%	5.8	19.0	22.9	27.8

**Biological replicate 3**

<b>Genotypes*</b>	<b>0% PEG</b>	<b>10% PEG</b>	<b>15% PEG</b>	<b>20% PEG</b>
WT	2.89 <sup>a</sup>	2.52 <sup>a</sup>	1.59 <sup>a</sup>	0.54 <sup>a</sup>
N3.9.1	4.01 <sup>a</sup>	2.68 <sup>a</sup>	1.84 <sup>a</sup>	0.47 <sup>a</sup>
N3.10.1	4.20 <sup>a</sup>	2.03 <sup>a</sup>	1.74 <sup>a</sup>	0.46 <sup>a</sup>
N4.1.3	6.17 <sup>b</sup>	4.78 <sup>b</sup>	3.36 <sup>b</sup>	0.97 <sup>b</sup>
N4.3.3	6.12 <sup>b</sup>	4.66 <sup>b</sup>	3.76 <sup>b</sup>	0.74 <sup>ab</sup>
Grand mean	4.68	3.30	2.46	0.63
CV%	17.2	18.8	16.0	23.4

\* Similar letters are not significantly different at the 0.05 level of probability according to Duncan Multiple Range Test.

**Table A.6. ANOVA tables of total lateral root length in response to PEG treatments**

**Biological replicate 1**

A. Variate: total lateral root length under 0% PEG

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	7.124	1.425	0.65	
Genotype	4	35.861	8.965	4.12	0.014
Error	20	43.508	2.175		
<b>Total</b>	<b>29</b>	<b>86.492</b>			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

B. Variate: total lateral root length under 10% PEG treatment

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Replication	5	6.9333	1.3867	2.23	
Genotype	4	15.7774	3.9444	6.35	0.002
Error	20	12.4202	0.6210		
<b>Total</b>	<b>29</b>	<b>35.1309</b>			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

C. Variate: total lateral root length under 15% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	1.7595	0.3519	0.86	
Genotype	4	19.1643	4.7911	11.64	<.001
Error	20	8.2288	0.4114		
Total	29	29.1526			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

D. Variate: total lateral root length under 20% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	3.0498	0.6100	1.96	
Genotype	4	3.4553	0.8638	2.78	0.055
Error	20	6.2223	0.3111		
Total	29	12.7273			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

E. Genotype x PEG treatment interaction on total LR length

Source of variation	df	SS	MS	F	P
Replication	5	30.049	6.010	3.57	
Genotype (G)	4	64.072	16.018	9.52	<.001
Treatment (PEG 0, 10, 15%) (T)	2	55.636	27.818	16.54	<.001
GxT	8	4.832	0.604	0.36	0.938
Error	70	117.741	1.682	70	117.741
Total	89	272.330			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

## Biological replicate 2

A. Variate: total lateral root length under 0% PEG

Source of variation	df	SS	MS	F	P
Replication	5	2.879	0.576	0.48	
Genotype	4	84.917	21.229	17.84	<.001
Error	20	23.804	1.190		
Total	29	111.599			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

B. Variate: primary root length under 10% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	30.150	6.030	2.38	
Genotype	4	133.930	33.483	13.20	<.001
Error	20	50.732	2.537		
<b>Total</b>	29	214.813			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

C. Variate: total lateral root length under 15% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	5.8370	1.1674	4.57	
Genotype	4	18.6976	4.6744	18.28	<.001
Error	20	5.1139	0.2557		
Total	29	29.6485			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

D. Variate: total lateral root length under 20% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	0.66125	0.13225	2.74	
Genotype	4	0.90829	0.22707	4.70	0.008
Error	20	0.96663	0.04833		
Total	29	2.53617			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

E. Genotype x PEG treatment interaction on total LR length

Source of variation	df	SS	MS	F	P
Replication	5	21.018	4.204	3.02	
Genotype (G)	4	205.086	51.271	36.81	<.001
Treatment (PEG 0, 10, 15%) (T)	2	273.689	136.845	98.25	<.001
GxT	8	32.459	4.057	2.91	0.007
Error	70	97.498	1.393	70	97.498
Total	89	629.750			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

### Biological replicate 3

A. Variate: total lateral root length under 0% PEG

Source of variation	df	SS	MS	F	P
Replication	5	16.239	3.248	2.08	
Genotype	4	48.991	12.248	7.85	<.001
Error	20	31.207	1.560		
Total	29	96.438			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

B. Variate: total lateral root length under 10% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	9.7907	1.9581	2.95	
Genotype	4	39.8785	9.9696	15.03	<.001
Error	20	13.2630	0.6632		
<b>Total</b>	29	62.9322			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

C. Variate: total lateral root length under 15% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	3.8764	0.7753	0.79	
Genotype	4	24.9771	6.2443	6.40	0.002
Error	20	19.5082	0.9754		
Total	29	48.3618			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

D. Variate: total lateral root length under 20% PEG treatment

Source of variation	df	SS	MS	F	P
Replication	5	0.57485	0.11497	1.95	
Genotype	4	1.13663	0.28416	4.81	0.007
Error	20	1.18122	0.05906		
Total	29	2.89270			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

E. Genotype x PEG treatment interaction on total LR length

Source of variation	df	SS	MS	F	P
Replication	5	10.687	2.137	2.0	
Genotype (G)	4	97.848	24.462	22.95	<.001
Treatment (PEG 0, 10, 15%) (T)	2	85.340	42.670	40.03	<.001
GxT	8	3.464	0.433	0.41	0.913
Error	69	73.556	1.066		
Total	88	270.520			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability



**Table A.7. Means and coefficients of variation for germination rate of Arabidopsis transgenic GmNAC003 (N3.10.1), GmNAC004 (N4.3.3), and the wild type (WT) lines in response to 1  $\mu$ M ABA treatment.**

<b>Genotype</b>	<b>Mean (%)</b>	<b>Duncan test*</b>
GmNAC003	57.82	a
Wild-type	86.38	b
GmNAC004	91.62	c
Grand mean	78.61	
CV%	5.7	

\* Similar letters are not significantly different at the 0.05 level of probability according to Duncan Multiple Range Test

**Table A.8. ANOVA tables of germination rate of transgenic GmNAC003, GmNAC004 and wild type lines in response to 1  $\mu$ M ABA treatment**

<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>
Genotype	2	5292.23	2646.11	132.19	<.001
Residual	21	420.36	20.02		
Total	23	5712.59			

df: Degree of Freedom, SS: Sum of Squares, MS: Mean Squares, F: variance ratio, P: false-rejection probability

## **VITA**

Truyen Ngoc Quach was born on October 15 th, 1974 in Vietnam. He received his B.S. degree from Hanoi Agriculture University, Vietnam in 1997 in agronomy. He got his M.Sc degree in plant science from the University of Adelaide, Australia in 2004. He is currently finishing his Ph.D. degree in plant science at the University of Missouri-Columbia.