REGULATION OF *ARABIDOPSIS* FLORAL DEVELOPMENT AND
TRANSCRIPTOME DURING DROUGHT STRESS BY NAC TRANSCRIPTION
FACTORS

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ABSTRACT

As sessile organisms, plants are exposed to adverse environmental factors that negatively affect their growth and development. Many plants are particularly sensitive and susceptible to drought conditions at their reproductive stage. There are a number of studies about drought effects on crop reproductive development, but the molecular mechanism underlying drought response during reproduction is still unclear. A previous study on the inflorescence of wild-type (WT) *Arabidopsis thaliana* (hereafter, *Arabidopsis*) found that many genes are induced by drought, including *NAC019*, which encodes a putative transcription factor that belongs to the NAC family. However, the function of *NAC019* in drought response during floral development is not clear. This study aimed to investigate *NAC019* function in the floral response to drought conditions, with a focus on its role in the stress regulatory network.

To achieve this objective, we subjected the *nac019* mutant to drought and characterized its phenotype. The stamens and pistils of *nac019*-1 were smaller than WT, but their length ratio was not significantly different. Despite the smaller size, the pollen produced under drought conditions in the mutant was viable and able to generate a pollen tube, indicating that pollen fertility in *nac019*-1 was not severely affected by drought. Moreover, when we examined the emergence of new open flowers from a newly recovered inflorescence, we found that, after drought-induced arrest of floral development, *nac019*-1 took several days longer than WT to produce a new open flower. This indicates that recovery of *nac019*-1 inflorescence from drought was delayed compared to WT. Additionally, *nac019*-1 could still produce siliques with normal number of seeds, but the silique number was reduced relative to WT. It also contained more undeveloped siliques than WT. These results suggest that *nac019*-1 floral meristem development was severely affected by drought, but not its floral morphology.
The observation that the \textit{nac019-1} phenotype was relatively weak suggested that \textit{NAC019} and its related genes might share redundant functions. Therefore, a triple mutant with additional mutations from two closely related genes was generated and subjected to drought. Our preliminary data indicated that the vegetative tissue of the triple mutant had a more severe phenotype than \textit{nac019-1}. Throughout drought stress, relative water content of this triple mutant was lower, and recovered more slowly than the single mutant.

To understand the regulatory role \textit{NAC019} plays, microarray analysis was performed on the inflorescence of \textit{nac019} during early drought treatment. In \textit{nac019-1}, fewer genes were induced by drought, indicating that the mutant might have a slower response to drought at the onset of this stress. We compared this transcriptomic profile to WT to identify putative \textit{NAC019} target genes. We found that many genes were associated with stress and hormone response as well as floral development. Some of the genes are \textit{DREB2B}, \textit{ARF2}, \textit{MYB21} and \textit{MYB24}. We also examined the expression of a few genes over a time course of drought stress. Interestingly, \textit{NAC019} was highly expressed throughout drought stress, indicating that it is essential for the inflorescence response to drought.

Based on our results, we propose a model that includes \textit{NAC019} function in a regulatory network for floral development under drought conditions. Future studies are required to test the hypotheses in this model, including the interaction between \textit{NAC019} and its target genes. Towards this goal, a GR-inducible system has been generated for \textit{NAC019}, providing a functional assay for \textit{NAC019} function in regulating the drought-responsive floral transcriptome.
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Chapter 1

INTRODUCTION AND LITERATURE REVIEW
1. ARABIDOPSIS FLORAL DEVELOPMENT

Flower formation and development

Most flowers are composed of four types of organs, namely sepals, petals, stamens and carpels. In *Arabidopsis thaliana* (hereafter *Arabidopsis*) and a number of other plants, sepals and petals are the sterile organs and they arise in the first and second whorl, respectively. The reproductive organs that develop to form male and female gametophytes arise in the two inner whorls. The third whorl contains stamens, which are responsible for producing pollen, and the carpels, which produce ovules, are in the fourth whorl. Flower formation involves the induction of floral meristem fate, floral organ patterning and cell differentiation, all of which are under strict genetic regulation (Krizek and Fletcher, 2005).

The transition from the vegetative stage to the reproductive stage requires signals from both developmental regulation and external environments (Koornneef et al., 1998). The transition of the inflorescence meristem into the floral meristem is the first step in floral development. *LEAFY (LFY), APETALA1 (AP1)* and *CAULIFLOWER (CAL)* are floral meristem identity genes (Weigel et al., 1992; Bowman et al., 1993). They ensure that primordia initiated along the periphery of an inflorescence meristem adopt a flower fate (Krizek and Fletcher, 2005). Both *AP1* and *LFY* encode transcription factors, and the expression of *AP1* is activated by *LFY* (Liljegren et al., 1999; Wagner et al., 1999; William et al., 2004). Their functions are antagonistic to the inflorescence meristem genes, *TERMINAL FLOWER 1 (TFL1)* and *AG-LIKE 24 (AGL24)*. In the inflorescence meristem, TFL1 and AGL24 repress the expression of *AP1* and *LFY*, whereas in the floral meristem, their expression is repressed by *AP1* and *LFY* (Ratcliffe et al., 1999; Yu et al., 2004). This indicates that positive and negative feedback interactions occur among genes.
regulating inflorescence- and floral-meristem identity during the transition from the inflorescence to the flower (Liljegren et al., 1999).

**Stages of floral development in Arabidopsis**

Regulation of cell division patterning during the early and late stages of floral development is controlled by different mechanisms. In the early floral stages, cell division patterning is dependent on the radial position of a cell within the floral meristem but not on future organ identity. Later, during organogenesis, cell division orientation is controlled by the ABC floral homeotic genes (Jenik and Irish, 2000). The developmental stages of Arabidopsis flowers have been described previously (Smyth et al., 1990) and this description is beneficial as a reference and also in interpreting the mechanism of genes controlling floral development. Based on the landmarks of different developing organs, floral development in Arabidopsis is divided into 20 stages (Fig. 1-1). The first 12 stages are defined from floral initiation until the bud opening (anthesis), and the remaining stages are defined from anthesis until silique dehiscence (Smyth et al., 1990; Alvarez-Buylla E.R., 2010).

For a description of the floral stages, please see Fig. 1-1. Stage 1 begins with the initiation of a floral buttress on the flank of the apical meristem (Smyth et al., 1990). The bract primordium is formed before the flower primordium, and organ boundaries can be observed at this stage. During stage 2, the flower primordium boundary becomes distinct from the inflorescence meristem and quickly grows larger in all directions (Kwiatkowska, 2006; Alvarez-Buylla E.R., 2010). The beginning of stage 3 is marked by the visibility of sepal primordia, which later overlay the dome-shaped portion of the flower primordium (stage 4). Then, the petal and stamen primordia become apparent, indicating the commencement of stage 5 (Alvarez-Buylla E.R., 2010)
During stage 6, sepals enclose the floral bud, the petal primordia grow slowly, and the stamen primordia enlarge very rapidly. In addition, a rim around the central dome of the flower primordium begins to grow upward to produce an oval tube that will become the gynoecium. Stage 7 commences as the long stamens become stalked at their base, which later give rise to the filaments and the wider upper region to the anthers. At stage 8, the anther locules become visible in the long and short stamens, and the petal primordia become apparent. Then, petal primordia and other organs elongate rapidly in stage 9. Both long and short stamens grow rapidly, but for the short stamens, most of the growth occurs in the anther region. In the same stage, nectary glands are emerged. When the petals reach the top of the short stamens, this stage is classified as stage 10. At stage 11, the stigmatic papillae are developed and become visible, and the petal primordia reach the top of the long stamens. At stage 12, the organs continue to lengthen. The petals and the filaments elongate rapidly, and the lateral sepals continue to grow while the stamens and gynoecium elongate coordinately. Furthermore, the upper part of the gynoecium differentiates into the style, and a boundary exists between the style and the cap of the stigmatic papillae. This stage ends when the sepals open (Smyth et al., 1990; Alvarez-Buylla E.R., 2010).

At stage 13, the petals become obvious and continue to elongate rapidly. The stigma is receptive at this stage and anthesis occurs. Furthermore, the filaments extend very fast so that the stamens can outstrip the gynoecium in length, and pollination can occur. At this stage, the gynoecium is fully mature, and three distinct regions (an apical stigma, a style and a basal ovary) can be distinguished. After pollination, the pollen tubes grow to fertilize the ovules, the stamens extend above the stigma and furrows at both valve/replum boundaries appear. Stage 14 is marked by the beginning of silique (fruit) and seed development. At this stage, cell division and expansion occur in the exocarp and mesocarp, and the chloroplast is also developed. At stage 15, the stigma extends above the long anthers and later on (stage 16), the siliques elongate, and the petals and sepals wither (Alvarez-Buylla E.R., 2010).
The floral organs senesce at stage 17, and the green silique widens and elongates to its final length. At this stage, a dehiscence zone is formed to prepare the fruit to open at maturity, and the thickening of cell walls of the valve margin lignified layer occurs. The silique begins to yellow from the top to the base at stage 18 and lignification occurs at the cell layer, which later contributes to the silique shattering process. When the silique dries (stage 19), the valves begin to separate due to a lack of cell cohesion at the separation layer. At stage 20, the valves separate from the dry silique and the mature seeds are released (Alvarez-Buylla E.R., 2010).

Figure 1-1. Stages of floral development in Arabidopsis
Schematic representation of the 20 stages of floral development and the duration for each stage in hours are shown. [Image is taken with permission from Alvarez-Buylla et al. (Alvarez-Buylla E.R., 2010)].
Molecular genetics of floral organ specification

Floral morphogenesis is regulated through several key factors. The ABC model of floral organ patterning was proposed in 1991 (Coen and Meyerowitz, 1991), and this model emphasizes the function of three classes (A, B and C) of homeotic genes that specify floral organs (Fig. 1-2). APETALA (AP) genes, PISTILLATA (PI) and AGAMOUS (AG) are the key regulators that define the identities of floral organs (Bowman et al., 1991). AP1 and AP2 belong to class A function, AP3 and PI are the class B genes, and the only C-function gene is AG (Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). These genes work in concert to regulate floral organ identity (Bowman et al., 1991). Class A genes specify sepal identity and together with class B, they specify petal identity. The formation of reproductive organs is regulated by class B and C genes. Stamen identity is conferred by the combination of class B and C genes, whereas carpel identity is specified by class C genes alone (Bowman et al., 1991; Coen and Meyerowitz, 1991). The functions of class A and C are mutually antagonistic (Drews et al., 1991). This is demonstrated by the formation of reproductive organs in the ap1 and ap2 mutant (Bowman et al., 1991; Gustafson-Brown et al., 1994; Jofuku et al., 1994), whereas in the ag mutant, perianth organs (sepals and petals) can be seen (Bowman et al., 1991).

Four SEPALLATA genes (SEP1, SEP2, SEP3 and SEP4) have been identified and are classified as E-function genes (Pelaz et al., 2000; Ditta et al., 2004). The ABC model is hence extended to the ABCE model. Instead of producing other types of floral organs, the sep1 sep2 sep3 triple mutant exhibited the formation of sepals only, suggesting that the SEP1/2/3 genes have overlapping functions required for petal, stamen and carpel development (Pelaz et al., 2000). Furthermore, protein-protein interaction studies revealed that the SEP class of protein interacts with the products of class B- and C-function genes. SEP3 not only interacts with class B proteins (AP3 and PI) but also mediates the interaction between AP3 and PI with AG (Honma and Goto,
Therefore, SEP proteins are required to form a higher-order complex with AP3, PI and AG as a tetramer to specify different floral organ identity (Honma and Goto, 2001; Melzer and Theißen, 2009). In addition, ectopic expression of SEP genes in transgenic plants overexpressing floral organ identity genes (API, AP3, PI and AG) confirmed that their combination is adequate to confer floral organ identity (Honma and Goto, 2001; Pelaz et al., 2001).

Recently, the (A)BC model that integrates a newly defined “(A)” function into the B- and C-functions has been proposed (Fig. 1-2) (Causier et al., 2010). The authors tried to clarify the controversial roles of class A function in defining perianth organ identity and the spatial regulation of C-function genes. In the previous ABC model (Coen and Meyerowitz, 1991), class A genes function in defining the first and second whorl identities (Bowman et al., 1991; Coen and Meyerowitz, 1991). Nonetheless, the formation of petals is still observed in plants harboring an ap1 mutation in combination with some other mutations, such as ag (Bowman et al., 1993) and agl24 (Yu et al., 2004). Moreover, development of bracts and the formation of new flowers in the outer whorls of ap1 and ap2 mutant flowers cannot be explained by the ABC model (Bowman et al., 1993; Amy Litt, 2007; Causier et al., 2010). According to the ABC model, class A- and C-functions are mutually antagonistic (Coen and Meyerowitz, 1991), and the absence of A-function should result in the formation of carpels in the outer whorl (Bowman et al., 1991; Gustafson-Brown et al., 1994). So far, only the ap2 mutant exhibits this phenotype, verifying that AP2 restricts C-function to the first and second whorls. However, this phenotype is only observed in Arabidopsis, suggesting that the A-function is not universal for the flowering model (Litt, 2007).

In the new (A)BC model, the (A)-function genes establish the floral meristem identity and thus, are expressed before the B- and C-functions genes. The SEP genes are one of the components in (A)-function genes, meaning that they are required for the establishment of floral context. Once the floral context is established, the (A)-function genes activate the floral organ
identity genes (class B and C). In short, the proposed (A)BC model emphasizes the multifaceted roles of (A)-function, which are necessary to enable the B- and C-function proteins to regulate floral organ specification (Causier et al., 2010).

Figure 1-2. Proposed models for *Arabidopsis* floral organ patterning
Models of floral organ patterning are shown above. I. The ABC model (Coen and Meyerowitz, 1991), II. The ABCE model (Pelaz et al., 2000) and III. The (A)BC model (Causier et al., 2010). Image adapted from provided citation.
2. PLANT RESPONSE TO ABIOTIC STRESS

Strategies for drought stress resistance in plants

Plant growth and development are affected by various environmental factors, such as drought, flood, high temperature, high salinity and soil nutrient deficiency. Among them, drought is the most common and wide-spread stress throughout the world. Plant response to drought stress is complex because it depends on the species, genotype, developmental stage, organ and cell type of the plants, as well as the duration and severity of the stress (Bray, 1997; Lafitte et al., 2007; Kamoshita et al., 2008; Skirycz and Inzé, 2010; Cramer et al., 2011). However, as a plant copes with stress conditions, several mechanisms are adopted to ensure its survival and reproduction. Plant resistance to drought conditions can be divided into escape, avoidance and tolerance strategies (Levitt, 1980).

Drought escape refers to the completion of the plant life cycle before water deficit occurs, and this strategy relies on successful reproduction before severe drought sets in (Harb et al., 2010). A plant ‘escapes’ drought by flowering earlier so that its photosynthetic product can be partitioned and mobilized to developing fruits and other organs (Meyre et al., 2001; Chaves et al., 2003). In wheat and rice, plants exhibit increased mobilization of nutrient reserves, where accelerated grain filling rate under water stress conditions has been observed (Yang et al., 2001; Yang et al., 2003; Guan et al., 2010).

Drought avoidance is another strategy to minimize water loss and maximize water uptake (Chaves et al., 2003). In the avoidance strategy, several adaptive traits such as decreased stomatal aperture (Cornic, 2000; Flexas and Medrano, 2002) and reduced radiation absorption (Ehleringer and Cooper, 1992) are employed in order to minimize water loss. Stomatal closure and inhibition of leaf growth protect plants from extensive water loss, which results in cell dehydration,
runaway xylem cavitation and death (Chaves et al., 2003). In addition, an enhanced root system such as deep, thick and highly branched roots can penetrate and maximize water uptake from the soil, by gaining access to water deep in the soil (Price et al., 2002; Gowda et al., 2011).

When drought conditions are prolonged and become severe, a plant adjusts itself so that it can tolerate low water availability. During this period, the plant is in dormant state and metabolic activities are reduced or minimized. This mechanism is known as dehydration tolerance, wherein the plant functions under sub-optimal water availability through adaptation or acclimation that permits metabolism to occur at low water potential (Wood, 2005). Plants that endure acclimation under drought stress maintain vegetative and reproductive growth over an extended period. At the cellular level, protection from the damaging effects of dehydration is mainly provided by dehydrins, late-embryogenesis-abundant (LEA) proteins and compatible solutes. These components have been regarded as cellular stabilizers that function in protecting proteins and membrane structure under dehydration (Ingram and Bartels, 1996). Moreover, ROS is an important signal transduction mediator involved in plant dehydration tolerance. ROS production is enhanced as a result of stomatal closure, and its elevated levels could trigger defense systems and subsequent acclimatory responses (Cruz de Carvalho, 2008).

**Acute response and adaptation to stress**

Plant responses to stress can be categorized as acute and adaptive. Plants first undergo a rapid and acute inhibition of growth as preparation for more severe conditions, followed by recovery and adaptation as the establishment of a new steady state to prolonged stress conditions (Skirycz and Inzé, 2010).

Salt stress imposed on barley causes an immediate reduction in leaf elongation rate (Fricke et al., 2006). Similarly, exposure to osmotic stress results in a rapid decrease in cell
division rates and followed by increasing division rates. In *Arabidopsis*, cell number is significantly reduced after 24 hours of osmotic stress, but within 72 hours, cell proliferation rates between stressed and control plants are identical, indicating that those leaves have adapted to the restrictive environment (Skirycz et al., 2011). Besides leaves, root growth also undergoes acute and adaptive growth responses under salt stress (West et al., 2004). All these findings suggest that the regulation of cell cycle under stress conditions is essential in modulation of plant growth. Acute and adaptation responses are also observed during *Arabidopsis* floral development under drought conditions. Our previous work has shown that floral organ growth is arrested after exposure to drought but resumes after several days of treatment (Su et al., submitted). This pattern is also correlated with the number of seeds produced in the silique, which gradually decreases during early periods of drought, followed by recovery of seed production as drought progresses (Su et al., submitted).

Since plant water status is maintained through water uptake from roots, the role of aquaporin is very important during drought. Under water-restricted conditions, changes in aquaporin expression in the root negatively affect hydraulic conductivity and cell turgor. This promotes short-term growth inhibition (Ehlert et al., 2009). However, during the recovery phase, both expression and protein levels of aquaporin are positively modulated by abscisic acid (ABA). The elevated level of aquaporins contributes to the recoveries of leaf water potential, cell elongation and the maintenance of a favorable water status (Parent et al., 2009).

Both transcriptomic and proteomic approaches could provide a broad picture of the dynamic molecular events during early onset of stress and the recovery. Global expression profiling on *Arabidopsis* treated with different stresses (salt, osmotic and cold) identified 118 differentially expressed genes during the acute phase of each treatment. These genes are either up- or down-regulated by all three stresses, and according to gene annotation, they are mainly involved in regulation of gene expression and phosphorylation (Kreps et al., 2002). This finding
supports the involvement of regulatory proteins such as transcription factors, phosphatases and protein kinases, which are rapidly and transiently induced during acute response to stress.

Phosphoproteome analysis performed on the growing zone of maize leaves under water stress revealed the co-variation between protein phosphorylation events and plant water status. The alteration of phosphorylation sites upon early changes of plant water status mainly occurred to the proteins that are involved in epigenetic and transcriptional regulation, cell cycle-related changes, hormone-mediated responses and carbohydrate metabolism adjustment (Bonhomme et al., 2012). Upon rehydration, recovery of phosphorylation status was observed in the proteins involved in the ABA-, ethylene-, auxin- or jasmonate-related responses (Kline et al., 2010; Skirycz et al., 2011; Bonhomme et al., 2012), protein kinases and phosphatases (Bonhomme et al., 2012). This suggests that phosphorylation modulates hormone-mediated response and cell signaling events during plant adaptive growth.

In conclusion, the information obtained from transcriptomics and proteomics approaches allows for a better understanding of plant response to stress conditions. However, knowledge of molecular mechanisms underlying plant acute and adaptive responses under stress conditions is still fragmentary. Moreover, interaction and crosstalk among molecular pathways increase the complexity of plant molecular responses to abiotic stress. Although analyses of time series reveal multiple phases of plant response to harsh environments, more analyses using different approaches are required to obtain a clearer picture of growth plasticity under stress conditions.

Effects of abiotic stress on plant reproductive development

Plants have evolved a plethora of strategies to sense and adapt their physiology and growth to a range of environmental changes. However, when the environmental changes exceed a certain threshold, plant growth may be adversely affected to various degrees. At the reproductive
stage, many crops are vulnerable to harsh environmental conditions. In rice, drought imposed during vegetative stages has mild effects on subsequent development and grain yield. However, when drought occurs during panicle development and grain filling, it severely affects seed production (Boonjung and Fukai, 1996). In addition, high temperature or drought during floral initiation or inflorescence development causes delay or complete inhibition of flowering, which eventually reduces plant yield (Wopereis et al., 1996; Winkel et al., 1997).

Depending on the species, type of abiotic stress or developmental stage affected, stress can have differential effects on plant reproduction (Barnabás et al., 2007; Lafitte et al., 2007; Kamoshita et al., 2008). For example, the cereals wheat (*Triticum aestivum*) and maize (*Zea mays*) respond differently to heat and drought stress. In maize, female organ development is delayed under drought but the male inflorescence is less affected (Herrero and Johnson, 1981). As photosynthesis is inhibited during drought, excessive starch reserves contained in the maternal tissues are not beneficial to maintain ovary growth, and thus cause abortion in female florets (Zinselmeier et al., 1995; Boyer and Westgate, 2004). However, the effect of heat stress on wheat female gametophyte is less prominent when compared to the male gametophyte. Some ovaries show abnormal development, where the embryo sac is absent or smaller, but in most cases, pollen tube growth is inhibited in the style and ovule (Saini et al., 1983). Furthermore, male sterility is observed in wheat subjected to high temperatures, where some anthers are small and fail to dehisce normally. Furthermore, the pollen are shriveled and have abnormal cytoplasm, showing that the male organ is more sensitive to heat stress (Saini and Aspinall, 1982).

In maize, the elongation rate of styles (silks) is reduced when suffering from drought (Westgate and Boyer, 1985). When drought occurred during megasporogenesis, the embryo sac develops abnormally (Moss and Downey, 1971). Likewise, salt stress causes ovule abortion in *Arabidopsis* as a result of excessive ROS (Sun et al., 2004; Sun et al., 2005). Ovary growth is maintained by carbohydrate status. Therefore, ovary growth inhibition is associated with a
decrease in glucose, depletion of starch, increase in sucrose concentration and inhibition of acid invertase activity (Zinselmeier et al., 1995). Invertase hydrolyzes sucrose to glucose and fructose. The elevated concentration from these sugars can increase the osmotic pressure within cells (Gibeaut et al., 1990) and hence, maintains reproductive sink strength (Zinselmeier et al., 1995). All of these observations demonstrate that drought stress can disrupt carbohydrate metabolism in maize ovaries. Depletion of starch implies that the embryo could not utilize this source of carbon, whereas lack of invertase activity in the ovary may disrupt its sink strength, and thus, cause abortion of the female gametophyte (Zinselmeier et al., 1995).

Pollen sterility caused by drought stress occurs as a result of abnormal microsporogenesis and microgametogenesis (Saini, 1997). Studies in wheat have shown that meiosis is completed in the microspore mother cells, but microspore development is arrested at various stages. The dislocation of microspores from their normal peripheral position can happen between the young microspore stage and the first pollen grain mitosis. Tapetal cells play an important role in providing nutrition for the male reproductive cells. Therefore, tapetum abnormal vacuolization could also lead to microspore disorientation (Lalonde et al., 1997; Saini, 1997). The disoriented pollen grains usually have dilute cytoplasm, little or no intine but normal exine, and they fail to accumulate starch (Sheoran and Saini, 1996; Lalonde et al., 1997). Without starch, the pollen tube could not reach the ovule because it serves as fuel for pollen tube growth in the female floret (Boyer and McLaughlin, 2007). Similar to the female organ under stress conditions, reduced invertase activity is also observed in the stressed anthers (Lalonde et al., 1997; Oliver et al., 2005). It is possible that altered invertase activity is involved in the failure of pollen development under stress (Saini, 1997). In addition to pollen dysfunction, filament elongation is also affected by stress. Stamen elongation rate is delayed under salt stress but still manage to pollinate the stigma (Sun et al., 2004). During drought stress, the under-developed stamens could not reach the
stigma, causing failure of pollen delivery to the top of the pistil. Consequently, fertilization in the female gametophyte is affected (Su et al., submitted).

Apart from altered carbohydrate metabolism that causes reproductive structural defects, hormones are also involved in the failure of the development of the male and female gametophytes. They act as chemical signals that control reproductive development under stress conditions. ABA is produced by vegetative tissues as part of the acclimation strategy under stress conditions. In crops such as wheat, barley and rice, ABA has been implicated as a cause of pollen sterility. It is also responsible for the changes to sugar metabolism in the maize female inflorescence (Boyer and Westgate, 2004).

It has been suggested that ABA accumulation in the reproductive organ originates from maternal tissues (Asch et al., 2001) or is translocated from other parts of the plants such as the leaf (Oliver et al., 2007). It affects the early reproductive development of crops in three ways (reviewed by (Liu et al., 2005)). First, ABA may inhibit cell division in the developing embryo or endosperm, resulting in a weak sink for assimilates and cause abortion of young ovaries (Setter and Flannigan, 2001). Second, ABA induces stomatal closure and indirectly, inhibits photosynthesis. This can reduce the rate of sugar supply to the sink organs (Liu et al., 2004). Finally, ABA may disrupt carbohydrate metabolism within the ovaries by affecting the activity of carbohydrate-catalyzing enzymes such as acid invertase (Trouverie et al., 2003). Hence, ABA affects early reproductive development by influencing both sink strength and assimilate partitioning from the source to sink tissues (Liu et al., 2005; Thakur et al., 2010).

Besides ABA, the increased level of methyl jasmonate (MeJA) due to drought stress also influences total yield production. Exogenous application of MeJA on morning glory (Pharbitis nil) (Maciejewska and Kopcewicz, 2002) and flowering tobacco (Nicotiana sylvestris) (Baldwin and Hamilton, 2000) results in reduced flower number. A similar observation was seen in transgenic Arabidopsis that overexpress JASMONIC ACID CARBOXYL
METHYLTRANSFERASE (JMT) (Cipollini, 2007). Furthermore, in rice, accumulation of MeJA causes reduced numbers of spikelets, low filling rate and alterations of floral organ numbers (Kim et al., 2009b). It was postulated that MeJA produced upon exposure to drought stress stimulates the production of ABA and together, leads to the loss of grain yield (Kim et al., 2009b). Positive feedback may exist between MeJA and ABA biosynthesis during drought stress, but further analysis is required to confirm this hypothesis (Kim et al., 2009a).

3. TRANSCRIPTIONAL REGULATION OF DROUGHT STRESS RESPONSE

Systems biology and omics approaches have been utilized to dissect some of the key regulatory pathways in plant responses to abiotic stress (Cramer et al., 2011). In general, plants respond to stresses by modulating gene expression, which eventually leads to the restoration of cellular homeostasis, detoxification and growth recovery (Xiong and Zhu, 2002). Genome-wide expression profiling analyses have identified hundreds of genes that are up- and down-regulated during stress conditions. This indicates that the expression of multiple genes was affected by stress and their induction or suppression is important in plant acclimation strategies.

Dehydration stress triggers the production of ABA, which subsequently induces the expression of various stress-responsive genes. Some genes respond very rapidly whereas others are induced slowly after the accumulation of ABA (Shinozaki and Yamaguchi-Shinozaki, 1997). Higher endogenous ABA levels in plants trigger expression of downstream target genes such as transcription factors, detoxification enzymes, metabolic enzymes and others (Shinozaki and Yamaguchi-Shinozaki, 2007). However, not all stress related genes are responsive to exogenous ABA application, suggesting that both ABA-dependent and ABA-independent signaling pathways are involved in stress response (Shinozaki and Yamaguchi-Shinozaki, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997).
Transcriptional regulation of stress via the ABA-dependent signaling pathway

ABA is a phytohormone that plays a pivotal role in plant growth and response to abiotic stresses. It is not only involved in seed germination and developmental process, but also in the regulation of plant water status. It acts specifically in the guard cells to control stomatal aperture, regulating the adjustment towards severe water shortage (Christmann et al., 2006). Under osmotic stress, it regulates the induction of many stress-inducible genes such as RESPONSIVE TO DEHYDRATIONs (RD29A, RD22 and RD20) (Yamaguchi-Shinozaki and Shinozaki, 1993a; Yamaguchi-Shinozaki and Shinozaki, 1993b; Aubert et al., 2010), EARLY RESPONSIVE TO DEHYDRATIONs (ERD5 and ERD10) (Seki et al., 2002a), genes encoding LEA proteins, heat shock proteins, galactinol synthases, glutathione transferase and others (Seki et al., 2002a). Many of these genes are involved in protecting macromolecules and membranes, protecting cells from reactive molecules and dehydration, repairing stress-induced damage and adjusting osmotic pressure (Seki et al., 2002a; Shinozaki and Yamaguchi-Shinozaki, 2006). This indicates that higher endogenous ABA levels are essential for acquiring stress tolerance. Therefore, the activation of ABA biosynthesis is an important component of the ABA-dependent signaling pathway.

The essential components of ABA signaling in plants have been identified, and its signaling module consists of three protein classes: Pyrabactin Resistance/Pyrabactin Resistance-Like/Regulatory Component of ABA Receptors (PYR/PYL/RCARs), Protein Phosphatases 2Cs (PP2Cs) and SNF1-Related Protein Kinase 2s (SnRK2s) (Hubbard et al., 2010). In this PYR/PYL/RCARs-PP2Cs-SnRKs model of ABA signaling transduction, PYR/PYL/RCARs act as ABA receptors, the PP2Cs act as negative regulators of the pathway, and SnRKs act as positive regulators of downstream signaling (Ma et al., 2009; Park et al., 2009; Hubbard et al., 2010). This PYR/PYL/RCARs-PP2Cs-SnRKs complex exhibits a double negative regulation system in ABA
signaling (Umezawa et al., 2010). The binding of ABA to the PYR/PYL/RCARs inhibits PP2Cs activity and results in SnRKs activation (Park et al., 2009; Umezawa et al., 2009; Vlad et al., 2009; Hubbard et al., 2010). Therefore, in the absence of ABA, the PP2Cs are active and constitutively repress SnRKs activity and downstream signaling events. By contrast, PYR/PYL/RCARs interact with PP2Cs in the presence of ABA and inhibits their phosphatase activity, allowing SnRK2s activation and hence, phosphorylation of target proteins, and consequently causing activation of ABA-responsive genes (Hubbard et al., 2010; Fujita et al., 2011) (Fig. 1-3).

Several transcription factors (TFs) involved in the ABA-dependent signaling pathway have also been identified and characterized. TFs play a central role because they regulate gene expression by interacting with the cis-regulatory elements located in the promoter region of the target genes. Since many stress-responsive genes have been studied, their regulation by TFs is also essential in elucidating plant stress response. Among the TFs that control the ABA-responsive gene expression are ABRE-BINDING PROTEINS/ABRE-BINDING FACTORS (AREBs/ABFs), AtMYB2, AtMYC2 and RESPONSIVE TO DEHYDRATION 26 (RD26).

The AREB/ABF family is the major class of TFs that control ABA-mediated gene expression under osmotic stress (Fujita et al., 2005). The members of this family (AREB1/ABF2, AREB2/ABF4, ABF1 and ABF3) are mainly expressed in the vegetative tissues under abiotic stress conditions (Choi et al., 2000; Fujita, 2005; Furihata et al., 2006; Yoshida et al., 2010). From yeast one-hybrid screening and gel-shift assays, AREB1/ABF2, AREB2/ABF4, ABF1 and ABF3 were isolated based on their strong affinity for ABA-responsive elements (ABRE) sequences: PyACGTGG/TC (Choi et al., 2000). Analyses of the promoters of ABA-responsive genes indicate that multiple ABREs or ABRE with its coupling element (CE) are required for their expression under stress conditions (Zhang et al., 2005; Gomez-Porras et al., 2007). This demonstrates the importance of the AREB/ABF family in regulating ABA-inducible gene
expression during abiotic stress. Furthermore, much evidence suggests their roles in acquiring drought tolerance (Kang et al., 2002; Kim et al., 2004; Fujita, 2005; Yoshida et al., 2010; Qin et al., 2011). Overexpression of AREB1/ABF2, ABF3 and ABF4 leads to induction of LEA class genes (RD29B and KIN2), regulatory genes (HIS1-3 and RD20) and other stress-responsive genes. They are involved in the alleviation of water stress and thus increase drought stress tolerance (Kang et al., 2002; Fujita, 2005). Recently, a study demonstrated that AREB1, AREB2 and ABF3 are the master TFs that cooperatively regulate ABRE-dependent gene expression in ABA signaling because many stress-responsive genes are drastically impaired in the areb1 areb2 abf3 triple mutant (Yoshida et al., 2010).

The expression of RD22 is induced by ABA, but its promoter region does not contain a typical ABRE consensus sequence (Yamaguchi-Shinozaki and Shinozaki, 1993b). This apparent paradox was resolved by the discovery of another regulator for ABA-response genes, a basic helix-loop-helix-ZIP (bHLH-ZIP) MYC-related protein (Abe et al., 1997). A MYC transcription activator, AtMYC2, and its associated R2R3-MYB transcription factor, AtMYB2, have been shown to bind to the cis-acting element of RD22 promoter (Abe et al., 1997; Abe et al., 2003). AtMYC2 binds specifically to the CACATG sequence whereas AtMYB2 binds to the TGGTTAG motif and they cooperatively activate RD22 expression (Abe et al., 1997). Transgenic plants overexpressing AtMYB2 and/or AtMYC2 show elevated levels of RD22 and ALCOHOL DEHYDROGENASE 1 (AtADH1). They are also hypersensitive to ABA and exhibit improved stress tolerance (Abe et al., 2003).

In addition to AREB/ABF, MYC and R2R3-MYB families, one member of the NAC (NAM, ATAF1, 2, and CUC2) (Aida et al., 1997) TF family has been identified as having a role in the ABA-dependent stress signaling pathway (Fujita et al., 2004). RD26 was among the NAC cDNA clones that were isolated from yeast one-hybrid screening as it binds to the promoter region of ERD1, which contains the CATGTG motif (Abe et al., 1997; Tran et al., 2004).
Promoter analysis of *RD26* suggests that its regulation is ABA-dependent based on the existence of several cis-elements such as ABRE, MYB and MYC recognition sites (Fujita et al., 2004). As its expression is induced by high salinity, drought and ABA, its overexpression also enhances stress tolerance and induces higher expression of stress-inducible genes such as *GLYOXALASE (GLY)* and *RD20* (Fujita et al., 2004; Tran et al., 2004).

Several other classes of TFs involved in ABA-mediated gene expression have also been reported, but for some TFs (Fujita et al., 2011), their expression is not significantly induced by exogenous ABA application. Among these are members of the AP2/ERF, HD-ZF, HD-Zip, C2H2-ZF, B3, WRKY and NF-Y classes (reviewed by (Fujita et al., 2011; Golldack et al., 2011)). Their induction is not only triggered by osmotic stress but also by several other abiotic stresses such as cold, ozone and UV radiation (Fujita et al., 2011). Furthermore, some of these genes have been shown to play a role in hormone-mediated responses, demonstrating their function in the crosstalk between ABA and other hormone signaling pathways under abiotic stress.

**Transcriptional regulation by the ABA-independent signaling pathway in response to stress**

Some stress-responsive genes are not induced by exogenous ABA application, suggesting an ABA-independent manner of regulation in the stress response. Promoter analysis of *RD29A* demonstrated the presence of a cis-acting element, TACCGACAT, which is responsible for dehydration-, high salinity- and low temperature-induced expression (Yamaguchi-Shinozaki and Shinozaki, 1994). This motif is known as the dehydration responsive element (DRE), and its core sequence, CCGAC is also shared with the C-repeat (CRT) and the low temperature responsive element (LTRE) (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994).
The key regulators involved in this pathway are C-REPEAT/DRE BINDING PROTEINs (CBFs/DREBs), which belong to the AP2/ERF TF family (Sakuma, 2002). These proteins activate downstream gene expression by specifically binding to their DRE/CRT sequence under stress conditions (Stockinger et al., 1997; Liu et al., 1998). DREB1A/CBF3, DREB1B/CBF1 and DREB1C/CBF2 expression is only induced by low temperature (Liu et al., 1998; Seki et al., 2002b), but the expression of DREB2A and DREB2B is induced by drought, salt and heat stress (Sakuma et al., 2006b; Sakuma et al., 2006a; Schramm et al., 2008; Yoshida et al., 2008; Chen et al., 2010a). These findings indicate that two independent families of DREB proteins function in two separate signaling pathways under cold and dehydration stress (Liu et al., 1998). Constitutive expression of DREB1s enhances tolerance to freezing and dehydration but causes growth retardation in transgenic plants (Liu et al., 1998). By contrast, the overexpression of intact DREB2A does not increase stress tolerance, but its active form results in significant drought and heat tolerance. This suggests that post-transcriptional modification is required for its activation (Liu et al., 1998; Sakuma et al., 2006b; Sakuma et al., 2006a).

The expression of ERD1 was strongly induced by osmotic stress but not by exogenous ABA application (Nakashima et al., 1997). The promoter region of ERD1 consists of NAC recognition site (NACRS), which contains the CATGTG element. Three NAC factors (NAC019, NAC055 and NAC072) have been shown to bind specifically to the NACRS (Tran et al., 2004). Overexpression of NAC genes leads to stress tolerance, but the induction of ERD1 is not up-regulated in these transgenic plants. This suggests that another factor is required for its activation under stress conditions (Tran et al., 2004). Further work identified another TF, known as ZINC FINGER HOMEODOMAIN 1 (ZFHD1), which specifically binds to the 14bp of RPS1 site 1-like sequence (CACTAAATTGTGCAC) and activates ERD1 expression (Tran et al., 2007). This RPS1 site 1-like sequence was then renamed the ZFHD recognition site (ZFHDRS). Transactivation assays have shown that both the ZFHDRS and NACRS are required for the activation of ERD1.
Overexpression of ZFHD alone is sufficient for acquiring stress tolerance, but the size of the transgenic plant is reduced (Tran et al., 2007). Interestingly, co-expression of ZFHD and NACs did not negatively affect plant growth, although they showed enhanced stress tolerance. This suggests a possible role for NAC019, NAC055 and NAC072 in other aspects of development under stress conditions (Tran et al., 2007).

In conclusion, the molecular mechanisms underlying plant stress response have been dissected based on the analysis of cis-acting elements and the identification of stress-responsive TFs. Undoubtedly, the crosstalk between ABA-dependent and -independent signaling pathways is important in activating stress-responsive genes. Their action provides additional coordination between stress signals and the regulatory role of ABA in the activation of downstream stress-responsive genes (Agarwal and Jha, 2010). Some TFs that are associated with stress are regulated at the transcriptional level, and hence create a transcriptional cascade (Shinozaki and Yamaguchi-Shinozaki, 2007; Hirayama and Shinozaki, 2010; Cramer et al., 2011). Furthermore, TF regulation is also controlled by post-translation modification such as ubiquitination, sumoylation and phosphorylation. Taken together, these observations point to TFs as hub components that integrate multiple signals under abiotic stress conditions. Regulation at both the transcriptional and post-translational levels is required for a rapid and fine-tuned regulation of TFs under abiotic stress conditions (Hirayama and Shinozaki, 2010).
PYR/RCARs are ABA receptors and PP2C is a negative regulator that suppresses SnRK2 activity. In the presence of ABA under stress conditions, the PYR/RCARs complex inhibits PP2C activity and thus, activates SnRK2 expression. SnRK2 positively regulates downstream signaling genes. ABA molecules are represented with pink diamonds and inhibitory interactions are marked with black connections. Figure is adapted from Hubbard et al. and Umezawa et al. (Hubbard et al., 2010; Umezawa et al., 2010).
Figure 1-4. Transcriptional regulatory networks of stress response in *Arabidopsis*

Two signaling pathways have been identified in plant stress response: the ABA-dependent and ABA-independent pathways. Transcription factors are shown in ellipses and their *cis*-acting elements are shown in boxes. Figure is adapted from Yamaguchi-Shinozaki & Shinozaki (Yamaguchi-Shinozaki and Shinozaki, 2006).
4. THEESIS OVERVIEW

In this thesis, a study of one of NAC TFs, NAC019 \((At1g52890)\), is described. Early studies on the identification of \textit{NAC019} suggested that it functions in drought stress response in the vegetative tissue. Previous work on wild type (WT) inflorescence development performed by Dr. Zhao Su showed \textit{NAC019} induction during early drought stress. Therefore, it was chosen for further functional studies. The goal of this study is to investigate the function of \textit{NAC019} in floral development under drought stress. We also would like to know its role in transcriptional regulation of downstream genes so that the connection between the stress transcriptional regulatory network and floral development can be deciphered.

In Chapter 2, I describe the phenotypic characterization of the \textit{nac019} mutant under drought stress, mainly focusing on flower morphology and seed production. Preliminary characterization of the \textit{nac019 nac55 nac072} triple mutant is also presented in Chapter 2. Then, further analysis on microarray-based transcription profiling of the \textit{nac019} inflorescence is described in Chapter 3. In this analysis, I aimed to identify the putative target genes of NAC019 under drought stress. Thus, a comparative analysis between WT and \textit{nac019} is included, as well as gene expression analysis in a time-course manner. However, identification of putative NAC019 target genes by microarray cannot distinguish between primary (direct) and secondary (indirect) targets. Therefore, we employed a GR-inducible system to test if genes are targeted directly by NAC019. In Chapter 4, the description of the transgenic line harboring the NAC019-GR construct is provided.
5. REFERENCES


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Chapter 2

PHENOTYPIC CHARACTERIZATION OF THE NAC MUTANTS UNDER DROUGHT STRESS CONDITIONS
1. ABSTRACT

Drought stress negatively affects yield in plants as a result of physiological and metabolic changes in response to this condition. Studies on the effect of drought to wild type (WT) Arabidopsis thaliana (hereafter Arabidopsis) inflorescence development revealed that abnormal flower morphology leads to silique sterility, subsequently reducing yield. NAC019 is a drought-responsive gene, and its overexpression has been shown to improve plant tolerance to drought.

To investigate NAC019 function in the inflorescence during stress response, the nac019-1 mutant was subjected to drought, and its flower, silique and seed production were examined. The pistils and stamens of nac019-1 were smaller than WT, but their length ratio did not differ significantly. Furthermore, nac019-1 pollen fertility was also not affected severely by drought. However, the nac019-1 inflorescence recovered from drought more slowly than WT, as determined based on the emergence of new open flowers from the newly recovered inflorescence. Both WT and nac019-1 inflorescence recovered from drought, and the siliques produced were categorized into three types: early, severe and recovered. The nac019-1 mutant produced fewer siliques with normal seeds under drought conditions, and it contained more undeveloped siliques than WT. This could be due to delayed floral meristem recovery in the mutant as well as a shorter recovery period. Overall, the nac019-1 inflorescence phenotype was not severely affected by drought.

This relatively weak phenotype under stress could be a result of NAC gene redundancy. Therefore, to further dissect NAC019 function, a nac019 nac055 nac072 triple mutant was generated. Observation of the vegetative tissue suggested that the triple mutant was greatly affected by drought conditions. Although the effect of drought on its inflorescence is not yet known, it is likely that its flower and seed production will be greatly reduced.
2. INTRODUCTION

The NAC gene family is named after NAM (NO-APICAL MERISTEM), ATAF1/2 (ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR) and CUC2 (CUP-SHAPED COTYLEDON). It is among the largest family of plant-specific transcription factors (TFs) (Aida et al., 1997; Olsen et al., 2005). To date, more than 100 NAC genes have been identified in both Arabidopsis and rice (Oryza sativa) (Ooka et al., 2003; Nuruzzaman et al., 2010). Additionally, its family members have been identified in other plant species such as soybean (Glycine max), wheat (Triticum aestivum), poplar (Populus trichocarpa), citrus (Citrus sinensis) and tobacco (Nicotiana tabacum) (Rushton et al., 2008; Liu et al., 2009; Hu et al., 2010; Xia et al., 2010; de Oliveira et al., 2011; Le et al., 2011). The abundance of NAC genes in some plant species contributes to their diversity in protein structure as well as in their biological functions (Puranik et al., 2012).

The NAC proteins have a conserved N-terminal region, known as a NAC domain (Aida et al., 1997). This domain is essential for DNA or protein binding, dimerization and nuclear localization [reviewed in (Puranik et al., 2012)]. Its C-terminus (transcription regulatory region) is highly diverse (Olsen et al., 2005; Puranik et al., 2012) and functions in activating (Xie et al., 2000; He et al., 2005; Zheng et al., 2009) or repressing transcription (Hao et al., 2010; Yamaguchi et al., 2010; Kim et al., 2012). Moreover, some NAC proteins contain a transmembrane motif, which is necessary for plasma or endoplasmic reticulum membrane anchoring (Seo et al., 2008). These characteristics support the roles of NAC genes in regulating biological process such as cell division, flowering and germination in response to environmental stress (Kim et al., 2007; Kim and Park, 2007; Kim et al., 2008).

Functional studies on several NAC members have revealed its role in developmental processes and stress responses (Olsen et al., 2005). NAM, CUC1 and CUC2 play a role in the
formation of the shoot apical meristem (SAM) and organ separation during embryonic and floral development (Aida et al., 1997). NAC-LIKE, ACTIVATED BY APETALA3/PISTILLATA (NAP) is the target of the AP3/PI heterodimer, and it regulates cell division and cell elongation in developing petals and stamens (Sablowski and Meyerowitz, 1998). In addition to embryonic and floral development, NAC genes also play roles in root development. For example, NAC1 functions in promoting lateral root formation through an auxin-mediated signaling pathway (Xie et al., 2000). Furthermore, secondary wall thickening in the endothecium of anthers is regulated by NAC SECONDARY WALL THICKENING PROMOTING FACTOR 1 (NST1) and NST2 (Mitsuda et al., 2005). This suggests that NAC TFs also play a role in cell wall biosynthesis.

Another member of the NAC family, VASCULAR NAC-RELATED DOMAIN (VND)-INTERACTING 2 (VNI2), not only is involved in the regulation of xylem vessel formation (Yamaguchi et al., 2010), but also functions in mediating signaling crosstalk between salt stress response and leaf senescence (Yang et al., 2011).

NAC proteins have been implicated in biotic and abiotic stress responses. Among the earliest isolated Arabidopsis NAC genes that function as regulators of abiotic stress are NAC019, NAC055 and NAC072 (Fujita et al., 2004; Tran et al., 2004). Their expression is mainly induced by drought and salt stress, as well as abscisic acid (ABA) and methyl jasmonate (MeJA) treatment (Tran et al., 2004). Additionally, NAC019 and NAC055 are also involved in defense response to pathogen attack through a JA-mediated signaling pathway (Bu et al., 2008). Another NAC-domain protein, ATAF1, possesses dual functions in biotic and abiotic stress response (Lu et al., 2006; Wu et al., 2009). Its overexpression enhances drought tolerance and increases susceptibility to necrotrophic pathogen (Wu et al., 2009). In other plant species such as rice, wheat, tobacco and soybean, the overexpression of several NAC members improves tolerance to drought, salt and cold stress (Hu et al., 2006; Jeong et al., 2010; Hao et al., 2011; Liu et al., 2011; Xue et al., 2011). Furthermore, their role in defense response to pathogens has been elucidated
through gene knockout and overexpression (Puranik et al., 2012). The multiple functions of NACs suggests that NAC genes are important mediators in the crosstalk between biotic and abiotic stress signaling pathways, and that they are beneficial for the production of genetically engineered, stress tolerant plants (Puranik et al., 2012). Interestingly, several NACs integrate environmental signals in the regulation of plant developmental processes (Xie et al., 2000; He et al., 2005; Kim et al., 2007; Kim et al., 2008; Hao et al., 2011; Yang et al., 2011). These versatile characteristics indicate the importance of NAC gene family members in the success of plant survival and reproduction under unfavorable conditions (Puranik et al., 2012).

Several studies have characterized the effect of abiotic stress on reproductive development, especially in crops such as rice (Boonjung and Fukai, 1996; Sheoran and Saini, 1996; Oliver et al., 2005), maize (Moss and Downey, 1971; Herrero and Johnson, 1981) and wheat (Lalonde et al., 1997). In Arabidopsis, environmental stress studies have mainly focused on vegetative development such as in the shoot, leaf and root, but details characterizing stress effect on floral development are less abundant. Our group has subjected Arabidopsis to drought during Arabidopsis reproductive stage to understand the developmental and transcriptional adaptation of its inflorescence. Under drought conditions, several developmental defects have been observed in the flower, such as ovule abortion, failure of flower opening, abnormal anther development and delayed filament elongation (Su et al., submitted). Through genome-wide expression analysis, we have identified several candidate genes that may function as regulators of stress response during floral development. One of the genes belongs to the NAC gene family, NAC019 (Su et al., submitted).

The role of NAC019 in stress response was first identified through the manipulation of its expression levels. The overexpressing NAC019 transgenic plant exhibits increased drought tolerance (Tran et al., 2004) and reduced resistance towards a necrotrophic fungus, Botrytis cinerea (Bu et al., 2008). NAC019 is also involved in the ABA- and JA-mediated signaling
pathways (Bu et al., 2008; Jensen et al., 2010), supporting the notion that it functions in plant response to biotic and abiotic stress. Under severe and mild drought conditions, $NAC019$ expression was induced in Arabidopsis inflorescences (Su et al., submitted; unpublished data). Therefore, it was hypothesized that under drought conditions, $NAC019$ may play a role in the regulation of genes related to floral development as well as stress-responsive genes.

We wanted to investigate the effects of $NAC019$ mutation on flower phenotype during drought stress. To this end, the flowers of $nac019-1$ subjected to drought were dissected to examine any defects caused by drought. The sizes of the floral organs were measured and compared to WT. In addition, we examined $nac019-1$ pollen viability and in vivo pollen germination to determine whether its pollen is susceptible to drought. Finally, the number of flowers, siliques and seeds produced by $nac019-1$ under drought conditions were assessed.

The inflorescence phenotype of $nac019-1$ was relatively weak, and we speculated that $NAC019$ might share redundant functions with other related genes. A BLAST alignment search showed that the NAC019 amino acid sequence has high similarity with NAC055 and NAC072. Therefore, to investigate if these genes function redundantly during floral development under drought stress, we created a $nac019 nac055 nac072$ triple mutant. At the time of writing, a complete description of the response of the triple mutant to drought had not yet been obtained. However, we describe here a preliminary analysis of the triple mutant based on its vegetative tissue.
3. MATERIALS AND METHODS

Plant materials and experimental conditions

The seeds of *nac019-1* (SALK_096295C) were obtained from the Arabidopsis Biological Resource Center (ABRC). Other T-DNA insertion lines used in this study were also obtained from ABRC: *nac019-2* (CS870199), *nac055-1* (SALK_014331C), *nac055-2* (SALK_152738), *nac072-1* (SALK_063576) and *nac072-2* (SALK_083756). WT and mutants were in the Columbia-0 (Col-0) background. The seeds were sown in pots filled with 100 g mixture of Metro-Mix 360 soils (Sun Gro Horticulture Canada Ltd., USA) and Turface Greens Grade (Profile Product LLC., USA) at a ratio of 3:2 by volume (Su et al., submitted). They were placed at 4°C for 2 days in the dark to break residual dormancy. After stratification, they were transferred and grown in a Conviron growth chamber (Conviron Inc., USA) at 22°C under a 16/8 hours light/dark photoperiod with 60% humidity and 300 µmol photon m$^{-2}$ s$^{-1}$.

Drought stress treatment

Plants were well watered until they reach bolting stage, and the length of main stem was about 1 cm. The water withholding indicated the beginning of drought treatment (Su et al., submitted), and the humidity was decreased to 30% in order to increase soil water evaporation of plants subjected to drought. The soil moisture for well-watered (WW) plants was maintained at 90% (90 g water/100 g soils) whereas the soil moisture content of drought-treated (DT) plants was gradually decreased to about 30% of the soil water-holding capacity. The humidity was set to the normal growth condition (60%) when the soil moisture of DT plants reached 30%. The soil water condition of WW and DT plants was maintained by daily watering until the fruits (siliques) matured (Su et al., submitted). In this study, the plants were divided for different purpose of
characterization. They were separated for the following purposes: 1) analysis of the number of mature flowers, siliques and seeds, 2) measurement of floral organ size and analysis on anther staining; and 3) *in vivo* pollen germination assay.

**Reproductive organ size measurement**

The stamens and pistils of the DT flower buds were dissected under a Nikon SMZ-U dissecting scope (Nikon, Japan). Images were photographed with an Optronics (Goleta, CA) digital camera, and they were edited using Photoshop CS4 (Adobe Systems Inc., USA). Measurement of pistil and stamen length was performed using ImageJ (http://rsbweb.nih.gov/ij/). The reproductive organs were measured from DT flower buds at Days 0, 3, 5, 7, 10, 14, 16, 20, 22 and 25 of drought treatment (hereafter labeled as T for day of drought treatment).

**Pollen grain viability**

The anthers were stained with Alexander’s stain (Alexander, 1969) to determine pollen grain viability under drought conditions. They were fixed in Carnoy’s fixative (Alcohol: Chloroform: Glacial acetic acid = 6: 3: 1) for 2 hours at room temperature. After the fixative solution was discarded, the Alexander’s stain was added to the tube and incubated at 50°C for 12 hours. The samples were washed with 10% glycerol for 8 hours at room temperature before they were placed on a microscope slide. Slides were examined using a Nikon Eclipse E400 microscope (Nikon, Japan) and photographed with an Optronics digital camera. All images were edited using Photoshop CS4.
**In vivo pollen germination**

Before performing hand-pollination, the outer floral organs (sepals, petals and stamens) from WT flower stage 12 (Smyth et al., 1990) were removed by dissection under the dissecting scope. The plant was placed in the growth chamber for 12 hours to allow for recovery. After that, the pollen grains from the mature flowers of DT plants grown at T0, T4, T7, T10, T14 and T18 were applied on the stigma of the emasculated flower.

Three hours after pollination, the stigmas were excised and fixed in Carnoy’s fixative for 2 hours at room temperature. The fixative solution was discarded, and the stigmas were incubated in 8M NaOH for 14 hours. Then, the samples were washed with distilled water and incubated in an aniline blue solution (0.05% in K3PO4, pH 8.5) for 2 hours in the dark. They were placed on microscope slide and observed using a Nikon Eclipse E800 microscope (Nikon, Japan). Digital images were acquired using a Hamamatsu C4742 (Hamamatsu Corp., Japan) digital camera with Image Pro Plus software version 4.5.1.27 for Windows (Media Cybernetics Inc., USA).

**Determination of NAC019, NAC055 and NAC072 expression by semi-quantitative RT-PCR**

RNA samples were collected from the inflorescences of WT and nac019-1 plants subjected to drought stress. Total RNA was extracted using the NucleoSpin RNA Plant kit (Macherey-Nagel, GmbH & Co., Germany) according to the manufacturer’s instructions. One microgram of total RNA from each sample was treated with RNase-free DNase I (Thermo Fisher Scientific Inc., USA) prior to reverse transcription to eliminate the possibility of genomic contamination. Reverse transcription was performed on the total RNA using the GoScript™ Reverse Transcription System (Promega Corp., USA) according to the manufacturer’s
instructions. The synthesized cDNA was used as a template for PCR amplifications, and the transcript of NAC019, NAC055 and NAC072 was amplified using the primers listed in Table 2-1.

**Generation of double and triple mutants**

To generate a double mutant, outcrosses were first performed between nac019-1 and the single mutants nac055-1, nac055-2, nac072-1 and nac072-2 by applying the pollen from mature flowers to the stigma of the emasculated flower. Three primers were used for genotyping each single mutant: a primer to the left border of the T-DNA insert (LBb1.3 or oMC3088) and a pair of primers that specifically amplified the region of genomic DNA containing the insert site. Their combination was used to confirm the presence of the T-DNA insertion. Primer sequences are listed in Table 2-1.

The F1 and F2 progenies were genotyped using gene-specific primers (Table 2-1). The triple mutant was generated by crossing the double mutants nac019-1 nac055-1 and nac019-1 nac055-2 with the nac019-1 nac072-2 double mutant. These genetic crosses produced a heterozygous triple mutant, the genotype of which was confirmed by PCR genotyping. The F2 progeny seeds were planted, and PCR genotyping was performed to identify homozygous triple mutants (nac019-1 nac055-1 nac072-2 and nac019-1 nac055-2 nac072-2).

**Relative water content (RWC) measurement**

A leaf from a DT plant was cut and its fresh weight was determined. Then, the leaf was immersed in water overnight and blotted-dry with paper towel. The leaf was weighed to obtain its turgid weight. The same leaf was then dried in an oven overnight to acquire its dry weight.
Relative water content (RWC) was determined by using the formula below (Smart and Bingham, 1974).

\[
RWC = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100
\]
Table 2-1. Primers used in the characterization of the NAC mutants

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Primer sequence from 5’ to 3’</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>oMC4235</td>
<td>TTTTACCCGACCGATGAAG</td>
<td>SALK_096295C genotyping (F)</td>
</tr>
<tr>
<td>oMC4236</td>
<td>CACCAACTTGCCCCCGA</td>
<td>SALK_096295C genotyping (R)</td>
</tr>
<tr>
<td>oMC1533</td>
<td>GGTAACATTTGTGCTCAGTGTTGG</td>
<td>ACT2 (F)</td>
</tr>
<tr>
<td>oMC1534</td>
<td>AACGACCTTAATCTTCATGCTGC</td>
<td>ACT2 (R)</td>
</tr>
<tr>
<td>oMC3088</td>
<td>ATTTTGGCGATTTTCGGAAC</td>
<td>Left border T-DNA insertion (SALK lines)</td>
</tr>
<tr>
<td>oMC4010</td>
<td>GCCTTTTCAGAAAATGGATAATAGCCTTGCTTCC</td>
<td>Left border T-DNA insertion (SAIL lines)</td>
</tr>
<tr>
<td>oMC4128</td>
<td>ACAACATTACCGGTGTCCCTTC</td>
<td>CS870199 genotyping (F)</td>
</tr>
<tr>
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<td>AGATCTATTTGCAGCGATGAGC</td>
<td>CS870199 genotyping (R)</td>
</tr>
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<td>SALK_014331C genotyping (F)</td>
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<td>SALK_014331C genotyping (R)</td>
</tr>
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<td>TGGCGAAATTTCTTTATGCCCAC</td>
<td>SALK_152738 genotyping (F)</td>
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<td>SALK_152738 genotyping (R)</td>
</tr>
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<td>oMC4984</td>
<td>GATGACGACCCATTCTGCT</td>
<td>SALK_063576 and SALK_083756 genotyping (F)</td>
</tr>
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<td>SALK_063576 and SALK_083756 genotyping (R)</td>
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<tr>
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<td>NAC019 cDNA (F)</td>
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<tr>
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<td>NAC019 cDNA (R)</td>
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<td>NAC055 cDNA (F)</td>
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<td>oMC8116</td>
<td>TCCGTTAGCCCGAGTGTAGGTT</td>
<td>NAC072 cDNA (F)</td>
</tr>
<tr>
<td>oMC4984</td>
<td>GATGACGACCCATTCTGCT</td>
<td>NAC072 cDNA (R)</td>
</tr>
</tbody>
</table>
4. RESULTS

T-DNA insertional mutants of nac019-1 and nac019-2

To ensure that the phenotypic changes observed in the mutant were caused by disruption of the gene and not by the insertion, we used two T-DNA insertion lines for NAC019. The insertion in the nac019 mutants were confirmed by PCR genotyping, and by sequencing; the insertion in nac019-1 was located in the second exon of NAC019 and the insertion in nac019-2 was located at 187 bp upstream of NAC019 start codon (Fig. 2-1).

![Figure 2-1. Schematic of T-DNA insertion locations in nac019-1 and nac019-2](image)

ATG is the start codon and TGA is the stop codon. Pink boxes represent exon, blue lines represent non-coding regions and blue inverted triangles indicate the T-DNA insertions.

To determine if both T-DNA lines were null mutants, we examined their expression in the leaf and inflorescence of WT, nac019-1 and nac019-2 under well-watered conditions. The samples were collected, and RNA extraction was performed before cDNA was synthesized through reverse transcription. NAC019-specific primers were used to amplify its transcript, and we found that it was expressed in WT and nac019-2 but not in nac019-1 (Fig. 2-2). Semi-quantitative RT-PCR results confirmed that nac019-1 is a null mutant as NAC019 expression was undetected. However, nac019-2 was not a null mutant because NAC019 transcript still could be seen. This is not surprising because the T-DNA insertion in nac019-2 is located at the promoter region and might not affect NAC019 transcription (Fig. 2-1).
Figure 2-2. Semi-quantitative RT-PCR on WT, *nac019-1* and *nac019-2*

The *NAC019* transcript was abolished in *nac019-1* but not in *nac019-2*. *NAC019* expression was assayed in the leaf and the inflorescence under well-watered conditions. *ACT2* was used as an internal control.

**Phenotypic characterization of *nac019-1* under drought stress**

*The number of flowers was reduced in the *nac019-1* mutant under drought conditions*

To investigate the function of *NAC019* in floral development under drought stress, we observed the response of *nac019-1* to drought. *NAC019* is a drought-responsive gene (Tran et al., 2004), and it was expected that the mutant would be hypersensitive to drought compared to WT. From our observations, flower production is disturbed once the leaf is wilted under progressive drought in WT. Severe drought causes abnormal flower morphology and some flowers are unable to mature (Su et al., submitted). For that reason, mature flowers were counted daily so that the effect of drought on flower production could be determined.

The average number of mature flowers produced by WT and *nac019-1* was obtained and summed up in order to get its cumulative number. Under well-watered conditions, WT and *nac019-1* continuously produced flowers until approximately 16 days of drought treatment. The number of total flowers produced by them was not differed significantly (Fig. 2-3A). Under
drought stress, both WT and \textit{nac019-1} produced almost similar numbers of mature flowers during the early days of drought. From T8 to T14 (T indicates the day of drought treatment), the number of flowers produced by \textit{nac019-1} was slightly more than WT but the difference was not significant (Fig. 2-3B). At T10 and T11, no mature flowers were produced in WT and \textit{nac019-1}, respectively. After two weeks of drought treatment, WT started to produce open flowers from the newly emerged inflorescence, which was 6 days earlier than \textit{nac019-1}. As WT acclimated from drought, its recovery was observed since it continued producing more flowers. In contrast to WT, only a few open flowers were produced by \textit{nac019-1} after it recovered at T20. Consequently, the total number of flowers produced by \textit{nac019-1} was fewer than WT.
Figure 2-3. The total number of mature flowers produced by WT and nac019-1
Open flowers (Flower stage: 13 and 14) under (A) well-watered and (B) drought conditions were counted daily and cumulative flower numbers are presented. Asterisks indicate that the difference between WT and nac019-1 was significant (T-test, p-value <0.05) and error bars represent SD (n=5).
The nac019-1 mutant produced fewer siliques under drought conditions

Under prolonged drought conditions, flower production was arrested for both WT and nac019-1, but both genotypes showed recovery. Therefore, we examined the number of siliques and seeds in both genotypes. In this experiment, dry seeds were counted after the silique matured.

The siliques produced under this condition can be divided into three types. The first type (Type I) was produced at the early period of drought (less than 10 days), and the siliques normally contained about 40 to 50 seeds. As drought progressed, the siliques became shorter, and the seed number decreased until it no longer produced seeds. This Type II silique produced fewer than 5 seeds and was observed when drought became severe. Finally, the Type III silique was produced when the plant had acclimatized to the drought conditions and recovered. There was variation in the seed number produced after recovery and thus, every silique that contained more than 5 seeds was categorized as a Type III silique (Fig. 2-4, Fig. 2-5).

Comparison between WT and nac019-1 showed that there was no significant difference in the number of the Type I siliques produced. This suggests that WT and nac019-1 possessed similar early responses to drought. However, drought conditions affected nac019-1 more than WT because nac019-1 contained more Type II siliques. This means that nac019-1 had more undeveloped flowers under drought conditions, which in turn, affected seed production. After acclimation, nac019-1 produced Type III siliques, but less than WT did. This indicates that nac019-1 recovered but could not produce as many siliques as WT after its recovery (Fig. 2-6).

Silique production represents the success of floral development under drought conditions, and reduced seed number in a silique could indicate that fertilization is disrupted by defective floral organs. Therefore, the total number of seeds for each type of silique was counted to determine the effect of drought on WT and nac019-1 seed production. There was no significant difference between Type I siliques in WT and nac019-1. However, the number of seeds produced in the Type III siliques in WT was more than in the Type III nac019-1 siliques. This is expected
because reduced number of Type III siliques in nac019-1 would give a reduced number of its total seeds (Fig. 2-7). Nonetheless, the number of seeds per silique of Type I and Type III showed no significant difference between WT and nac019-1 (Fig. 2-5). This indicates that in nac019-1, drought had more severe effect on silique production than on seed production.

Figure 2-4. The type of siliques produced by (A) WT and (B) nac019-1 under drought conditions

Three types of siliques were produced under drought conditions: Type I (early phase of drought), Type II (severe phase of drought) and Type III (recovered phase of drought).
Figure 2-5. The number of seeds produced in Type I, Type II and Type III siliques of WT and nac019-1 under drought conditions

There was no significant difference between the number of seeds produced in the Type I, Type II and Type III siliques of WT and nac019-1. Bars represent mean ± SD (n≥5).

Figure 2-6. The number of siliques produced by WT and nac019-1 under drought conditions

Bars represent mean ± SD (n=5) and significant differences between WT and nac019-1 are marked with an asterisk (T-test, p-value < 0.05).
Figure 2-7. The total seeds produced in WT and nac019-1 under drought conditions

Bars represent mean ± SD (n=5) and significant differences between WT and nac019-1 are marked with asterisk (T-test, p-value < 0.05).

Stamens and pistils in the nac019-1 mutant are shorter under drought conditions

In addition to flower production, we also asked if the flower morphology of nac019-1 was affected by drought. Therefore, the stamens and the pistils of WT and nac019-1 were measured to determine whether their size is changed under drought conditions. To obtain this information, we used a different set of plants and measurements on stage 13 flowers were made at several time points.

The mature flowers of WT and nac019-1 showed no differences in stamen and pistil length at T0, but their lengths gradually decreased under prolonged drought conditions (Fig. 2-8)
and Fig. 2-9). After a week of drought treatment, the reproductive organs of \textit{nac019-1} were smaller than WT, although both genotypes showed size reduction under these conditions. At T14, WT had produced a few open flowers from the newly emerged inflorescence, whereas \textit{nac019-1} still had no new inflorescences or open flowers. This continued until T20, when \textit{nac019-1} started to produce an open flower from the newly emerged inflorescence (Fig. 2-3). All flowers produced from the newly emerged inflorescence were classified as recovered flowers. Measurements of pistil and stamen length in the recovered flowers (WT at T14, \textit{nac019-1} at T20) were made, but the differences between them were not significant. After drought recovery, the length of pistil and stamens of WT and \textit{nac019-1} was restored to their normal size (Fig. 2-8 and Fig. 2-9).

The \textit{nac019-1} mutant had shorter organ length than WT and thus, we investigated if the size could affect its pollination under drought conditions. Successful pollination requires a synchronization of pistil and stamen length so that the pollen can be delivered to the papillae stigma. Therefore, we measured the ratio of stamen to pistil in WT and \textit{nac019-1}. A ratio of more than 1 indicates that stamens were longer than the pistil whereas shorter stamens would give a ratio of less than 1. For all the time points we examined, the ratio was close to 1 (Fig. 2-10), indicating that the coordination between stamens and pistil was not severely affected by drought conditions and that pollination was still permitted.
Figure 2-8. The pistil and stamens of WT and nac019-1 at different days of drought treatment

Flower stage 13 was used for dissection and floral organ measurement, except at T10, where the oldest closed buds were used for comparison. T represents the day of drought treatment and the bar size is 1 mm.
Figure 2-9. The length of stamens and pistils in WT and nac019-1 under drought conditions
The length of (A) long stamens and (B) pistil is shown at different days of drought treatment. A T-test was used to determine the significance of the differences between WT and nac019-1. Significant differences are marked with an asterisk (p-value < 0.05) and error bars represent SD (n=3).
Figure 2-10. The ratio of stamen length to pistil length in WT and nac019-1 under drought conditions

A ratio of more than 1 indicates that stamens were longer than the pistil whereas shorter stamens give a ratio of less than 1. Bars represent the mean ratio ± SD (n=3).

We further examined pollen viability through Alexander’s staining and in vivo pollen germination. The results suggest that the pollen was viable and able to germinate in vivo under drought conditions (Fig. 2-11 and Fig. 2-12). Thus, drought might slightly affect the size of flowers but not pollen fertility. In nac019-1, fertile siliques were still produced after the plant recovered from prolonged drought (Fig. 2-4 and Fig. 2-6). Although the seed number in the silique was reduced after recovery (Fig. 2-5), nac019-1 fertility was not severely affected by drought. Overall, it was obvious that drought greatly affected the number of siliques produced in nac019-1. This occurs as a result of reduced flower number during progressive drought, instead of defective floral organs.
Figure 2-11. Alexander’s staining on the anthers of WT and nac019-1

All anthers were dissected from flower stage 13 except for T10 and T14, where the anthers from the oldest closed flower bud were used. In each panel, the WT anther is shown on the right whereas the nac019-1 anther is shown on the left. C represents well-watered conditions, and T indicates the day of drought treatment.
Figure 2-12. Aniline blue staining for evaluation of pollen tube growth of WT and nac019-1
Pollen from WT and nac019-1 at selected time points were applied on the pistil of WT grown under well-watered conditions. In each panel, three pistils are shown from each of WT and nac019-1. T indicates the day of drought treatment.

NAC055 and NAC072 are closely related with NAC019

The phenotype shown by nac019-1 under drought stress indicates that mutation of NAC019 caused delayed inflorescence recovery and thus, reduction in silique production. Decreased silique number reflects the number of flowers that can be produced by nac019-1 under
drought stress (Fig. 2-3B and Fig. 2-6). In addition, we found that the nac019-1 flower size was slightly smaller than WT under drought stress (Fig. 2-8 and Fig. 2-9). We assumed that the weak phenotypes shown by nac019-1 might be a result of NAC gene redundancy. In Arabidopsis, there are about 100 genes belonging to the NAC family, and NAC019 is closely related to NAC055 and NAC072 (Ooka et al., 2003; Jensen et al., 2010). Amino acid sequence alignment among NAC019, NAC055 and NAC072 indicates that these genes possess high similarity (Fig. 2-13). Therefore, we speculated that they might have redundant functions during drought stress response.

NAC019, NAC055 and NAC072 are stress-responsive genes whose expression in vegetative tissue is induced by drought, high salinity and ABA (Fujita et al., 2004; Tran et al., 2004). Likewise, NAC019, NAC055 and NAC072 expression is also induced by drought in the WT inflorescence (Su et al., submitted). Moreover, NAC019 and NAC055 expression is up-regulated in stamens treated with JA, suggesting that they may be involved in stamen maturation (Mandaokar et al., 2006). Although NAC072 expression in the floral organs has not been reported, transgenic plants overexpressing it have reduced floral apical dominance, fewer flowers and reduced seed numbers (Fujita et al., 2004). Thus, generating a triple mutant may shed light on the function of these proteins in floral development under drought stress.
Figure 2-13. Amino acid sequence alignment of NAC019, NAC055 and NAC072

Letters highlighted in black color indicate identical amino acids in all NAC proteins whereas letters highlighted in gray color indicate sequence similarity in two of the three NAC proteins.

**Generation of the nac019 nac055 nac072 triple mutant**

To exclude the effects of genetic redundancy on the nac019-1 drought phenotype, a nac019 nac055 nac072 triple mutant was generated. It was expected that the triple mutant will have significant phenotype changes under drought. We used two T-DNA insertion lines for NAC055 (nac055-1 and nac055-2) and NAC072 (nac072-1 and nac072-2). Out-crossing was performed between two single mutants to obtain the double mutants nac019 nac055 and nac019 nac072. From genotyping analysis on the F2 generation, we isolated three lines of homozygous double mutants: nac019-1 nac055-1, nac019-1 nac055-2 and nac019-1 nac072-2.

To create a nac019 nac055 nac072 triple mutant, crossing was performed between the double mutants nac019 nac055 and nac019 nac072. Two lines of F1 triple mutant generation, nac019-1 nac055-1 nac072-2 and nac019-1 nac055-2 nac072-2 were obtained. Further genotyping was done on the F2 generation, and out of 66 plants for each genotype, we obtained three homozygous nac019-1 nac055-1 nac072-2 triple mutant and two homozygous plants from nac019-1 nac055-2 nac072-2. In addition, sequencing also was done so that the location of the T-
DNA insertion could be determined. We found that the insertion in \textit{nac055-1} was located in the third exon whereas the insertion of \textit{nac055-2} was located in the 3´-UTR (untranslated region). The T-DNA insertion in \textit{nac072-2} was located in the first exon of \textit{NAC072} (Fig. 2-14).

To determine if the triple mutants are null mutants, RT-PCR was performed on both lines. Total RNA from their inflorescence was used for cDNA synthesis, and gene-specific primers were used for amplification (Table 2-1). From our data, expression of \textit{NAC019} and \textit{NAC072} could not be detected in the mutants. \textit{NAC055} transcript was also undetected in \textit{nac019-1 nac55-1 nac072-2}, but its expression was observed in the \textit{nac019-1 nac055-2 nac072-2} mutant (Fig. 2-15). The presence of \textit{NAC055} transcript in \textit{nac019-1 nac055-1 nac072-2} might occur as a result of the T-DNA insertion location (Fig. 2-14). Therefore, only \textit{nac019-1 nac055-1 nac072-2} was chosen for further drought analysis.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2-14.png}
\caption{Schematic showing the location of T-DNA insertions}
\end{figure}

The T-DNA insertion locations of (A) \textit{nac055} and (B) \textit{nac072} are shown above. ATG is the start codon and TGA is the stop codon. Pink boxes represent exons, blue lines represent non-coding regions and blue inverted triangles indicate the insertions of T-DNA.
Figure 2-15. Semi-quantitative RT-PCR on the inflorescence of WT and nac019 nac055 nac072

NAC019 and NAC072 expression was abolished in both triple mutants. The NAC055 transcript was undetected in nac019-1 nac055-1 nac072-2, but it was observed in nac019-1 nac055-2 nac072-2. WT was used as control and ACT2 served as an internal standard.

Phenotypic characterization of the nac019-1 nac055-1 nac072-1 triple mutant under drought conditions

Drought causes water loss in plants, affecting their physiology. Relative water content (RWC) is a useful indicator of plant water status because it expresses the absolute amount of water that the plant requires to reach artificial full saturation. Moreover, it represents relative cellular volume, and any changes will affect the interaction between macromolecules and organelles. Water deficit reduces RWC values and hence, causes metabolic and physiological changes. Consequently, photosynthesis activity in plants is reduced and ABA accumulation is triggered (González and González-Vilar, 2001).
To estimate the plant water status in WT, single mutant and triple mutant under drought stress, we determined their leaf RWC over a time course. Three leaves were collected from drought-treated plants at different time points (T0, T4, T6, T8, T10, T13 and T16). For each time point, their fresh, turgid and dry weights were recorded for RWC calculation. As drought continued, the RWC for all genotypes gradually decreased (Fig. 2-16). However, among these genotypes, only nac019-1 nac055-1 nac072-2 exhibited lower RWC at all selected time points. Moreover, the RWC of the triple mutant did not increase, although WT and nac019-1 showed improvement in their water status at T16 (Fig. 2-16). These results suggest that metabolic and physiology events in the triple mutant were severely affected by drought. It also indicates that the triple mutant is more sensitive to drought stress than WT and nac019-1.

We also observed that most nac019-1 nac055-1 nac072-1 stems were bent and leaned onto the soil surface at T10. By contrast, WT and nac019-1 still stood upright (Fig. 2-17). Stem bending might be due to low water status in the triple mutant itself. We noticed that the leaf lost its turgidity under drought conditions. Hence, the stem was not well supported and could not stand upright. This was consistent with our data, where nac019-1 nac055-1 nac072-2 had the lowest RWC compared to WT and nac019-1 (Fig. 2-16). Overall, this result suggests that the triple mutant was severely affected by drought.
Figure 2.16. Relative water content of WT, nac019-1 and nac019-1 nac055-1 nac072-2 under drought stress

Detached leaf samples were used for RWC measurement. Asterisks indicate that the difference between WT and the triple mutant was significant (T-test, p-value <0.05). Three replicates were used in RWC determination, and the error bars represent S.D.
Figure 2-17. The condition of WT, single mutant and triple mutant at T10

A tray of (A) WT, (B) nac019-1 and (C) nac019-1 nac055-1 nac072-2 is shown. In each tray, on the left are the plants under well-watered conditions and on the right are the plants under drought treatment.
5. DISCUSSION

In this study, we examined the phenotype of NAC mutants to investigate the function of NAC019 in floral development under drought conditions. First, we evaluated nac019-1 response to drought by observing its flowers, siliques and seeds. Additionally, we observed nac019-1 pollen fertility by examining its pollen viability and germination under drought stress. Comparison with WT suggests that floral morphology of nac019-1 was not greatly affected by drought. The size of nac019-1 stamens and pistils was smaller than WT, but its pollen was fertile (Fig. 2-8 until Fig. 2-12). Interestingly, nac019-1 had a slower recovery from drought, based on the time it took to produce an open flower from the newly emerged inflorescence (Fig. 2-3B).

The success of floral development can be reflected by the production of siliques and seeds. Therefore, we counted the number of siliques and seeds in WT and nac019-1. From our data, drought had minimal effects on nac019-1 seed production (Fig. 2-7), but it greatly affected the number of nac019-1 siliques produced (Fig. 2-6). The total number of WT siliques produced was 30.5 ± 3.32 whereas in nac019-1, it was about 27.8 ± 1.30. This difference was not significant but the results indicate that nac019-1 had more undeveloped siliques (Type II) under drought conditions (Fig. 2-6). This could happen because of delayed inflorescence recovery of nac019-1 from drought, which prevents the emergence of nac019-1 newly opened flowers. Moreover, nac019-1 had fewer recovered (Type III) siliques than WT (Fig. 2-6). It is possible that nac019-1 had a shorter recovery period and its floral development could no longer sustain due to its developmental stage. In this study, drought treatment was started when the plants were about three weeks old and drought progressed for four weeks. Therefore, nac019-1 might have adapted to drought for a while, but it was too old to maintain its floral meristem. From these observations, we conclude that nac019-1 is more sensitive to drought compared to WT since
The *nac019-1* recovery response was delayed and it could only produce several recovered siliques (Type III).

Our results suggest that drought might affect *nac019-1* by decelerating its floral meristem recovery. However, the possibility of a slower response of *nac019-1* during the beginning of drought stress cannot be excluded. This can be seen from the total number of flowers produced by *nac019-1* at T8 through T14, which was slightly more than WT (Fig. 2-3B). The delayed response of *nac019-1* towards drought might shift or delay its inflorescence recovery. During the early days of drought treatment, it is possible that *nac019-1* had not yet perceived the stress. Therefore, it still produced a regular number of flowers (normally 2 or 3 flowers per day). Later on, severe drought conditions were sensed by *nac019-1* and thus, it responded to drought by slowing down and further arresting flower production. As drought progressed, *nac019-1* acclimatized to the severe environment and started to produce open flowers at T20, which was 6 days later than the first recovered flower in WT (Fig. 2-3B).

In the characterization of the *nac019-1* single mutant, we lacked information from its vegetative tissue. It is well known that water deficit is initially sensed by roots and then leaf growth is inhibited. Due to reduced stomatal conductance and photosynthetic activities, photosynthate is not efficiently translocated to other sink organs such as fruit and seed, which then causes yield reduction (Barnabás et al., 2007). To assess *nac019-1* vegetative tissue sensitivity to drought, we examined its RWC in another set of experiments to infer plant water status. However, the RWC of WT and *nac019-1* were indistinguishable as both genotypes showed a similar pattern (Fig. 2-16). This might be due to variance in the growth conditions in the different sets of experiments. One factor that could influence the result was the nutrient content in the soil used. We believe that the nutrient content was different and that affected plant response in two separate experiments. Thus, we could not determine if *nac019-1* vegetative tissue is more sensitive to drought, although previously, flower recovery was delayed longer than WT.
Our results indicated that the inflorescence phenotype of nac019-1 under drought stress was relatively weak. Therefore, we created a triple mutant to exclude possible compensatory effects from other NAC genes. We chose NAC055 and NAC072 because they are closely related to NAC019 and have roles in drought response (Ooka et al., 2003; Fujita et al., 2004; Tran et al., 2004). Examination of the RWC of nac019-1 nac055-1 nac072-2 suggests that the leaf tissue of the triple mutant was very sensitive to drought since it had the lowest RWC (Fig. 2-16). In addition, most of the nac019-1 nac055-1 nac072-2 stems were bent and almost fell down at T10 (Fig. 2-17).

Based on our observations, the nac019-1 nac055-1 nac072-2 leaf was larger than WT and nac019-1. It is possible that its larger size caused drastic reduction in RWC. Drought reduces the transpiration rate in plants by stomatal closing in order to minimize dehydration (Cornic, 2000; Aroca et al., 2006). However, transpiration rate is dependent on leaf area, and a larger leaf will have a higher transpiration rate and vice versa (Eavis and Taylor, 1979). Under drought conditions, the leaves with larger surface area may undergo rapid water loss and subsequently, reduces plant water status. Having a large leaf might not be beneficial for plants because small, narrow leaves are more adapted to dry environments and lose less water than large leaves (Abrams, 1994; Teklehaimanot et al., 1998).

An additional nac019 mutant, nac019-2 should be treated with drought to see if its phenotypic changes are similar to nac019-1. In addition, a complementation test should be performed to ascertain whether two mutations occur independently (non-complementary) in the NAC019 gene. This can be achieved by crossing the two alleles of nac019 together and subjecting the progeny to drought treatment and evaluating the phenotypic changes. If the phenotype shown by the progeny is similar to nac019-1 or nac019-2, it would confirm that the phenotypic changes under drought are caused by the mutant alleles of NAC019. Alternatively, a construct containing the putative promoter of NAC019 fused to the NAC019 cDNA could be introduced into nac019-1.
or nac019-2 to rescue their phenotype under drought. By performing these experiments, a more convincing conclusion about the biological role NAC019 in floral development under drought stress can be reached.

6. REFERENCES


Su, Z., Ma, X., Guo, H., Sukiran, L., and Ma, H. Developmental and transcriptomic adaptations to drought in the Arabidopsis flower. (Submitted)


Chapter 3

TRANSCRIPTOME AND EXPRESSION ANALYSES OF THE nac019-1 MUTANT UNDER DROUGHT STRESS

(Liyana Sukiran, Zhao Su and Hong Ma)
1. ABSTRACT

Global expression profiling may shed light on the role of NAC019 regulation in drought-response and floral development. We performed microarrays on the inflorescence of nac019 during early drought stress (T3, T4 and T5) to examine the global transcriptional changes. T3 was used as the baseline and a total of 96 and 1602 genes were differentially expressed in nac019-1 at T4 and T5, respectively. A small number of differentially expressed genes at early drought treatment time points indicated that transcriptional changes are occurring in nac019 although its phenotype was relatively weak under drought stress.

Then, we compared nac019 expression to WT in order to identify the putative NAC019 target genes under drought stress. We found that many target genes were associated with abiotic stress and floral development. NAC019 may function in different signaling pathways because most of the candidate genes were responsive to abscisic acid (ABA) and jasmonic acid (JA). We analyzed NAC019 expression throughout drought stress, and the results indicated that NAC019 is preferentially expressed in the inflorescence under these conditions. The expression of several stress-responsive and floral-related genes was also examined to predict their relationship with NAC019 during drought stress. These genes might be regulated by NAC019 under drought conditions, but their interaction has not yet been definitely shown. Based on the microarray analysis, we propose a model that includes NAC019 in the stress regulatory network for floral development.
2. INTRODUCTION

Microarrays are a powerful tool used in the analysis of whole-genome expression profiles. They are widely used in investigating gene expression in various species under different treatments and in different tissue types. In Arabidopsis thaliana (hereafter Arabidopsis), many stress-inducible genes have been identified through microarray analysis (Kreps et al., 2002; Seki et al., 2002a; Seki et al., 2002b). Functional analysis of the stress-inducible genes is critical for a full understanding of the molecular mechanisms of stress response as well as for improving crop stress tolerance through gene manipulation (Seki et al., 2010).

Microarrays are used to detect gene expression levels by measuring the hybridization of mRNA to thousands of genes immobilized on a glass surface, known as a chip (Watson et al., 1998). A probe is an essential component of the microarray because its specificity and affinity determine the recognition of the target molecules on the solid surface. Hybridization between the probe and its target molecules occurs in high ionic strength buffers at relatively high temperatures. After hybridization, washing is performed to remove any cross-hybridization. Target molecules are labeled using fluorescent molecules or dye so that probe-target interactions can be detected. The intensity of fluorescent signals is detected and quantified to determine the abundance of transcripts in the target molecules. Then, normalization is performed and further data analysis is performed (Dufva, 2009).

The role of a TF in stress response can be dissected by microarray analysis because it helps identify the TF target genes. Furthermore, stress co-regulated genes can be identified through microarray analyses and hence, they are very useful in determining cis-regulatory elements. Furthermore, microarrays aid in the identification of downstream genes of a regulatory gene. This can be achieved by combining the expression data with the genomic sequence data (Seki et al., 2010). Therefore, obtaining these kinds of information can provide insight into the
transcriptional regulatory network of stress response and the molecular mechanisms underlying plant stress response.

Microarray analysis has been used to investigate expression profiling for different purposes. In the case of stress-related studies, microarrays have been performed on WT plants that were subjected to stress treatments in order to examine the global transcriptional changes associated with a particular stress phenotype. Through microarray analysis, many ABA-, drought-, cold- and salt-inducible genes were identified. Furthermore, crosstalk among these stress responses has been identified (Seki et al., 2002a; Seki et al., 2002b). The functions of genes encoding known and putative TFs in hormone signaling and stress response have been deduced by performing microarrays (Chen, 2002). Stress-responsive genes have been categorized into functional and regulatory proteins, and the information obtained from microarrays has assisted in the construction of stress regulatory network (Shinozaki and Yamaguchi-Shinozaki, 2007).

In addition to WT plants, microarrays have also been performed on transgenic plants or mutants that possess improved stress tolerance or hypersensitivity to stress in order to analyze the regulation and function of the gene of interest (Seki et al., 2010). As an example, the eskimo1 (esk1) mutant possesses freezing tolerance and a microarray conducted on the mutant identified the altered expression of TFs, signaling components and stress-responsive genes. Expression data obtained from the analysis provides hints as to how ESK1 functions as a negative regulator in cold stress response (Xin et al., 2007).

Previously, microarray analysis was performed on transgenic plants overexpressing NAC019. The 35S::NAC019 transgenic plants possess improved drought tolerance, and microarray analysis revealed that several stress-inducible genes, including GLYOXALASE 1, SUPEROXIDE DISMUTASE and KINESIN-LIKE CALMODULIN BINDING PROTEIN were up-regulated. Those genes were considered as NAC019-dependent genes because the core sequence CACG was found at their promoter regions (Tran et al., 2004).
The role of *NAC019* in drought response is particularly interesting because *NAC019* is a TF and its up-regulation could activate downstream stress-related genes. To date, only a few genes have been identified as NAC019 target genes (Tran et al., 2004), and our goal is to find more stress- and development-related target genes. In this study, we utilized the ATH1 GeneChip array to find putative NAC019 target genes under drought stress conditions in the inflorescence. Candidate genes were identified by comparing gene expression profiles between WT and *nac019* in a time-course manner (from T3 to T5). In this study, the function of *NAC019* in the inflorescence during drought stress was deduced based on the identification of its putative target genes.
3. MATERIALS AND METHODS

Sample collection, RNA isolation and microarray experiment

Wild type (WT) and *nac019-1* plants were subjected to similar drought stress conditions as described in Chapter 2. Their inflorescences were collected at days 3, 4 and 5 of drought treatment (labeled as T3, T4 and T5). These samples were frozen in liquid nitrogen and stored at -70°C until further RNA extraction. Total RNA from the inflorescences was extracted using the RNeasy Plant Mini kit (Qiagen, USA) according to the manufacturer’s instructions. Its concentration and purity were quantified with Agilent 2100 Bioanalyzer (Agilent Technologies Inc., USA) prior to cDNA preparation. In this study, we used a GeneChip microarray (ATH1 Genome Array, Affymetrix). Labeling, hybridization, washing, staining, scanning and data collection were performed at the Genomics Core Facility, at the Pennsylvania State University.

Microarray analysis

Microarray data was analyzed using the Bioconductor package in R (http://www.r-project.org). Data normalization was performed using the RMA method, and the expression values were converted to logarithm base 2 (log2). The LIMMA package was used to compare signals from WT and mutant inflorescences treated with drought at different days. Genes with at least two-fold changes and q-values (FDR) less than 0.05 were considered as differentially expressed genes. By contrast, non-differentially expressed genes were regarded as genes that have less than two-fold changes and/or q-values more than 0.05. In WT and *nac019-1*, we compared the ratio of expression values at T4 and T5 to the values at T3.

To identify putative genes that are regulated by NAC019, comparison between the genes induced in WT and down- and/or non-differentially regulated genes in *nac019-1* was performed.
To assess the biological functions of the genes, GO enrichment analysis was performed using the agriGO tools (Du et al., 2010).

Expression analyses by semi-quantitative RT-PCR and quantitative real-time PCR

One microgram of total RNA was reverse-transcribed using the GoScript™ Reverse Transcription System (Promega Corp., USA) according to the manufacturer’s instructions. The synthesized cDNA was used as a template for PCR amplifications. For semi-quantitative RT-PCR, the reaction mixture was diluted 5-fold. To determine NAC019, NAC055 and NAC072 expression in WT and nac019-1, primers listed in Table 3-1 were used. Actin2 (At3g18780) was served as an internal control.

The reaction mixture was diluted 20-fold, and quantitative real-time PCR (qPCR) was performed to determine the reliability of the microarray data as well as gene expression patterns over the time course of drought treatment. The reaction was carried out in the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Life Technologies Corp., USA), and SYBR® Select Master Mix (Life Technologies Corp., USA) was used. The reaction was performed in triplicate, and values were normalized using the internal standard, ACT2 (At3g18780). A list of primers used in the qPCR experiments is given in Table 3-1. Relative gene expression values were calculated using the delta-delta-CT method (Livak and Schmittgen, 2001).
Table 3-1. Primers used for expression analysis in WT and \textit{nac019}

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<th>Primer sequence from 5' to 3'</th>
<th>Description</th>
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<td>oMC1533</td>
<td>GGTAACATTGTGCTCAGTGGTG</td>
<td>ACT2 (F)</td>
</tr>
<tr>
<td>oMC1534</td>
<td>AACGACCCTTAATCTTCATGCTG</td>
<td>ACT2 (R)</td>
</tr>
<tr>
<td>oMC4130</td>
<td>GGGTATCCAAGAAACTGACCC</td>
<td>NAC019 cDNA (F) ¹</td>
</tr>
<tr>
<td>oMC4131</td>
<td>AACCTTTTTAGCTTAACCTC</td>
<td>NAC019 cDNA (R) ¹</td>
</tr>
<tr>
<td>oMC7818</td>
<td>CTGCTTTGAGTCTCTGTTCCA</td>
<td>ARF2 (F)</td>
</tr>
<tr>
<td>oMC7819</td>
<td>AGGGCTCATGTTTGCCTTAG</td>
<td>ARF2 (R)</td>
</tr>
<tr>
<td>oMC7824</td>
<td>GGTCTGGTACACCTTTCTT</td>
<td>SUB (F)</td>
</tr>
<tr>
<td>oMC7825</td>
<td>TAGGTTGACCCCTGACACAGAG</td>
<td>SUB (R)</td>
</tr>
<tr>
<td>oMC8112</td>
<td>CGACTGACGAAGAAGCTGATG</td>
<td>NAC055 cDNA (F) ¹</td>
</tr>
<tr>
<td>oMC8133</td>
<td>ATCCCTTCAGGTTTGTGCTG</td>
<td>NAC055 cDNA (R) ¹</td>
</tr>
<tr>
<td>oMC8116</td>
<td>TCCGTTAGCCCAAGTGTTGGTT</td>
<td>NAC072 cDNA (F) ¹</td>
</tr>
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<td>oMC4984</td>
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<td>oMC8168</td>
<td>GCATCAGACCGAAGGAAATTA</td>
<td>DREB2A (R)</td>
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¹ Semi-quantitative RT-PCR
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<td>oMC8206</td>
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1 Semi-quantitative RT-PCR
4. RESULTS

Global expression profiles of the nac019 inflorescence at T4 and T5 of drought stress

From previous microarray analysis on the WT inflorescence treated with drought, many stress-responsive genes, including NAC019, are induced at days 3, 4 and 5 (labeled as T3, T4 and T5) (Su et al., submitted). NAC019 encodes a TF (Tran et al., 2004) and was suspected to be involved in the regulation of drought acclimation-related genes. To investigate how NAC019 functions in drought stress response, we treated the nac019-1 mutant with drought. Two independent RNA samples from nac019-1 inflorescence at T3, T4 and T5 were collected and subjected to microarray analysis.

The R-value of the Pearson’s correlation coefficient was determined to investigate the relationship among genes expressed in WT and nac019 under drought stress. Before that, a pairwise comparison was subjected to all data sets before a hierarchical clustering was performed, and a heat map was generated (Fig. 3-1). For most of the data sets, their expression value was highly correlated except for WT at T3 and T5. This suggests that gene expression did not change very much in the inflorescence during the early phase of drought response.

To identify genes that were differentially expressed in WT and nac019, we compared the ratio of expression values at T4 and T5 to T3. Genes that had at least 2 fold-changes and q-values (FDR) less than 0.05 were considered as differentially expressed. In WT, a total of 1168 genes were differentially expressed at T4, with 759 and 409 genes up- and down-regulated, respectively. The number of differentially expressed genes in WT increased at T5; of 3428 of total genes, 1817 were up-regulated, and 1611 were down-regulated. In nac019, 96 (57 up-regulated, 39 down-regulated) and 1602 (979 up-regulated and 623 down-regulated) were differentially expressed at T4 and T5, respectively (Fig. 3-2).
The small number of differentially expressed genes identified in nac019 at T4 supports the relationship indicated by the correlation coefficient (Fig. 3-1). Gene expression might not change drastically, and thus only several genes could be detected at T4. Nonetheless, these results indicate that drought stress can still cause transcriptional changes in nac019, although no obvious inflorescence or floral phenotypic changes were observed. In nac019, less than 100 genes were differentially expressed at T4, but more genes were up- and down-regulated at T5. Differentially expressed genes at T4 are important for early drought response, and their up- or down-regulation may also be required for the induction or repression of late response genes at T5.

**Figure 3-1. The heat map for the expression correlation coefficients in WT and nac019**

Correlation coefficient values are indicated in the heat map bar. WT represents wild type; N19 represents nac019. T3, T4 and T5 are the days of drought treatment.
Figure 3-2. The number of differentially expressed genes in the WT and nac019 inflorescence at T4 and T5
Purple and green represent WT and nac019, respectively. T3 was used as the baseline for the expression ratio.

Identification of putative NAC019 target genes by microarray

NAC019 expression is induced in the inflorescence under drought conditions (Su et al., submitted). If NAC019 acts as a transcriptional activator, genes regulated by NAC019 should also be induced under drought stress. Therefore, to identify genes that might be regulated by NAC019, we compared the transcriptomes of WT and nac019 under drought stress. In this study, we only focused on the activator role of NAC019 during drought stress. We reasoned that the expression of the genes that were induced by NAC019 would either be repressed or unchanged in the absence of NAC019. Thus, we selected genes that were up-regulated in WT (with fold-change ≥ 2 and q-value ≤ 0.05) and down-regulated/not differentially expressed in nac019 (fold-change value < 2 and q-value > 0.05).
At T4, we identified 22,300 genes whose expression was repressed/unchanged in the mutant (Fig. 3-3). Of these, 731 genes were induced in WT and repressed/unchanged in \textit{nac019} (Fig. 3-3A). At T5, there were 21,378 genes were repressed/unchanged in \textit{nac019}, and we found more genes (1037) were up-regulated in WT and down-regulated/unchanged in \textit{nac019} (Fig. 3-3B). Then, we compared both sets to determine which genes were induced and repressed/unchanged at both T4 and T5. This yielded 274 genes that were induced at both T4 and T5, and 457 and 763 genes were specifically induced in WT at T4 and T5, respectively (Fig. 3-4). Genes induced exclusively at T4 may be transiently expressed and regulated by NAC019 whereas genes induced at T5 only might be the further downstream genes of NAC019. Therefore, GO analysis was performed using agriGO (Du et al., 2010) to examine which biological process these NAC019-regulated genes were associated with.

**Figure 3-3. Venn diagram of the genes that were induced in WT and repressed/unchanged in \textit{nac019}**

The total number of genes identified at (A) T4 and (B) T5 is shown in brackets. Up-regulated genes in WT are indicated with red circles and down-regulated/unchanged genes in \textit{nac019} are indicated with gray circles.
NAC019 positively regulates transcription factors that are involved in stress response

TFs play an important role in gene regulation because of their ability to bind to the upstream region of target genes and promote gene activation or repression. Induction of regulatory proteins is essential in the early stress response as it triggers signal transduction pathways and consequently activates the expression of genes that are involved in slow and adaptive stress responses (Yamaguchi-Shinozaki and Shinozaki, 2006). Through GO term analysis, we found a significant over-representation of genes in the ‘regulation of transcription’ category (55/454, p-value = 4.73e-04). This result suggests that NAC019 activates other TFs in the WT inflorescence during early drought stress.

MAPMAN analysis (Thimm et al., 2004) classified those genes into several groups of TF gene families that are known to be involved in stress response (Table 3-2). There were six members of the AP2/ERF family that were induced in WT but not in nac019. One of them was
DREB2A, which functions in salt and drought stress by regulating stress-responsive genes through the DRE/CRT core sequence (Nakashima et al., 2000; Sakuma, 2002). This suggests that part of the rapid stress response by NAC019 regulation may also be mediated through an ABA-independent signaling pathway. Furthermore, other AP2/ERF members, such as DREB26, RAP2.6 and RAP2.6L, were also induced in WT but not in nac019. These genes were induced by stress hormones (JA and SA) and abiotic stresses (drought, heat and salt). The abundance of their transcripts in the inflorescence suggests that they might function in floral development under stress conditions (Krishnaswamy et al., 2011).

Another TF family with many members found in this group is the WRKY family, which is known to play a role in plant defense response and regulation of abiotic stress response through the ABA signaling pathway (Rushton et al., 2012). WRKY18, WRKY33 and WRKY40 increase plant tolerance to salt stress (Jiang and Deyholos, 2009; Chen et al., 2010b) whereas WRKY46, WRKY53 and WRKY70 positively regulate basal resistance to Pseudomonas syringae (Hu et al., 2012). Induction of WRKY genes in the WT inflorescence but not in nac019 suggests that NAC019 may be involved in biotic stress by regulating WRKY gene expression. Therefore, this result supports previous findings suggesting a role for NAC019 in plant defense response (Bu et al., 2008).

Other TFs including members of the MYB family such as MYB73 and MYB77 were induced in WT but not in nac019. Both of these genes are associated with stress response (Jung et al., 2008), and MYB77 modulates auxin signal transduction during lateral root growth formation (Shin et al., 2007). NAC019 regulation may also be involved in the activation of other gene families such as basic helix-loop-helix (bHLH), HB (homeobox leucine zipper), C3H zinc fingers, heat shock factor (HSF) and NAC itself (Table 3-2).
Table 3-2. Transcription factors that were induced in WT and repressed/unchanged in *nac019* at T4 only

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<th>TF gene family</th>
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<th>Expression Ratio</th>
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<td></td>
<td></td>
<td>WT</td>
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<tr>
<td>AP2/ERF</td>
<td>At5g05410</td>
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<td>At5g13330</td>
<td><em>RELATED TO AP2 6-LIKE</em> (<em>Rap2.6L</em>)</td>
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<td></td>
<td>At1g21910</td>
<td><em>DRE-BINDING PROTEIN 26</em> (<em>DREB26</em>)</td>
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<td>At1g43160</td>
<td><em>RELATED TO AP2 6</em> (<em>RAP2.6</em>)</td>
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</table>
**NAC019 regulates hormone-mediated and stress-responsive genes during early drought response**

Consistent with the role of *NAC019* as a stress-responsive gene, we found that the GO term ‘response to stimulus’ was highly enriched in the set of genes that were induced in WT but not in *nac019*. Under this category, these genes were mainly associated with stress and hormone response. Among 457 genes that were exclusively up-regulated at T4, 70 of them were involved in response to water deprivation, cold, wounding, defense and oxidative stress (*Table 3-3*). By contrast, only 37 out of 274 induced genes at both T4 and T5 were involved in response to water deprivation, cold and high salinity (*Table 3-4*). Different stresses may trigger genes that are involved in some common signaling pathways (Seki et al., 2002b). Therefore, *NAC019* may be involved in the crosstalk between abiotic stress signaling pathways because it is able to regulate the expression of genes involved in various stresses. This is also supported by previous studies that have shown the involvement of *NAC019* in ABA and jasmonate response during abiotic and biotic stress (Bu et al., 2008; Jiang et al., 2009).

Several genes that are regulated by hormone signaling might also be affected by *NAC019* during early drought stress. Of the 457 genes that were exclusively induced at T4, 36 of them were hormone-responsive. These genes were mainly associated with response to JA, auxin and ethylene (*Table 3-5*). Of the genes that were induced at both T4 and T5, 23 of them were mainly responsive to ABA and JA (*Table 3-6*). Additionally, 25 genes induced exclusively at T5 were ABA-responsive genes only (*Table 3-7*).

The over-representation of JA-responsive genes was expected because *NAC019* expression is influenced by methyl jasmonate (MeJA), which is the precursor of JA (Tran et al., 2004). JA has been shown to be involved in plant defense, stress response and male reproductive development (Creelman and Mullet, 1995). Therefore, it is tempting to speculate that *NAC019*
plays a role in floral development under drought stress through its regulation of JA-regulated genes such as *MYB21* and *MYB24*.

Moreover, the over-representation of ABA-responsive genes also suggests that their induction might be regulated by *NAC019*. Not only is ABA production essential during stress response but *NAC019* is also one of the genes that are induced by ABA (Tran et al., 2004). NAC019 might be involved in the ABA-dependent signaling pathway during drought stress response in the inflorescence. Among ABA-responsive genes, we identified two calcium-dependent protein kinases (CDPKs), *CPK6* and *CPK32*. Previous studies demonstrated that *CPK6* is involved in ABA and MeJA signaling in guard cells (Mori et al., 2006; Munemasa et al., 2011). *CPK6* induction in the WT inflorescence suggests that ABA and MeJA crosstalk signaling also occurs in the reproductive tissue. Additionally, CPK32 was found to interact with ABF4 and positively modulate its expression, which demonstrates that *CPK32* has a role in ABA signaling during stress response (Choi et al., 2005). In WT, induction of both *CPK6* and *CPK32* could trigger the expression of downstream ABA-responsive genes. However, their expression was not induced in *nac019*. This suggests that *NAC019* may be involved in ABA and/or JA signaling pathways through its regulation of *CPK6* and/or *CPK32*.

Our analysis also revealed that *DREB2B* was induced in the WT inflorescence but not in *nac019* at both T4 and T5. Its role in response to drought has been characterized previously (Nakashima et al., 2000; Sakuma, 2002). Additionally, *RD19* (RESPONSIVE TO DEHYDRATION 19), which encodes a cysteine protease, was also identified. Neither *DREB2B* nor *RD19* requires ABA for their induction during stress response (Yamaguchi-Shinozaki et al., 1992; Nakashima et al., 2000). This suggests that *NAC019* might be involved in the ABA-independent signaling pathway in the inflorescence via mediation of *DREB2B* and *RD19* expression.
<table>
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<tr>
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<th>Stress</th>
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Table 3-4. Genes induced in WT and repressed/unchanged in nac019 at T4 and T5 (GO category: Response to stress)

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Table 3-5. Genes induced in WT and repressed/unchanged in \textit{nac019} at T4 only (GO category: Response to hormone)

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<td>2.23</td>
</tr>
<tr>
<td>At4g17490</td>
<td>\textit{ETHYLENE RESPONSIVE FACTOR 6 (ERF6)}</td>
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<td>Ethylene At2g44840</td>
<td>\textit{ETHYLENE-RESPONSIVE FACTOR 13 (ERF13)}</td>
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<td>At4g37260</td>
<td>\textit{MYB73}</td>
<td>1.85</td>
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<td>2.06</td>
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<tr>
<td>At5g67300</td>
<td>\textit{MYBR1}</td>
<td>1.77</td>
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Table 3-6. Genes induced in WT and repressed/unchanged in *nac019* at T4 and T5 (GO category: Response to hormone)

<table>
<thead>
<tr>
<th>AGI</th>
<th>Gene name</th>
<th>Expression ratio</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>WT T4/T3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nac019 T5/T3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T4/T3 T5/T3</td>
</tr>
<tr>
<td>At2g17290</td>
<td><strong>CALCIUM DEPENDENT PROTEIN KINASE 6 (CPK6)</strong></td>
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<tr>
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<td><strong>CALCIUM-DEPENDENT PROTEIN KINASE 32 (CPK32)</strong></td>
<td>-0.26 0.49</td>
</tr>
<tr>
<td>At3g57530</td>
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<td></td>
<td><strong>CORONATINE INDUCED 1 (CORI1)</strong></td>
<td>-0.09 0.71</td>
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<tr>
<td>At1g29395</td>
<td><strong>EARLY FLOWERING 3 (ELF3)</strong></td>
<td>1.16 1.23</td>
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<td></td>
<td><strong>GATED OUTWARDLY-RECTIFYING K+ CHANNEL (GORK)</strong></td>
<td>0.50 0.73</td>
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<tr>
<td>At4g23600</td>
<td><strong>MYB6</strong></td>
<td>1.33 1.43</td>
</tr>
<tr>
<td></td>
<td><strong>PROTEIN PHOSPHATASE 2CA (PP2CA)</strong></td>
<td>0.04 0.70</td>
</tr>
<tr>
<td>At3g05880</td>
<td><strong>RARE-COLD-INDUCIBLE 2A (RCI2A)</strong></td>
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<td><strong>RECEPTOR-LIKE PROTEIN KINASE 1 (RPK1)</strong></td>
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<tr>
<td>At1g69270</td>
<td><strong>REVEILLE 2 (RVE2)</strong></td>
<td>1.54 2.08</td>
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<td></td>
<td><strong>RING/U-box superfamily protein</strong></td>
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<td>At5g37260</td>
<td><strong>SIZI</strong></td>
<td>2.30 2.96</td>
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<td><strong>TRIACYLGLYCEROL BIOSYNTHESIS DEFECT 1 (TAG1)</strong></td>
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<td></td>
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<td>-0.08 0.16</td>
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<td>At2g19450</td>
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<tr>
<td></td>
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<td>0.32 0.97</td>
</tr>
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<td>At1g19180</td>
<td><strong>JASMONATE-ZIM-DOMAIN PROTEIN 1 (JAZ1)</strong></td>
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</tr>
<tr>
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<td></td>
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<tr>
<td>At3g27810</td>
<td><strong>MYB21</strong></td>
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<td></td>
<td>0.19 0.79</td>
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<td><strong>MYB24</strong></td>
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<td>-0.18 0.25</td>
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<td><strong>MYB6</strong></td>
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<td>0.04 0.70</td>
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<tr>
<td>At2g28900</td>
<td><strong>OUTER PLASTID ENVELOPE PROTEIN 16-1 (OEP16-1)</strong></td>
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<td></td>
<td></td>
<td>0.27 -0.67</td>
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<tr>
<td>At5g37260</td>
<td><strong>REVEILLE 2 (RVE2)</strong></td>
<td>3.20 4.75</td>
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<tr>
<td></td>
<td></td>
<td>-0.56 0.75</td>
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Table 3-7. Genes induced in WT and repressed/unchanged in nac019 exclusively at T5 (GO category: Response to ABA stimulus)

<table>
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<th>Gene name</th>
<th>Expression ratio</th>
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<tr>
<td>At2g36270</td>
<td>ABA INSENSITIVE 5 (ABI5)</td>
<td>1.87</td>
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<tr>
<td>At1g54100</td>
<td>ALDEHYDE DEHYDROGENASE 7B4 (ALDH7B4)</td>
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<td>At4g25000</td>
<td>ALPHA-AMYLASE-LIKE (AMY1)</td>
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<td>0.42</td>
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<td>At1g35720</td>
<td>ANNEXIN 1 (ANNAT1)</td>
<td>1.08</td>
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<td>At1g01720</td>
<td>ATAF1</td>
<td>1.61</td>
<td>0.79</td>
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<td>At4g38230</td>
<td>CALCIUM-DEPENDENT PROTEIN KINASE 26 (CPK26)</td>
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<td>At1g74740</td>
<td>CALCIUM-DEPENDENT PROTEIN KINASE 30 (CPK30)</td>
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<td>0.67</td>
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<td>At1g20630</td>
<td>CATALASE 1 (CAT1)</td>
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<tr>
<td>At1g76180</td>
<td>EARLY RESPONSE TO DEHYDRATION 14 (ERD14)</td>
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<td>At2g18960</td>
<td>$H(+)\text{-ATPASE 1 (HA1)}$</td>
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<td>IRON-REGULATED TRANSPORTER 1 (IRT1)</td>
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<td>LIPID TRANSFER PROTEIN 3 (LTP3)</td>
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<tr>
<td>At1g55020</td>
<td>LIPXYGENASE 1 (LOX1)</td>
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<td>At3g63210</td>
<td>MARD1</td>
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<td>At3g06490</td>
<td>MYB108</td>
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<td>0.53</td>
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<td>At1g22640</td>
<td>MYB3</td>
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<td>0.62</td>
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<td>At5g16600</td>
<td>MYB43</td>
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<td>MYB94</td>
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<td>0.76</td>
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<td>At5g62470</td>
<td>MYB96</td>
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<tr>
<td>At4g33950</td>
<td>OPEN STOMATA 1 (OST1)</td>
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<td>RELA/SPOT HOMOLOG 2 (RSH2)</td>
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<td>0.86</td>
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<td>At2g33380</td>
<td>RESPONSIVE TO DESSICATION 20 (RD20)</td>
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<td>0.70</td>
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<td>At1g15100</td>
<td>RING-H2 FINGER A2A (RHA2A)</td>
<td>1.47</td>
<td>-0.02</td>
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<tr>
<td>At3g50500</td>
<td>SNF1-RELATED PROTEIN KINASE 2.2 (SNRK2.2)</td>
<td>1.27</td>
<td>0.65</td>
</tr>
</tbody>
</table>
NAC019 regulates genes involved in floral development during early drought response

Among the 270 genes induced in WT during early drought stress, 14 genes were over-represented in the floral development category (Table 3-8). This suggests that NAC019 may also be involved in flower-related gene expression under drought stress. Several genes in this category are encoding TF. One of them is a basic helix-loop-helix (bHLH) TF known as BIGPETALp (BPEp). It is regulated by AP3 and PI, and controls petal size by restricting cell growth (Szcscsi et al., 2006). Other TFs included MYB21 and MYB24, which play a role in mediating jasmonate response during petal and stamen maturation (Mandaokar et al., 2006; Mandaokar and Browse, 2009), and gynoecium growth (Reeves et al., 2012). Moreover, AUXIN RESPONSE FACTOR 2 (ARF2), a repressor of cell division, was also induced in WT but not in nac019. The arf2 flower mutant displays elongated sepals and gynoecium, which restricts the anther to deliver its pollen to the stigma surface and thus causes infertility (Okushima et al., 2005; Schruff et al., 2006).

Additionally, STRUBBELIG (SUB), a receptor-like kinase that functions in floral development was also identified. SUB is essential for the formation of the outer integument and the correct shape of the gynoecium and petals, as well as for the control of cell shape and cell proliferation in the L2 layer of floral meristems and the L1-derived outer integument of ovules (Chevalier et al., 2005). Moreover, SUB also participates in the coordination of cell morphogenesis between cell layers during floral development (Yadav et al., 2008). ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1) was induced in WT but not in nac019. ADPG1 is a polygalacturonase that is required in cell separation during reproductive development. Its loss of function delays anther dehiscence and floral organ senescence.

All the genes described here are involved in floral organ development. BPEp, SUB and ADPG1 play a role in cell morphogenesis and cell separation whereas reduced MYB21, MYB24
and ARF2 expression in the flower cause sterility. The coordination between filament elongation and pistil length in Arabidopsis is crucial to ensure that the pollen can be delivered to the stigma for further fertilization. The induction of these genes in the WT inflorescence during early drought stress may be necessary to sustain functional flower structure. Although there was no significant change in stamens and pistils at early time points (Fig. 2-7 and Fig. 2-8), it is possible that they are regulated by NAC019 under prolonged drought stress. It is also possible that their expression was not completely reduced in the nac019 mutant, ameliorating phenotypic changes.

Table 3-8. Genes induced in WT and repressed/unchanged in nac019 at T4 and T5 (GO category: Floral development)

<table>
<thead>
<tr>
<th>AGI</th>
<th>Gene name</th>
<th>Expression Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T4/T3</td>
</tr>
<tr>
<td>At3g57510</td>
<td>ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1)</td>
<td>1.45</td>
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<tr>
<td>At5g62000</td>
<td>AUXIN RESPONSE FACTOR 2 (ARF2)</td>
<td>3.62</td>
</tr>
<tr>
<td>At1g59640</td>
<td>BIG PETAL P (BPEP)</td>
<td>1.13</td>
</tr>
<tr>
<td>At5g15840</td>
<td>CONSTANS (CO)</td>
<td>1.38</td>
</tr>
<tr>
<td>At2g25930</td>
<td>EARLY FLOWERING 3 (ELF3)</td>
<td>1.41</td>
</tr>
<tr>
<td>At1g77300</td>
<td>EARLY FLOWERING IN SHORT DAYS (EFS)</td>
<td>1.35</td>
</tr>
<tr>
<td>At1g68050</td>
<td>FLAVIN-BINDING, KELCH REPEAT, F BOX 1 (FKF1)</td>
<td>1.56</td>
</tr>
<tr>
<td>At1g65480</td>
<td>FLOWERING LOCUS T (FT)</td>
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<td>At3g05120</td>
<td>GA INSENSITIVE DWARFIA (GID1A)</td>
<td>1.13</td>
</tr>
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<td>At1g19180</td>
<td>JASMONATE-ZIM-DOMAIN PROTEIN 1 (JAZ1)</td>
<td>1.81</td>
</tr>
<tr>
<td>At3g27810</td>
<td>MYB 21</td>
<td>1.72</td>
</tr>
<tr>
<td>At5g40350</td>
<td>MYB 24</td>
<td>1.00</td>
</tr>
<tr>
<td>At5g37260</td>
<td>REVEILLE 2 (RVE2)</td>
<td>3.20</td>
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<tr>
<td>At1g11130</td>
<td>STRUBBELIG (SUB)</td>
<td>2.88</td>
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</table>
Expression analysis on WT and nac019-1 during early drought response

To determine the expression of putative NAC019 target genes, we first performed semi-quantitative RT-PCR on the WT and nac019 inflorescence collected at T3, T4 and T5. Three genes were selected: NAC019, ARF2 and SUB (Fig. 3-5). Then, we performed qPCR to confirm their expression. The NAC019 expression pattern was examined, and the result indicated that it has a similar expression pattern as was seen in our microarray data. Induction of NAC019 was detected at T3, and its expression gradually increased at T4 and T5 (Fig. 3-6A). This result also further confirmed that NAC019 expression was abolished in the nac019 mutant.

ARF2 and SUB were selected because both genes play a role in floral development and because they were induced in WT but not in nac019 at both T4 and T5 (Table 3-7). ARF2 expression in WT was dramatically induced by drought at T3 and T4, but it was greatly reduced at T5. Likewise, the SUB expression pattern was similar to ARF2, although its overall expression was lower than ARF2. By contrast, their expression was repressed in nac019 at all selected time points (Fig. 3-6B and Fig. 3-6C). qPCR analysis indicated that both genes were highly expressed in WT at T3 and T4, and then reduced at T5. However, based on the microarray result, their expression gradually increased from T3 to T5. The results may be inconsistent because we used different samples for the microarray and qPCR. Hence, their expression might have been affected by the growth conditions at that time.
Figure 3-5. Semi-quantitative RT-PCR on the WT and *nac019* inflorescence at T3, T4 and T5

The expression of *NAC019*, *ARF2* and *SUB* was determined by RT-PCR. Twenty-eight cycles were used for amplification of all genes except for *ACT2*, where 23 cycles were used.
Figure 3-6. Expression profiles of NAC019, ARF2 and SUB from T3 to T5 in the WT and nac019 inflorescence

The expression of (A) NAC019, (B) ARF2 and (C) SUB in WT (purple) and nac019-1 (green) was determined by qPCR. Bars represent the mean of gene expression from three replicates and error bars represent the SD value.
Expression analysis of the WT and nac019-1 inflorescence over a time course of drought stress

By examining gene expression patterns at different time points, we can decipher NAC019 function and regulation in the development of the inflorescence during drought stress. In order to identify gene expression pattern changes from the beginning through the end of the drought treatment, we divided the time points into several stages: early, intermediate, severe and recovery stage. Early drought stage was represented by T3 and T5, and T7 was chosen as intermediate stage. At all these points, no phenotypic changes were observed for either the flower or the inflorescence of WT and nac019. T10 was regarded as the severe stage because the inflorescence was no longer developing in WT and nac019. In WT, the recovery stage was represented by T14 because new flowers were produced from the newly recovered inflorescence. In contrast, T14 was still considered as the severe stage for nac019 because its inflorescence had not yet recovered. T20 was regarded as the recovery stage for nac019 when new open flowers emerged (Fig. 2-7 and Fig. 2-8).

NAC019 is preferentially expressed in the inflorescence throughout drought stress

Semi-quantitative RT-PCR indicated that NAC019 was present in the inflorescence throughout drought stress (Fig. 3-7A). To determine the difference of NAC019 transcript levels in the vegetative and reproductive tissues, we examined its expression in the leaf and the inflorescence from WT plants that were subjected to drought. From qPCR analysis, the results showed that NAC019 was induced in both organs throughout drought period. However, its expression in the leaves was lower than in the inflorescences. NAC019 was slightly induced in the leaves at T5 before its expression decreased at T7, and gradually increased until T20. By contrast, NAC019 expression in the inflorescences was progressively induced by drought, and the highest
expression was observed at T10. During recovery stage (T14 and T20), NAC019 expression was gradually decreased (Fig. 3-7B). These results indicate that NAC019 is preferentially expressed in the inflorescence throughout drought period, suggesting that it is important in regulating floral development during drought stress response.

It is surprising that NAC019 was highly induced at T10, when the inflorescence was small and undeveloped. Overexpression of NAC019 confers drought tolerance to transgenic plant vegetative tissue, but its effect on the flower and the inflorescence is unknown (Tran et al., 2004). It is possible that high expression of NAC019 in the inflorescence inhibits floral meristem or floral organ growth in WT. Additionally, induction of NAC019 at T14, although was relatively lower than T10, demonstrates that it may be important for inflorescence recovery. NAC019 may be required for the functional restoration of floral organs so that young flower buds can continue to develop as the plant acclimatizes to drought conditions. However, how NAC019 expression affects inflorescence growth remains unclear because its high expression at T10 seems to have negative effects on inflorescence development. At recovery stage, NAC019 was reduced. At this time, the inflorescence might have adapted to drought stress, and NAC019 may be essential only for the regulation of stress-responsive genes, rather than flower related genes.
Figure 3-7. \textit{NAC019} expression pattern in the WT inflorescence under drought stress

(A) Semi-quantitative RT-PCR of \textit{NAC019} expression in the inflorescence. (B) qPCR analysis of \textit{NAC019} expression in the leaf (dash line) and the inflorescence (solid line). \textit{ACT2} was used as an internal standard, and T0 was used as a control. The mean of the expression values from three replicates was shown and error bars represent the S.D. value.

\textit{NAC055} and \textit{NAC072} were expressed in the nac019 inflorescence under drought conditions

Based on the phylogenetic analysis of 108 NAC proteins in \textit{Arabidopsis}, NAC019 is closely related with NAC055 and NAC072, and they were classified in group III-3 (Jensen et al., 2010). We speculated that they may have overlapping function in drought stress response. Therefore, we performed qPCR to examine \textit{NAC055} and \textit{NAC072} expression changes in WT and nac019 under drought stress. Interestingly, the expression pattern of \textit{NAC019}, \textit{NAC055} and \textit{NAC072} were almost similar in WT (Fig. 3-7B and Fig. 3-8A). Their transcripts can be seen as
drought progressed, and the highest expression was observed at T10; when the WT inflorescence had severely affected by drought. Finally, their expression was gradually reduced from T14 to T20 (Fig. 3-8A). This result suggests that, other than NAC019, its homologs are also important for the inflorescence response to drought. In the nac019 mutant, induction of NAC055 and NAC072 can be seen at T10 and T14. At these days, nac019 had not yet recovered from drought. It is possible that their high expression during severe stage might be the reason why delayed inflorescence recovery was observed in nac019. By contrast, reduced NAC055 and NAC072 expression at T20 might promote the recovery of the nac019 inflorescence (Fig. 3-8B).

It is very likely that NAC055 and NAC072 expression during drought stress is not regulated by NAC019 because their expression in nac019, especially at T10 and T14 was not completely abolished. This supports our hypothesis that NAC019, NAC055 and NAC072 might function redundantly during drought stress response, and why such a relatively weak phenotype of nac019-1 was observed. Hence, generating a nac019 nac055 nac072 triple mutant may assist us in determining the biological function of NAC019 under drought conditions.
Figure 3-8. Expression patterns of NAC genes in the WT and nac019 inflorescence under drought stress

The expression of NAC055 (purple line) and NAC072 (orange line) throughout drought stress in (A) WT and (B) nac019 was examined by qPCR. ACT2 was used as an internal standard and T0 was used as a control. The mean of the expression values from three replicates was shown and error bars represent the S.D. value.

Expression analysis of putative NAC019 target genes

To address the question whether NAC019 is necessary in regulating its putative target genes involved in floral development under drought stress, we examined the time course expression of these genes in the inflorescence of WT and nac019 under drought conditions. These genes were selected based on their relative expression in microarray data and their functional category.

Quantitative real-time PCR (qPCR) analyses revealed that stress-responsive genes such as DREB2A, DREB2B, ARF2 and MYC2 (Nakashima et al., 2000; Abe et al., 2003; Sakuma et al., 2006; Wang et al., 2011) were slightly induced in the WT inflorescence at early and intermediate stages of drought conditions. The expression of DREB2A and DREB2B was highly induced at T10 (severe stage), and gradually decreased at the recovery stage (T14 and T20). Note that, the expression pattern of these genes was similar as the expression pattern showed by NAC019 under
drought stress (Fig. 3-7B). Additionally, the expression of ARF2 and MYC2 was progressively increased and reached their highest expression at T14 before decreased at T20 (Fig. 3-9A). At T10, all stress-responsive genes we examined exhibited a slight induction in nac019, except for ARF2. However, compared to WT, their expression was relatively lower in the nac019 mutant (Fig. 3-9A). These results suggest that the presence of NAC019 in the inflorescence is required for the elevated expression of stress-responsive genes during drought.

The transcript of floral development-related genes was also examined by qPCR to determine how NAC019 affects their expression under drought stress. We still found that the expression of these genes in WT were higher than in nac019. This also suggests that NAC019 is required for the induction of floral-related genes under drought stress. BPEp, MYB21 and MYB24 are involved in the development of petals and stamens in Arabidopsis (Mandaokar et al., 2006; Szecsi et al., 2006; Cheng et al., 2009). Their expression was gradually increased until at T7, and was reduced at severe stage of drought stress. However, BPEp was re-induced at T14, whereas both MYB21 and MYB24 were induced again at T20 (Fig. 3-9B). Reduced expression of these genes may prevent floral buds maturation at T10, when no open flowers were produced and the inflorescence was undeveloped. Besides that, ADPG1 was gradually induced in WT until T20, and its expression level was greater than in nac019 (Fig. 3-9B). ADPG1 is mainly expressed in the mature anthers and mature siliques, and encodes a polygalacturonase, which involved in cell separation during anther dehiscence and pod shatter (Ogawa et al., 2009). It is unclear how ADPG1 functions under drought stress but its high expression may promote premature dehiscence of the anthers in the inflorescence.

Altogether, our expression analyses suggest that NAC019 regulation is required for the expression of drought-responsive genes and floral development genes under drought conditions. NAC019 may regulate the expression of these genes throughout drought periods to maintain the functional floral structure so that the effects of drought on reproductive development can be
minimized. However, the fact that other factors may be involved in the regulation of NAC019-regulated genes under drought stress cannot be excluded because their expression still can be detected in the nac019 mutant.

Figure 3-9. Expression patterns of several putative NAC019 target genes in the WT and nac019 inflorescence under drought stress.

The expression of genes involved in (A) stress response and (B) floral development was examined by qPCR. ACT2 was used as an internal standard and T0 was used as a control. WT and nac019 is coded by blue and green lines, respectively. The mean of the expression values from three replicates was shown and error bars represent the S.D. value.
5. DISCUSSION

We conducted microarray analysis on the nac019 mutant that had been subjected to drought, in order to investigate nac019 transcriptomic changes and to identify putative NAC019 target genes. We examined transcriptional changes at different time points, using T3 as a baseline for comparison with T4 and T5. For the purpose of identifying putative NAC019 target genes, we compared expression profiles of nac019 and WT at similar time points.

We identified genes that were differentially expressed in the inflorescence of WT and nac019 at T4 and T5. Relative to WT, only a small number of genes were identified in nac019 at T4, but that number increased at T5 (Fig. 3-2). This is consistent with the correlation coefficient heat map that showed a high correlation between the nac019 data set at T3 and T4 but not between T3 and T5 (Fig. 3-1). It is possible that nac019 has a slower response during the beginning of drought stress. Thus, only small changes in gene expression occur in the mutant. Alternatively, using T0 as the baseline instead of T3 might reveal greater expression changes.

To examine the possible relationship between the biological function of putative NAC019 target genes and drought response time, GO analysis was performed. We regarded genes that were induced exclusively at T4 as NAC019 transiently regulated and found that they were mainly associated with transcription regulation and response to stress and hormones (Table 3-2, Table 3-3 and Table 3-5). This is not surprising because the rapid and emerging response to stress involves the up-regulation of TFs that can induce downstream gene expression for further adaptive responses (Yamaguchi-Shinozaki and Shinozaki, 2006). Interestingly, we found members of several TF families that might be regulated by NAC019 (Table 3-2). This suggests that NAC019 transcriptional regulation during early drought response affects several TF gene families that are also involved in various responses to stress and hormones.
Furthermore, identification of these genes suggests that *NAC019* plays an important role in the inflorescence during the response to drought stress. Many putative NAC019 downstream genes were not only responsive to drought stress but also responsive to cold, wounding, defense and oxidative stress (*Table 3-3*). Moreover, our data supports a role for NAC019 in regulating stress-responsive genes through the ABA-dependent and ABA-independent signaling pathways, as well as the JA signaling pathway (*Table 3-6* and *Table 3-7*). This suggests that the involvement of *NAC019* is necessary in various stress response and signaling pathways. Besides that, our expression analyses also demonstrated the importance of NAC019 in regulating the expression of floral-related and stress-responsive genes under drought conditions (*Fig. 3-9*). This is because the genes we examined had shown high expression in WT but reduced expression in *nac019*.

*DREB2A* and *DREB2B* were highly induced in WT but not in *nac019* (*Fig. 3-9A*). Both genes are involved in osmotic stress response by binding to the DRE/CRT sequence in the promoter regions of their target genes (Sakuma et al., 2006a; Sakuma et al., 2006b). To date, many functional studies have been performed on *DREB2A* but not on *DREB2B* (Sakuma et al., 2006b; Sakuma et al., 2006a; Schramm et al., 2008; Yoshida et al., 2008). Recently, a study found that the *dreb2b* mutant has smaller pollen grains, suggesting that it may function in male gametophyte development but the mechanism remains unknown (Reňák et al., 2012). Identification of these genes suggests that *NAC019* may play a role in the ABA-independent signaling pathway by regulating their expression in the inflorescence during drought stress.

In addition, we also observed induction of several genes related to the JA signaling pathway in WT but not in *nac019* (*Table 3-6* and *Table 3-8*). Among them are *JAZ1*, *MYC2*, *MYB21* and *MYB24*, which are involved in jasmonate response during floral maturation (Reeves et al., 2012). Additionally, *BPEp*, a gene involved in mediating JA signaling during petal growth was also found (Szecsi et al., 2006; Brioudes et al., 2009). Reduced expression of these genes in
nac019 suggests that JA signaling in the inflorescence might be affected by drought, which consequently could affect jasmonate-regulated floral development.

MYC2 is a central regulator in the JA signaling pathway in Arabidopsis and its expression is regulated in a CORONATINE INSENSITIVE1 (COI1)-dependent manner (Lorenzo et al., 2004). The JAZ proteins, repressors of JA-responsive genes, interact with MYC2 in the absence of jasmonate-isoleucine (JA-Ile) and prevent MYC2 from activating the expression of JA-responsive genes (Chini et al., 2007). JA-Ile is a conjugated form of JA and binds to COI1, which leads to ubiquitination and degradation of JAZ proteins. Consequently, the inhibitory effect on MYC2 is released, and downstream target gene expression is activated (Chini et al., 2007; Thines et al., 2007). It was unexpected that MYC2 expression in nac019 was lower than in WT (Fig. 3-9A) because NAC019 is the downstream target of MYC2 in the JA signaling defense response (Bu et al., 2008). However, its reduced expression in nac019 might be due to the repressive effect of TIME FOR COFFEE (TIC). TIC is a circadian-clock component that interacts with MYC2 and inhibits its activity in JA signaling (Shin et al., 2012). In our microarray data, TIC was highly expressed in the WT and nac019 inflorescence under drought stress.

Apart from the function of MYC2 in defense response, its role in floral development is unknown although its expression in the stamen is induced by jasmonate (Mandaokar et al., 2006). In the reproductive organ, MYB21 and MYB24 are the components of JA signaling that interact with the JAZ proteins (JAZ1, JAZ8 and JAZ11) and regulate male fertility, particularly filament elongation and anther development (Mandaokar et al., 2006; Song et al., 2011). Both genes had reduced expression in nac019, suggesting that their expression might be dependent on NAC019 (Fig. 3-9B). To date, there is no data on the relationship between MYC2 and MYB21 or MYB24 in jasmonate response. Under drought conditions, it is possible that MYC2 regulates MYB21 and MYB24 expression through NAC019.
ARF2 was expressed throughout drought stress in WT, and this was consistent with the expression pattern of NAC019 (Fig. 3-7B and Fig. 3-9A). Previous studies have demonstrated the functions of ARF2 in floral organ development (Ellis et al., 2005; Okushima et al., 2005). However, our qPCR analysis suggests that ARF2 behaved more like stress-responsive gene since its expression pattern was similar as other stress-responsive genes we examined (Fig. 3-9A). ARF2 is also involved in ABA mediating seed germination and primary root growth (Wang et al., 2011). In our study, it is possible that ARF2 may be involved in drought stress by mediating ABA response in the inflorescence. Under drought conditions, the inflorescence might contain high levels of ABA, which can induce NAC019 and subsequently activate ARF2 expression. However, the relationship between NAC019 and ARF2 has not been clearly demonstrated. Although ARF2 is a NAC019-regulated gene, it is also possible that other factors are required for its activation.

Based on our data, we propose a model showing a role for NAC019 in the stress transcriptional regulatory network for floral development under drought conditions (Fig. 3-11). First, NAC019 may function in the ABA-independent signaling pathway through its regulation of drought-responsive genes, DREB2A and DREB2B. Low DREB2B expression levels cause smaller pollen grains but how it affects fertility is still unknown (Reňák et al., 2012). Second, NAC019 may play a role in the ABA-dependent signaling pathway in the inflorescence by regulating ARF2 expression, which is also an ABA-responsive gene (Wang et al., 2011). Finally, NAC019 may be involved in JA-signaling during floral development because jasmonate-responsive genes such as BPEp, MYB21 and MYB24 were highly induced in WT but not in nac019. The activation of these genes may be essential for sustaining the coordination between pistil and stamens, restoration of floral organs and successful pollination during drought stress.

Overall, our microarray data suggests that NAC019 may be involved in different signaling pathways by regulating genes such as DREB2B, ARF2, MYB21, MYB24, BPEp and others. However, whether this interaction is direct or indirect needs to be determined for the
validation of the model. Furthermore, chromatin immunoprecipitation (ChIP) should be conducted to confirm the NAC019 direct target genes under drought stress.

![Diagram showing the role of NAC019 in drought stress regulatory networks during floral development](image)

Figure 3-10. A model showing the role of NAC019 in drought stress regulatory networks during floral development

NAC019 may be involved in the (A) ABA-independent, ABA-dependent and (B) JA signaling pathways through regulation of the expression of genes indicated above. ARF2, MYB21, MYB24 and BPEp have been reported to play a role in floral development. DREB2B function in drought response is known but has not been shown for floral development.
6. ACKNOWLEDGEMENTS

I would like to thank the individuals in the Genomic Core Facility at University Park, including Dr. Craig Paul, who helped us perform the microarray hybridizations and Dr. Deborah Grove, for her assistance in using the qPCR machine. I also would like to thank Dr. Zhao Su and Dr. Kuangyu Yen for their suggestions in analyzing microarray data.

7. REFERENCES

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Su, Z., Ma, X., Guo, H., Sukiran, L., and Ma, H. Developmental and transcriptomic adaptations to drought in the Arabidopsis flower. (Submitted).


Chapter 4

GENERATION OF TRANSGENIC PLANTS CARRYING 35S::NAC019-GR-3xFLAG AND pNAC019::NAC019-GR-3xFLAG CONSTRUCTS FOR THE IDENTIFICATION OF NAC019 DIRECT TARGET GENES

(Liyana Sukiran, Zhao Su and Hong Ma)
1. ABSTRACT

The transcriptome profile of *nac019* was analyzed, and we identified putative genes regulated by NAC019 under drought stress. However, it is unknown whether those genes are primary or secondary targets of NAC019. Therefore, we employed a glucocorticoid receptor (GR)-inducible system to facilitate the identification of NAC019 direct targets. To achieve this objective, we fused the coding sequence for the N-terminus of NAC019 to the coding sequence for the GR domain, and drove the construct either under the *NAC019* native promoter or the 35S promoter. The construct was transformed into the *nac019* mutant, and the genotype of the transgenic lines was determined. We examined *NAC019-GR* expression and found that most of the transgenic plants expressed the *NAC019-GR* transgene. This indicates that our transgenic plants were successfully generated and thus, the presence of NAC019-GR protein was determined by Western blot.

Due to time constraints, this chapter only describes the initial stage of work using the GR-inducible system. The procedure for generating the *NAC019-GR-3xFlag* construct is explained, and the examination of *NAC019-GR* transgene in the transgenic lines is described. In addition, the Western blot results are also shown. As this project is still in the early phase, further experiments that can be done to identify NAC019 direct targets are discussed. These include chemical treatments for GR induction and chromatin immunoprecipitation (ChIP) applications.
2. INTRODUCTION

Transcription factors (TFs) play an important role in plant response to stress conditions as they regulate many stress-inducible genes that are essential in stress acclimation and the adaptive response. They are able to bind to the gene promoters and either activate or repress transcription. Many TF gene families have been identified through microarray approaches and their functions in stress responses have been further dissected. Microarrays provide information on transcriptome changes in the loss- and gain-of-function mutants and thus, can facilitate the placement of a TF in a developmental pathway or process (Gorte et al., 2011). Moreover, information obtained from microarray analysis is important as it assists researchers in the identification of direct and indirect target genes of the TF.

In this study, we sought to identify NAC019 direct target genes in order to gain a better understanding of NAC019 function in the inflorescence during drought stress. We would like to link the role of NAC019 in the stress regulatory network with floral developmental genes. Although our microarray data provided putative NAC019 target genes and their expression changes under drought stress, the microarray results alone are insufficient because they do not distinguish indirect and direct target genes. It is known that induction of a TF not only activates its primary target genes, but also other secondary target genes (Gorte et al., 2011).

Identification of TF target genes can be performed by using a chemical induction system. This system requires the construction of a TF fused to a nuclear receptor, such as the glucocorticoid receptor (GR), so that the TF can be induced chemically (Gorte et al., 2011). GR is classified as a ligand-dependent TF because its ability to bind DNA and regulate gene expression requires the presence of a ligand. It has a modular structure, consisting of an N-terminal domain (NTD), DNA binding domain (DBD) and ligand binding domain (LBD) (Kumar and Thompson, 2005). In the absence of its ligand (i.e., hormone), the GR protein remains in the cytosol due to its
dimerization with heat shock proteins (HSPs). However, when the hormone is present, GR disassociates from the HSP complex and translocates from the cytosol to the nucleus. Then, it binds to a glucocorticoid response element (GRE) and regulates gene expression. To ensure that the TF can induce gene expression changes, its expression level should be considered. If TF gene expression driven by its native promoter is too weak, a constitutive promoter such as the cauliflower mosaic virus 35S (CaMV 35S) can be used to increase its expression (Gorte et al., 2011).

Dexamethasone (DEX) is the agonist of GR, and its presence will release GR from the HSP complex. Without DEX induction, the TF-GR chimeric protein is sequestered with HSP proteins, and its activity is disabled. When DEX is added, the TF-GR interaction with HSPs is disrupted. The GR-TF fusion protein will be released from the HSP complex and will enter the nucleus to activate or repress gene expression (Bamberger et al., 1996; Gorte et al., 2011). In order to perform this experiment, one needs to consider the suitable concentration of DEX to be applied to the plant and the time required for the TF can achieve full activation after DEX addition (Gorte et al., 2011).

Treatment with DEX alone cannot differentiate between direct and indirect targets of a TF because TF induction will induce primary and secondary target genes altogether. Thus, cycloheximide (CHX) can be used to distinguish the target genes. CHX is a drug that inhibits protein translation by blocking the movement of peptidyl-tRNA from the acceptor site to the donor site on ribosomes (McKeehan and Hardesty, 1969). In the presence of CHX, transcriptional activation will still occur, but subsequent translation of primary target genes will be blocked. Hence, activation of secondary target genes will be prevented. However, the concentration of CHX and the length of application should be considered because failure to completely block protein synthesis will contaminate the candidate direct target genes with secondarily induced
genes. In contrast, if protein synthesis is blocked for too long, it will lead to cell death (Gorte et al., 2011).

The GR-inducible system has been successfully applied in plant biology research. It has been widely used to determine the target genes of a TF of interest (Wagner et al., 1999; Ito et al., 2007; Feng et al., 2012). For example, the APETALA3 (AP3): GR fusion protein was used to identify AP3 target genes in the inflorescence. Its target gene NAP (NAC-like, ACTIVATED BY AP3) was discovered through this method (Sablowski and Meyerowitz, 1998).

ChIP is a method used to investigate the interaction between a specific protein and a genomic DNA region, such as TF and its putative target gene (Carey et al., 2009). In this approach, cells are treated with formaldehyde to crosslink the TF with specific DNA regions that contain the TF binding site. Next, the nuclei are isolated and sonicated to shear the genome randomly. This allows for the determination of a more refined location of the DNA-protein interaction. Then, an immunoprecipitation is performed by using a specific antibody. The immunoprecipitated material is collected and the crosslinking is reversed. The DNA is purified and its identity can be determined with standard PCR or quantitative real-time PCR (Weinmann, 2004; Carey et al., 2009).

A few years ago, the ChIP method was expanded to ChIP-chip (microarray hybridization) and ChIP-seq (high-throughput sequencing), which allow global discovery of protein-DNA interactions (Ho et al., 2011). However, ChIP-seq is preferable to ChIP-chip because it offers higher resolution, less background noise and greater coverage. Moreover, it is cost-effective and requires little DNA starting material (Park, 2009).

We took advantage of the inducible TF system and generated a construct that consists of a NAC019-GR fusion gene driven by the NAC019 native promoter (pNAC019) or the 35S promoter (35S). Then, the constructs were transformed into WT and nac019-1, and positive transgenic lines were obtained through screening on an MS plate supplemented with hygromycin.
During the preparation of this thesis, only the T1 generation of \( p\text{NAC019}:\text{NAC019-GR-3xFlag} \) and \( 35S:\text{NAC019-GR-3xFlag} \) transgenic lines were obtained. The \( \text{NAC019-GR} \) transcript and its fusion protein were further examined to ensure that the transgenic plants are able to express the transgene. It is important to assess the production of the \( \text{NAC019-GR} \) fusion protein in the cytosol to prepare for later induction experiments using this construct.

3. MATERIALS AND METHODS

Construction of the \( 35S:\text{NAC019-GR-3xFlag} \) plasmid

To produce a \( 35S:\text{NAC019-GR-3xFlag} \) construct, the \( \text{NAC019} \) coding sequence (CDS) without the stop codon was amplified with the primers oMC8090 and oMC8091, and the 35S promoter was amplified from the pBI221 vector using primers oMC8086 and oMC8087. The GR-3XFlag fragment was amplified with the primers oMC7453 and oMC7456. These fragments were cloned into pCAMBIA1301, which was linearized with \( Kpn1 \). The cloning was performed using the In-Fusion® HD Cloning Kit (Clontech, USA) according to manufacturer’s instructions. The reaction mixture was transformed into \( E. \text{coli DH5}\alpha \) using the heat-shock transformation method, and the transformant culture was grown on LB agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaOH, 15 g/L agar, pH 7) supplemented with kanamycin (Kan). Colony PCR was performed using the primers oMC8086 and oMC7456 to verify the presence of the gene fragment in the vector. To further validate the insertion, the clone was digested with \( Kpn1 \) and \( Pml1 \), and sequenced. The vector map is provided in the Appendix A.
Construction of the pNAC019::NAC019-GR-3xFlag plasmid

To create a pNAC019::NAC019-GR-3xFlag plasmid, the 3.8 kb of NAC019 upstream sequence was first amplified with the primers oMC8096 and oMC8097. Using the 3.8 kb promoter as a template, another fragment was amplified using oMC8105 and oMC8106 primers to introduce Kpn1 and Xba1 sites for further cloning reactions. Next, the fragment containing the NAC019 CDS without the stop codon was amplified with the primers oMC8107 and oMC8091. To perform cloning reaction, the promoter fragment with Kpn1 and Xba1 linkers was mixed together with NAC019 CDS (from oMC8107 and oMC8091 amplification), GR-3xFlag and pCAMBIA1301 using In-Fusion® HD Cloning Kit (Clontech, USA). Subsequent steps were performed as similar as described for the generation of the 35S:NAC019-GR-3xFlag construct. However, the fragment was verified by colony PCR using the primers oMC8105 and oMC7456 and through digestion with Sac1 and Pml1 because of the presence of two Kpn1 sites in the pNAC019::NAC019-GR-3xFlag plasmid. The vector map is provided in the Appendix B.

Agrobacterium-mediated transformation by the floral dip method

Both constructs were transformed into Agrobacterium tumefaciens through heat shock transformation, and plasmid digestion was performed to verify the presence of the construct. Plasmid transformation into Arabidopsis was performed based on floral dip method (Clough and Bent, 1998). The positive A. tumefaciens carrying the 35S:NAC019-GR-3xFlag or pNAC019::NAC019-GR-3xFlag construct was grown in YEB medium (5 g/L sucrose, 1 g/L yeast extract, 10 g/L peptone, 0.5 g/L MgSO₄·7H₂O, 2% agar, pH 7.2) supplemented with kanamycin (Kan) and rifampicin (Rif), shaking at 200 rpm at 28°C overnight. The culture was sub-cultured in YEB + Kan + Rif medium overnight until the OD₆₀₀ reached 1.2 to 1.6. The culture was
centrifuged at 5000xg for 10 min, and the supernatant was suspended with inoculation medium (0.5X Murashige-Skoog (MS), 5% (w/v) sucrose, 0.05% Silwet L-77) until the OD
was 0.8. The inflorescences of WT and nac019-1 were dipped into the solution for a few seconds, and the plants were kept in the dark at room temperature for 24 hours to retain the humidity after inoculation.

Screening of putative transformants using an antibiotic selection marker

Seeds from transformed plants were harvested and surface-sterilized with 10% bleach. The seeds were washed six times with water and submerged in 0.05% agarose before planting on MS plates supplemented with hygromycin (Hyg). The seeds were vernalized for 2 days at 4°C. After stratification, they were transferred to a growth chamber and grown vertically at 22°C under a 16/8 hours light/dark photoperiod and 300 µmol photon m⁻² s⁻¹. After 10 days, the green seedlings with elongated roots were selected and transplanted onto Metro-Mix 360 soils (Sun Gro Horticulture Canada Ltd., USA). These seedlings were considered as positive putative transformants because they carried resistance to hygromycin, an antibiotic that inhibits root growth.

Identification of positive transgenic lines

To further confirm the presence of the construct in the transgenic lines, genotyping was performed. Genomic DNA was extracted, and PCR was performed by using oMC7456 and oMC8107 primers. To examine the expression of NAC019-GR in the transgenic plants, total RNA from leaf tissue was extracted with the NucleoSpin RNA Plant kit (Macherey-Nagel, GmbH & Co., Germany) according to the manufacturer’s instructions. For each sample, 0.5 µg of total
RNA was treated with RNase-free DNase I (Thermo Fisher Scientific Inc., USA) prior to reverse transcription to eliminate the possibility of genomic contamination. Reverse transcription was performed on total RNA using the M-MLV Reverse Transcriptase kit (Promega Corp., USA) following the manufacturer's instructions. The cDNA synthesized was used as a template for PCR amplifications. To detect NAC019-GR expression, primers similar to those used for genotyping were used for the amplification. The program used to amplify the NAC019-GR fragment was 94°C, 3 min; followed by 30 cycles of (94°C, 30 sec; 62°C, 30 sec; 72°C, 2 min) and 72°C, 3 min.

**Protein extraction and SDS-PAGE sample preparation**

For each transgenic line, about 0.05 g leaf was collected for protein extraction. The leaf was grinded into a fine powder and total protein was extracted using the Plant Total Protein Extraction kit (Sigma-Aldrich, USA). Before performing SDS-PAGE, 10 µl samples were added with SDS-PAGE sample buffer (40% glycerol, 0.24M Tris-Cl (pH 6.8), 8% SDS, 0.04% bromophenol blue and 0.1M DTT) and boiled for 5 min. For SDS-PAGE, 10% resolving gel (40% acrylamide, 1.5M Tris-Cl (pH 8.8), 10% SDS, 2% ammonium persulfate and 0.005% TEMED) and 5% stacking gel (40% acrylamide, 1M Tris-Cl (pH 6.8), 10% SDS, 2% ammonium persulfate and 0.005% TEMED) were used.

**Western blot**

The resolving gel was rinsed briefly in the semi-dry Transfer Buffer (sdTB) (48 mM Tris, 39 mM glycine, 20% methanol and 0.0375% SDS). For protein transfer purpose, a Hybond-P polyvinylide difluoride (PVDF) membrane (GE Healthcare Life Scince, USA) and a Whatmann
Filter paper were used. The PVDF membrane was soaked in the methanol for 5 seconds, and then, in the sdTB for 10 min. Three filter papers were wetted in the sdTB before they were placed onto a Trans-Blot SD electroblotter machine (Bio-Rad, USA). On top of the filter papers was the PVDF membrane, followed by the gel and finally, the additional three filter papers. About 150 mA was used for protein transfer and the process took about an hour.

After semi-dry protein transfer, blocking was performed to avoid non-specific protein binding. Beforehand, a washing buffer (PBST) which contained 1X PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄) and 0.1% Tween-20 was prepared. Blocking buffer used in this process contained 1X PBST and 5% non-fat dry milk. The PVDF membrane was wetted with methanol for a few seconds and rinsed with PBST for 5 min. Then, the membrane was placed in the blocking buffer for one hour at room temperature. A primary antibody (1:1000) was added into the blocking buffer and the membrane was incubated in this solution at 4°C overnight.

On the next day, the membrane was washed three times with PBST for 5 min. Then, it was incubated in the blocking buffer containing a secondary antibody (1:100 000) for an hour at room temperature. The membrane was rinsed briefly with PBST, and developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) for 5 min. Finally, the membrane was scanned with ChemiDoc System (Bio-Rad, USA) for signal detection from the protein of interest.
Table 4-1. List of primers used in generating the 35S::NAC019-GR-3xFlag and pNAC019::NAC019-GR-3xFlag plasmids

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Primer sequence from 5’ to 3’</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>oMC7453</td>
<td>ATTCAGCAAGCCACTGCAGG</td>
<td>GR-3xFlag (F)</td>
</tr>
<tr>
<td></td>
<td>ACCTGTAATTCACACGTGTACTTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCATCGTCTCCTTGTAATC</td>
<td></td>
</tr>
<tr>
<td>oMC7456</td>
<td>ACGAATTCTCGAGCTCGGTACCATAAG</td>
<td>GR-3xFlag (R)</td>
</tr>
<tr>
<td></td>
<td>ATTAGCCTTTTTCAATTTCA</td>
<td></td>
</tr>
<tr>
<td>oMC8086</td>
<td>TCTAGAGTCCCCCGTGTCTCTT</td>
<td>35S promoter with Kpn1 linker (F)</td>
</tr>
<tr>
<td>oMC8087</td>
<td>ACGGGGGGACTCTAGAATGGGTATCC</td>
<td>35S promoter with Xba1 linker (R)</td>
</tr>
<tr>
<td></td>
<td>CAAGAAAACCTGACCC</td>
<td></td>
</tr>
<tr>
<td>oMC8090</td>
<td>TTGCTGAATGTAAACCATAAAACCC</td>
<td>NAC019 CDS with Xba1 linker (F)</td>
</tr>
<tr>
<td></td>
<td>ACCCACCACCT</td>
<td></td>
</tr>
<tr>
<td>oMC8091</td>
<td>ATTAACCTCCAAAAACTAAATGCTTCTC</td>
<td>NAC019 3.8kb promoter (F)</td>
</tr>
<tr>
<td></td>
<td>TAAA</td>
<td></td>
</tr>
<tr>
<td>oMC8096</td>
<td>TCGGTGGGTAATAACTCGGAA</td>
<td>NAC019 3.8kb promoter (R)</td>
</tr>
<tr>
<td>oMC8097</td>
<td>ACGAATTCTCGAGCTCGGTACCATAAA</td>
<td>NAC019-GR-3xFlag fragment with Kpn1 linker (F)</td>
</tr>
<tr>
<td></td>
<td>TTAACTCCACGTAAATGCTTCTC</td>
<td></td>
</tr>
<tr>
<td>oMC8105</td>
<td>TCTAGAGGCTCGTCTCGGTACTTATA</td>
<td>NAC019-GR-3xFlag fragment with Xba1 linker (R)</td>
</tr>
<tr>
<td></td>
<td>TATGG</td>
<td></td>
</tr>
<tr>
<td>oMC8106</td>
<td>ACGAGACCCTCTAGAATGGGTATCTCC</td>
<td>NAC019-GR-3xFlag fragment with Xba1 linker (R)</td>
</tr>
<tr>
<td></td>
<td>CAAGAAAACCTGACCC</td>
<td></td>
</tr>
</tbody>
</table>

1. oMC7456 and oMC8086 were used in colony PCR for the amplification of the 35S-NAC019-GR-3xFlag fragment.
2. oMC8107 and oMC8091 were used to amplify NAC019 CDS from the 35S::NAC019-GR-3xFlag plasmid for pNAC019::NAC019-GR-3xFlag cloning.
3. oMC7456 and oMC8105 were used in colony PCR for the amplification of pNAC019-NAC019-GR-3xFlag.
4. RESULTS

Determination of NAC019-GR expression in the transgenic plants

We first screened the seeds that harbored the engineered plasmid by using hygromycin selection. We obtained about 130 positive transformants of 35S:NAC019-GR-3xFlag and 100 of pNAC019::NAC019-GR-3xFlag. The seedlings were transferred onto soil and their genotype was further confirmed by genotyping.

To examine the expression of NAC019-GR, we randomly chose 30 plants from each of the positive transgenic lines and performed semi-quantitative RT-PCR. The NAC019-GR transcript in 10 plants from each of 35S::NAC019-GR-3xFlag and pNAC019::NAC019-GR-3xFlag transformants is shown in Fig. 4-1. Almost all plants had detectable NAC019-GR transcript, although its expression level was different in the individual plants. In the 35S::NAC019-GR-3xFlag line, plant #1 had the highest NAC019-GR expression compared to other plants. NAC019-GR was highly expressed in the pNAC019::NAC019-GR-3xFlag #14.

It was surprising that the expression of NAC019-GR was higher under the NAC019 native promoter than under the 35S promoter. The transgene might be silenced due to post-transcriptional process such as methylation (reviewed by Matzke and Matzke, 1995). Higher NAC019 expression in the pNAC019::NAC019-GR-3xFlag #14 may be because the plants underwent stress caused by the growth conditions. It is known that NAC019 expression is induced by dehydration (Tran et al., 2004). Thus, when compared to other plants, it is possible that plant #14 might have absorbed less water from its surroundings and thus, induced NAC019 expression.
Figure 4-1. NAC019-GR transcript was detected in almost all the transgenic lines for both 35S::NAC019-GR-Flag and pNAC019::NAC019-GR-Flag.

Semi-quantitative RT-PCR was performed to examine NAC019-GR transcript levels in the transgenic plants. Expression levels were determined from leaf tissue of 3-week-old plants.

**Determination of the NAC019-GR protein in the transgenic plants**

We selected several samples for total protein extraction and performed Western blot to determine the presence of NAC019-GR protein in the transgenic plants. We expected to see a band with size about 70 kDa, which consists of NAC019, GR and Flag proteins. However, in all samples, most of them had a 35 kDa band (Fig. 4-2). The band observed might be a GR-3xFlag fusion protein (34.5 kDa) only, instead of NAC019-GR-3xFlag. The NAC019-GR-3xFlag protein might undergo post-translational cleavage, which creates smaller protein size. It is also possible that the fusion protein was not stable and degraded by proteases.
Figure 4-2. Western blot analysis of NAC019-GR fusion protein

Western blot analysis for (A) 35S::NAC019-GR-3xFlag and (B) pNAC019::NAC019-GR-3xFlag transgenic lines. The ladder with size in kDa is shown on the left. Each transgenic plant is numbered on top of the membrane.
5. DISCUSSION

To gain a better understanding of the biological function of \textit{NAC019} and its transcriptional regulation under drought stress, it is necessary to identify its direct target genes. Therefore, we took advantage of the GR-inducible system and generated constructs that may assist us in identifying the direct targets of NAC019. The construct was successfully generated, and transgenic lines that carried the plasmid were obtained (Fig. 4-1). However, due to time limitations, further experiments are not included.

Here, I discuss the future experiments that should be performed to identify direct target genes of \textit{NAC019}. The experiments consist of chemical induction by DEX and CHX for the activation of \textit{NAC019} transcription in the plant cell, followed by determining the NAC019 binding site using ChIP. Then, evaluation of the quality of the ChIP sample should be performed by qPCR (Kaufmann et al., 2010; Gorte et al., 2011).

Due to limited materials from T1 generation, the T2 of transgenic lines will be used. They will be treated with mock solution, DEX, CHX and combination of DEX and CHX. DEX induction will translocate the NAC019-GR chimeric protein from the cytosol to the nucleus and activate transcription of downstream genes. Subsequently, the primary and secondary target genes of NAC019 will be induced. To distinguish NAC019 direct target genes, CHX treatment will be performed as it inhibits translation of NAC019 downstream genes (Bamberger et al., 1996). The solutions will be applied to the transgenic lines by watering so that they can be absorbed by the roots and transported to the shoots. To ensure that NAC019 is fully active after chemical treatments, leaf and inflorescence samples will be collected at several time points. This tissue will be used for ChIP experiments and the DNA bound by NAC019 in these samples will be purified. Following that, qPCR will be performed to examine the enrichment of DNA fragments bound by NAC019. Next, the samples will be processed for ChIP-seq analysis.
By performing these experiments, direct interactions between NAC019 and its recognition site in the promoter of its putative target genes can be verified. Furthermore, the model proposed in Chapter 3 can be validated from these results, and these findings will provide a better understanding of the molecular function of *NAC019* in regulating stress-responsive and floral-related genes.

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7. REFERENCES


Appendix A

Vector map of the 35S::NAC019-GR-3xFlag plasmid

**pCAMBIA 1301-35S-NAC019-GR-3xFlag**

Diagram showing the structure of the plasmid with various genetic elements labeled, such as CaMV 35S promoter, NAC019 CDS, pLSI-REP, and others.
Appendix B

Vector map of the pNAC019::NAC019-GR-3xFlag plasmid
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