Dissection of Drought Responses in Arabidopsis

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(ABSTRACT)

Plants as sessile organisms are susceptible to many environmental stresses such as drought, and salinity. They have therefore evolved mechanisms to acclimate and tolerate environmental stresses. Knowledge of the molecular aspects of abiotic stress gleaned from extensive studies in Arabidopsis has provided much information on the complex processes underlying plant response to abiotic stresses. Nevertheless, there is a need for integration of the knowledge gained and a systematic molecular genetic dissection of the complex responses to abiotic stress. In this study in Arabidopsis, comparative expression profiling analysis of progressive (pDr) and moderate (mDr) drought treatments revealed common drought responses, as well as treatment specific signatures responses to drought stress. Under prolonged moderate drought plants develop different mechanisms for acclimation: induction of cell wall loosening at early stage, and a change in hormonal balance (ABA: JA) at late stage of moderate drought. Taking a reverse genetics approach, a MYB transcription factor (*MYB109*) has been identified as a regulator of growth under drought and salt stress. Global expression profiling showed possible mechanisms of how *MYB109* modulates growth under drought conditions: as a regulator of RNA processing and splicing and as a negative regulator of jasmonic acid biosynthesis and signaling. A forward genetics screen for drought and salt tolerance of transposon activation tag (ATag) lines led to the discovery of novel genes, which shed light on unexplored areas of abiotic stress

biology. Utilizing this strategy, a potential role for cell wall modification and MATE transporters in response to drought and salt stress has been discovered, which needs further analysis to integrate this information on the role of these biological processes in plant stress biology.

This dissertation is dedicated to my wonderful parents

(*Mariam and Mohammad Harb*)

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1. INTRODUCTION

Global climate changes are predicted towards an increase in temperature and water scarcity. About 70% of the available fresh water is used in agriculture, and it will be highly affected by the water shortage. Therefore, one of the most detrimental factors that plants face is water deficit or drought. Drought is a major abiotic stress, abiotic stress also includes: salinity, cold and high temperature. Drought affects plants by low water potential, which leads to a low yield in the most important crops. Indeed, it is the time to find effective ways to increase crop yield without consuming too much water in agriculture.

Prediction and simulation studies show that the climate is getting warmer, and large areas of the earth are getting drier (Hopkin, 2007; Overpeck and Cole, 2007). The world's population is increasing, and more water and food are needed (Rosegrant and Cline, 2003). To get more food we need higher yielding crops, which however will be threatened by drought conditions. Because of the global water scarcity especially in developing countries with the highest density of population, there is a pressure to compromise between high potential yield of crops and the scarcity of available water. One way to achieve stable crop production is by adopting methods and techniques, which enable crops to produce high yield using low amount of water (Passioura, 2004). This can be achieved by crops with higher water use efficiency and resistance to drought without decreasing their yield. In order to be able to develop crops with improved stress tolerance, a comprehensive understanding of plants response to stress is required.

Arabidopsis as a Model Plant

The large genome size and high ploidy level of most crop plants make them difficult for genetic and molecular analysis. Therefore, *Arabidopsis thaliana* has been chosen as the plant model (Koornneef and Meinke, 2010), because of the many characteristics that facilitate analysis and investigation of many aspects of plants functioning: developmental, biochemical, physiological, and molecular. It has small size, short life cycle, high seed production per generation, small genome size (~145 Mb), complete sequenced genome, with high percentage of annotated genes, and easy genetic manipulation and transformation. In addition, Arabidopsis has an excellent supporting infrastructure in terms of analysis tools: comprehensive databases with extensive information about genetic maps, physical maps, and whole genome sequence. Moreover, among plants, Arabidopsis has the largest collections of mutants (including T-DNA

and transposon insertional mutants) with a good coverage of the genome, which facilitates the molecular genetics dissection of many aspects of growth and development. Hence, this led to the efficient and fruitful applications of forward and reverse genetics in gene discovery and the elucidation of gene function.

Arabidopsis is an attractive model system for the dissection of many complex developmental, biochemical and physiological processes. Arabidopsis has helped enrich our understanding of how complex and vital biological processes are regulated at many levels to fine tune plant growth and response to the surrounding environment. As a result, of detailed studies in Arabidopsis extensive and broad knowledge has been gained of the early embryonic development, photosynthesis, light signal transduction, hormone biology and biochemistry, the regulation of flowering and the transition between the vegetative to reproductive phases, response to biotic and abiotic stress and many others (De Smet et al., 2010; Chory, 2010; Santner and Estelle, 2010; Amasino, 2010; Nishimura and Dangl, 2010; Hirayama and Shinozaki; 2010).

In the area of plant response to abiotic stress, knowledge gained from Arabidopsis about stress signaling pathways, downstream functional and regulatory responses paved the way to the investigations and applications in crop plants (Nakashima et al., 2009; Hirayama and Shinozaki; 2010). For example, similar ABA and non-ABA stress signaling pathways have been identified in rice (Nakashima et al., 2009). Much of the difference in genome size between Arabidopsis and crop plants such as rice and maize, are in intergenic DNA comprising repetitive DNA and transposons, while the gene number has not increased much. The increase in the genome size due to polyploidy (increase in gene number) in crops such as wheat, leads to gene duplication with a slow change in gene identity by divergence of duplicated gene functions. Therefore, in most cases our knowledge from the basic set of Arabidopsis genes can be transferrable to other important crop plants, and this helps in the deciphering of many biological problems, which otherwise are difficult to investigate and understand using complex plants.

Plant Response to Abiotic Stress

Plants are the primary producers of biomass for food and fuel, and all environmental stresses affect them. Plants are dependent on water, and because of unpredictable weather and climate changes, water scarcity and drought will severely affect plant growth and yield. As sessile organisms, plants are highly susceptible to the changes in the environment around them,

amd so need a mechanism to cope with extreme or unfavorable conditions. Under drought stress, plants have two responses, which are escape or resistance to drought stress (Price et al., 2002). Drought resistance is further distinguished into drought tolerance and drought avoidance (Levitt, 1980). In drought escape mechanisms, plants complete their life cycle during the period of abundant water, before the onset of stress (Mitra, 2001). Avoidance of drought stress is the ability of plants to maintain high internal water content. Using drought stress tolerance, plants are able to withstand low internal water content through various mechanisms. Plant protective mechanisms to drought stress can be generally considered under drought resistance, until distinguished into precise mechanisms, and also because functionally most drought resistance mechanisms are useful to protect plants to drought stresses.

Drought research has provided some basic knowledge of the biochemical and molecular drought response components and pathways (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 2007), including drought regulated genes, with the identification of some signaling pathways (Bartels and Sunkar, 2005, Hirayama and Shinozaki, 2010). However, little is known about the interaction among the different components of drought response, which can be best described in a network of interacting pathways involved in drought response.

Much molecular data has been obtained on the response and acclimation of plants to drought stress (Kreps et al., 2002; Ramanjulu and Bartels, 2002; Seki et al, 2002; Zhu, 2002;; Bray, 2004; Chinnusamy et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2007; Zeller et al., 2009). Plants respond to environmental signals through pathways that are initiated by perception of the signal, leading to signal amplification and into signal transduction cascades, and finally exhibited in biochemical and molecular changes that take place in the cell (Nakashima et al., 2009). The different types of abiotic stresses have multiple initial inputs on plants. This causes a complexity in the sensing components and in the signaling pathways in response to the multiple inputs (Xiong et al., 2002).

Signal Perception and Signaling Transduction

Studies on the initial sensing of plants to stress and identity of sensor molecules are at its infancy, but several candidate osmosensors have been found. For example, in Arabidopsis

AtHK1 was found to function as osmosensor when tested in mutated yeast cells (Urao et al., 1999; Ueguchi et al., 2001; Tran et al., 2007; Wohlbach et al., 2008).

Signal transduction is a well studied area in plant response to abiotic stress. The initial signals that are perceived by sensors or receptors lead to the activation of secondary messengers. One of the major secondary messengers in plants as well as in animals is calcium (Luan, 2002). Multiple cascades of phosphoproteins (kinases, and phosphatases) were found to activate regulatory genes downstream in the response pathways (Bartels and Sunkar, 2005). MAPK, CDPK, and phosphatases are examples of these phosphoproteins. Three major functional groups in the MAPK pathway were identified in Arabidopsis: MAPKKK, MAPKK, and MAPK (Chinnusamy et al., 2004; Mishra et al., 2006).

Functional stress-responsive genes, which code for products to protect the cells from the consequences of the stress, are activated under stress (Tuberosa et al., 2003). The products of these genes have different roles in the stress resistance process. Functional genes could be involved in the production of osmoprotectants such as the amino acid proline, sugars (mannitol), polyols, and glycine betaine (Ingram and Bartels, 1996; Yancey, 2001). Antioxidants and reactive oxygen species scavengers are a group of functional products that alleviate the effects of reactive oxygen species (ROS) resulted from osmotic stress (Valliyodan and Nguyen, 2006; Miller et al., 2010). Other products: LEA-like proteins, aquaporins and chaperones are used by plants for repair and protection from the detrimental consequences of abiotic stresses (Ramanjulu and Bartels, 2002; Bartels and Sunkar, 2005).

The plant hormone ABA plays an important role in the physiology of plants, and is also found to have a vital role in plants response to water deficit (Iuchi et al., 2001; Bray, 2002; Umezawa et al., 2006b; Zhang et al., 2006; Acharya and Assmann, 2009; Kim et al., 2010). In addition to ABA-dependent pathway, an ABA-independent pathway was found to be functional in plants in response to osmotic stress (Shinozaki and Yamaguchi Shinozaki, 1997; Zhu, 2002; Hirayama and Shinozaki, 2010), showing the diversity in the network of stress responses.

Changes in Gene Expression

The basis for different environmental responses in plants is the differential expression of their genes, which is a result of the interaction between transcriptional factors and their corresponding cis- elements located in the promoters of the genes. Using transcriptome and

expression profiling tools, a number of genes regulated by drought stress have been discovered (Seki et al., 2002; Matsui et al., 2008). These genes have been classified into two major groups: regulatory genes (transcriptional factors), and functional genes (Shinozaki and Yamaguchi-Shinozaki, 1997; Valliyodan and Nguyen, 2006; Shinozaki and Yamaguchi – Shinozaki, 2007).

The identification and annotation of transcription factors in the Arabidopsis genome revealed about 1,978 TFs grouped into approximately 50 gene families (http://rarge.psc.riken.jp/rartf/, Iida et al., 2005; Mitsuda et al., 2009). Transcriptional factors (trans- elements) are proteins that bind to specific DNA regions called the cis-elements in the gene and regulate its expression. They either increase or decrease the expression of genes (up-regulation), or (down-regulation), respectively (Riechmann et al., 2000). Members of different transcription factors families were found to be involved in the response to drought stress (Ramanjulu and Bartels, 2002; Shinozaki and Yamaguchi – Shinozaki, 2006). These gene families are AP2/ERF, bZIP, NAC, MYB, MYC, Cys2His2 zinc-finger and WRKY (Umezawa et al., 2006a, Singh et al., 2002).

Thesis Overview

In this study, utilizing reverse and forward genetics strategies, plant responses to drought stress have been explored in a step towards a better understanding of the complex biology underpinning these responses. The findings, presented here, have been divided into three chapters. In Chapter 1, the dissection of plant response to drought stress at physiological, biochemical, and molecular levels has been described, where it is shown that plant responses to drought is divided into three stages: early stage of stress perception, signaling and preconditioning, intermediate stage in preparation to acclimation, and late stage of new homeostasis with reduced growth. Chapter 2 describes a reverse genetics approach in integration with physiological assays that explores the role of a MYB transcription factor in growth under drought and salt stress. Chapter 3 presents the utility of transposon activation tagging as a forward genetics tool for the discovery of novel genes, which revealed new unexplored aspects of plant reactions to abiotic stress, ascribing cell wall modification and transporters as functional genes in protection against abiotic stress.

2. A. Molecular and Physiological Analysis of Drought Stress in Arabidopsis Reveals Early Responses Leading to Acclimation in Plant Growth

B. ABSTRACT

Plant drought stress response and resistance are complex biological processes that need to be analyzed at a systems-level. Genomics combined with physiological approaches must now be used to dissect experimental models of drought stress often encountered by crops in the field. Towards this goal a controlled, sub-lethal, moderate drought (mDr) treatment system was developed using the model flowering plant *Arabidopsis thaliana*, as a reproducible assay for the dissection of responses to drought. Arabidopsis mutants deficient in Abscisic acid (ABA) biosynthesis and signaling (*aba1* and *abi1*) displayed sensitivity and reduced growth under mDr, indicating a crucial role for ABA in drought response and acclimation. The dissection of mDr stress responses using a time course analysis of biochemical, physiological and molecular processes revealed early accumulation of ABA and induction of associated signaling genes, coinciding with a decrease in stomatal conductance as an early avoidance response to drought stress. The comparative transcriptome analysis of drought stress treatments revealed the similarity of early stage mDr to progressive drought, identifying common and specific stress responsive genes. Bionformatics was used to subsequently identify corresponding promoter cis-regulatory elements. During these studies, a peak in the expression of expansin genes previously shown to be involved in cell wall expansion, was found to be a preparatory step toward drought acclimation. The time course analysis of mDr provides a model with three stages of plant responses: an early priming and preconditioning stage, followed by an intermediate stage preparatory for acclimation, and a late stage of new homeostasis with reduced growth.

C. INTRODUCTION

Drought is a major environmental stress factor that affects the growth and development of plants. Drought or soil water deficit can be chronic in climatic regions with low water availability, or random and unpredictable due to changes in weather conditions during crop growing seasons. The effects of drought are expected to increase with deleterious climate change and growing water scarcity. Water is an increasingly scarce resource given current and future human population and societal needs, putting an emphasis on sustainable water use. Thus, an understanding of drought stress and water use in relation to plant growth is of importance for sustainable agriculture.

Plants are sessile organisms having evolved specific acclimation and adaptation mechanisms to respond to and survive short- and long- term drought stresses. Analysis of these protective mechanisms will contribute to our knowledge of tolerance and resistance to abiotic stress in a more general sense. The complex responses to environmental stress, from perception, to transcriptional, translational, and ultimately physiological changes need to be considered at a global systems biology level to study the multiple interactive components in this biological process.

In response to drought brought about by soil-water deficit, plants can exhibit either drought escape or drought resistance mechanisms, with resistance further classified into drought avoidance (maintenance of tissue water potential) and drought tolerance (Levitt, 1980; Price et al., 2002). Drought escape is described as the ability of plants to complete the life cycle before exposure to severe stress. Drought avoidance is characterized by maintenance of high tissue water potential despite a soil water deficit. Mechanisms such as improved water uptake under stress, capacity of plant cells to hold acquired water and further reduce water loss, confer drought avoidance. Plants respond to water deficit using mechanisms of avoidance by improved root traits (Price et al., 2002), by reducing water loss through reduced epidermal (stomatal and cuticular) conductance, reduced radiation absorption and reduced evaporative surface (leaf area). Drought tolerance is the ability to withstand water-deficit with low tissue water potential (Ingram and Bartels 1996). Plants under drought stress may survive by also maintaining cell turgor and reducing evaporative water loss by accumulating compatible solutes (Yancey et al., 1982).

In recent years much molecular information has been generated on the response of plants to environmental stresses. Plants respond to environmental stresses such as drought by the

induction of both regulatory and functional sets of genes (Ingram and Bartels, 1996; Ramanjulu and Bartels, 2002; Bartels and Sunkar, 2005). Very little is known about the early events in the perception of stress signals (Urao et al., 1999; Ueguchi et al., 2001; Tran et al., 2007; Wohlbach et al., 2008). Several common stress signaling pathways have been categorized as ABA-dependent or ABA-independent processes (Shinozaki and Yamaguchi-Shinozaki, 1997 Shinozaki and Yamaguchi-Shinozaki, 2007). Most of the key genes in these pathways have been identified such as transcription factors belonging to the class of: dehydration-responsive element/C-repeat-binding (DREB/CBF), Abscisic acid binding factor (ABF), and transcription factors of MYC and MYB families (Abe et al., 1997; Bartels and Sunkar, 2005; Sakuma et al., 2006). Corresponding stress responsive cis-elements such as ABRE, and DRE have also been identified (Yamaguchi-Shinozak and Shinozaki, 1994; Ramanjulu and Bartels, 2002; Bartels and Sunkar, 2005). Downstream signaling genes and molecules acting as secondary messengers have been identified following early perception of stress, revealing the role of Ca^{+} and reactive oxygen species (ROS) as secondary messengers (Bartels and Sunkar, 2005). These regulatory mechanisms induce downstream functional genes, which are needed to establish new cellular homeostasis that leads to drought tolerance and/or resistance (Yancey, 2001; Ramanjulu and Bartels, 2002).

Most of our knowledge of drought adaptation/resistance at the molecular level is based on plant responses to dehydration and/or osmotic treatments under laboratory conditions (Yamaguchi-Shinozaki and Shinozaki, 1994; Abe et al., 1997; Oono et al., 2003; Umezawa et al., 2004). Although these conditions are somewhat different from soil water deficit/drought met by plants under field conditions, valuable knowledge has been gained from such studies. Dehydration studies revealed the common stress signaling pathways, both ABA-dependent and ABA-independent, which have become a paradigm in plant stress biology (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 2007). These pathways were initially discovered in Arabidopsis, which paved the way to the characterization of parallel pathways in other crop plants such as in rice as a model for monocot plants (Nakashima et al., 2009).

A number of drought treatment regimes have been used to test the response of plants for improved tolerance/resistance. One method is progressive drought (pDr), in which water is withheld for a certain period of time until symptoms of wilting are observed. Usually, this

method of drought treatment has been used to determine survival rate or to monitor transcriptional changes, of wild-type plants or genotypes engineered to overexpress candidate genes for drought tolerance (Sakuma et al., 2006; Catala et al., 2007; Nelson et al., 2007;Yu et al., 2008; Ning et al., 2010). These studies have shed some light on plant responses to drought at both physiological and molecular levels. However, one of the drawbacks of pDr treatment, because of the uncontrolled soil water moisture, is that it cannot be used to compare the performance of different genotypes with different growth characteristics, such as smaller plants. Unlike pDr simulations, in nature drought that develops during a growing season occurs for a short period, from which tolerant plants can manage to survive and complete their growth cycle. Methods to simulate field-like conditions and quantify drought responses will provide a better understanding of drought resistance mechanisms.

Soil water deficit resulting in drought stress in crop plants has been recently tested in Arabidopsis using a controlled, sub-lethal soil moisture treatment. Controlled drought treatment exposing plants to uniform levels of soil moisture, enables evaluations between genotypes/ecotypes responses to sub-lethal drought (Granier et al., 2006; Bouchabke et al., 2008). PHENOPSIS has been developed as an automated type of controlled drought screen, which was used to compare the performance of different Arabidopsis ecotypes, and resulted in the identification of the naturally resistant ecotype An1 (Granier et al., 2006). Controlled drought was also used to study the response of the Arabidopsis *erecta* mutant and *ERECTA* gene overexpression (Masle et al., 2005), the overexpression of the Arabidopsis *ESKIMO1* gene (Bouchabke-Coussa et al, 2008), and overexpression of the proline biosynthesis gene in chickpea (Bhatnagar-Mathur et al., 2009).

Comprehensive physiological and molecular studies have not yet been performed on the response of plants to moderate drought (mDr). A transcriptome study in loblolly pine, treated with cycles of mild drought and recovery (Watkinson et al., 2003; Vasquez-Robinet et al., 2010), showed a photosynthetic acclimation pattern in contrast to photosynthesis inhibition under severe drought. A comprehensive understanding of the response of plants to mDr with physiological and molecular tools would provide us with a better understanding of the acclimation process. We present here an analysis of controlled mDr in Arabidopsis under soil water deficit treatment simulating field conditions of crop plants. A semi-automated, controlled mDr testing system was developed and used to compare to pDr treatment for physiological and molecular responses. This

revealed differential gene expression reprogramming under the two drought treatments. The dissection of mDr treatment is presented using a time course study to provide a picture of physiological and molecular responses towards acclimation in plant growth.

D. MATERIALS AND METHODS

Growth Conditions and Drought Treatments

Arabidopsis ecotype (Columbia) seeds were sown in moistened peat pellets (Jiffy Products, Shappagan, Canada), stratified at 4°C for 2 days, and then transferred to a growth room kept at 10 hours light (100 µmole m^{-2} s^{-1}) and 22°C. For drought treatment pellets were weighed before sowing to determine the amount of water in pellets at the beginning of the experiment. Controlled moderate drought (mDr) was maintained by giving plants water to keep the soil moisture level at 30% of field capacity, which is 200% or 2 g H_2O g^{-1} dry soil. To do this, a semi-automated system was developed; a balance (GF-1000, A&D, and California) was connected to the computer utilizing software for communication, which enabled entering of the weights directly into an Excel worksheet file. On the Excel worksheet file a set of equations were used to calculate the water content in each weighed pellet, the required final water content, and the amount of water to be added. The pellets were weighed daily, and were supplemented with the calculated amount of water to reach 30% of field capacity (mDr level).

The sensitivity of different developmental stages to mDr was tested utilizing the same drought treatment as described above. Water was withheld at 25 days after sowing (DAS) for the first group, 30 DAS for the second group, and 35 DAS for the third group. After around 5-7 days a mDr stress is achieved as soil moisture level of 2 g g^{-1} dry soil is reached, and the plant stages for the 3 groups are: 8-leaf (1.08), 10-leaf (1.10), and 12-leaf (1.12), respectively (Boyes et al. 2002). The mDr treatments are referred to by the initiation date (25, 30, 35 DAS) in the experiments reported. The three groups were exposed to mDr for 10 days, and then the sensitivity was assessed by calculation of the RB. Relative Reduction in Biomass (RB) = $(B_{WW}-B_{DRT})/B_{WW}$; where B_{WW} is Biomass under well-watered (WW) conditions; B_{DRT} is Biomass under mDr conditions. The effect of the duration of mDr was also tested by harvesting plants at day 5 and 10 of mDr.

For the determination of leaf relative water content (LRWC), 10 plants were sampled per each time point starting from day -2 of mDr until day 3 of mDr. The weight of the whole cut rosette was measured at the time of cutting, and then the cut rosettes were soaked in deionized water for 16 hrs. After that, they were weighed and allowed to dry in oven at 75°C. The dry weight was taken, and %LRWC was calculated as follows: %LRWC= (turgid weight- weight at time x)/ (turgid weight- dry weight) *100.

For progressive drought (pDr) treatment, plants were grown in a growth room as described above, water was withheld at 35 DAS, and pellets were allowed to dry and monitored by weighing the pellets until the required pDr level was reached; two levels were tested in this study: wilting, and 1 day before wilting (pre-wilting).

Measurement of Growth Rate during Vegetative Stages

The rate of growth of Arabidopsis ecotype (Columbia) at two different developmental stages was determined as follows: plants were grown under normal growth conditions as described above, and then harvested for biomass measurement at different stages. Plants were harvested at 25 days after sowing (DAS) for group one, 30 DAS for group two and at 35 DAS for group three. These dates are the actual dates of harvest, unlike for drought treatments described above. The rate of growth during two developmental stages: 25-30 DAS and 30-35 DAS, was calculated using the formula: Relative growth rate (RGR) = $(\ln W_2 - \ln W_1)/(t_2 - t_1)$ (Hoffman and Poorter, 2002). The growth rate was assessed based on biomass and leaf area. Leaf area was determined using ImageJ (NIH, USA), which was used to analyze the scanned rosettes of the drought and well-watered treatments. Relative expansion rate (RER) was calculated as described for biomass.

ABA and JA Mutants under mDr screens

Abscisic acid deficient and signaling and JA signaling mutants were tested under moderate drought conditions. The following mutants were ordered from Arabidopsis Biological Resource Center (Ohio State University, USA): *abscisic acid insensitive1* (*abi1*) (SALK_076309C), *coronatine-insensitive 1 (coi1)* (SALK_095916C), *Jasmonate-insensitive 1* (*jin1*) (SALK_061267C), *Jasmonate resistant 1* (*jar1*) (CS8072) in Col background, *abi1* (CS22), and *abscisic acid deficient 1* (*aba1*) (CS21) in L*er* background. Eight replications per

mutant and the corresponding wild type were tested, and the performance assessed by comparing the biomass under drought to that under well-watered treatment and the relative reduction in biomass (RB) was calculated as mentioned above.

Gas Exchange Measurements

Gas exchange measurements were done under mDr conditions in a time course study, in which five time points of mDr were tested: -1, 0, 1, 2, and 3 days of mDr For the gas exchange measurements, a LICOR 6400XT (LICOR, Nebraska, USA) and an Arabidopsis Extended chamber were used, and the following conditions were set for LICOR measurement: flow rate 150 μmole s^{-1}, CO_2 400 μmole, humidity 50%.

Biochemical Analyses

Starch analysis was performed on plants treated for drought and well-watered conditions. Samples were taken at different time points of drought treatment: -1, 0, 1, 2, and 3 days of mDr, and from well-watered controls at same time, and stored at -80 ºC. Sampling was done in the late afternoon, during the period of highest starch concentration (Caspar et al., 1985). The samples were ground to a fine powder under liquid nitrogen, the weight of each sample determined, and starch was quantified using EnzymChrom starch assay kit (BioAssay Systems, USA) following the instructions of the manufacturer.

For ABA quantification, plant samples were harvested at different time point of mDr: 0, 1, and 2 days of mDr, and were stored at -80 ºC. ABA was extracted from plant samples as described (Bray and Beachy, 1985), and ABA was quantified using the Phytodetek ABA test kit (Agdia, Indiana, USA) following the manufacturer's instructions. Proline was quantified in plants samples at 3 and 4 days of mDr as described (Bates et al., 1973).

Expression Profiling of Early and Late mDr and pDr

For mDr, plants at 30 DAS stage were treated as described above under moderate drought treatment. For RNA isolation, three biological samples of 5 pooled plants were collected at early stage (day1), and at late stage of mDr (day10). RNA was isolated using RNeasy Kit (Qiagen, USA). After that, genomic DNA was eliminated using DNase kit (Qiagen, USA). For gene expression analysis 4 ug RNA samples, with 2-3 replications/treatment were used for Affymetrix

(ATH1 25K) GeneChip hybridization analysis at the VBI Core Lab Facility (VBI, Blacksburg, VA, USA).

For pDr, five weeks WT plants were drought-treated until the first day of wilting, and a control group of each genotype kept well-watered. Three biological samples of 5 pooled plants from drought-treated and well-watered control of each genotype were collected for RNA isolation. RNA isolation and Affymetrix GeneChip hybridization analysis were performed as described above.

Analysis of Gene Expression Profiles

For each of the drought experiments, mDr-Day1, mDr-Day10 and pDr, raw data were background corrected, normalized and summarized according to the custom CDF (see below) using RMA (Irizarry et al., 2003; Ihaka and Gentleman, 1996; Gentleman et al., 2004), followed by non-specific filtering of genes that do not have enough variation (interquartile range (IQR) across samples < IQR_{median}) to allow reliable detection of differential expression. A linear model was then used to detect differential expression of the remaining genes (Smyth 2004). The *p*-values from the moderated *t*-tests were converted to *q*-values to correct for multiple hypothesis testing (Storey and Tibshirani, 2003), and genes with *q*-value <0.1 were considered as differentially expressed in response to the drought treatments.

Reannotation of Arabidopsis GeneChip Probe-Gene Mapping

A high-quality custom chip definition file (CDF) was built for the Arabidopsis GeneChip array by uniquely mapping 232,697 probe sequences (http://www.affymetrix.com/analysis/downloads/data/) to 21,389 Arabidopsis (TAIR8) gene-based probe sets in the following manner: (i) probes that have perfect sequence identity with a single target gene were selected, (ii) probes mapping to reverse complements of genes were annotated separately as antisense probes (not used in the above counts), and finally, (iii) probes were grouped into probe sets, each corresponding to a single gene, and probe sets having at least 3 unique sequences were retained (>99% probe sets have >=5 probes). Note that these stringent criteria used to construct the CDF make it possible to reliably measure expression values of members of multigene families (free from cross-hybridization between paralogs showing high sequence similarity).

Promoter Analysis

For analysis of potential promoter-resident cis-regulatory elements (CREs), FIRE (Elemento et al., 2007) was used to discover motifs specific to different sets of differentially expressed genes by comparing the motif content of 1kb upstream sequences of these genes to that of the rest of the genome, followed by comparison to known cis-elements (Higo et al., 1999; Davuluri et al., 2003; Galuschka et al., 2007; Mahony and Benos, 2007). All sequences were obtained from TAIR. Sequence logos were drawn using WebLogo (Crooks et al., 2004). This *de-novo* approach was taken since i) CREs could diverge far more quickly than coding sequences across species, making them hard to find simply by searching, and ii) searching based on known elements in Arabidopsis is limited by the scope of experimental identification in a select set of genes, making identification of degenerate yet potentially functional positions in the element hard.

Gene Functional Enrichment Analysis

Gene function descriptions and GO annotations were downloaded from TAIR (TAIR8; Swarbreck et al., 2008). For enrichment analysis, Biological process (BP) and Cellular component (CC) branches of GO were further used. Applying the true-path-rule, a gene annotated with a particular GO term was also annotated with all its parents. To avoid very generic, non-informative terms for analysis, only terms annotating <=500 genes ('specific GO-terms') were retained. Genes annotated with a given specific GO-term were considered as a gene set. ABA-response gene sets were obtained from Nemhauser et al. (2006). Genes containing CREs discovered de-novo were further included as additional gene sets.

All the gene sets described above (GO_BP, GO_CC, ABA-response, and CRE) were tested for the statistical significance of enrichment among the experimentally identified drought gene sets (mDr-Day1, mDr-Day10 and pDr up- and down-regulated genes) and among themselves using the cumulative hypergeometric test. For a pair of gene sets *i* and *j*, if *N* is the total number of genes, n_i and n_j are the number of genes in gene set *i* and *j*, and *m* is the number of genes common to the gene sets, the probability (*p*-value) of an overlap (enrichment) of size equal to or greater than observed is given by the formula below.

$$P(X = x \geq m) = \sum_{x=m}^{\min(n_i,n_j)} \frac{\binom{n_i}{x}\binom{N-n_i}{n_j-x}}{\binom{N}{n_j}}$$

To adjust for multiple comparisons, a Benjamini-Hochberg false discovery rate (FDR; *q*-value) was calculated from the *p*-values, and a *q*-value threshold of 0.01 was used for significance.

The results from the enrichment analysis were visualized in the form of a gene set-graph, where pairs of significantly overlapping gene sets (nodes) are connected to each other by edges. The graph was augmented with information about gene set size (node size), source/type (node color), and extent of overlap between gene sets (edge width). The graph was visualized using Cytoscape (Shannon et al., 2003).

Gene Expression Analysis by qRT-PCR

RNA was isolated using RNeasy Kit (Qiagen, USA). After that, genomic DNA was eliminated using DNase kit (Qiagen, USA). The first strand cDNA was synthesized using iScript cDNA synthesis Kit (BioRad, USA). BioRad SYBER green was used to quantify the expression of the genes (Table 1). Fold change of expression was calculated relative to UBQ10 (AT4G05320) and SAND (AT2G28390) reference genes (Czechowski et al., 2005), and relative to the corresponding well-watered control as described in (Livak abd Schmittgen, 2001).

Table 1.1. List of genes and primers

Gene	Forward Primer 5' ------- 3'	Reverse Primer 5' ------- 3'
ABF3 (ABA binding factor)	ACGGCGGTGGTAACAACATTG	GCTCCTTCCAGACATCATCAACC
DREB2A (dehyration element binding factor)	CACGAAAGCGATTTATCAACTC	AAACTGCCATCTCCTTCCC
RD22 (Responsive to dehydration)	TGGCGATTGCGGCTGATTTAAC	GGAGAGAGTTGGGAATGGGAGTG
RD29A (Rreposnive to dessication 29A)	GTGGGCTTTGGTGACGAGTC	GTGTCCATTCCAGTTTCAGTCTTC
RD29B (Rreposnive to dessication 29A)	GACCACACCAAACCCATTGAGC	TCGTTCTTACAGGATCAGCCAGTG
RAB18 (Responsive to ABA 18)	GAGGAGGAAGAAGGGAATAACAC	CGTAGCCACCAGCATCATATC
NCED3 (ABA biosynthesis)	AATCATCAAACTCTCCCGCCATTG	GCCGCCGCTCTCTGGAAC
GORK (Outward K+ Channel)	TTGGTGTGGGCAATATACTCCTC	GCGATAGGCAACAAAGAACTGAAG
PLDα (ABA signaling)	ATTAACGGCGAGGAAGTGG	GTCTGGAATATGAGCATCTTGG
GPA1 (ABA signaling)	CGACTTCTATGTTATTACCTGTGG	AGCAGCCTGTGTATTCTCATC
RPK1 (ABA signaling)	GTCTTTGTGCTTCTTGTTCTTGTG	CGTCAGAGGAATACCAATGTCAAC
HAB1 (ABA signaling)	TTGGAGCAAGGATTCTTCAACATC	CACACCACACCACAACAAAGC
ABI1(ABA signaling)	TCTCAGGTAGCGAACTATTGTAG	AGCATCGGTTTCTCCTTAGC
ABI2 (ABA signaling)	CGGTTCTCAGGTAGCGAATTATTG	AGCCTTCTTCCACTTCTCTTGC
MYB60 (Stomatal regulation)	CACAGCGATCAGTTTCCATACG	TCTTTATACCCTGGTCATCATCCC
PsbW (Photosynthesis PSII)	ACGATTCTCCCGCCACCAC	CGACCGAACACAAGCCATATCTG
PsbQA (Photosynthesis PSII)	TCAGCAGAGTGAGGAGAC	AATAGCATCGGCGAGGAC
PSAH2 (Photosynthesis PSI)	AAGGCAAGGACAAGTACAAGTG	ATAGATTCAAATGGATGCGAGAAC
PsbQO2 (Photosynthesis PSII)	ACAAGAACAGAGGCTGACACC	AGTTTCGGAAGAGACGGTAAAGTG
EXPA3 (Expansin A3)	TCAAAGACAGATTGGGTGAGGATG	AGAGGCGGTGACTCGGAAAG
EXPA4 (Expansin A4)	GAGTTGTGGAGCCTGCTTTGAG	AGAAATTGGTTGCGGTGATGAGG
EXPA8 (Expansin A8)	GCTACAAACTTTTGCCCACCTAAC	GCACGATACTGAGCGATCTGAAG
EXPA10 (Expanisn A10)	GCTCAATCGTTGTAACCGCTAC	GCAAGGTCAAAGTGTTCAAGAGG
EXLB1 (Expansin-like B1)	TTTGCCTCTCCTTTGTCTATCCG	CACCACTCACTTCACCGTTATTG
APX1 (Ascorbate peroxidase)	TGTAATCTTCGCTCACGGTTGG	TGTAATCTTCGCTCACGGTTGG
TPX1 (Thioredoxin-dependent peroxidase)	GCTGTCGGCGATGTCGTACC	GCACCAGGAACACCAAAGAGAATG
GPX6 (Glutathione peroxidase)	ATTCAATGGCTGCTTCTTCCG	AACCTTCCCTTTGTAGATGCTTAG
CSD1 (Cu/Zn dismutase 1)	AACTGCCACCTTCACAATCAC	GCCTGCGTTTCCAGTAGCC
CSD2 (Cu/ Zn dismutase 2)	GCGGCGAAGAAGGCTGTTG	GGAGTGAGACCAGTGATACGAAC
FSD1 (Fe dismutase 1)	CCCTTGTGCTCGGCTCTTTC	ATGTAATCTGGTCTTCGGTTCTGG

E. RESULTS

Plant Temporal Responses to Moderate Drought (mDr)

To study the response of Arabidopsis to controlled soil water deficit (drought), the effect of mDr (Fig. 1) was tested at different vegetative developmental stages. Plants were grown under well-watered conditions and drought stress applied by withholding water at different growth stages to three sets of plants, at 25 days after sowing (DAS), 30 DAS, and 35 DAS. These growth stages, as defined for Arabidopsis (Boyes et al., 2002), of drought initiation correspond to the 6-leaf (1.06), 8-leaf (1.08), and 10-leaf (1.10) stages, respectively. Around 5-7 days after drought initiation and evapo-transpirational water loss, mDr stress is achieved and then maintained by adding water daily to reach soil moisture level of 2 g g^{-1} dry soil, with the plant stages at drought initiation at 8-leaf (1.08), 10-leaf (1.10), and 12-leaf (1.12), respectively (Boyes et al. 2002). The soil moisture level was maintained at a level that was non-lethal and above wilting point, at 30 % field capacity by replenishing the evapo-transpired water, and the reduction in biomass taken as a quantitative measure of growth calculated as described in methods. Figure 2 shows that the highest relative reduction in biomass (RB) of mDr treated compared to well watered plants, was at initiation of drought at the 30 DAS stage, with the 25 DAS treatment also significant, and the 35 DAS treatment least responsive to the drought treatment (Fig. 2A). In addition, the effect of varying the duration of mDr treatment was tested, and the data showed that 5 or 10 days mDr treatment gave similar RB (data not shown). The growth rate of Arabidopsis ecotype Columbia plants was determined for two developmental stages: 25-30 DAS, and 30-35 DAS. The rate of growth (both in terms of biomass and leaf area) during the first developmental stage 25-30 DAS, was higher than that during the second one 30-35 DAS (Fig. 2B).

To determine at what time point plants start to sense drought stress, a time-course experiment was conducted and plant samples were taken starting at 2 days before mDr treatment (-2), 1 day before mDr (-1), 0, 1, 2, and 3 mDr. The mDr stress (+1) is defined when the plants reach 2 g. g^{-1} H_2O/dry soil and water supplemented (if needed), to maintain the controlled drought as described in Methods. The relative water content in plant samples and in the soil was determined for each time point (Fig. 2C, D). Leaf relative water content (LRWC) measurements showed that plants begin to sense drought one day before mDr treatment is stabilized, designated

as -1 (Fig. 2C), and further analysis was done beginning at this time point. At day 0 of mDr (beginning of mDr) the LRWC decreases, and it continues to decrease at day 1 of mDr (Fig. 2 C, D). However, at day 2 the LRWC starts to increase to a normal level like that of the well-watered control (Fig. 2C). The soil water content is held constant from day 1 until the end of mDr treatment (Fig. 2D).

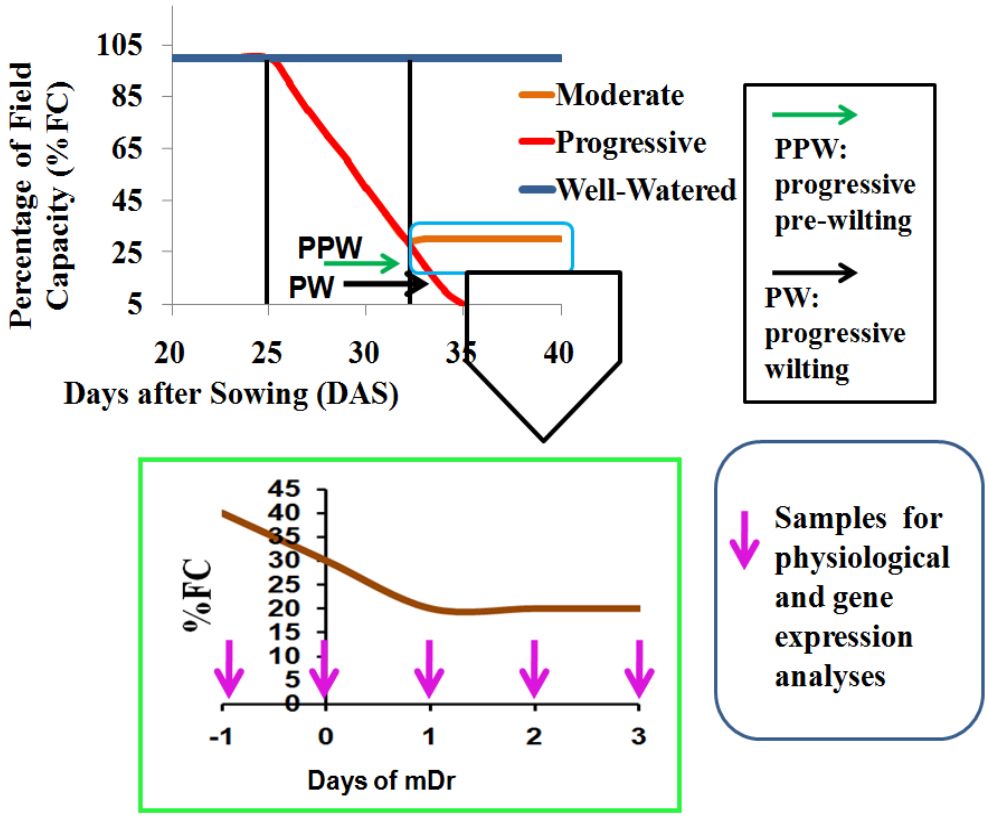

Figure 1.1. Schematic illustration of the drought treatments and sampling in this study. For drought treatments, water was withheld at day 25 after sowing (DAS), and the progress of drought monitored by soil moisture shown here as % Field capacity. Two progressive drought (pDr) treatments were done: wilting and pre-wilting (1 day before wilting, predicted based on soil moisture content). Controlled moderate drought (mDr) was used to study plant responses at physiological and molecular levels, with sampling times indicated (-1, 0, 1, 2, and 3), showing cycles of soil moisture equilibration.

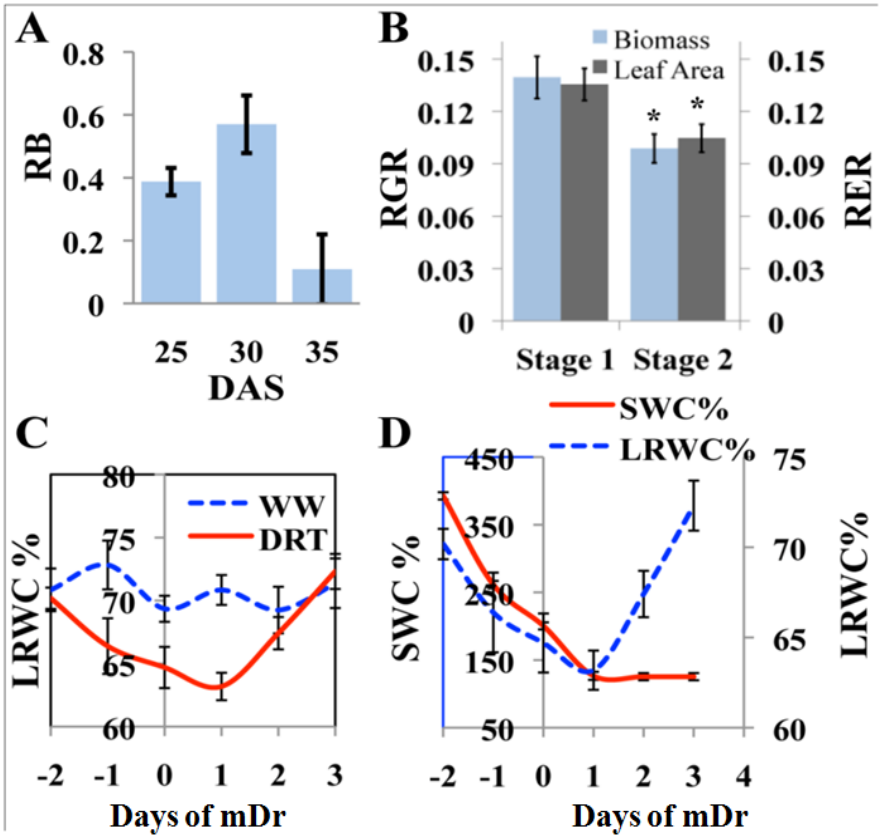

Figure 1.2. Growth of Arabidopsis ecotype Columbia (Col-0) in response to mDr. A, Response of different developmental stages to mDr, in terms of relative reduction in biomass (RB), the experiment was repeated, (n= 7; P <0.001). B, Relative growth rate (RGR), shown in biomass and relative expansion rate (RER) in leaf area during two developmental stages, Stage 1: (25- 30) days after sowing (DAS), Stage 2: (30- 35) DAS. Error bars represent SE, * indicate significant difference (n=16, P < 0.01). C, Leaf relative water content (LRWC %) at different days of mDr under well-watered (WW), and drought (DRT) conditions, (the experiment was repeated, n=12, p-value <0.0001). D, Leaf relative water content (LRWC %, n=12), and corresponding soil water content (SWC %, n=20) at different days of mDr, experiments were repeated.

Drought Responses of Hormonal Pathway Mutants

To validate the drought screen, genotypes previously known to be affected by drought, were selected. Specifically, the response of ABA signaling and biosynthesis knockout mutants (*abi1* and *aba1*) in two Arabidopsis backgrounds (Col-0 and Ler, respectively) was tested under mDr conditions. The reduction in growth (measured as biomass) under controlled mDr stress compared to well watered controls provides a parameter to compare different genotypes to the wild-type, and distinguish genotypes with altered sensitivity/resistance to drought. The ABA signaling mutant (*abi1*) and biosynthesis mutant *(aba1*) showed higher sensitivity to drought stress compared to their corresponding wild type controls (Fig. 3A and B).

In another experiment, jasmonate signaling mutants were tested. The jasmonate signaling mutants *coi1* and *jin1* displayed significant drought resistance at the end of mDr treatment (day 10) compared to control plants (Fig. 3C and D). Another jasmonate response mutant *jar1*, showed similar drought response to the WT. Indeed, this mutant was not completely insensitive to jasmonate compared to the *coi1* and *jin1* mutants, probably because *jar1* is not a complete gene knockout but an amino acid substitution mutant (Staswick et al., 2002). In this mutant jasmonic acid is synthesized but not conjugated with isoleucine.

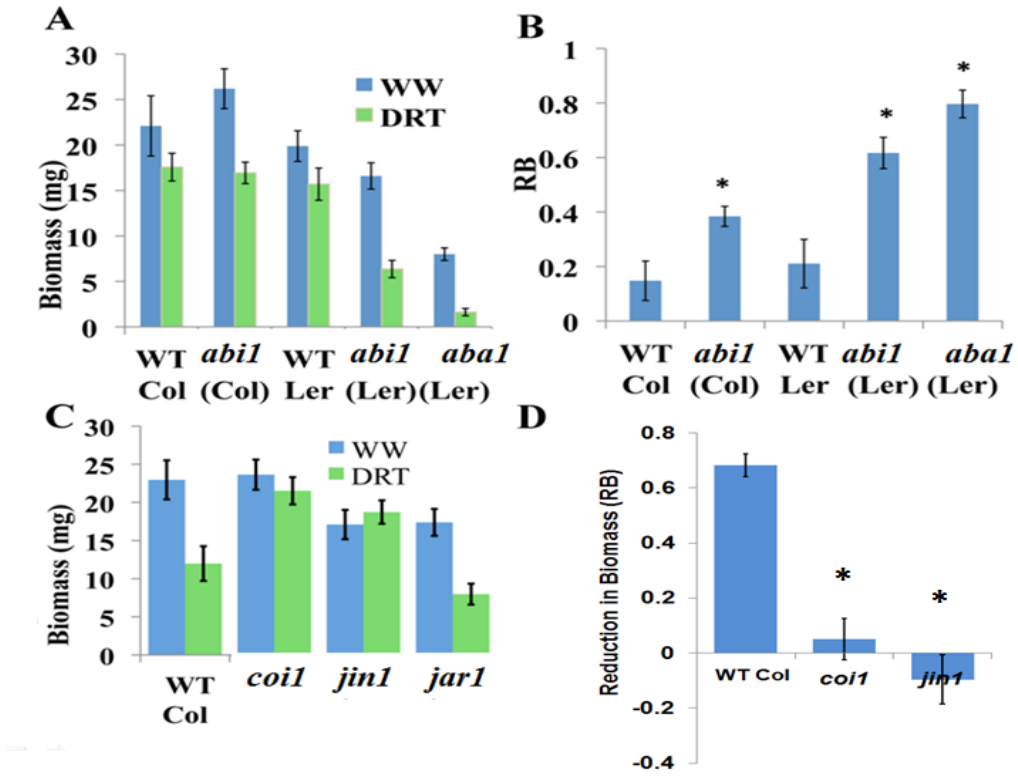

Figure 1.3. Response of ABA and JA mutants to mDr treatment. A, Biomass of ABA mutants under well-watered (WW) and drought (DRT) conditions. B, Relative reduction in biomass (RB) of ABA mutants. C, Biomass of JA mutants under well-watered and drought conditions. D, Relative reduction in biomass (RB) of JA mutants. Error bars represent SE, (n=8; $P < 0.001$), * indicate significant difference to WT control.

Gas Exchange Parameter Changes in Response to mDr

Stomatal conductance showed a decrease at 1 day of mDr, reaching 59% of the well-watered control (Fig. 4A). An approximate 50 % reduction was observed at day 1 of mDr (Fig. 4A), and continued to decrease till day 2 of mDr, with approximately 40% reduction to that of the well-watered control. At day 3 of mDr, it increased to the level of well-watered control. The same trend was shown for the internal CO_2 (Ci) concentration (Fig. 4A). However, photosynthesis showed a different trend, as it did not decrease at day 1 of mDr, and at day 2 it

showed a 10% decrease compared to the well-watered control (Fig. 4A). Instantaneous water use efficiency (WUEi) was higher than the well-watered control at day 1 and 2 of mDr (Fig. 4B). It was above 4 µmol $mmol^{-1}$ at day 1 and 2 of mDr while the well-watered WUEi was around 2 µmol $mmol^{-1}$. At day -1, 0, and 3 WUEi was the same as that of the well-watered control (Fig. 4B).

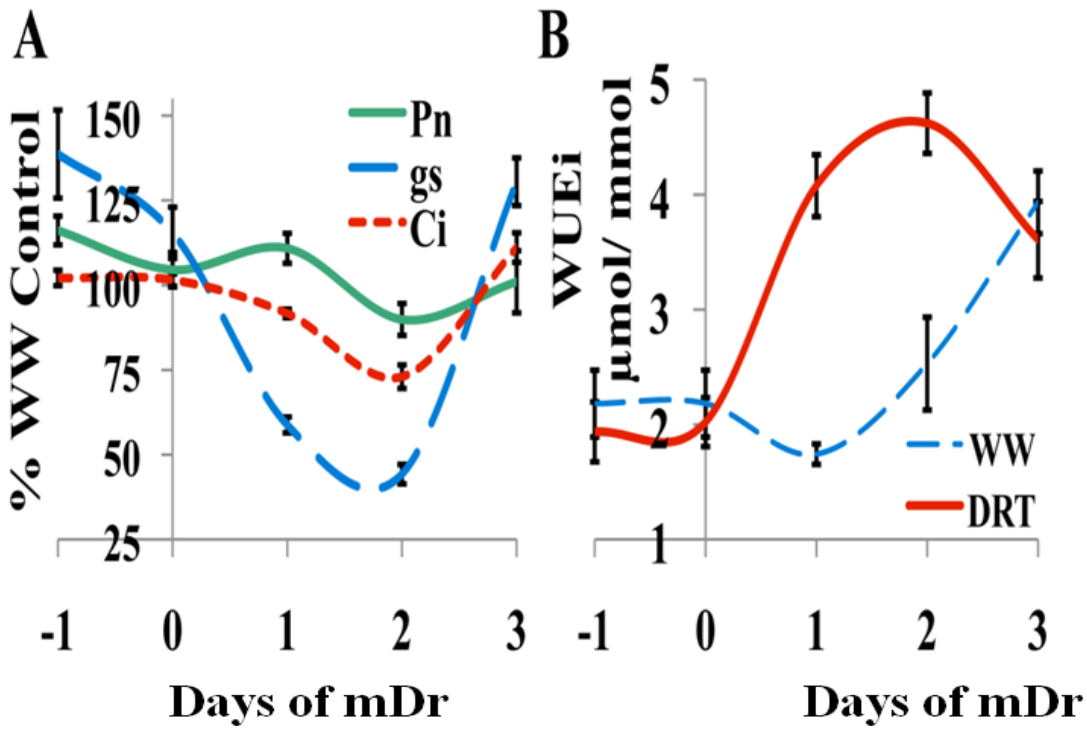

Figure 1.4. Gas exchange measurements and water use efficiency (WUEi) in a time course of mDr. A, Photosynthesis (Pn), stomatal conductance (gs), and internal CO_2 (Ci). B, Instantaneous water use efficiency (WUEi) in a time course of mDr. N=5 per treatment per time point, 3 leaves measured/plant, the experiment was repeated , error bars represent SE, $P < 0.001$.

A Portrait of Plant Transcriptional Response to Soil Water Deficit

In order to understand the global effects of drought stress on gene expression, microarrays were used to profile gene expression levels under mDr (Day1 and Day10) and pDr conditions and their corresponding controls in samples from young leaves. Analysis of differential expression showed that a large number of genes (2039) were significantly perturbed very early (Day1) in response to mDr. In contrast, after a prolonged moderate drought treatment

(Day10), a far less number of genes (728) were differentially expressed. Compared to the two mDr treatments, severe effects of drought on gene expression were revealed by the response to pDr (wilting): 7648 differentially expressed (DE) genes, about 30% of the genome.

Comparison of the three drought treatments (mDr-Day1, mDr-Day10 and pDr) was carried out first at the gene-level (Fig. 5). One hundred and seventy eight (178) genes responded to mDr and pDr treatments (91 up- and 87 down-regulated), while 1083 (545 up- and 538-down regulated) genes were specific to mDr. All the drought response genes from each of these treatments were functionally characterized using enrichment analysis of gene sets, mostly as described by Gene Ontology (GO; Ashburner et al., 2000) biological process terms, but also including gene sets associated with ABA-response and obtained from a previous publication (Table S1 in Nemhauser et al., 2006).. Among the genes up-regulated in both mDr-Day1 and pDr (646 genes) were predominantly water deprivation response genes (*q*-value ~1E-15), with overlapping sets of genes known to respond to ABA stimulus (*q*~1E-12.6), osmotic (*q*~1E-8.1), cold (*q*~1E-4.1) and oxidative (*q*~1E-2.5) stresses. Expression dynamics of several of these genes has been verified using qRT-PCR (*see below*). Fundamental processes of the cell known to be grossly affected by drought including DNA packaging (*q*~1E2.6), ribosome biogenesis (*q*~1E-2.9) and protein folding (*q*~1E-3.2) were concomitantly down-regulated in mDr-Day1 and pDr. The plants are, thus, mounting an early response to mDr that is very similar to the classical response to progressive soil water deficit (pDr). However, severe effects including down-regulation of photosynthesis (*q*~1E-20.7) and related processes are restricted to pDr.

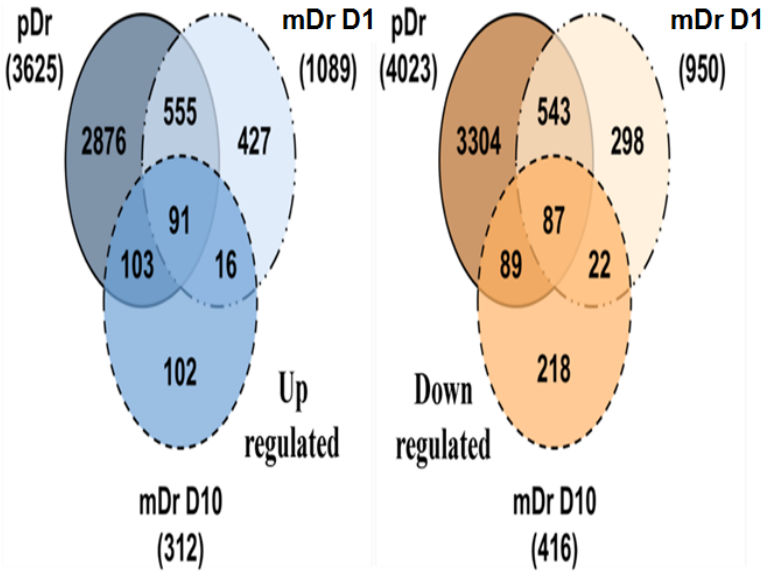

Figure 1.5. Gene expression analysis under moderate (mDr) and progressive (pDr) drought. Venn diagrams comparing up- and down-regulated genes of pDr, mDr 1 day (mDr D1) and 10 day (mDr D10) treatments.

The distinctive reaction of the plant to mDr-Day1 was the activation of plant cell wall modification genes that underlie cell growth. In contrast, these genes are down-regulated by pDr. This aspect of mDr response was pursued experimentally.

The expression of most of the common drought responsive genes was up-regulated by mDr-Day1 and pDr, whereas, at late stage of mDr (Day10) a few drought responsive genes were up-regulated, but the majority was down-regulated. Among the genes up-regulated only at late stage of mDr stage (Day 10) are a few hormone- (ABA) mediated signaling genes, possibly mediating acclimation. On the other hand, genes of: glucosinolate synthesis (*q*~1E-4), auxin biosynthesis and metabolism (*q*~1E-2.9), jasmonic acid (JA) biosynthesis and signaling (*q*~1E-2.8) and cell wall thickening (*q*~1E-2.9) were among those solely down-regulated in mDr-Day10. Few others involved in regulation of cell growth (*q*~1E-2.8) were down-regulated mDr-Day10-and-pDr.

Cis-Rgulation of Drought Response Genes

Several *cis*-regulatory elements (CREs) have been identified previously that are known to mediate response to environmental stresses including drought. To identify sequences of CREs potentially mediating the transcriptional regulation of drought response genes identified in our data sets, we devised a CRE-discovery pipeline using the de-novo motif discovery tool, FIRE (Elemento et al., 2007). We subsequently compared the newly identified sequences to known *cis*-elements in prominent databases (PLACE, AGRIS and AthaMap) (Higo et al., 1999; Davuluri et al., 2003; Galuschka et al., 2007; Mahony and Benos, 2007). Sequence logos of CREs of interest were then produced (Crooks et al., 2004). Applying this pipeline to mDr-Day1, mDr-Day10 and pDr (further separated into up- and down-regulated) gene sets, led to the identification of several known and novel CREs.

The CRE that was most prominent among the genes up-regulated in mDr-Day1 and pDr was one highly similar to the experimentally identified ACGT-containing ABRE-motif ACGTG(G/T)C (Fig. 6, Hattori et al., 2002). At position 6, the (G/T) degeneracy is exactly preserved in the pDr-ABRE, while it is strictly T in the mDr-Day1-ABRE, thus, making it more similar to the functionally equivalent coupling element 3 (Hobo et al., 1999). Interestingly, an element very similar to the ABRE – A(A/C)(A/C)RCGTG – was found among genes down-regulated in mDr-Day10. This class of ABRE-like CREs, hence, is probably mediating the ABA-dependent water deprivation response that is found to be up-regulated in mDr-Day1 and pDr, but down-regulated in mDr-Day10.

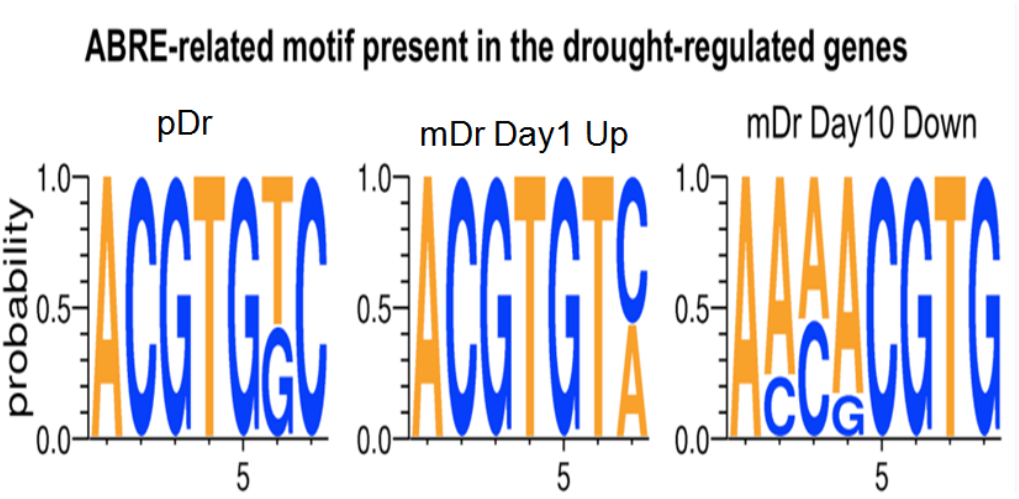

Figure 1.6. Sequence logos of *cis*-elements derived by de novo promoter analysis with similarity to the ABRE element in the three drought treatments: progressive drought (pDr), early stage of moderate drought (mDr Day1) and late stage of moderate drought (mDr Day10).

Another element that supports this inverse regulation was one highly similar to the DRE/CRT-motif (A/G)CCGAC recovered from genes up-regulated in mDr-Day1 and down-regulated in mDr-Day10, intriguingly, with opposite orientation biases, backward and forward, respectively. DRE/CRT and ABRE have been found to be interdependent in the dehydration-responsive expression of the *rd29A* gene in Arabidopsis (Narusaka et al., 2003).

Yet, another classical stress-response was found in early mDr, comprising slowing down of protein folding in the ER that triggers the unfolded protein response (Martinez and Chrispeels, 2003), suggested by the identification of the UPRE-like element among genes up-regulated in mDr-Day1. In contrast, an UPRE-like element was identified among genes down-regulated by pDr. Here, it is important to note that the category 'protein folding' was enriched among the down-regulated genes in mDr-Day1 and pDr. Moreover, several genes involved in cell elongation and division are down-regulated by UPR (Martinez and Chrispeels, 2003), genes that are down-regulated in pDr, but up-regulated in mDr-Day1.

Two elements, both AT-rich, were discovered among the up-regulated genes in mDr-Day1 and among up- and down-regulated genes in mDr-Day10: the former similar to CREs present in photo-responsive genes (AT1BOX and CCA1 motif2; Terzaghi and Cashmore, 1995; Wang et al., 1997) and the latter similar to the Evening Element involved in circadian control of

gene expression in Arabidopsis (Harmer et al., 2000). However, in the promoters of down-regulated genes in mDr-Day10, the Evening Element was found to be significantly co-localized with the DRE/CRT-like element

A novel CRE – (G/T)(A/C)CAGCT(A/C/G)(A/T) – has been identified to be uniquely enriched among genes with unknown function and down-regulated in mDr-Day10.

Cellular Metabolism under Drought Stress

Global gene expression analysis showed a substantial down-regulation of many photosynthetic genes under pDr drought compared to fewer changes under mDr (Table 2). In Arabidopsis more than 50% of the photosynthate is stored as starch (Zeeman and Rees, 1999). We therefore examined the gene expression data for effects of both drought treatments on starch biosynthesis and degradation. Two enzymes in starch biodegradation, α-amylase and β-amylase, were induced under pDr with expression $\log_2$ ratios of 1.5 and 3, respectively compared to control plants. In contrast, β-amylase was only induced with $\log_2$ ratio of 0.4 under mDr. To validate these observations, plants were sampled for starch quantification from both drought treatments. The highest accumulation of starch in wild type Arabidopsis plants was previously found to be in the late afternoon (at the end of the daily photoperiod) (Caspar et al., 1985). Therefore, individual plants of the same age (30 DAS), were collected at the late afternoon from plants treated to 1 day of wilting, and plants of 1 day of mDr. Starch analysis showed no accumulation of starch in the wilting plants, compared to normal starch accumulation in plants exposed to 1 day of mDr (data not shown).

Table 1.2. Expression of photosynthesis genes of photosystem I and photosystem II under progressive wilting (pDr) and moderate (mDr) drought

	Log2 Ratio		
Gene ID	**pDr**	**mDr**	**TAIR Annotation**
At1g30380	-1.036		Photosystem I subunit K (PSAK)
At1g03130	-1.0078	0.22	Photosystem I subunit D-2 (PSAD-2)
At3g16140	-0.712		Subunit H of photosystem I (PSAH-1)
At1g08380	-0.742		Subunit O of photosystem I (PSAO)
At4g12800	-0.695		Photosystem I subunit L (PSAL)
At4g28750	-0.789		PSAE-1 (PSA E1 KNOCKOUT); catalytic
At4g02770	-0.74		Photosystem I subunit D-1 (PSAD-1)
At1g52230	-1.872		Photosystem I subunit H-2 (PSAH-2/PSAH2/PSI-H)
At3g01440	-2.266	-0.106	Oxygen evolving enhancer 3 (PsbQ) family protein
At5g64040	-1.723		Photosystem I subunit PSI-N (PSAN)
At2g06520	-0.872		Photosystem II subunit X (PSBX)
At1g06680	-0.8		Oxygen –evolving enhancer protein 2(PSBP-1)
At5g01530	-1.063		Chlorophyll A-B binding protein CP29 (LHCB4)
At1g44575	-1.471		Nonphotochemical quenching (NPQ4)
At1g67740	-1.513		Photosystem II BY (PSBY)
At4g05180	-0.896		Photosytem II subunit Q-2 (PSBQ/PSBQ-2/PSII-Q)
At1g14150	-2.03	0.167	Oxygen evolving enhancer 3 (PsbQ) family protein
At4g21280	-1.888	-0.145	PSBQ/PSBQ-1/PSBQA; calcium ion binding.
At4g28660	-2.594	-0.542	Photosystem II reaction center PSB28 protein (PSB28)
At3g21055	-1.739		Photosystem II subunit T (PSBTN)

Gas exchange measurements in the time course of mDr treatment showed that plants have almost normal photosynthetic rates (Fig. 4A). Since Arabidopsis stores more than 50% of the photosynthate as starch, we wanted to confirm the gas exchange measurements by quantifying starch accumulation under time course of mDr. Starch concentration was determined at 5 time points: -1, 0, 1, 2, and 3 days of mDr, and showed no significant differences for these time points compared to their corresponding well-watered control (data not shown).

To test for evidence of osmotic adjustment under mDr treatment, proline content was determined at days 3 and 4 of mDr. We found no significant change in proline in drought treated plants compared to well-watered controls (data not shown). Moreover, GC-MS analysis also showed no significant changes in proline concentration during the time course of mDr (Joel Shuman, personal communication).

Because ABA is one of the most important stress-related hormones, we also tested the change in ABA concentration in the time-course experiment of mDr. At day 0 of mDr, plants accumulated high ABA concentration compared to well-watered control. The concentration continued to increase until day 1 of mDr (Fig. 7A), and at day 2 the ABA concentration started to decrease.

Expression of Stress Signaling Pathway Genes under Drought Stress

On exposure of plants to drought stress, ABA-dependent and ABA-independent signaling pathways have been shown to be induced (Shinozaki and Yamaguchi- Shinozaki, 1997). To test the effect of soil water deficit achieved using two different schemes: the expression levels of key genes in the signaling pathways were quantified during pDr pre-wilting (1 day before wilting) drought and controlled mDr treatments. Results of these experiments revealed that the ABA biosynthesis gene, under pDr pre-wilting (PPW), *NCED3* was induced 4-fold higher than mDr (Fig. 7B). The drought responsive transcription factor *DREB2A* was induced at a higher level under pDr compared to mDr. Under pDr the expression level of *DREB2A* was about 10 fold, whereas it was about 4 fold under mDr. Another gene showing differential expression between the two drought treatments is *RD29B*, which is induced almost 100 fold under pDr compared to 20 fold under mDr (Fig. 7B). The drought responsive expression levels of the rest of the tested stress signaling genes were not significantly different between the two drought treatments (Fig. 7B).

The expression level of key genes in the stress signaling pathways, ABA-dependent and ABA-independent, was quantified in the time course analysis of mDr. *NCED3* was highly induced to 4 fold at day 0 and 1 of mDr compared to 0.5 fold at day 2 and 3 of mDr (Fig. 7C). The expression of *ABF3* (ABA-dependent pathway) was high at 0 day of mDr of about 4 fold, its expression decreased to about 2 fold at day 1 and 2 of mDr. . As a representative of an ABA-independent pathway, *DREB2A* showed induction at day 0 to 4 fold and stayed induced at day 1 of mDr. After day 1, its expression was back to well-watered control level (Fig. 7D).

Downstream of *NCED3*, *ABF3*, and *DREB2A*, there are many described genes that are recognized as stress marker genes, such as: *RD22*, *RD29A*, *RD29B*, and RAB18 (Yamaguchi-Shinozaki K, Shinozaki K, 1993a; Yamaguchi-Shinozaki K, Shinozaki K, 1993b**;** Seki et al.,

2002). The expression profile of these marker genes in a time course of mDr (Fig. 7E) showed induction at day 0 of mDr that continued at day 1, and then decreased in expression.

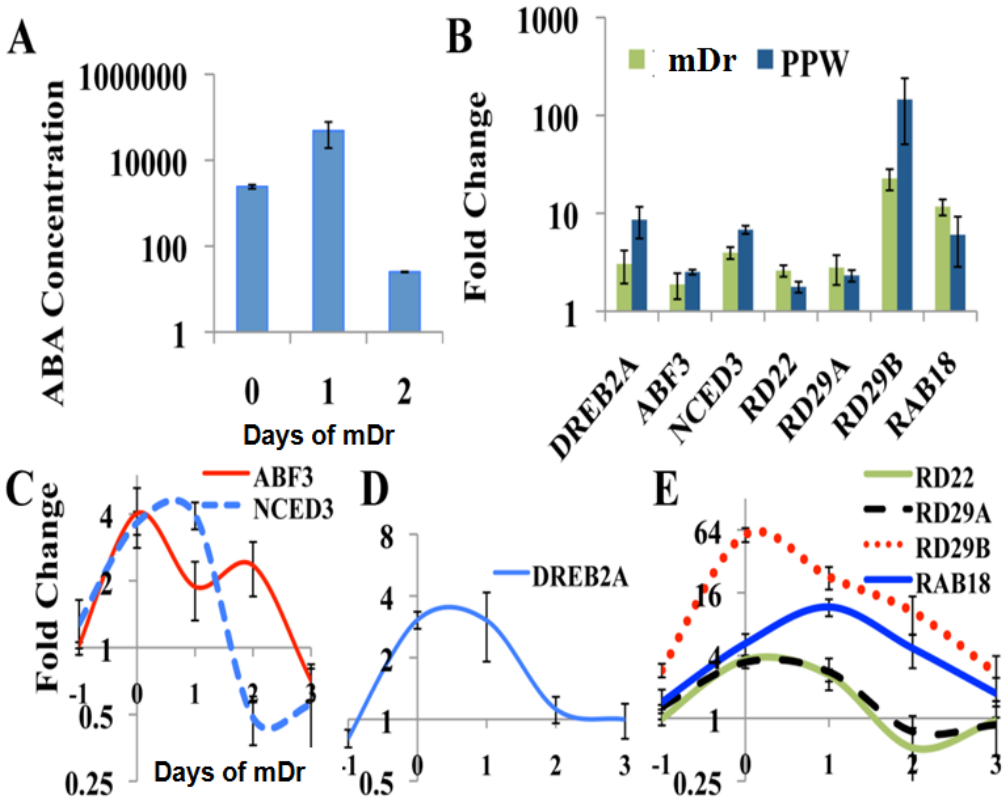

Figure 1.7. Drought stress responses in ABA levels and ABA-related genes. A, ABA quantification (in % of well watered control) from day 0 to day 2 of mDr B, qRT-PCR analysis of stress signaling genes and stress marker genes under pDr pre-wilting (PPW) drought and mDr. C-E, Time course response in days of mDr of stress related genes using qRT-PCR, showing fold change (y-axis). C, ABA-signaling pathway genes *NCED3* and *ABF3*. D, *DREB2A* (ABA-independent signaling pathway). E, Stress marker genes. A-D, 3 replications with 5 plants pooled/replication, error bars represent SE.

Stomatal Responses in a Time Course Analysis of mDr

To understand the stomatal responses to mDr treatment at the molecular level, a set of stomatal-related genes were chosen based on our microarray data of mDr, to study their kinetics of expression changes in the time-course study of mDr. For *PLDα* and *GPA1*, two positive regulators of ABA signaling in the stomata, the expression levels under mDr started increasing to

2 fold from day -1 (one day before mDr) to peak at day 1 with 3 fold change, and then decreased thereafter (Fig. 8A). Since outward potassium channels have an important role in stomatal response to the surrounding environment, we quantified the expression of *GORK*, an outward K^+ channel gene. The highest induction of *GORK* was at day 1 of mDr of 3 fold expression level, then its expression started to decrease from day 2 onwards (Fig. 8A).

Another group of genes with a major role in stomatal response and ABA signaling belong to the family of type C protein phosphatases (PP2Cs). We therefore tested the expression profile of three main PP2Cs: *ABI1*, *ABI2*, and *HAB1*. The expression of *ABI1* and *ABI2* started to increase to about 3 fold at day -1 of mDr, continued to day 1, and then decreased (Fig. 8B). *HAB1* showed a decrease in expression at day -1, followed by an induction of 4 fold at day0 and day1 and a decrease thereafter (Fig. 8B). A receptor-like kinase 1 (*RPK1*), described to be active in the early response to ABA signaling in the stomata (Osakabe et al., 2005), was induced in our mDr microarray. In the mDr time course analysis *RPK1* showed induction of about 2 fold at day -1, which continued to day1, followed by a decrease (Fig. 8C). In summary, PP2Cs genes (*ABI1* and *ABI2)* and *RPK1* were induced at an early stage of mDr (day -1 to day 1), and after that their expression started to normalize similar to the well-watered control.

In our drought microarrays we found *MYB60* was repressed at day 1 of mDr, and under pDr. This gene was found to be specifically expressed in guard cells, and the corresponding *myb60* null mutant plant exhibited reduced stomatal opening (Cominelli et al., 2005). We therefore quantified *MYB60* expression at 5 time points during mDr. *MYB60* was induced at day -1 and 0 of mDr (Fig. 8D), and repressed at day 1, which stabilized from day 2 onwards to that of the well-watered control (Fig. 8D).

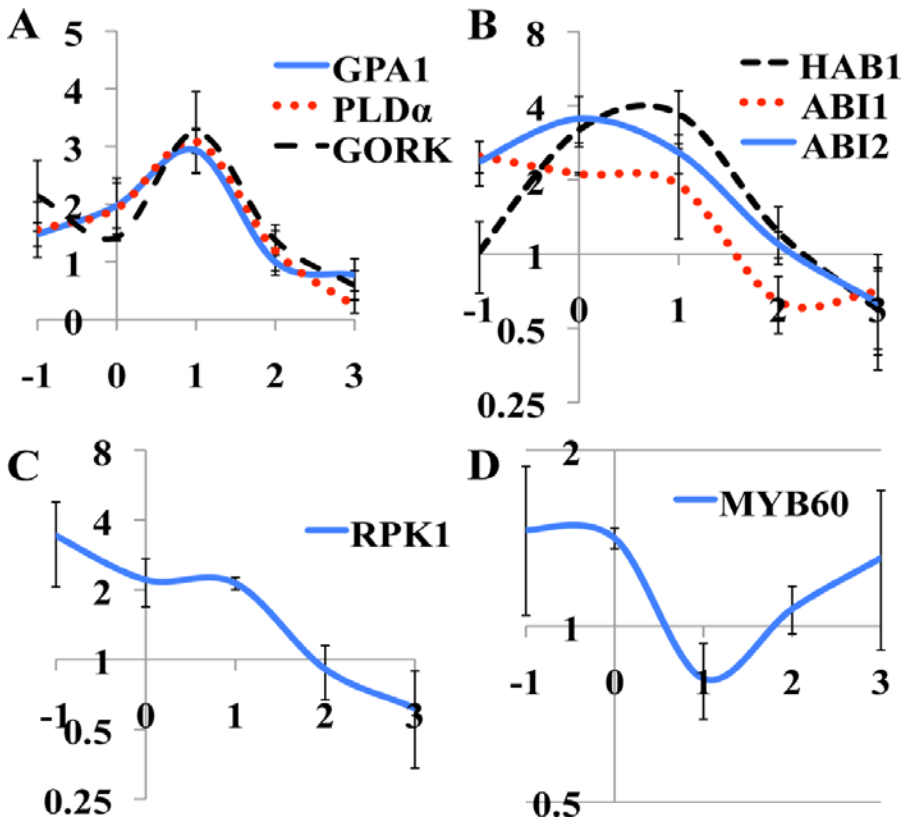

Figure 1.8. Gene expression profiles of stomatal-related genes during mDr treatment. The y-axis shows the fold change using qRT-PCR, and x-axis the days of mDr. A, *PLDα*, *GPA1*, and *GORK*. B, Type C protein phosphatase genes (PP2Cs). C, Receptor-like kinase 1 (*RPK1*). D, *MYB60* transcription factor. A-D, 3 replications with 5 plants pooled/replication, error bars represent SE.

Expression of Photosynthesis and Antioxidant Genes under Drought Stress

The comparison of the mDr and pDr microarray data revealed that many photosynthesis genes were significantly repressed under wilting, in contrast to a brief effect of one day under mDr (Table 2). We selected a few photosynthesis genes, whose corresponding proteins are part of PSI (*PsQO2*, *PSAH2*) and PSII (*PsbW*, *PsbQA*), and profiled their expression under the mDr time course experiments (Fig 9A). The expression of *PsbW* significantly decreased from day 1 to lowest level at day 2 of mDr while *PsbQA* expression was normal at day -1, 0, and 1, and

started to decrease at day 2. The PSI subunit gene *PsQO2* showed normal expression level at days 1, 0, 1, and 2 and at day 3 started to decrease. *PSAH2* was induced at day -1, reducing to normal level at day 0, and then induced at days 1 and 2, followed by repression at day 3 of mDr (Fig. 9A).

To test for oxidative stress related molecular events in response to mDr, six enzymes with antioxidant activity were chosen: ascorbate peroxidase 1 (APX1), thioredoxin peroxidase 1 (TPX1), glutathione peroxidase 6 (GPX6), cytosolic Cu/Zn dismutase (CSD1), chloroplastic Cu/Zn dismutase (CSD2), and Fe dismutase (FSD1). *APX1* was slightly decreased at day (-1) of mDr, then slightly increased at day 1, after that it showed a slight decrease at day 2 of mDr (Fig. 9B). *TPX1* was slightly increased during the time course of mDr (Fig. 9B). GPX6 showed a drastic decrease in expression at day 1 of mDr. Both *CSD1* and *CSD2* were significantly repressed at day 1 and day 2 of mDr, while *FSD1* was slightly changed over the time course period (Fig. 9B).

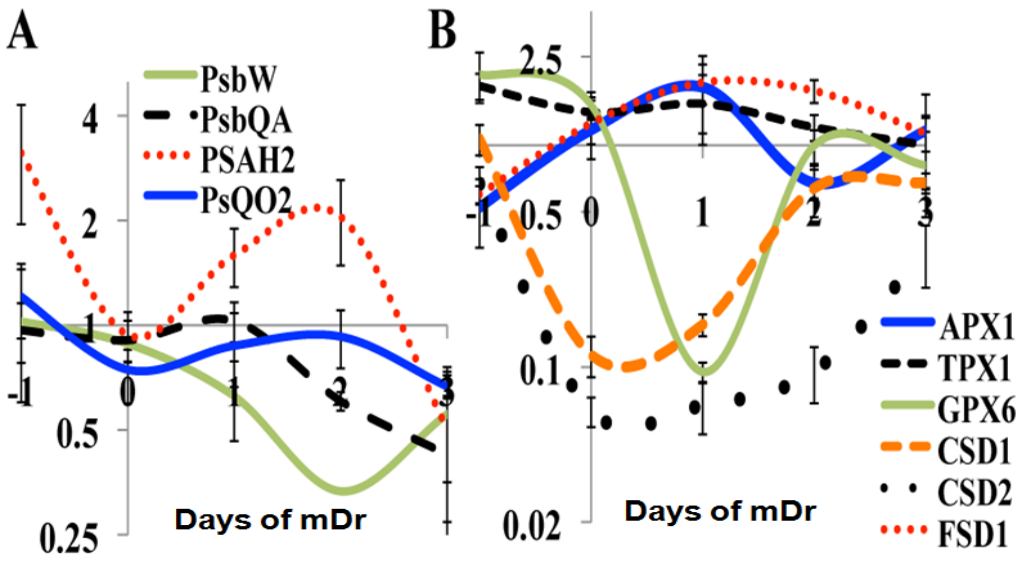

Figure 1.9. Gene expression profiles of drought responsive genes. Time course in days mDr (Days of mDr, x-axis) showing gene expression fold change (y-axis). A, Photosynthesis related genes. B, Antioxidant enzyme genes. A-B, 3 replications with 5 plants pooled/replication, error bars represent SE.

Expression of Cell Expansion Genes under Drought

GO enrichment analysis of mDr differentially expressed (DE) genes showed the enrichment of cell expansion related genes. The induction of cell expansion genes was specific to mDr, and the same genes were down-regulated or not differentially expressed in pDr. In addition, the comparison of mDr and pDr microarray data with ABA responsive genes from AtGeneExpress (Table S1 in Nemhauser et al., 2006), showed that cell expansion genes were only up-regulated in mDr compared to pDr wilting drought and ABA treatments. We therefore quantified the effect of pDr pre-wilting (PPW) and mDr treatments on the expression level of the expansin genes: *EXPA3*, *EXPA4*, *EXPA8*, *EXPA10*, and *EXPLB1* (Fig. 10A). After one day of mDr most of the expansin genes were induced, while under pDr *EXPA3* and *EXPA8* showed repression and *EXPA4* remained unchanged (Fig. 10A).

In the time course analysis of mDr the *EXPA3*, *EXPA4*, *EXPA8*, and *EXPA10* genes were repressed at day 0, induced at day 1, followed by a decrease at day 2 and 3 of mDr (Fig. 10B). *EXPLB1* showed a steady increase in expression until day 1, and then a gradual decline to day 3 reaching normal expression level relative to well-watered control (Fig. 10B).

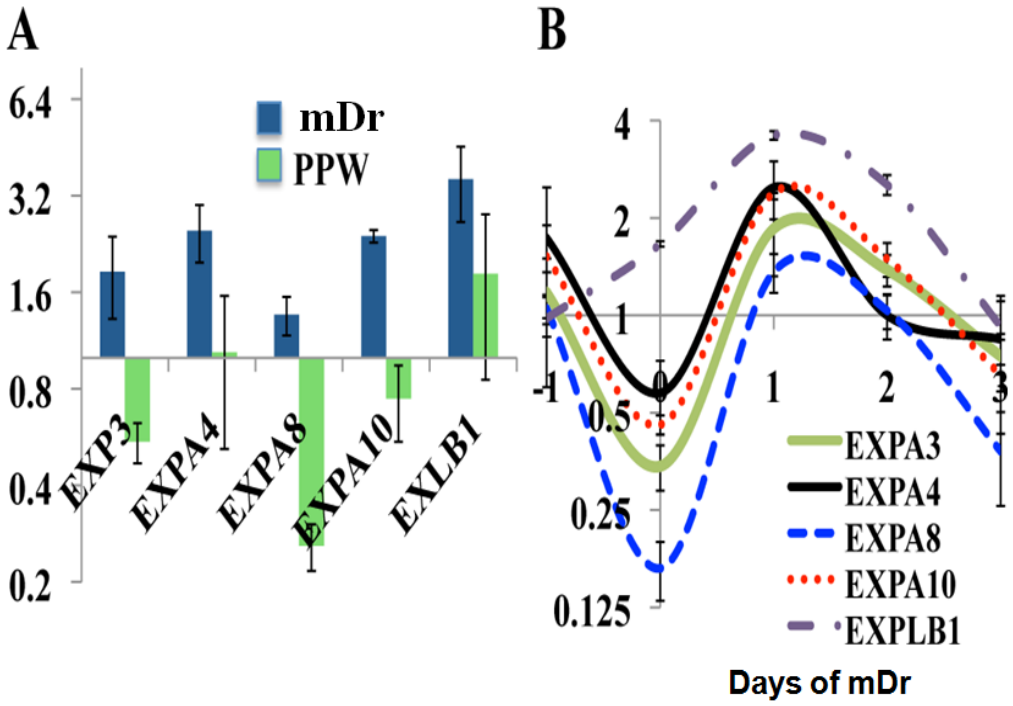

Figure 1.10. Gene expression profiles of expansin genes in drought acclimation response. A, Expression of expansin genes under pDr pre-wilting (PPW) and mDr, showing fold change (y-axis). B, Expression profiles of expansin genes shown in fold change (y-axis) in a time course of mDr (x-axis, days of mDr). A-B, 3 replications with 5 plants pooled/replication, error bars represent SE.

F. DISCUSSION

Growth Reduction under Drought Stress

The application of controlled mDr stress on Arabidopsis plants enabled us to evaluate many physiological and molecular parameters in relation to the drought stress treatment. mDr, maintained by daily replenishing evapo-transpired water, was applied at plant growth stage 1.08-1.10 corresponding to 8-10 leaf stage (Boyes et al., 2002), for 5-10 days and caused a significant reduction in growth as observed by dry matter accumulation and leaf expansion. The reduction was dependent on the developmental stage of the plants, drought initiation at stage 1.08 and 1.10 corresponding to 25 and 30 days after sowing (DAS) showed highly significant growth reduction compared to later developmental stages. Moreover, both 5 and 10 days duration of drought, gave a significant reduction in growth. To understand the biological processes involved in the response of plants to drought that inhibit plant growth, gene expression analysis of plants treated to 10 days of mDr was done. The perturbation in expression was much reduced at the later time point, suggesting a stabilization or acclimation in responses. Our interest therefore was to find out the causes in reduction of growth due to drought stress, the time when the responses start and the physiological, biochemical, and molecular changes responsible for the reduction of growth under drought.

Drought Transcriptome Analysis

A comparative transcriptome analysis of pDr-wilting and mDr (1 and 10 days) revealed common drought responsive targets. This was substantiated by GO analysis, which showed enrichment for genes involved in stress response processes such as desiccation, stress and water deprivation in the two drought treatments. In addition, common stress responsive *cis*-elements were enriched in promoters of genes up-regulated under pDr-wilting and mDr-Day1, and between mDr-Day1 and mDr-Day10. Moreover, qRT-PCR analysis showed that most of the characteristic stress signaling and stress marker genes were similarly induced under pDr pre-wilting and mDr Day1 treatments. In conclusion, these expression analyses revealed expected drought responses at the early stage of mDr. Hence, Arabidopsis plants under mDr sense and respond to drought stress in a similar way to the more drastic progressive, wilting or dry-down drought treatments, which lead to lethality. The mDr treatment is therefore a good model system to dissect the response and resistance of plants to drought.

Prominent genes up-regulated early and late were the homeobox genes *ATHB7* and *ATHB12*, which have been shown to be involved as regulators of plant growth under drought stress (Olsson et al., 2004). Mutants of these genes displayed reduced sensitivity to ABA, and overexpression showed ABA hypersensitivity and phenocopy of WT Arabidopsis under drought treatment. These previous results suggested that the *ATHB7* and *ATHB12* genes probably maintain the reduced growth of plants under drought, which is an acclimation response of plants to survive prolonged drought stress. Other genes co-expressed with these homeobox regulators in this study, may have similar roles, such as transcription factors belonging to the NAC and CCAAT binding (CBF-B/NF-YA) families, protein phosphatases and kinases. These expression studies therefore revealed a number of drought responsive genes that might be important in protecting plants from drought stress, and are candidates for future genetic analysis.

In our Affymetrix array analysis known ABA up-regulated genes (Table S1 in Nemhauser et al., 2006) made up 26% (944/3625) of the pDr up-regulated genes, 31% (341/1089) of mDr-Day1 and 22% (93/416) of mDr-Day10 differentially regulated genes (Fig. 5). These results of mDr and pDr are consistent and reveal the similarity in significance of ABA and non-ABA related or dependent pathways in drought responses. In genome-wide oligonucleotide microarray studies of Arabidopsis soil water deficit pDr responses (Huang et al., 2008), a higher level of drought regulated genes were found in comparison to ABA responses using a more active ABA analog (Huang et al., 2007).

Stress Perception and Signaling is Transient and Occurs at Early Stage of Drought

Plant responses to different stresses were shown to be mediated by ABA-dependent and ABA-independent stress signaling pathways (Shinozaki, 1997; Hirayama and Shinozaki, 2010). In addition, extensive studies of ABA signaling reveal the central role of ABA in response to different environmental stimuli (Cutler et al., 2010; Kim et al., 2010). To assess the role of ABA in mDr stress treatment, ABA biosynthesis and signaling mutants were tested under mDr stress, and showed significant reduction in growth (higher sensitivity) compared to the wild type. Therefore, ABA is needed for normal drought response, and any perturbation in ABA biosynthesis or signaling will negatively affect plant growth under drought.

To determine the time course of ABA accumulation under drought, ABA was quantified at three time points (day 0, 1, and 2) of mDr, showing highest concentration at days 0 and 1.

Consistent with this, the expression pattern of some characteristic genes in stress signaling pathways: *DREB2A*, *ABF3*, and *NCED3*, showed induction at an early stage of the drought stress. Moreover, the same expression pattern was shown for a group of downstream regulated genes that are designated as stress markers: *RD22*, *RD29A*, *RD29B*, and *RAB18*. These experiments thus show that drought stress perception and signaling occur at an early stage of mDr treatment, and in the future will enable a molecular genetic and physiological dissection of subsequent responses to the stress.

Drought Avoidance by Stomatal Closure at an Early Stage of Drought Stress

Stomatal closure under drought is an avoidance response/strategy adopted by plants to save water and maintain turgor (Levitt, 1980; Chaves and Oliveira, 2004; Skirycz and Inze, 2010). Under mDr treatment plants showed an early response with a drastic decrease in leaf relative water content (LRWC) and stomatal conductance (gs), but photosynthesis rates remained normal. Moreover, the expression pattern of stomatal-related genes showed a peak at an early stage of drought stress. The α-subunit of the heterotrimeric G protein gene (*GPA1*) and *PLDα*, are known to play a critical role in the inhibition of stomatal opening (Mirsha et al., 2006; Nilson and Assmann, 2010; Zhao et al., 2010), exhibited high expression, consistent with their role in the inhibition of stomatal opening. Outward and inward potassium channels regulate the movement of K^+ across the membrane of guard cells in response to ABA signals (Schroeder et al., 2001; Nilson and Assmann, 2007). Under stress, the outward channels are induced, and inward channels repressed (Schroeder et al., 2001). In agreement with these previous findings, the outward channel gene GORK showed the highest expression at day 1 of drought stress treatment.

Another important group of genes in ABA signaling in the guard cells, are the type C protein phosphatase (PP2C) genes, which act as negative regulators in ABA signaling in stomata (Pedro, 1998; Gosti et al., 1999; Saez et al., 2006). Some of the PP2Cs were found to be induced under drought, salt stress, and low temperature (Tahtiharju and Palva, 2001; Bray 2004). Here, three PP2Cs: *ABI1*, *ABI2*, and *HAB1* were found to be induced early during drought treatment. Another gene *RPK1* (leucine-rich repeat receptor-like kinase1) was found to be induced at an early stage of drought stress. This is consistent with its role in mediating an early response in ABA signaling and regulation of guard cells under stress, with a function in improvement of

abiotic stress tolerance (Osakabe et al., 2010). *MYB60* has an important role in the regulation of guard cells, with the knockout mutation resulting in stomatal closure (Cominelli et al., 2005). Under mDr time course experiments, *MYB60* expression is lowest at day 1, corresponding with the drastic decrease in stomatal conductance under drought treatment. The mediation of drought responses through regulation of a stomatal drought response network resulting in induction of *GPA1*, *PLDα, GORK* and the *PP2C*s, and repression of the TF *MYB60* are consistent with the available data.

Normal Photosynthesis and No Oxidative Stress Under mDr

Photosynthesis rate determined by instantaneous gas exchange measurements was not affected by the mDr stress treatment, which was supported by the expression profile of photosynthesis-related genes. Starch accumulation was also normal during mDr treatment (data no shown). In agreement with these observations, previous studies found that photosynthesis usually is not affected by mild and mDr (Cornic and Massacci, 1996; Flexas and Medrano, 2002). Moreover, analysis of the publicly available expression profiling data under drought and salt stress, showed a non-significant effect of mild drought on the expression of photosynthetic genes both qualitatively and quantitatively (Chaves et al., 2009).

ROS are produced in different compartments of the plant cell, both under normal and stressful conditions (Grene, 2002). When plants are challenged by drought or other abiotic stresses, ROS are generated as a result of the inhibition of photosynthesis and the predominance of photorespiration (Noctor et al., 2002). ROS are found to have a dual function in plants, they are needed as signaling molecules, but a high concentration is also detrimental (Kwak et al., 2003; Slesak et al., 2007). High ROS concentration is hence a stress symptom, and plants have to maintain the ROS within a certain level that is required for normal cellular homeostasis. ROS concentration in the cell is maintained by the antioxidant system, which is made up of antioxidant molecules ascorbate, glutathione, and α-tocopherol; in addition to the antioxidant enzymes peroxidases, catalases, and superoxide dismutases (Alscher et al., 2002; Grene, 2002). The induction of members of the antioxidant system is highly correlated with the severity of the stress. Under severe abiotic stresses such as high light, low temperature, high temperature, salt stress, severe drought, and a combination of stresses; antioxidant enzymes are differentially and

highly induced (Kliebenstein et al., 1998; Noctor et al., 2002; Rodriguez Milla et al., 2003; Miao et al., 2006**;** Miller et al., 2010).

mDr did not exhibit acute oxidative stress, as shown by the nearly-normal expression levels of three antioxidant enzyme genes *APX1*, *TPX1*, and *FSD1*; and repression of the other tested antioxidant enzyme genes *GPX6*, *CSD1*, and *CSD2*. This is consistent with the normal photosynthesis rate under mDr, proven both at physiological and molecular levels. However, *GPX6* and *TPX1* are slightly, yet significantly, up-regulated (~ 2 fold) at the very early stage of drought stress, and these peroxidases might be involved in reducing very early ROS responses, partly from stress signaling (Kwak et al., 2003; Pham and Desikan, 2009). Indeed, the promoter of *GPX6* was found to have the common stress cis-elements (ABRE and CRT/DRE), and it was responsive to osmotic stress (Rodriguez Milla et al., 2003). In conclusion, the mDr level is below the threshold required for the generation of high destructive concentration of ROS. Therefore, there was a net normal level of antioxidant enzymes except for the dismutases, which were repressed. The repression of the dismutases can be explained by the low concentration of superoxide, and efficient photosynthesis, due to the incomplete closure of the stomata under stress conditions (Cruz de Carvalho, 2008). In addition, studies on many crops showed a discrepancy regarding the expression of the antioxidant enzymes in response to drought. In some cases they were induced, in other cases they were repressed (Cruz de Carvalho, 2008), suggesting that different ROS balance and levels are required at different responses.

Acclimation to mDr by Cell Wall Adjustment

One of the first acclimation responses to drought is the decrease in leaf growth, which results in maintenance of cell turgor, and reduces the transpiration area (Mathews et al., 1984; Neumann, 1995). In addition to cell turgor, cell wall biochemical and biophysical characteristics play an important role in cell growth (Mathews et al., 1984; Neumann, 1995). In Arabidopsis, leaf size is a result of both cell division and cell expansion (Horiguchi et al., 2006), and under mild drought Arabidopsis leaves compensate for low expansion rate by the extension of expansion duration (Aguirrezabal et al., 2006). Cell expansion is a process of cell wall modification and loosening catalyzed by enzymatic and non-enzymatic protein components of the cell wall (Cosgrove, 2005), which is composed of cellulose and hemicelluloses in a matrix of pectins and proteins (Cosgrove, 2005). Expansins are the key cell wall loosening proteins, which

act by the breakage of hydrogen (non-covalent) bonds between cellulose and the surrounding matrix leading to slippage of the cell wall components under acidic pH (acid growth), and consequently, the increase in extensibility of the cell wall (McQueen-Mason et al., 1992).

Physical properties of the cell wall play a crucial role in the response of plants to water deficit (Bacon, 1999). Transcriptome analysis of pDr showed the repression of many expansin genes (Bray, 2004), while mild osmotic stress revealed the induction of expansin genes (Skirycz et al., 2010). Cell expansion in response to drought was characterized in the maize root system as an adaptation to low water potential (Wu and Cosgrove, 2000). In addition, there are many studies on leaf growth under water deficit in maize leaves, and other crop plants such as sunflower, and Arabidopsis (Mathews et al., 1984; Aguirrezabal et al., 2006; Bouchabke´ et al., 2006; Granier and Tardieu, 2009). Despite the plethora of studies on cell expansion in response to drought, very little is known about the molecular basis of this process in plant responses to internal and external stimuli.

Both the microarray data and qRT-PCR analyses reported here revealed the up-regulation of expansin genes at day 1 of mDr treatment. In contrast, under pDr pre-wilting and pDr wilting drought treatments most of the expansin genes were down-regulated. The expression profile of 4 expansin genes in the time course analysis of mDr showed a pattern of repression at the beginning of drought (day 0), induction at day 1, and repression thereafter. A fifth expansin gene *EXPLB1* has a different expression pattern, with a peak in expression at day 1, and a decrease starting at day 2. This early peak in expansin expression can be interpreted as an acclimation to mDr by cell wall adjustment. This is a common type of acclimation response, which can proceed by loosening and/or tightening of cell wall structure depending on the species, organ, and tissue (Neumann, 1995; Moore et al., 2008). A study of the resurrection plant (*Craterostigma plantagineum*) showed an increase in expansin expression and activity at an early stage of dehydration, resulting in a flexible cell wall as an adaptation to dehydration (Jones and McQueen-Mason, 2004). Consistently, in our study there was no significant RB at an early stage of mDr compared to later stage (day 5 and afterward), supported in part by the slight increase in expansin expression as an adaptation to stress that occurs as an early response. Differential spatial expression of expansins was shown in maize leaves, tomato shoot apex, and tomato embryos (Chen et al., 2001; Vogler et al., 2003; Muller et al., 2007), and in Arabidopsis the spatial expression of *EXPA10* in leaf growth and development has been described (Cho and

Cosgrove, 2000). Therefore, the spatial and temporal patterns of expansin expression and activity need to be studied in response to drought.

The plant cell wall is required not only for mechanical support, but for growth and adaptation to the hostile environments. There is still a lot to be learned about cell wall modification under different abiotic stresses at molecular, cellular, tissue, and whole-plant level. Studies on the effects of overexpression of expansin genes show enhanced growth in rice, and high sensitivity to hormones and salt stress in Arabidopsis (Choi et al., 2003; Kwon et al., 2008). These suggest the important role of expansins in acclimation and adaptive responses of plants to abiotic stresses.

Drought Acclimation Processes in Late Stages of mDr

Gas exchange measurements at late stage of mDr (Day10) showed no significant difference in stomatal conductance and photosynthesis of drought-treated plants compared to the well-watered control (data not shown). Moreover, no stomatal-related genes were differentially expressed at this stage. In contrast, microarray and qRT-PCR analyses of the early stage of mDr (Day1) showed that many stomatal-related genes were either up-or down-regulated. Hence, early stage (Day1) showed reduced stomatal conductance, whereas normal stomatal conductance and reduced growth was shown at late stage of mDr (Day10).

Jasmonic acid biosynthesis and signaling were among the main enriched GO categories in the down-regulated genes at late stage of mDr. Jasmonates have been found to have a potential role in response to drought stress in soybean as they showed an early increase within 2 hrs of dehydration and decrease in concentration afterward (Creelman and Mullet, 1995). Moreover, jasmonates were found to cause stomatal closure (Raghavedra and Reddy, 1987). This role was confirmed by the impaired stomatal response to exogenous jasmonates in jasmonate insensitive mutants (*jar1* and *coi1*) (Suhita et al., 2004; Munemasa et al, 2007). There is cross talk between jasmonates and ABA as they utilize similar cascade of events to stimulate stomatal closure (Suhita et al., 2004).

Under our mDr treatment the *coi1* and *jin1* mutants were found to be significantly resistant (or insensitive to drought stress) compared to the wild type, with biomass accumulation under drought not different from well-watered control. This suggests that the reduced growth as a response to drought stress, as a developmental program for acclimation, is not switched on in

the absence of JA signal perception and response. This is supported by studies that show the JA-mediated inhibition of seedling and root growth is suppressed in the *coi1* mutant (Xie et al., 1998).

In experiments on stomatal closure, a characteristic drought response; jasmonates induce closure that is suppressed in the *coi1* mutant, which retains normal ABA responsiveness (Munemasa et al, 2007). Likewise, studies on barley genotypes and ABA-deficient tomato mutant plants revealed the role of JA in stomatal modulation through ABA (Herde et al., 1997; Bandurska et al., 2003). In the tomato ABA-deficient mutant, exogenous ABA was sufficient to close stomata and reduce transpiration (Herde et al., 1997). Since JA was also shown to repress photosynthesis genes (Reinbothe et al., 1994), one can expect photosynthesis to be unaffected in JA insensitive mutants through a JA-mediated signaling program, although ABA response would still be active.

At the early stage of mDr (Day1), plants accumulate high ABA concentration with induction of ABA biosynthesis and signaling genes, but with no significant differential expression of JA pathway genes. At late stage of mDr (day10), ABA level is normal with biosynthesis genes not up-regulated. However, JA signaling and biosynthesis genes are significantly down-regulated. The negative correlation in expression of the ABA and JA pathway genes, is also seen in transcription profiling studies of Methyl jasmonic (MeJA) treated plants which show repression of ABA/drought responsive genes such as *ATHB12* and *ABF3* (Devoto et al., 2005).

We propose that at the early stage of mDr, endogenous JA in combination with high ABA level is enough to stimulate the preparatory response needed for drought acclimation (examples: stomatal closure, and cell wall modification). JA is probably not required at high concentration under drought stress, and an increase in its concentration might negatively affect plants response in growth. To minimize the inhibitory effect of JA on plant growth under prolonged drought (late mDr), the down-regulation of JA biosynthesis and signaling pathways can act in establishing new homeostasis in the acclimation process.

Model of Plant Responses to Drought

The response to mDr extended over a period of time, can be distinguished into multiple stages, from early to intermediate and late (Fig. 11). During the early priming or preconditioning stage, stress perception, signaling and reprogramming of gene expression takes place. Many of these immediate responses, such as ABA response genes and ROS scavengers, probably involved in signaling responses, are also observed in pDr. The drought response pathways can be traced by the expression pattern of individual genes of known function. The differentially expressed genes at the early stage are characterized by the induction of a set of enzymes, channel protein genes, and transcription factors, which interact to control the stomatal aperture in response to internal and external stimuli. RPK1, a receptor-like kinase functions upstream in the ABA signaling pathway in the stomata. Downstream, PLDα interacts with GPA1 to inhibit stomatal opening, and frees phosphatidic acid, which in turn interacts with the PP2C (ABI1) to stimulate stomatal closure (Mirsha et al, 2006). Moreover, the induced outward K+ channel (GORK) extrudes K+ outside the guard cells, resulting in the loss of turgor and stomatal closure. Another key protein is the transcription factor MYB60 which is repressed at the early stage of drought, regulating stomatal closure. In the intermediate stage responses, there is an onset of reduction in growth, although no changes in growth are measurable. However, cell wall adjustments take place, as part of the acclimation response. At the late stage, plants reach a new homeostasis status, reaching an altered jasmonate: ABA pathway balance, having reduced growth with reduced levels of energy consuming processes, but with stabilized metabolism and physiology similar to the well-watered control.

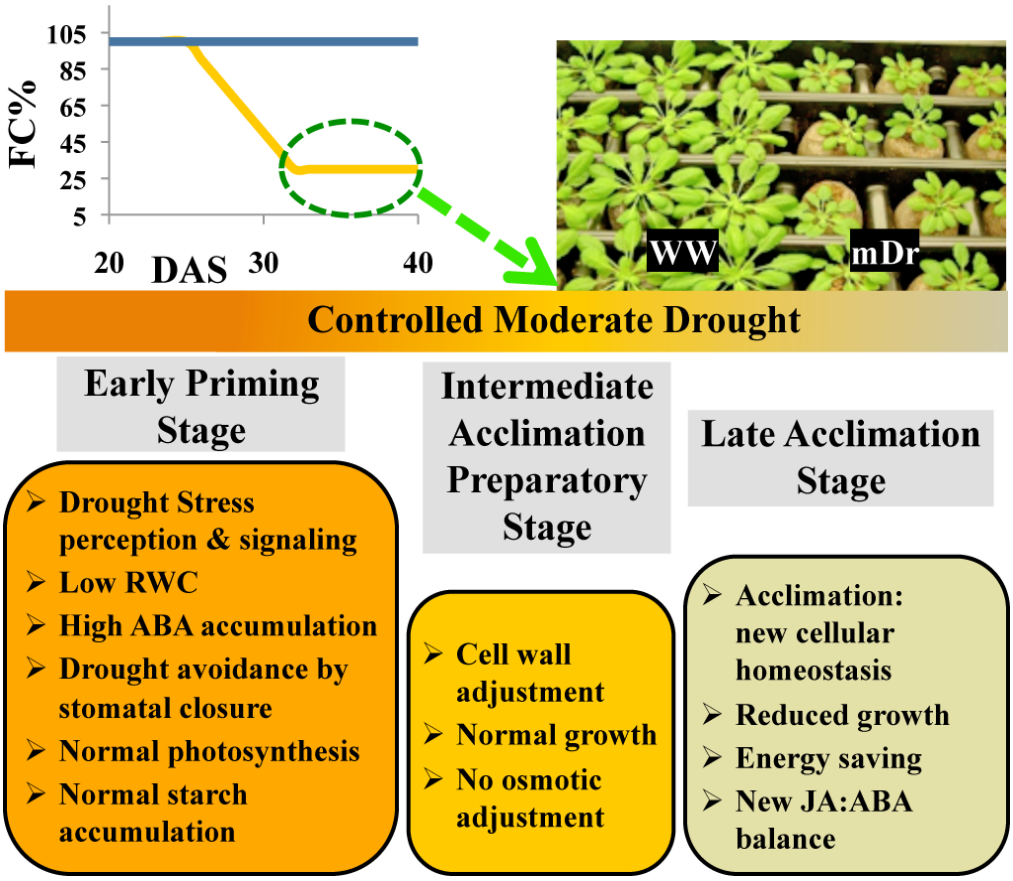

Figure 1.11. Physiological, biochemical, and molecular plant responses to mDr. Plant response to moderate drought (mDr) is dissected into three stages. Early priming (preconditioning) stage, at which all stress signaling and avoidance processes take place. Intermediate stage, is preparatory for acclimation, as plants modify and adjust cell walls for reprogrammed growth response at a later stage. At the late stage, plants are set to a new homeostasis with altered hormonal signaling, and reduction in energy demanding processes, leading to acclimated plants with reduced growth.

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3. A. The Arabidopsis *MYB109* Gene is Required for Growth under Drought and Salt Stress

B. ABSTRACT

In response to external environmental stresses, and internal signal transduction, regulation of gene transcription is the central level of control to fine tune plant responses to the changing environment. The MYB transcription factor superfamily is largest gene family in Arabidopsis, with the R2R3 MYB group being the most common in plants, shows diverse expression patterns and functions. Indeed, signaling through MYB transcriptional factors plays a crucial role in ABA-dependent stress signaling. In this study, *MYB109* (R2R3 MYB) was shown to be induced by progressive and moderate drought treatments. The function of *MYB109* was assessed by analysis of two knockout mutants (*myb109-1*and *myb109-2*), which displayed sensitivity to drought and salt stress, quantified as lower accumulation of biomass compared to the wild type. In addition, the mutants were ABA insensitive at the vegetative stage. The altered response to drought, salt, and ABA could be explained by the constitutive down-regulation of *MYB60* in *myb109-1* under normal and drought conditions, shown by global gene expression profiling and qRT-PCR analysis. Other possible mechanisms for the role of *MYB109* under drought stress were revealed by transcriptome analysis, suggesting that *MYB109* might act as a positive regulator of RNA processing and splicing, which is required to enhance plant response to drought stress. In addition, *MYB109* might be a negative regulator of jasmonic acid (JA) biosynthesis and signaling, which decreases plant growth and resistance under drought stress.

C. INTRODUCTION

Drought and salt stress are major abiotic stresses, which cause high losses in crop plants in large portion of the world. Extensive studies have addressed the molecular regulation under drought and salt stress (Chinnusamy and Zhu, 2006; Nakashima et al., 2009; Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu, 2002). In response to environmental stresses both regulatory and functional sets of genes are induced (Ingram and Bartels, 1996; Ramanjulu and Bartels, 2002; Bray, 2004; Bartels and Sunkar, 2005). Our knowledge about the early events in the perception of stress signals is still scarce (Urao et al., 1999; Ueguchi et al., 2001; Tran et al., 2007; Wohlbach et al., 2008). Two main stress signaling pathways, ABA-dependent and ABA-independent, pathways have been described (Shinozaki and Yamaguchi-Shinozaki, 1997). Stress signaling pathways function through a set of transcription factors, which modulate the expression of downstream functional genes that ultimately result in the expression of drought tolerance (Shinozaki and Yamaguchi-Shinozaki, 2007).

Transcription factors (TFs) are crucial hubs in the regulatory network(s) of stress response. The *Arabidopsis thaliana* genome has about 1,978 TFs grouped into approximately 50 gene families (http://rarge.psc.riken.jp/rartf/, Iida et al., 2005; Mitsuda et al., 2009), which play a crucial role in development, growth, and response to environmental stimuli. In general, TFs that belong to the AP2/ERF, bZIP, MYB, and WRKY gene families play a critical role in response to abiotic and biotic stress (Singh et al., 2002), with specific members being early responders in ABA-dependent and ABA-independent stress signaling pathways (Abe et al., 1997; Bartels and Sunkar, 2005; Sakuma et al., 2006). Expression profiling of 402 TFs in response to several biotic and abiotic stresses, revealed specific and common responsive TFs (Chen et al., 2002). Moreover, in the expression profile of 7000 Arabidopsis genes under drought, cold, and salt stress, 40 transcription factors were up-regulated by the three stresses. In the same study, only 2 transcription factors were down-regulated by the three stresses (Seki et al, 2002). Moreover, expression profiling of progressive and moderate drought showed 426 TFs were induced and 272 were repressed under progressive drought, whereas under moderate drought the numbers were 200 and 108, respectively (Harb et al., submitted). This suggests the important role of transcription regulation in response to different stresses. *AtHB7* and *AtHB12* are two transcription factors with homeodomain and leucine zipper motif, which were found to be up-

regulated by drought stress (Olsson et al., 2004), and function in the regulation of plant growth under drought conditions. Overexpression analysis of several transcription factors resulted in enhanced drought tolerance and resistance: SHINE (AP2/ERF transcription factor), SNAC1(Stress-responsive NAC1), HRD (AP2/ERF-like transcription factor), TSRF1 (Ethylene responsive transcription factor (ERF)) (Aharoni et a.a, 2004; Hu et al, 2006; Karaba et al., 2007; Quan et al. 2010).

MYB TF genes are found in animals, insects, fungi and plants (Stracke et al., 2001), and are divided into subfamilies based on the number of repeats in the binding domain. In animals, the binding domain of MYB genes has 3 repeats R1R2R3. The majority of MYB genes in plants have two repeats R2R3, with around 125 R2R3-MYB genes in Arabidopsis (Yanhui et al., 2006). Therefore, the R2R3-MYB family is one of the largest gene families in plants. In addition to R2R3-MYB, plants have R1R2R3-MYB and MYB-related proteins (Yanhui et al., 2006). MYB genes have diverse functions in plants involved in phenylpropanoid metabolism, cell shape, response to hormones, and cellular proliferation (Martin and Paz-Ares, 1997). The first plant MYB to be cloned was the *C1* gene in maize, which is required for the regulation of anthocyanin biosynthesis (Paz-Ares et al., 1986, 1987). Other examples of MYB genes in plants are: *ANTHOCYANIN2* (*PhMYBAN2*) in petunia for the regulation of anthocyanin biosynthesis (Quattrocchio et al., 1999), *PHANTASTICA* (*PHAN*) gene in snapdragon for the regulation of axes asymmetry (Waites et al., 1998), NtMYB2 in tobacco is induced by wounding (Sugimoto et al., 2000), *JAMYB* in rice is induced by JA against fungal infection (Lee et al., 2001), *NtAn2* in tobacco for anthocyanin biosynthesis in reproductive tissues (Pattanaik et al., 2010), *ZmMYB42* in maize in the regulation of cell wall structure (Sonbol et al., 2009), and so on.

In Arabidopsis, the first R2R3-MYB to be identified was *GLABROUS1* (*GL1*/*MYB0*), which was found to function in the differentiation of leaf trichomes (Oppenheimer et al., 1991)). Other examples are: *MYB75* that functions in anthocyanin biosynthesis (Borevitz et al., 2000), whereas *MYB26* and *MYB46* play a role in secondary cell wall thickening (Yang et al., 2007; Zhong et al., 2007). Many MYB genes were found to have important roles in response to biotic and abiotic stress. *MYB41* functions in response to salinity, desiccation, cold, and ABA (Lippold et al., 2009). *MYB15* has a role in cold stress, and *MYB102* is induced by osmotic, ABA, and wounding (Agarwal et al., 2006; Denekamp and Smeekens, 2003).

We identified a drought responsive gene *MYB109* that belongs to the R2R3-MYB gene family. The analysis of *MYB109* function in abiotic stress response is described here, using knockout (KO) mutants showing sensitivity to drought and salt stress. In addition, we show that the KO mutant has an altered stomatal response to ABA, and provide an analysis of the *MYB109* downstream regulated genes.

D. MATERIALS AND METHODS

Plant Growth and Genotypes

The Arabidopsis ecotype Columbia (Col) was used in all experiments, either as wild type (WT) or mutants. Arabidopsis seed were sown in moistened Jiffy peat pellet (Jiffy Products, Shippagan, Canada), stratified at 4 °C for 2 days, and then grown under light (100 μmole m^{-2} s^{-1}) for 10 hrs at 22 °C in a growth room.

Two T-DNA insertion mutant alleles of the *MYB109* gene (SALK_148462C, and SALK_047505C) and *MYB25* (SAIL_557_G02/CS874842) were obtained from Arabidopsis Biological Resource Center (Ohio State University, USA). For characterization of the *MYB109* mutants at the seedling stage, seed were surface sterilized in 10% bleach (NaClO) for 15 min, rinsed three times with sterile water, and spread on half concentration Murashige Skoog (MS) plates. To screen for plant phenotype, the mutants were grown in superfine soil (Fafard, Massachusetts, USA) in plastic pots under light (100 μmole m^{-2} s^{-1}) for 16 hrs at 22°C.

Genotyping of the Two *MYB109* Insertion Mutants

DNA from the two mutant lines (*myb109-1, myb109-2*) and WT was isolated (Pereira and Aarts, 1998), and genotyping done using T-DNA and gene specific primers, using 10 ng DNA for PCR. To detect presence of the un-interrupted gene, two gene specific primers were used (*myb109-1* Forward primer: CGGTGCAGTGACATAACACAC, Reverse primer: CGTAATTTCGTGGAAATGAATG), (*myb109-2* Forward primer: TTTCGATTAAGCATAA-TGGCG, reverse primer: CTTGAGACAAGGGTCAAGCTG). To check presence of the T-DNA insert, the T-DNA left border (LBb1 GCGTGGACCGCTTGCTGCAACT) and reverse gene specific primer were used. Amplification was done under the following PCR cycling protocol: initial denaturation at 95° C for 5 min, then 30 cycles (95 °C for 30 sec, 57 °C for 30 sec, and 72 °C for 1 min), and final extension at 72° C for 5 min.

Expression of *MYB109* in the Mutants

RNA was extracted using RNeasy Mini Kit (Qiagen, USA), the extracted RNA treated with DNase using RNase-Free DNase Set (Qiagen, USA), and cDNA synthesized using iScript cDNA Synthesis Kit (BioRad, USA). For expression analysis the gene primers: Forward primer TTGTGAGGAAACTAGGACCGAGGAA, and Reverse primer: CCGCGTGAGCAGAAAT-TATCATACG were used. The expression level of *MYB109* was quantified by qPCR using BioRad SYBR green kit (Biorad, USA), and UBQ10 (At4G05320) used as a reference gene. The PCR protocol was: initial denaturation at 95 °C 5 min, 30 cycles (95 °C 30 sec, 57 °C 30 sec, 72 °C 1 min), and final extension at 72 °C 5 min. Fold change of expression was calculated relative to the UBQ10 reference gene and relative to the well-watered control (Livak and Schmittgen, 2001).

Moderate Drought Stress Screen

Plants of the three genotypes (two mutants and WT Col) were grown as described above for 30 days after sowing (DAS). For drought treatment pellets were weighed before sowing to determine the amount of water in pellets at the beginning of the experiment. At 30 DAS, watering was stopped for the drought-treated plants, and continued for the well-watered control. After 5 -7 days plants reached moderate drought level, which was maintained by giving plants water to keep the soil moisture level at 30% of field capacity, which is 200% or 2 g H_2O g^{-1} dry soil. To do this, a semi-automated system was developed; a balance (GF 1000, A &D, California, USA) was connected to the computer utilizing software for communication, which enabled entering of the weights directly onto an Excel worksheet file. On the Excel worksheet file a set of equations were used to calculate the water content in each weighed pellet, the required final water content, and the amount of water to be added. The pellets were weighed daily, and were supplemented the calculated amount of water to reach 30% of field capacity (moderate drought level). After 10 days of moderate drought treatment, plants were harvested and the relative reduction in biomass (RB) was calculated = (B_{WW}- B_{DRT})/ B_{WW}; where B_{WW} is biomass under well-watered (WW) conditions, and B_{DRT} is biomass under moderate drought conditions. Plant performance under moderate drought was also assessed by Leaf area, which was calculated from scanned images using ImageJ software (NIH, USA).

For progressive drought and survival test, plants were grown in a mix of peatmoss and profile (2:1) in clay pots under 10 hrs light at 22°C. At 30 days after sowing (DAS), water was withheld, and plants were allowed to lose water until day 2 of wilting (determined gravimetrically). Then, they were rewatered and survival percentage was calculated as follows: (number of recovered plants/ total number of drought-treated plants) *100.

Salt Stress Screen at Seedling and Old Vegetative Stage

Arabidopsis seeds of the three genotypes (*myb109-1*, *myb109-2*, and WT Col) were surface sterilized as described above and plated on half-MS plates. Seeds were kept to grow under light (100 μmole m^{-2} s^{-1}) for 16 hrs at 22 °C for 5 days. For salt treatment, one group of seedlings was transferred to half-MS plates plus 150 mM NaCl, while a control group was transferred to NaCl-free half MS media. Seedling plates of both groups were kept under 16 hrs light at 22 °C for 7 days, and then the fresh weight of all seedlings measured.

For the vegetative stage, Arabidopsis plants of the three genotypes (*myb109-1*, *myb109-2*, and WT Col) were grown in peat pellets under 10 hrs light at 22 °C for 30 days. The plants were then separated into two groups: salt-treatment and well-watered control. The salt-treatment group plants were kept in 150 mM NaCl solution for 5 days, then in water for 7 days (recovery period), while the control group plants kept in water all the time. Plant performance under salt and control treatments was evaluated by measuring leaf area and change in growth (biomass) as described for drought test.

Response to ABA at Germination, Seedling, and Vegetative Stage

Surface sterilized seeds of *myb109-1* and WT Col were plated on half-MS with 1.5 μM ABA and without ABA. Plates were cold treated for 2 days, and then kept under light for 16 hrs at 22°C. After five days, germination was evaluated under a dissecting microscope and pictures were taken using a Leica camera (DFC310 FX) (Leica-Microsystems, USA).

Arabidopsis seed of three genotypes (*myb109-1*, *myb109-2*, and WT Col) were surface sterilized and spread on half-MS plates. After 2 days cold treatment, plates were kept under 16 hrs light at 22°C for 5 days. For ABA treatment, one group of seedlings was transferred to half-MS plates containing 10 μM ABA, and the control group transferred to ABA-free half-MS

media. Both groups were kept to grow vertically under 16 hrs light at 22°C for 7 days. After that, the fresh weight of all plants was measured.

For vegetative stage, five-week old plants of *myb109-1*, *myb109-2*, and WT Col were separated into control and the ABA-treatment groups. The control group was sprayed with water, and the ABA-treatment group sprayed with 50 μM ABA solution. The rosettes from the two groups of plants were cut and their change in fresh weight monitored: initially at time of spraying (0 time point) and then measured every 20 min for 2 hrs. The cut rosette water loss (CRWL%) was calculated = (initial fresh weight-final fresh weight)/(initial fresh weight-dry weight)*100.

Stomatal Response to ABA

The whole rosette of three plants at 35 days after sowing (DAS) (5 week old) of *myb109-1* and WT Col were cut and soaked in MES buffer (pH 6.15) as control group, and in 50 μM ABA in MES buffer (pH 6.15) as ABA-treated group (Osakabe et al., 2005). To study stomatal response to ABA, plants of both groups were kept under light for 2 hrs, pictures of the stomata were taken, and then images analyzed for the number of closed stomata and stomatal aperture size under both treatments. For the analysis, measurements were made on 3 plants/ genotype were taken for a total of 120 stomata/ genotype/treatment.

For the determination of whole plant ABA responses, *myb109-1* and WT plants at 35 DAS (5 week old) were divided into two groups: one group was treated with 50 μM ABA in MES buffer (pH 6.15), and another group treated with MES buffer (pH 6.15) only. After 30 min, gas exchange measurements were taken for 5 plants of each group. LICOR 6400XT (LICOR, Nebraska, USA) and the Arabidopsis Extended chamber were used with the following conditions set for gas exchange measurements: flow rate 150 μmole s^{-1}, CO_2 400 μmole, humidity 50%.

Construction of *MYB109* Vectors and Plant Transformation

The predicted promoter of *MYB109* (http://mendel.cs.rhul.ac.uk/mendel.php), was amplified as a 2 Kb upstream region using the PHUSION high fidelity PCR kit (New England BioLabs, USA). The Amplified sequence was digested with suitable restriction enzymes and fused to GUS and GFP in two different constructs. MYB109::GUS and MYB109::GFP were integrated into pBIN+ binary vector, and transformed into Agrobacterium EHA105 strain by

electroporation. WT (Col) plants at the suitable stage were transformed with the construct by floral dip (Clough and Bent, 1998).

The complete genomic DNA sequence of *MYB109* was ampilified using PHUSION high fidelity PCR kit (New England BioLabs, USA). The amplicon was fused into pGEM-T vector (Promega, USA), and was sequenced. The *MYB109* gene was cut out with suitable restriction enzymes and cloned into pCambia1301 binary vector (Cambia, Brisbane, Australia). The binary vector was electroporated into Agrobacterium strain EHA105 strain and used for transformation into WT Col plants by floral dip (Clough and Bent, 1998).

Selection of transformants, for the *MYB109* promoter-reporter (GUS and GFP) constructs was done on 50 μg/ml kanamycin MS media plates, and for *MYB109* overexpression construct on 25 μg/ml hygromycin MS plates.

Expression Profiling of *myb109-1* mutant and Wild Type Columbia under Progressive Drought

Five weeks old *myb109-1* mutant and WT plants were drought-treated until the first day of wilting, and a control group of each genotype kept well-watered. Three samples of 5 pooled plants / sample from drought-treated and well-watered control of each genotype were collected for RNA isolation (as described above). For gene expression analysis 4 ug RNA samples, with 2-3 replications/treatment were used for Affymetrix GeneChip hybridization analysis at the VBI Core Lab Facility (VBI, Blacksburg, VA, USA).

Analysis of gene expression profiles

Analysis of gene expression was done as described in (Harb et al., submitted); for each of the drought experiments, pDr *myb109-1*, and pDr WT raw data were background corrected, normalized and summarized according to the custom CDF using RMA (Ihaka and Gentleman, 1996; Irizarry et al., 2003; Gentleman et al., 2004), followed by non-specific filtering of genes that do not have enough variation (interquartile range (IQR) across samples < IQR_{median}) to allow reliable detection of differential expression. A linear model was then used to detect differential expression of the remaining genes (Smyth 2004). The *p*-values from the moderated *t*-tests were converted to *q*-values to correct for multiple hypothesis testing (Storey and Tibshirani, 2003),

and genes with *q*-value <0.1 were declared as differentially expressed (DE) in response to the drought treatments.

qRT-PCR Analysis of *MYB109* under Progressive and Moderate Drought

WT Col plants were grown as described above and water withheld for drought treatment. For progressive drought samples were collected from plants at the first day of wilting, and simultaneously for well-watered control for RNA isolation. To quantify the expression of *MYB109* in a time course of moderate drought, WT samples were collected at different days of moderate drought. RNA isolation, cDNA synthesis, and quantification of *MYB109* gene were done as described above. *MYB109* was quantified by SYBR Green mix, SAND family gene (At2G28390) gene was used as a reference (Czechowski et al., 2005), and the fold change was calculated as described above. The expression of several stress signaling, stress marker, and stomatal genes were quantified in *myb109-1* and WT treated for 1 day of moderate drought as described above.

E. RESULTS

MYB109 is Induced under Progressive and Moderate Drought Treatments

From our previous progressive drought expression data (Harb et al., submitted), and the publicly available expression data, *MYB109* was known to be one of the drought up-regulated genes. Therefore, qRT-PCR was carried out to test the expression of *MYB109* under both progressive and moderate drought. For progressive drought treatment, watering was stopped for 5-week old plants that were allowed to lose water by evapo-transpiration till the first day of wilting, and samples were collected for RNA isolation and qRT-PCR analysis. For moderate drought, at 30 days after sowing (DAS), watering was withheld and the soil moisture levels monitored by weighing. When the pellets reached a soil moisture level equivalent to 30% Field capacity (FC), which was considered sub-lethal, the moisture level was maintained by providing the amount of lost water. Samples were collected daily (day 0, 1, 2, 3) from multiple replications for RNA isolation and qRT-PCR analysis. Quantification of *MYB109* expression under both drought treatments showed the induction of the gene by almost 4 fold (Fig. 1A). Moreover, the expression of *MYB109* was quantified in a time course of moderate drought, and showed the highest expression at day 1 of moderate drought (Fig.1B).

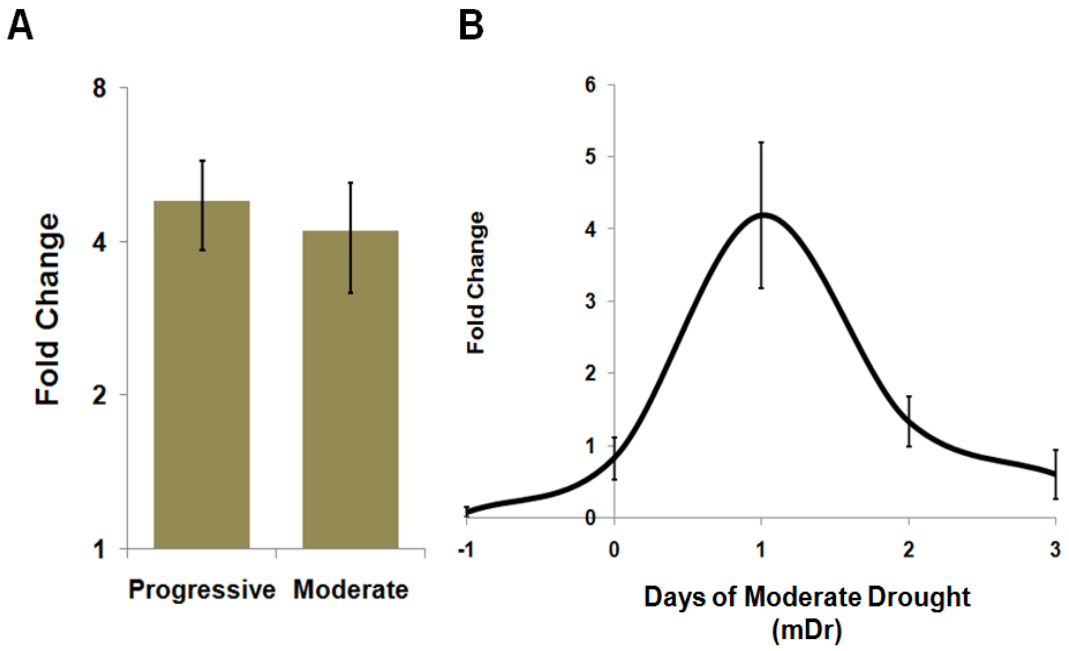

Figure 2.1. Induction of *MYB109* by drought stress treatments. A, qRT-PCR analysis of *MYB109* under progressive and moderate drought. B, The expression profile of *MYB109* in a time course of moderate drought. Bars represent ± SE, n=3 biological replications (5 pooled plants/ replication).

Morphological and Molecular Characterization of Two *MYB109* Mutants

To test the function of the *MYB109* gene in plant drought response and resistance, two available insertion knockout (KO) mutant lines, *myb109-1* and *myb109-2* (Fig. 2A), were confirmed to be homozygous (data not shown) and used for further analysis. The two mutants are morphologically similar to the wild type (WT), but accumulate higher biomass under well-watered conditions compared to the WT at the vegetative stage (Fig. 2B). This higher biomass is significant, since in similar screens with a set of 200 KO mutant lines under well-watered conditions (unpublished), most were similar to WT. Moreover, qRT-PCR analysis showed that under normal growth conditions *MYB109* is not expressed in *myb109-1* (Fig. 2C). Surprisingly, *myb109-2* showed 3-fold induction in expression of *MYB109* compared to the WT (data not shown). In this mutant the T-DNA insert is 129 bp upstream of the ATG of *MYB109* (Fig. 3A). The T-DNA insert contains a 35S promoter followed by NOS terminator, about 1 kb from the left border (Fig. 3B). This 35S promoter (with enhancer) would probably be able to enhance

promoters in the proximity, as well as initiate transcription from the 35S promoter towards the *MYB109* gene. To check the nature of transcripts in *myb109-2*, two T-DNA left border primers were tested in combination with *MYB109* specific primers. The analysis revealed a weak transcript that started in the T-DNA and continued through the gene. Examining the DNA sequence downstream of the 35S promoter, we found multiple start and stop codons in all reading frames (Fig. 3 and 4), suggesting that transcripts initiating from the 35S promoter or spurious transcription starts within the T-DNA would not be translated. However, we cannot rule out that the 35S enhancer acts on the *MYB109* minimal promoter (129 bp sequence upstream of ATG) and activate the gene. Since we see multiple transcripts running in from the T-DNA into the gene, it is difficult to resolve the multiple transcription starts and the role truncated proteins might play. However, the observation of a mutant phenotype in *myb109-2*, similar to *myb109-1* suggests that *myb109-2* is a KO mutant too.

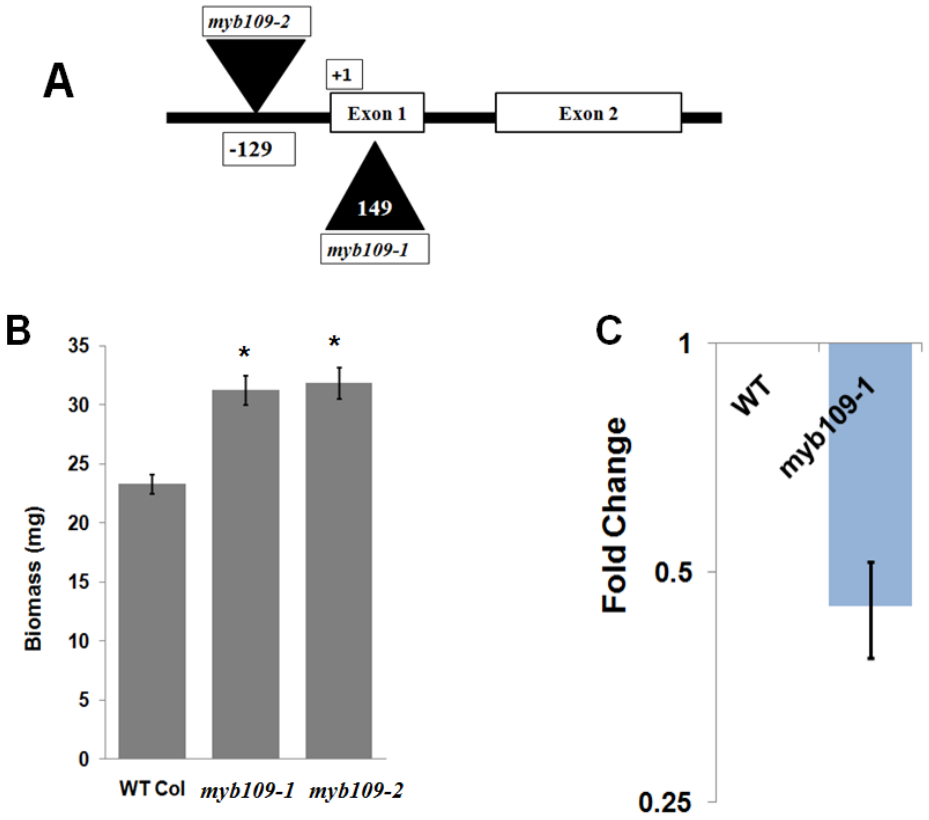

Figure 2.2. Characterization of the two *MYB109* insertion mutants A, Schematic illustration of T-DNA insertion in the two mutants (*myb109-1*, and *myb109-2*). B, Biomass in mg of *myb109-1*, *myb109-2*, and WT. Bars represent ± SE, n=8. * indicates significance compared to the WT at P-value <0.001, the experiment was repeated. C, qRT-PCR showing reduced expression of *MYB109* in *myb109-1*. N= 3 biological replications (5 pooled plants/replication). Bars represent ± SE.

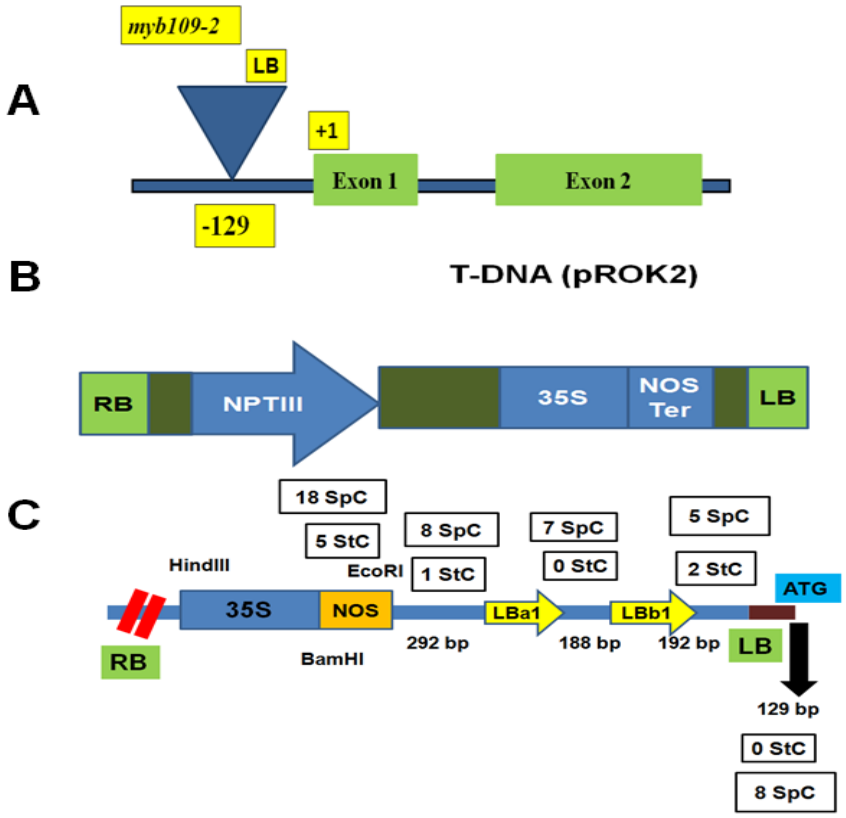

Figure 2.3. Analysis of the *myb109-2* insertion mutant genome structure A, Schematic illustration of the T-DNA insertion in *myb109-2* (129 bp upstream of the *MYB109* gene). B, T-DNA in pROK2 vector , which was used in the T-DNA insertional mutagenesis (Baulcombe et al., 1986; Alonso et al., 2003). C, Illustration of part of T-DNA and the upstream region of *MYB109* showing the number of start codons (StC) and stop codons (SpC).

Figure 2.4. Analysis of the *myb109-2* insertion mutant genome structure. Nucleotide sequence of the insertion site showing part of the 35S promoter to T-DNA left border (close to ATG of *MYB109*) highlighting start codons (StC) (blue color), stop codons (SpC) (pink color). BamHI restriction site (RE) (red underline), EcoRI (RE) (green underline), left border (LB) (green color), 2 left border primers (LBa1, and LBb1) (yellow color), and ATG of *MYB109*.

_MYB109 M_utants are Drought Sensitive, but the Paralog _MYB25_ Mutant is Similar to Wild Type

Since *MYB109* is induced by drought treatments, it might be expected to have a biological function in response to drought. To test this, the two *MYB109* mutants and the WT plants were exposed to a constant moderate drought (soil water content equivalent of 30% of field capacity) for 5-10 days, and measurements made for leaf area and biomass. Both *MYB109* mutants showed a significantly higher loss in biomass and leaf area under moderate drought compared to the WT (Fig. 5A and B). The two mutants lost about 50% of their biomass and 10-15% of their leaf area under moderate drought treatment compared to 30% loss of the WT biomass and less than 5% of leaf area.

MYB25 was predicted to be the paralog of *MYB109* (Stracke et al., 2001). To test if there is a functional redundancy between these two genes under drought treatment, the response of the *MYB25* KO mutant was tested along with the WT under drought. However, the relative reduction in biomass in m*yb25* was not significantly different from that of the WT (Fig. 5C and D).

Under progressive drought, *myb109-1* and WT were grown in clay pots. At 35 DAS progressive drought was initiated, and *myb109-1* started to wilt at soil water content of 13% g g^{-1} dry soil compared to 9% g g^{-1}dry soil for WT. Permanent wilting for the genotypes was determined as the point where plants do not recover from wilting, which was at 4% g g^{-1} dry soil for *myb109-1* and 2% g g^{-1} dry soil for WT. Moreover, under progressive wilting drought, *myb109-1* showed 40 % survival rate compared to 70% of WT.

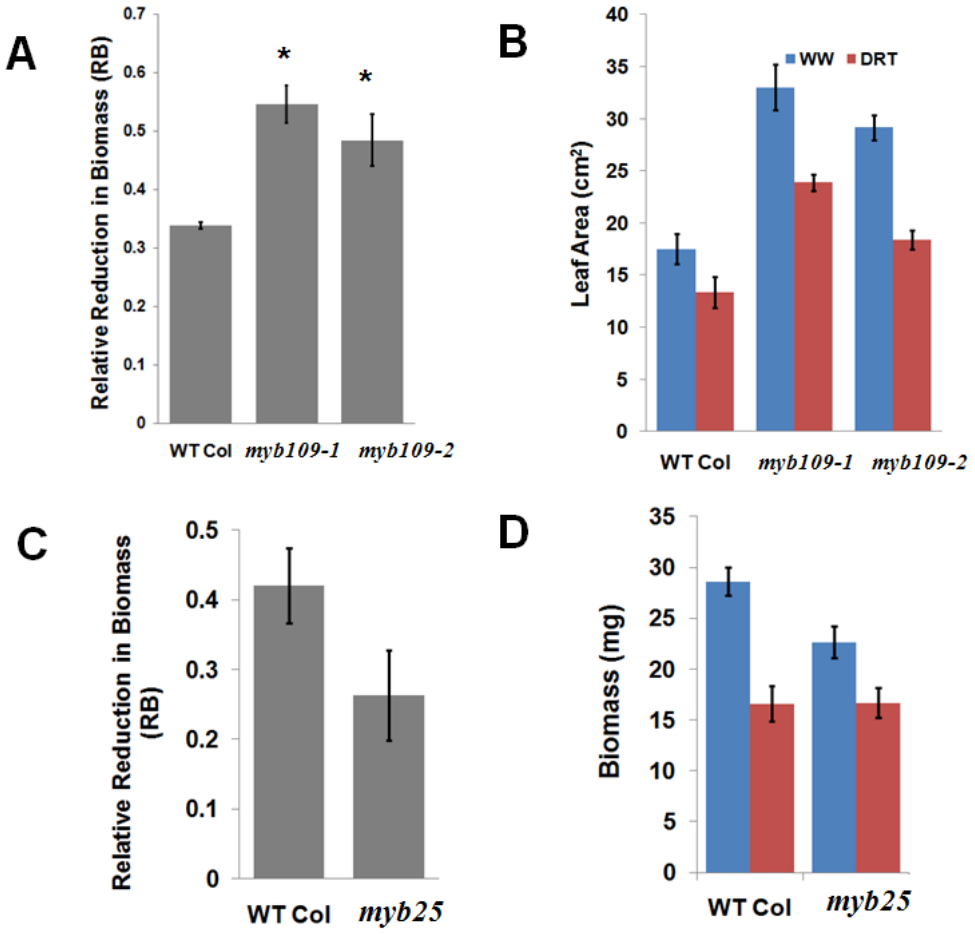

Figure 2.5. Response of *MYB109* and *MYB25* KO mutants to moderate drought treatment. A, Relative reduction in biomass (RB) of *myb109-1*, *myb109-2*, and WT under moderate drought. N=12, * indicates significance at p <0.01. B, Leaf area of *myb109-1*, *myb109-2*, and WT under moderate drought (DRT) compared to well-watered conditions (WW). N=8, p <0.01. A and B, Experiment was repeated. C, Relative reduction in biomass (RB) of *myb25* and WT under moderate drought. D, Leaf area of *myb25* and WT under moderate drought (DRT) compared to well-watered conditions (WW). C and D, N= 10. Bars represent ± SE.

Gene Expression Analysis of *myb109-1* under Progressive Drought

Gene expression analysis of WT Col and *myb109-1* under progressive drought (sampled at the first day of wilting) was done using RNA from rosette leaves, as described in methods. The differentially expressed (DE) genes were extracted using q-value cutoff of 0.1. The Venn diagrams (Fig. 6A and B) summarize the numbers of common and genotype specific drought DE

genes that are up- and down- regulated, respectively. Gene expression analysis revealed quantitative and qualitative differences under progressive drought as a result of loss of function of *MYB109*. In the WT 3625 genes were up-regulated, in contrast to 2552 genes in *myb109-1*. About 44% of up-regulated genes were WT-specific, and only 14% *myb109-1* specific. For down-regulated genes, 4023 and 3196 genes were DE in WT and *myb109-1*, respectively. Percentages of genotype specific down-regulated genes were 33, and 12%, for WT and *myb109-1*, respectively.

GO enrichment analysis revealed well-known stress and ABA responsive GO categories enriched in the common up-regulated genes between WT and *myb109-1* such as: water deprivation, osmotic stress, abscisic acid stimulus, salt stress, and so on. In addition, the common down-regulated DE genes between the WT and *myb109-1* were enriched for photosynthesis, protein synthesis, and other metabolic processes. GO analysis of genotype specific up-regulated genes showed the enrichment of RNA processing and splicing in WT (Table 1), and response to jasmonic acid (JA), and JA biosynthesis in *myb109-1* (Table 2). Genotype specific down-regulated genes were enriched for protein and amino acid biosynthesis in WT, and for chlorophyll biosynthesis and metabolic processes in *myb109-1*.

Table 2.1. RNA splicing genes up-regulated in WT under progressive drought compared to well- watered control

Gene ID	Log2 (Ratio)	TAIR Annotation
AT1G77180	1.114318405	Chromatin protein family
AT3G19670	0.704272519	FF domain-containing protein
AT2G41500	0.538230215	Lachesis (EMB2776)
AT2G29210	0.569203461	Splicing factor PWI domain-containing protein
AT3G45590	0.540157239	Splicing endonuclease 1 (ATSEN1)
AT1G23860	0.522353976	SRZ-21
AT2G37340	0.650445499	Arginine/serine- rich zinc knuckle-containing protein 33
AT4G37120	0.385463879	Swellmap 2 (SMP2)
AT4G25500	0.71887034	Arginine/serine-rich splicing factor 35 (ATRSP35)
AT3G50670	0.686981551	Spliceosomal protein UIA
AT5G52040	0.564459255	Arginine/serine-rich splicing factor 41(ATRSP41)
AT4G36690	0.572932416	RNA binding (ATU2AF65A)
AT3G55460	0.767357541	SC35-like splicing factor 30 (SCL30)
AT5G64200	0.761092353	Arginine/serine-rich splicing factor 35 (ATSC35)
AT3G53500	0.527548226	Nucleic acid binding (RSZ32)
AT1G32490	0.499863453	Embryo defective 2733/ATP-dependent helicase (EMB2733)

Table 2.2. Jasmonic acid (JA) signaling and biosynthesis genes up-regulated in *myb109-1* under progressive drought compared to well-watered control

Gene ID	Log2 (Ratio)	TAIR Annotation
AT3G17860	0.563384527	Jasmonate-ZIM-domain protein 3 (JAZ3)
AT1G30135	1.463894196	Jasmonate-ZIM-domain protein 8 JAZ8)
AT1G70700	1.827619831	Jasmonate-ZIM-domain protein (JAZ9)
AT1G72450	1.885988454	Jasmonate-ZIM-domain protein 6 (JAZ6)
AT5G13220	2.052041106	Jasmonate-ZIM-domain protein 10 (JAZ10)
AT1G74950	2.508942186	Jasmonate-ZIM-domain protein 2 (JAZ2)
AT2G34600	3.232602321	Jasmonate-ZIM-domain protein 7 (JAZ7)
AT1G17380	3.641504763	Jasmonate-ZIM-domain protein 5 (JAZ5)
AT1G32640	2.685874161	Jasmonate insensitive 1 (ATMYC2/ JIN1)
AT5G42650	1.242622549	Allene oxide synthase (AOS)
AT2G06050	2.910198694	OPDA-reductase 3 (OPR3)
AT1G17420	3.569235506	Lipoxygenase 3 (LOX3)
AT1G72520	4.005241597	Lipoxygenase, putative

Cis-element analysis (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi) revealed the common stress and ABA responsive motifs in the up-regulated genes shared by WT and *myb109-1* (genes not affected by the KO mutation) under progressive drought: ABF binding site, ABRE like binding, DRE core motif, etc (Table 3) (Guiltinan et al., 1990; Busk et al., 1997). The up-regulated WT specific genes (genes that require functional *MYB109*) were enriched for ABRE-binding motif, ABF binding site, and ACGTABREMOTIFA2OSE. Up-regulated genes in *myb109-1* were enriched for ABRE binding site motif, DRE core motif, and CACGTGMOTIF.

Table 2.3. Cis-elenments enriched in the differentially expressed (DE) genes in WT and *myb109-1* under progressive drought. IUPAC nucleotide alphabet: M=A or C (i.e A=0.5 C=0.5 G=0 T=0), R= A or G, W= A or T, S= C or G, Y= C or T, K= G or T, V= not T (i.e A=1/3 C=1/3 G= 1/3 T=0), H= not G, D= not C, B= not A, N= A= C= G= T= 0.25

Motif	No. of Genes	P-value	Sequence
WT X myb109-1			
ABFs binding site	160	< 10 - 10	CACGTGGC
ABRE-like binding	787	< 10 - 10	BACGTGKM
ACGTABREMOTIFA2OSE	629	< 10 - 10	ACGT-core
CACGTGMOTIF	575	< 10 - 10	CACGTG
DREB1A/CBF3	199	< 10 - 10	RCCGACNT
GBF1/2/3 BS in ADH	67	< 10 - 10	CCACGTGG
TGA1 binding site	88	< 10 - 4	TGACGTGG
UPRMOTIFIIAT	108	< 10 - 8	CCNNNNNNNNNNNCCACG
ABRE binding site	225	< 10 - 10	YACGTGGC
ABREATRD22	126	< 10 - 10	RYACGTGGYR
ATHB5ATCORE	87	< 10 - 4	CAATNATTG
DRE core motif	528	< 10 - 10	RCCGAC
GADOWNAT	416	< 10 - 10	ACGTGTC
GBOXLERBCS	128	< 10 - 10	MCACGTGGC
Z-box promoter motif	110	< 10 - 10	ATACGTGT
WT Up			
ABFs binding site	72	< 10 - 4	CACGTGGC
ABRE-like binding	376	< 10 - 9	BACGTGKM
CAGGTGMOTIF	263	< 10 - 4	CACGTG
GBOXLERBCS	66	< 10 - 5	MCACGTGGC
ACGTABREMOTIFA2OSE	290	< 10 - 10	YACGTGGC
GADOWNAT	174	< 10 - 6	ACGTGKC
ABRE binding site	107	< 10 - 6	ACGTGTC
myb109-1 Up			
ABRE binding site	39	< 10 - 4	YACGTGGC
DRE core motif	137	< 10 - 7	RCCGAC
CACGTGMOTIF	107	< 10 - 7	CACGTG
W-box promoter motif	318	< 10 - 4	TTGACY
WT X myb109-1 Down			
Ibox promoter motif	1035	< 10 - 10	GATAAG
UPRMOTIFIAT	108	< 10 - 4	CCACGTCA
TGA1 binding site	106	< 10 - 4	TGACGTGG

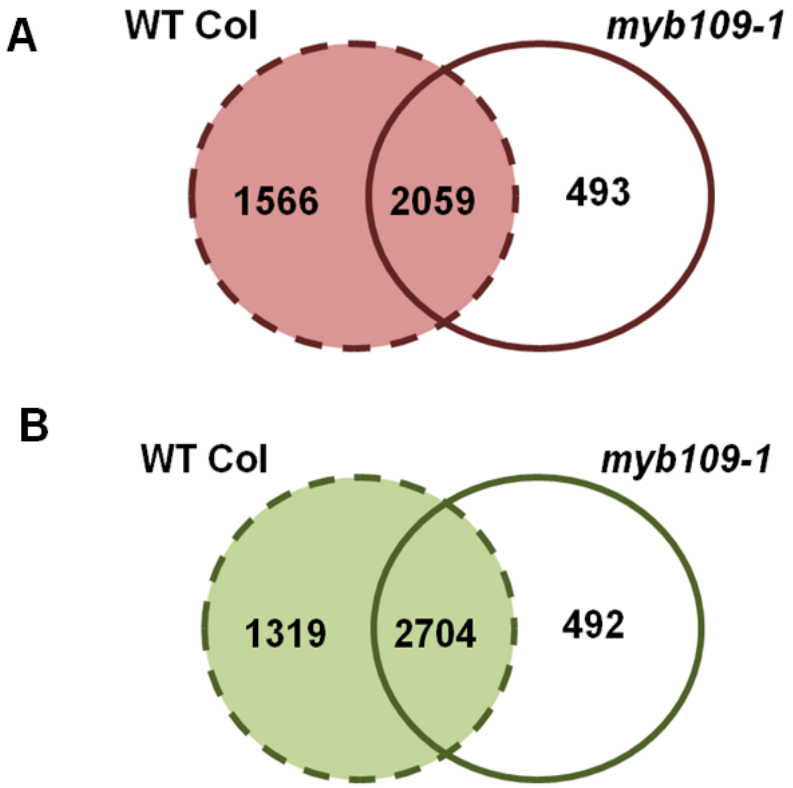

Figure 2.6. Differentially expressed genes (DE) in WT and *MYB109* KO in response to progressive drought. A, Common and genotype specific up-regulated genes. B, Common and genotype specific down-regulated genes.

Since *MYB109* is responsive to ABA, the ABA responsive genes from AtGeneExpress (Table S1 in Nemhauser et al., 2006) were compared to DE genes in WT and *myb109-1* under progressive drought (Fig. 7A). The number of common DE (up- and down-regulated) genes between drought and ABA was higher in WT compared to *myb109-1*. In the WT 181, and 182 genes were up- and down-regulated, respectively. For *myb109-1*, the numbers were 44, and 47, respectively.

Expression profiling revealed the up-regulation of JA biosynthesis and signaling genes under progressive drought in *myb109-1*, so we compared our expression data with methyl jasmonate (MJ) responsive genes in AtGeneExpress (Table S6 in Nemhauser et al., 2006). The analysis showed 208 up- and 236 down-regulated genes are common between progressive

drought and MJ regardless of the functionality of *MYB109* (Fig. 7B). Moreover, *myb109-1* specific up-regulated genes share more genes with MJ than the WT (Fig. 7B). One hundred ten genes are common between the up-regulated genes in MJ and *myb109-1* under progressive drought, whereas only 32 genes are common between MJ and WT. For the down-regulated genes, *myb109-1* and MJ shared 34 genes, and 58 genes are common between WT and MJ.

GO analysis (http://bioinfo.cau.edu.cn/agriGO) was done in a step to understand the biology of the cross talk between MJ and drought in the two genotypes WT and *myb109-1*. Common up-regulated genes between progressive drought and MJ were enriched for response to wounding, ABA stimulus, osmotic stress, and water deprivation. *myb109-1* and MJ were enriched for JA biosynthesis and metabolism, in addition to response to wounding, biotic stress, and so on. Regardless the loss of function of *MYB109*, the down-regulated genes shared between progressive drought and MJ are enriched for cell growth, cell wall loosening and modification, which was not found in down-regulated genes under progressive drought only.

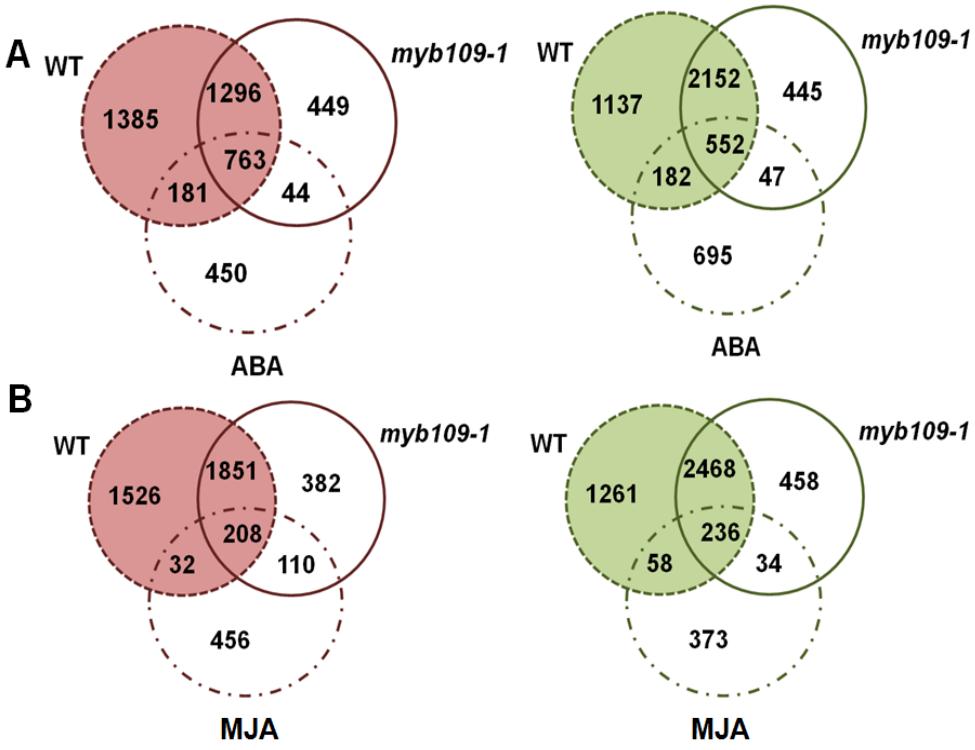

Figure 2.7. Cross talk between drought and hormone responsive genes. A, WT and *myb109-1* (MYB) under progressive drought and ABA, showing up-regulated genes (left), and down-regulated genes (right). B, WT and *myb109-1* (MYB) under progressive drought and MJA, comparing up-regulated genes (left), and down-regulated genes (right).

In Silico Promoter Analysis of *MYB109, MYB25, and MYB60*

To investigate the promoter of *MYB109* for drought and /or ABA-related cis-elements, the predicted 2-kb promoter of *MYB109* (http://mendel.cs.rhul.ac.uk) was analyzed using the PLACE cis analysis tool (http://www.dna.affrc.go.jp/PLACE). The analysis revealed a number of dehydration and ABA cis-responsive elements in the promoter of *MYB109*: ABRELATERD1, AGGTATERD1, MYB2AT in *RD22*, DRECRTCOREAT, MYB2CONSENSUS, MYCCONSENSUS, LTRE, and MYB1AT (Busk et al., 1997; Abe et al., 2003; Simpson et al., 2003). In addition, the guard cell specific motif (TAAASTKST1) was found in the *MYB109* promoter (Plesch et al., 2001).

A promoter scan of *MYB25* (*MYB109* paralog) revealed dehydration responsive cis-elments: AGGTATERD1, CBFHV, DRECRTCOREAT, MYB1AT, MYB2AT, MYB2-CONSENSUSAT, MYBATRD22, MYCATERD1, MYCATRD22, MYCCONSENSUSAT, and TAAAGSTKST1. *MYB60* promoter was enriched for 2 dehydration responsive cis-elements and one guard cell specific motif: MYCCONSENSUSAT, AGGTATERD1, and KAAAGSTKST1

MYB109 Mutants are Sensitive to Salt Stress

Analysis of publicly available expression data indicated that *MYB109* was induced by salt stress. To test the functional role under salt stress, the two *MYB109* KO mutants were tested under 150 mM NaCl salt stress treatment at seedling and vegetative stages. Media screens at the seedling stage showed that the mutants had a significantly higher reduction in biomass compared to the WT (Fig.8A). At the vegetative stage, the mutants had higher reduction in biomass and leaf area under salt stress compared to the WT (Fig. 8B and C), respectively. The *MYB109* mutants showed more than 30% reduction in biomass, whereas the WT had about 10% reduction in biomass.

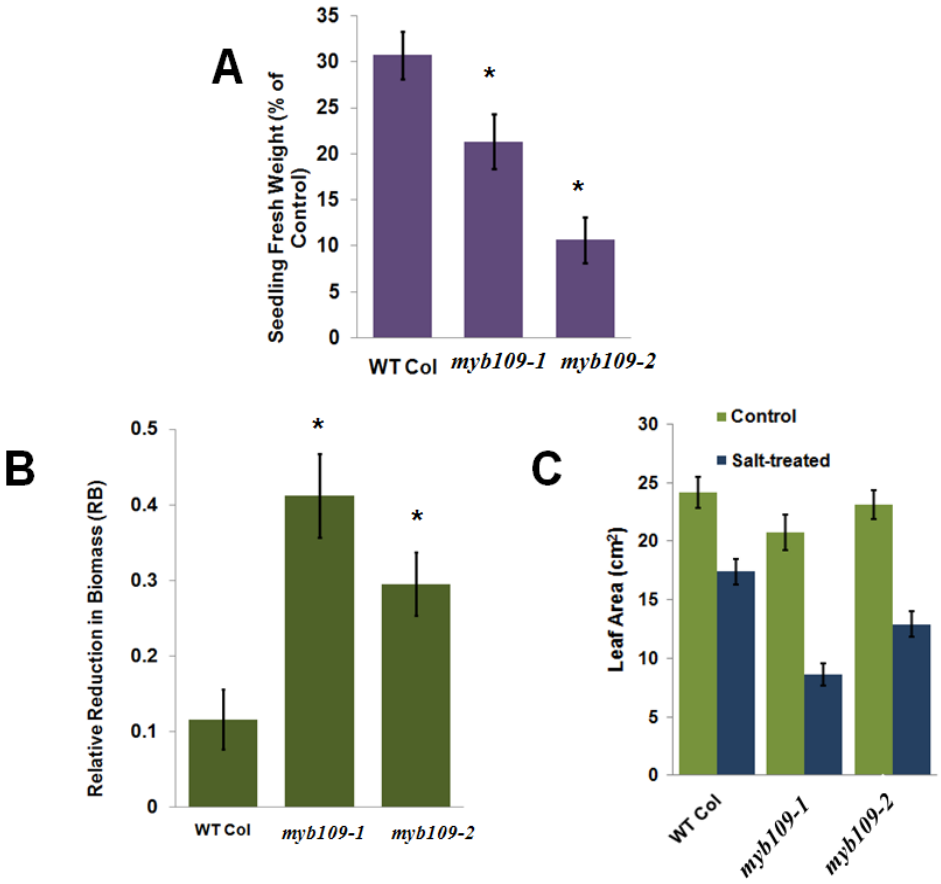

Figure 2.8. Response of the *MYB109* KO mutants to salt stress. A, Seedling relative fresh weight of *myb109-1*, *myb109-2*, and WT under salt stress. N=25, p-value <0.01. B, Relative reduction in biomass (RB) of *myb109-1*, *myb109-2*, and WT at vegetative stage. N=10, p-value <0.01. C, Leaf area under salt stress and control conditions. N=8, p-value <0.01. B and C, the experiment was repeated. Bars represent ± SE. * indicates significance.

Response of MYB109 Mutants to ABA at Different Developmental Stages

The publicly available data showed that *MYB109* is induced in response to ABA. At seed germination stage *myb109-1* showed the same sensitivity to ABA as the WT (Fig. 9A-C). At the seedling stage, the reduction in the seedling fresh weight of *myb109-1* and *myb109-2* was not significantly different from that of the WT (Fig. 9D- F).

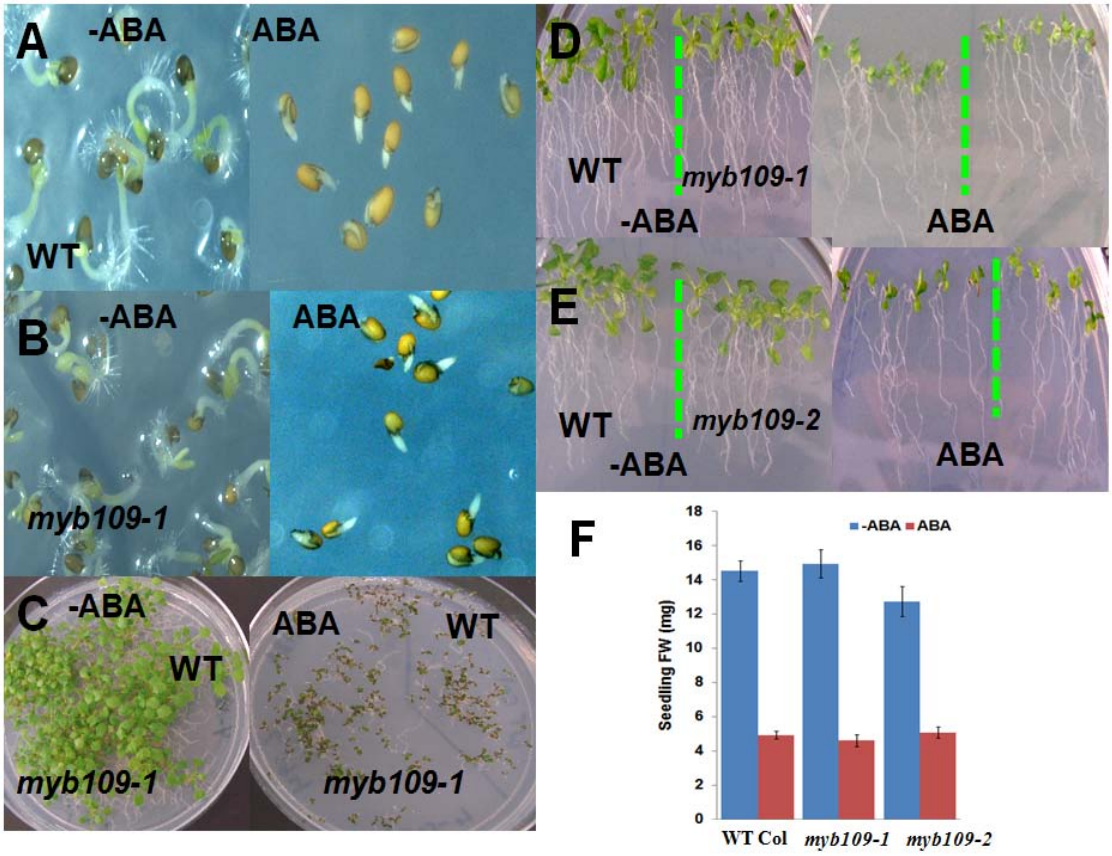

Figure 2.9. ABA response at seed germination and seedling stages. A, WT on -ABA and on ABA MS media. B, *myb109-1* on -ABA and on ABA MS media. C, WT and *myb109-1* on -ABA and on ABA MS media 15 days after sowing (DAS). D, WT and *myb109-1* response to ABA at seedling stage. E, WT and *myb109-2* response to ABA at seedling stage. F, Seedling growth (fresh weight in mg) on -ABA and ABA MS media ABA , N=30. Bars represent ± SE.

Analysis of *MYB109* expression as exhibited by the Arabidopsis eFP browser (http://bbc.botany.utoronto.ca/efp) showed expression in the guard cells under normal conditions and in response to ABA. Therefore, the sensitivity of guard cells to ABA was evaluated in terms of stomatal conductance, cut rosette water loss (CRWL%), and measurement of stomatal opening. In response to ABA stomatal conductance was changed by 0.1 mmol m^{-2} s^{-1} in *myb109-1* compared to untreated control, whereas the change was 0.4 mmol m^{-2} s^{-1} in the WT (Fig. 10A). CRWL was tested by spraying *myb109-1*, m*yb109-2* and WT plants with ABA and ABA-free solution. Both mutants were insensitive to ABA compared to the WT, as they lost the same

amount of water under ABA-free and ABA-treated conditions (Fig. 10B and C). Stomatal closure was tested in response to exogenous ABA, with WT showing 80% closed stomata, and *myb109-1* only 40% closed (Fig.10D). In addition, the change in the size of stomatal aperture in response to ABA was measured as percentage of the untreated control. The stomatal aperture in WT showed 40% closure and less than 10% in *myb109-1* (Fig. 10E- G).

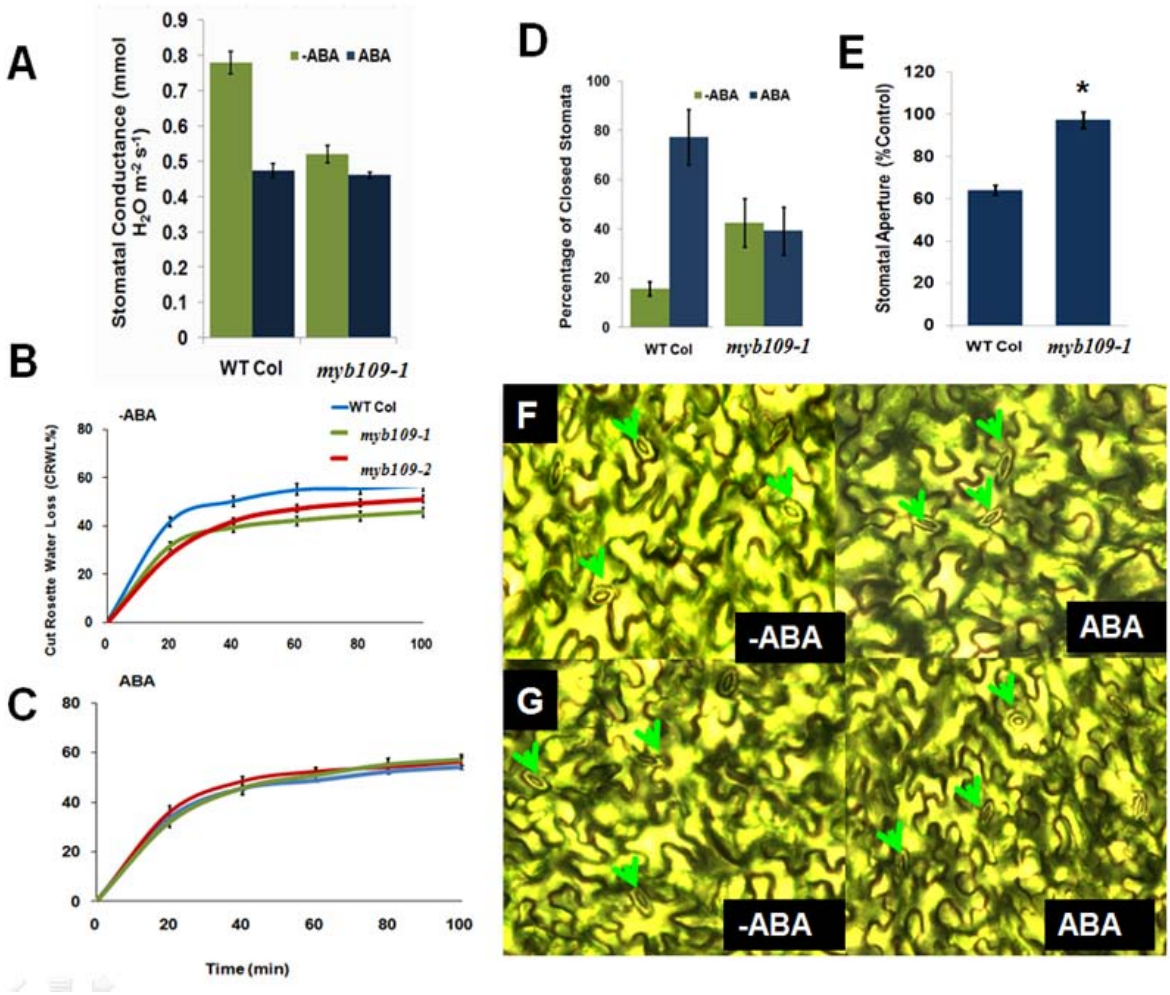

Figure 2.10. Response to ABA at vegetative stage. A, Stomatal conductance in response to ABA, N=4, P-value for WT <0.000001, <0.01 for *myb109-1*. B, Cut rosette water loss (CRWL%) of *myb109-1*, *myb109-2*, and WT without exogenous ABA. N=8. C, CRWL% of *myb109-1*, *myb109-2*, and WT with exogenous ABA. N=8. B and C, the experiment was repeated. D, Percentage of closed stomata of *myb109-1* and WT in response to ABA. 120 stomata of 3 plants were studied, p-value <0.05 for WT and not significant for *myb109-1*. E, Size of the stomatal aperture relative to the untreated control. 120 stomata of 3 plants were studied, p-value <0.00001 for WT and not significant for *myb109-1*. D and E, the experiment was repeated. F, Stomata of WT in the absence (-ABA)) and in presence of ABA (ABA). G, Stomata of *myb109-1* in the absence (-ABA) and presence of ABA (ABA). Bars represent ± SE. * indicates significance.

Expression of Stress Signaling, Stress Marker, and Stomatal-Related Genes in *myb109-1* under Moderate Drought

To test if the sensitivity to ABA and drought of *myb109* mutants is caused by defects in gene regulation at the upstream stress signaling response or at the level of stress marker or functional genes, the expression level of a group of stress signaling and marker genes was quantified in *myb109-1* and WT after one day of moderate drought. The expression level of *ABF3*, *RD22*, *RD29A*, *RD29B*, and RAB18 was not significantly different in *myb109-1* compared to WT under drought and well-watered conditions (Fig.11A). Consistent with microarray results, qRT-PCR showed a lower expression level of *DREB2A* in *myb109-1* than in WT under moderate drought (Fig. 8A). In WT DREB2A was induced by about 3 fold compared to 1.5 fold in *myb109-1*.

Since *MYB109* is expressed in the guard cells (publicly available expression data), we reasoned that it might also be involved in ABA signaling in the guard cells. Therefore, the expression levels of phospholipase D alpha (*PLDα*), alpha subunit of the G-protein (*GPA1*), type C protein phosphatases (PP2Cs) (*ABI1*, and *ABI2*), outward K+ channel (*GORK*), and receptor-like kinase (*RPK1*) were quantified in the *myb109-1* mutant and WT after one day of moderate drought. The expression level of these genes was not significantly different in *myb109-1* compared to the WT under drought and well-watered conditions (Fig. 11A).

MYB60 is a guard cell expressed gene, which was found to be repressed in response to drought stress leading to stomatal closure (Cominelli et al., 2005). Microarray analysis under progressive drought revealed the down-regulation of this gene in WT and *myb109-1*. To test the effect of *MYB109* KO mutant on the expression of *MYB60* under moderate drought, the expression of *MYB60* was quantified in the WT and *myb109-1* after 1 day of moderate drought. In contrast to progressive drought, the expression of *MYB60* was not significantly different under moderate drought compared to well-watered conditions (Fig. 11A). Although, microarray analysis did not show any effect of *MYB109* KO mutant on global gene expression under normal growth conditions, the qRT-PCR analysis revealed a significant repression of *MYB60* in the *myb109-1* mutant compared to WT (Fig. 11B).

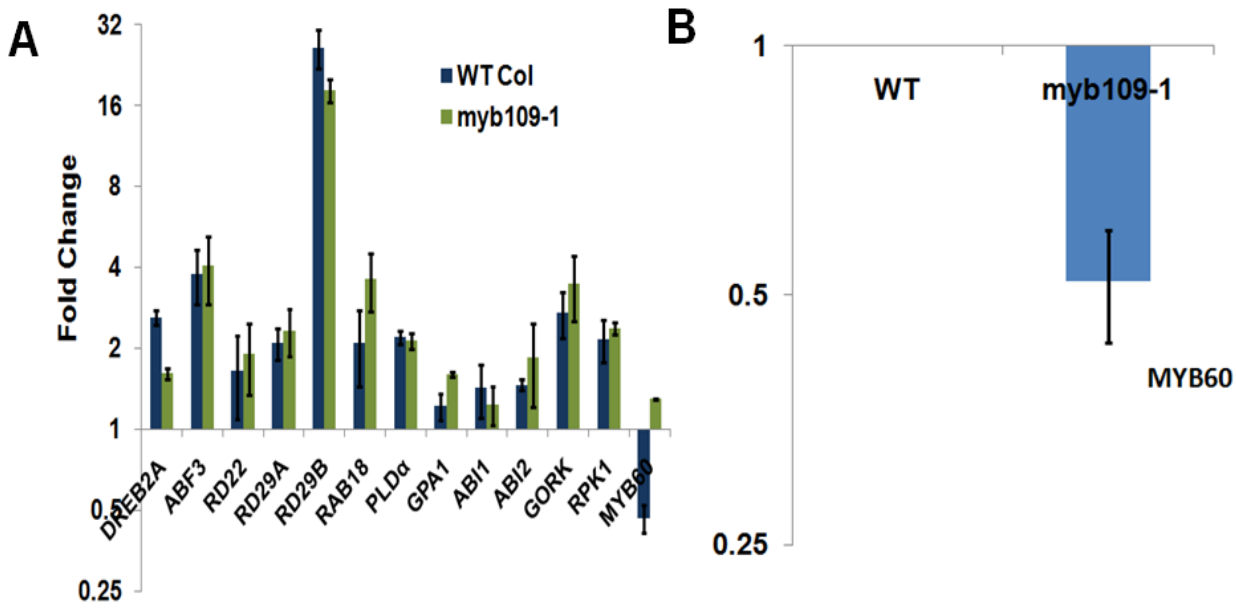

Figure 2.11. Quantification of the expression of genes in ABA signaling under moderate drought stress. A, Stress signaling, marker genes, and stomatal-related genes in WT and *myb109-1* under moderate drought. B, Expression level of *MYB60* in WT and *myb109-1* under normal growth conditions. N=3 biological replications, bars represent ± SE.

Gene Expression Localization and Overexpression

Two promoter-reporter constructs (MYB109::GUS, and MYB109::sGFP) were made to investigate the expression pattern of *MYB109* in planta. Microscopy and biomchemical analyses of the first positive transformants (T_0) of both reporter consturcts did not show any expression. For the constructuon of the promoter-reporter fusion, we amplified the closest predicted promoter (2kb) (http://mendel.cs.rhul.ac.uk). But, we found that the upstream region of *MYB109* is 12kb with no annotated genes, and in addition to the 2kb we amplified in this study; there are 3 other predicted promoters for *MYB109*: 2.5 kb, 3 kb, and 4 kb (Fig. 12). So, it could be that there are improtant cis-elements for gene expression, which are not included in the 2 kb region but in 3 or 4 kb. Another possible explanation, other cis-elements in the 3' UTR or in the intronic region are needed for the expression of *MYB109*, which has been found in the *GL1* gene (Larkin et al., 1993).

In overexpression analysis of *MYB109* under 35S promoter, two lines generated were confirmed positive at the DNA and RNA expression level. The overexpression line 1 (L_1)

showed higher expression of *MYB109* compared to line 3 (L_3) (Fig. 13). Neither overexpression line showed obvious morphological differences compared to the empty vector control, similar to what was observed for overexpression of TF genes such as *DREB1A* (dehydration-responsive element/C-repeat-binding), *AtHB7* and *AtHB12* (homeodomain leucine-zipper), *DREB2A* (dehydration-responsive element/C-repeat-binding); and HRD (AP2/ERF-like), (Maruyama et al., 2004; Olsson et al., 2004; Sakuma et al., 2006; Karaba et al., 2007). The response of these *MYB109* OX lines will be tested later to drought, salt, and ABA.

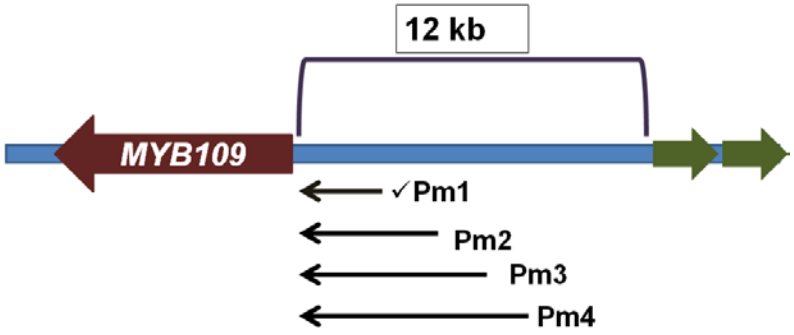

Figure 2.12. The upstream region and the 4 predicted promoters of *MYB109*. Pm: promoter. Pm1, promoter 1 is 2 kb upstream of *MYB109* and was tested in this study.

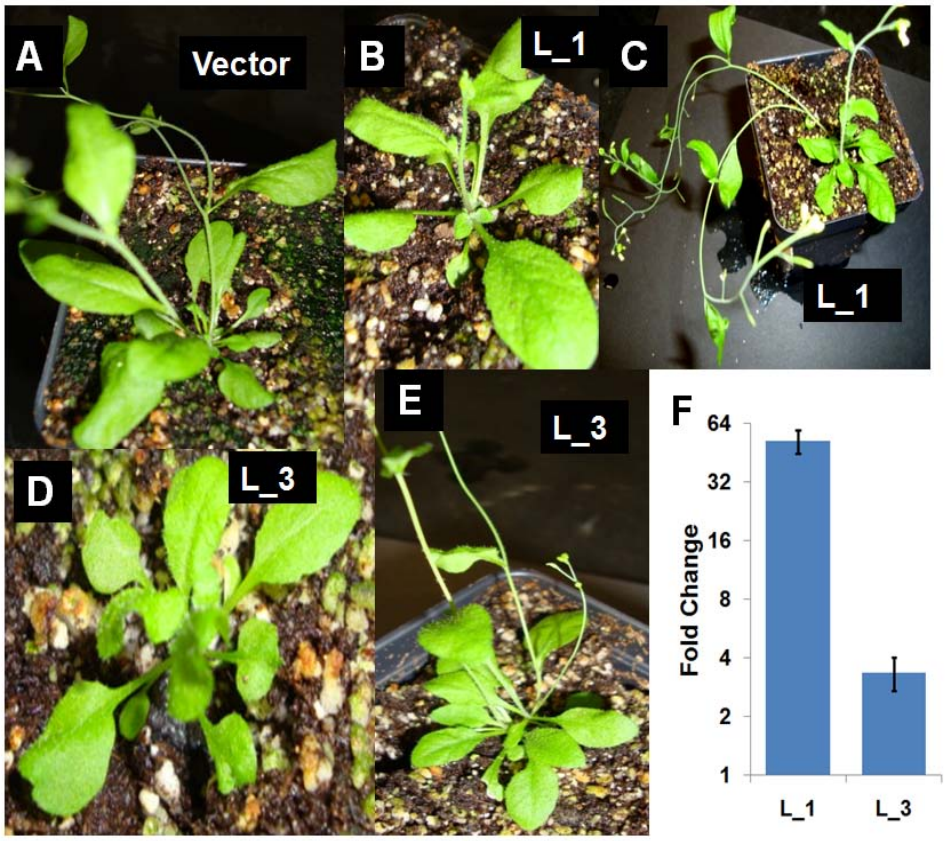

Figure 2.13. Overexpression lines of *MYB109* under 35S promoter. A, Empty vector (pCambia1301). B, and C, 35S:*MYB109* transgenic line 1 (L_1) at 30 and 40 days after sowing (DAS), respectively. D, and E, 35S:*MYB109* line 3 (L_3) at 30 and 40 DAS, respectively. F, qRT-PCR of *MYB109* expression in L_1 and L_3 relative to empty vector (control) and reference gene.

F. DISCUSSION

MYB109 Knockout Mutants are Morphologically Normal but Have Higher Biomass

Phenotypic characterization of *my109-1* and *myb109-2* showed normal morphology compared to their WT (Col) background, but exhibited higher biomass in a quantitative analysis. This normal phenotype was observed for other MYB genes, for example the *MYB44* KO mutant was similar to the WT (Jung et al., 2008). Another example is the KO mutant of *MYB96*, which has normal morphology compared to its WT background (Seo et al., 2009). This indicates the specificity of function of those MYB genes, and the lack of pleiotropic effects of the KO mutants. This makes these KO mutants an attractive system to dissect the function of a specific gene under a specific condition.

The higher biomass of the *MYB109* KO lines is unexpected. The *myb109* mutants were first screened in batches KO mutants of a set of 200 Arabidopsis drought responsive regulatory genes, with the average of the mutant lines as normal compared to the Col WT. Therefore, the higher biomass is not a common occurrence among the KO mutants, the *myb109* mutants among the few which were followed and confirmed to have higher biomass.

The reduced stomatal conductance of the *myb109* mutants might be expected to reduce CO_2 exchange and thus fixation into biomass, thus contrary to what is expected. Therefore, we believe the reduced stomatal conductance is not a factor in reducing CO_2 assimilation. Other mechanisms such as alterations in post-transcriptional processing or hormonal signaling, described below, might be involved in biomass accumulation in the *myb109* genotype.

MYB109 is Induced by Drought and the Knockout Mutants are Drought and Salt Sensitive

Expression profiling revealed that *MYB109* is induced under progressive drought. Furthermore, the induction of *MYB109* under drought was confirmed by qRT-PCR analysis, which showed 4 fold change in the expression of this gene under two drought treatments, progressive and moderate drought. Moreover, the expression profile of *MYB109* in a time course of moderate drought revealed a peak of expression at day 1 of moderate drought. Previously, we showed that at day 1 most of the early stress response and signaling events take place as priming and preconditioning stage for drought acclimation (Harb et al., submitted). This indicates that *MYB109* is among the early stress responsive genes, which are needed in the preconditioning and preparation for drought acclimation in the next stages. Consistent with this, under moderate

drought treatment the two KO mutants of *MYB109* showed higher sensitivity -in terms of reduction in biomass- compared to the WT control. *MYB109* is a member of R2R3-MYB TF family, represented by about 125 gene family members (Yanhui et al., 2006). Previous studies showed the involvement of members of this family in response to abiotic and biotic stresses (Stracke et al., 2001). For example, *MYB2* was shown to be induced in response to drought and salt stresses (Urao et al., 1993), *MYB30* induced in response to pathogens (Stracke et al., 2001, Raffaele et al., 2008), *MYB102* by ABA, osmotic stress, and wounding, *MYB41* by salinity, desiccation, cold, and ABA (Denekamp and Smeekens, 2003; Lippold et al, 2009). *BOS1* was found to have a role in biotic (fungal infection) and abiotic stress (water deficit, salinity, and oxidative stress) (Mengiste et al., 2003). *MYB15* was found to be involved in the regulation of CBF under cold stress (Agarwal et al., 2006). From the above examples, one can conclude that many R2R3-MYB genes are responsive to biotic and abiotic stress. In addition, many MYB genes respond not only to one stress, but a number of stresses. Here we found that in addition to responsiveness to drought, *MYB109* is also responsive to salt stress, as the mutants showed higher reduction in biomass compared to the WT under salt stress.

Testing the performance of the KO mutant of *MYB25* (the paralog of *MYB109*) ((Stracke et al., 2001) under moderate drought revealed non-significant change in biomass compared to the WT. This is consistent with our expression analysis of WT and *myb109-1* under progressive drought, since *MYB25* was significantly repressed in both genotypes. This also suggests that under drought stress *MYB109* has a specific role, which is not redundant with the paralog gene (*MYB25*). However, MYB genes have been shown to have partial redundancy. This was explained by the differences in the expression patterns of those genes, resulting from differences in the cis-elements and interactions at DNA and protein level (Jin and Martin, 1999). However, promoter analysis of *MYB109* and *MYB25* showed that both contain dehydration responsive motifs, as well as other gene specific motifs. One possible explanation for the absence of drought phenotype in *myb25* is that the gene is needed at a different developmental stage (earlier or later) than the stage we tested in this study. Another possibility is the nature of drought stress and severity that may play a role in the differential induction of those MYB genes.

Transcriptome Analysis Reveals Potential Mechanisms of *MYB109* Function under Drought

Expression profiling analysis of the WT and *myb109-1* revealed no direct effect of the KO mutant under normal (well-watered) growth conditions. However, the comparison of gene expression of WT and the *myb109-1* mutant under progressive drought in relation to the corresponding well-watered control showed the *myb109* mutant had a lower number of DE genes. In addition, *myb109-1* showed a lower number of DE genes that were ABA responsive compared to the WT, suggesting a role in ABA response. The reduction of DE genes in the mutant genotype indicates that *MYB109* is needed for a complete (normal) gene reprogramming in response to drought stress.

GO analysis of DE genes revealed the enrichment of common stress functional categories: response to stress, response to water deprivation, and response to ABA stimulus. Consistent with GO analysis, cis-element analysis also revealed several stress responsive elements such as: ABF binding site, ABRE-like binding, DRE core motif, and ABREATRD22 (Guiltinan et al., 1990; Iwasaki et al., 1995; Busk et al., 1997; Abe et al., 2003).

Interestingly, GO analysis of the up-regulated genotype specific genes showed the enrichment of RNA splicing and processing in the WT but not in *myb109-1*. Posttranscriptional modifications are considered as a crucial level of gene expression regulation and quality control of protein production (Matlin et al., 2005). RNA splicing is a major posttranscriptional modification step, which has been shown to be important in growth and development (Reddy, 2007). In addition, it increases the diversity and complexity of protein products needed to enhance the plant (organism) efficiency in response to the environmental changes/stimuli (Kazan, 2003; Hirayama and Shinozaki, 2010). In Arabidopsis, global mapping of alternative splicing revealed that about 42% of the intron-containing genes are alternatively spliced (Filichkin et al., 2010). RNA splicing and processing has been shown to be an important part of plant response to different abiotic stresses such as cold, heat, salt, and drought (Ali and Reddy, 2008; Floris et al., 2009). A genome-wide profiling of alternative splicing in Arabidopsis under different developmental and stress conditions revealed differential splicing patterns among the different developmental and stress conditions (Iida et al., 2004). In Arabidopsis and crop plants, many examples of differential gene splicing show the importance of this modification and regulation in response to different biotic and abiotic stresses. *STABILIZED1* (*STA1*) is an RNA

splicing and processing protein, which was induced by cold stress and the *sta1* KO mutant was shown to be incapable of proper splicing of *COR15A*, resulting in increased sensitivity to cold stress (Lee et al., 2006). Alternative splicing of Toll-like proteins was shown to be critical part for their functionality in the innate immunity (Jordan et al., 2002). In addition, in many crops alternative splicing was shown to be critical in response to different abiotic stresses: wheat *DREB2* under cold, salt and drought, maize *DREB2A* under cold, rice alternative oxidase (*OsIM*) under salt stress, grape dehydrin (*DHN1*) under drought stress (Kong et al., 2003; Egawa et al., 2006; Xiao and Nasssuth, 2006; Qin et al., 2007; Ali and Reddy, 2008).

The importance of RNA processing in the regulation of ABA signaling has been shown by the dissection of RNA processing mutants (Kuhn and Schroeder, 2003). *ABA HYPERSENSITIVE GERMINATION 2* (*AHG2*) is a poly (A)-specific ribonuclease, which is induced by cold, salt, and osmotic stress (Nishimura et al., 2005), and the *ahg2* mutant has increased sensitivity to ABA and osmotic stress. *ABA HYPERSENSITIVE 1* (*ABH1*) is an mRNA cap binding protein (*CBP80*), which is required for the proper assembly of the splicesome (Ru et al., 2008). This gene was found to regulate ABA signaling, as *abh1* showed increased ABA sensitivity and enhanced stomatal closure resulting in an improved drought tolerance (Hugouvieux et al., 2001). In this study, and consistent with previous findings *AHG2* was up-regulated in the WT but not in *myb109-1* under progressive drought, whereas *ABH1* was down-regulated. This implicates one possible explanation for the suppressed ABA sensitivity (ABA insensitivity) in the stomata of *myb109-1* (in the absence of functional *MYB109*).

In an overexpression of *ABF3* (*ABA BINDING FACTOR 3*) in the ABA-dependent pathway genes for RNA splicing and processing were DE -both up and down-regulated- that could be an important mechanism explaining the enhanced drought tolerance in *ABF3* overexpression (Abdeen et al., 2010). Taken together, this suggests a possible role of *MYB109* as a regulator of proper RNA splicing and processing to fine tune plant responses to drought stress.

Another exciting finding was the induction of JA biosynthesis and signaling in *MYB109* KO under progressive drought, with about 20 genes in JA biosynthesis and signaling were induced. Studies on the cross talk between JA and abiotic stresses are scarce. JA was shown to be induced within 2 hrs of dehydration in soybean (Creelman and Mullet, 1995). Moreover, in cross talk with ABA it was found to play a role in stomatal closure in many plants based on

studies on JA and ABA insensitive mutants (Raghavedra and Reddy, 1987; Herde et al., 1997; Bandurska et al., 2003; Suhita et al., 2004; Munemasa et al, 2007).

Exogenous Jasmonic acid (JA) was also found to inhibit growth, and photosynthesis in barley, tomato and Arabidopsis (Reinbothe et al., 1994; Xie et al., 1998). Moreover, expression profiling showed an antagonistic interaction between ABA and JA, as many ABA responsive genes such as *ATHB12* and *ABF3* were repressed under high JA accumulation (Devoto et al., 2005). Comparing our progressive drought data to MJA responsive genes from AtGeneExpress (Nemhauser et al., 2006); revealed genes involved in the down-regulation (inhibition) of growth, cell wall processing and modification in the overlap between genes that responded to progressive drought and MJA stimulus. Consistent with this, a combination of high JA concentration and a mutation in *CELLULOSE SYNTHASE 3* (*CESA3*), *cev1*, showed reduced growth and enhanced plant resistance to fungal pathogens (Ellis et al., 2002). In our progressive drought expression data, *CESA3* was repressed in WT and *myb109-1*. In contrast to the WT, *myb109-1* has the combination of JA responsive gene induction and the repression of *CESA3*, which is associated with reduced growth, described above, and might explain the higher sensitivity or reduced growth of the mutant in response to drought stress. Moreover, increased accumulation of JA inhibits cell cycle and growth (Zhang and Turner, 2008; Onkokesung et al., 2010). All of these indicate a possible negative role of JA in response to drought stress. Indeed, we have shown that plants under prolonged moderate drought stress repressed JA biosynthesis and signaling, which could play a role in the acclimation process under prolonged drought treatment (Harb et al., submitted). In conclusion, *MYB109* might act as a negative regulator of JA biosynthesis and signaling under drought stress to improve plant response and resistance to drought.

MYB109 is Required for Normal Response to ABA at Vegetative Stage

ABA has a central role in response to different abiotic and biotic stresses (Zhang et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2007). Indeed, many studies revealed an increase in ABA biosynthesis upon exposure to water deficit, and osmotic stresses (Iuchi et al., 2001; Ko et al., 2006). ABA-dependent stress signaling pathways are major pathways that function through transcription factors such as ABF, MYB, MYC, and NAC (Shinozaki, 1997; Nakashima et al., 2009; Hirayama and Shinozaki, 2010). *MYB2* was found to up-regulate the expression of *RD22* under drought stress in an ABA-dependent manner (Abe et al., 1997; Abe et al., 2003).

From publicly available gene expression data, *MYB109* was found to be induced by ABA. We therefore tested the sensitivity of *myb109-1* to ABA at different developmental stages. *myb109-1* has the same sensitivity to ABA as that of the WT at seed germination and seedling stages compared to the WT. However, several lines of evidence showed an altered stomatal response of *myb109-1* to ABA. Stomatal conductance was unchanged in *myb109-1* in response to ABA compared to untreated control, whereas the WT showed a highly significant reduction in stomatal conductance in response to ABA. In addition, cut rosette water loss (CRWL%) showed no change in water loss in *myb109-1* and *myb109-2* in response to ABA compared to reduced water loss in the WT. Moreover, the WT has higher percentage of closed stomata in response to ABA compared to *myb109-1*. Stomatal opening and closure is under the control of a complex network of receptors, channels, enzymes, and transcription factors (Schroeder et al., 2001, Nilson and Assmann, 2007; Kim et al., 2010). Plant hormones, mainly the stress hormone ABA play a crucial role in stomatal functioning (Acharya and Assmann, 2009). Many R2R3-MYB genes have been shown to regulate the development and/or the behavior of the stomata. *MYB88* and *FOUR LIPS* (*FLP*) play a critical role in stomatal division and patterning (Lai et al., 2005). The overexpression of *MYB44* resulted in the stimulation of stomatal closure and stress tolerance (Jung et al., 2008), whereas *MYB61* overexpression led to a decrease in the stomatal apertures (Liang et al., 2005). Through mutant analysis, *MYB60* was found to function as a negative regulator of stomatal aperture (Cominelli et al., 2005). In conclusion, *MYB109* is required for a normal ABA signaling.

To find out how *MYB109* is regulating sensitivity to ABA, we quantified the expression of some of the key genes in stress signaling pathways and stress marker genes under moderate drought: *DREB2A*, *ABF3*, *RD22*, *RD29A*, *RD29B*, and *RAB18* (Yamaguchi-Shinozaki and Shinozaki, 1993a; Yamaguchi-Shinozaki and Shinozaki,1993b; Choi et al., 2000; Sakuma et al., 2006; Hirayama and Shinozaki, 2010). With the exception of *DREB2A*, the expression level of these genes was not significantly different in *myb109-1* from the WT. The expression of *DREB2A* was lower in *myb109-1* under progressive (microarray data) and moderate drought (qRT-PCR) compared to the WT. To test how reduction in the expression level of *DREB2A* plays a role in the high drought sensitivity of *myb109-1*, we checked the expression of the direct targets of *DREB2A* such as: *RD29A*, and *RD29B*. Expression profiling under progressive drought showed a reduction in *RD29A* expression, whereas the expression of *RD29B* were the same as

that of the WT. However, quantification of the expression level of *RD29A* and *RD29B* under moderate drought revealed similar induction in *myb109-1* and WT. Some possible explanations and consequences for the reduction in expression of *DREB2A* in the *MYB109* KO in response to drought are: a) the repressed level of *DREB2A* is above the threshold level needed for normal functioning of this gene; b) *DREB2A* regulation modulates downstream expression that is dependent on additional control mechanisms; c) *DREB2A* has other unknown (undiscovered) targets.

The expression of critical genes in ABA signaling in the guard cells was quantified under moderate drought, showing that with the exception of *MYB60*, all genes have similar expression level as the WT. *MYB60* was not DE in *myb109-1* under moderate drought compared to the well-watered control of the mutant. However, our progressive drought expression profiling data showed the repression of *MYB60* in *myb109-1* and WT. Hence, the effect of *myb109-1* was tested on the expression of *MYB60* under normal (well-watered) and drought conditions. *MYB60* was significantly repressed in the mutant under normal (well-watered) conditions compared to the WT. This indicates that *MYB60* is repressed in a constitutive manner in *myb109-1*. On the other hand, repression of *MYB60* in *myb109-1* under progressive drought, which showed low expression of *MYB60* under normal conditions, might indicate more suppression (repression) of *MYB60* with the increase in drought severity. *MYB60* is a stomatal gene, which was repressed by drought stress resulting in stomatal closure (Cominelli et al., 2005). *MYB109* is also reported to be a stomatal expressed gene. Therefore, repression of *MYB60* in *myb109-1* is consistent with the altered stomatal response to ABA, and suggests a role for *MYB109* in the regulation of *MYB60* expression under normal and stress conditions.

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4. A. Activation Tagging Forward Genetics Screen for Drought and Salt Resistance in Arabidopsis

B. ABSTRACT

Activation tagging is a high-throughput method of overexpressing genes, using an enhancer present in insertion sequences that are randomly inserted in the genome and enhance the expression of adjacent genes. Gain-of-function approaches are advantageous to identify functions of redundant genes, not identifiable by knockout (KO) mutations, and for identification of phenotypes with small effects, which are enhanced by activation. An activation tag (ATag) library of 800 lines was generated in Arabidopsis ecotype Columbia using the *En-I* (*Spm*) transposon system. The ATag lines were used in a forward genetics strategy to identify novel genes that confer resistance/tolerance to abiotic stresses. The ATag lines were screened for altered drought and salt stress response phenotypes using quantitative assays for biomass accumulation under stress, revealing a number of resistant and sensitive ATag mutants. The detailed morphological, histological, physiological, and molecular characterization of the ATag lines designated as C65 and C437 are described. C65 is a bushy, pale green, early flowering, early senescencing, and is salt resistant. The putative tagged genes in C65 include a gene of unknown function and a MATE transporter gene. The ATag mutant C437 is compact with short petiole, and round leaves, and is drought and salt resistant. The putative tagged genes in C437 are 2 genes from the xyloglucan endotransglucosylase/transferase gene family (XTH), predicted to be involved in cell wall modification and cell expansion during growth. These mutants reveal a potential role for cell wall modification and MATE transporters in response to abiotic stress, which requires further characterization.

C. INTRODUCTION

Being sessile, plants are susceptible to the changes in the surrounding environment. Therefore, they have to be equipped with mechanisms that enable them to cope with environmental challenges. Drought and salt stress are the two main abiotic stresses, which challenge crop production and plant survival. Abiotic stress research has provided some basic knowledge of the biochemical and molecular drought response components and pathways (Ingram and Bartels, 1996; Chinnusamy et al., 2003; Shinozaki and Yamaguchi-Shinozaki, 2007; Munns and Tester, 2008), including stress-regulated genes, and stress signal transduction pathways (Bartels and Sunkar, 2005; Nakashima et al., 2009). However, little is known about the interaction among the components of stress response, and about the network of the pathways involved in stress response.

In order to improve abiotic stress resistance in crop plants, a comprehensive understanding of plant response to stress is needed. Much molecular data has been obtained on the response and adaptation of plants to abiotic stress (Kreps et al., 2002; Ramanjulu and Bartels, 2002; Seki et al, 2002; Zhu, 2002; Chinnusamy et al., 2003; Bray, 2004; Shinozaki and Yamaguchi-Shinozaki, 2007; Zeller et al., 2009). Plants respond to environmental signals in response pathways that are initiated by perception of the signal, leading to signal amplification through signal transduction cascades, and finally biochemical and molecular changes take place in the cell (Nakashima et al., 2009). The different types of abiotic stresses have multiple initial inputs on plants (Xiong et al., 2002). This causes a complexity in the sensing components and in the signaling pathways in response to the multiple inputs (Hirayama and Shinozaki, 2010).

Forward and reverse genetics techniques have been used to understand plant response to the different stresses (Pereira, 2001; Papdi et al., 2010). Insertion mutagenesis using T-DNA and transposon knockout (KO) mutants as well as RNAi knockdown lines are useful methods to dissect the pathways that are involved in plant response to stress (Xiong and Zhu, 2002). Although loss-of-function mutagenesis is a direct way to reveal gene function, gene redundancy and lethality in Arabidopsis make the loss-of-function mutations of less practical use (Pereira, 2001). About 2/3rd of the Arabidopsis genome is duplicated (Bounchè and Bouchez, 2001). Thus a gain-of-function strategy using activation tagging was developed (Marsch- Martinez et al., 2002; Hirschi, 2003; Nakazawa et al., 2003). Activation tagging systems using T-DNA insertions bearing a 35S CaMV enhancer (Weigel et al., 2000; Tani et al., 2004), generated over thirty

dominant mutants out of more than thirty thousand transformed plants (Weigel et al., 2000). However, the low frequency of activating tag mutants in Arabidopsis due to multiple inserts and methylation makes it less attractive for genome saturation of the Arabidopsis genome (Chalfun-Junior et al., 2003).

Transposon-based activation tagging is an efficient mutagenesis method. The *En-I* (*Spm*) system has been used for activation tagging in Arabidopsis (Marsch-Martinez et al., 2002; Schneider et al., 2005). The system uses the herbicide selectable markers, the BAR and SU1 genes, for greenhouse-based selection of stable transposon insert (Tissier et al., 1999; Marsch-Martinez et al., 2002; Schneider et al., 2005). The *En-I* system revealed a high frequency of dominant mutations of about 1%, with a population of 2,900 insertions revealing 31 dominant mutants (Marsch-Martinez et al., 2002). Later unpublished work with more precise screening of progeny lines revealed around 200 independent mutations in 8000 ATag lines giving a frequency of 2.5%.

Using the technique of activation tagging many novel mutants have been isolated (Kardailsky et al., 1999; Borevitz et al., 2000; Marsch Martinez et al., 2002; Karaba et al., 2007). These include mutations for flowering time (Kardailsky et al., 1999), parthenocarpy (Ito and Meyerowitz, 2000), reduced apical dominance (Nakazawa et al., 2003), multiple leaf phenotypes (Marsch- Martinez et al., 2002) as well as other developmental (Marsch Martinez et al., 2006; Schneider et al., 2005) and biochemical mutants (Aharoni et al., 2004; Schneider et al., 2005; Seo and Park, 2010) as well as abiotic and biotic stress resistance mutants (Aharoni et al., 2004; Chini et al., 2004; Karaba et al., 2007; Yu et al., 2008; Aboul-Soud et al., 2009; Seo and Park, 2010).

An attractive tool to investigate genes with redundant function or minor mutant phenotypes with small genetic effects is activation tagging, a gain-of-function approach. In activation tagging, a DNA insertion sequence, such as transposon or T-DNA contains an enhancer sequence is used. When this activation tag (ATag) element inserts near genes in the genome, it can increase the expression of adjacent genes (to a distance of ~10 kb) and often displays an overexpression phenotype of the tagged gene. In this study, an ATag population of about 800 lines was generated in Arabidopsis ecotype Columbia (Col) as has been described previously for ecotype Ws (Marsch Martinez et al., 2002). Activation tagging mutant lines were screened for drought and salt stress phenotype. Drought and salt resistant lines were analyzed

molecularly, and the tagged gene(s) were identified. In addition, two ATag mutant lines were studied in detail, C65 and C437. C65 is salt resistant, and C437 is drought and salt resistant. The detailed characterization of the two mutants is presented here as a method to identify novel stress resistant genes by a forward genetics strategy.

D. MATERIALS AND METHODS

Generation of Activation Tagging Population in Arabidopsis Columbia Ecotype

Seeds of T_2 transformants were generated in Arabidopsis ecotype Columbia (Col-0) at Plant Research International (Raffaella Greco unpublished; Wageningen, Netherlands) (Marsch-Martinez et al., 2002). T_2 seeds were sown in peat: perlite: vermiculite mix in plastic flats and grown under 16 hrs (100 μmole m^{-2} s^{-1}) light at 22° C. After germination, seedlings were sprayed with Basta herbicide (Thompson et al., 1987), plants with SU1 phenotype (small dark green) were grown to maturity and seeds were collected. To generate the stable activation tag population, seeds collected from SU1 plants were sown at high density in peat:perlite:vermiculite mix in flats. After 3-5 days of germination, seedlings were sprayed with Basta and R7402 (O'keefe et al., 1994; Marsch-Martinez et al., 2002) twice a week for 2-3 wks. Double resistant seedlings were counted to estimate the stable transposition frequency (STF), which is the ratio of surviving plants to the total number of seed sown (Marsch-Martinez et al., 2002). Only double resistant seedlings with STF less than 3% (expected to contain unique insertions) were transferred to plastic pots to grow to maturity for morphological and phenotypic characterization (Marsch-Martinez et al., 2002). Seeds from stable insertion lines were sown to study segregation and dominance.

Drought Screen of Columbia Activation Tagging Mutants

For testing drought response phenotypes, the plant genotypes from activation tag lines, knockout (KO) mutants and wild type (WT) were grown in Jiffy peat pellets (Jiffy Products, Shippagan, Canada) under 10 hr light (100 μmole m^{-2} s^{-1}) at 22°C. For drought treatment, pellets were weighed before sowing to determine the amount of water in pellets at the beginning of the experiment. At 30 days after sowing (DAS), watering was stopped for the drought-treated

plants, and maintained for the well-watered control. After 5 -7 days plants reached moderate drought (mDr) level, which was maintained by giving plants water to keep the soil moisture level at 30% of field capacity, which is 200% or 2 g H_2O g^{-1} dry soil. To do this, a semi-automated system was developed; a balance (GF 1000, A &D, California, USA) was connected to the computer utilizing software for communication, which enabled entering of the weights directly onto an Excel worksheet file. On the Excel worksheet file a set of equations were used to calculate the water content in each weighed pellet, the required final water content, and the amount of water to be added. The pellets were weighed daily, and supplemented with the calculated amount of water to reach 30% of field capacity (mDr level). After 10 days of mDr treatment, plants were harvested and the relative reduction in biomass (RB) was calculated as follows: Relative Reduction in Biomass (RB) = $(B_{WW} - B_{DRT})/ B_{WW}$

B_{WW}, Biomass under well-watered (WW) conditions; $B_{DRT,}$ Biomass under MD conditions.

Salt Stress Treatment

Plants of activation tag lines, KO mutants and the WT were grown in Jiffy peat pellets as described above. After 30 DAS, a group of plants was treated with 150 mM NaCl treatment, and another group was kept in water as a control. Plants were treated for 7 days, after which they were harvested and their biomass was determined as shown above.

For salt stress at seedling stage, the specific activation tag (ATag) lines and WT were tested under salt stress. Seeds of both genotypes were surface sterilized by 70% ethanol, and 10% bleach (NaClO), and then rinsed 3 times with sterile distilled water. After that, seeds were spread on MS media, stratified in dark for 2 days, and allowed to grow under 100 µmole m^{-2} s^{-1} light/16 hrs for 15 days. Then, 15 days old ATag seedlings and WT were transferred to 150 mM NaCl MS media, and another group of seedlings transferred to MS media. Seedlings were grown under the same conditions as above for 7 days, and at the end of salt stress treatment fresh weight of the seedlings were measured in mg.

Identification of Candidate Genes in Activation Tag Mutants by TAIL PCR

DNA of ATag mutants was isolated as described (Pereira and Aarts, 1998), and TAIL PCR carried out (Liu et al., 1995a; Liu et al., 1995b; Tsugeki et al., 1996) using 10 ng of DNA. Table 1 shows the set of primers that was used for TAIL PCR. Amplicons after the third PCR

reaction were purified and sequenced in the core lab facility (VBI, VT, USA) using the right border primer of the transposon (DSpm1, 5'CTTATTTCAGTAAGAGTGTGGGGTTTTGG 3').

Table 3.1. List of primers used in TAIL PCR analysis

Primer Name	**Primer Sequence (5' ---------- 3')**
Int2	CAGGGTAGCTTACTGATGTGCG
Irj-201	CATAAGAGTGTCGGTTGCTTGTTG
DSpm1	CTTATTTCAGTAAGAGTGTGGGGTTTTGG
AD1	TG(A/T)G(A/T/G/C)AG(A/T)A(A/T/G/C)CA(G/C)AGA
AD2	(G/C)TTG(A/T/G/C)TA(G/C)T(A/T/G/C)CT(A/T/G/C)TGC
AD3	CA(A/T)CGIC(A/T/G/C)GAIA(G/C)GAA
AD4	TC(G/C)TICG(A/T/G/C)ACIT(A/T)GGA
AD5	(A/T)CAG(A/T/G/C)TG(A/T)T(A/T/G/C)GT(A/T/G/C)CTG
AD6	AG(A/T)G(A/T/G/C)AG(A/T)A(A/T/G/C)CA(A/T)AGG

Expression Analysis of Candidate Genes by RT-PCR

RNA from Col activation tagging mutants and the WT was isolated using RNAeasy kit (Qiagen, USA), DNA eliminated by DNAse RNAase free kit (Qiagen, USA), and cDNA synthesized using iScript cDNA synthesis kit (Biorad, USA). Expression analysis of candidate genes was done using gene specific primers (Table 2), using PCR protocol: initial denaturation at 95 °C for 5 min, 30 cylces (denaturation 95 °C for 1 min, annealing 56 °C for 30 s, extension 72 °C for 1 min), and final extension at 72 °C for 5 min. UBQ10 (AT4G05320) was used as a reference gene. In addition, expression of the candidate genes was quantified by SYBR green, the amplification done as described above. The fold change of expression was calculated relative to UBQ10 reference gene and relative to the WT control (Livak and Schmittgen, 2001).

To test expression under drought, the ATag mutant plants and WT were grown for 35 DAS as described above. After that, watering was stopped for one group and plants kept to dry until one day before wilting. For the other group, plants were kept in water as well-watered control group. At one day before wilting -determined based on gravimetric monitorning of the soil water content-, plants were harvested for RNA isolation. RNA isolation and cDNA synthesis were done as described above. Expression was tested both by RT-PCR and by quantitative PCR (qRT-PCR) as described above.

Table 3.2. List of primers used to test the expression of *EN-I* (transposon) tagged genes

Primer Name	**Primer Sequence (5' ---------- 3')**
C394_80 LP	AGAGTGTGGGGTTGATTTCG
C394_80 RP	AAGCTGATGGAGAGGCTGAA
C394_90 LP	ACGGCTCTGATCGTTGACTT
C394_90 RP	TTTGCACTGTGTGCTCTTCC
C394_700 LP	AAGGTTCATCAGCCACCAAC
C394_700 RP	ACCAAACAGCCACCTGTCTC
C437_70 LP	GGCCGTGGGAAGATACTCAACAAC
C437_70 RP	GGGTGTGTCATCGACGGTCAATATG
C437_80 LP	AGGCTCCTTTCACCGCTTTCTA
C437_80 RP	ACTCTGTACCCCTTTCATTCTTGTCTG
C437_90 LP	TTTGCAGCACAATCTATCAGCGTCT
C437_90 RP	TCCACGTGACTCGGCATTCATAAAC
C65_20 LP	CAACACAGAGGAATGCGAGA
C65_20 RP	GCTCAAGCCGTACCAAACTC
C65_30 LP	TACTTAACCCGCAAGCAACC
C65_30 RP	ACAATCTTGCCCAGCAATTC
C144_10 LP	GATACTGGACCAAGGGTGGA
C144_10 RP	AGCTCCATCTGTTCGTTGCT
C144_20 LP	GAGGGGAATGGAGAAAGAGTGAAAG
C144_20 RP	TGTGTGGGGAGGGTGTTGAATTGG
C144_30 LP	GGGGTTTGTGTTGAGATGAAAGAGG
C144_30 RP	TGGAGGTGGTAGATGATATGGGTTG
C19-1_20 LP	AAACCGATATCAGCGTTTGG
C19-1 _20 RP	GGTGCGGTAACAAGGGTAGA
C19-1_400 LP	ACTGTGTGAAGGGCAAGTCC
C19-1_400 RP	TGTCAGGAGCAGATCCACAG
UBQ10 LP	TGTGTTTTGGGGCCTTGTAT
UBQ10 RP	CAAGTTTCGCAGAACTGCAC

Histological Characterization of ATag mutants and WT

Plants of the ATag mutants and WT were grown for 40 DAS under 100 µmole m^{-2} s^{-1} light /16 hrs light. Samples were fixed in FAA fixative [8.5 (70%) ethanol: 1 (40%) formaldehyde: 0.5 glacial acetic acid] (Cutler, 1978). Samples were prepared for staining through a series of rehydration, dehydration, infiltration, embedding in paraffin, and casting in

plastic boats. After that, they were microtome-sectioned into 12 µM sections. Tissue sections were fixed on slides and stained with Safranin O, and counterstained with Fast Green (Sass, 1958). Safranin O gives red color to the nuclei, lignin, suberin and waxes. The counterstain Fast Green gives bluish green color to the cytoplasm and cellulose.

Characterization of the Insertion Mutants of Candidate Genes

Insertion KO mutants of candidate genes of C437 and C65 in Col-0 background were obtained from Arabidopsis Biological Resource Center (Ohio State University, USA). For C437 and C65 ATag insertions, KO mutants for two candidate genes per ATag line were ordered: C437_X70, C437_ X80, C65_X20, and C65_X30 (SALK_005941, SALK_025862C, SALK_000823, and SALK_127812C), respectively.

The KO mutants were genotyped using T-DNA and gene specific primers (Table 3). Two PCR reactions were prepared for each genotype using 10 ng DNA. In the first reaction, the two gene specific primers were used to check the presence of the intact gene. To check for the interruption of the gene, the left border T-DNA primer (LBb1) and the reverse gene specific primer were used in the second reaction. The amplification was done under the following PCR cycling conditions: initial denaturation at 95º C for 5 min, then 30 cycles (95 ºC for 30 sec, 57 ºC for 30 sec, and 72 ºC for 1 min), and final extension at 72º C for 5 min.

Table 3.3. List of primers used for genotyping of T-DNA insertion mutants

Primer Name	**Primer Sequence (5’ ---------- 3’)**
KO-C437_X70 LP	GCTTGTTGTGCATTCCTTAGG
KO-C437_X70 RP	TCCTCACATTCCTCACCAAAC
KO-C437_X80 LP	ACTATACGAGTGCATGGGTGG
KO- C437_X80 RP	CGTGGGCTGTATTCTAGTTGG
KO-C65_X20 LP	AGAGTTTGGTACGGCTTGAGC
KO-C65_X20 RP	AGGGGAATTCGGTTTATTTCC
KO-C65_X30 LP	AACTTTTAGGCCTTGCTTTGC
KO-C65_X30 RP	GAAACGAATCTCGTTAGTGCC
T-DNA left border (LBb1)	GCGTGGACCGCTTGCTGCAACT

Gas Exchange Measurements and Isotopic Carbon Discrimination

For the gas exchange measurements, a LICOR 6400XT (LICOR, Nebraska, USA) and an Arabidopsis Extended chamber were used, and the following conditions were set for LICOR measurement: flow rate 150 µmole s^{-1}, CO_2 400 µmole, humidity 50%.

For isotopic discrimination analysis, oven dried rosette leaves were ground to a fine powder using a mortar and pestle, and packed in tin capsules in 96-well tray. The samples were sent to UC Davis Stable Isotope Facility (http://stableisotopefacility.ucdavis.edu/). The carbon isotope ratio is expressed as δ in part per million (‰) and calculated relative to PDB (PeeDee Belemnite) standard as follows:

δ (‰) = (Rsample/Rstandard - 1)1000; where R is the ratio of heavy to light isotope.

Four replications were analyzed per genotype, and each replication is a pool of 3 plants.

Preparation of Overexpression Constructs and Plant Transformation

The genomic sequence of each candidate gene was amplified using PHUSION high fidelity PCR kit (New England BioLabs, USA), using a set of primers to amplify the genes for overexpression (Table 4). The amplicons were cloned into pGEM-T vector (Promega, USA), and sequenced in the core lab facility (VBI, VT, USA). The cloned genes were integrated into the plant binary vectors pCambia1301 or pBIN-plus using suitable restriction sites, under control of the CaMV35S promoter, and the recombinant plasmids electroporated into Agrobacterium EHA105 strain. Wild type (Col-0) plants at suitable flowering stage were transformed by floral dip method (Clough and Bent, 1998). Arabidopsis transformants were selected on 50 µg/ml kanamycin MS media for pBin-plus vectors and 25 µg/ml hygromycin MS media for pCAMBIA vectors.

Table 3.4. List of primers used for overexpression of *EN-I* transposon tagged genes

Primer Name	**Primer Sequence (5’ ---------- 3’)**
C437_X70_LP	ACG CAC ACA CAC AAA GAC
C437_X70_RP	CTC GGA CAT AAT AGA CAA GC
C437_X80_LP	CAT TTG AGA TAT CAA TAC ACC TCC
C437_X80_RP	CAC AAA CAC CGC ATA CAT ATG
C65_OX20 LP	CTC ACA CAT CAG ACA TCA CT
C65_OX20 RP	CTC TCA TTA CTC ATT AAA CAT TC
C65_OX30 LP	CAC CTT CTT CAT CAT CTC CT
C65_OX30 RP	GAA ACG AAT CTC GTT AGT GCC

E. RESULTS

Arabidopsis *En-I* ATag Mutant Population Generation in Ecotype Columbia and Mutant Screen

Selection of the *En-I* transposon ATag population in ecotype Col, by positive- and counter- selection with the Basta and R7402 herbicides respectively, resulted in about 800 stable activation tag lines (ATag). Most of the generated ATag lines were WT looking, and about 200 showed putative morphological phenotypes different from the wild type (Fig.1). A screen for inheritance of mutants was conducted for the 200 ATag lines, with 15 lines showed dominance inheritance as judged by the segregation and similar morphology to the parent line (Fig. 2). These 15 ATag lines were screened for drought and salt stress resistance.

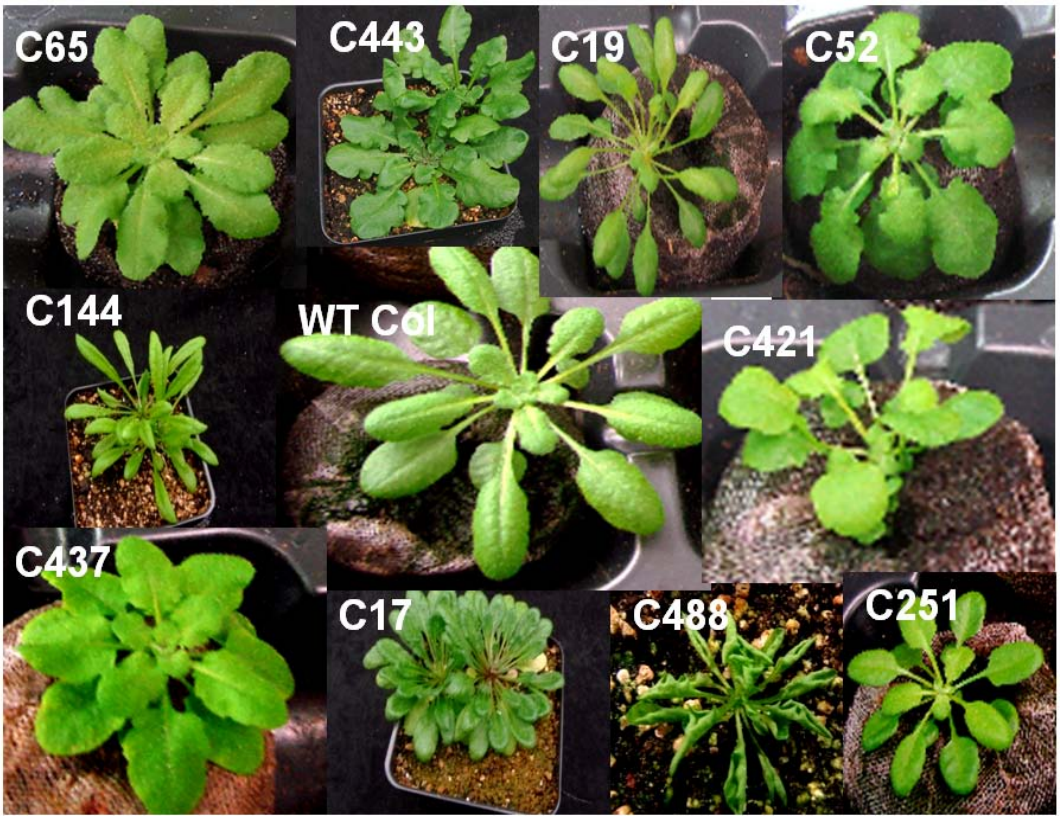

Figure 3.1. Columbia activation tag (ColATag) mutants showing morphological phenotypes.

Figure 3.2. Inheritance test of some Col ATag mutants. Col ATag lines were sown 4-8 plants per/line, then the progeny was scored for the parent phenotype to check the dominance level of the ATag gene (s). The mutants are labeled C for Columbia ecotype followed by a serial number. p :parent, pg: progeny

Drought and Salt Tolerance Screens

To select ATag lines with drought phenotype, a preliminary (first) drought screen was done. In the first drought screen 15 ATag lines with visible morphological phenotypes were tested, 3 lines scored as drought-resistant as they showed less reduction in biomass than the WT (Fig.3B). The remaining lines showed non-significant reduction in biomass compared to the WT. In a repetition second drought screen, 10 of the 15 lines were tested, including the drought resistant lines from the first (preliminary) screen. The second drought test was to confirm the drought response phenotype of the resistant lines, and to reselect lines with altered drought response phenotype (resistant or sensitive) from among the segregating ATag lines, to evaluate lines that are the same zygosity (hemi- or hetero-zygous) for the insertion allele. The second drought screen revealed 6 resistant lines with less reduction in biomass, and 2 sensitive lines with more reduction in biomass compared to the WT (Fig. 3C).

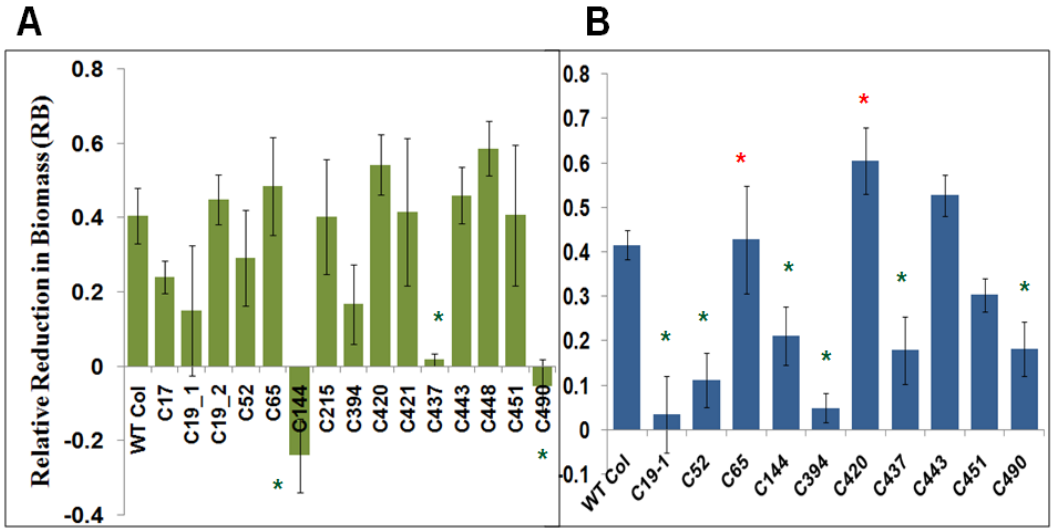

Figure 3.3. Drought screen of ColATag mutants. A, First drought screen of 15 ATag lines showing relative reduction in biomass (RB). B, Second drought screen of 10 ATag lines showing relative reduction in biomass (RB). Bars represent ± SE, N=6. * indicates significance at p-value <0.05. Red * indicates sensitive lines, green * indicates resistant lines.

Ten ATag lines at 30 DAS were tested under salt stress (150 mM NaCl) for 1 week (Fig. 4A). Five lines were salt resistant as they showed less reduction in biomass compared to the WT (Fig. 4B). One line was sensitive as it showed more reduction in biomass than the WT.

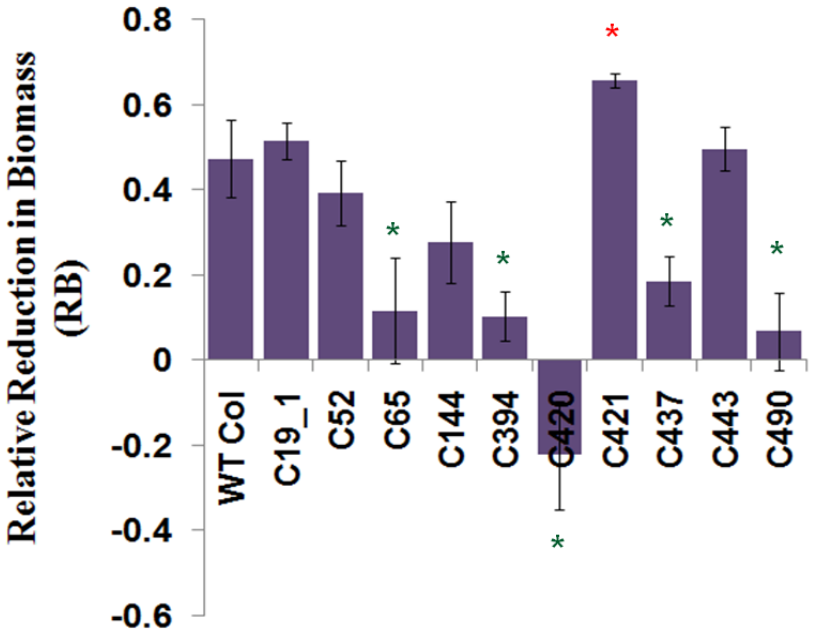

Figure 3.4. Salt stress screen of ColATag mutants. Relative reduction in biomass (RB) of 10 ATag compared to WT Col. Bars represent ± SE, N= 4. * indicates significance at p-value <0.05. Red * indicates sensitive lines, green * indicates resistant lines.

Identification of Candidate Genes in ColATag Lines

The ColATag lines with altered drought or salt responses were further analyzed molecularly to identify candidate genes responsible for both the morphological and abiotic stress phenotypes. TAIL PCR was conducted as described in methods, the resultant amplicons were sequenced, and candidate genes surrounding the transposon insertion were identified using Blastn to the Arabidopsis genome sequence (TAIR9, TAIR; Altschul et al., 1997). Based on the Blast results, models were drawn to show the candidate genes surrounding the transposon insertion (Fig. 5). Next, the most likely activated gene candidates –genes within 10 kb from the transposn insert- were tested for expression (Fig. 6). The expression analysis revealed some candidate genes are overexpressed, other were not different from the WT. The genes showing overexpression in the ATag mutant line were then used for further analysis and characterization.

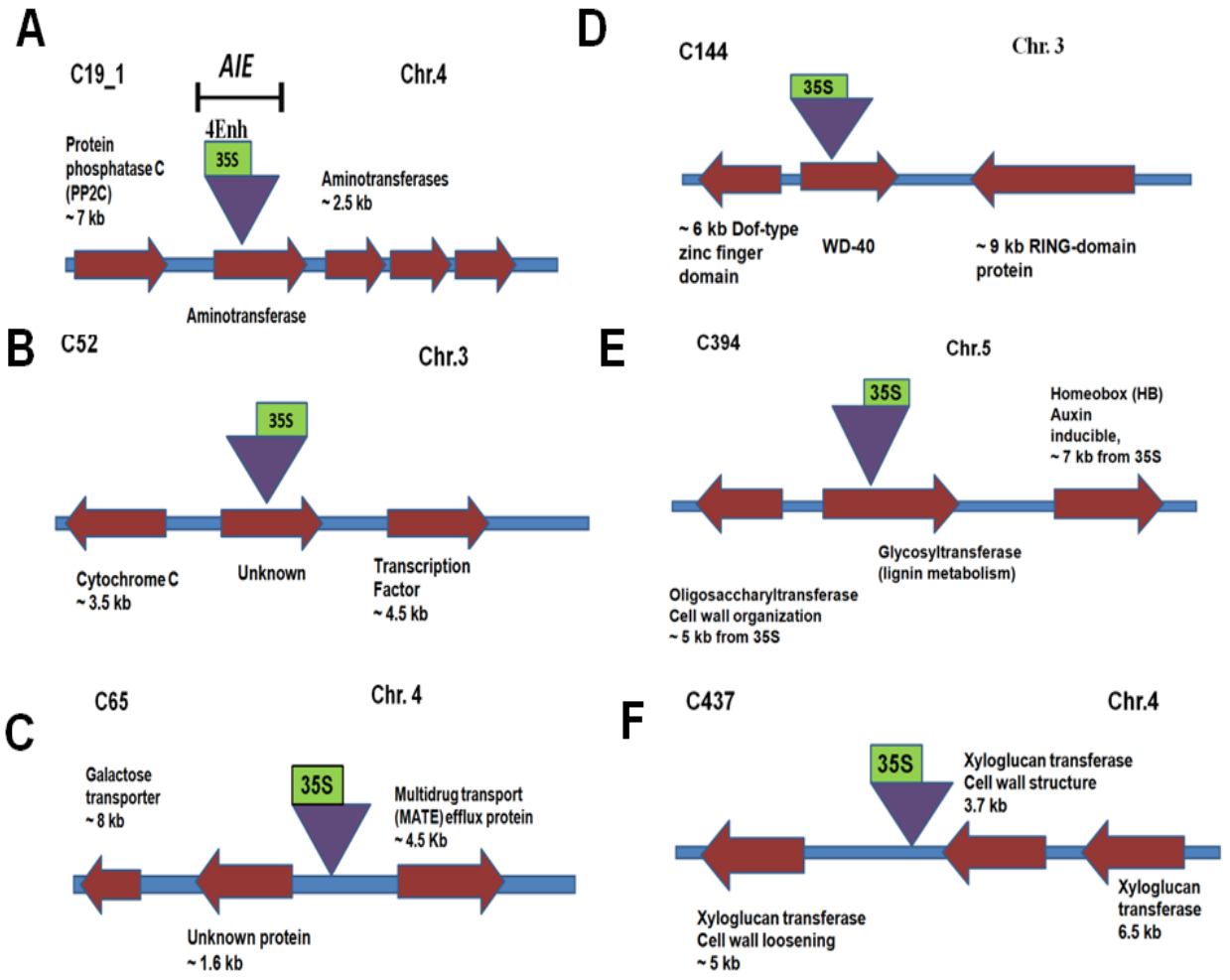

Figure 3.5. Schematic illustration depicting the position of stable transposon (AIE) insertion in Arabidopsis genome in ColATag mutants. The thick red arrows show the coordinates and direction of candidate genes labeled alongside. A-F, Insertion in 6 different ColATag mutants. kb shown is the distance from 35S enhancer in the AIE.

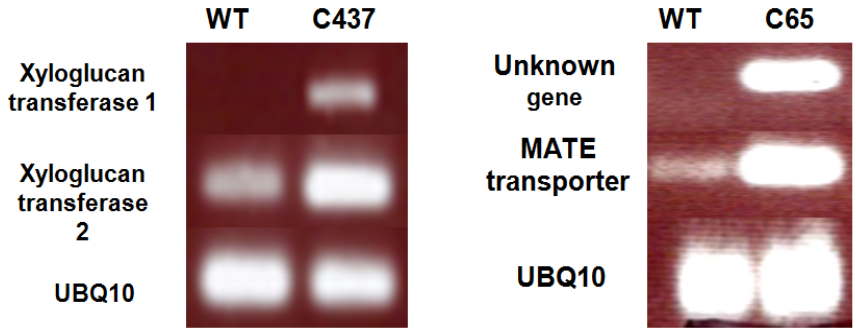

Figure 3.6. RT-PCR expresssion analysis of candidate genes in two ColATag lines. Two mutants are shown: C65 and C437 and the candidate genes for each mutant. UBQ10 is the reference gene. The two xyloglucan transferases are from the same gene family, and therefore designated 1 and 2.

Characterization of C437 ATag Mutant

From the first drought screen of ColATag mutant population, line C437 showed highly significant resistance to mDr and was chosen for further detailed analysis. The C437 ATag mutant is compact with short petiole, round leaves, and has less biomass than the WT (Fig. 7A and B). The mutant has similar flowering time as that of the WT (Fig. 7B). TAIL-PCR analysis followed by sequencing and Blast analysis revealed the position of the AIE transposon insertion in the C437 ATag mutant. The AIE insertion is on chromosome 4 around 3.7 kb downstream of a xyloglucan transferase enzyme gene, C437_X80 (Fig. 7C). In addition, the insert is surrounded by 2 other xyloglucan transferase genes, C437_X70 and C437_X90 (Fig. 7C). These 3 genes belong to xyloglucan endotransglucosylase/hydrolase (XTH) gene family (Yokoyama and Nishitani, 2001). Expression analysis of by RT-PCR and quantitative RT-PCR (qRT-PCR) showed high expression of two genes designated as C437_X70 and C437_X80, whereas C437_X90 showed similar expression to that of the WT (Fig. 7D). For histological analysis of this mutant at flowering stage (40 DAS), root and leaf sections were stained with Safranin O that stains nuclei, lignin, suberin, and counterstained with Fast Green that stains cellulose. C437 showed higher lignin accumulation (deeper Safranin staining) in cross sections of the root and leaves of the mutant compared to WT (Fig. 7E-J).

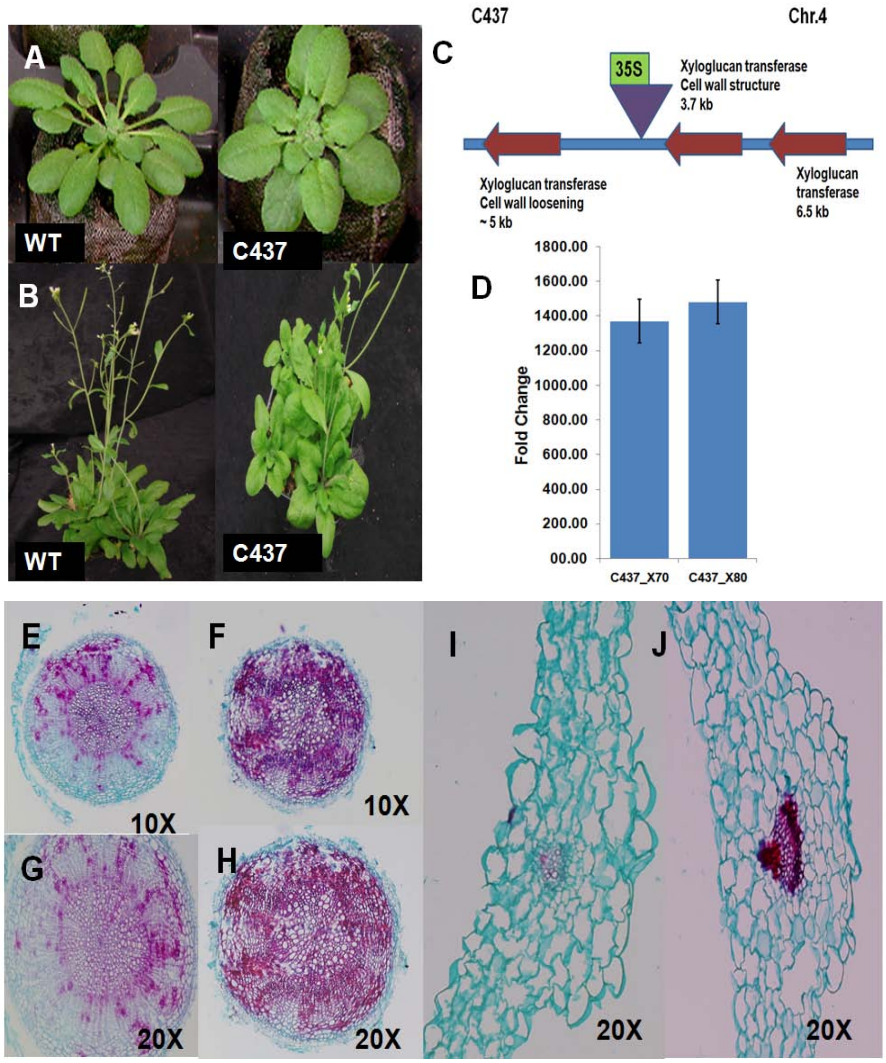

Figure 3.7. Phenotypic and molecular characterization of the C437ATag mutant. A, Morphological phenotype of C437 ATag mutant compared to the WT at vegetative stage (30 DAS) under normal growth conditions. B, Morphological phenotype of C437 ATag mutant compared to the WT at flowering stage (40 DAS) under normal growth conditions. C, Illustration of transposon insertion in C437 ATag mutant showing 3 genes surrounding the transposon insert from the xyloglucan transferase gene family (XTH). D, Quantification of two candidate genes in C437 ATag by qRT-PCR. Bars represent ± SE, N=3 biological replications. E-J, Histological analysis for anatomical characterization of C437 ATag, Root and leaf sections were stained with Safranin O that stains lignin, and counterstained with Fast Green that stains cellulose. E, Cross section of WT root at 10X. F, Cross section of C437 ATag root at 10X. G, and H same as E and and F, respectively, at 20X magnification. I, Cross section in WT leaf at 20X. J, Cross section in C437 ATag leaf at 20X.

Under mDr, C437ATag was highly resistant compared to wild type (Fig. 8A, and B-D). Leaf area of C437 ATag was reduced by 50% under drought compared to well-watered control, whereas the WT showed 75% decrease in leaf area (Fig. 8B). Moreover, the relative reduction in biomass (RB) for C437 ATag and WT was 0.2 and 0.5, respectively, showing a significant difference in the mutant over WT (Fig. 8C). Expression under progressive drought (pDr) of the two candidate genes in C437 ATag did not show any significant induction in the WT background (Fig. 8E). However, in the C437 ATag mutant line, the candidate gene C437_X70 was induced by about 4-fold and candidate gene C437_X80 by 2-fold (Fig. 8F).

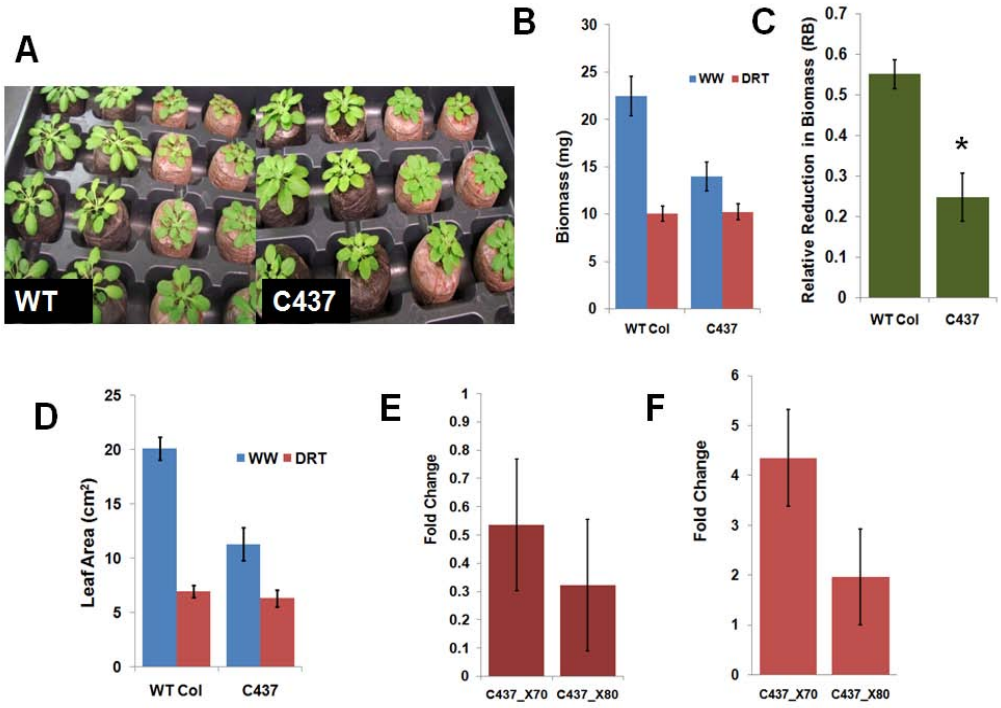

Figure 3.8. Response of C437 ATag mutant to drought stress. A, C437 ATag line shown at the end of drought treatment compared to the WT. B, Biomass in mg of WT and C437 ATag under mDr. C, Relative reduction in biomass (RB) in WT and C437ATag under mDr compared to well-watered control. D, Leaf area of WT and C437ATag under mDr compared to well-watered control. B-D, n=10, p-value <0.01. Leaf area, WT p-value <0.0001, and C437 ATag <0.05. E, qRT-PCR of C437_X70 and C437_80 genes in the WT under pDr. F, qRT-PCR of C437_X70 and C437_80 genes in C437 ATag under pDr. E and F, n=3 biological replications of 5 pooled plants/replication. * indicates significance. Bars represent ± SE.

Consistent with the drought phenotype of C437 ATag, expression profiling analysis of pDr and mDr (Harb et al., submitted) revealed the differential expression of many xyloglucan transferases in response to drought stress (Table 5).

Table 3.5. The expression of xyloglucan transferases under pDr and mDr drought.
mDr treatment with two stages: early stage mDr1 (day 1), and late stage mDr10 (day 10)

Gene	pDr LogRatio	mDr1 LogRatio	mDr10 LogRatio	TAIR Annotation
AT2G01850			-0.652	Endo-xyloglucan transferase A3 (*EXGT-A3*)
AT5G57550		-0.52943	-2.193	Endo-xyloglucan transferase (*XTR3*)
AT4G30290		0.358136		Endo-xyloglucan transferase (*ATXTH19*)
AT2G36870		0.612891		xyloglucan:xyloglucosyl transferase, putative
AT1G14720	0.743			Endo-xyloglucan transferase (*XTR2*)
AT5G13870	0.353			Endo-xyloglucan transferase (*EXGT-A4*)
AT4G37800	-3.01		-0.919	xyloglucan:xyloglucosyl transferase, putative
AT1G11545	-2.14		-0.679	xyloglucan:xyloglucosyl transferase, putative
AT1G32170	-0.532			Endo-xyloglucan transferase (*XTR4*)
AT5G65730	-4.641	-0.724	-0.779	xyloglucan:xyloglucosyl transferase, putative
AT2G06850	-3.452	-0.566	-1.018	Endo-xyloglucan transferase (*EXGT-A1*)
AT4G03210	-4.050	-0.564		Endo-xyloglucan transferase (*XTH9*)
AT3G23730	-2.119	-0.729	-1.450	xyloglucan:xyloglucosyl transferase, putative
AT4G14130	-0.458	-0.871		Endo-xyloglucan transferase (*XTR7*)
AT4G30270	-1.476	-0.420		Endo-xyloglucan transferase (*MERI5B*)

In addition to drought resistance phenotype, the C437 ATag line showed resistance to salt stress. Ten plants of WT and C437 ATag were tested under salt stress (150 mM NaCl) for 1 week. The reduction in biomass (RB) was less in C437 ATag compared to the WT (Fig. 9A and B).

In response to ABA, C437 ATag showed hypersensitivity (Fig. 9F) at seed germination stage compared to the WT (Fig. 9D).

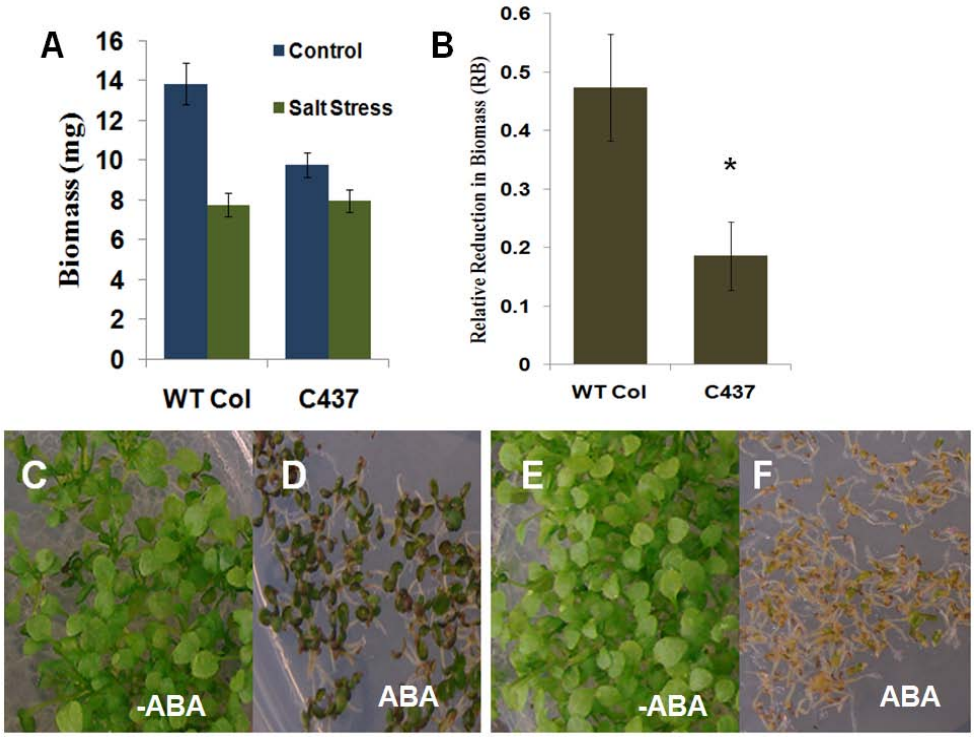

Figure 3.9. Response of C437 ATag to salt stress and ABA. A, Biomass in mg of WT and C437 ATag under salt stress. B, Relative reduction in biomass (RB) of WT and C437 ATag under salt stress. N= 20 WT, n= 10 C437 ATag, *indicates significance at <0.01. Bars represent ± SE. C and D, Germination test of WT response to ABA at seed germination stage. E and F, C437 ATag response to ABA stimulus at seed germination stage. C and E are control germination on MS media, D and F on ABA media, with F showing low germination compared to D.

To test the effect of loss of function of C437_X0 and C437_X80 on plant performance under drought, T-DNA insertion mutants in C437_X70 and C437_X80 genes were tested. The KO mutant of C437_X70 was morphologically similar to the WT (Fig. 10A), whereas the KO mutant of C437_X80 showed higher biomass compared to the WT (Fig. 10A, D, and E). The T-DNA insert in KO of C437_X70 is in exon 2 (~375 bp from ATG), and that of C437_X80 at 502 bp upstream of the ATG (Fig. 10B). Under mDr, C437_X70_KO was highly resistant compared to the WT (Fig. 10A and C-E), whereas the C437_X80 KO mutant showed similar response to drought as that of the wild type (Fig. 10A and C-E). This might be explained by the possibility that the KO of C437_X80 forms a functional protein as that of the WT (see explanation below).

The two mutants showed the same sensitivity to ABA as the WT at the seed germination stage (data not shown).

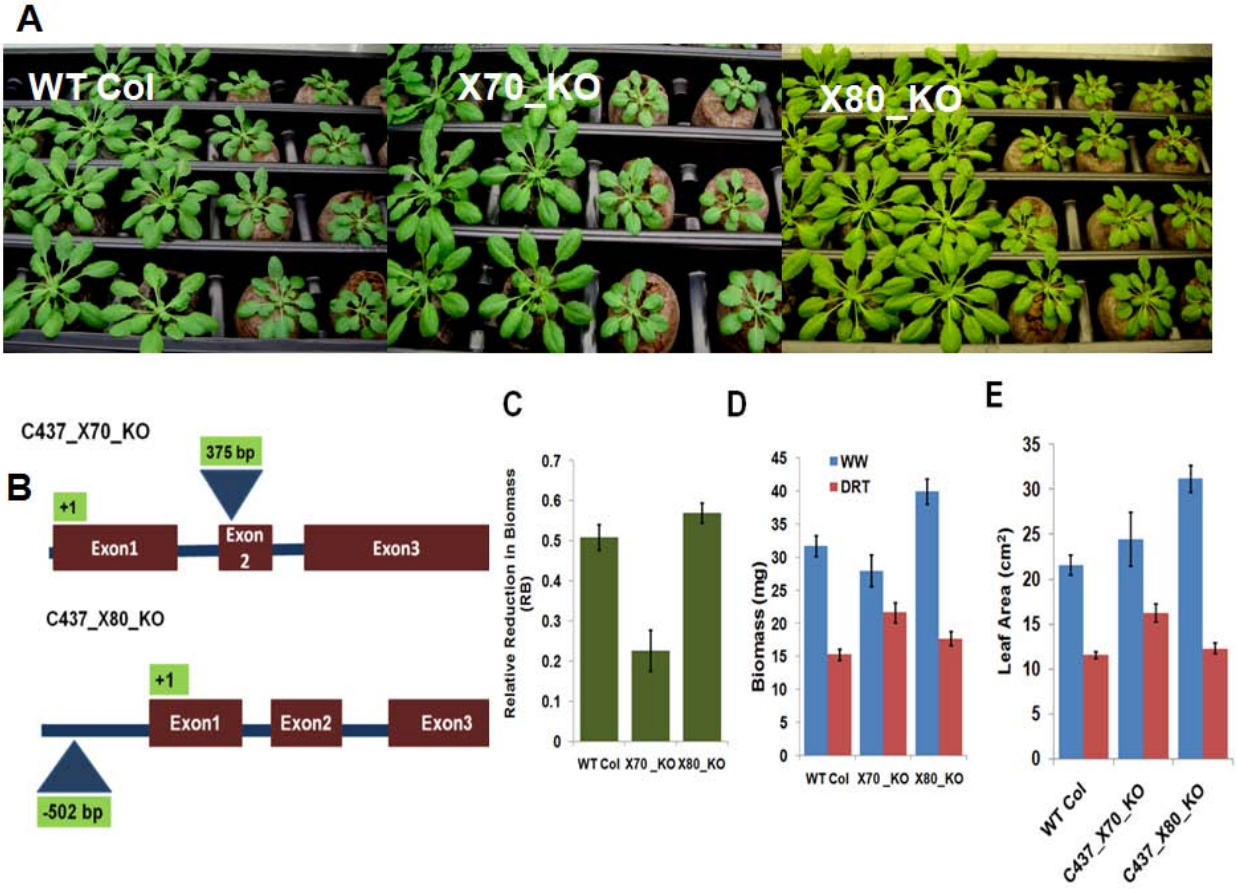

Figure 3.10. Characterization of KO mutants of C437_X70 and C437_X80. A, Morphology of the two mutants under well-watered and drought conditions compared to the WT. B, Illustration of T-DNA insertions in C437_X70 and C437_X80. C, Relative reduction in biomass (RB) under drought of the 2 KO mutants compared to the WT. D, Biomass in mg under drought and well-watered conditions of the 2 KO mutants compared to the WT. E, Leaf area under drought and well-watered conditions of the 2 KO mutants compared to the WT. C-E, Bars represent ± SE, N= 16. * indicates significant difference, p-value <0.01. The KO mutant of C437_X80 showed higher biomass than the WT, n= 16 and p-value= 0.002.

To confirm gene interruption in C473_X70 and C437_80 KO (T_DNA insertion) mutants, individual plants of both KO mutants were genotyped as described in methods. Homozygous plants of each KO mutant were further tested at the expression level. The KO

mutant of C437_X70 was confirmed both at DNA (genotyping) and RNA (expression) level (data not shown), whereas, homozygous plants of C437_X80 KO mutant showed the same expression of the C437_X80 gene as the WT (data not shown). The expression level of C437_X70 was lower in C437_X70 KO mutant compared to that in the WT. The T-DNA insert contains a 35S promoter followed by NOS terminator, about 1 kb from the left border (Fig. 11A). This 35S promoter (with enhancer) would probably be able to enhance promoters in the proximity, as well as initiate transcription from the 35S promoter towards the C437_X80 gene. To check the nature of transcripts in the KO of C437-X80, two T-DNA left border primers were tested in combination with C437_X80 specific primers. The analysis revealed a weak transcript that started in the T-DNA and continued through the gene. Examining the DNA sequence downstream of the 35S promoter, we found multiple start and stop codons in all reading frames (Fig. 11B) and (Fig. 12), suggesting that transcripts initiating from the 35S promoter or abnormal transcription starts within the T-DNA would not be translated. However, we cannot rule out that the 35S enhancer acts on the C437_X80 minimal promoter (502 bp sequence upstream of ATG) and activate the gene. Since we see multiple transcripts running in from the T-DNA into the gene, it is difficult to resolve the multiple transcription starts and the role truncated proteins might play.

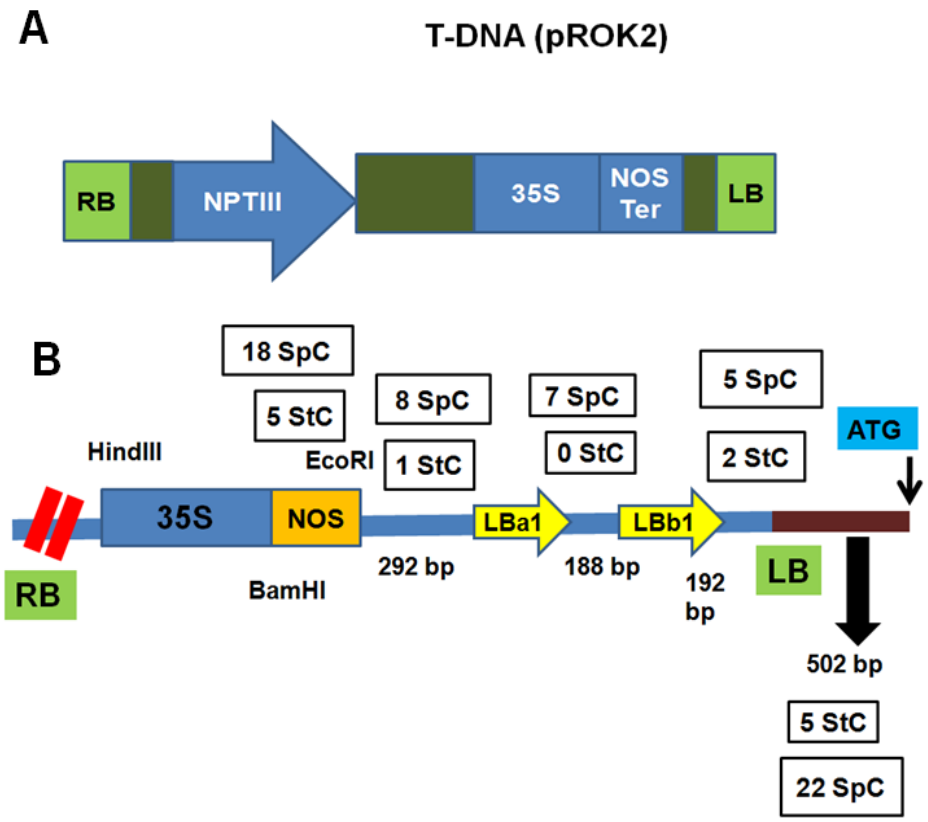

Figure 3.11. Analysis of the C437_X80 insertion mutant (SALK_025862C) genome structure. A, T-DNA in pROK2 vector that was used in T-DNA insertional mutagenesis (Baulcombe et al., 1986; Alonso et al., 2003). B, Illustration of part of T-DNA and the upstream region of C437_X80 showing the number of start codons (StC) and stop codons (SpC).

Figure 3.12. Analysis of the C437_X80 T-DNA insertion mutant (SALK_025862C) genome structure. Nucleotide sequence of the insertion site showing part of the 35S promoter to T-DNA left border (close to ATG of C437_X80) highlighting start codons (StC) (blue color), stop codons (SpC) (pink color). BamHI restriction site (RE) (red underline), EcoRI (RE) (green underline), left border (LB) (green color), 2 left border primers (LBa1, and LBb1) (yellow color), and ATG of C437_X80.

To test if highly significant drought resistance phenotype of the KO of C437_X70 was because of the overexpression (activation) of one of the surrounding genes (Fig. 13), which could be driven by the 35 enhancer in the T-DNA insertion in C437_X70; the expression of C437_X80

in the KO of C437_70 was tested, no activation of C437_X80 was detected. This might be explained by that C437_X80 is only induced under drought treatment (conditional induction or expression). Another possibility, is that C437_X60 (integral membrane Yip1 family protein) on the other side of C437-_X70 was activated; this was not tested, because the gene was not one of the candidate genes overexpressed in C437 ATag line, but we cannot eliminate the possibility of its action under drought stress. Since this gene is induced by osmotic and drought stress as shown by Arabidopsis eFP browser (Winter et al., 2007, http://bbc.botany.utoronto.ca/efp).

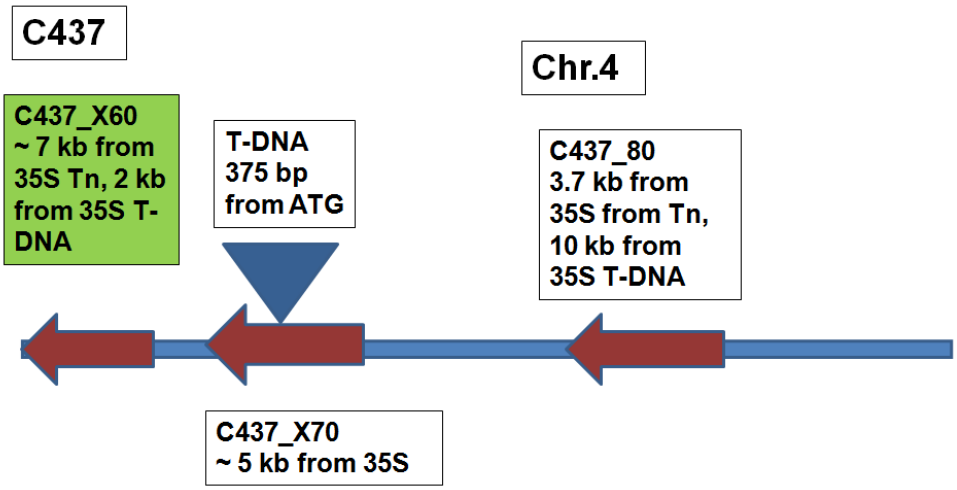

Figure 3.13. T-DNA insertion in C437_X70 (SALK_005941) showing the genes surrounding C437_X70, which could be activated by the action of 35S in the T-DNA insert. The expression of C437_X80 was tested, but C437_X60 was not (this gene was not one of the candidate genes chosen for C437 ATag, but cannot eliminate the possibility of its action). Tn: transposon.

To test which gene of the two candidate genes will recapitulate the morphological phenotype of the mutant, overexpression lines of C437_70 and C437_X80 under CaMV35S promoter were generated. The lines were checked for overexpression both at DNA and RNA expression level. Three overexpression lines were proven positive at both DNA and RNA levels for C437_X70 and 4 lines for C437_X80 (Fig. 14A and B). None of the generated overexpression lines for both genes recapitulated the morphological phenotype of C437 ATag (original activation tag mutant) (Fig. 13A and B). One of the possible explanations for this is the overexpression of both genes is needed for recapitulation, because both genes were highly overexpressed in the original ATag mutant. Another explanation is the possibility of microRNA activity in the genome region surrounding the transposon insertion. To test this we used 5 kb on

both sides of the 2 genes in Blast screens to plant microRNA database (http://bioinformatics.cau.edu.cn/PMRD/) applying a relaxed p-value of 10, but no match was found. Therefore, the first possibility is most probable. Yet, we cannot eliminate other factors playing role in this observation.

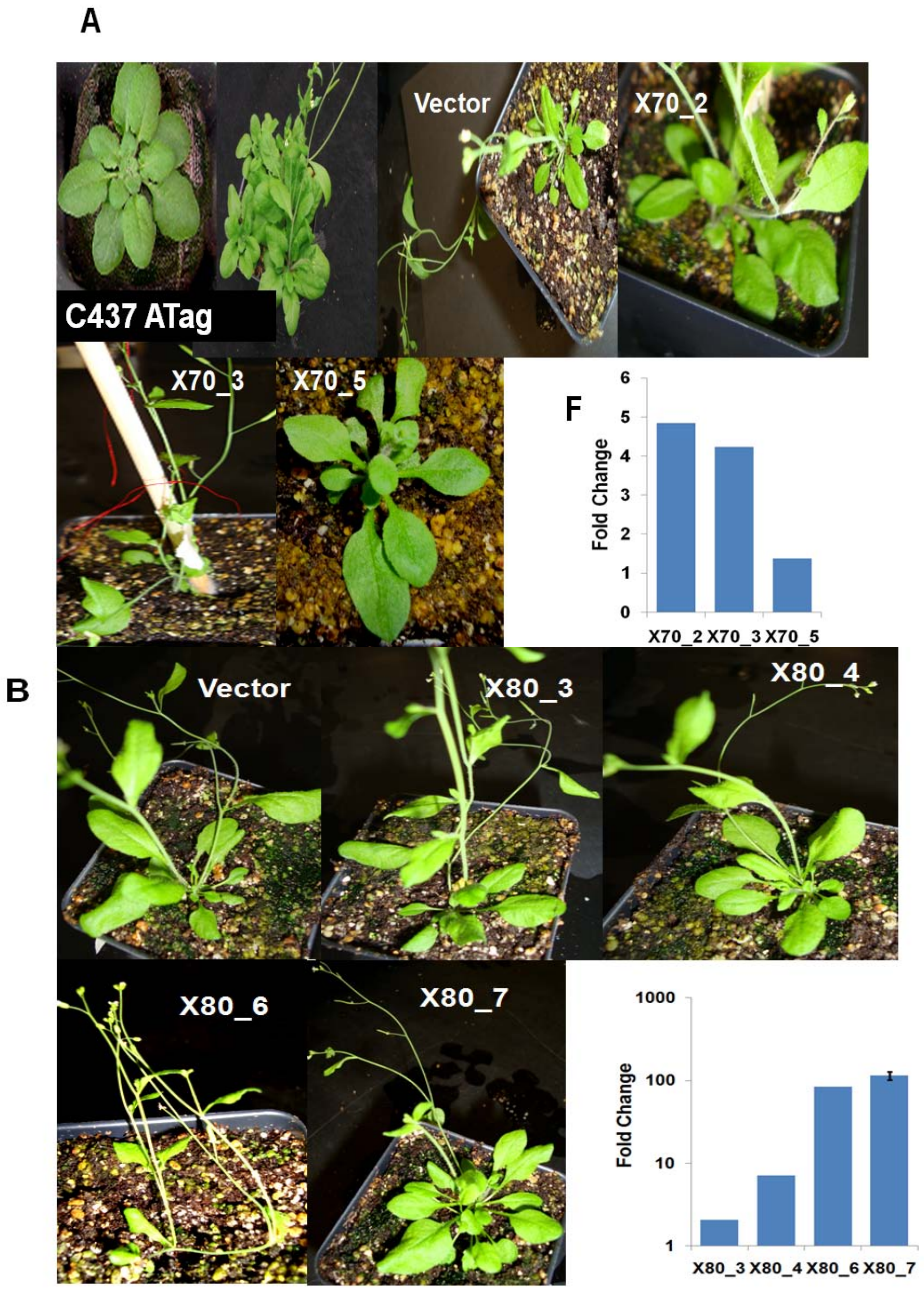

Figure 3.14. Overexpression of C437_X70 and C437_X80. A, Morphology and expression of C437 ATag (original activation tagging mutant), and overexpression lines of C437_X70 (X70_2, X70_3, and X70_5) compared to empty pBIN+ vector. B, Morphology and expression of overexpression lines of C437_X80 (X80_3, X80_4, X80_6, and X80_7) compared to empty pCambia1301 vector.

Characterization of C65 ATag Mutant

C65 ATag mutant showed a significant resistant response compared to the WT, and was chosen for detailed study. C65 is bushy, with light green color, early flowering (it flowers 1 week before the WT) (Fig. 15A). In addition, at flowering stage older leaves showed early senescence under normal growth conditions (Fig. 15B). The transposon insertion was in chromosome 4 around 4.5 kb upstream a MATE transporter gene and 1.5 kb upstream of a gene of unknown function (Fig. 15C). The 2 genes were designated as C65_X30, and C65_X20, respectively. In addition, a galactose transporter gene was found 8 kb from the 35S enhancer tetramer of the transposon insert (Fig. 15C), but was not included as a candidate gene, although we cannot eliminate this possibility. Quantification of the expression of C65_X20 (X20) and C65_X30 (X30) showed the overexpression of both genes, but C65_X30 had higher expression than C65_X20 (Fig. 15D). The expression level of C65_X30 was about 70 fold, whereas that of C65_X20 about 12 fold enhanced compared to the WT control under normal growth conditions.

For anatomical characterization of C65 ATag; sections in the root, hypocotyl, and leaf were stained with Safranin O for lignin, and counterstained with Fast Green for cellulose. C65 ATag showed higher abundance of lignin in the root and hypocotyl compared to the WT (Fig. 15E and F), and the same level in the leaf (Fig. 15G).

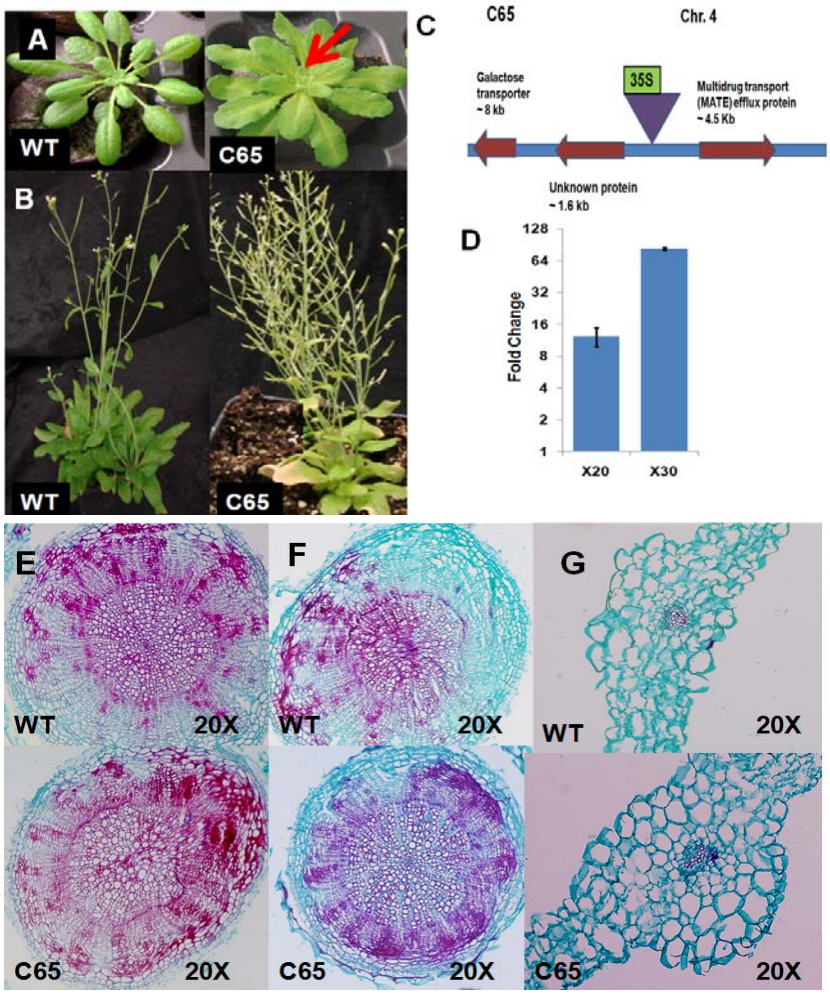

Figure 3.15. Morphological, anatomical and molecular characterization of C65ATag mutant. A, Morphological phenotype of C65 ATag mutant compared to wild type Col at vegetative stage (30 DAS) under normal growth conditions, C65 ATag shows early flowering (red arrow). B, Morphological phenotype of C65 ATag mutant compared to wild type Col at flowering stage (40 DAS) under normal growth conditions. C, Illustration of transposon insertion in C65 ATag mutant; the insertion in chromosome 4 around 4.5 kb upstream MATE transporter gene, and 1.5 kb upstream a gene of unknown function. D, Quantification of two candidate genes (X20 and X30) in C65 ATag by qRT-PCR. Bars represent ± SE, N= 3 biological replications. Histological analysis for anatomical characterization of C65ATag, Root, hypocotyl and leaf sections were stained with Safranin O that stains lignin, and counterstained with Fast Green that stains cellulose. E, Cross section of wild type root, and C65 ATag. F, Cross section of wild type hypocotyl, and C65 ATag. G, Cross section of wild type leaf, and C65 ATag.

The performance of C65 ATag under mDr in terms of reduction in biomass was similar to the WT (Fig. 16A and B).

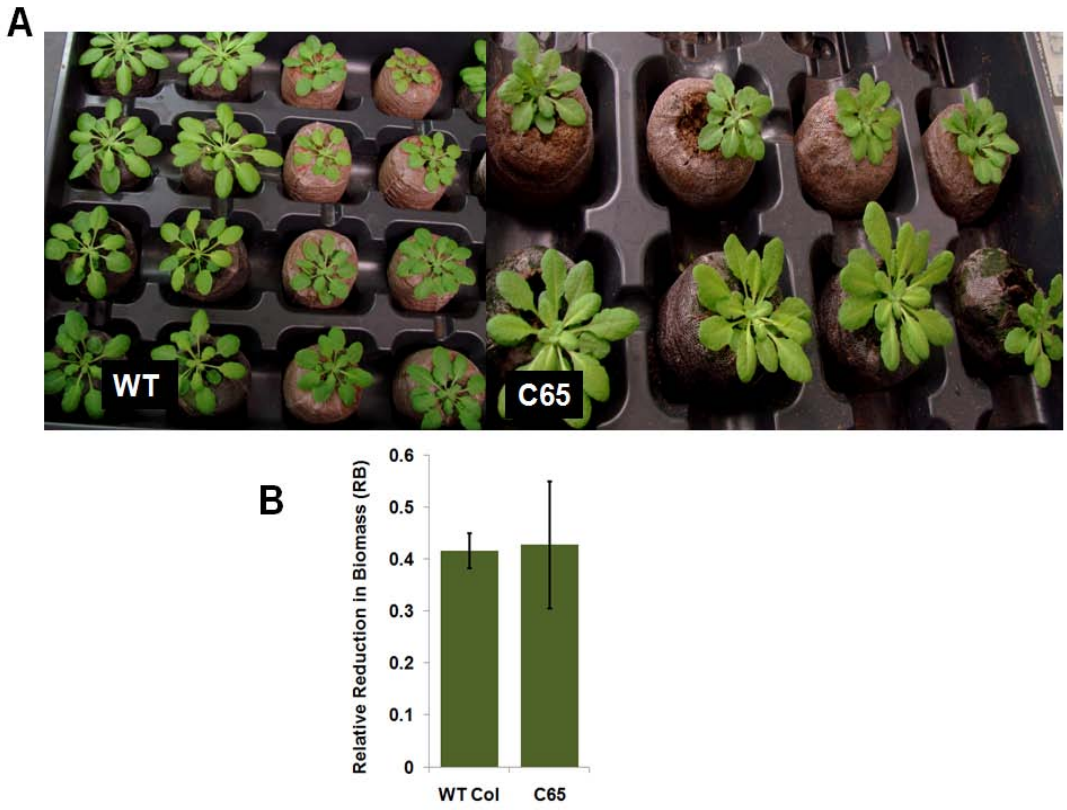

Figure 3.16. Response of C65 ATag to mDr. A, C65 ATag at the end of drought treatment compared to WT. B, Relative reduction in biomass (RB). N= 6. Bars represent ± SE.

KO mutants of C65_X20 and C65_X30 were ordered for testing under salt stress (Fig. 17A). As described by SALK and TAIR, the T-DNA insertion in C65_X20_KO should be in the exon, but checking the sequence viewer nucleotide view on TAIR, the insertion was in the 3 'UTR (5 bp downstream of stop codon) (Fig. 17B). The insertion of C65_X30_KO was found in the exon (Fig. 17C). The two mutants were genotyped; the genotype of all the tested individual plants of C65_X20_KO was same as the WT with no interruption of the gene. This might be explained by an error in the localization of the T-DNA insertion in this mutant, and thereby the primers that were used for genotyping were not the correct primers to use. The KO mutant of C65_X20 was disregarded and not used for further analysis. Most of the tested individual plants of C65_X30_KO were homozygous (data not shown). Moreover, they showed no gene

expression of C65_X30 gene (data not shown). Based on this characterization, only the KO of C65_X30 was used for further analysis.

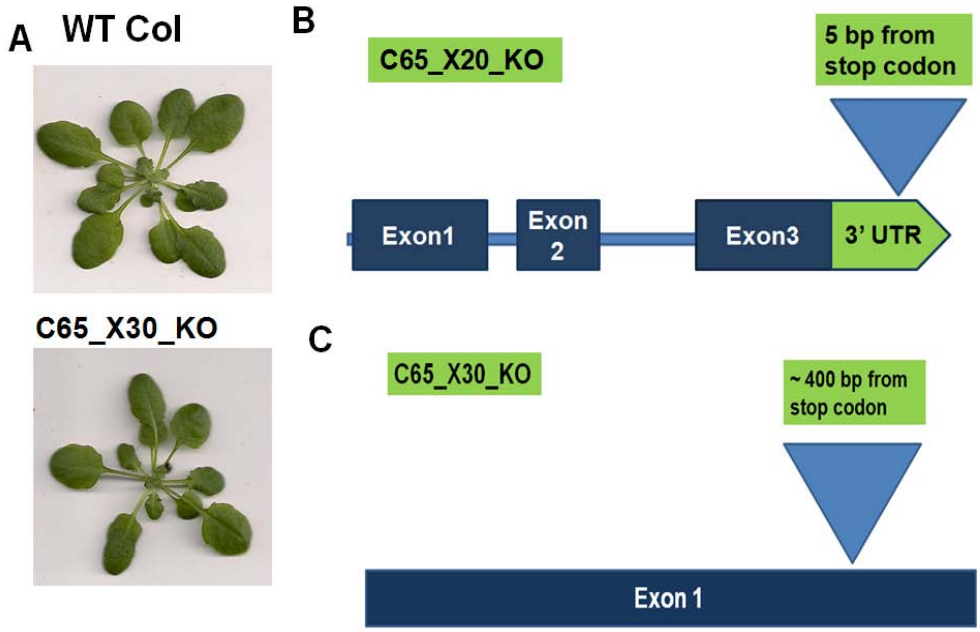

Figure 3.17. Characterization of KO mutants of C65_X20 (SALK_000823) and C65_X30 (SALK_127812C). A, Morphology of C65_X30_KO compared to the WT. B and C, Illustration of T-DNA insertion in C65_X20_KO and C65_X30_KO, respectively.

C65 ATag and C65_X30_KO were tested under salt stress. The C65 ATag was significantly resistant to salt stress at seedling and vegetative stages (Fig. 18AE). At seedling stage, the reduction of biomass was 20% in C65 ATag, whereas 50% in the WT (Fig.18A). At vegetative stage, C65 ATag showed less than 0.05 relative reduction in biomass (RB) compared to 0.25 RB in the WT (Fig. 18D and E). However, the reduction in biomass of C65_X30_KO under salt stress was not significantly different from the WT (Fig. 18F and G).

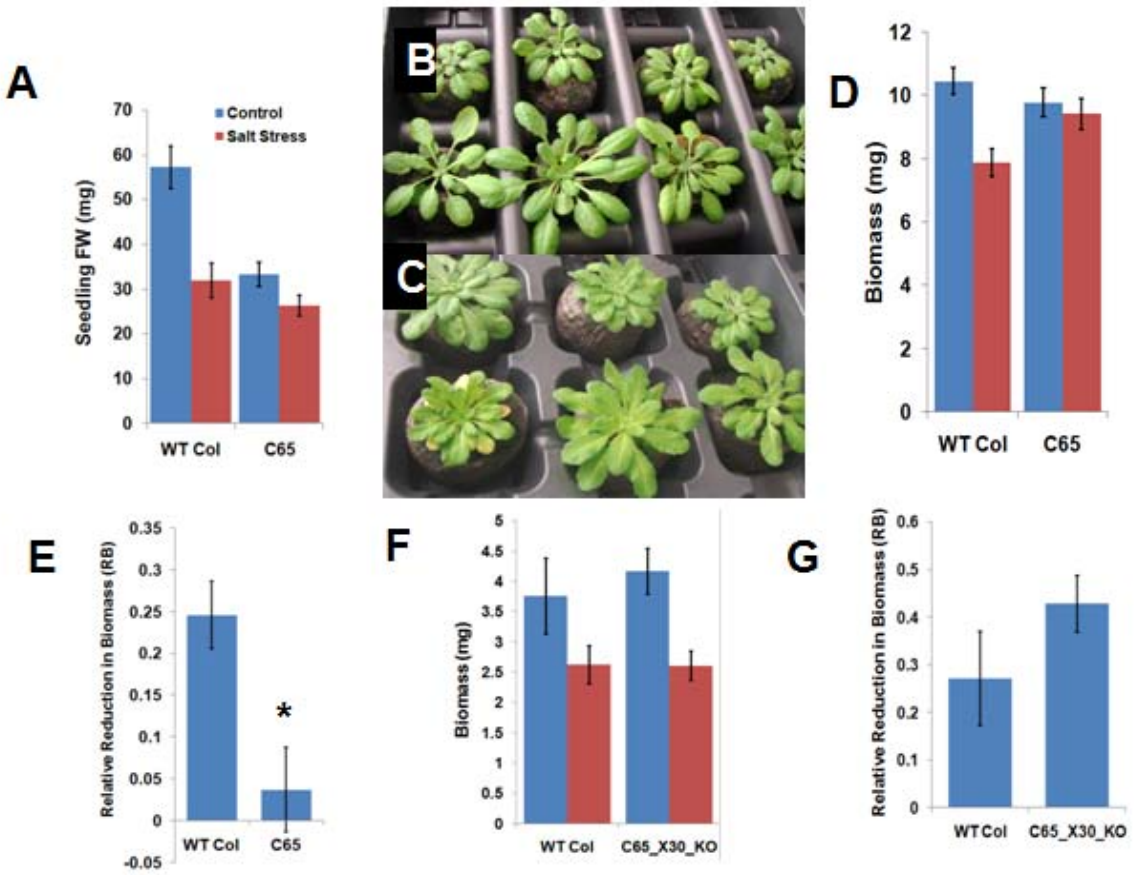

Figure 3.18. Response of C65 ATag and C65_X30 KO mutant to salt stress. A, Seedling fresh weight in mg of C65 ATag and WT under salt stress compared to untreated control. N=12, WT p-value <0.001. B, WT plants under salt stress (top) and control conditions (bottom). C, C65 ATag plants under salt stress (top) and untreated-control (bottom). D, Biomass in mg of WT and C65 ATag under salt stress and control at vegetative stage. C65 ATag n=8, WT n= 18, WT p-value < 0.001. E, Relative reduction in biomass (RB) of WT and C65 ATag at vegetative stage. C65 ATag N= 8, WT N= 18, * indicates significant difference at p value < 0.01. F, Biomass in mg of WT and C65_X30_KO under salt stress and control at vegetative stage. N=12, C65_KO_X30 p-value < 0.01. G, Relative reduction in biomass (RB) of WT and C65 ATag at vegetative stage. N=12. Bars represent ± SE.

For gas exchange measurements, 4 plants of C65 ATag and WT at 40 DAS were measured under normal growth conditions. The C65 ATag showed higher stomatal conductance (gs) and transpiration, but normal photosynthesis compared to the WT (Fig. 19 A and B).

Consistent with gas exchange results, carbon isotopic discrimination analysis revealed increased δ13C discrimination (lower ^{13}C to ^{12}C ratio) in the C65 ATag line under normal conditions (Fig. 19C). Under salt stress the mutant was more efficient in stomatal closure than the WT, as it showed reduced discrimination (higher ^{13}C to ^{12}C ratio) compared to the untreated control (Fig. 19C).

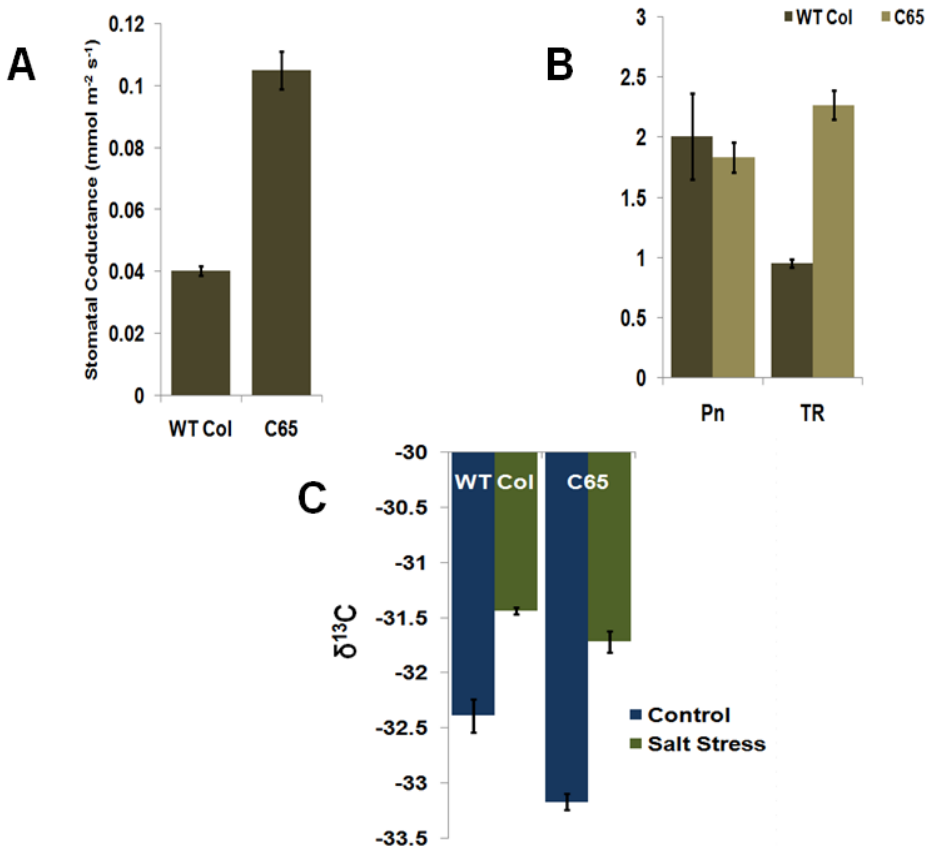

Figure 3.19. Gas exchange and carbon isotopic discrimination of C65 ATag and WT Col. A, Stomatal conductance (gs) of C65 ATag and WT under normal growth conditions. B, Photosynthesis (Pn) (µmole m^{-2} s^{-1}), and transpiration (mmol m^{-2} s^{-1}) of C65 ATag and WT under normal growth conditions. Gas exchange measurements 4 plants and 2 leaves/ plant were measured, $p<10^{-5}$ for gs and TR. C, ^{13}C to ^{12}C ratio ($\delta^{13}C$) of C65 ATag and WT under salt stress compared to control conditions. For carbon isotopic discrimination 4 replications and 3 pooled plants/replication were analyzed. Under normal conditions $\delta^{13}C$ is significant between C65 ATag and WT at p= 0.01. Under salt conditions p = 0.001 for C65 ATag, and 0.02 for WT.

The two candidate genes surrounding the transposon insert were overexpressed under the CaMV 35S promoter in the pBIN+ vector. Selection of >4000 seeds on kanamycin media resulted in a single overexpression line of C65_X20 (X20_1) and 2 lines of C65_X30 (X30_1 and X30_2). The overexpression lines were checked at DNA level using 35S primer and the reverse gene specific primer, but were found not positive (data not shown).

F. DISCUSSION

En-I Activation Tagging in Columbia Ecotype an Efficient Tool for the Dissection of Abiotic Stress

A population of activation tagging (ATag) mutants in ecotype Columbia was generated. The frequency of easily distinguishable morphological phenotype mutants observed was 15 from 800 ATag lines, or almost 2%, which is much higher than observed for T-DNA activation tagging and in the range of that observed previously for the *En-I* ATag system (Marsch-Martinez et al., 2002). The mutants with morphological mutant phenotypes, indicated presence of an activated gene, and were tested for drought and salt stress response phenotype: namely resistance/tolerance or sensitivity. The ATag mutants with drought and /or salt phenotype were further analyzed molecularly, and the candidate tagged genes in 6 ATag mutants were identified. The molecular analysis revealed a diverse group of tagged genes from the 6 mutants distributed across the Arabidopsis genome, on chromosomes 3, 4 and 5. Genes of different functional groups were identified: protein phosphatase type 2C (PP2C), aminotransferase, cytochrome C, Dof transcription factor (TF), RING-domain TF, homeobox domain (HB) TF, MATE transporter, and cell wall modification enzymes.

In this study the main focus was on two main findings: on the role of functional genes in cell wall modification and transporter genes in relation to abiotic stress.

Cross Talk between Cell Wall Modification and Abiotic Stress

An activation tag (ATag) mutant C437 with short petiole, and compact leaves was identified. The C437 ATag mutant showed drought and salt resistance phenotype, and hypersensitivity to ABA at seed germination stage. These are characteristic phenotypes of a drought and salt resistance gene and were studied further. Molecular analysis of the mutant revealed that the transposon insert is in chromosome 4, and the putative tagged genes are members of the xyloglucan endotransglucosylase/hydrolase/transferase (XTH) gene family. In this study, 3 genes surrounding the transposon insertion were identified in C437 ATag: C437_X70 about 5 kb from 35S enhancer in the transposon, C437_X80 about 3.7 kb, and C437_X90 about 6.5 kb. Expression analysis of the 3 genes showed that C437_X70 and C437_X80 were overexpresed, whereas C437_X90 was not. In previous study, C437_X70 and C437_X80 showed different spatial expression: C437_X70 showed high expression in the stem, whereas C437_X80 was specifically expressed in the root (Yokoyama and Nishitani, 2001). Genetic analysis using T-DNA insertion and RNAi loss-of-function mutants of the root specific XTH genes, showed a major role of the C437_X80 gene in primary root growth in comparison to other XTH members expressed in the root (Osato et al., 2006). This might explain the drought resistance phenotype of C437 ATag, with the high activity of C437_X80 in the root enhancing root growth and consequently water uptake under drought stress. The root phenotype was not tested in this study, as we analyzed only the rosette leaves, but this is a highly probable hypothesis that might explain the drought resistance phenotype of C437 ATag mutant. Moreover, transcriptome analysis of Arabidopsis root tissue under salt stress revealed the induction of C437_X80 by more than 4 fold (Jiang and Deyholos, 2006), and could explain the salt stress resistance phenotype of C437 ATag. Consistent with the drought and salt resistance phenotype of C437 ATag shown in this study, analysis of C437_X70 and C437_X80 expression by Arabidopsis eFP browser revealed both genes are induced by osmotic and drought stress (Winter et al., 2007, http://bbc.botany.utoronto.ca/efp).

Xyloglucans are a major component of the cell wall in dicotyledonous and monocotyledonous plants, and in all different tissues and organs at different developmental stages (Vissenberg et al., 2005). Chemically xyloglucan is a polymer of glucans and side chain of glucosyl molecules (Nishitani and Tominaga, 1992). Members of XTH gene family are required

to connect cellulose microfibrils in the cell wall, which makes the cell wall matrix, so they function in cell wall modification and reconstruction, which allow flexibility during plant growth (Nishitani and Tominaga, 1992; Nishitani, 1997; Van Sandt et al., 2007). In Arabidopsis, about 33 genes were identified in this family, with extensive gene duplications arranged in tandem as well as distributed across the genome (Yokoyama and Nishitani, 2001; Vissenberg et al., 2005). Members of XTH are differentially expressed in different tissues and organs (Yokoyama and Nishitani, 2001), and in response to plant hormones (Yokoyama and Nishitani, 2001). This suggests diverse roles of members of XTH gene family, which might indicate non-redundant gene functions. For example, 4 phylogenetically related genes: *XTH17*, *XTH18*, *XTH19*, and *XTH20* were found to be expressed in Arabidopsis root, but showed differential tissue expression pattern, which plays a critical role in the structuring of the cell wall in the root (Vissenbrerg et al., 2005). This suggests that the closely related members of the XTH family are not redundant, but have specific activities and expression patterns, and the additive activity of the genes (enzymes) is required for the cumulative biological effect.

The histological analysis of C437 ATag showed high accumulation of lignin in the root and leaves of the mutant. Lignin accumulation is a common plant response to different stresses (Moura et al., 2010; our drought expression data unpublished). In maize lignin content was found to be an indicative measure of drought tolerance (Hu et al., 2009). The drought and salt resistance phenotype of C437 ATag can be attributed to a higher accumulation of lignin in vascular tissues, which strengthen these tissues and improve their functionality under stress. Indeed, the overexpression of Cu/Zn-superoxide dismutase was shown to increase lignin accumulation in vascular tissues and enhance salt tolerance in Arabidopsis (Gill et al., 2010). Moreover, in poplar, in situ enzyme characterization revealed a role for xyloglucan transferases in the formation of secondary cell wall of vascular tissue (Bourquin et al., 2002).

The loss-of-function T-DNA insertion KO mutants of C437_X70 and C437_X80 were tested under mDr, with the C437_X70 KO showing highly significant resistance to drought stress, although the mutant showed a loss-of-function proven by insertion genotyping and RNA expression level. One possible explanation for the enhanced drought resistance of this mutant might be the overexpression of one of the surrounding genes (neighboring genes of C437_X70) driven by the adjacent 35S enhancer present in the T-DNA insertion. To test this, we studied the expression of the adjacent genes C437_X60 and C437_X80, in the C437_X70 mutant

background. This gene was one of the candidate genes identified close to the transposon insertion in the original C437 ATag, which was overexpressed in C437. The expression analysis showed that C437_X80 was not overexpressed in the KO mutant of C437_X70 under normal well-watered conditions. Another explanation, C437_X70 gene might negatively regulate some aspects of plant response under drought stress, so it is normally down-regulated under that stress. Indeed, in our drought expression data C437_X70 was repressed both under pDr and mDr treatments. The T-DNA insertion in C437_X80 is in the promoter region, and shows normal level as the wild type. This suggests that the mutant is not a true loss-of-function mutant, although we showed some bioinformatics analysis supporting that it might be a KO or knockdown mutant. Nevertheless, the mutant was tested under mDr, but showed similar response to drought as the wild type. However, since the C437_X80 insertion mutant shows higher biomass than the wild type, it suggests that it might be involved in activating other adjacent genes as a gain-of-function mutant.

In the overexpression analysis of C437_X70 and C437_X80, none of the 35S overexpression lines of the two genes recapitulated the morphological phenotype of the original C437 ATag mutant. This could be explained by the fact that both genes were overepxressed in C437 ATag, so in order to recapitulate the mutant phenotype both genes have to be overexpressed. Another possible explanation is the role of microRNA, since plant microRNAs were shown to have a major role in the regulation of leaf development, morphogenesis and other growth aspects, in addition to their role in response to different environmental stresses (Palatnik et al., 2003; Zhang et al., 2006; Rodriguez et al., 2010). Therefore, the presence of microRNA in the surrounding region of the transposon insertion and the genes was tested by using 5 kb region on both sides of the 2 genes in a Blast screen for miRNA, but no match was found.

Cell wall biochemical and biophysical characteristics play an important role in cell growth (Mathews et al., 1984; Neumann, 1995). Physical properties of the cell wall play a crucial role in the response of plants to water deficit (Bacon, 1999). Under mild drought Arabidopsis leaves compensate for low expansion rate by the extension of expansion duration (Aguirrezabal et al., 2006). Cell expansion is a process of cell wall modification and loosening catalyzed by enzymatic and non-enzymatic protein components of the cell wall (Cosgrove, 2005), which is composed of cellulose and hemicelluloses in a matrix of pectins and proteins (Cosgrove, 2005). Xyloglucan transferases (XTHs) in addition to other cell wall modification

proteins such as expansin genes play a crucial role in the construction and reconstruction of the cell wall during growth and development. We have shown the importance of cell wall expansion as an early response to mDr; at early stage of mDr the expression of many expansin genes was increased and then decreased at later stages in the process of acclimation to drought stress (Harb et al., submitted). Moreover, under pDr many cell wall modification genes were repressed (Bray, 2004, Harb et al., submitted). Our expression profiling analysis of both mDr and pDr revealed several members of XTH gene family were differentially expressed, both up- and down-regulated, some genes were common to both drought treatments, while others were specific to each drought treatment (Table 5). In response to water deficit, XTH genes were among the major players to enhance root growth in maize plants (Wu and Cosgrove, 2000). In the rice root, a xyloglucan transferase (EXT) gene was found to be a candidate cell wall modification gene for a QTL identified for increased root elongation under water stress (Zheng et al., 2008).

The cross talk between cell wall modifications and abiotic stress is poorly explored area, and still a lot to be learned about this important aspect of growth in response to different abiotic stresses at molecular, cellular, tissue, and whole-plant level. A forward genetics strategy such as applied here is therefore a good tool to identify and elucidate such novel functions.

A Possible Role for MATE Transporters in Response to Salt Stress

An activation tag mutant C65 was identified, which exhibits a rosette that looks like an ice crystal with pale green color, compact elongated leaves, and early flowering compared to WT. Under normal growth conditions, the mutant has higher stomatal conductance and transpiration, but photosynthesis is normal. The mutant is also salt resistant both at seedling and vegetative stage. Molecular analysis of C65 ATag revealed the transposon insert in chromosome 4, surrounded by two genes: C65_X20 annotated as a gene of unknown function about 1.6 kb from the ATag 35S enhancer, and C65_X30 a MATE transporter gene about 4.5 kb from the ATag 35S enhancer. Expression analysis of the two genes showed both are overepxressed, with the MATE transporter showing higher induction level than the unknown gene. We were not able to determine which gene is responsible for the phenotype of C65 ATag, since no overexpression line of the genes could be recovered after transformation.

Consistent with the salt resistance phenotype of C65 ATag shown here, analysis of C65_X30 expression by Arabidopsis eFP browser revealed the gene is highly induced by

osmotic and salt stress (Winter et al., 2007, http://bbc.botany.utoronto.ca/efp). Under salt stress the loss-of-function T-DNA insertion mutant of C65_X30 (MATE transporter) showed similar response as the wild type. In the C65_X20 T-DNA insertion line obtained from the SALK collection, no T-DNA insertion was found at the predicted position, and the line was not tested further.

In agreement with the gas exchange measurements, carbon isotopic discrimination analysis showed increased discrimination in the C65 ATag line - low ^{13}C to ^{12}C ratio - compared to the WT under normal growth conditions. Under salt stress the C65 mutant showed higher transpiration efficiency, with higher reduction in carbon isotope discrimination compared to the WT, and more efficient in stomatal closure in response to salt stress than the WT. A strong correlation between carbon isotopic discrimination ratio ($\delta^{13}C$) and transpiration efficiency (TE) in plants was found (Farquhar *et al*., 1989). TE is defined as biomass produced per unit of water transpired. In Arabidopsis, the *ERECTA* gene was found to encode a QTL that controls transpiration efficiency and carbon isotope discrimination (Masle et al., 2005). In addition, carbon isotope discrimination was used as a tool to study the differential drought response among different Arabidopsis ecotypes (Bouchabke et al., 2008). Quantitative genetics analysis was used to dissect five QTLs for carbon isotope discrimination in Arabidopsis (Juenger et al., 2005). Recently, a correlation between $\delta^{13}C$ and transpiration efficiency was shown in the analysis of ABA and drought response of GPA1 loss-of-function mutants (Nilson and Assmann, 2010).

Although we were not able to identify which gene is responsible for the salt resistance phenotype of the C65 ATag mutant, the MATE transporter gene is a very likely candidate. Multidrug and toxic compound extrusion (MATE) transporters are a group of transporters that are found in all kingdoms of life, functioning in the detoxification of exogenous and endogenous metabolites (Omote et al., 2006). Plant MATE transporters are not well studied, with 58 MATE transporters present in Arabidopsis. They are localized in the plasma and vacuolar membranes, several are functional in development, anthocyanin biosynthesis, flavonoid transport and pollen development (Diener et al., 2001; Marinova et al., 2007; Zhao and Dixon et al., 2009; Thompson et al., 2010). Moreover, in different plants many MATE transporters were found to enhance tolerance to different toxic chemicals and stresses: response to pathogens and UV stress in Arabidopsis, aluminum stress in sorghum and maize (Nawrath et al., 2002; Magalhaes et al.,

2007; Maron et al., 2010). Moreover, transcriptome analysis of Arabidopsis root tissue under salt stress revealed the induction of 13 MATE transporter genes by more than 2-fold, (Jiang and Deyholos, 2006), supporting a function in the salt stress resistance phenotype of C65 ATag. Checking the expression data of (Jiang and Deyholos, 2006), our MATE gene was not among the differentially expressed MATE genes under salt stress. In their expression analysis, they analyzed root tissue under salt stress, whereas our expression analysis was based on leaf tissue. So, it might be that our MATE has a role in response to salt stress specifically in the leaf tissue, differential spatial expression and functioning.

Salt stress increases reactive oxygen species (ROS), and in response to increase in ROS plants induce many enzymatic and non-enzymatic scavenging proteins (Miller et al., 2010). Recently, the overexpression of Cu/Zn-superoxide dismutase was shown to increase lignin accumulation in vascular tissues and enhance salt tolerance in Arabidopsis (Gill et al., 2010). Histological analysis of the C65 ATag showed high lignin accumulation in the root and hypocotyl, which could similarly explain the salt resistance phenotype in the mutant. We cannot determine which gene is responsible for the high accumulation of lignin C65 ATag, as there were no previous studies that show the involvement of MATE transporters in lignin biosynthesis in plants. But, we cannot exclude the possibility that this could be a new function for the C65_X30 (MATE) gene, which was highly overexpressed in C65 ATag. On the other hand, it could be the action of the unknown gene C65_X20, which was also overexpressed, although at a lower level of expression compared to C65_X30 in the C65 ATag.

In this forward genetics strategy to identify novel genes that function in abiotic stress, we suggest the significance and interaction of cell wall modification and transporter proteins to plant abiotic stress response and resistance. These reveal new aspects of plants interaction with the surrounding environment, which need more exploration and dissection.

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5. SUMMARY

Abiotic stresses are a serious challenge to plant growth and development. Plants are continuously encountering one or more stresses, so they have to be equipped with mechanisms to cope with these challenges. Under drought stress, plants exhibit either escape or resistance response mechanisms. Plants respond to stresses by the perception of stress signals, amplification of the stress signal via complex and interconnected signaling pathways, and subsequently the regulation of downstream regulatory and functional target genes. Different abiotic stresses affect osmotic and ion homeostasis in plants, which are the signals sensed by osmosensors. The signal transduction cascades then lead to changes in the expression of regulatory genes. Regulatory genes (transcriptional factors) control the expression of functional genes. Activation of functional genes leads to the accumulation of osmoprotectants, repair and detoxifying chemicals, which help in maintaining cellular homeostasis, and consequently lead to stress tolerance or resistance.

In this study, a physiological, biochemical, and molecular dissection of Arabidopsis plant responses to controlled prolonged moderate drought (mDr) revealed stage-wise responses, which can be divided into: early perception signaling stage, preparatory intermediate stage, and late stage of acclimation with reduced growth. Relative water content (RWC%), and gas exchange measurements showed lower values at early stage of mDr in drought-treated plants compared to the well-watered control. In addition, at this stage plants accumulate high concentration of ABA. Consistent with the physiological and biochemical analysis, expression profiling and quantitative PCR (qRT-PCR) revealed extensive reprogramming of gene expression at the early stage of mDr. Moreover, transcriptome comparison between two stages of mDr showed molecular mechanisms for acclimation under prolonged controlled drought treatment. At late stage most of the common stress responsive genes are repressed compared to early stage of mDr. In addition, jasmonic acid and signaling is repressed at late stage of mDr, which can be a mechanism for acclimation by the change of hormonal balance under prolonged drought treatment. Transcriptome analysis of plant response under two different drought treatments: moderate (mDr) and progressive drought (pDr) showed common and differential responses. In mDr and pDr, common genes are induced that are responsive to: water deprivation, ABA stimulus, osmotic stress, cold stress, and oxidative stress. The differential response is demonstrated

quantitatively by the difference in the number of differentially expressed (DE) genes, higher numbers of genes are differentially expressed (up- and down-regulated) under pDr compared to mDr. Moreover, qualitative difference between the two drought treatments has been shown by Gene Ontology (GO) enrichment analysis. At early stage of mDr, many genes involved in cell wall modification are induced, whereas under pDr they are repressed.

A reverse genetic approach revealed the role of an R2R3 MYB transcription factor (*MYB109*) in plant growth under drought and salt stress. The knockout (KO) mutant of *MYB109* displayed higher sensitivity to drought and salt stress, measured in terms of reduction in biomass. In addition, the KO mutant was insensitive to ABA assessed by: stomatal conductance, cut rosette water loss (CRWL), and stomatal aperture in response to exogenous ABA. Quantitative PCR (qRT-PCR) revealed constitutive repression of *MYB60* in the KO mutant. *MYB60* is a stomatal gene, which is normally repressed in response to ABA and drought treatment resulting in stomatal closure and consequently the protection of plants against stress. The repressed function of *MYB60* is one possible explanation for the high sensitively to drought and salt stress, and the insensitivity to ABA in the *MYB109* KO mutant. Moreover, transcriptome analysis under pDr showed additional possible mechanisms of the function of *MYB109* under drought stress: a) as a regulator of RNA processing and splicing, and b) a negative regulator of jasmonic acid biosynthesis and signaling.

The utility of forward genetics as a strategy for gene discovery in response to abiotic stress was explored. A number of drought resistant and sensitive transposon activation tag (ATag) mutants were identified. Detailed characterization of two of these ATag mutants uncovers the role for new unexplored aspects of plant growth and development in response to abiotic stress. One ATag mutant (C437) is compact, having short petioles, with high lignin accumulation in the leaf and root, and resistant to drought and salt stress. The tagged genes in this mutant are from the xyloglucan endotransglucosylase/transferase gene family (XTH), members of which play a crucial role in cell wall loosening and modification during growth. The research on cell wall modification in response to abiotic stress is in its infancy, and a lot need to be discovered about the cross talk between cell biochemistry and modification, and abiotic stress. Another ATag mutant (C65) is bushy, with pale green leaves, early flowering, has high lignin in the hypocotyl and the root, and salt resistant. One of the putative tagged genes in this mutant is a MATE transporter gene. Expression profiling analysis of salt-treated Arabidopsis

roots revealed a number of induced MATE transporters, suggesting a potential role for MATE transporters in Arabidopsis salt stress response and tolerance, which needs further investigations.

Despite the plethora of studies and information on plants response to abiotic stress, there is still a lot unexplored, and a careful integrative analysis needs to be done for efficient improvement of crop plants under environmental stress conditions such as drought, which is expected to exacerbate in the future.

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