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Analysis of Drought Stress in Arabidopsis

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

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ABSTRACT

Plant drought stress response and resistance are complex biological processes that need to be analyzed at a systems-level using genomics and physiological approaches to dissect experimental models that address drought stresses encountered by crops in the field. Towards this goal a controlled, sub-lethal, moderate drought (mDr) treatment system was developed in Arabidopsis (*Arabidopsis thaliana*) as a reproducible assay for the dissection of plant responses to drought. The drought assay was validated using Arabidopsis mutants in ABA biosynthesis and signaling displaying drought sensitivity, and jasmonate response mutants showing drought resistance, indicating the crucial role of ABA and jasmonate signaling in drought response and acclimation. A comparative transcriptome analysis of soil water deficit drought stress treatments revealed the similarity of early stage mDr to progressive drought, identifying common and specific stress responsive genes and their promoter cis-regulatory elements. 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. This is accompanied by a peak in expression of expansin genes involved in cell wall expansion, as a preparatory step toward drought acclimation by the adjustment of the cell wall. 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.
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 the period of plant growth. The effects of drought are expected to increase with 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 (Rosegrant and Cline, 2003). Thus, an understanding of drought stress and water use in relation to plant growth is of importance for sustainable agriculture.

Plants being sessile have evolved specific acclimation and adaptation mechanisms to respond and survive short- and long-term drought stresses. Analysis of these protective mechanisms will contribute to our knowledge of tolerance and resistance to stress. The complex responses to environmental stress, from perception, to transcriptional and physiological changes need to be considered at a global systems biology level to study the multiple interactive components in this biological process (Krishnan and Pereira, 2008).

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 severe stress sets in. Drought avoidance is 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, among other mechanisms, 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). The common stress signaling pathways have been distinguished into ABA-dependent and ABA-independent (Shinozaki and Yamaguchi-Shinozaki, 1997, 2007). Most of the key genes in these pathways have been identified such as transcription factors belonging to the class of DREB/CFB, ABF, MYC and MYB (Abe et al., 1997; Bartels and Sunkar, 2005; Sakuma et al., 2006), including the identification of stress responsive cis-elements ABRE, and DRE (Yamaguchi-Shinozaki and Shinozaki, 1994; Ramanjulu and Bartels, 2002; Bartels and Sunkar, 2005). Downstream of the early signal perception events, signaling genes and molecules acting as secondary messengers have been identified, revealing the role of Ca\(^{2+}\) 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 responses at the molecular level is based on plant responses to molecular laboratory experimental conditions of dehydration and/or osmotic treatments (Yamaguchi-Shinozaki and Shinozaki, 1994; Abe et al., 1997; Oono et al., 2003; Umezawa et al., 2004). Although these conditions are far from soil water deficit/drought met by plants under field conditions, there has been valuable knowledge gained from such studies. Dehydration studies revealed the common stress signaling pathways of 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 discovered in Arabidopsis as a model system, which paved the way to the discovery 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 treatments 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 gene expression changes, of wild-type or of plant genotypes overexpressing 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 into 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. In nature drought often develops during a growing season and 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 causing drought stress in crop plants has been recently tested in Arabidopsis using controlled soil moisture treatment that is not lethal. Controlled drought treatment exposing plants to constant levels of soil moisture deficit, enables the evaluation between genotypes/ecotypes for plant responses to sub-lethal drought (Granier et al., 2006; Bouchabke et al., 2008). PHENOPSIS has been developed as an automated controlled drought screen, which was used to compare the performance of different Arabidopsis ecotypes (accessions), and resulted in the identification of a resistant accession An1 (Granier et al., 2006). Controlled drought was also used to study the response of the Arabidopsis *erecta* mutant and ERECTA gene complementation (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 done on the response of plants to moderate drought (mDr). A transcriptome study in Loblolly pine, treated for cycles of mild drought and recovery (Watkinson et al., 2003; Vasquez-Robinet et al., 2010), showed a photosynthetic acclimation pattern in response to mild drought 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 employed to compare with pDr treatment for physiological and molecular responses. This revealed differential gene 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.
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 batches 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 6-leaf (1.06), 8-leaf (1.08), and 10-leaf (1.10) stages when watering was withheld, 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 for the 3 batches 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 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 duration of mDr treatment was tested, and showed that 5 or 10 days of moderate drought (DMD) 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 DMD. The mDr stress (+1) is defined when the plants reach 2 g. g\(^{-1}\) H\(_2\)O/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. 2 C and D). Leaf relative water content (LRWC) measurements showed that plants start 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 and 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).

**Drought Responses of Hormonal Pathway Mutants**

To validate the drought screening on genotypes known to be affected by drought, the response of ABA signaling and biosynthesis knockout mutants in two Arabidopsis backgrounds (Col, and Ler) 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 \((aba1)\), show higher sensitivity to drought stress compared to their respective wild type controls (Fig. 3A and B).

In other experiments additional hormone response mutants were tested. The jasmonate response mutants \(coi1\) and \(jin1\) display significant drought resistance in the screen at 10 days of mDr (Fig. 3C and D). The other jasmonate response mutant \(jar1\), shows a drought response phenotype not significantly different from WT. The response of this mutant to jasmonate is also moderate compared to the \(coi1\) and \(jin1\) mutants, probably because this \(jar1\) allele is not a complete gene knockout but an amino acid substitution mutant (Staswick et al., 2002).

**Gas Exchange Parameter Changes in Response to mDr**

Stomatal conductance showed a decrease at 1 day of mDr (1 DMD), reaching 59% of the well-watered control (Fig. 4A). It reduced by almost 50% from that at day -1 and 0 day of mDr (Fig. 4A), and continued to decrease till day 2 of mDr, with about 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)

**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 (Day01 and Day10) and pDr conditions with corresponding controls in samples from young leaves. Analysis of differential expression showed that a large number of genes (2039) are significantly perturbed very early (Day01) in response to mDr. However, after a prolonged spell of moderate stress (Day10), a far lesser number of genes (728) show any response. Compared to both these responses, the backdrop of severe effects of drought on gene expression is revealed by the response to pDr (wilting): 7648 differentially expressed (DE) genes – ~30% of the genome – replete with well-known stress response genes and processes.

Comparison of the three responses (mDr Day01, mDr Day10 and pDr) was carried out first at the gene-level (Fig. 5 and Supplemental Table S1). The mDr and pDr treatments share a set of 178 differentially expressed genes (91 up- and 87 down-regulated), while 1083 (545 up- and 538-down regulated) genes are specific to mDr. All the drought response genes from each of these treatments were functionally characterized using enrichment analysis of genesets, mostly as described by Gene Ontology (GO; Ashburner et al., 2000) biological process terms, but also including genesets concerning ABA-response obtained from previous publications (Nemhauser et al., 2006). Results from these analyses are summarized in Figure 6 and details provided in Supplemental Table S2. Among the genes up-regulated in both mDr Day01 and pDr (646 genes) are predominantly water deprivation response genes ($q$-value $\sim$1E-15), with overlapping sets of genes known to respond to ABA stimulus ($q$ $\sim$1E-12.6), osmotic ($q$ $\sim$1E-8.1), cold ($q$ $\sim$1E-4.1) and oxidative ($q$ $\sim$1E-2.5) stresses. Expression dynamics of several of these genes has been verified using qRT-PCR (*see below*). Fundamental processes of the cell known to be grossly affected by drought including DNA packaging ($q$ $\sim$1E2.6), ribosome biogenesis ($q$ $\sim$1E-2.9) and protein folding ($q$ $\sim$1E-3.2) were concomitantly down-regulated in mDr Day01 and pDr. The plants are, thus, mounting an early response to mDr that is very similar to the classical response to progressive soil water deficit (pDr). However, severe effects including down-regulation of photosynthesis ($q$ $\sim$1E-20.7) and related processes are restricted to pDr.
The distinctive reaction of the plant to mDr Day01 is the activation of plant cell wall modification genes that underlie cell growth \((q \approx 1E-4.4)\), which is, in fact, down-regulated by pDr. This aspect of mDr response was pursued experimentally. On the other hand, expression of most of the typical ‘response to water deprivation’ genes, found to be up-regulated by mDr Day01 and pDr, were either similar to control, while some were even down-regulated \((q \approx 1E-2.8)\) as the plants grew under sustained mDr (mDr Day10). This ‘quenched’ response is most probably due to acclimation of the plant to the continued stress. Among the genes up-regulated only at this stage are a few hormone- (ABA) mediated signaling genes \((q \approx 1E-2.1)\), possibly mediating acclimation. Glucosinolate \((q \approx 1E-4)\), IAA-derivative \((q \approx 1E-2.9)\), JA \((q \approx 1E-2.8)\) and very-long-chain fatty acid \((q \approx 1E-2)\) metabolism genes are among those solely down-regulated in mDr Day10. Some genes involved in cell wall thickening \((q \approx 1E-2.9)\) and few others involved in regulation of cell growth \((q \approx 1E-2.8)\) are also down-regulated in mDr Day10-only and mDr Day10-and-pDr, respectively. This supports the idea that cell growth, in general, is hampered by prolonged water-deficit.

Cis-regulation 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 CREs potentially mediating the transcriptional regulation of drought response genes identified here, we devised a CRE-discovery pipeline: discover novel elements using a de-novo motif discovery tool FIRE (Elemento et al., 2007), compare the found elements to known cis-elements in prominent databases (Higo et al., 1999; Davuluri et al., 2003; Galuschka et al., 2007; Mahony and Benos, 2007) and draw out sequence logos of CREs of interest (Crooks et al., 2004). Applying this pipeline to mDr Day01, mDr Day10 and pDr (further separated into up- and down-regulated) genesets, led to the identification of several known and novel CREs (Supplemental Table S3). The CREs discovered to be associated with mDr and pDr are represented in Figure 7. A consolidated view of the enrichment analyses showing cis-elements related to specific stresses and genesets is provided in Figure 6. Motifs discussed below are referred to by the name of the most similar ‘known’ CRE (see the ‘PLACE motifs’ table in Figure 7 for the key).

The CRE standing out among the genes up-regulated in mDr Day01 and pDr was one highly similar to the experimentally identified ACGT-containing ABRE-motif ACGTG(G/T)C (Fig. 5;
At position 6, the (G/T) degeneracy is exactly preserved in the pDr-ABRE, while it is strictly T in the mDr Day01-ABRE, thus, making it more similar to the GADOWN motif. Interestingly, an element very similar to the ABRE – A(A/C)(A/C)RCGTG – was found among genes down-regulated in mDr Day10, which is more similar to the functionally equivalent coupling element 3 (Hobo et al., 1999). 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 Day01 and pDr, but down-regulated in 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 Day01 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 in early mDr, comprising slowing down of protein folding in the ER that triggers the unfolded protein response (UPR; Martínez and Chrispeels, 2003), is vindicated by the identification of the UPRE-like element among genes up-regulated in mDr Day01. In contrast, an UPRE-like element was identified among genes down-regulated by pDr. Here, it is important to note that ‘protein folding’ was enriched among the down-regulated genes in mDr Day01 and pDr, supporting the fact that, in both treatments, protein folding is affected. As for the association of the UPRE-element, upset protein folding causes UPR involving the up-regulation of genes that help in remedying the situation, an early signal captured by the UPRE-element in mDr Day01 genes, while severe drought stress (pDr) pushes the system beyond repair, causing the down-regulation of the whole protein machinery including. Moreover, several genes involved in cell elongation and division are down-regulated by UPR (Martínez and Chrispeels, 2003), genes that are down-regulated in pDr, but up-regulated in mDr Day01.

Two elements, both AT-rich, were discovered among the up-regulated genes in mDr Day01 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). It is unclear how photo-response and circadian cycling play a role in drought response. However, in the promoters of down-regulated genes in
mDr Day10, the Evening Element was found to be significantly colocalizing 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 down-regulated in mDr Day10 with as yet unknown function. Overall, the CREs discovered de-novo corroborate our understanding about the various facets of drought response concerning the mDr and pDr treatments, and point way to several associations of processes and pathways perturbed in drought to their transcriptional regulation.

**Cellular Metabolism under Drought Stress**

Global gene expression analysis showed a substantial down-regulation of many photosynthetic genes under pDr-wilting drought compared to a subtle change under mDr (Supplemental Table S1). 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 log2 ratios of 1.5 and 3, respectively. Under mDr only β-amylase was induced with log2 ratio of 0.4. 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 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).

Gas exchange measurements in the time course 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 day 3 and 4 of mDr. We found no significant change in proline in drought treated
plants compared to well-watered control (data not shown). Moreover, GC-MS metabolomic analysis also showed no significant changes in proline concentration over the time course of mDr (Joel Shuman, personal communication).

Because ABA is the most important stress hormone, 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, the concentration continued to increase until day 1 of mDr (Fig. 8A), 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 by two different ways: pDr pre-wilting (1 day before wilting) drought and controlled mDr Day01 treatments, on the response of the signaling pathways at a molecular level, the expression levels of key genes in the signaling pathways were quantified. ABA biosynthesis gene NCED3 was induced 4-fold more under pDr pre-wilting (PPW) compared to mDr (Fig. 8B). The drought responsive transcription factor DREB2A was induced at a higher level under pDr compared to 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 change under mDr Day01 (Fig. 8B). The drought responsive expression levels of the rest of the tested stress signaling genes were not significantly different between the two drought treatments (Fig. 8B).

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, an important enzyme in ABA biosynthesis, was highly induced at day 0 and 1 of mDr compared to day -1, 2, and 3 (Fig. 8C). The expression of ABF3 (ABA-dependent pathway) was high at 0 day of mDr, and after that, its expression started to decrease. In the ABA-independent pathway, DREB2A showed induction at day 0 and stayed induced at day 1 of mDr. After day 1, its expression was decreased (Fig. 8D).

Downstream of NCED3, ABF3, and DREB2A, there are many responsive genes that are recognized as stress marker genes: RD22, RD29A, RD29B, and RAB18. The expression profile
of these marker genes in a time course of mDr (Fig. 8E) showed induction at day 0 of mDr that continued at day 1, and then decreased in expression.

**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 PLDα1 and GPA1, two positive regulators of ABA signaling in the stomata, the expression under mDr started increasing from day -1 to peak at day 1, and then decreased thereafter (Fig. 9A). 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, which reduces from day 2 onwards (Fig. 9A).

Another group of genes with a major role in stomatal response and ABA signaling belong to the family of protein phosphatases type C (PP2Cs). We therefore tested the expression profile of three main PP2Cs: ABI1, ABI2, and HAB1. The expression of ABI1 and ABI2 started to increase at day -1 of mDr, continued to day 1, and then decreased (Fig. 9B). HAB1 showed a decrease in expression at day -1, followed by an induction at day1 and a decrease thereafter (Fig. 9B). 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 at day -1, which continued to day 1, followed by a decrease (Fig. 9C).

In our drought microarrays we found MYB60 was repressed at day 1 of mDr, and under pDr. This gene has been found to be specifically expressed in guard cells, and its null mutant reduces stomatal opening (Cominelli et al., 2005). We therefore tested the response during the time course of mDr, quantifying its expression at 5 time points. MYB60 was induced at day -1 and 0 of mDr (Fig. 9D), and repressed at day 1, which stabilized from day 2 onwards to that of the well-watered control (Fig. 9D).

**Expression of Photosynthesis and Antioxidant Genes under Drought Stress**

The comparison of mDr and pDr microarrays revealed that many photosynthesis genes were significantly repressed under wilting, in contrast to subtle effect of one day under mDr
(Supplemental Table S1). We selected a few photosynthesis genes, whose proteins are part of PSI (PQL2, PSAH2) and PSII (PSBW, PSBQA) to profile their expression under the mDr time course experiments (Fig 10A). 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 PQL2 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. 10A).

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). The expression of APX1 and TPX1 was slightly changed over the time course period (Fig. 10B). 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. 10B).

Expression of Cell Expansion Genes under Drought

The induction of cell expansion genes was specific to mDr, and the same genes were down-regulated or not differentially expressed in pDr. GO enrichment analysis of mDr DE genes showed the enrichment of cell expansion related genes. In addition, the comparison of moderate and pDr microarray data with ABA responsive genes, showed that cell expansion was upregulated in mDr compared to pDr wilting drought and ABA treatments. We therefore quantified the effect of pDr pre-wilting (PPW) and mDr (MD) treatments on the expression level of the expansin genes: EXPA3, EXPA4, EXPA8, EXPA10, and EXBL1 (Fig. 11A). 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. 11A).

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. 11B). EXBL1 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. 11B).
DISCUSSION

Growth Reduction under Drought Stress

The application of controlled mDr stress on Arabidopsis plants enabled us to evaluate many parameters in relation to the drought stress treatment as well as the response of plants at the physiological and molecular level simultaneously. mDr, maintained by daily replenishing evapotranspired 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, 5 or 10 days duration of drought, both 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 processes. 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 (Fig. 6). In addition, common stress responsive cis-elements were enriched in promoters of genes up-regulated under pDr-wilting and mDr Day01, and down-regulated 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 Day01 treatments. In conclusion, these expression analyses revealed regular 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. mDr treatment is therefore a good model system to dissect the response and resistance of plants to drought.

The analysis of the late stage of drought in mDr Day10 compared to the earlier mDr Day01 indicates the continuity of stress responses, which were examined for evidence of plant acclimation responses. Prominent among 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 in 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 coexpressed with these homeobox regulators are probably also involved in a similar role, such as transcription factors belonging to the NAC and CCAAT binding (CBF-B/NF-YA) family, protein phosphatases and kinases. These expression studies therefore reveal 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 the ABA up-regulated genes (Nemhauser et al., 2006) made up 26% (944/3625) of the pDr up-regulated genes, 31% (341/1089) of mDr Day01 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 response-eliciting ABA analog (Huang et al., 2007).

**Stress Perception and Signaling is Transient and occurs at Early Stage of Drought**

Plant responses to different stresses have been shown to be mediated by ABA-dependent and ABA-independent stress signaling pathways (Shinozaki and Yamaguchi-Shinozaki, 1997; Hirayama and Shinozaki, 2010). In addition, extensive studies on 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 plants 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 occurs at an early stage of mDr treatment, and 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 stratégie 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 LRWC and stomatal conductance (gs), but photosynthesis rate 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 PLDα1, which were found to play a critical role in the inhibition of stomatal opening (Mishra et al., 2006; Nilson and Assmann, 2010; Zhao et al., 2010), exhibit a 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. In agreement with this, 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 protein phosphatase type C (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, PLDα1, GORK and the PP2Cs, and repression of the TF MYB60 are consistent with 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 dismutases (Alschger 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 \textit{APX1}, \textit{TPX1}, and \textit{FSD1}; and repression of the other tested antioxidant enzyme genes \textit{GPX6}, \textit{CSD1}, and \textit{CSD2}. This is consistent with the normal photosynthesis rate under mDr, proven both at physiological and molecular levels. However, \textit{GPX6} and \textit{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 \textit{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.

In this research, microarray and qRT-PCR analyses revealed the up-regulation of cell expansion genes under 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 *EXLB1* 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 at 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 (Day01) many stomatal-related genes were either up-or down-regulated. Hence, early stage (Day01) 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 *coil* and *jin*1 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 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 *coil* mutant (Xie et al., 1998).

In experiments on stomatal closure, a characteristic drought response; jasmonates induce closure that is suppressed in the *coil* 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 (Day01), 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 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. Indeed, interaction between cellulose synthesis and high concentration of JA revealed a negative effect of JA on cell wall modification and plant growth, which enhanced plant resistance to fungal pathogens (Ellis et al., 2002). Moreover, JA induction in response to wounding and herbivory freezes cell cycle, inhibits cell expansion, and results in stunted growth.
(Zhang and Turner, 2008; Onkokesung et al., 2010). 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. 12). 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, PLDα1 interacts with GPA1 to inhibit stomatal opening, and frees phosphatidic acid, which in turn interacts with the PP2C (ABI1) to stimulate stomatal closure (Mishra 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.
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 H₂O g⁻¹ dry soil. To do this, a semi-automated system was developed; a balance (GF-1000, A&D, 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 MD conditions. The effect of the duration of mDr was also tested by harvesting plants at 5 days of mDr (DMD) and at 10 DMD.

For pDr treatment, plants were grown in a growth room as described above, water was withheld at 35 DAS, and pellets were kept 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) = (lnW2-lnW1)/(t2-t1) (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 our moderate drought conditions. The following mutants were ordered from Arabidopsis Biological Resource Center (Ohio state university, USA): abi1 (SALK_076309C), coi1 (SALK_095916C), jin1 (SALK_061267C), jar1 (CS8072) in Col background, abi1 (CS22), and aba1 (CS21) in Ler 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 calculation of the relative reduction in biomass (RB) done 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 (DMD). 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, CO₂ 400 μmole, humidity 50%.

Biochemical Analyses

Starch analysis was done 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 DMD, and from well-
watered controls at same time, and stored at -80 °C. 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 (DMD), and were stored at -80 °C. 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).

**Analysis of gene expression profiles**

For each of the drought experiments, mDr Day01 (mDr_Day01), mDr Day10 (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 < IQRmedian) 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 in response to the drought treatments.

**Reannotation of Arabidopsis GeneChip probe-gene mapping**

The mapping of Affymetrix ATH1 probe sets to Arabidopsis loci provided TAIR is arrived at using the following procedure (ftp://ftp.arabidopsis.org/Microarrays/Affymetrix/README on 7/30/09): The mapping to the TAIR8 Transcripts was performed using the BLASTN program with E-value cutoff <= 9.9e-6. For the 25-mer oligo probes used on the Affymetrix chips, the required match length to achieve this E-value is 23 or more identical nucleotides. To assign a probe set to a given locus, at least 9 of the probes included in the probe set were required to match a transcript at that locus. Disregarding probe sets that map to more than one locus, this procedure results in mapping 21,180 probe sets to 21,019 genes.
TAIR as a database will wish to preserve the probe-‘probe set’ definitions provided by Affymetrix for users to map probe sets to genes after performing microarray analysis using the default chip definition file (CDF). But strictly, there are two issues in this procedure that could lead to significant inaccuracies in estimation of gene expression: a) A probe set mapped to a locus can contain up to 2 probes that do not match the locus at all and other probes that do not match the locus uniquely; b) Since multiple probe sets can map to the same locus, during analysis one has to make an ad hoc procedure to either combine information from all the mapping probe sets or choose one of the probe sets based on an arbitrary criterion. Both choices have been used in previous studies frequently.

To get around these issues and improve to the mapping generally, we sought to: a) Increase the stringency of mapping a 25-mer probe from 23 or more identical nucleotides to a perfect match; b) Assign a probe to a locus only when it uniquely maps to that locus; c) Combine all the probes that uniquely map to a given locus into a single probe set, identified after the locus. Previous studies have shown that such a reannotation procedure to achieve correct mapping of probes to genes in GeneChips lead to significantly altered quantification of gene expression (Dai et al., 2005).

Thus, a high-quality CDF was built for the Arabidopsis ATH1 array by uniquely mapping 232,697 probe sequences (http://www.affymetrix.com/analysis/downloads/data/) to 21,389 Arabidopsis (TAIR8; Swarbreck et al., 2008) gene-based probesets 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 with at least 3 probes 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). This new custom CDF is available from NCBI with the GEO accession number……

Promoter analysis

For analysis of potential promoter-resident cis-regulatory elements (CREs), FIRE (Elemento et al, 2007) was used to discover motifs informative about the different sets of differentially
expressed genes compared to the rest of the genes in the genome. Briefly, FIRE seeks to discover motifs whose patterns of presence/absence across all considered regulatory regions (motif profile) are most informative about the expression of the corresponding genes (expression profile). To measure these associations, FIRE uses mutual information (MI) (Cover & Thomas, 2006). FIRE performs a randomization test and considers an observed MI value (for a motif-expression profile pair) to be significant only when it is greater than all the random MI values calculated by randomly assigning the expression values to genes. A Z-score reflecting how far the observed MI value is, in number of standard deviations, from the average random MI is calculated. These are the Z-scores presented for each motif in Figure 7. Moreover, it also performs jack-knife resampling (Efron, 1979), where, in each of 10 trial the above randomization test is carried out. Only motifs that are statistically significant in at least 6 trials are reported. Newly discovered motifs were compared to known cis-elements in the PLACE, AGRIS and AthaMap databases (Higo et al, 1999; Davuluri et al., 2003; Galuschka et al., 2007) and to each other using STAMP (Mahony & Benos, 2007). All upstream sequences were obtained from TAIR. 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 geneset. ABA-response genesets were obtained from Nemhauser et al. (2006). Genes containing CREs discovered de-novo were further included as additional genesets.

All the genesets described above (GO_BP, GO_CC, ABA-response, and CRE) were tested for the statistical significance of enrichment among the experimentally identified drought
genesets (mDr_Day01, mDr_Day10 and pDr up- and down-regulated genes) and amongst themselves using the cumulative hypergeometric test. For a pair of genesets \( i \) and \( j \), if \( N \) is the total number of genes, \( n_i \) and \( n_j \) are the number of genes in geneset \( i \) and \( j \), and \( m \) is the number of genes common to the genesets, the probability (\( p \)-value) of an overlap (enrichment) of size equal to or greater than observed is given by the formula below.

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

To adjust for multiple comparisons, a Benjamini-Hochberg false discovery rate (FDR; \( q \)-value; Benjamini and Hochberg, 1995) 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 geneset-graph, where pairs of significantly overlapping genesets (nodes) are connected to each other by edges. The graph was augmented with information about geneset size (node size), source/type (node color), and extent of overlap between genesets (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 I (Qiagen, USA) digestion. 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 (Supplemental Table S4). 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 and Schmittgen, 2001).

**Accession Numbers**

The Arabidopsis Genome Annotation or the locus ID numbers for the genes investigated in this study are \( ABI1 \) (At4g26080), \( ABI2 \) (At5g57050), \( APX1 \) (At1g07890), \( CSD1 \) (At1g08830), \( CSD2 \) (At2g28190), \( EXLB1 \) (At4g17030), \( EXPA10 \) (At1g26770), \( EXPA3 \) (At2g37640), \( EXPA4 \) (At2g39700), \( EXPA8 \) (At2g40610), \( FSD1 \) (At4g25100), \( GORK \) (At5g37500), \( GPA1 \)
(At2g26300), GPX6 (At4g11600), HAB1 (At1g72770), MYB60 (At1g08810), NCED3 (At3g14440), PLDa1 (At3g15730), PQL2 (At3g01440), PSAH2 (At1g52230), PSBQA (At4g21280), PSBW (At2g30570), RAB18 (At5g66400), RD29A (At5g52310), RD29B (At5g52300), RPK1 (At1g69270) and TPX1 (At1g65980). The gene expression data reported here is available from NCBI with the GEO accession numbers ……….

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LEGENDS TO FIGURES

Figure 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 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).

Figure 2. Growth of Arabidopsis ecotype Columbia in response to mDr. A, Response of different developmental stages to mDr, in terms of relative reduction in biomass (RB), the experiment was repeated twice, (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 (DMD) under well-watered (WW), and drought (DRT) conditions, (experiment repeated twice, 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 moderate drought (DMD), experiments were repeated twice.

Figure 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.

Figure 4. Gas exchange measurements and water use efficiency (WUEi) in a time course of mDr. A, Photosynthesis (Pn), stomatal conductance (gs), and internal CO₂ (Ci). B, Instantaneous water use efficiency (WUEi) in a time course of mDr. N=5 per treatment per time point, 3 leaves measured/plant, experiment repeated twice, error bars represent SE, P < 0.001.

Figure 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 D01) and 10 day
(mDr D10) treatments. Sequence logos of cis-elements derived by de novo promoter analysis with similarity to the ABRE element in the three drought treatments.

**Figure 6.** Differentially expressed mDr Day01 genes in relation to other factors. The up- and down-regulated mDr Day01 genes compared to other genesets including mDr Day10, pDr, GO biological process, GO cellular components, ABA-related and cis-element-based genesets. Only geneset-pairs with significant enrichment have been connected to each other. The key describes the identity of nodes, where colors refer to the type of geneset. Node size corresponds to the number of genes in that geneset (indicated in the parentheses on each node) and edge thickness corresponds to the level of overlap between the genesets connected.

**Figure 7.** Cis-regulatory elements identified in the upstream regions of mDr and pDr-regulated genes. Each element identified along the rows was identified using *de novo* motif discovery to find short degenerate DNA sequences whose presence or absence in the 1Kb upstream regions of genes is highly informative about the expression of the given gene set (e.g. up-regulated genes in mDr Day 01) given the background distribution of the sequence in the upstream sequences of all the genes in the genome. The colored matrix indicates which motifs were identified using genes regulated in which drought treatment, with yellow indicating down-regulation and blue up-regulation. Motifs informative about up- and down-regulation together are indicated by green. In the adjoining table, the sequence of the *de novo* motifs are given in the nucleotide IUPAC nomenclature along with the Z-score of the information value of the motif reflecting how far the observed value is, in number of standard deviations, from the average random information (see Materials and Methods). Known elements in the PLACE database with significant match to each *de novo* motif are presented in the ‘PLACE motifs’ table in the form of the database ID, DNA sequence and E-value of sequence match with the *de novo* motif. Motifs with no match to any known element are novel putative regulatory elements.

**Figure 8.** 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 (DMD). B, qRT-PCR analysis of stress signaling genes and stress marker genes under pDr pre-wilting (PPW) drought and mDr Day01 (MD). C-E, Time course response in days of mDr (DMD, x-axis) of stress related genes using qRT-PCR, showing fold change (y-axis). C, ABA-signaling pathway genes
GORK. B, Protein phosphatase C genes (PP2Cs). C, Receptor-like kinase 1 (RPKI). D, MYB60 transcription factor. A-D, 3 replications with 5 plants pooled/replication, error bars represent SE.

**Figure 10.** Gene expression profiles of drought responsive genes. Time course in days mDr (DMD, 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.

**Figure 11.** Gene expression profiles of expansin genes in drought acclimation response. A, Expression of expansin genes under pDr pre-wilting (PPW) and mDr (MD), 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 drought). A-B, 3 replications with 5 plants pooled/replication, error bars represent SE.

**Figure 12.** Physiological, biochemical, and molecular plant responses to mDr. Plants response to mDr 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 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.
SUPPLEMENTAL DATA

**Supplemental Table S1.** Differentially expressed genes identified using expression profiling of moderate (mDr) and progressive (pDr) drought treatments

**Supplemental Table S2.** GO enrichment analysis of mDr- and pDr-regulated genes

**Supplemental Table S3.** Cis-regulatory element analysis of mDr- and pDr-regulated genes

**Supplemental Table S4.** List of genes and primers used in the study
ABRE-related motif present in the drought-regulated genes

- pDr Up
  - ACGTGTTC
- mDr Day01 Up
  - ACGTGTTC
- mDr Day10 Down
  - ACGTGTTC

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Controlled Moderate Drought

Early Priming Stage
- Drought Stress perception & signaling
- Low RWC
- High ABA accumulation
- Drought avoidance by stomatal closure
- Normal photosynthesis
- Normal starch accumulation

Intermediate Acclimation Preparatory Stage
- Cell wall adjustment
- Normal growth
- No osmotic adjustment

Late Acclimation Stage
- Acclimation: new cellular homeostasis
- Reduced growth
- Energy saving
- New JA:ABA balance