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

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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. Toward this goal, a controlled, sublethal, 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 abscisic acid (ABA) biosynthesis and signaling displaying drought sensitivity and in 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 the 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.

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 to 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 and the 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) and 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; Wohlbach et al., 2008). The common stress signaling pathways have been distinguished into abscisic acid (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 DRE-binding protein (DREB)/C-repeat-binding factor (CBF), ABA-binding factor (ABF), MYC, and MYB (Abe et al., 1997; Bartels and Sunkar, 2005; Sakuma et al., 2006), including the identification of the stress-responsive cis-elements ABA-responsive element (ABRE) and dehydration-responsive element (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 Ca2+ 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 the 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, 2007). These pathways were discovered in Arabidopsis (Arabidopsis thaliana) as a model system, which paved the way for the discovery of parallel pathways in other crop plants such as in rice (Oryza sativa) 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 plants 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 on plant responses to drought at both the 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, 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 sublethal 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 Pro biosynthesis gene in chickpea (Cicer arietinum; 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 (Pinus taeda), treated for cycles of mild drought and recovery (Watkinson et al., 2003; Vásquez-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 semiautomated, 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 toward acclimation in plant growth.
RESULTS

Plant Temporal Responses to 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 was applied by withholding water at different growth stages to three batches of plants: at 25 d after sowing (DAS), 30 DAS, and 35 DAS. These growth stages, as defined for Arabidopsis (Boyes et al., 2001), of drought initiation correspond to six-leaf (1.06), eight-leaf (1.08), and 10-leaf (1.10) stages when watering was withheld, respectively. Around 5 to 7 d after drought initiation and evapotranspirational water loss, mDr stress is achieved and then maintained by adding water daily to reach a soil moisture level of 2 g g\(^{-1}\) dry soil, with the plant stages for the three batches at drought initiation at eight leaf (1.08), 10 leaf (1.10), and 12 leaf (1.12), respectively (Boyes et al., 2001). The soil moisture level was maintained at a level that was nonlethal and above the wilting point, at 30% field capacity, by replenishing the evapotranspired water, and the reduction in biomass was taken as a quantitative measure of growth calculated as described in “Materials and Methods.” Figure 2 shows that the highest relative reduction in biomass (RB) of mDr-treated compared with 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 duration of mDr treatment was tested, and 5 or 10 d 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 to 30 DAS and 30 to 35 DAS. The rate of growth (both in terms of biomass and leaf area) during the first developmental stage, 25 to 30 DAS, was higher than that during the second stage, 30 to 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 d before mDr treatment (−2), 1 d before mDr (−1), and 0, 1, 2, and 3 DMD. The mDr stress (+1) is defined when the plants reach 2 g water g\(^{-1}\) dry soil and water supplemented (if needed) to maintain the controlled drought, as described in “Materials and 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 1 d 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

Figure 1. Schematic illustration of the drought treatments and sampling in this study. For drought treatments, water was withheld at 25 DAS, and the progress of drought was monitored by soil moisture, shown here as percentage field capacity (FC). Two pDr treatments were done: wilting and prewilting (1 d before wilting, predicted on soil moisture content). Controlled mDr was used to study plant responses at physiological and molecular levels, with the sampling times indicated (−1, 0, 1, 2, and 3). [See online article for color version of this figure.]

Figure 2. Growth of Arabidopsis ecotype Columbia in response to mDr. A, Response of different developmental stages to mDr in terms of 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 DAS) and stage 2 (30–35 DAS). Error bars represent SE; asterisks indicate significant differences (n = 16, P < 0.01). C, LRWC (%) at different DMD under well-watered (WW) and drought (DRT) conditions. The experiment was repeated twice (n = 12, P < 0.0001). D, LRWC (%) n = 12 and corresponding soil water content (SWC%); n = 20) at different DMD. The experiments were repeated twice. [See online article for color version of this figure.]
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 (Columbia and Landsberg erecta) was tested under mDr conditions. The reduction in growth (measured as biomass) under controlled mDr stress compared with well-watered controls provides a parameter to compare different genotypes with the wild type and to distinguish genotypes with altered sensitivity/resistance to drought. The ABA signaling mutant (ABA insensitive1 [abi1]) and biosynthesis mutant (aba1) show higher sensitivity to drought stress compared with their respective wild-type controls (Fig. 3, A 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 d of mDr (Fig. 3, C and D). The other jasmonate response mutant, jar1, shows a drought response phenotype not significantly different from the wild type. The response of this mutant to jasmonate is also moderate compared with 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 d of mDr (1 DMD), reaching 59% of the well-watered control (Fig. 4A). It was reduced by almost 50% from that at day −1 and 0 d of mDr (Fig. 4A) and continued to decrease until day 2 of mDr, with about 40% reduction from that of the well-watered control. At day 3 of mDr, it increased to the level of the well-watered control. The same trend was shown for the internal CO2 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 with the well-watered control (Fig. 4A). instantaneous water use efficiency (WUEi) was higher than in the well-watered control at days 1 and 2 of mDr (Fig. 4B). It was above 4 μmol mmol−1 at days 1 and 2 of mDr, while the well-watered WUEi was around 2 μmol mmol−1. At days −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 (2,039) 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 with both these responses, the backdrop of severe effects of drought on gene expression is revealed by the response to pDr (wilting); 7,648 differentially expressed genes, approximately 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; Supplemental Table S1). The mDr and pDr treatments share a set of 178 differentially expressed genes, approximately 30% of the genome, replete with well-known stress response genes and processes.
response genes (q value approximately 1E-15), with overlapping sets of genes known to respond to ABA stimulus (q approximately 1E-12.6), osmotic (q approximately 1E-8.1), cold (q approximately 1E-4.1), and oxidative (q approximately 1E-2.5) stresses. Expression dynamics of several of these genes have been verified using quantitative reverse transcription (qRT)-PCR (see below). Fundamental processes of the cell known to be grossly affected by drought, including DNA packaging (q approximately 1E2.6), ribosome biogenesis (q approximately 1E-2.9), and protein folding (q approximately 1E-3.2), were concomitantly down-regulated in mDr Day01 and pDr. Thus, the plants are mounting an early response to mDr that is very similar to the classical response to pDr. However, severe effects, including down-regulation of photosynthesis (q approximately 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 approximately 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, was similar to control, while some were even down-regulated (q approximately 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 approximately 1E-2.1), possibly mediating acclimation. Glucosinolate (q approximately 1E-4), indole-3-acetic acid derivative (q approximately 1E-2.9), jasmonic acid (JA; q approximately 1E-2.8), and very-long-chain fatty acid (q approximately 1E-2) metabolism genes are among those solely down-regulated in mDr Day10. Some genes involved in cell wall thickening (q approximately 1E-2.9) and a few others involved in the regulation of cell growth (q approximately 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 responses 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 the de novo motif discovery tool FIRE (Elemento et al., 2007), compare the found elements with 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-regulated and down-regulated) gene sets 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 gene sets is provided in Figure 6. Motifs discussed below are referred to by the name of the most similar “known” CRE (for the key, see the “PLACE motifs” table in Fig. 7).

The CRE standing out among the genes up-regulated in mDr Day01 and pDr was one highly similar to the experimentally identified ACCTG(T/C) motif. 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 (for GA down-regulated) motif. Interestingly, an ele-

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Gas-exchange measurements and WUEi in a time course of mDr. A, Photosynthesis (Pn), stomatal conductance (gs), and internal CO2 (Ci). B, WUEi in a time course of mDr. n = 5 per treatment per time point, three leaves were measured per plant, the experiment repeated twice, and error bars represent ±s (P < 0.001). DRT, Drought; WW, well watered. [See online article for color version of this figure.]

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Gene expression analysis under mDr and pDr. Venn diagrams comparing up-regulated and down-regulated genes of pDr, mDr D01, and mDr D10 treatments are shown at top. Sequence logos of cis-elements derived by de novo promoter analysis with similarity to the ABRE element in the three drought treatments are shown at bottom. [See online article for color version of this figure.]
ment 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/C-repeat (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 RESPONSIVE TO DEHYDRATION29A (RD29A) gene in Arabidopsis (Narusaka et al., 2003).

Yet another classical stress response in early mDr, comprising slowing down of protein folding in the endoplasmic reticulum that triggers the unfolded protein response (UPR; Martínez and Chrispeels, 2003), is vindicated by the identification of the UPR element-like element among genes up-regulated in mDr Day01. In contrast, a UPR element-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 UPR element, upset protein folding causes UPR involving the up-regulation of genes that help in remedying the situation, an early signal captured by the UPR element in mDr Day01 genes, while severe drought stress (pDr) pushes the system beyond repair, causing the down-regulation of the whole protein machinery. 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 photoresponsive genes (AT1BOX and CCA1 motif2; Terzaghi and Cashmore, 1995; Wang et al., 1997) and the latter similar to the Evening Element involved in the circadian control of gene expression in Arabidopsis (Harmer et al., 2000). It is unclear how photoresponse 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 the 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 with a subtle change under mDr (Supplemental Table S1). In Arabidopsis, more than 50% of the photosynthate is stored as starch (Zeeman and Rees, 1999). Therefore, we 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 a 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; Casper et al., 1985). Therefore, individual plants of the same age (30 DAS) were collected at the late afternoon from plants treated to 1 d of wilting and plants of 1 d of mDr. Starch analysis showed no accumulation of starch in the wilting plants, compared with normal starch accumulation in plants exposed to 1 d 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 the time course of mDr. Starch concentration was determined at five time points, 2, 1, 0, 1, 2, and 3 d of mDr, and showed no significant differences for these time points compared with their corresponding well-watered control (data not shown).

To test for evidence of osmotic adjustment under mDr treatment, Pro content was determined at days 3 and 4 of mDr. We found no significant change in Pro in drought-treated plants compared with well-watered controls (data not shown). Moreover, gas chromatography-mass spectrometry metabolomic analysis also showed no significant changes in Pro concentration over the time course of mDr (J. 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 in two different ways, pDr prewilting (1 d before wilting) drought and controlled mDr Day01 treatments, on the response of the signaling pathways at the molecular level, the expression levels of key genes in the signaling pathways were quantified. The ABA biosynthesis gene NINE-CIS-ÉPOXYCAROTENOID DÍÓXYGENASE3 (NCED3) was induced 4-fold more under pDr prewilting compared with mDr (Fig. 8B). The drought-responsive transcription factor DREB2A was induced at a higher level under pDr compared with mDr. Another gene showing differential expression between the two drought treatments is RD29B, which is induced almost 100-fold under pDr compared with mDr with a 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 levels of key genes in the stress signaling pathways, ABA dependent and ABA independent, were quantified in the time-course analysis of mDr. NCED3, an important enzyme in ABA biosynthesis, was highly induced at days 0 and 1 of mDr compared with days −1, 2, and 3 (Fig. 8C). The expression of ABF3 (ABA-dependent pathway) was high at 0 d of mDr, and after that its expression started to decrease. In the ABA-independent pathway, DRE-B2A 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 RESPONSIVE TO ABA18 (RAB18). The expression profiles 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.

Stomatal Responses in a Time-Course Analysis of mDr

To understand the stomatal responses to mDr treatment at the molecular level, a set of stomatically related genes were chosen based on our microarray data of mDr to study the 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 was then reduced from day 2 onward (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). Therefore, we tested the expression profiles of three main PP2Cs: ABI1, ABI2, and HOMOLOGY TO ABI1 (HABI). The expression of ABI1 and ABI2 started to increase at day −1 of mDr, continued to day 1, and then decreased (Fig. 9B). HABI showed a decrease in expression at day −1, followed by an induction at day 1 and a decrease thereafter (Fig. 9B). RECEPTOR-LIKE PROTEIN KINASE1 (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 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). Therefore, we tested the response during the time course of mDr, quantifying its...
MYB60 was induced at days -1 and 0 of mDr (Fig. 9D) and repressed at day 1; it stabilized from day 2 onward 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 the subtle effect of 1 d 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 its lowest level at day 2 of mDr, while PSBQA expression was normal at days -1, 0, and 1 and started to decrease at day 2. The PSI subunit gene PQL2 showed normal expression levels at days -1, 0, 1, and 2, and at day 3 it started to decrease. PSAH2 was induced at day -1, reduced to a 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 copper/zinc dismutase (CSD1), chloroplastic copper/zinc dismutase (CSD2), and iron 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 days 1 and 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 differentially expressed genes showed the enrichment of cell expansion-related genes. In addition, the comparison of mDr and pDr microarray data with ABA-responsive genes showed that cell expansion was up-regulated in mDr compared with pDr wilting drought and ABA treatments. Therefore, we quantified the effect of pDr prewilting and mDr treatments on the expression levels of the EXPANSIN genes EXP3, EXP4, EXP8, EXP10, and EXPANSIN-LIKE B1 (EXLB1; Fig. 11A). After 1 d of mDr, most of the expansin genes were induced, while under pDr, EXP3 and EXP8 showed repression and EXP4 remained unchanged (Fig. 11A).

In the time-course analysis of mDr, the EXP3, EXP4, EXP8, and EXP10 genes were repressed at day 0, induced at day 1, followed by a decrease at days 2 and 3 of mDr (Fig. 11B). EXLB1 showed a steady increase in expression until day 1 and then a gradual decline to day 3, reaching a 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 levels simultaneously. mDr, maintained by daily replenishing evapotranspired water, was applied at plant growth stages 1.08 to 1.10, corresponding to

Figure 9. Gene expression profiles of stomatally related genes during mDr treatment. The y axis shows the fold change using qRT-PCR, and the x axis shows the days of mDr. A, PLDa1, GPA1, and GORK. B, PP2Cs. C, RPK1. D, MYB60 transcription factor. For all panels, three replications with five plants pooled per replication were used; error bars represent se. [See online article for color version of this figure.]
the eight- to 10-leaf stages (Boyes et al., 2001), for 5 to 10 d 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 stages 1.08 and 1.10, corresponding to 25 and 30 DAS, showed a highly significant growth reduction compared with later developmental stages. Moreover, 5 or 10 d 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 d of mDr was done. The perturbation in growth due to drought stress, the time when the responses start, and the physiological, biochemical, and molecular changes responsible for the reduction in growth under drought.

Drought Transcriptome Analysis

A comparative transcriptome analysis of pDr wilting and mDr (1 and 10 d) 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 under mDr Day10. Moreover, qRT-PCR analysis showed that most of the characteristic stress signaling and stress marker genes were similarly induced under pDr 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, therefore, is 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 with 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 wild-type 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 of 3,625) of the pDr up-regulated genes, 31% (341 of 1,089) of mDr Day01, and 22% (93 of 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 was found in comparison with ABA responses using a more response-eliciting ABA analog (Huang et al., 2007).

Stress Perception and Signaling Are Transient and Occur at an 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 with 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...
points (days 0, 1, and 2) of mDr, showing highest concentrations 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 they 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 LRWC and stomatal conductance, but photosynthesis rate remained normal. Moreover, the expression pattern of stomatically 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 critical roles in the inhibition of stomatal opening (Mishra et al., 2006; Nilson and Assmann, 2010; Zhang et al., 2009; Zhao et al., 2010), exhibit high expression, consistent with their roles 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 are 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 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 (Tähtiharju and Palva, 2001; Bray, 2004). Here, three PP2Cs, AB11, AB12, and HAB1, were found to be induced early during drought treatment. Another gene, RPK1, 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 the regulation of guard cells under stress, with a function in the 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 the regulation of a stomatal drought response network, resulting in the induction of GPA1, PLDa1, GORK, and the PP2Cs and repression of the transcription factor MYB60, is 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 drought 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 nonsignificant 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 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 the antioxidant molecules ascorbate, glutathione, and α-tocopherol in addition to the antioxidant enzymes peroxidases, catalases, and 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 the 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 the physiological and molecular levels. However, GPX6 and TPX1 are slightly, yet significantly, up-regulated (approximately 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...
Acclimation to mDr by Cell Wall Adjustment

One of the first acclimation responses to drought is the decrease in leaf growth, which results in the 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 nonenzymatic 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 (noncovalent) 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 (Zea mays) 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 (Helianthus annuus) as well as in 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 prewilt- ing and pDr wilting drought treatments, most of the expansin genes were down-regulated. The expression profiles of four 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 the 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 with a 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 (Solanum lycopersicum) 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 hostile environments. There is still a lot to be learned about cell wall modification under different abiotic stresses at the molecular, cellular, tissue, and whole-plant levels. 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 the late stage of mDr (Day10) showed no significant differences in stomatal conductance and photosynthesis of drought-treated plants compared with the well-watered control (data not shown). Moreover, no stomataly related genes were differentially expressed at this stage. In contrast, microarray and qRT-PCR analyses of the early stage of mDr (Day01) showed that many stoma-
tally related genes were either up-regulated or down-regulated. Hence, the early stage (Day01) showed reduced stomatal conductance, whereas normal stomatal conductance and reduced growth were shown at late stage of mDr (Day10).

JA biosynthesis and signaling were among the main enriched GO categories in the down-regulated genes at the late stage of mDr. Jasmonates have been found to have a potential role in response to drought stress in soybean (Glycine max), as they showed an early increase within 2 h of dehydration and a decrease in concentration afterward (Creelman and Mullet, 1995). Moreover, jasmonates were found to cause stomatal closure (Raghavendra 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 a 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 with the wild type, with biomass accumulation under drought not different from the 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 that 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 (Hordeum vulgare) 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 the 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 the late stage of mDr (Day10), the 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 mDr, in 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 (e.g. 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 plant response in growth. Indeed, interaction between cellulose synthesis and a 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 the 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 to late (Fig. 12). During the early priming or preconditioning stage, stress perception, signaling, and reprogramming of gene expression take 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

![Figure 12. Physiological, biochemical, and molecular plant responses to mDr. Plant responses to mDr are dissected into three stages: early priming (preconditioning) stage, in which all stress signaling and avoidance processes take place; intermediate stage, which is preparatory for acclimation, as plants modify and adjust cell walls for reprogrammed growth responses at later stages; and late stage, in which plants are set to a new homeostasis with altered hormonal signaling and reduction in energy-demanding processes, leading to acclimated plants with reduced growth. FC, Field capacity. [See online article for color version of this figure.]](image-url)
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 (Arabidopsis thaliana ecotype Columbia) seeds were sown in moistened peat pellets (Jiffy Products), stratified at 4°C for 2 d, and then transferred to a growth room kept at 10 h of light (100 µmol 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 mDr treatments were maintained by giving plants water to keep the soil moisture level at 30% of the amount of water in pellets at the beginning of the experiment. For drought treatment, pellets were weighed before sowing to determine the controlled mDr (0, 1, and 2 DMD) and were stored at

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 DMD. For the gas-exchange measurements, a LICOR 6400XT and an Arabidopsis Extended chamber were used, and the following conditions were set for LICOR measurement: flow rate of 150 mol m⁻² s⁻¹, CO₂ at 400 µmol, and humidity of 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 the same times 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 was determined, and starch was quantified using the EnzymChrom starch assay kit (BioAssay Systems) following the instructions of the manufacturer. For ABA quantification, plant samples were harvested at different times of mDr (0, 1, and 2 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 Phytodekt ABA test kit (Agdia) following the manufacturer’s instructions. Pro was quantified in plant samples at 3 and 4 DMD as described (Bates et al., 1973).

Analysis of Gene Expression Profiles

For each of the drought experiments, mDr Day01, mDr Day10, and pDr, raw data were background corrected, normalized, and summarized according to the custom chip definition file (CDF; see below) using robust multichip average (Affymetrix, 2001). The mapping of Affymetrix ATH1 probe sets to Arabidopsis loci provided by The Arabidopsis Information Resource (TAIR) is arrived at using the following procedure: Affymetrix ATH1 probe sets to Arabidopsis loci provided by The Arabidopsis Information Resource (TAIR) is arrived at using the following procedure (http://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 ≤ 9.0e-6. For the 25-mer oligonucleotide 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 nine 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 CDF. But strictly, there are two issues in this procedure that could lead to significant inaccuracies in the estimation of gene expression: (1) a probe set mapped to a locus can contain up to two probes that do not match the locus at all and other probes that do not match the locus uniquely; (2) since multiple probe sets can map to the same locus, during analysis one has to use 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 the mapping generally, we sought to (1) increase the stringency of mapping a 25-mer probe from 23 or more identical nucleotides to a perfect match; (2) assign a probe to a locus only when it uniquely maps to that locus; and (3) 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 GeneChip leads 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 probe sets in the following manner: (1) probes that have perfect sequence identity with a single target gene were selected; (2) probes mapping to reverse complements of genes were annotated separately as antisense probes (not used in the above counts); and finally, (3) probes were grouped into probe sets, each corresponding to a single gene, and probe sets with at least three probes were retained (more than 99% of probe sets have five or more probes). Note that these stringent criteria used to construct the CDF make it possible to reliably measure the expression values of members of multigene families (free from cross-hybridization between paralogs showing high sequence similarity). This new custom CDF is available from the National Center for Biotechnology Information with the Gene Expression Omnibus accession number GPL10948.

Promoter Analysis
For analysis of potential promoter-resident CREs, FIRE (Elemento et al., 2006) was used to discover motifs informative about the different sets of differentially expressed genes compared with 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 and 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 sds, 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 trials, the above randomization test is carried out. Only motifs that are statistically significant in at least six trials are reported. Newly discovered motifs were compared with known cis-elements in the PLACE, AGRIS, and AthaMap databases (Higo et al., 1999; Davuluri et al., 2003; Galuszka et al., 2007) and with each other using STAMP (Mahony and Benos, 2007). All upstream sequences were obtained from TAIR. This de novo approach was taken since (1) CREs could diverge far more quickly than coding sequences across species, making them difficult to find simply by searching and (2) searching based on known elements in Arabidopsis is limited by the scope of the dataset.

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 the RNeasy Kit (Qiagen). After that, genomic DNA was eliminated using DNase I (Qiagen) digestion. The first-strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). Bio-Rad SYBER Green was used to quantify the expression of the genes. The 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 by Livak and Schmittgen (2001).

The Arabidopsis Genome Initiative locus identifier numbers for the genes investigated in this study are as follows: ABA1 (At4g26080), ABA2 (At5g75050), APX1 (At1g07890), CSD1 (At1g88830), CSD2 (At2g29190), EXL1 (At1g70300), EXPD10 (At1g26770), EXPD3 (At2g37460), EXPD4 (At2g39700), EXD8 (At2g40610), FSD1 (At4g25100), GORK (At5g37900), GPA1 (At2g26300), GPX6 (At4g11600), HAB1 (At1g72770), MYB60 (At1g88830), NCED3 (At3g14440), PLDv1 (At3g15730), PQL2 (At3g01440), PSAH2 (At1g52230), PSBQ4 (At4g21200), PSBv (At2g30700), RAB18 (At5g66400), RDA29A (At5g52310), RDA29B (At5g52300), RPK1 (At1g69270), and TPH1 (At1g69980).

Gene function annotations 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, noninformative terms for analysis, only terms annotating 500 or fewer 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_Day01, mDr_Day10, and pDr up-regulated 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 sets i and j, and m is the number of genes common to the gene sets, the probability of an overlap (enrichment) of size equal to or greater than observed is given by the formula below.

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

To adjust for multiple comparisons, a Benjamini-Hochberg false discovery rate (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 the 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).

Supplemental Data
The following information is available in the online version of this article.

Supplemental Table S1. Differentially expressed genes identified using expression profiling of mDr and pDr 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 this study.

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