Running title: Development mediates leaf stress response

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Developmental stage specificity and the role of mitochondrial metabolism in the response of Arabidopsis leaves to prolonged mild osmotic stress\(^{[\text{w}]}\)

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ABSTRACT

When subjected to stress, plants reprogram their growth by largely unknown mechanisms. To provide insights into this process, growth of Arabidopsis (Arabidopsis thaliana) leaves that develop under mild osmotic stress was studied. Early during leaf development, cell number and size were reduced by stress, but growth was remarkably adaptable, as division and expansion rates were identical to control levels within a few days of leaf initiation. To investigate the molecular basis of the observed adaptability leaves with only proliferating, exclusively expanding, and mature cells were analyzed by transcriptomics and targeted metabolomics. The stress response measured in growing and mature leaves was largely distinct; several hundred transcripts and multiple metabolites responded exclusively in the proliferating and/or expanding leaves. Only a few genes were differentially expressed across the three stages. Data analysis showed that proliferation and expansion were regulated by common regulatory circuits, involving ethylene and gibberellins, but not abscisic acid. The role of ethylene was supported by the analysis of ethylene-insensitive mutants. Exclusively in proliferating cells, stress induced genes of the so-called “mitochondrial dysfunction regulon”, comprising alternative oxidase. Up-regulation for eight of these genes was confirmed with promoter: β-glucuronidase reporter lines. Furthermore, mitochondria of stress-treated dividing cells were morphologically distinct from control ones and growth of plants overexpressing the alternative oxidase gene was more tolerant to osmotic and drought stresses. Taken together, our data underline the value of analyzing stress responses in function of development and demonstrate the importance of mitochondrial respiration for sustaining cell proliferation under osmotic stress conditions.
INTRODUCTION

Drought stress causes reduced plant growth and, consequently, often dramatic decreases in crop yield (Boyer, 1982). With the rapidly growing world population, environmental deterioration and the increasing need for bio-energy crops, sufficient food has to be produced on less arable land, while fresh water resources become more restricted. Therefore, sustainable and equitable global food, feed, and bio-energy security relies on the development of high-yielding crop plants that can resist adverse environmental conditions. For these reasons, understanding the mechanisms underlying plant adaptation to stress is not only of primary scientific, but also of socio-economical, importance.

Plants have evolved numerous adaptation responses to minimize the harmful effects of drought stress, summarized in the avoidance/tolerance model (reviewed by Verslues et al., 2006). To avoid dehydration, mechanisms that help to balance water uptake and loss such as stomatal closure and accumulation of compatible solutes (e.g. proline and raffinose) are activated. These changes consequently result in reduced transpiration and a lower water potential, respectively. Additionally, tolerance mechanisms are triggered to protect cells against damage; for instance, protective proteins are synthesized, such as dehydrins and late-embryogenesis abundant (LEA) to restrict damage to other proteins and cellular membranes, while production of antioxidants reduces the levels of harmful reactive oxygen species (ROS). Long-term water stress can be accompanied by changes, such as cuticula thickening, changing root architecture, or the hardening of cell walls. Until now, most of these drought responses have been only studied in mature tissues and under rather extreme stress conditions. In contrast, drought-associated growth restriction has received little interest, initially being considered as a secondary effect of stress related to the reduced photosynthetic activity and stomatal closure. However, after the onset of stress, growth rates have been shown to decrease rapidly, independently of photosynthesis, (referred to as "short-term adjustment"), followed by growth recovery and adaptation to the new condition (referred to as "long-term adaptation") (Burssens et al., 2000; Veselov et al., 2002; West et al., 2004; Fricke et al., 2006). Therefore, although it is now accepted that plants actively reduce their growth as part of stress response, the underling
mechanisms are still only poorly understood. These growth changes allow plants
to save and redistribute resources that can become limited, for example smaller
leaves loose less water due to a reduced transpiration area, while differential
growth recovery leads to beneficially higher root-to-shoot ratios (Hsiao and Xu,
2000).

In Arabidopsis (Arabidopsis thaliana), leaf primordia emerge as rod-like
structures (20-40 μm in size) on the flank of the vegetative shoot apical meristem
(SAM). Abaxial and adaxial domains become specified in this primordium and
begin to form the flat lamina via lateral and distal cell proliferation. Final leaf
shape is achieved during the following developmental phase in which organ
growth and histogenesis are typified by extensive cell expansion. The cell
expansion phase is initiated at the distal tip, proceeds gradually in a distal-
proximal manner (Donnelly et al., 1999) and coincides with the onset of post-
mitotic endoreduplication and the sink-to-source transition. By targeting the right
age, one can harvest leaves that are entirely proliferating, expanding, or mature
(Beemster et al., 2005).

The main aim of this study was to provide insight into how both proliferating
and expanding leaves of Arabidopsis adapt to prolonged mild osmotic stress
when compared to mature leaves. Therefore, a relatively mild stress assay was
developed and leaves only with proliferating, expanding, or mature cells were
microdissected before expression and metabolite profilings. This experimental
set-up differed from published drought gene expression studies in which mature
leaves or complete plant shoots were submitted to relatively severe drought
treatments with complete growth arrest or even plant death as a result (Seki et
al., 2002; Catala et al., 2007; Kilian et al., 2007; Bouchabke-Coussa et al., 2008;
Giraud et al., 2008; Matsui et al., 2008; Perera et al., 2008; Weston et al., 2008;
Zhang et al., 2008; Zeller et al., 2009). Stage-specific sampling together with
detailed growth analysis provided candidate biological processes, genes, and
metabolites that supported growth under stress-limiting conditions. These data
are discussed in the context of current models of plant performance under water-
limiting conditions.

RESULTS
Development of a mild osmotic stress assay

To decipher the mechanisms by which water deficit affects plant growth, an experimental set-up was developed that reproducibly reduced leaf size by 50% (Fig. 1, A-C). The best results were obtained with a low concentration (25 mM) of mannitol that decreases the water potential of the growth medium and, consequently, water uptake of the exposed roots. As an alternative to mannitol, the high-molecular weight osmoticum polyethylene glycol (PEG) 3500 was also tested, however in this instance the observed growth reduction was highly variable. Even when grown on the same plate, some plants developed very severe phenotypes, while others appeared unaffected (Supplemental Fig. S1), complicating further analysis and data interpretation. Therefore, mannitol was used for the detailed profiling analysis, while PEG was used to confirm selected results. More specifically, Arabidopsis seedlings were germinated and grown on medium with or without mannitol up to 22 days after stratification (DAS) (Fig. 1A). In contrast to the responses reported for higher concentrations of mannitol (usually 100-300 mM), no changes in seed germination, frequency of leaf initiation (data not shown) or leaf morphology (curling and narrowing) were observed (Fig. 1, A and B). Importantly, also the operating efficiency of photosystem II did not change, when measured with non-destructive imaging at 15 and 22 DAS, reflecting the mild character of the applied stress (Fig. 1, D and E).

Leaf growth adapts to the osmotic stress

To identify the mechanisms underlying the 50% reduction of the final leaf size when grown under osmotic stress, the third leaf that initiates and subsequently develops under stress conditions (Fig. 1C) was used for further analysis. As the final leaf size depends on the developmental window and rates of cell division and expansion (Beemster et al., 2005), these parameters were assessed using kinematic analysis (De Veylder et al., 2001). This analysis provides information on cell area, cell number, number of guard cells, and cell
division and expansion rates, throughout leaf development (Fig. 2). To obtain these data, leaves were harvested daily from early meristematic stage (9 DAS and approximately 0.1 mm² in size) to maturity (22 DAS). Leaf primordia younger than 9 DAS were too small to dissect. At 9 DAS, growth of leaf 3 was driven exclusively by cell division as demonstrated by expression of the B-type cyclin CYCB1;2 in the whole-leaf primordia (Fig. 3B).

Cellular measurements demonstrated that both reduction in cell number and size contributed equally to the reduced area of mature leaves grown on mannitol (Fig. 2, A-C). Already at the first measurements at 9 DAS, both leaf size and cell number had significantly decreased, while cell size was not affected by the treatment (Fig. 2, A-C). Intriguingly, neither cell division rates nor the developmental cell proliferation window were reduced (Fig. 2E), but, on the contrary, division rates were approximately 10% higher between 12 and 16 DAS, partially compensating for the initial reduction in cell number (Fig. 2E; Supplemental Fig. S2). As the frequency of leaf initiation was also unchanged, the observed reduction in cell number had to arise very early, during the first few days after leaf initiation, thus escaping analysis. Cell differentiation was unaffected, starting from day 10 both under normal and stress conditions. Cell expansion rates were initially reduced in stressed leaves but reached control levels at day 15, and exceeded them between day 18 and 20, partially compensating the initial reduction in cell size (Fig. 2, C and F; Supplemental Fig. S2). Leaf growth rates gradually decreased and leaf 3 approached maturity by day 22 (Fig. 2D). As alterations in cell area can be associated with changes in endoreduplication (Inzé and De Veylder, 2006), the ploidy distribution was determined, but no significant differences were measured either in onset or in level of endoreduplication (Supplemental Fig. S3). Similarly to mannitol, PEG also influenced both cell number and size, contributing to the reduced leaf area; however, the effects were predominantly on cell number, while cell size was reduced only marginally (Supplemental Fig. S4).

Cell drawings revealed further differences between control and mannitol-stressed leaves. The shape of the epidermal cells was clearly affected: cells were not only smaller but also less lobed (Fig. 2H), relating to approximately a 15% decrease in cell perimeter when calculated per cell area (Fig. 2I). Moreover,
although the first stomata appeared simultaneously in both control and stressed leaves, their number had decreased when calculated per total cell numbers (stomatal index, SI) (Fig. 2G). The maximum SI reduction was calculated at 13 DAS (more than 65%), but the difference diminished to 23% at 22 DAS (Supplemental Fig. S2). In contrast to the SI, trichome density increased in mannitol-grown plants when calculated per leaf area. Changes in SI and trichome density could be measured for PEG-grown plants as well (Supplemental Fig. S4).

**Experimental set-up**

To obtain molecular insight into growth adaptation to osmotic stress, samples of exclusively proliferating leaf primordia (P); expanding (E), and mature (M) leaves were subjected to whole-genome transcript profiling (Fig. 3A). Additionally, the aerial parts of whole seedlings at stage 1.03 (Boyes et al., 2001) were included and compared to the leaf samples. To complement the transcript data, metabolites were measured in 1.03 seedlings, E, and M leaf samples. The small size of the P leaves prompted the development of a micro-dissection method with RNAlater (see “Materials and Methods”) that made the samples unsuitable for metabolite measurements. At the time of harvest, growth rates of control and stressed plants were identical, allowing us to study growth adaptation to drought rather than growth inhibition. Statistical analysis was used to identify significant changes with respect to developmental stage (P, E, and M; “significant leaf stage effect”) (Supplemental Table S1) and treatment (with and without 25 mM mannitol; “significant osmotic stress effect”) (Supplemental Tables 2-5 online). Differential transcripts were investigated with MapMan for pathway visualization (Thimm et al., 2004) and PageMan to calculate functional over-representation of MapMan categories (Usadel et al., 2006) (Fig. 4; Supplemental Fig. S5). Data were also compared to selected publically available microarray experiments, mainly from AtGenExpress (see “Materials and Methods”; Supplemental Table S3).

**Transcript and metabolite changes during normal leaf development**
To establish a baseline of changes associated with normal leaf development, transcripts and metabolites with significant leaf stage effect were identified (0-0 mM) providing details on proliferating-to-expanding (P/E) and expanding-to-mature (E/M) stage transitions. In total, 2800 transcripts could be assigned to eight expression clusters with the 2-fold expression and global test (<0.05) cut-offs between any of the two stages and the abundance of 12 metabolites varied between E and M leaves (Supplemental Fig. S5; Supplemental Tables S1 and S4). As expected, the transcriptome of proliferating leaves (group 1 in Supplemental Fig. S5) was enriched for categories linked to mitotic cell cycle and cell division; expanding leaves were characterized by high expression of cell wall-related genes (group 2), while senescence markers (e.g. SEN1 and NAC92) appeared exclusively in mature leaves. The expression of genes associated to the mitotic cell cycle, such as B-type cyclins and kinesins, was reduced sharply at the P/E transition (group 1), whereas transcripts related to DNA and protein synthesis declined gradually across the three developmental stages (group 7). Transcripts related to photosynthesis, primary and secondary metabolisms, light signaling, transport, and auxin and redox regulation increased at the P/E transition and remained high in M leaves (group 4). Exit from expansion into maturity resulted in further changes: on the one hand, transcripts associated with cell wall and fatty acid metabolism (group 6), a number of amino acids (e.g. glutamine and asparagine) and organic acids (such as citric acid) decreased and, on the other hand, abiotic stress related pathways, such as genes encoding enzymes from raffinose metabolism, peaked in the M leaves (Supplemental Fig. S5). In summary, these data demonstrate extensive differences, consistent with known processes, between the three developmental stages confirming the growth analysis and verifying the sample selection and harvesting protocols.

**Adaptation to osmotic stress depends on the leaf developmental stage**

Mild osmotic stress (25 mM mannitol) resulted in alterations in the levels of several hundred transcripts. In total, 399, 741, and 614 transcripts were up-regulated and 97, 632, and 374 down-regulated in P, E, and M leaves,
respectively (Fig. 4A; Supplemental Table S2). Based on the Venn diagram, we delineated lists of genes with altered expression levels due to osmotic stress in one or more developmental stages, revealing hundreds of transcripts affected by mannitol exclusively in one leaf stage and only very few genes that were differentially expressed across the three stages. Overall, the stress response in P and E leaves was more similar, while in fully-grown M leaves it was most distinct, especially when compared to P leaves (Fig. 4B). Similarly, metabolite analysis of E and M leaves revealed distinct metabolite profiles (Fig. 5, A-C; Supplemental Table S4). Expression of 24 genes that were differentially up- or down-regulated by osmotic stress in one or more of the leaf stages and belonged to different functional categories was checked with quantitative reverse-transcription PCR (qRT-PCR) (Supplemental Fig. S6). The majority of the changes could be validated in independent mannitol and PEG experiments.

**Seedling samples are most similar to the expanding leaves**

To learn how many stress-regulated genes and metabolites would have been detected with whole seedlings, osmotic stress effects on transcriptome and metabolome of three leaf stages were compared to seedling shoots at stage 1.03. For the seedling, 244 up-regulated and 59 down-regulated genes, and four up-regulated and 12 down-regulated metabolites were detected, altogether considerably fewer than for any of the leaf developmental stages (Supplemental Table S2). Overall the changes were most consistent with those of growing and particularly E leaves; more than 80% of the changes were also measured in the E samples (Supplemental Fig. S7). Importantly, almost none of the transcripts that changed exclusively in the P or M stages were found in the seedling samples. In summary, utilizing whole shoots substantially diluted information and stressed the value of sampling leaves at different developmental stages.

**Mature leaves are characterized by classical drought response while biotic stress genes are up-regulated in the growing leaves**

In addition to the dissection of responsive genes through the
overrepresentation analysis of MapMan functional categories, we investigated whether these genes were affected in other microarray experiments (see “Materials and Methods”). Comparison with the publicly available stress expression data revealed significant overlap, irrespective of the leaf stage (Supplemental Table S3). However, only M and, to a lesser extent, E leaves showed the classical water stress response as determined in previous whole-plant studies. Expression of abiotic stress markers (e.g. RD29B, RD29A, RD22, and ATHVA22E), LEA genes (e.g. COR15 and ERD10), genes involved in fatty acid biosynthesis (e.g. CER1 and KCS1) and for lipid transfer important for cuticle thickening was induced (Fig. 4C; Table I; Supplemental Tables S2 and S3). The role of abscisic acid (ABA) during abiotic stress is well documented and comparison with ABA addition data revealed a significant overlap for both E and M leaves (Supplemental Table S3), and expression of genes encoding the ABA biosynthetic enzymes, AAO1 and AAO2, was also induced (Table I). Proline, a typical drought-induced metabolite, its precursor glutamine, and other metabolites classically associated with drought stress, erythritol and putrescine, accumulated exclusively in M leaves (Fig. S5; Supplemental Table S4). Moreover, induced expression of CHS, FLS, and PAP1 genes suggested a possible accumulation of flavonol compounds, again specifically in the M leaves (Table I; Supplemental Table S2).

In contrast to M leaves, both P and E leaves were enriched for genes classically associated with biotic stress and comparison with available microarrays of wounded plants or plants treated with pathogens, flagellins, oxidative stress, ethylene, and salicylic acid revealed a significant overlap (Fig. 5; Table I; Supplemental Tables S2 and S3). This overlap consisted of WRKY transcription factors (e.g. WRKY30 and WRKY33), methyltransferases, cytochrome P450 enzymes, pathogen-related proteins (chitinases, PR4, and PR5), mildew resistance locus proteins (MLO3, MLO6, and MLO12), disease resistance proteins, MAP kinases (MPK2 and MPK3), and indole glucosinolates/camalexin biosynthesis (MYB51 transcription factor, CYP79B2, CYP83B1, CYP81F2, CYP71A13, and CYP71B15).

Osmotic stress affects sugar and amino acid content
Sugars and amino acids are expected to play a major role in plant adaptation to stress (reviewed by Seki et al., 2007). A comparison of the stress response of E and M leaves with publicly available glucose addition and carbon starvation experiments revealed a significant enrichment of sugar-responsive genes (Supplemental Table S3). This increase in glucose predicted from the transcript data could be experimentally confirmed by metabolite profiling (Fig. 5). Similarly to glucose, also starch accumulated in E and M leaves, as showed by Lugol's staining (Fig. 6). Furthermore, genes encoding enzymes involved in starch synthesis (such as starch-branching enzyme [SBE2]) and the ADP-glucose pyrophosphorylases (APL2, APL3, and APL4) were up-regulated only in M leaves, while those encoding starch-degrading enzymes (e.g. the β-amylases BMY-3, BMY-5, and BMY-8) were down-regulated in both E and M leaves (Fig. 6; Supplemental Table S2). In contrast to sugars, levels of 9 out of 16 measured amino acids were reduced in E, but not in the M, leaves (Fig. 5). Summarizing, prolonged osmotic stress caused accumulation of soluble sugars and starch in E and M leaves, whereas the amino acid content was reduced in the E tissues.

**Osmotic stress and hormonal crosstalk in the growing leaves**

As hormonal crosstalk plays an essential role for both growth and environmental responses, transcriptome data were compared with available hormone addition experiments (Goda et al., 2008). This comparison revealed that ethylene-responsive genes were enriched among the transcripts affected in P and E leaves (Supplemental Table S3). Osmotic stress significantly enhanced the expression of genes encoding ethylene signaling components, such as ethylene receptors (ESR1 and ESR2) and ethylene response factors (ERF2, ERF4 and ERF5) (Fig. 7A; Supplemental Table S2). Consistent with the expression data, the growth of ethylene-insensitive mutants was more sensitive to 25 mM mannitol and mutants developed severe phenotypes characterized by growth arrest and curled, pale leaves. As anticipated, the *ein2.5* plants, for which the ethylene insensitivity was the strongest, also responded most clearly to the mannitol treatment (Fig. 7, B and C). Moreover, we found a possible involvement of
gibberellin (GA) signaling in growth adaptation as predicted targets of the DELLA transcription factors were very significantly enriched in the P and E samples (Table I; Supplemental Table S3). In contrast, auxin-responsive genes were specifically down-regulated in E leaves (e.g. SHY1, SHY2, BDL, and PIN1) and, accordingly, the response of P and E leaves shared a significant similarity with the transcriptional response to chemical treatments employing auxin inhibitors (2,3,5-triiodobenzoic acid, 2,4,6-trichlorophenoxyacetic acid) (Fig. 7A; Supplemental Table S3).

**Cell wall metabolism is affected in the expanding leaves**

To explain the reduced growth of mannitol-grown seedlings, genes involved in cell division and cell expansion are among the prime suspects. Osmotic stress resulted in the differential expression of many cell wall-related genes of which the majority in the E leaves (Fig. 8A; Supplemental Table S2). Overall, genes related to cell wall synthesis (cell wall precursors and cellulose synthesis) and those encoding arabinogalactan proteins were down-regulated while genes involved in conferring cell wall extensibility (xyloglucan transferases and expansins) and cell wall degradation were evenly distributed among up- and down-regulated transcripts. Interestingly, expression was induced for three pectin methyl-esterase (PME) genes that can mediate changes in cell wall pH. Additionally, considering the proposed role of superoxide in affecting cell wall extensibility, we utilized nitrobluetetrazolium (NBT) staining to investigate superoxide accumulation in the growing and M leaves. This analysis revealed that superoxide levels increased exclusively in mannitol-grown E leaves (Fig. 8B) while no accumulation could be detected in P or M leaves. No significant differences were found in transcripts of either the core cell cycle (Vandepoele et al., 2002) or associated with cell division (Menges et al., 2003) is consistent with identical cell proliferation rates at the time P samples were taken.

**Alternative respiration is of key importance for cell division**

Of the interesting transcripts induced by mannitol exclusively in the P
leaves was the gene encoding an alternative oxidase (AOX1a) that plays a major role in the alternative respiratory pathway in Arabidopsis mitochondria (Giraud et al., 2008, 2009; Ho et al., 2008). AOX1a was strongly co-expressed with a number of genes referred to as "mitochondrial dysfunction regulon" (Van Aken et al., 2007), containing other mitochondrial genes (mitochondrial small heat shock protein HSP23.5, AAA-type ATPase BCS1, NADH dehydrogenase NDB4, and unknown proteins targeted to the mitochondria), MATE transporters, glutathione-S-transferases, transcription factors, cytochrome P450, and steroid sulfotransferase. Sixteen out of these 25 genes were also up-regulated by mannitol, of which 13 responded only in the P leaves (Fig. 9A). To confirm these changes and to provide further spatial resolution, promoter:β-glucuronidase (GUS) reporter lines were constructed for eight of the 13 overlapping "mitochondrial dysfunction regulon" genes (see “Materials and Methods). The obtained GUS staining agreed very well with the microarray data, confirming induction for seven out of eight genes in the P or P and E leaves (Fig. 9, A and B; Supplemental Fig. S8). At the spatial level, GUS induction was not just observed in fully P leaves, but also in leaves that were partially proliferating, i.e. had already started to differentiate at their tip (Fig. 9B; Supplemental Fig. S8). Moreover, this analysis allowed the response of other leaves to be studied. Induction was not restricted to leaf three but could be observed in young leaves throughout plant development. To investigate whether changes in expression of genes from the “mitochondrial dysfunction regulon” translated into a cellular phenotype, mitochondria of SAM and P leaves were examined by transmission electron microscopy (Fig. 9, C and D). A significant difference in the SAM sections of the mannitol-grown seedlings was the presence of large-sized mitochondria. In addition, in both SAM and leaf sections mitochondria were rounder that could be expressed as increased circularity index.

The proposed role of AOX is to prevent over-reduction of mitochondrial electron transport chain (mETC) under stress conditions and, therefore, the formation of ROS. Accordingly, superoxide levels were comparable in control and stressed P leaves visualized with NBT staining (Fig. 8B). Another proposed role of alternative respiration is to enable ATP production directly from the glycolysis and support growth under conditions in which the mETC is inhibited, such as
drought stress. To determine whether AOX plays a role in growth adaptation, the growth of AOX1a-overexpressing plants (AOX-OE) was measured with or without mannitol. Leaf areas were recorded daily between 9 and 23 DAS and the obtained data were used to calculate the percentage of reduction of leaf area caused by stress. Early on and under optimal conditions, the leaf area of AOX-OE plants was reduced by approximately 20%, but it caught up with control plants by 19 DAS (Fig. 9E). At the same time, the leaf area measured for plants grown on mannitol was the same or even larger for AOX-OE plants, which translated into a lower percentage of reduction, on average by approximately 7-8% (Fig. 9F). To investigate whether the enhanced growth of AOX-OE plants under osmotic stress would translate into better performance under drought, an in soil drought assay was developed. Water-saturated soil was used to germinate seeds, allowed to dry until it reached a relative water content (RWC) of 68% (control conditions), 60% (mild drought), or 55% (severe drought) and afterwards RWC was kept constant (Fig. 10A). Similarly to the in vitro AOX-OE, plants were smaller under normal conditions, but we measured no differences in daily changes in soil water status between wild-type and AOX:OE plants (Fig. 10B). Importantly relative growth rates of wild-type and AOX-OE plants measured under control conditions were not significantly different indicating that observed size reduction arose during early seedling establishment. Mild and severe drought resulted in decreased growth rates and, as a consequence, the final size of the wild-type rosettes was reduced by 16% and 27% (at 20 DAS), respectively (Fig. 10, C and D). Likewise to osmotic stress, this reduction was lower for the two independent AOX-OE lines due to higher growth rates (Fig. 10, C and E). In addition to over-expressors, also aox1a knockout plants were included in the experiment. These plants were 15% larger under normal conditions but had no growth phenotype under drought stress (Fig. 10B), possibly due to the redundancy with other AOX genes. In conclusion, our data indicate that changes in mitochondrial metabolism support cell proliferation under stress conditions.

DISCUSSION

Phenotypic plasticity of Arabidopsis leaves to water-limiting conditions
Phenotypic plasticity allows a plant to manage its resources under changing environmental conditions. Reduction of the final leaf size is an important adaptive response to many abiotic and biotic stresses. Stable soil water deficits affect both the final cell number and size of Arabidopsis leaves, however, it is still unclear how these changes arise (Aguirrezabal et al., 2006). Here, we provide insight into the kinetics of leaf growth under steady and relatively mild osmotic stress that similarly to drought reduces both cell number and size. Reduction of cell number was shown to occur during very early leaf development. Approximately from 4 days after leaf initiation (9 DAS) onward, cell division rates were indistinguishable or even slightly higher in stressed than in control plants. It is plausible that stress affects the duration of the first few divisions in the emerging leaves and/or fewer cells are recruited from the meristem into the leaf primordia. The latter would imply regulation of the leaf size at the shoot apical meristem (SAM) level that, to our knowledge, has not been described previously. On the contrary, the SAM size has previously been associated with number rather than with size of initiated leaves (Clark et al., 1993; Mauseth, 2004; Boucheron et al., 2005). Similarly to cell number, cell size was also reduced by stress early in leaf development with expansion rates that were lower during the first few days after cell differentiation and became indistinguishable or higher in stressed versus control plants from 15 DAS onward. These higher cell division and expansion rates measured late during the proliferation and expansion stages, respectively, marginally compensated the initial reduction in cell number and size. This observation is in agreement with previous studies demonstrating compensation occurring in Arabidopsis leaves subjected to drought (Aguirrezabal et al., 2006; Pereyra-Irujo et al., 2008). Stress not only affected final leaf size, but also the stomatal index, resulting in decreased stomatal density that can be interpreted as an adaptation to minimize water loss. Similar observations had been made previously for birch (Pääkkönen et al., 1998) and senna (Ratnayaka and Kincaid, 2005). While the number of stomata decreased, trichome density increased. Trichomes are known to positively affect the water economy in leaves, primarily through the increased reflection of solar radiation, and reduction of leaf temperature and, hence, transpiration rate. Accordingly, trichome density has proved to be an important
adaptive trait to drought stress (Gianoli and González-Teuber, 2005). It would be interesting to investigate how mild drought stress affects the signaling networks involved in stomata and trichome development. In conclusion leaf growth rate can very well adapt to stress conditions and the initial reduction of cell proliferation and cell expansion can be considered as an important adaptive response to generate smaller organs. Importantly, the obtained growth data provided a basis for sample selection for further profiling analysis. As at the time of sampling, growth rates of stressed and control plants were identical, growth adaptation rather than growth repression was clearly under investigation.

Response of Arabidopsis leaves to osmotic stress depends on the developmental stage

Developmental input into stress response of growing and mature leaves is the major contribution of the presented work and allowed the identification of candidate processes involved in growth adaptation to stress. Although it could be anticipated that the response to osmotic stress would differ between leaves at different developmental stages, the extent of these differences was greater than expected. Only 20 genes, mainly general stress markers such as CYP79B2 and chitinase (At2g43570), were up-regulated by osmotic stress at all three leaf stages, while hundreds of transcripts and a number of metabolites changed exclusively at one leaf stage. Importantly, osmotic stress response of proliferating (P) and expanding (E) leaves shared high overlap, pointing to common mechanisms that allow growth adaptation to stress in addition to stage-specific processes. A much smaller, but significant, overlap was also measured between stress response of E and mature (M) leaves, both at the transcript and metabolite level, most probably illustrating their photosynthetic status. Virtually no overlap was detected between P and M leaves. Additionally, the obtained results strongly argue that by zooming in on selected developmental stages, a serious dilution of information is avoided, and that even relatively mild stress results in hundreds of transcript and multiple metabolite changes that would not be identified using whole-seedling data. Finally our work demonstrated that young Arabidopsis seedlings are most similar to the expanding leaves. In conclusion, similarly to the
transcriptional responses to high salinity of specific root cell types (Dinneny et al., 2008), the presented work argues for the importance of spatial and developmental resolution to fully understand different aspects of stress tolerance of multicellular organisms.

**Classical stress response dominates in mature leaves**

Published profiling experiments, performed mainly on M leaves (Bouchabke-Coussa et al., 2008; Giraud et al., 2008; Perera et al., 2008; Zhang et al., 2008) or complete plant shoots (Kilian et al., 2007; Seki et al., 2002; Catala et al., 2007; Matsui et al., 2008; Weston et al., 2008; Zeller et al., 2009) and under relatively severe drought or osmotic conditions (Seki et al., 2002; Catala et al., 2007; Matsui et al., 2008; Urano et al., 2009), have led to the identification of a set of transcriptional and metabolic responses associated with drought stress. Also in our experiments, M leaves showed the classical drought stress response, validating the treatment (for an overview, see Fig. 10), but the response was much less pronounced in E leaves and only marginal in P leaves. Accumulation of LEA proteins, proline, and flavonoids in the M leaves could be related to their role in protecting the photosynthetic and enzymatic machinery of source leaves against oxidative damage that would be less important in E, but particularly, in P leaves (Havaux and Kloppstech, 2001; Mowla et al., 2006; Verslues et al., 2006 and references within). Accordingly, diaminobenzidine (DAB) and NBT staining revealed no differences in either hydrogen peroxide (H₂O₂) or superoxide (O₂⁻) levels in M leaves or in the operating efficiency of photosystem II. In fact, both starch and reducing sugars (glucose and fructose) accumulated in both E and M leaves indicating carbon abundance. Glucose could contribute to a decrease in cell water potential and has been observed to increase in Arabidopsis and in grapevine (Vitis vinifera), subjected to long-term drought (Rizhsky et al., 2004; Cramer et al., 2007). The role of starch is less clear and is possibly the indirect affect of glucose accumulation. In addition, production of waxes and cuticle thickening, as circumstantially suggested by the microarray data, would further prevent water loss from the leaf surface (Samuels et al., 2008). In conclusion, M and, to a lesser extent, E leaves tolerate osmotic stress by exploiting classical
drought responses, validating the osmotic stress assay as a good proxy for drought stress.

DELLA and ethylene crosstalk in growth regulation of P and E leaves

Only few genes and metabolites have been shown to be involved in regulation of leaf growth under adverse environmental conditions (Granier and Tardieu, 2009). Among these, a prominent role belongs to the DELLA TFs. Different abiotic and biotic stresses affect GA levels, stabilizing DELLA which in turn restrict plant growth and activate tolerance mechanisms - possibly by modulating the ROS metabolism (Achard et al., 2006, 2008; Navarro et al., 2008). Microarray analysis revealed a clear involvement of DELLA and ethylene-responsive genes in the adaptation of the P and E leaf tissues to mild osmotic stress. Ethylene, which accumulates under stress, has importantly been shown to crosstalk with DELLA factors, promoting their stabilization in both GA -dependent and -independent manners (reviewed by Yoo et al., 2009). Thus, ethylene and GA seemingly play a major role in regulating both cell proliferation and expansion under stress conditions, whereas ABA is mainly important in the mature tissues. Further support of this finding was provided by the proposal that ethylene is a primary drought signal for growing leaves, while ABA for the M leaves of tomato (*Solanum lycopersicum*) (Sobeih et al., 2004). The importance of ethylene signaling could be further validated with ethylene-insensitive mutants that were much more sensitive to mild osmotic stress. The strength of the phenotype corresponded very well with the degree of ethylene insensitivity. While *ein2.5* plants were characterized by almost complete growth arrest, the performance of the weak ethylene-insensitive mutant *eil1* was comparable with that of wild-type plants. Activation of the ethylene signaling could also explain the up-regulation of at least proportion of genes classically associated with biotic stress measured in the growing leaves and these will be interesting targets to study for their role is osmotic stress tolerance. Extensive overlap between abiotic and biotic signaling has been reported previously, so it is not surprising that genes classically associated with biotic stresses could also play an important role under abiotic conditions (Narusaka et al., 2004; Kankainen et al., 2006; reviewed by Fujita et
al., 2006; Walley et al., 2007). Good examples are the disease resistance regulator OCP3, PR-3, and GmERF3, recently shown to confer tolerance not only to biotic, but also abiotic, agents including drought, salt and osmotic stress (Seo et al., 2008; Ramírez et al., 2009; Zhang et al., 2009). In summary, although abscisic acid, ethylene, and DELLA proteins have been implicated in stress tolerance previously, our data provide a temporal context, showing that they are developmentally separated. Additionally, our work provided a number of further candidate genes classically associated with biotic stress, such as WRKY (WRKY53, WRKY15, and WRKY33) and ERF (ERF5 and ERF2) transcription factors, to test their function in stress tolerance and growth adaptation to osmotic stress. Interestingly, WRKY15 and WRKY33 were also among the list of DELLA target genes (Navarro et al., 2008).

Cell wall loosening drives cell expansion at the lower turgor pressure

Water loss changes turgor pressure and, thus, directly affects cell expansion. Previous work, done primarily on maize roots, has demonstrated that cell wall loosening facilitates growth at low turgor pressures. Abundance and activity of cell wall loosening enzymes (expansins and xyloglucan endotransglucosylase /hydrolases [XETs]), pH, ROS accumulation, and cellulose production have been proposed to be involved in the increasing cell wall extensibility (Wu and Cosgrove, 2000; Sharp et al., 2004; Zhu et al., 2007; Moore et al., 2008). We provide evidence for similar mechanisms acting in E leaves of Arabidopsis subjected to osmotic stress. Superoxide levels were exclusively elevated in the E leaves, consistent with earlier findings showing that the generation of hydroxyl radicals from superoxide and H2O2 plays a direct role in cell wall loosening via polysaccharide cleavage under both favorable and drought stress conditions (Liszkay et al., 2003; Zhu et al., 2007). Furthermore, up-regulated was expression of a number of genes involved in regulation of cell wall extensibility (reviewed by Moore et al., 2008) encoding expansins and XTHs including EXP10 and XTH19 (Cho and Cosgrove, 2000; Vissenberg et al., 2005; Osato et al., 2006). Wall-loosening activity of expansins has been linked to acidic growth (Cosgrove, 2000) and microarray data revealed up-regulation of three
genes encoding pectin methyl-esterase (PME) (AT4G02330, AT2G26440, and AT4G33220) that can reduce the cell wall pH by converting methoxyl groups of pectins to carboxyl groups accompanied by proton release (Wen et al., 1999). Finally, expression of cellulose synthesis genes was reduced which might result in further cell wall weakening. Decrease of cellulose content was observed in tobacco (*Nicotiana tabacum*) cell cultures treated with high concentration of PEG, which had been proposed to divert carbon from cell walls into compatible solute synthesis, contributing to the osmotic adjustment (Iraki et al., 1989). In conclusion, the presented data emphasize the importance of cell wall for growth adaptation of Arabidopsis leaves subjected to long-term osmotic stress and identify a number of candidate genes involved in cell wall loosening, such as XTH19 and three pectin methylesterases.

**Alternative respiration is essential in the proliferating leaves**

The “mitochondrial dysfunction regulon” comprises 25 genes that are tightly co-expressed under a number of stress treatments that affect mitochondrial electron transport chain and up-regulated in the transgenic lines over-expressing mitochondria-associated prohibitin genes (Van Aken et al., 2007). Although the exact role of the mitochondrial dysfunction regulon is still subject of debate, proteins encoded by the regulon are probably involved in the maintenance of mitochondrial function and morphology. One of the regulon genes encodes an alternative oxidase that shortcuts the mitochondrial electron transport chain (mETC) by transferring electrons directly to oxygen, providing plants with an alternative electron transport pathway. As alternative respiration limits oxidative phosphorylation and ATP production it is mainly employed under stress conditions that negatively affect components of the mETC and ATP-synthase complex, thereby preventing ROS formation (Sweetlove et al., 2002; Umbach et al., 2005; Rhoads et al., 2006; Giraud et al., 2008; Ho et al., 2008). Moreover, together with cytosolic or mitochondrial NADH dehydrogenase, AOX provides a path to recycle cytosolic NAD⁺-supporting glycolysis and ATP synthesis, via substrate phosphorylation, or to recycle NAD⁺ required for the TCA cycle and, hence, nucleotides and amino acid synthesis, respectively (reviewed by Fernie et
al., 2004). As energy, nucleotides, and amino acids are indispensable for cell division and ROS can cause serious damage to replicating DNA, alternative respiration would be certainly of central importance for growth maintenance in the P leaves. In accordance, expression of AOX1a, NDB4, and other genes of the mitochondrial dysfunction regulon was induced in the P, but not E or M leaves. Moreover, larger and rounder mitochondria found in the actively proliferating cells of the SAM provide further evidence to link “mitochondrial dysfunction regulon” with maintenance of cell divisions rates under stress conditions. Promoter:GUS lines confirmed the induction of genes of the mitochondrial dysfunction regulon in young P and differentiating leaves throughout plant growth. As anticipated, growth of AOX-OE plants was less reduced by mild osmotic stress already very early on during leaf development, indicating that cell proliferation was responsible for the observed phenotype. Importantly, better growth performance was also measured under controlled drought treatment in soil, providing good evidence that osmotic stress can be used as a good proxy for drought. Importantly we measured no differences in daily changes in soil water status between wild type and AOX:OE plants indicating that all plants experienced the same control and stress conditions and so observed growth improvement can not be solely attributed to smaller AOX:OE rosette size. Smaller and larger rosettes of AOX-OE and aox1a plants, respectively, further indicate that mETC is a preferable energy source under favorable conditions and alternative oxidation under stress conditions. Interestingly, enhanced growth was also observed for AOX-OE under cold, although the cellular basis of this finding was not investigated (Fiorani et al., 2005) and the aox1a mutant was much more sensitive to combined drought and light stress (Giraud et al., 2008). In conclusion profiling data followed by targeted analysis of the transgenic plants proved that alternative respiration supports cell proliferation under stress conditions. Importantly; these results also indicate a prominent role for the other “mitochondrial dysfunction regulon” genes that will be further studied in relation to growth and stress regulation.

Conclusions

This study demonstrates that rather than being a secondary effect of
compromised photosynthesis and carbon limitation, reduction of leaf growth is an important adaptive response to osmotic stress. Molecular profiling of actively growing and mature leaves offered developmental resolution to stress responses allowing the distinction of biological processes important for mature and/or growing leaves. By zooming in on selected developmental stages a serious dilution of information was avoided, revealing new insights and demonstrating that many changes measured in whole plants are in fact developmentally separated. Obtained data indicate that both cell proliferation and expansion are regulated by common regulatory cascades involving ethylene and GAs but not ABA signaling while down-stream effector genes are stage specific. Among these are enzymes supporting cell expansion under low turgor pressure and mitochondrial genes crucial for maintaining cell proliferation. To validate profiling data the latter was confirmed by using transgenic lines over-expressing the AOX1a gene with both osmotic and drought stress assays. Notably, data mining identified further candidate genes that would be interesting to study in relation to growth and stress regulation, such as those from the “mitochondrial dysfunction regulon” and those encoding WRKY and ERF transcription factors. In summary, this work significantly contributes to the understanding of growth and stress physiology by providing a developmental input into stress responses and demonstrating the importance of mitochondrial metabolism in the proliferating leaves.

MATERIALS AND METHODS

Plant growth

Seedlings of Arabidopsis thaliana (L.) Heyhn. ecotype Columbia-0 (Col-0) were grown in vitro in half-strength Murashige and Skoog medium (Murashige and Skoog, 1962), supplemented with 1% sucrose under a 16-h day (110 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) and 8-h night regime. Before autoclaving, 25 mM mannitol (Sigma) was added to the agar medium, while PEG-infused plates were prepared according to Van der Weele et al. (2000) with 10 mM overlay of PEG 3500 (Sigma).
Growth analysis

Leaf 3 was harvested daily from 9 until 22 DAS from 8-10 plants in three independent experiments. After clearing with 70% ethanol, leaves were mounted in lactic acid on microscopic slides. Epidermal cells (40-100 cells) were drawn for four leaves with a DMLB microscope (Leica) fitted with a drawing tube and a differential interference contrast objective, while leaves were photographed with a binocular. Photographs of leaves and drawings were used to measure the leaf area and the cell size, respectively, with the ImageJ software. Leaf area and cell size were subsequently used to calculate cell numbers. The stomatal index is percentage of stomata per all cells. Means of leaf area, cell size, and cell number were transformed logarithmically and locally fitted to a quadratic function of which the first derivative was taken as the relative growth rate (De Veylder et al., 2001). Mean values of the three biological experiments were used for statistical analysis.

Drought assay

Water deficit was imposed by controlling and stabilizing the soil water status during development of soil-grown plants. Plants were germinated in cylindrical polypropylene pots (200 ml, diameter 53 mm, height 88 mm; VWR International) cut on their sides to allow faster desiccation and filled with water-saturated soil. The soil was allowed to dry until it reached an RWC of 68%, 60%, and 55%, and subsequently watered daily to maintain a constant water status based on the pot weight. RWC was defined as the ratio of % water:dry soil, which in this case was always 30 g±0.5 g (RWC=mass of water/mass of water + mass of dry soil). Photographs were taken daily and used to extract total rosette areas that were subsequently used to calculate relative growth rates [(RLGR= (ln rosette area day2- ln rosette area day1)/time)]. Plants were grown under 110-120 μmol m⁻² s⁻¹ light and 8-h night regime. A detailed description of the drought assays will be published elsewhere.

Sampling for profiling analysis
Leaf 3 was harvested from plants at 9, 15, and 22 DAS, while seedlings reached stage 1.03 (third leaf, 1 mm in size) around 11 DAS. All samples were from three independent experiments and from multiple plates within the experiment. Complete harvest was done in growth chambers starting at 2 h into the day and took less than 15 min. As leaf initiation and developmental timing were not affected, samples were harvested simultaneously from both control and mannitol-treated plants. Because of their small size, leaves from plants at 9 DAS were dissected under a binocular microscope. Briefly, whole seedlings were harvested in an excess of RNAlater solution (Ambion) and, after overnight in 4°C, dissected on the cooling plate under the binocular microscope with precision microscissors. Dissected leaves were transferred to a new tube, frozen in liquid nitrogen, and ground with a Retsch machine and 3-mm metal balls. Microarray analysis, qRT-PCR confirmation, and metabolite measurements were carried out on material harvested from separate experiments.

RNA extraction

RNA was extracted with TriZol according to the manufacturer's protocol (Invitrogen) and 4 μg of glycogen as carrier during the precipitation step. RNA samples were subjected to DNA digestion (Roche) and subsequently cleaned-up with RNeasy clean-up kit (Qiagen).

ATH1 expression profiling and data analysis

RNA samples (three biological replicas for each treatment and stage) were hybridized to single Affymetrix ATH1 Genome arrays at the VIB Microarray Facility (Leuven, Belgium). Expression data were processed with Robust Multichip Average (RMA) background correction, normalization, summarization, and BioConductor (Gentleman et al. 2004; Irizarry et al. 2003a, 2003b). An alternative cdf ("tinesath1cdf") was used, in which each probe is uniquely assigned to one transcript (Casneuf et al., 2007) http://www.bioconductor.org/packages/release/data/experiment/html/tinesath1cdf.
BioConductor package *Limma* was used to identify differentially expressed genes (Smyth, 2004). A factorial design (mannitol treatment 25 mM-0 mM and developmental stage P-E-M) was applied to analyze the data. For comparisons of interest, moderated t statistics with the eBayes method were done and P values were corrected for multiple testing (for each contrast separately using topTable; Hochberg and Benjamini, 1990). In addition, we applied a more stringent correction for multiple testing across genes and across contrasts with decideTests (global) (Supplemental Tables S1 and S2). Importantly lists of significant genes obtained with both methods were very similar leading to identical conclusions. Besides the moderated t statistics for each pairwise contrast, we calculated global F statistics to identify the genes that were affected in at least one contrast (Supplemental Tables S1 and S2). Two-fold change in expression (only for 0-0 mM comparisons) and decideTest (global) <0.05 cut-offs were used to delineate gene lists of interest. Further subsets of these differentially expressed gene lists were identified and subjected to overrepresentation analysis.

**Comparison to publicly available microarray data**

Selected public microarray data were grouped according to experiment type (e.g. abiotic stress and hormone treatment) (see Supplemental Table S3). Groups of experiments were RMA processed and subjected to *Limma* analysis, as described above. Sets of responsive genes were delineated always with a 2-fold expression change and false discovery rate (FDR)-corrected P value < 0.05 cut-offs. Although these cut-offs were chosen somewhat arbitrarily, we assessed the robustness of the results by testing more and less stringent cut-offs. All tests gave very similar results (data not shown). The lists of responsive genes were compared to those identified in our microarray experiment to identify global trends in the functional repertoire of the affected genes that were used as hints to explore the results in more detail. Overrepresentation was tested by means of Fisher exact tests followed by Bonferroni P value correction.

**Metabolite analysis**
Metabolite data were obtained from nine samples harvested from three independent experiments. The relative levels of metabolites were determined with an established gas chromatography time-of-flight mass spectrometry protocol exactly as described by Lisec et al. (2006). Chromatograms and mass spectra were evaluated by Chroma TOF 1.6 (Leco, St Joseph, MI) and TagFinder 4.0 (Luedemann et al. 2008). The amount of metabolites was analyzed as relative metabolite abundance calculated by normalization of the signal intensity to that of 13C-sorbitol, which was added as an internal standard and fresh weight.

qRT-PCR

For cDNA synthesis, 100 ng-2 μg of RNA was used with the SuperScript Reverse III reagent (Invitrogen) according to the manufacturer's instructions. Primers were designed with the QuantPrime (Arvidsson et al., 2008) website (Supplemental Table 5 online). qRT-PCR was done on a LightCycler 480 (Roche Diagnostics) in 384-well plates with LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's recommendations. Melting curves were analyzed to check primer specificity. Normalization was done against the average of housekeeping genes UBQ10, GAPDH, and CBP20; \( \Delta Ct = Ct \text{ (gene)} - Ct \text{ (mean (housekeeping genes))} \) and \( \Delta \Delta Ct = \Delta Ct \text{(control)} - \Delta Ct \text{(mannitol or PEG)} \). \( \Delta Ct \) values for the three biological replicates were used for statistical analysis. Ct refers to the number of cycles at which SYBR Green fluorescence reaches an arbitrary value during the exponential phase of the cDNA amplification.

Lugol's, NBT, and GUS staining

Multiple plants at 15 and 22 DAS were cleared in 70% ethanol, stained for 20 min with excess of Lugol's solution (Sigma), and subsequently washed with water. Multiple leaves from three independent experiments were stained for 1 h with 0.1% nitrobluetetrazolium (NBT) (Sigma) solution in complete darkness and, subsequently, cleared in 80% boiling ethanol.
Whole plantlets were harvested after 9, 15 and 22 days and incubated in 90% acetone (4°C) for 30 minutes, washed in 100 mM Tris.HCl/50 mM NaCl (pH 7.0), and subsequently incubated in 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) buffer [100 mM Tris.HCl/50 mM NaCl buffer (pH7.0), 2 mM K3[Fe(CN)6] and 4 mM X-gluc] at 37°C for 2.5 hours. Seedlings were washed in 100 mM Tris.HCl/50 mM NaCl (pH 7.0) and cleared overnight in 90% lactic acid. Samples were photographed with a differential interference contrast microscope (Leica, Vienna, Austria).

Transmission electron microscopy

Young leaves and SAM of 9-DAS-old seedlings were excised and immersed in a fixative solution of 2% paraformaldehyde and 2.5% glutaraldehyde and postfixed in 1% OsO4 with 1.5% K3Fe(CN)6 in 0.1 M Na-cacodylate buffer, pH 7.2 for 1 h under vacuum infiltration at room temperature and 4 h rotation at room temperature, followed by overnight fixation at 4°C. After washing 3 times for 20 min with the buffer, samples were dehydrated through a graded ethanol series, including a bulk staining with 2% uranyl acetate at the 50% ethanol step, followed by embedding in Spurr’s resin. Ultrathin sections of a gold interference color were cut with a ultramicrotome (Leica EM UC6), post-stained with uranyl acetate and lead citrate (Leica EM AC20), collected on formvar-coated copper slot grids, and viewed with a transmission electron microscope 1010 (JEOL, Tokyo, Japan).

Transgenic lines and mutants

Seeds of AOX-overexpressing lines were kindly provided by Prof. James E. Siedow (Duke University, USA) (Fiorani et al., 2005). CYCB1;2:DB-GUS lines were a kind gift of Dr. Peter Doerner (University of Edinburgh). Generation of promoter:GFP-GUS lines. Genomic DNA was isolated from Arabidopsis (Col-0) with DNeasy plant kits (Qiagen) according to the manufacturer’s instructions. 1500 base pair genomic regions (or the intergenic region in the case of near-by coding sequences) upstream of the specific start codon were amplified by PCR
with the Platinum Taq High Fidelity DNA polymerase (Invitrogen) and the forward and reverse primers (Supplemental Table S5). The PCR products were cloned into pDONR221 and cloned by recombination to pBGWFS7, generating a transcriptional GFP-GUS fusion. The constructs were transformed into Arabidopsis Col-0 by Agrobacterium-mediated floral dipping. Transformants with bar resistance gene were selected by spraying with 40 mg L\(^{-1}\) Pestanal (glufosinate ammonium; Sigma-Aldrich). Transformants with a single insertion locus, and subsequently homozygous lines, were selected by segregation analysis on MS medium containing 5 mg L\(^{-1}\) glufosinate ammonium. All analyses were performed with non-segregating homozygous T3 transgenic lines. Ethylene-insensitive and aox1a mutants were obtained from the Arabidopsis Seed Stock Center ((ein2.5 (N8844), ein3.1 (N8052), eil1 (655070), etr1.3 (N3070), ein4 (N8053), and aox1a (N584897)).

**Operating efficiency of photosystem II**

Operating efficiency of photosystem II was measured using IMAGING-PAM Chlorophyll Fluorometer (Heinz Walz) in three independent experiments.

**Accession numbers**

Microarray data were deposited in the GEO database (GSE16474).

**Supplement data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Variable phenotypes of PEG-grown plants.

**Supplemental Figure S2.** Percent reduction of cell number, size, and stomatal index measured for leaf three dissected from plants grown without or with 25 mM mannitol.

**Supplemental Figure S3.** Leaf ploidy is not affected by mannitol.

**Supplemental Figure S4.** Cellular analysis of PEG-grown plants.

**Supplemental Figure S5.** Classification of transcripts differentially
expressed during leaf development (0-0 mM).

**Supplemental Figure S6.** Validation of microarray data by q-RT-PCR.

**Supplemental Figure S7.** Overlap between osmotic stress and leaf profiling data.

**Supplemental Figure S8.** Validation of microarray data with promoter:GUS lines of the genes from the mitochondrial dysfunction regulon.

**Supplemental Table S1.** Genes differentially regulated during development.

**Supplemental Table S2.** Expression data for the mannitol treatment.

**Supplemental Table S3.** Comparison with publically available expression datasets.

**Supplemental Table S4.** GC/MS metabolite data.

**Supplemental Table S5.** List of primers used in q-RT-PCR experiments and cloning of promoter:GUS lines.

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**Figure Legends**

**Figure 1.** Mannitol set-up.
A, Plants at 22 DAS grown in the absence (left) or presence (right) of 25 mM mannitol. B, Leaf series (third leaf encircled). C, Leaf area calculation. Arrow marks leaf 3 used in further analysis. D, Measurement of operating efficiency of photosystem II. E, Images of the effective PS II quantum yield (left 0 mM/right 25 mM).

**Figure 2.** Kinematic analysis of leaf 3 dissected from plants grown with or without 25 mM mannitol from 9 to 22 DAS.
A, Leaf area. B, Cell number. C, Cell size. D, Relative leaf growth rate. E, Relative cell division rate. F, Relative cell expansion rate. G, Stomatal index. H, Example of cell drawings of epidermal cells. I, Cell perimeter per cell area (perimeter/area) from epidermal cells. Data ± SE are means of three independent experiments. Leaves (8-10) were used to measure leaf area. Cellular data are from four leaves in each experiment.

**Figure 3.** Experimental set-up.
A, Schematic representation of Arabidopsis leaf development. Proliferating (P) cells, red; expanding (E) cells, green; and mature (M) cells, white. Scale bar only applies for the leaves, but not to the representation of the SAM. Leaf 3 initiates approximately at 5 DAS; all cells proliferate at 9 DAS, expand exclusively around 15 DAS, and approach maturity at 22 DAS, both under control and stress conditions. Samples for profiling analysis were dissected at 9, 15, and 22 DAS. B, GUS activity staining of leaf 3 from CYCB1;2-GUS plants at 9 DAS grown without or with 25 mM mannitol. The expression of CYCB1;2 is closely related to cell division activity.

**Figure 4.** Functional analysis of transcripts that are significantly affected by osmotic stress.
A, Venn diagram grouping of genes differentially regulated by osmotic stress in P, E, and M leaves (global test, p-value<0.05). B, Log_{2} fold changes (25 mM-0 mM)
for all analyzed genes (>20000) used to construct scatter-plots. Note the similarity between responses of P and E leaves and almost no overlap between P and M stage. 

C, Functional analysis with MapMan categories and PageMan overrepresentation tool. Significantly enriched or depleted functional groups are represented in blue or red, respectively.

**Figure 5.** Distinct metabolite profiles of expanding and mature leaves subjected to osmotic stress are distinct. 
A, Relative abundance of all the measured metabolites. B, Log$_2$ fold changes (25 mM-0 mM) for all analyzed metabolites used to construct a scatter-plot. C, Venn diagram with metabolites listed.

**Figure 6.** Increased starch levels in mannitol-grown plants.
A, MapMan representation of starch metabolism. Red and blue, down-regulated and up-regulated genes, respectively. E25-E0 and M25-M0, expanding and mature leaves (25 mM-0 mM), respectively. B, Lugol’s staining of 15-DAS-old plants harvested 8 h into the day. Note intense blue staining of the mannitol-grown seedling.

**Figure 7.** Importance of ethylene signaling for stress tolerance of growing leaves.
A, Expression changes for the selected genes involved in ethylene, gibberellin and auxin signaling, and metabolism. Bold font indicates significance (global test, $p$-value<0.05). B, Analysis of ethylene insensitive mutants Plants that developed a severe phenotype (curled pale leaves, growth arrest) were scored and used to calculate percentage of the “healthy” looking seedlings. Star indicates significance (TTEST, $p$-value<0.05). Data ± SE are means of multiple plates. C, Wild type and *ein2.5* plants grown on medium without or with 25 mM mannitol.

**Figure 8.** Cell wall-related genes and superoxide levels affected by osmotic stress in the expanding leaves.
A, Expression changes for the selected genes involved in cell wall metabolism. Bold font indicates significance (global test, $p$-value<0.05). B, Superoxide levels
visualized in leaf 3 from 9 and 15-DAS-old plants grown without (left) or with mannitol (right) stained with NBT.

**Figure 9.** Importance of alternative respiration in proliferating leaves.  
A, Log$_2$-fold change of genes from the mitochondrial dysfunction regulon in long-term mannitol experiments measured in P, E, and M leaves. Arrows indicate genes used for promoter:GUS analysis; green, orange, and red arrows, expression validated by GUS staining, in one of two GUS lines, and not validated, respectively. B, Photographs of 15-DAS-old plants of two promoter:GUS lines, with arrow marked young, proliferating leaves (note induction of both genes). The red dot indicates the third expanding leaf (note induction of *At4g37370* gene only). Photographs of the third leaf from 9-DAS-old *At4g37370*:GUS line, showed a strong induction. C, Transmission electron (TEM) micrographs of control and mannitol-grown plants (proliferating leaf 3 and SAM) were used to calculate area and circularity of the mitochondria. Data ±SE are means of 40-60 mitochondria. D, TEM micrographs of SAM from control and mannitol-grown seedlings. Arrows point mitochondria clusters. E and F, Leaf area measured for leaf 3 dissected from control (Col-0) and *AOX1a*-overexpressing plants grown without or with 25 mM mannitol from 9 DAS until 22 DAS. Obtained data were used to calculate % reduction under (E) control (*AOX-OE* 0 mM; Col-0, 0 mM) and (F) stress conditions (25 mM/0 mM). Data ± SE are means of 8-10 leaves.

**Figure 10.** Growth of the *AOX-OE* plants is less reduced by in soil drought.  
A, Relative soil water content (RWC) determined throughout the experiment (from 6 until 20 DAS) based on pot weight taken before watering. Stress treatment started at 13 DAS (arrowhead) when control plants were first watered to RWC of 68%. Black and grey lines, Col-0 and *AOX-OE* plants, respectively. B, Percent reduction of *AOX:OE* and *aox1a* rosette area compared to wild-type (Col-0) plants measured under control conditions (RWC 68%). C, Percent reduction of Col-0 and *AOX-OE* rosette area under mild (RWC 60%) and severe (RWC 55%) drought compared to control conditions from 13 until 20 DAS (duration of stress treatment). D, Relative growth rates (RLGR) of Col-0 under control and drought conditions. Note reduction of RLGR associated with stress onset (arrowhead).
RLGR of Col-0 and AOX:OE plants under control and drought conditions. Arrowhead indicates start of stress treatment. Notice less reduced RLGR measured for AOX-OE plants (stars). Data ± SE are means from 8-10 plants for each genotype and treatment. AOXOE_X3 and AOXOE_XXL are two independent AOX1a-overexpressing lines while aox1a stands for AOX1a knockout mutant.

Figure 11. Schematic representation of processes, genes, and metabolites (bold) affected by stress (25-0 mM). TFs, transcription factors, PMEs, pectin methyl-esterases. Dark grey (red, online) represents proliferating, light grey (green, online) expanding and white mature cells.
Table I. List of selected key genes involved in the biological processes affected by osmotic stress. Expression changes are presented as LOG₂. Significance (global test, \( p \)-value<0.05) is in bold. DELLA targets identified by Navarro et al. (2007) are marked with X.

| Locus     | Name       | \( \log_2 P25-0 \) | \( \log_2 E25-0 \) | \( \log_2 M25-0 \) | DELLA |
|-----------|------------|---------------------|---------------------|---------------------|-------|
| **Abiotic stress markers** |            |                     |                     |                     |       |
| AT5G52300 | RD29B      | -0.19               | 0.31                | 1.61                |       |
| AT5G52310 | RD29A      | -0.59               | 1.52                | 1.40                |       |
| AT2G42540 | COR15A     | -0.13               | 0.88                | 1.12                |       |
| AT5G25610 | RD22       | 0.71                | 1.38                | 1.09                |       |
| AT2G42530 | COR15B     | -0.09               | 1.25                | 1.03                |       |
| AT1G20450 | ERD10      | -0.22               | 1.25                | 0.99                |       |
| AT2G17840 | ERD7       | -0.09               | 0.36                | 0.94                |       |
| AT1G01470 | LEA14      | -0.24               | 0.59                | 0.83                |       |
| AT2G30870 | ERD13      | 0.71                | 1.20                | 0.79                |       |
| AT4G19120 | ERD3       | -0.25               | 0.12                | 0.52                |       |
| **Fatty acids/cuticle**      |            |                     |                     |                     |       |
| AT1G01120 | KCS1       | 0.41                | 0.03                | 0.59                |       |
| AT2G38530 | LTP2       | 0.83                | 6.09                | 1.12                |       |
| AT2G38540 | LTP1       | 0.06                | 0.61                | 0.49                |       |
| AT1G02205 | CER1       | -0.07               | -0.09               | 1.01                |       |
| AT1G68530 | CUTICULAR 1| 0.27                | 0.61                | 0.46                |       |
| AT1G67730 | GLOSSY8    | 0.07                | 0.44                | 0.45                |       |
| **ABA synthesis**            |            |                     |                     |                     |       |
| AT5G20960 | AAO1       | 0.50                | 1.21                | 0.79                |       |
| AT3G43600 | AAO2       | 0.07                | 0.77                | 0.55                |       |
| **Flavonoids**               |            |                     |                     |                     |       |
| AT5G13930 | CHS        | -0.36               | -0.02               | 1.16                |       |
| AT5G08640 | FLS        | -0.46               | -0.31               | 0.87                |       |
| AT1G56650 | PAP1 TF    | -0.01               | 0.73                | 0.84                |       |
| **Biotic stress markers**    |            |                     |                     |                     |       |
| AT2G39200 | ATMLO12    | 0.93                | 3.08                | 0.40                |       |
| AT3G45290 | ATMLO3     | 1.55                | 1.54                | 0.29                |       |
| AT1G61560 | ATMLO6     | 0.56                | 0.82                | 0.00                |       |
| AT2G44490 | PEN2       | 1.60                | 1.01                | -0.04               | X     |
| AT4G01700 | Chitinase  | 2.26                | 1.94                | -0.11               | X     |
| AT1G33590 | Disease resistance. | 1.30 | 0.93 | 0.51 | X |
| AT3G45640 | MPK3       | 0.62                | 0.97                | 0.05                | X     |
| Gene       | Protein/Enzyme | Fold Change | F1 | F2 |
|------------|----------------|-------------|----|----|
| AT1G59580  | MPK2           | 0.42        | 0.47| 0.26|
| AT1G75040  | PR-5           | 2.19        | 3.65| 0.47|
| AT4G23810  | WRKY53         | 1.34        | 1.52| 0.04|
| AT2G38470  | WRKY33         | 1.08        | 2.75| 0.00|
| AT2G23320  | WRKY15         | 0.76        | 0.59| 0.17|

**Indole Glucosinolates/Calmexin**

| Gene       | Protein/Enzyme | Fold Change | F1 | F2 |
|------------|----------------|-------------|----|----|
| AT5G57220  | CYP81F2        | 1.82        | 3.80| 0.04|
| AT2G30770  | CYP71A13       | 2.42        | 2.65| 0.37|
| AT4G39950  | CYP79B2        | 1.61        | 2.14| 0.91|
| AT2G22330  | CYP79B3        | 0.19        | 1.28| 0.84|
| AT4G31500  | CYP83B1        | 0.24        | 0.89| 0.39|
| AT1G18570  | MYB51          | 0.99        | 1.68| 0.22|
### A

| Locus       | Name     | Log$_2$ P25-0 | Log$_2$ E25-0 | Log$_2$ M25-0 |
|-------------|----------|---------------|---------------|---------------|
| Ethylene    |
| AT3G23240   | ATERF1   | 0.12          | 0.40          | -0.11         |
| AT5G47220   | ATERF2   | 0.71          | 0.51          | -0.11         |
| AT3G15210   | ATERF4   | 0.17          | 0.87          | 0.53          |
| AT5G47230   | ATERF5   | 1.40          | 1.06          | 0.06          |
| AT2G40940   | ERS1     | 0.50          | 0.40          | 0.36          |
| AT1G04310   | ERS2     | 0.71          | 0.53          | -0.28         |
| AT5G61600   | ethylene res. | 0.24 | 0.71 | 0.14 |
| AT2G22300   | ethylene res. | 0.53 | 0.39 | 0.11 |
| Gibberellins|
| AT5G25900   | GA3      | -0.07         | 0.66          | 0.69          |
| AT3G03450   | RGL2     | -0.10         | -0.52         | 0.20          |
| AT4G25420   | ATGA20OX1| -0.13         | -0.70         | -0.29         |
| Auxin       |
| AT1G04550   | IAA12    | 0.00          | -0.41         | -0.24         |
| AT2G33310   | IAA13    | -0.17         | -0.50         | -0.33         |
| AT2G28350   | ARF10    | -0.18         | -0.53         | -0.25         |
| AT1G73590   | PIN1     | -0.20         | -0.54         | -0.02         |
| AT5G43700   | IAA4     | 0.10          | -0.55         | 0.35          |
| AT4G13260   | YUCCA2   | -0.72         | -0.55         | -0.79         |
| AT1G44350   | ILL6     | -0.11         | -0.97         | 0.03          |
| AT1G52830   | IAA6     | -0.12         | -0.99         | -0.46         |

### B

![Graph showing % healthy plants 25mM mannitol](image)

### C

![Images comparing Col-0, ein2.5, and ein3.1](image)
### Cell wall

| Locus         | Name                          | Log₂ P25-0 | Log₂ E25-0 | Log₂ M25-0 |
|---------------|-------------------------------|------------|------------|------------|
| AT5G55730     | FLA1                          | -0.29      | -0.39      | 0.31       |
| AT4G12730     | FLA2                          | -0.49      | -0.73      | -0.11      |
| AT4G39350     | CELLULASE SYNTHASE 2          | 0.13       | -0.31      | -0.03      |
| AT5G64740     | CELLULASE SYNTHASE 6          | -0.23       | -0.32      | 0.01       |
| AT5G09870     | CELLULASE SYNTHASE 5          | -0.36       | -0.46      | -0.16      |
| AT4G30290     | XTH19                         | 0.59       | 1.76       | 0.45       |
| AT1G26770     | EXP10                         | 0.31       | 0.56       | 0.29       |
| AT3G55500     | EXP16                         | -0.11       | 1.00       | -0.09      |
| AT4G02330     | PME                           | 2.07       | 4.94       | 0.65       |
| AT4G33220     | PME                           | 0.29       | 0.93       | 0.48       |
| AT2G26440     | PME                           | 0.27       | 1.24       | -0.04      |

**B**

NBT staining 15_DAS

NBT staining 9_DAS

0mM

25mM
A: Bar chart showing Log2 fold change for various genes.

B: Images illustrating various stages of plant development.

C: Graphs showing changes in area and circularity for different treatments.

D: Microscopic images highlighting changes in tissue structure.

E: Bar chart showing percentage reduction for different treatments.

F: Bar chart showing percentage reduction for different treatments.
Stress (25mM-0mM)

Up: mitochondrial metabolism – alternative respiration

Up: biotic stress markers e.g. WRKY TFs; ethylene and GAs signalling

Up: cell wall loosening enzymes, PMEs, $O_2^-$

Down: auxin signalling, cellulose synthesis, amino acids

Up: lipid metabolism, ABA signalling, LEA proteins, starch, glucose

Up: flavonoids, proline, putrescine, erythritol