Short title: Reduced drought sensitivity through cell expansion

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Distinct Cellular Strategies Determine Sensitivity to Mild Drought of Arabidopsis Natural Accessions¹ [OPEN]

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One-sentence summary:

Efficient closure of stomata and maintenance of cell expansion during drought conditions are crucial to maximally preserve plant growth during water deficit.

Footnotes:

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Author Contributions

Y.C., H.V. and D.I. conceived the original screening and research plans; H.V., M.D. and D.I. supervised the experiments; Y.C. performed most of the experiments; M.V. provided technical assistance to Y.C.; Y.C.,

M.D. and H.V. designed the experiments and analyzed the data; Y.C. conceived the project and wrote the article with contributions of all the authors; M.D, D.I. and H.V. supervised and completed the writing. D.I. agrees to serve as the author responsible for contact and ensures communication.

Abstract

The world-wide distribution of *Arabidopsis* (*Arabidopsis thaliana*) accessions imposes different types of evolutionary pressures, which contributes to various responses of these accessions to environmental stresses. Responses to drought stress have mostly been studied in the Columbia accession, which is predominantly used in plant research. However, the reactions to drought stress are complex and our understanding of the responses that contribute to maintaining plant growth during mild drought is very limited. Here, we studied the mechanisms with which natural accessions react to mild drought at a physiological and molecular level during early leaf development. We documented variations in mild drought responses among natural accessions and used transcriptome sequencing of a drought-sensitive accession, ICE163, and a drought-insensitive accession, Yeg-1, to gain insights into the mechanisms underlying this discrepancy. This revealed that ICE163 preferentially induces jasmonate- and anthocyanin-related pathways, which are beneficial in biotic stress defense, whereas Yeg-1 has a more pronounced activation of abscisic acid signaling, the classical abiotic stress response. Related physiological traits, including content of proline, anthocyanins and reactive oxygen species, stomatal closure and cellular leaf parameters, were investigated and linked to the transcriptional responses. We can conclude that most of these processes constitute general drought response mechanisms that are regulated similarly in drought-insensitive and -sensitive accessions. However, the capacity to close stomata and maintain cell expansion under mild drought appeared to be major factors that allow to better sustain leaf growth under mild drought.

INTRODUCTION

With the increasing effects of global warming and climate change, agricultural crop production is facing major challenges. Drought is one of the main abiotic stresses across the world that limits agricultural yield by reducing plant growth (Araus et al., 2002). Drought stress can occur in a broad spectrum, ranging from mild drought, which leads to a decrease in growth, to severe drought in which plants suffer from desiccation and wilting, eventually leading to their death. In the previous decades, numerous studies have focused on severe drought stress (Iuchi et al., 2001; Cominelli et al., 2005; Fujita

et al., 2005; Yoo et al., 2010), but recent studies have demonstrated that plants apply different regulatory strategies to cope with mild drought compared to severe drought stress (Harb et al., 2010; Skirycz et al., 2011; Ma et al., 2014; Clauw et al., 2015). For example, only one third of differentially expressed genes overlap between severe drought and mild drought treatments in young developing *Arabidopsis* (*Arabidopsis thaliana*) leaves (Clauw et al., 2015) and plants that were described to be tolerant to severe drought stress did not perform better under mild drought conditions (Skirycz et al., 2011). Exploring the molecular and physiological strategies that plants apply to cope with mild drought stress is therefore essential to ensure our future agricultural productivity.

Being sessile, plants have evolved complex regulatory, transcriptional, metabolic, physiological and morphologic strategies to overcome drought stress (Claeys and Inzé, 2013; Tardieu et al., 2014). Under water-deficient conditions, plants limit their shoot growth to reduce water losses. The development of leaves, which is strictly controlled by cell proliferation and cell expansion (Gonzalez et al., 2012), can be significantly and rapidly affected by drought stress (Skirycz and Inzé, 2010); water deficit greatly suppresses cell expansion due to the low turgor pressure (Feng et al., 2016). Because leaves contribute to a large part of the plant aerial tissue and are the source of carbohydrates through photosynthesis, reductions in their size will lead to a significant penalty in biomass.

Phytohormones play an important role in responses to drought stress. The most well-known drought-related hormone, abscisic acid (ABA), accumulates rapidly during drought conditions (Christmann et al., 2005). The mechanisms of ABA-mediated drought stress responses have been well studied (for a review, see Nakashima and Yamaguchi-Shinozaki, 2013; Yoshida et al., 2014). During drought stress, several key elements of the ABA signaling pathway, such as *SNF1-RELATED PROTEIN KINASES 2* (*SnRK2s*) (Boudsocq et al., 2004), *ABSCISIC ACID-RESPONSIVE ELEMENT BINDING PROTEIN 1* (*AREB1*) and *AREB2* (Fujita et al., 2005) are upregulated. In addition, ABA accumulation by water deficit leads to the rapid closure of stomata (Israelsson et al., 2006), which increases water use efficiency and, hence, results in an improved survival during drought stress. On the other hand, jasmonates (JAs) and ethylene (ET), which are known to play essential roles in orchestrating plant defense against pests and pathogens, also influence abiotic stress tolerance (for a review, see Kazan, 2015). Under water-deficient conditions, JA interferes with redox processes and enhances the synthesis of antioxidative stress compounds, such as ascorbate and glutathione (Shan and Liang, 2010). Moreover, transcriptome studies have shown that JA-related genes are the most relevant class of non-ABA genes that are responsive to severe drought stress (Huang et al., 2008; Harb et al., 2010). The effect of ET on drought stress is bidirectional: it inhibits ABA-induced stomatal closure (Tanaka et al., 2005), but, on the other hand,

promotes stomatal closure by prompting NADPH oxidase-mediated production of scavenging reactive oxygen species (ROS) in stomatal guard cells (Desikan et al., 2006).

Some metabolites, such as anthocyanins and proline, also act in the defense against drought (Bhaskara et al., 2015; Li et al., 2017a). Anthocyanins are synthesized via the phenylpropanoid pathway in plants and have been described to play an important role in biotic and abiotic stress tolerance by absorbing ultra violet, high light irradiation and ROS (Landry et al., 1995; Mittler et al., 2004). During drought stress, an increased biosynthesis and decreased catabolism result in a higher accumulation of proline, limiting the retardation of growth (Kishor et al., 1995). Proline counteracts osmotic stress in several aspects: as an osmolyte (Handa et al., 1986), as a stabilizer of protein structures (Wu and Bolen, 2006), as a scavenger of free radicals (Smirnoff and Cumbes, 1989), as a sink for energy (Hare and Cress, 1997) and by maintaining the $\text{NADP}^+/\text{NADPH}$ balance (Sharma et al., 2011). Recent research also revealed a dynamic and accession-dependent proline accumulation under drought stress (Kesari et al., 2012; Bhaskara et al., 2015).

Adverse conditions can also trigger the production of ROS in plants, including superoxide anion radicals (O_2^-), hydroxyl radicals ($\text{OH}^\bullet$), hydrogen peroxide (H_2O_2) and singlet oxygen (O^1_2). ROS can act as signal transduction molecules in plants, but a high accumulation of ROS will lead to severe cellular damage, the inhibition of photosynthesis and even plant death (Dat et al., 2000). ROS accumulation during stress largely depends on the balance between ROS production and ROS scavenging (Mittler et al., 2004). Superoxide dismutases (SODs) act as primary enzymes in ROS scavenging. Overexpression of SOD-encoding genes can enhance the tolerance of plants to severe drought (Liu et al., 2013). In addition, the ROS balance in plants under stress conditions can also be regulated by JAs (Miller et al., 2010), anthocyanins (Li et al., 2017a) and proline (Liu et al., 2015).

These different, interconnected stress response strategies provide a broad regulatory potential to preserve plant fitness during unfavorable conditions (Pieterse et al., 2009; Berens et al., 2019). All of these responses have most often been studied in the context of moderate to severe drought that was applied to adult plants, but little is known about how plants that originate from different natural environments react to and coordinate their growth during mild drought stress. Arabidopsis has been used as a model for plant research for a long time and many natural accessions are widely distributed in various geographical areas. These diverse natural habitats each exert different evolutionary pressures, which allowed the accessions to develop different molecular and physiological strategies to adapt to their specific environment (Weigel, 2012). Hence, specific strategies to cope with water deficit can be expected between natural accessions. Exploring the specific responses of natural accessions to mild

drought can therefore give more insight into the diverse regulatory networks, which can be useful for plant breeders. Here, we compared the response of fifteen natural accessions to mild drought stress and we observed a strong diversity in their reduction of final leaf size. To identify the discriminating factors that underlie these phenotypic differences, we explored the transcriptomic changes of representative sensitive and insensitive accessions shortly after this mild drought treatment and identified different responses. Subsequent physiological experiments showed that the efficiency in stomatal closure and especially the capacity to maintain cell expansion during drought are crucial to preserve leaf growth.

RESULTS

Natural Arabidopsis Accessions Reduce Leaf Growth to a Different Extent under Mild Drought

To explore how genetic diversity in Arabidopsis can affect the response to mild drought stress, we screened the growth of fifteen natural accessions from different origins (Fig. 1A, Supplemental Table S1.1) on the automated Weighing, Imaging and Watering Machine (WIWAM) (Skirycz et al., 2011). The mild drought (MD) treatment was initiated for half of the plants at six days after stratification (DAS), when the third true leaf (L3) is emerging from the shoot apical meristem (Supplemental Fig. S1). A progressive soil drying was maintained for five days, until the soil moisture dropped from $2.2 \text{ g}_{\text{water}}/\text{g}_{\text{soil}}$ to $1.2 \text{ g}_{\text{water}}/\text{g}_{\text{soil}}$. The other half of the plants was kept under well-watered (WW) conditions to serve as a control (Fig. 1B, for the experimental details, see Material and Methods). Although natural accessions are known to display differences at the end of their vegetative stage, they only showed minor developmental differences during early development and the onset of drought initiation (Fig. 1B, Supplemental Fig. S1-16 and Supplemental Table S2), based on the description of Boyes et al. (2001). The plants were harvested at 22 DAS and the area of the mature L3 was measured. The average leaf area (LA) already differed between the accessions under WW conditions (Fig. 1C), but except for EY15-2, all accessions showed a significant relative reduction in LA under MD compared to WW conditions (Fig. 1C). Remarkably, the reduction in LA was very different depending the accession ($p=2.2\text{E-}16$; ANOVA, Ecotype x Treatment interaction), ranging from 14% to 61% (Fig. 1C, Supplemental Table S3). The sensitivity to MD (% reduction) did not depend on the size of the leaf under WW conditions, since no correlation between LA under WW conditions and relative reduction by MD could be observed (Supplemental Fig. S17). We identified drought-sensitive accessions, such as Oy-0, Ler-0, ICE97 and ICE163, as well as more insensitive accessions, including C24, Yeg-1, An-1, Sha and EY15-2. Finally, we wanted to verify if there is a correlation between the observed reduction in LA in our accessions and their geographic location of origin. Therefore, we integrated a large dataset detailing the local seasonal historical

frequency of drought weeks (HFDW) of a large collection of *Arabidopsis* natural accessions, calculated with satellite remote sensing (Monroe et al., 2018). However, no significant correlation could be observed between the reduction in LA in the accessions and their HFDW during winter ($p = 0.9926$), spring ($p = 0.49$), summer ($p = 0.5884$), fall ($p = 0.5509$) (Supplemental Table 1.2, Supplemental Fig. S18).

Mild Drought Triggers Different Transcriptomic Changes in ICE163 and Yeg-1

To gain further insights into the molecular mechanisms that underlie the different leaf growth responses under MD stress, we selected two accessions that were significantly affected by drought, but at opposite ends of the spectrum. ICE163 and Yeg-1 were chosen as representatives of sensitive and insensitive accessions, respectively (Fig. 1C, Supplemental Table S3). Using the same drought setup as described above, we isolated the actively growing L3 at five days after water retention (11 DAS), when the target weight of the $1.2 \text{ g}_{\text{water}}/\text{g}_{\text{soil}}$ was reached in the MD pots, for transcriptome profiling by RNA-sequencing. A multidimensional scaling (MDS) plot showed that the samples clustered mainly by ecotype (Supplemental Fig. S19A), illustrating the profound effect of genetic background on the transcriptome. Even in the absence of the stress treatment (WW conditions), ICE163 and Yeg-1 had very different basal gene expression levels. In total, 22.1% of all expressed genes were differentially expressed (DE) between both accessions, including 1853 and 2736 genes with higher expression in Yeg-1 and ICE163, respectively (Supplemental Fig. S19B). GO enrichment analysis of these DE genes showed different enriched biological processes in ICE163 and Yeg-1. For example, genes involved in the negative regulation of meiosis regulation and cell cycle DNA repair were enriched in Yeg-1, while in ICE163 photosynthesis and defense responses were overrepresented (Supplemental Table S4).

Next, we compared the transcriptomic responses that were altered by the MD treatment in ICE163 and Yeg-1, as shown in the workflow in Supplemental Fig. S20. First, we identified 514 genes that were DE in ICE163 and 430 genes in Yeg-1 (MD vs. WW per accession, $\text{FDR} \leq 0.05$, $|\log_2\text{FC}| \geq 0.6$). In the second step, common responsive genes were identified. In total, 178 genes (143 upregulated, 35 downregulated) were in commonly expressed between these accessions in the same direction (Fig. 2, A and B, Supplemental Fig. S21A). Within the common MD-induced genes, GO categories such as superoxide radicals ($\text{FDR} = 0.000213$), JA biosynthesis ($\text{FDR} = 0.0252$), cell wall modification ($\text{FDR} = 0.035$) and the ABA signaling pathway ($\text{FDR} = 0.0171$) were overrepresented (Supplemental Table S5). Overrepresented GO categories of the common downregulated genes included the response to ozone ($\text{FDR} = 0.0102$) and superoxide ($\text{FDR} = 0.00313$) (Supplemental Table S5).

To search for transcriptional mechanisms that could underlie the different phenotypic responses of Yeg-1 and ICE163 to drought, we further explored the specific DE genes in these accessions

(Supplemental Fig. S20, step 4). First, we selected genes that were DE in at least one accession and with a $\log_2FC$ (MD vs. WW) difference between two accessions equal or larger than 1. As such, 138 genes were identified (Fig. 2C, Supplemental Table S6). Among these genes, 11 genes were DE in both accessions (but to a clearly different extent), and 79 and 48 genes were DE only in ICE163 or Yeg-1, respectively. Second, we identified 12 genes that showed a significant ecotype to treatment (E x T) interaction in the statistical model, with an FDR lower than 0.05 (Fig. 2D, Supplemental Fig. S21B). This set might include genes that were in the overlap of Figure 2A-B, but that were affected by MD to a much larger extent in one of the two accessions. To further interpret the differences in transcriptional responses between ICE163 and Yeg-1, we combined the genes identified with these two methods, which resulted in a list of 143 genes (Supplemental Fig. S21C). To get a better understanding of the specificity of our dataset, we compared our common and ecotype-specific genes with those of previous reports that studied the effect of mild drought on mature leaves (Des Marais et al., 2012) and proliferating leaves (Clauw et al., 2015; Clauw et al., 2016) of different accessions. Interestingly, an overlap of the accession-specific genes within each study showed almost no overlap with the other studies (Supplemental Fig. S22A). More surprisingly, very few overlap was found between the common genes from each study (Supplemental Fig. S22B), in which different GO categories were enriched (Supplemental Table S7).

A GO enrichment analysis was performed to gain further insight into the functional categories of these 143 genes that were different in both transcriptomes (Supplemental Fig. S20, step 5). The significantly top-enriched categories included anthocyanin synthesis, JA signaling, response to ROS and response to abiotic stimulus (Supplemental Table S8). We selected genes based on their annotated functions in one of these GO categories (Fig. 2E, Supplemental Fig. S20 step 6, Supplemental Fig. S21D, Supplemental Table S9). Interestingly, more JA- and anthocyanin-related genes were induced in ICE163, while more ABA-related genes were induced in Yeg-1 by MD. With reverse transcription quantitative PCR (RT-qPCR), we could confirm the observed differential expression of the majority of selected genes (Supplemental Fig. S23). As these results highlight that differences in drought sensitivity might be attributed to selective activation of anthocyanins, ROS, proline, and ABA mechanisms, we aimed at validating these transcriptomic findings by measuring these responses at physiological level. Wherever feasible, we included additional accessions that responded in an insensitive (EY15-2 and Sha) or sensitive (Oy-0 and ICE97) manner to the MD treatment.

Anthocyanins Are Effective ROS Scavengers and Proline Acts as General Response Compound to Mild Drought

Drought is known to induce oxidative stress in plants by generating ROS (Miller et al., 2010). ROS can act as signaling molecules, but a strong accumulation can severely hamper plant growth and lead to cell death.

Our GO analysis of genes that were DE in ICE163 and Yeg-1 during MD showed a potential involvement of ROS, as illustrated by the differential regulation of *COPPER/ZINC SUPEROXIDE DISMUTASE 2 (CSD2)* and *FE SUPEROXIDE DISMUTASE 1 (FSD1)* (Fig. 2E). Therefore, we examined the abundance of H₂O₂, one of the most prominent ROS, by performing a 3,3-diaminobenzidine (DAB) staining under WW and MD conditions. H₂O₂ levels, visualized as deep brown precipitates, were increased in the cotyledons of two insensitive accessions, Sha and Yeg-1, and two sensitive accessions, ICE97 and Oy-0 under MD, but not in EY15-2 and ICE163 (Fig. 3A). Therefore, no obviously consistent differences between the insensitive and sensitive accessions could be observed.

To keep the homeostasis of ROS, plants have evolved complex enzymatic and non-enzymatic antioxidant systems, such as SODs and metabolites that act as antioxidants (Gill and Tuteja, 2010; Soares et al., 2019). Proline accumulation is known to play a positive role in abiotic stress (Bhaskara et al., 2015). In addition to proline, anthocyanin-related genes were overrepresented in our GO analysis. Because both proline and anthocyanins are able to scavenge ROS (Gould et al., 2002; Signorelli et al., 2014), we measured their abundance in seedlings at five days after water limitation. Most accessions, except for Sha, accumulated proline to a similar level after the MD treatment (Fig. 3B). On the other hand, anthocyanins measurements revealed that the ecotypes that accumulated fewer H₂O₂, ICE163 and EY15-2 (Fig. 3A), had a significant increase in anthocyanin content during MD (Fig. 3C). These results suggest that in our MD conditions, proline, ROS or anthocyanin levels are not discriminating factors between sensitivity and insensitivity, but that anthocyanins might be effective in counteracting ROS.

Insensitive Accessions Close Their Stomata More Efficiently

The closure of stomata under drought conditions is tightly controlled by ABA (Israelsson et al., 2006), one of the hormones that was highlighted by the differential transcriptomic analysis in sensitive and insensitive accessions. We therefore measured the effect of drought on the stomatal closure of the insensitive and sensitive accessions at five days after water limitation. In WW conditions, Oy-0 and ICE163 (drought-sensitive accessions) showed already a higher ratio of open stomata than the other sensitive accession ICE97 and the three insensitive accessions Sha, Yeg-1 and EY15-2 (Fig. 4, A and B). Under MD, stomatal opening was significantly reduced in all accessions (Fig. 4, A and B), but we found that the insensitive accessions had fewer open stomata (10.3%, 4.7% and 8% for Sha, Yeg-1 and EY15-2, respectively) than the sensitive accessions (20.3%, 14.3% and 23.3% for ICE163, ICE97 and Oy-0,

respectively) (Fig. 4B). We also investigated other physiological parameters that are related to dramatic changes in stomatal opening, such as leaf surface temperature, photosynthetic efficiency and relative water content (RWC) (Li et al., 2017b) at 11 DAS. Under MD, the differences of leaf surface temperature were very minor between the examined natural accessions, ranging from 20.68°C in Sha, to 20.99°C in Oy-0 (Supplemental Fig. S24A). In addition, no significant difference in photosynthetic efficiency could be detected in the accessions (Supplemental Fig. S24B) and only Sha showed a decrease in RWC (Supplemental Fig. S24C) at 11 DAS.

On top of the stomatal closure, it is reasonable to think that accessions with a higher number of stomata in a given leaf area can experience more stress when the soil water content decreases. Previous work has shown that under MD conditions, plants with a lower stomatal density (SD, number of stomata per mm²) showed a lower transpiration and higher water use efficiency (Yoo et al., 2010; Franks et al., 2015). The SD of all sensitive and insensitive accessions was therefore analyzed at 22 DAS. Remarkably, the sensitive accessions ICE163 and ICE97 showed a significant increase in SD during MD treatment (Fig. 4C), whereas the SD was unaltered in the insensitive accessions.

The increase in SD in sensitive accessions could potentially result from a higher production of stomata during drought. To explore this, we calculated the stomatal index (SI, the number of stomata per total number of epidermal cells) at 22 DAS. From all accessions, Sha had the highest SI in both WW and MD conditions (32% and 29%, respectively), while Oy-0 had the lowest (23% and 22%, respectively) (Fig. 4D). However, we could not observe any significant effect on the SI by the MD treatment in all six accessions (Fig. 4D), suggesting that the development of stomata was not altered during drought.

Taken together, the insensitive accessions were found to close their stomata more efficiently than the sensitive accessions. The latter had a higher SD under MD stress, which is not resulting from an increased stomatal production but likely results from a decreased growth of non-stomatal cells.

Drought-Insensitive Accessions Arrest Cell Proliferation but Maintain Cell Expansion

Our transcriptome data revealed that several cell wall-modifying enzymes, such as XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASES 19 (XTH19) and XTH24, were differently affected by drought in the sensitive accession ICE163 vs. the insensitive accession, Yeg-1 (Fig. 2E). Because leaf growth is strictly controlled by cell proliferation and cell expansion, we examined the effect of the MD treatment on these cellular characteristics in the selected sensitive and insensitive accessions. We found a significant reduction in average pavement cell numbers during drought in most accessions (Fig. 5A), and in all ecotypes, the cell number was reduced to a similar extent (Fig. 5A, Supplemental Fig. S25A). On the other hand, the average pavement cell area was dramatically reduced by the MD treatment in the

sensitive accessions, whereas surprisingly no reduction could be observed in the insensitive accessions (Fig. 5B, Supplemental Fig. S25B). More specifically, the sensitive accessions showed an increased proportion of smaller cells or a decreased proportion of large pavement cells during MD treatment, but no significant differences could be observed in the insensitive accessions (Fig. 5C, Supplemental Fig. S26). These data indicate that the reduction in cell expansion is the major discriminating phenotypic factor between MD sensitive and insensitive accessions.

DISCUSSION

Arabidopsis natural accessions, which originated from different geographic regions, have evolved under different environmental conditions and therefore vary in many phenotypic and physiological traits, such as flowering time (Stinchcombe et al., 2004), circadian period length (Michael et al., 2003), hypocotyl length (Maloof et al., 2001) and root and leaf architecture (Pérez-Pérez et al., 2002; Chevalier et al., 2003; Rosas et al., 2013). Adapted to local habitats, they also show various responses to abiotic stresses, such as salt stress (Katori et al., 2010), drought (Des Marais et al., 2012), and low temperature (Hannah et al., 2006). In this study, we used fifteen natural accessions and indeed observed this diversity in the drought-triggered reductions in LA, which ranged from 14% to 61%. We did not find a correlation between the sensitivity of these examined accessions with their geographic origin and the respective environmental conditions (Monroe et al., 2018). This could be caused by our limited sample set, but likely also by other environmental factors that were kept constant in our experimental setup, such as temperature, light conditions and soil composition. The long-term effects of maintaining leaf size during mild drought on other important within-generational traits, such as seed yield, remain to be explored further. A previous studies have shown that descendants of mild drought-stressed and non-stressed plants were phenotypically indistinguishable, regardless of their growth condition (Van Dooren et al., 2020). Moreover, within-generation changes in DNA methylation are not related to known drought-associated transcriptome responses (Van Dooren et al., 2020) and transgenerational drought stress appears to have few effects on the DNA methylome (Ganguly et al., 2017; Van Dooren et al., 2020).

In *Arabidopsis*, molecular mechanisms that contribute to stress defences can be identified by performing transcriptomic analysis of natural accessions, as the difference in sensitivity between accessions can be achieved by the activation of specific stress-induced pathways. As such, mild drought induces a limited number of common genes in mature leaves of different accessions (Des Marais et al., 2012). Similarly, our transcriptomic analysis performed on leaves at an early developmental stage identified much more accession-specific genes than those shared between the profiled insensitive (Yeg-1)

and sensitive (ICE163) accession. However, a previous report (Clauw et al., 2015) found fewer specifically DE genes between six tested accessions, whereas the majority of genes were shared. In contrast to our study, the drought treatment had a similar effect on the reduction of L3 area in these six accessions (Clauw et al., 2015), which might explain this general molecular response. When comparing the ecotype-specific genes described in this study with these previously identified ecotype-specific genes, we found only few overlaps. Curiously, we found also very few overlaps when comparing the common genes, illustrating that the complex interaction of the genetic background, the developmental stage and slight changes in the spectrum of drought stress lead to unique transcriptome profiles. Transcriptional and physiological responses of plants to drought conditions are therefore very dynamic processes. Under severe drought or progressive drought conditions, plants are desiccated, resulting in wilting and even death. During such conditions, photosynthesis is strongly inhibited (Galmés et al., 2007; Harb et al., 2010), the expression of senescence genes is upregulated and genes involved in ribosomal synthesis are downregulated (Huang et al., 2008; Alqurashi et al., 2018). As ribosomes are essential to *de novo* protein synthesis, perturbation of their biogenesis can result in cell cycle arrest and apoptosis (Kalinina et al., 2018). During mild drought stress, the absence of such drastic responses allow the plant to at least partially continue their growth. Stomatal closure in response to severe drought can restrict photosynthesis (Chaves et al., 2003), but at the same time limit excessive decreases in water potential in plants (Van Houtte et al., 2013), thereby ensuring their water content. The RWC of plants strongly depends on the composition of their leaf surface wax and by their stomatal closure, and modifications of these can influence their sensitivity to severe drought stress (Cominelli et al., 2005; Patwari et al., 2019; Qian et al., 2019). Under mild drought stress, however, changes in RWC are not strongly correlated with drought sensitivity (Bac-Molenaar et al., 2016). Our data also showed no significant changes in photosynthetic efficiency or RWC under mild drought in most accessions, except for Sha.

Even if other, different transcriptional and physiological regulations of *Arabidopsis* natural accessions by mild drought treatment have been described before (Des Marais et al., 2012; Clauw et al., 2015), it is of great importance to reveal some of the discriminating factors that render plants either sensitive or insensitive to mild drought stress. Our study gives some insights into this by analyzing the differences between the sensitive accession, ICE163, and the insensitive accession, Yeg-1, at the transcriptomic, physiological, cellular and phenotypic level. First, some of the tested factors were equally affected by drought in the sensitive and the insensitive accessions. This was true for the accumulation of proline. High and equal proline accumulations were observed in most accessions, except in Sha, which is among the insensitive accessions. Former research has shown that proline accumulation is compromised

in Sha because of defective alternative splicing of *DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1* (*P5CS1*), which encodes a proline biosynthesis enzyme (Kesari et al., 2012). Our observations confirmed their findings that high accumulation levels of proline do not necessarily lead to an increased resistance to drought stress (Kesari et al., 2012). At the phenotypic level, another trait that was affected by drought similarly in sensitive and insensitive accessions, is the reduction of cell division. Leaf growth is strictly coordinated by cell proliferation and cell expansion (Gonzalez et al., 2012), and a reduction in cell division can be observed soon after exposure to mild drought conditions (Dubois et al., 2017). However, this reduction is not less affected in insensitive accessions. Together, these data suggest that sustaining cell division or proline accumulation during drought stress might not be a suitable strategy to maintain the growth of young leaves.

Second, our approach revealed accession-specific responses at transcriptome level that could be linked to the level of sensitivity to mild drought conditions. For example, our transcriptomic data suggested that the insensitive accession, Yeg-1, shuts down JA and anthocyanin biosynthesis, while these were activated in the sensitive accession, ICE163. These results are different from previous observations for severe drought experiments, showing that plants that accumulate higher JA or anthocyanin levels have an increased survival rate or suffer less from leaf area reduction (Savchenko et al., 2014; Li et al., 2017a). However, strong mutants of *CORONATINE INSENSITIVE 1* (*coi1*) and *JASMONATE INSENSITIVE 1* (*jin1*) that are JA-insensitive, have been shown to suffer less biomass losses during mild drought treatments (Harb et al., 2010). This suggests that the effectiveness of specific molecular responses can be dependent on the intensity of the drought treatment. Application of exogenous JA can enhance drought resistance in plants (Anjum et al., 2011) and overaccumulation of anthocyanins, as ROS scavenger, is positively associated with oxidative stress and drought resistance (Nakabayashi et al., 2014). Several anthocyanin biosynthesis genes, such as *ANTHOCYANIDIN SYNTHASE* (*ANS*) and *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*), were upregulated while the biosynthesis repressor-encoding gene *MYB-LIKE 2* (*MYBL2*) was downregulated in ICE163 by mild drought. Instead, JA signaling repressor-encoding genes, *JASMONATE-ZIM-DOMAIN PROTEIN 1* (*JAZ1*) and *JAZ7*, were upregulated in Yeg-1 by mild drought. In accordance with these expression data, we detected more anthocyanin accumulation in ICE163 compared to Yeg-1, under mild drought conditions. Antioxidant molecules, such as anthocyanins, can be efficient in keeping ROS homeostasis under mild drought. According to DAB staining, ICE163 plants accumulated less ROS in their cotyledons than Yeg-1 upon mild drought treatment, which is opposite to their detected anthocyanin levels. Both proline and anthocyanins have been reported to scavenge ROS under abiotic stress (Sperdouli and Moustakas, 2012; Signorelli et al., 2014) and we found

that the lower levels of ROS in EY15-2 and ICE163 correspond with a higher anthocyanin content, suggesting an important role of anthocyanins in reducing ROS levels in these accessions. However, this ability to scavenge ROS more efficiently does not, or not sufficiently, contribute to the maintenance of growth under mild drought stress.

One of the molecular pathways that was specifically induced in the insensitive accession Yeg-1 was ABA, one of the most important drought-responsive phytohormones regulating stomatal movements (Daszkowska-Golec and Szarejko, 2013). Yeg-1 showed a higher expression of the key biosynthesis gene *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3)* under mild drought, indicating a potentially higher ABA content, as overexpressing *NCED3* has been shown to lead to an increased ABA level, as well as an improvement in drought resistance (Iuchi et al., 2001; Daszkowska-Golec and Szarejko, 2013). In accordance, we could also observe more closed stomata in Yeg-1 during mild drought. Natural variations in stomatal response to closing stimuli have been observed before (Aliniaieifard and van Meeteren, 2014). Here, we also observed various degrees of stomatal closure in natural accessions under mild drought, which is related to their ability to cope with mild drought stress.

On the other hand, ABA was previously also linked with cell expansion (Wu et al., 2018; Qiu et al., 2019) and, interestingly, this was another cellular parameter than we found to be differently affected in sensitive versus insensitive accessions. As mentioned earlier, cell expansion greatly contributes to leaf growth (Gonzalez et al., 2012) and is also decreased soon after exposure to mild drought conditions (Dubois et al., 2017). Genes from the *XTH* gene family encode cell wall-loosening enzymes, and some of them, such as *XTH19* and *XTH24*, are thought to positively influence cell elongation (Miedes et al., 2013; Lee et al., 2018). Under mild drought, *XTH19* was induced in Yeg-1, the insensitive accession, while *XTH24* was repressed in the sensitive accession ICE163. These differences in expression might explain the differences in cell area of L3 observed at the mature stage. Related to this, the maximal leaf expansion rate and the duration of leaf expansion were previously reported to be variable among natural accessions (Aguirrezabal et al., 2006). As such, An-1 is an insensitive accession that shows a prolonged leaf expansion upon drought, while Oy-0, a drought-sensitive accession, has a decreased duration of leaf expansion (Aguirrezabal et al., 2006). We also observed that Oy-0 showed a decreased pavement cell area under mild drought, which might be caused by a shorter expansion duration, as reported.

In summary, our results indicate that different natural accessions in *Arabidopsis* use specific strategies to cope with mild drought, illustrated by the contrasting transcriptomic responses and the diverse physiological responses in insensitive and sensitive accessions. Interestingly, our analysis suggests that the ability to efficiently close stomata and maintain cell expansion under drought could be

major determinants for the capacity to maintain leaf growth upon mild drought treatment. Our results further demonstrate that early responses in the transcriptome can reflect physiological responses at a later stage, such as *XTH* genes that can predict cell expansion phenotypes, or ABA biosynthesis genes that correspond with stomatal closure and improved resistance at a later stage. Further research is needed to elucidate how insensitive accessions trigger more pronounced ABA responses, and to find key elements of this regulatory network. In addition, further examination of leaf growth, stomatal opening and cell expansion in mutants or overexpression lines of known ABA signaling pathway elements in different genetic backgrounds during mild drought would help to reveal common and accession-specific elements in mild drought resistance.

MATERIALS AND METHODS

Plant Material, Growth Conditions and Mild Drought Treatment

Fifteen *Arabidopsis* (*Arabidopsis thaliana*) natural accessions were selected to cover a broad range of genetic diversity of *Arabidopsis*. For the experiment of MD treatment in soil, seeds were sown in pots, which were filled up to $85 \text{ g} \pm 0.5 \text{ g}$ of Saniflor soil (Van Isreal N.V., Geraardsbergen, Belgium) with an absolute water content of on average 70%. After 5 nights of stratification at 4°C in darkness, the pots were moved to a growth chamber (21°C and 16-h day/8-h night cycles). At 4 DAS, the pots were transferred to the automated phenotyping platform WIWAM (21°C and 16-h day/8-h night cycles) and randomized to homogenously mix the natural accessions and treatments. The controlled MD treatment was initiated at 6 DAS. Control-treatment pots were kept at $2.2 \text{ g}_{\text{water}}/\text{g}_{\text{soil}}$. The MD-treated pots initially contained $2.2 \text{ g}_{\text{water}}/\text{g}_{\text{soil}}$ and their relative water content was further decreased to $1.2 \text{ g}_{\text{water}}/\text{g}_{\text{soil}}$, which was reached at 11 DAS.

Leaf Size Measurement

Leaf size measurements were performed on the third true leaf (L3) of the rosette at 22 DAS. The leaves were harvested, photographed, cleared in ethanol and subsequently mounted on microscopic slides in lactic acid. The leaf area was measured based on the pictures using ImageJ v1.45. Three biological replicates were performed for all accessions. Eight to twelve leaves were measured per treatment per replicate for all accessions, except for ICE197, for which four to nine leaves per treatment were measured.

Statistical Analysis

All statistical analyses were performed with R, version 4.0.1 or Graphpad prism 8.4 (www.graphpad.com). ANOVA analyses were used for all leaf phenotypic and physiological data. Ecotype, treatment, replicate and their interactions were included as fixed effects of the model. Significant differences were assumed when with adjusted p-values lower than 0.05. The significance of the relative reduction in leaf area was determined by using the Phia package (<https://cran.r-project.org/web/packages/phia/phia.pdf>). The correlation between the relative reduction in leaf area and climates of origin (drought frequency (DF)) was based on a multiple linear regression model: L3 area reduction percentage ~ winter DF + Spring DF + Summer DF + Fall DF. The significant differences in relative frequencies of LN-transformed pavement cell area distribution was determined by ANOVA, using the Sidak's multiple comparisons test.

RNA-Sequencing Sampling

Eighty seedlings (distributed over nine to ten pots) were grown per ecotype per treatment per replicate. At 11 DAS, seedlings were harvested at 6 PM and stored in RNA-later (Ambion) solution. The third leaf was harvested by micro-dissecting, then pooled and flash frozen in liquid nitrogen. RNA extraction was done using Trizol (Invitrogen) combined with the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. In total, three biological replicates were performed.

RNA-Sequencing and Differential Expression Analysis

The sequencing was performed at the Nucleomics Core Facility (VIB, Leuven, Belgium, www.nucleomics.be), using a single-end mode with a read length of 75 bp on an Illumina NextSeq500. The quality control was done in the Galaxy platform with FastQC, alignment was done with Salmon. The Arabidopsis reference genome (TAIR10) was used.

The RNA-seq data was analyzed using R (version 3.6.0). Counts data of all splice variants were combined using the tximport package (<https://bioconductor.org/packages/release/bioc/html/tximport.html>). Then, the counts were normalized based on the library size of the samples. Genes that were detected less than three times with expression levels lower than 5 counts per million were removed. The new library was normalized by the TMM (trimmed mean of M values) method. A generalized linear model was applied with ecotype and treatment as factors using the glmQLFit function. Next, significant interactions were identified using the glmQLFTest function with the interaction term as a coefficient. The differential expression analysis of RNA-seq data was performed with the EdgeR package (<https://bioconductor.org/packages/release/bioc/html/edgeR.html>) with R studio (Version 1.1.463).

Differentially expressed genes of MD versus WW conditions for each ecotype were calculated using predefined contrasts. The cut-off of DE genes was set on a $FDR < 0.05$ and $\log_2FC \geq 0.6$ or $\log_2FC \leq -0.6$. The Venn diagrams were created with jvenn (Bennett et al., 2016).

RT-qPCR

The iScript cDNA Synthesis Kit (Bio-Rad) was used to synthesize the cDNA. The RT-qPCR was done on a LightCycler 480 (Roche Diagnostics) on 384-well plates with a LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's instructions. Melting curves were analyzed to check the primer specificity. Normalization was done against the average of housekeeping genes AT1G13320, AT2G32170 and AT2G28390: $\Delta Ct = Ct(\text{gene}) - Ct(\text{mean}[\text{housekeeping genes}])$ and $\Delta\Delta Ct = \Delta Ct(\text{WW condition}) - \Delta Ct(\text{MD condition})$. Ct refers to the number of cycles at which the SYBR Green fluorescence reaches an arbitrary value during the exponential phase of amplification.

Primers were designed with the QuantPrime website (<https://quantprime.mpimp-golm.mpg.de/main.php?page=home>) and the conservation of their sequence in ICE163 and Yeg-1 was verified with POLYMORPH (http://polymorph.weigelworld.org/cgi-bin/webapp.cgi?page=show_aln;plugin=show_aln;project=MPICao2010). AT code, name of genes and primers used in this study are listed in Supplemental Table S10.

Proline Content Measurements

Seedlings grown in control and drought conditions were harvested at 11 DAS. Free proline was measured using the ninhydrin assay (Bates et al., 1973).

3,3-Diaminobenzidine (DAB) Staining

Seedlings were harvested at 11 DAS. For each treatment, eight seedlings were used for DAB staining per replicate. DAB was dissolved in a 10-mM PBS solution (1 mg/ml). Seedlings were submerged into the staining solution and vacuum-infiltrated three times for 5 min. Subsequently, the samples were incubated overnight in the dark. Chlorophyll was removed by adding ethanol to the samples and a boiling step for 10 min to obtain a better staining contrast. The cleared seedlings were transferred to 90% (v/v) lactic acid and photographed.

Anthocyanins Measurements

Seedlings were harvested, their fresh weight was measured and they were frozen in liquid nitrogen. The samples were homogenized and 1 ml of extraction buffer (45% (v/v) methanol, 5% (v/v) acetic acid) was added, followed by a thorough mixing. The mixtures were centrifuged at $12,000 \times g$ for 5

min at room temperature. The supernatant was transferred to a new tube and centrifuged again for 5 min at room temperature. Then, 200 µl supernatant was transferred to 96-well plates (VWR tissue Culture Plates, No. 734-2328). The absorbance of the supernatant was measured at 530 and 657 nm with a VersaMax™ Absorbance Microplate Reader. The extraction buffer was used as blank. The total anthocyanin content was calculated by $(\text{Abs } 530 - 0.25 \times \text{Abs } 657)/\text{g fresh weight}$.

Stomatal Closure

Imprints of the third leaf were made at 11 DAS at 6 PM. The nail polish copy of the imprint was analyzed using a tabletop scanning electron microscope (TM-1000, Hitachi). Seven to eight leaves were analyzed per accession per treatment in each biological replicate. Three random spots were checked for each leaf to calculate the average number of closed and open stomata.

Cellular Analysis

For the cellular analysis, the total leaf blade area of cleared leaves was measured for at least eight representative leaves per replicate under a dark-field binocular microscope (MZ16, Leica). Abaxial epidermal cells in the center of the leaves were drawn using a microscope equipped with differential interference contrast optics (DM LB with 403 and 633 objectives; Leica) and a drawing tube. Scanned cell drawings were used to measure the pavement cell area as described before (Andriankaja et al., 2012). Cells were colored according to their area in Inkscape (<https://inkscape.org/>, RRID: SCR_014479).

Leaf surface temperature measurement

Leaf surface temperature was determined using a thermal Optris PI 200 camera (www.optris.com). At 11 DAS, a 30 seconds video of mild drought-treated pots was recorded. A stable 10-s fragment of the temperature of the first leaf pair was generated by Optris PI Connect to calculate their average temperature. Per accession, 25 to 40 seedlings were measured per replicate and in total, 3 biological replicates were performed.

Relative Water Content

The relative water content (RWC, %) was calculated according to this formula: $(\text{FW}-\text{DW})/(\text{TW}-\text{DW})$. The fresh weight (FW) was obtained by harvesting and weighing freshly harvested seedlings at 11 DAS. Turgid weight (TW) was obtained by incubating these seedlings into a six-well plate with de-ionized water overnight at room temperature, removing excess water by wiping with absorbent paper and weighing the plant material. Dry weight (DW) was recorded after incubating these seedlings at 60°C for

two nights in a drying oven. Per accession, about 40 seedlings were pooled per replicate; in total, three biological replicates were harvested.

Photosynthetic efficiency

Chlorophyll α fluorescence was measured using the Imaging-PAM M-series Chlorophyll Fluorescence System (Heinz Walz, Effeltrich, Germany) and used to quantify the maximum efficiency of photosystem II (PSII) (F_v/F_m), in which F_v and F_m denote variable fluorescence (ability of PSII to perform photochemistry) and maximal fluorescence (PSII centers closed) from light-adapted leaves, respectively (Baker, 2008).

Accession Numbers

Accessions numbers are available in Supplemental Table S10.

Supplemental Data

Supplemental Figure S1. Primordia of leaf 3 at 6 DAS.

Supplemental Figure S2. Rosettes of An-1 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S3. Rosettes of Blh-1 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S4. Rosettes of C24 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S5. Rosettes of Col-0 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S6. Rosettes of Cvi-0 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S7. Rosettes of EY15-2 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S8. Rosettes of ICE61 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S9. Rosettes of ICE75 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S10. Rosettes of ICE97 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S11. Rosettes of ICE163 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S12. Rosettes of Ler-0 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S13. Rosettes of Oy-0 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S14. Rosettes of Sha under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S15. Rosettes of WalhaesB4 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S16. Rosettes of Yeg-1 under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S17. Correlation plot between leaf size and sensitivity to mild drought.

Supplemental Figure S18. Scatterplot matrix of seasonal historical frequency of drought weeks (HFDW) of the home environment of natural accessions and their sensitivity to mild drought (percentage reduction of leaf 3 area).

Supplemental Figure S19. General overview of the RNA-seq data.

Supplemental Figure S20. Workflow for the analysis of common and ecotype-specific drought-responsive genes.

Supplemental Figure S21. Venn diagrams and expression levels of accession-specific differentially expressed genes.

Supplemental Figure S22. Venn diagrams of identified accession-specific genes and common genes in previous studies on natural accessions.

Supplemental Figure S23. RT-qPCR analysis of candidate genes in ICE163 and Yeg-1.

Supplemental Figure S24. Leaf surface temperature, photosynthetic efficiency and relative water content under well-watered (WW) and mild drought (MD) conditions.

Supplemental Figure S25. Relative reduction of pavement cell number and cell area under mild drought conditions.

Supplemental Figure S26. Relative frequencies of LN-transformed pavement cell area distribution of natural accessions under well-watered (WW) and mild drought (MD) conditions.

Supplemental Table S1: Original regions of natural accession, and multiple linear analysis of relative L3 area reduction and seasonal drought frequency.

Supplemental Table S2: Developmental stages of natural accessions at WW and MD conditions.

Supplemental Table S3: Significance of L3 relative reduction.

Supplemental Table S4: GO enrichment of DE genes between ICE163 and Yeg-1 under WW condition.

Supplemental Table S5: Common DE genes between ICE163 and Yeg-1 under MD and GO enrichment categories.

Supplemental Table S6: Genes with different regulation trends between ICE163 and Yeg-1 under MD.

Supplemental Table S7: GO enrichment of genes from different drought studies.

Supplemental Table S8: GO enrichment categories of selected 143 genes.

Supplemental Table S9: GO enrichment categories of selected genes.

Supplemental Table S10: Primers for RT-qPCR.

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Figure Legends

Figure 1. Different *Arabidopsis* accessions show a different leaf growth reduction under mild drought.

(A) Rosettes of 15 natural accessions under well-watered (WW) and mild drought (MD) conditions at 22 days after stratification (DAS). Scale bar = 5 cm. **(B)** Soil moisture content of WIWAM pots from 4 to 22 DAS. **(C)** Average area of the third leaf under WW and MD conditions at 22 DAS. Error bars represent the SE, n = 3 biological replicates. Percentages represent relative reductions in leaf area under MD conditions

compared to the WW control. Asterisks indicate a significant difference as determined by ANOVA, TukeyHSD (** p adj <0.01). Significance of relative reduction in leaf area differences between accessions is shown in Supplemental Table S3 (* p adj < 0.05; ANOVA, Ecotype X Treatment interaction).

Figure 2. ICE163 and Yeg-1 react differently to mild drought at the transcriptome level. (A) Venn diagram of upregulated genes by mild drought (MD; FDR $\leq$ 0.05, $\log_2FC \geq$ 0.6). **(B)** Venn diagram of downregulated genes by MD (FDR < 0.05, $\log_2FC \leq$ -0.6). **(C)** Scatter plot of differentially expressed genes in ICE163 and Yeg-1. $\log_2FC$ (MD vs. well-watered (WW)) of gene expression levels in Yeg-1 are plotted against those in ICE163. Gray: genes whose $\log_2FC$ (MD vs. WW) difference between Yeg-1 and ICE163 is less than 1; orange: genes whose $\log_2FC$ (MD vs. WW) difference between Yeg-1 and ICE163 is equal or larger than 1 and their expression is only significant in ICE163; green: genes whose $\log_2FC$ (MD vs. WW) difference between Yeg-1 and ICE163 is equal or larger than 1 and their expression is only significant in Yeg-1; purple: genes whose $\log_2FC$ (MD vs. WW) difference between Yeg-1 and ICE163 is equal or larger than 1 and their expression is significant in both Yeg-1 and ICE163. **(D)** Heat map of $\log_2FC$ of genes that show a significant (FDR < 0.05) Ecotype x Treatment (E x T) interaction. Red and blue correspond to increased or decreased expression upon drought, respectively. **(E)** Heat map of the $\log_2FC$ of selected genes from the E x T interaction and scatterplot. Red and blue correspond to increased and decreased expression (MD vs. WW), respectively. In D and E, asterisks indicate that the expression levels are significantly different from the control condition (WW) as determined by ANOVA, Tukey HSD (* FDR < 0.05; ** FDR <0.01; *** FDR <0.001). For gene names and abbreviations, see Supplemental Table S10.

Figure 3. Proline, anthocyanin and ROS accumulation upon mild drought stress. (A) H₂O₂ accumulation in natural accessions seedlings detected by 3,3-diaminobenzidine (DAB) staining under well-watered (WW) and mild drought (MD) conditions. Scale bar = 5 mm. **(B)** Proline concentration of sensitive and insensitive accessions under WW and MD conditions at 11 DAS. **(C)** Anthocyanin concentration in sensitive and insensitive accessions under WW and MD conditions at 11 DAS. In B and C, error bars represent the SE, n = 3 biological replicates. Different letters above the error bar indicate statistical differences (p adj < 0.05; ANOVA, TukeyHSD). Insensitive accessions are in bold font. FW, fresh weight.

Figure 4. Stomatal opening, density and index after mild drought treatment. (A) Open and closed stomata in the first leaf pair under well-watered (WW) and mild drought (MD) conditions at 11 DAS. Blue circles indicate open stomata, yellow circles indicate closed stomata. Scale bar = 100 μ m. (B) Ratio of open stomata in sensitive and insensitive accessions under WW and MD conditions at 11 DAS. Error bars represent the SE, n = 3 biological replicates with ≥ 7 leaves per treatment, per ecotype, per replicate. (C) Stomatal density at 22 DAS. (D) Stomatal index at 22 DAS. Error bars in C and D represent the SE, n = 3 biological replicates, with three leaves per treatment, per ecotype, per replicate. Different letters above the error bars indicate statistical significances (p adj < 0.05; ANOVA, TukeyHSD). Insensitive accessions are in bold font.

Figure 5. Mild drought differently affects pavement cell number and area in sensitive and insensitive accessions. (A) Average pavement cell number of leaf 3 under well-watered (WW) and mild drought (MD) conditions at 22 DAS. (B) Average pavement cell area of leaf 3 under WW and MD conditions at 22 DAS. Error bars represent the SE, n = 3 biological replicates. Different letters above the error bar indicate statistical differences (p adj < 0.05; ANOVA, TukeyHSD). Insensitive accessions are in bold font. (C) Representations of abaxial epidermal cell sizes of leaf 3 of natural accessions at 22 DAS under WW and MD conditions.

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Figure 1. Different *Arabidopsis* accessions show a different leaf growth reduction under mild drought. (A) Rosettes of 15 natural accessions under well-watered (WW) and mild drought (MD) conditions at 22 days after stratification (DAS). Scale bar = 5 cm. **(B)** Soil moisture content of WIWAM pots from 4 to 22 DAS. **(C)** Average area of the third leaf under WW and MD conditions at 22 DAS. Error bars represent the SE, n = 3 biological replicates. Percentages represent relative reductions in leaf area under MD conditions compared to the WW control. Asterisks indicate a significant difference as determined by ANOVA, TukeyHSD. (** p adj < 0.01). Significance of relative reduction in leaf area differences between accessions is shown in Supplemental Table S3 (* p adj < 0.05; ANOVA, Ecotype X Treatment interaction).

Figure 2. ICE163 and Yeg-1 react differently to mild drought at the transcriptome level. (A) Venn diagram of upregulated genes by mild drought (MD; FDR ≤ 0.05 , $\log_2FC \geq 0.6$). (B) Venn diagram of downregulated genes by MD (FDR < 0.05 , $\log_2FC \leq -0.6$). (C) Scatter plot of differentially expressed genes in ICE163 and Yeg-1. $\log_2FC$ (MD vs. well-watered (WW)) of gene expression levels in Yeg-1 are plotted against those in ICE163. Gray: genes whose $\log_2FC$ (MD vs. WW) difference between Yeg-1 and ICE163 is less than 1; orange: genes whose $\log_2FC$ (MD vs. WW) difference between Yeg-1 and ICE163 is equal or larger than 1 and their expression is only significant in ICE163; green: genes whose $\log_2FC$ (MD vs. WW) difference between Yeg-1 and ICE163 is equal or larger than 1 and their expression is only significant in Yeg-1; purple: genes whose $\log_2FC$ (MD vs. WW) difference between Yeg-1 and ICE163 is equal or larger than 1 and their expression is significant in both Yeg-1 and ICE163. (D) Heat map of $\log_2FC$ of genes that show a significant (FDR < 0.05) Ecotype x Treatment (E x T) interaction. Red and blue correspond to increased or decreased expression upon drought, respectively. (E) Heat map of the $\log_2FC$ of selected genes from the E x T interaction and scatterplot. Red and blue correspond to increased and decreased expression (MD vs. WW), respectively. In D and E, asterisks indicate that the expression levels are significantly different from the control condition (WW) as determined by ANOVA, TukeyHSD (* FDR < 0.05 ; ** FDR < 0.01 ; *** FDR < 0.001). For gene names and abbreviations, see Supplemental Table S10.

A

WW

ICE163

ICE97

Oy-0

Sha

Yeg-1

EY15-2

MD

B **C**

Figure 3. Proline, anthocyanin and ROS accumulation upon mild drought stress. (A) H_2O_2 accumulation in natural accessions seedlings detected by 3,3-diaminobenzidine (DAB) staining under well-watered (WW) and mild drought (MD) conditions. Scale bar = 5 mm. **(B)** Proline concentration of sensitive and insensitive accessions under WW and MD conditions at 11 DAS. **(C)** Anthocyanin concentration in sensitive and insensitive accessions under WW and MD conditions at 11 DAS. In B and C, error bars represent the SE, $n = 3$ biological replicates. Different letters above the error bar indicate statistical differences (p adj < 0.05; ANOVA, TukeyHSD). Insensitive accessions are in bold font. FW: fresh weight.

Figure 4. Stomatal opening, density and index after the mild drought treatment. (A) Open and closed stomata in the first leaf pair under well-watered (WW) and mild drought (MD) conditions at 11 DAS. Blue circles indicate open stomata, yellow circles indicate closed stomata. Scale bar = 100 μ m. **(B)** Ratio of open stomata in sensitive and insensitive accessions under WW and MD conditions at 11 DAS. Error bars represent the SE, $n = 3$ biological replicates with ≥ 7 leaves per treatment, per ecotype, per replicate. **(C)** Stomatal density at 22 DAS. **(D)** Stomatal index at 22 DAS. Error bars in C and D represent the SE, $n = 3$ biological replicates, with three leaves per treatment, per ecotype, per replicate. Different letters above the error bars indicate statistical significances ($p_{adj} < 0.05$; ANOVA, TukeyHSD). Insensitive accessions are in bold font.

Figure 5. Mild drought differently affects pavement cell number and area in sensitive and insensitive accessions. (A) Average pavement cell number of leaf 3 under well-watered (WW) and mild drought (MD) conditions at 22 DAS. Error bars represent the SE, $n = 3$ biological replicates. Different letters above the error bar indicate statistical differences ($p \text{ adj} < 0.05$; ANOVA, TukeyHSD). Insensitive accessions are in bold font. **(B)** Average pavement cell area of leaf 3 under WW and MD conditions at 22 DAS. Error bars represent the SE, $n = 3$ biological replicates. Different letters above the error bar indicate statistical differences ($p \text{ adj} < 0.05$; ANOVA, TukeyHSD). Insensitive accessions are in bold font. **(C)** Representations of abaxial epidermal cells sizes of leaf 3 of natural accessions at 22 DAS under WW and MD conditions.

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