

# ETHYLENE RESPONSE FACTOR6 Acts as a Central Regulator of Leaf Growth under Water-Limiting Conditions in Arabidopsis<sup>1[C][W][OA]</sup>

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Leaf growth is a complex developmental process that is continuously fine-tuned by the environment. Various abiotic stresses, including mild drought stress, have been shown to inhibit leaf growth in Arabidopsis (*Arabidopsis thaliana*), but the underlying mechanisms remain largely unknown. Here, we identify the redundant Arabidopsis transcription factors ETHYLENE RESPONSE FACTOR5 (ERF5) and ERF6 as master regulators that adapt leaf growth to environmental changes. *ERF5* and *ERF6* gene expression is induced very rapidly and specifically in actively growing leaves after sudden exposure to osmotic stress that mimics mild drought. Subsequently, enhanced *ERF6* expression inhibits cell proliferation and leaf growth by a process involving gibberellin and DELLA signaling. Using an *ERF6*-inducible overexpression line, we demonstrate that the gibberellin-degrading enzyme GIBBERELLIN 2-OXIDASE6 is transcriptionally induced by ERF6 and that, consequently, DELLA proteins are stabilized. As a result, *ERF6* gain-of-function lines are dwarfed and hypersensitive to osmotic stress, while the growth of *erf5erf6* loss-of-function mutants is less affected by stress. Besides its role in plant growth under stress, ERF6 also activates the expression of a plethora of osmotic stress-responsive genes, including the well-known stress tolerance genes *STZ*, *MYB51*, and *WRKY33*. Interestingly, activation of the stress tolerance genes by ERF6 occurs independently from the ERF6-mediated growth inhibition. Together, these data fit into a leaf growth regulatory model in which ERF5 and ERF6 form a missing link between the previously observed stress-induced 1-aminocyclopropane-1-carboxylic acid accumulation and DELLA-mediated cell cycle exit and execute a dual role by regulating both stress tolerance and growth inhibition.

Drought stress is one of the most destructive environmental cues that affect plant growth and crop productivity (Boyer, 1982; Yang et al., 2010). In response to water deprivation, leaf growth is shut down

by a fast and active mechanism initiated in order to save energy, as the duration and extent of the stress are unknown (Skirycz and Inzé, 2010; Skirycz et al., 2011a). This growth inhibition upon stress, however, is expected to cause yield losses that are unnecessary when the stress only lasts for short periods or when the stress is too mild to threaten the plant's survival.

The molecular processes by which mature plant organs respond to water shortage are extensively documented and are characterized by increasing abscisic acid levels activating stress-avoidance mechanisms (Xiong et al., 2002; Verslues et al., 2006; Seki et al., 2007; Schachtman and Goodger, 2008). However, the mechanisms by which stress affects actively growing plant organs are largely unknown. Although there have been many reports of transgenic Arabidopsis (*Arabidopsis thaliana*) lines with enhanced survival after severe water stress, an analysis of 27 of these showed no improved growth under milder, nonlethal drought conditions (Skirycz et al., 2011b). Thus, tolerance to severe drought stress and the ability of plants to continue to grow under mild stress conditions are very different traits mediated by different molecular processes. Furthermore, recent studies pointed out the importance of analyzing stress responses at the organ or tissue level, as the responses to stress both at the

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transcriptional level (Harb et al., 2010; Skirycz et al., 2010) and at the protein level (Baerenfaller et al., 2012) are much dependent on organ developmental stage or even on cell type identity (Dinnyen et al., 2008).

In *Arabidopsis*, leaf development consists of three major phases during which cell proliferation, driving the growth of very young leaves, gradually switches toward cell expansion. The transition between cell proliferation and cell expansion occurs gradually, from leaf tip to leaf base, and is generally paired with a switch from the mitotic cell cycle to endoreduplication (Donnelly et al., 1999; Vlieghe et al., 2005; Anastasiou and Lenhard, 2007; Andriankaja et al., 2012; Gonzalez et al., 2012). In plants undergoing mild osmotic stress, both cell proliferation and cell expansion are affected, and as a result, leaves have fewer and smaller cells (Skirycz et al., 2010; Tardieu et al., 2011). Proliferating leaves were shown to be affected in a two-step process, previously denominated the “pause-and-stop” mechanism (Skirycz et al., 2011a). In *Arabidopsis*, when stress occurs during early leaf development (leaves approximately 0.1 mm<sup>2</sup> in size), cell cycle progression is first arrested in a reversible manner by posttranslational inhibition of CYCLIN-DEPENDENT KINASE A (CDKA) activity. Only later, if the stress persists, the cell cycle pause will be converted into a definitive cell cycle exit. Cells then enter cell expansion, which is accompanied by the well-documented activation of endoreduplication and an increased DNA copy number. The exit out of the cell cycle was previously shown to be dependent on GA and DELLAs (Achard et al., 2009; Claeys et al., 2012).

In the first phase of a mild stress response in growing leaves (“pause”), we previously observed an early stress-induced increase in 1-aminocyclopropane-1-carboxylic acid (ACC) levels. ACC is the direct precursor of ethylene, a gaseous plant hormone that previously has been implicated in regulating, either positively or negatively, growth upon stress treatments. For example, ethylene was shown to either stimulate or inhibit primary root growth under low phosphate availability or under deficiency of other nutrients, respectively (Ma et al., 2003; Pierik et al., 2007). Shoot growth was shown to be positively regulated by ethylene during flooding as well as during shade avoidance, the latter as a result of increased cell expansion (Bailey-Serres and Voesenek, 2008; Jackson, 2008; Pierik et al., 2011). On the other hand, using mutants in the ethylene signaling pathway, ethylene was reported to confer growth inhibition. The *ctr1* mutant, in which the ethylene signaling pathway is constitutively active, has a dwarf phenotype due to a reduction of both cell size and cell number (Roman et al., 1995; Kieber, 1997). Moreover, ethylene-insensitive mutants are generally reported to be larger than wild-type plants (Roman et al., 1995). Consistently, overexpression of the ethylene receptors increases rosette size (Cao et al., 2006, 2007; Wuriyangan et al., 2009). Together, these seemingly contradictory observations can be explained by a biphasic model (Pierik et al., 2006), presenting ethylene as

a growth-stimulating hormone until an optimal concentration is reached, after which ethylene inhibits growth. This optimum varies according to environmental signals, internal signals, and species-dependent factors.

Here, we identify two redundant transcription factors, ETHYLENE RESPONSE FACTOR5 (ERF5) and ERF6, as being the central regulators of leaf growth inhibition upon mild osmotic stress. ERF5 and ERF6 belong to the class of APETALA2 (AP2)/ERF transcription factors (Fujimoto et al., 2000; Nakano et al., 2006) and are situated downstream of the ethylene signaling cascade, where they regulate ethylene-responsive genes (Yoo et al., 2009). Recently, these two transcription factors were shown to be able to interact with each other at the protein level, although further investigations are necessary to confirm an in planta interaction (Son et al., 2012). Although ERF5 and ERF6 have not yet been extensively characterized, they recently have been described as being important regulators of biotic stress defense (Moffat et al., 2012; Son et al., 2012). Besides its function in response to biotic stress, ERF6 was recently shown to control the expression of reactive oxygen species-responsive genes after activation by MPK3/MPK6 (Wang et al., 2013).

In this study, we focus on ERF6, demonstrating that it affects cell cycle exit by triggering the expression of the *GA2-OXIDASE6* (*OX6*) gene and consequently the inactivation of GAs. Thus, ERF6 provides a link between ACC and DELLA signaling in the cell cycle pause-and-stop model, improving our understanding of growth inhibition in the proliferating leaf primordia of plants subjected to water limitation. In addition, ERF6 regulates, in a GA- and DELLA-independent manner, the expression of multiple genes associated with abiotic and biotic stress conditions, such as genes encoding the transcription factors WRKY33, MYB51, and STZ (for salt tolerance zinc finger). Thus, ERF6 plays a dual role under stress, as it activates both stress tolerance and growth inhibition, and, importantly, these two roles occur independently from each other.

## RESULTS

### ERF5 and ERF6 Are Transcriptionally Induced in Actively Growing Leaves within 1 h of Stress Exposure

We recently investigated the effects of mild osmotic stress on the transcriptome of very young, small (approximately 0.1 mm<sup>2</sup> in size), and thus still actively growing leaves (Skirycz et al., 2010, 2011a). Among the more than 1,500 genes differentially expressed following exposure to mild osmotic stress, the transcription factors *ERF5* (AT5G47230) and *ERF6* (AT4G17490) were induced very early after stress onset, already 1 h after stress exposure specifically in actively growing leaves, and their induction was further maintained over time (Supplemental Fig. S1A). Furthermore, analysis of publicly available transcriptome data revealed that *ERF5* and *ERF6* are induced by several other, often

severe abiotic stresses, including drought (Supplemental Fig. S1B; Hruz et al., 2008). These data prompted us to investigate the role of ERF5 and ERF6 in integrating environmental signals into leaf growth regulation.

Within the large class of more than 120 AP2/ERF transcription factors, ERF5 and ERF6 belong to group IXb, a small group of transcriptional activators containing several other stress-responsive ERFs, such as ERF1 and ERF2 (Supplemental Fig. S2; Nakano et al., 2006; Skirycz et al., 2010). ERF5 and ERF6 share 51% amino acid similarity, with high conservation of three functional domains: CMIX-2, CMIX-5, and the AP2/ERF domain (Thompson et al., 1994).

### *erf5erf6* Loss-of-Function Mutants Grow Better under Osmotic Stress

To investigate the importance of *ERF5* and *ERF6* in leaf growth under various conditions, we used single and double mutants. Single *erf5* and *erf6* mutants were obtained from the SALK collection and have transfer DNA insertions in the 3' untranslated region and coding sequence, respectively (Supplemental Fig. S3A; Alonso et al., 2003; Wang et al., 2013). The *ERF5* and *ERF6* expression levels were strongly decreased both under normal and osmotic stress conditions (Supplemental Fig. S3B). The *erf5* and *erf6* single mutants had no obvious growth phenotype, most likely because of functional redundancy; thus, the *erf5erf6* double mutant was used for further analysis.

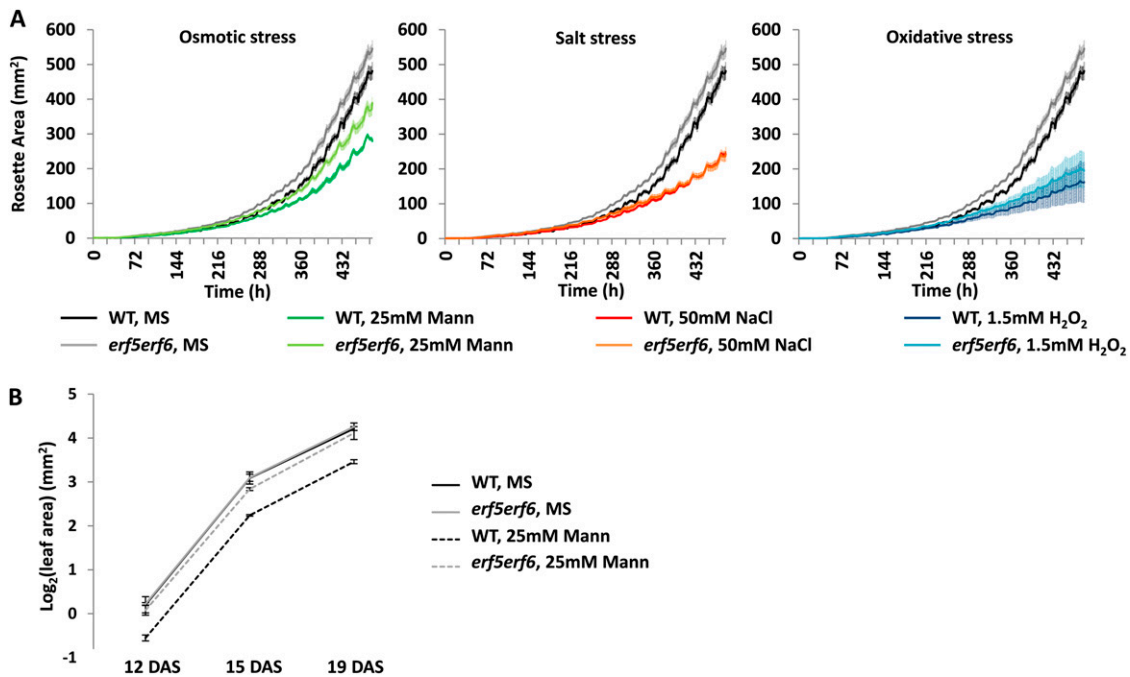
First, we explored how *erf5erf6* plants behave under standard conditions and when exposed to various long-term abiotic stress conditions. As we were interested in measuring growth dynamics in response to stress instead of limiting our analysis to end-point measurements, we chose to grow the *erf5erf6* mutant and the wild-type line on an automated phenotyping platform named the In Vitro Growth Imaging System (IGIS; see "Materials and Methods"). The IGIS platform allows for a continuous measurement of rosette size of in vitro-grown Arabidopsis plants by taking photographs every hour from germination onward until 20 d after stratification (DAS) and extracting the rosette area. The *erf5erf6* mutant and the wild type were exposed to different mild abiotic stresses: osmotic stress (25 mM mannitol, mimicking mild drought stress; Skirycz et al., 2011a), oxidative stress (1.5 mM hydrogen peroxide), and salt stress (50 mM NaCl). Interestingly, growth curves representing rosette area over time (Fig. 1A) demonstrate that under standard conditions, the *erf5erf6* mutant grows faster than the wild type. At the end point (20 DAS), *erf5erf6* was 13% larger (Supplemental Table S1). When grown on osmotic stress from germination onward, *erf5erf6* mutants tolerated the stress better than wild-type plants. The *erf5erf6* mutants were less affected by the stress-induced growth inhibition and showed an increase in final rosette area of 33% compared with wild-type plants exposed to this stress (Supplemental Table S1).

Importantly, the same tendency was observed for *erf5erf6* plants exposed to long-term oxidative stress and was consistent in two out of the three experiments. However, the long-term hydrogen peroxide treatment introduced significant variability between the experiments, making them poorly reproducible and thus rather difficult to interpret (details per experiment are provided in Supplemental Table S1). Finally, when exposed to long-term mild salt stress (50 mM NaCl), the *erf5erf6* plants were not larger than wild-type plants, suggesting that both transcription factors have no role in tolerance to salt stress. This hypothesis is further supported by the observation that exposure of plants to 50 mM NaCl does not induce *ERF5* and *ERF6* expression in very young, small proliferating leaves (Supplemental Fig. S4). Together, these data show that *ERF5* and *ERF6* are central regulators that orchestrate leaf growth under long-term mild osmotic and possibly oxidative stress conditions but not under salt stress.

Next we investigated the growth of *erf5erf6* when exposed to a short-term mild osmotic stress (25 mM mannitol). Briefly, the assay consists of growing plants on a nylon mesh covering control Murashige and Skoog (MS) medium until the third leaf has completely emerged from the shoot apical meristem but is still in a fully proliferative stage, at 9 DAS. At this time point, the mesh is transferred to MS medium containing 25 mM mannitol, and the effect of the stress is analyzed daily by measuring the growth of the third leaf. As previously shown, wild-type plants exposed to 25 mM mannitol show a reduction of leaf area of about 50%, caused by a reduction of both cell number and cell size (Skirycz et al., 2010, 2011a; Fig. 1B). Importantly, the growth of *erf5erf6* was significantly ( $P = 0.0004$ ) less affected than that of the wild type (Fig. 1B): on average, wild-type plants showed a 42% leaf size reduction, while the leaf size of *erf5erf6* was only decreased by 11% (Supplemental Table S2, C and D). As a consequence, third leaves harvested at 19 DAS from *erf5erf6* plants exposed to stress are 59% larger in comparison with those of wild-type plants exposed to stress ( $P = 0.025$ ). Thus, the third leaf of *erf5erf6* plants exposed to short-term mild osmotic stress continued to grow almost indistinguishably from that of wild-type plants grown on standard medium. Interestingly, this reduced leaf growth inhibition is already visible at 12 DAS (Fig. 1B), suggesting that ERF5 and ERF6 act early in leaf development.

### ERF6 Represses Leaf Growth by Inhibiting Cell Division and Cell Expansion

To investigate the cellular basis of ERF6-mediated growth inhibition, inducible gain-of-function *ERF6* lines were analyzed. To this end, the *ERF6* sequence was C-terminally fused to that of a glucocorticoid receptor (GR) domain and expressed in transgenic plants under the control of the cauliflower mosaic virus 35S promoter

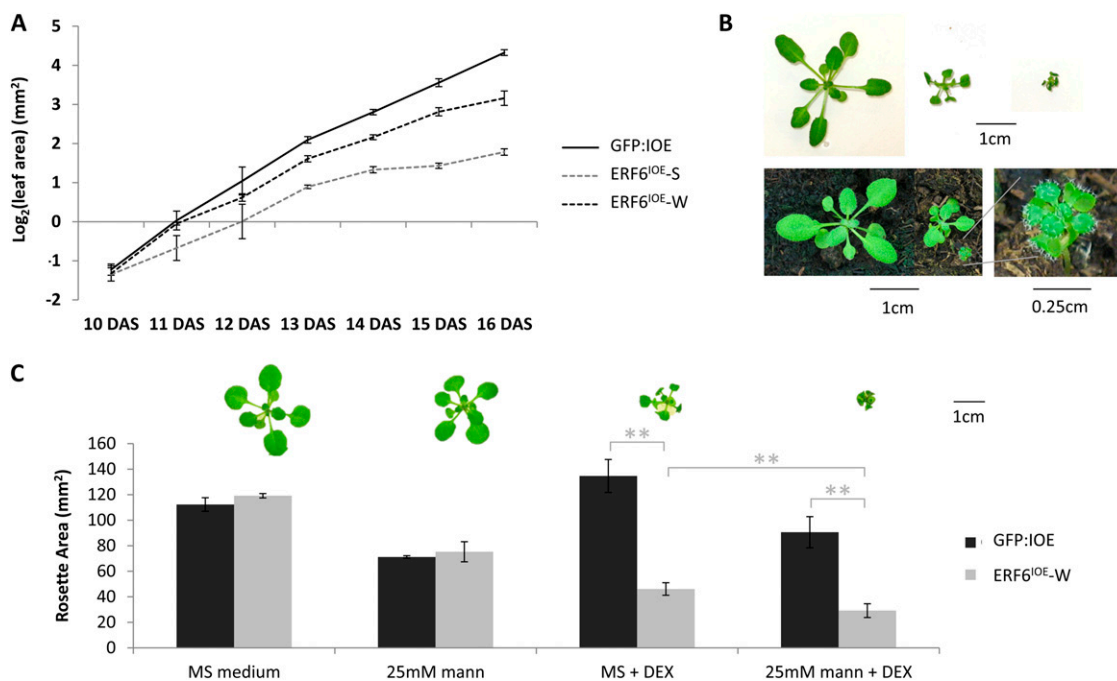


**Figure 1.** The *erf5erf6* double mutant is more tolerant to mild osmotic stress conditions. A, Rosette area over time of wild-type plants (WT) and *erf5erf6* double mutants under standard MS medium and different stress conditions. The *erf5erf6* mutant shows significant tolerance to osmotic stress (MS medium supplemented with 25 mM mannitol) only. Colored shadows indicate SE. Three biological repeats were performed with at least 12 seedlings per line per treatment. B, Leaf area measurements (third leaf) of the *erf5erf6* mutant and the wild type upon transfer at 9 DAS to standard or mild osmotic stress conditions. On osmotic stress, the *erf5erf6* mutant is always about 50% larger than the wild type (for detailed measurements, see Supplemental Table S2). Error bars indicate SE. Three biological repeats were performed with 16 leaves per repeat. H<sub>2</sub>O<sub>2</sub>, Hydrogen peroxide.

(p35S). This chimeric ERF6-GR protein is expected to stay in the cytoplasm, but after addition of the steroid hormone dexamethasone (DEX), it undergoes conformational changes and migrates to the nucleus, where it becomes functional as a transcription factor (Corrado and Karali, 2009). Two glucocorticoid-inducible overexpression (IOE) lines were used for further analysis: a strong *ERF6*-overexpressing line referred to as ERF6<sup>IOE-S</sup> (fold change = 7,000 as compared with the p35S-GFP-GR line, measured in young seedlings) and a weaker line denominated ERF6<sup>IOE-W</sup> (fold change = 220). As a control, a DEX-inducible 35S::GFP-GR (GFP:IOE) line was used. To examine the effect of *ERF6* overexpression specifically on actively growing leaves, plants were germinated on a nylon mesh overlaying MS medium and transferred to medium containing 5  $\mu$ M DEX at 9 DAS, when the third leaf is fully proliferating. *ERF6* activation from 9 DAS onward drastically reduced the growth of this leaf: at 16 DAS, leaf area reductions of 83% and 55% were measured for ERF6<sup>IOE-S</sup> and ERF6<sup>IOE-W</sup>, respectively, and significant reductions were already observed 48 and 72 h after DEX treatment of ERF6<sup>IOE-S</sup> and ERF6<sup>IOE-W</sup>, respectively (Fig. 2A). At the cellular level, the severe growth reduction of ERF6<sup>IOE-S</sup> at 16 DAS was caused by both smaller (57%) and fewer (59%) leaf cells (Supplemental Fig. S5). As expected, this cellular

phenotype was less pronounced in the weaker ERF6<sup>IOE-W</sup> line, in which mainly cell area was affected (Supplemental Fig. S5). Flow cytometry showed an increase in endoreduplication upon strong *ERF6* overexpression, suggesting that *ERF6* pushes cells from mitosis into endoreduplication and differentiation (Supplemental Fig. S6). When left on DEX for longer times (until 22 DAS), *ERF6*-overexpressing plants remained dwarfed and dark green, with stunted rosettes (Fig. 2B). Similar phenotypes were observed for soil-grown plants sprayed daily with a 5  $\mu$ M DEX solution from 9 DAS onward (Fig. 2B). Taken together, these data show that *ERF6* expression levels inversely correlate with leaf growth.

To further investigate the role of *ERF6* in stress-mediated growth inhibition, we exposed the ERF6<sup>IOE-W</sup> line to short-term osmotic stress. We only used the ERF6<sup>IOE-W</sup> line, as the ERF6<sup>IOE-S</sup> line shows a very severe phenotype making the accurate measurement of subtle growth changes rather difficult. The ERF6<sup>IOE-W</sup> line was grown on MS medium until 9 DAS and then transferred to medium with or without 25 mM mannitol, in combination with or without DEX. In the presence of DEX, the ERF6<sup>IOE-W</sup> line was found to be hypersensitive to osmotic stress as compared with the wild type (Fig. 2C). When first germinated on MS medium and then transferred to either DEX or



**Figure 2.** ERF5 and ERF6 negatively regulate leaf growth. **A**, Growth measurements of the third leaf of inducible *ERF6* overexpression plants transferred to DEX at 9 DAS to induce *ERF6* overexpression. Leaf size becomes significantly smaller than that of the control at 11 DAS for *ERF6*<sup>IOE</sup>-S and at 12 DAS for *ERF6*<sup>IOE</sup>-W. **B**, Rosettes of *ERF6*-overexpressing plants in vitro (growth medium supplied with DEX; top panel) and in soil (plants sprayed daily with DEX; bottom panel). From left to right: GFP:IOE control line, *ERF6*<sup>IOE</sup>-W, and *ERF6*<sup>IOE</sup>-S. Plants are 22 d old. **C**, Nineteen-day-old rosettes of *ERF6*<sup>IOE</sup>-W lines upon *ERF6* overexpression with DEX at 9 DAS, exposure to osmotic stress (25 mM mannitol), or the combination of mannitol and DEX. \*\**P* < 0.01. For **A** and **C**, error bars indicate se of three repeats with 16 plants per repeat. [See online article for color version of this figure.]

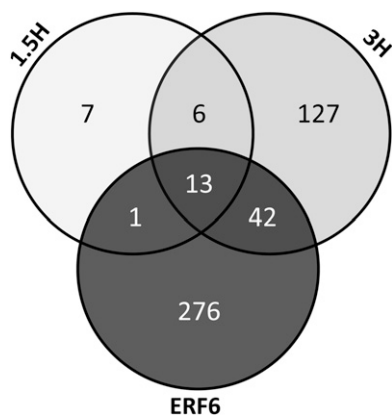
mannitol, *ERF6*<sup>IOE</sup>-W plants showed a reduction in growth but were still able to develop normally. However, when transferred to mannitol + DEX, the plants failed to develop correctly and were extremely dwarfed. Together, our data confirm that *ERF6* plays an important role in modulating leaf growth under stress.

### ERF6 Inhibits Growth through a GA/DELTA-Dependent Mechanism

To elucidate how *ERF6* reduces leaf growth, we performed a genome-wide analysis of genes rapidly induced by the activation of *ERF6* overexpression. To this end, 9-d-old *ERF6*<sup>IOE</sup>-S plants were transferred for 4 h to DEX, and subsequently, third leaf primordia (which are then smaller than 0.1 mm<sup>2</sup> in size) were microdissected and subjected to AGRONOMICS1 tiling arrays (Rehrauer et al., 2010). Already 4 h after DEX treatment, 344 genes were differentially expressed (false discovery rate-corrected *P* < 0.05), of which 332 were induced (Supplemental Table S3), suggesting that *ERF6* acts as an activator of gene expression. Gene Ontology annotation analysis of the 332 up-regulated genes using BiNGO (Maere et al., 2005) revealed that the putative *ERF6* targets are highly enriched in several

stress-related and biological signaling process categories, such as “response to water stress,” “response to chemical stimulus,” “response to biotic stimulus,” and “response to ethylene” (Supplemental Fig. S7), again strongly suggesting a role for *ERF6* in early stress response. Importantly, when comparing the putative *ERF6* target genes with the previously identified list of genes specifically induced in leaf initials within 3 h of exposure to osmotic stress (Skirycz et al., 2011a), we observed a highly significant overlap (18.5 times higher than expected by chance; Fig. 3). Out of the 332 putative *ERF6* targets, 56 were found to be induced within 3 h of mannitol treatment (Supplemental Table S4). Interestingly, 14 of the 27 genes induced after 1.5 h of mannitol treatment are differentially expressed upon *ERF6* induction (62.3 times more than expected by chance), again underlining the central role for *ERF6* in early stress response. An additional 56 *ERF6*-induced genes are also found to be induced 12 and 24 h following mannitol treatment in proliferating leaves.

Genes that are rapidly induced by DEX-mediated activation of *ERF6* are putative target genes. One of these genes, *GA2-OX6*, encoding an oxidase involved in GA inactivation, deserves particular attention because the *ERF6*<sup>IOE</sup> dwarf phenotype phenocopies that of plants insensitive to GAs (Peng et al., 1997; Thomas and Sun, 2004). Moreover, from our previously established



**Figure 3.** Overlap between ERF6 targets and genes rapidly induced by 25 mM mannitol. Comparison was performed of the 332 putative ERF6 targets with the previously identified list of genes specifically induced in leaf initials within hours upon exposure to osmotic stress (Skirycz et al., 2011a). Values indicated in the Venn diagram represent the number of genes induced upon 1.5- and 3-h mannitol treatment and the genes induced in leaf initials 4 h following DEX application in ERF6<sup>IOE-S</sup>.

list of osmotic stress-responsive genes (Skirycz et al., 2011a), we observed that *GA2-OX6* is the only GA 2-oxidase activated by mild osmotic stress (Supplemental Fig. S8). We subsequently tested whether the ERF6-mediated activation of *GA2-OX6* expression could explain the growth retardation caused by ERF6 activation. Time-course quantitative PCR analysis confirmed the induction of *GA2-OX6* expression within 2 h after the activation of strong *ERF6* overexpression (Fig. 4A). Consistently, there is a correlation between the timing and level of *GA2-OX6* induction and the observed growth inhibition, as seen in the ERF6<sup>IOE-W</sup> line, where upon DEX treatment the growth is less affected and the *GA2-OX6* induction by ERF6 is slower and less pronounced (Supplemental Fig. S9). Importantly, in the *erf5erf6* mutant, the growth of which is less affected by osmotic stress, the induction of *GA2-OX6* following stress exposure is delayed (Supplemental Fig. S10).

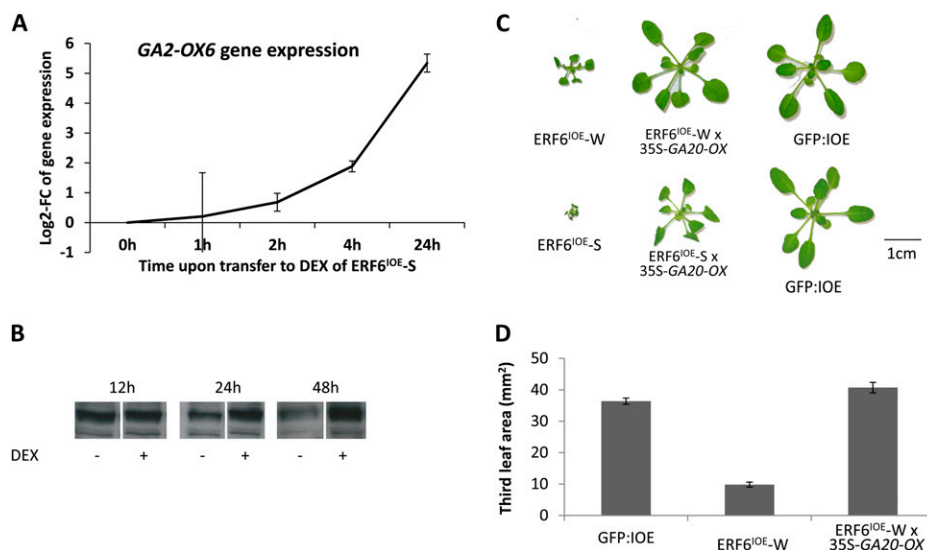
Our data support a model in which ERF6 is able to activate *GA2-OX6* expression, triggering GA breakdown and consequently stabilizing DELLA proteins, which are known to negatively affect growth. To further confirm this model, we investigated whether DELLAs were stabilized after *ERF6* activation by crossing the ERF6<sup>IOE</sup> lines with a GFP-tagged DELLA reporter line (pRGA::GFP-RGA; Silverstone et al., 2001). Stabilization of RGA, the major DELLA expressed in developing leaves (Dill et al., 2001), following ERF6 activation can be followed by measuring GFP protein levels. Western-blot analysis using a primary antibody against GFP demonstrated the stabilization of RGA between 12 and 24 h after ERF6 activation (Fig. 4B). Finally, to confirm the involvement of GA in the ERF6-mediated growth arrest, we tested whether the growth inhibition activated by ERF6 could be abolished by crossing the ERF6<sup>IOE-W</sup> line with a transgenic line overexpressing the rate-limiting GA

biosynthetic enzyme GA 20-oxidase1 (35S-*GA20-OX1*; Coles et al., 1999; Gonzalez et al., 2010). When grown on DEX, the resulting plants (ERF6<sup>IOE-W</sup> × 35S-*GA20-OX1*), in contrast to ERF6<sup>IOE-W</sup> plants, did not show any growth retardation (Fig. 4C), and the final size (at 21 DAS) of the third leaf was similar to that of control GFP:IOE plants (Fig. 4D). A similar, albeit more partial, restoration of growth was obtained for ERF6<sup>IOE-S</sup> × 35S-*GA20-OX1* plants (Fig. 4C). Together, these data confirm that, under osmotic stress, ERF6 induces the expression of the gene encoding the GA-inactivating enzyme *GA2-OX6*, thereby reducing the bioactive GA levels and stabilizing the DELLA proteins.

### ERF6 Activates a Plethora of Stress-Responsive Genes

We subsequently further explored the ERF6 regulon of 332 putative ERF6 target genes, which is enriched for stress-responsive genes, suggesting that ERF6 plays a role in orchestrating, besides growth, also early stress-induced gene expression. Multiple genes encoding stress-related transcription factors were identified within the overlap of putative ERF6 target genes and genes transcriptionally induced within 24 h after osmotic stress exposure (Skirycz et al., 2011a). To further uncover the link between ERF6 and this stress-related transcription factor network, we chose three representative genes, *STZ*, *WRKY33*, and *MYB51*, as these were previously shown to have a role in biotic and abiotic stress signaling (Sakamoto et al., 2000, 2004; Gigolashvili et al., 2007; Jiang and Deyholos, 2009; Birkenbihl et al., 2012; Li et al., 2012; Niu et al., 2012). Additional quantitative PCR analysis confirmed the induction of these genes within 2 h after DEX-mediated ERF6 activation using the ERF6<sup>IOE-S</sup> (Fig. 5A) and ERF6<sup>IOE-W</sup> (Supplemental Fig. S9) transgenic lines, thereby rendering them primary candidates for being direct ERF6 targets. This is supported using a protoplast activation assay with promoter-luciferase reporter constructs (pSTZ::*fLUC*, pWRKY33::*fLUC*, and pMYB51::*fLUC*), in which the respective promoters were cloned upstream of the *fLUC* gene (encoding the firefly luciferase enzyme) and expressed together with a 35S-*ERF6* or 35S-*ERF5* construct in tobacco (*Nicotiana tabacum*) Bright Yellow-2 (BY-2) protoplasts. Binding of ERF6 or ERF5 to the promoter of interest triggers the expression of the *fLUC* gene and the production of the luciferase enzyme. A significant increase in luciferase activity shows the activation of pSTZ, pWRKY33, and pMYB51 by both ERF5 and ERF6 (Fig. 5B). In summary, these data strongly suggest that ERF5 and ERF6 directly activate the expression of the stress-related transcription factor genes *STZ*, *WRKY33*, and *MYB51*.

Finally, we investigated whether the two functions of ERF6, being on the one hand leaf growth regulation and on the other hand the activation of a stress defense transcriptional cascade, are interdependent. For this purpose, we used the ERF6<sup>IOE-W</sup> × 35S-*GA20-OX1* and ERF6<sup>IOE-S</sup> × 35S-*GA20-OX1* lines, in which the



**Figure 4.** ERF6 regulates GA levels through transcriptional control of the *GA2-OX6* gene. A, Induction of *GA2-OX6* following the activation of *ERF6* overexpression. Two hours after transfer to DEX of *ERF6*<sup>IOE-S</sup>, *GA2-OX6* is significantly induced. Expression was measured in proliferating third leaves, and values are normalized to their expression in the GFP:IOE control line exposed to the same treatment. Error bars indicate se of three repeats with 64 young third leaves per repeat. B, Stabilization of the DELLA protein RGA upon the activation of *ERF6* overexpression shown by western blot, targeting the GFP domain of the RGA-GFP fusion protein in pRGA:GFP-RGA × *ERF6*<sup>IOE-S</sup> seedlings. DELLA stabilization first clearly appears 24 h after *ERF6* activation. Three biological replicates were performed. C, Growth complementation assay. By crossing the two independent *ERF6*:IOE lines with a 35S-*GA20-OX* line (ectopic GA overproduction), the dwarfed phenotype could be partially and fully complemented in *ERF6*<sup>IOE-S</sup> and *ERF6*<sup>IOE-W</sup> lines, respectively. Treatment with DEX was applied at 9 DAS, and photographs were taken at 21 DAS. D, Measurements of third leaves at 21 DAS of GFP:IOE, *ERF6*<sup>IOE-W</sup>, and *ERF6*<sup>IOE-W</sup> × 35S-*GA20-OX* upon treatment with DEX at 9 DAS. Error bars indicate se of three repeats with 12 leaves per repeat. [See online article for color version of this figure.]

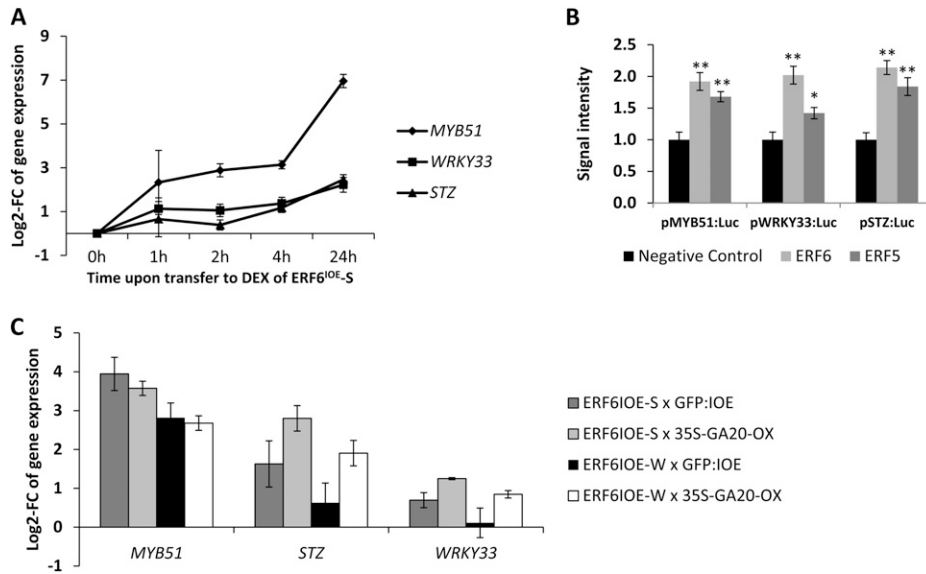
growth inhibition is entirely and partially abolished, respectively. Importantly, when *ERF6* overexpression was activated at 9 DAS by DEX treatment for 8 h, the stress defense transcriptional cascade (represented here by the expression of *STZ*, *WRKY33*, and *MYB51*) was still activated at least as highly as in the positive control lines (Fig. 5C). The expression of *STZ* and *WRKY33* was even considerably higher in the *ERF6*<sup>IOE-W</sup> × 35S-*GA20-OX1* plants (without growth reduction) as compared with that in *ERF6*<sup>IOE-W</sup> × 35S-*GFP* plants. Thus, although the growth inhibition by *ERF6* was suppressed, the stress-related transcription network was still active. These results are of significant importance, as they demonstrate that growth reduction caused by mild stress can be uncoupled from the stress defense response.

## DISCUSSION

### ERFs Are Rapidly Induced by Osmotic Stress

Osmotic stress was previously demonstrated to induce the expression of a large number of genes in actively growing leaves (more than 1,500 genes). Within 1.5 and 3 h of stress exposure, only a small number (27 and 193, respectively) were rapidly induced (Skirycz et al., 2011a). This suggests that stress signaling occurs

through cascades in which, in a simplified view, a few rapidly activated transcription factors orchestrate other transcription factors, which in turn switch on their own targets. In this context, *ERF5* and *ERF6*, two transcription factors that are transcriptionally induced within 1 h of osmotic stress, belong to a very confined group of early stress regulators. Intriguingly, the expression of the *ERF6* targets (*STZ*, *WRKY33*, and *MYB51*) is induced within 1 h of osmotic stress as well, suggesting that there is yet another mechanism than *ERF6* transcription that triggers *STZ*, *WRKY33*, and *MYB51* expression. A possible explanation for the fast induction of *STZ*, *WRKY33*, and *MYB51* could be that *ERF6* is expressed at basal levels under standard conditions (Andriankaja et al., 2012; Wang et al., 2013) and that *ERF6* is posttranscriptionally activated by osmotic stress. A similar mechanism was recently shown to occur during oxidative stress, where *ERF6* is phosphorylated by two mitogen-activated protein kinases (MPK3 and MPK6; Wang et al., 2013). Both kinases act downstream of ACC and independently of the EIN2 signaling pathway (Yoo and Sheen, 2008), and we have previously demonstrated their presence during very early osmotic stress (Skirycz et al., 2011a). Importantly, MPK3 and MPK6 were recently shown to physically interact with *ERF5* and *ERF6*, and phosphorylation of *ERF5* and *ERF6* by MPK3 and MPK6



**Figure 5.** ERF6 regulates the stress-related transcription factors STZ, MYB51, and WRKY33. A, Induction of STZ, MYB51, and WRKY33 following the activation of ERF6 overexpression. Within 2 h of the transfer of ERF6<sup>IOE-S</sup> to DEX, STZ, WRKY33, and MYB51 are significantly induced. Expression was measured in proliferating third leaves, and values are normalized to their expression in the GFP:IOE control line exposed to the same treatment. Error bars indicate  $\pm$  SE of three repeats with 64 young third leaves per repeat. B, ERF5/ERF6-dependent activation of the promoters of MYB51, WRKY33, and STZ by the protoplast activation assay. Indicated values are luciferase detection levels normalized to the negative control. Asterisks indicate significantly different values from the control at the 1% (\*\*) and 5% (\*) significance levels. Error bars indicate  $\pm$  SE, and eight biological repeats were performed. C, Induction of STZ, MYB51, and WRKY33 expression 8 h after the activation of ERF6 overexpression in ERF6<sup>IOE</sup>  $\times$  35S-GA20-OX plants. Although the dwarfed growth phenotype is partially and completely rescued in ERF6<sup>IOE-S</sup>  $\times$  35S-GA20-OX and ERF6<sup>IOE-W</sup>  $\times$  35S-GA20-OX plants, respectively (Fig. 4B), the stress-related transcription factors are still induced to the same extent as in the positive control lines (ERF6<sup>IOE-S</sup>  $\times$  GFP:IOE and ERF6<sup>IOE-W</sup>  $\times$  GFP:IOE, respectively). Expression values are normalized to their expression in the control line (GFP:IOE). Error bars indicate  $\pm$  SE of three repeats with 64 young third leaves per repeat.

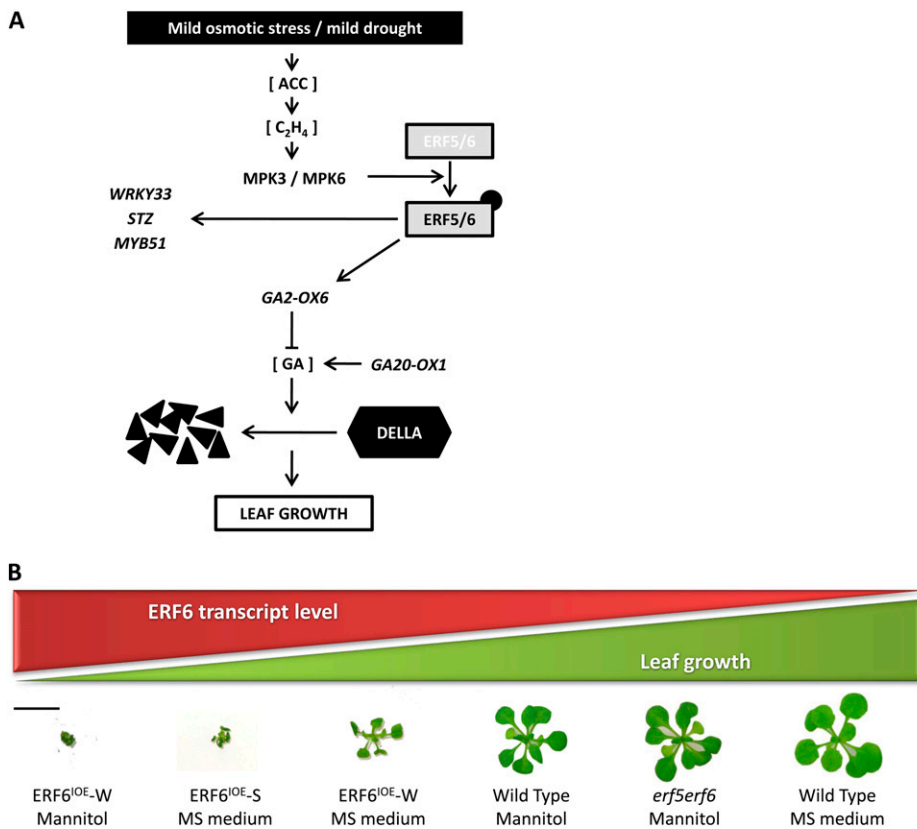
could be demonstrated (Popescu et al., 2009; Son et al., 2012; Wang et al., 2013). The very rapid transcriptional induction of ERF6 following stress exposure results from an autoactivation loop in which phosphorylated ERF6 activates its own expression, independent from induction by the upstream EIN3 and EIL1 transcription factors (Supplemental Fig. S11). Therefore, we propose the hypothesis that ERF5 and ERF6, present at the basal level prior to stress exposure, are upon stress treatment rapidly phosphorylated through MPK3 and MPK6 and thereby converted into active transcription factors, able to rapidly regulate their own expression and the expression of STZ, MYB51, and WRKY33.

#### ERF5 and ERF6 Form the Connection between ACC Accumulation and the GA/DELLA Response in Leaves Subjected to Mild Drought Stress

Recently, we have shown that growth inhibition by mild osmotic stress response occurs in two steps: first, a pause step, in which the cell cycle is temporally arrested in an ACC-dependent manner by inhibition of CDKA; and later, if stress is maintained, a stop mechanism, which pushes cells irreversibly out of the

cell cycle and into cell differentiation (Skirycz et al., 2011a). The last step was shown to be mediated by DELLAs, driving cells into early endoreduplication and thus mitotic exit (Claeys et al., 2012). However, it was not yet clear how the early ACC accumulation causes DELLA stabilization. Here, we propose that ERF5 and ERF6 form the connection between stress sensing and GA/DELLA signaling (Fig. 6A). The presence of GA2-OX6 among the putative ERF6 targets strongly supported this hypothesis. The GA2-OX6 gene encodes a GA-inactivating enzyme and its induction thus decreases the levels of bioactive GA, thereby stabilizing the DELLAs (Fig. 6A). An analysis of various transgenic lines with altered ERF6 levels and exposure of these lines to standard and mild osmotic stress conditions revealed a remarkable correlation between the levels of ERF6 expression and the severity of growth inhibition (Fig. 6B). ERF6<sup>IOE-S</sup> seedlings, which are characterized by very strong ERF6 overexpression, are completely dwarfed. As osmotic stress transcriptionally induces ERF6 expression and most likely triggers ERF6 activation, it is expected that osmotic stress would aggravate the growth phenotype. This is exactly what was observed for ERF6<sup>IOE-W</sup> plants, which are smaller but still develop normally. Exposing these weak





**Figure 6.** How ERF5 and ERF6 regulate leaf growth and stress defense under osmotic stress. **A**, Immediately upon exposure to osmotic stress, ACC accumulates in the actively growing leaves, where it is converted to ethylene. Ethylene further activates the signaling pathway involving MPK3 and MPK6. These kinases phosphorylate the basal amount of ERF5 and ERF6 present in the cell prior to stress exposure. The activated ERF5 and ERF6 then execute their dual functions: on the one hand, the activation of the stress defense transcriptional cascade with direct transcriptional activation of *WRKY33*, *STZ*, and *MYB51*, and on the other hand, the activation of leaf growth inhibition. This occurs through the transcriptional activation of the gene encoding the GA-inactivating enzyme GA2-OX6, thereby decreasing the bioactive GA concentration and stabilizing the DELLA proteins. **B**, In accordance with the model presented in **A**, *ERF6* transcript levels and stress-mediated activity inversely correlate with leaf growth (for discussion, see text). Bar = 1 cm. [See online article for color version of this figure.]

*ERF6* overexpression plants to mild osmotic stress (*ERF6*<sup>IOE</sup>-W + DEX + mannitol) completely abolishes plant growth.

Interestingly, the phenotype of *ERF6*-overexpressing plants strongly resembles that of the 35S:*gai*-GR line, in which a mutant GA-insensitive version of the DELLA protein GAI is overexpressed (Claeys et al., 2012). Both lines show the same cellular phenotype in the presence of DEX, with less and smaller epidermal cells. Importantly, the cellular phenotype observed in *ERF6*-overexpressing plants matches that of the epidermal cells of plants exposed to mild osmotic stress (Skirycz et al., 2010). The reduced cell number, however, could only be obtained by strong *ERF6* overexpression and was not clear after weak *ERF6* overexpression, indicating that ERF6 mainly works on cell expansion and to a lesser extent on cell division, in accordance with how DELLAs inhibit root and probably shoot growth (Achard et al., 2009). Finally, in the *ERF6*-overexpressing plants, proliferating cells of young leaves are pushed faster into endoreduplication, a process mediated through GA/DELLA signaling that was also observed under osmotic stress (Skirycz et al., 2011a). In conclusion, several lines of evidence show that ERF5 and ERF6 provide a link between ACC accumulation and DELLA signaling in the pause-and-stop model.

The involvement of DELLA and/or ethylene signaling in growth and stress responses is not restricted to osmotic stress. A well-known example is the involvement of C-REPEAT/DROUGHT-RESPONSIVE ELEMENT

BINDING FACTOR1 (CBF1) in freezing acclimation and growth inhibition under cold stress, upstream of several GA 2-oxidases (GA2-OX3, GA2-OX6, and GA2-OX1), thereby causing DELLA accumulation (Achard et al., 2008). Another AP2 transcription factor, DDF1, was shown to play a similar function under salt stress by regulating the expression of the GA2-OX7 gene (Magome et al., 2008). The data presented here demonstrated an analogous role for ERF6 in regulating growth under mild osmotic and oxidative stress. Moreover, whereas CBF1 acts upstream of several GA 2-oxidases (GA2-OX3, GA2-OX6, and GA2-OX1), we provide evidence that osmotic stress specifically involves GA2-OX6 (Supplemental Fig. S8). Thus, although hormonal interactions between ethylene and GA/DELLA might regulate general growth mechanisms that are shared between different abiotic cues, the identity of the molecular players involved is highly condition and tissue specific. We speculate that, although these transcription factors all activate, on the one hand, the DELLA-mediated growth inhibition, they each activate, on the other hand, a specific cluster of stress tolerance genes according to the type of stress.

#### ERFs Regulate Many Stress Resistance Genes in a GA/DELLA-Independent Way

Genome-wide identification of putative ERF6 target genes provided a list of 332 genes highly enriched for

genes involved in stress response and signaling. Among them were the well-known stress-related transcription factors *STZ*, *WRKY33*, and *MYB51*. Cotransfection of protoplasts with a promoter-luciferase reporter and 35S-*ERF5* or 35S-*ERF6* shows that *STZ*, *WRKY33*, and *MYB51* are most likely direct *ERF5* and *ERF6* target genes. *STZ* is a transcriptional repressor activated under severe salt stress to control survival mechanisms (Sakamoto et al., 2000, 2004; Mittler et al., 2006). *MYB51* is a homeodomain-like transcription factor known to regulate the biosynthesis of indole-glucosinolates, a class of secondary metabolites involved in defense against herbivores (Gigolashvili et al., 2007). Finally, *WRKY33* is a transcriptional activator involved in plant survival under high-salt, cold, and severe osmotic stress (Jiang and Deyholos, 2009). Furthermore, *WRKY33* has a role in biotic stress defense, where it regulates the balance between necrotrophic and biotrophic pathogen responses (Lippok et al., 2007; Pandey and Somssich, 2009; Birkenbihl et al., 2012). In another recent study dealing with biotic stress, *WRKY33* also was found downstream of *ERF5*, but surprisingly, and in contrast to the data presented here, *ERF5* overexpression appeared to down-regulate *WRKY33* (Son et al., 2012). A possible reason for this discrepancy is that in the latter study, plants constitutively overexpressing *ERF5* were used, possibly activating negative feedback loops suppressing *ERF5* activity. In contrast, using the inducible *ERF6* overexpression line, we could show that *WRKY33* expression increases gradually over time, demonstrating that under osmotic stress conditions, *ERF6* works as a transcriptional activator to regulate *WRKY33* expression. Supporting our observations, *ERF6* was recently shown to be necessary for *WRKY33* induction under oxidative stress (Wang et al., 2013). Consistent with the activation of stress tolerance genes by *ERF5* and *ERF6*, overexpression of *SIERF5* in tomato (*Solanum lycopersicum*) plants was recently shown to confer tolerance to drought stress (Pan et al., 2012).

*ERF6* regulates two diverse processes: on the one hand, the activation of the stress defense transcriptional cascade, and on the other hand, the regulation of growth inhibition. Our data show that both processes can be uncoupled. Overexpression of the gene encoding the GA biosynthetic enzyme GA20-OX1 (Coles et al., 1999) in *ERF6*-overexpressing plants suppressed the growth reduction phenotype but left the *ERF6*-mediated induction of stress response genes intact. Thus, in plants in which the stress signaling pathway is activated through *ERF6* overexpression, the stress tolerance factors remain activated even when growth inhibition is completely suppressed. This is similar to *CBF1*, which regulates a cluster of cold-responsive genes in a *DELTA*-independent way (Achard et al., 2008). From an agricultural point of view, this means that it should be possible to generate crops that are less affected by mild drought in terms of growth but are still able to activate their stress defense mechanisms.

## ***ERF5* and *ERF6* Regulate Growth under Multiple, But Not All, Abiotic Stresses**

The *erf5erf6* double mutant is more tolerant to both short-term and long-term osmotic stress, most likely because *GA2-OX6* expression is no longer activated (Supplemental Fig. S10). Although less clear due to experimental variability, a similar tendency is observed for plants exposed to long-term oxidative stress. Surprisingly, although salt stress is generally known to be closely related to osmotic stress, *erf5erf6* plants do not tolerate mild salt stress better than wild-type plants. This observation was supported by expression analysis demonstrating that *ERF5* and *ERF6* have no role in salt stress signaling in actively growing leaves. Consistently, in proliferating leaves, mild salt stress does not induce the expression of the *GA2-OX6* gene, in contrast to osmotic and oxidative stress. It is thus likely that growth inhibition induced by mild salt stress occurs independently of the *ERF5/ERF6*-centered growth regulatory pathway. Both drought and salt stress are characterized by the reduced ability to take up water, causing cellular dehydration and wilting. In addition, since salt ions are taken up by plant cells, plants have to cope with toxic levels of  $\text{Na}^+$ . In the majority of species,  $\text{NaCl}$  concentrations above 40 mM cause toxicity (Munns and Tester, 2008). In this study, plants were exposed to 50 mM  $\text{NaCl}$  and the observed growth reduction was probably mainly caused by  $\text{NaCl}$  toxicity and thus less related to osmotic stress defense. Although salt and osmotic stress show similarities, genome-wide expression studies revealed that large sets of genes are specifically induced by only one of these stresses (Denby and Gehring, 2005). Depending on the duration and exact conditions by which the stresses were applied, the overlap of genes responding to both abiotic stresses varied on average between 10% and 40%. When comparing only expression analyses performed on shoot tissue, the overlap was reduced to 3%. Thus, early stress-sensing and signaling responses in *Arabidopsis* shoots are mainly specific to either salt or osmotic stress, and our data clearly support this notion in actively growing leaves. Both osmotic stress and salt stress implicate the ethylene precursor ACC as an early signal (Zhang et al., 2011), and the molecular mechanisms by which this difference between salt and osmotic stress response is established in actively growing leaves are far from resolved. We speculate that the intermediate regulator acting between ACC and *ERF5/ERF6* in the cascade is active after either osmotic or salt stress and activates or inhibits *ERF5/ERF6* specifically in this condition. A putative candidate for such a regulator is *NEK6*, a kinase transcriptionally induced by ACC and by salt stress (Skirycz et al., 2011a; Zhang et al., 2011) but not by osmotic stress (Skirycz et al., 2011a). We further speculate that upon salt stress, the *NEK6* kinase either rapidly phosphorylates and thereby inactivates *ERF5/ERF6* or inhibits the ethylene biosynthesis pathway (Zhang et al., 2011), establishing a slower but stable *ERF5/ERF6* inhibition. It is likely that *CBF1* is the

functional equivalent of ERF6 under salt stress conditions (Achard et al., 2008). This probably allows the activation of genes conferring tolerance to sodium toxicity, which are not activated by ERF6.

## CONCLUSION

In this study, we provide a missing link between ACC accumulation and DELLA stabilization in the pause-and-stop mechanism by which Arabidopsis leaf growth is shut down under osmotic stress. We uncovered a dual regulatory role for the transcription factors ERF5 and ERF6 and propose them to be central elements in a signaling network summarized in Figure 6A. Mild osmotic stress triggers the accumulation of ACC and, most likely, initiates an ACC-dependent signaling cascade involving MPK3 and MPK6. These mitogen-activated protein kinases activate ERF5 and ERF6, which in turn initiate transcription of the *GA2-OX6* gene encoding a GA-degrading enzyme. GA breakdown stabilizes DELLA proteins and represses growth. In parallel, ERF5 and ERF6 activation triggers the expression of stress tolerance factor genes such as *STZ*, *WRKY33*, and *MYB51*. Interestingly, the ERF5- and ERF6-mediated growth inhibition and the activation of the stress-responsive network can be uncoupled. This uncoupling holds great potential for engineering crops that are less inhibited by mild stress while maintaining stress tolerance.

## MATERIALS AND METHODS

### Plant Lines

The single *erf5* and *erf6* mutants of Arabidopsis (*Arabidopsis thaliana*) were obtained from the SALK collection, references SALK\_076967 (*erf5*) and SALK\_030723 (*erf6*). The pRGA:RGA-GFP line was a kind gift of Prof. Dr. Tai-ping Sun (Duke University). The 35S-*GA2-OX1* line used was previously described by Gonzalez et al. (2010) and originally was a gift from P. Hedden (Coles et al., 1999). All lines used are in the Columbia background.

### In Vitro Plant Growth Conditions

Seedlings were grown in vitro on one-half-strength MS medium (Murashige and Skoog, 1962) containing 1% Suc at 21°C under a 16-h-day (110  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 8-h-night regime. For long-term experiments where no transfer was needed, 9 g L<sup>-1</sup> agar was added to the medium. For short-term experiments involving transfer, 6.5 g L<sup>-1</sup> agar was used, and the growth medium was overlaid with nylon mesh (Prosep) of 20- $\mu\text{m}$  pore size to facilitate transfer. For expression analysis and growth experiments, 64 and 16 seeds, respectively, were equally distributed on a 14-cm-diameter petri dish. The different *ERF6* gain- and loss-of-function lines were always grown together with the appropriate control on one plate to enable correct comparisons.

### Exposure to Short-Term Osmotic Stress and/or Glucocorticoid-Induced Activation of ERF6

Plants were grown on a nylon mesh covering control MS medium until the third leaf had completely emerged from the shoot apical meristem but was still in a fully proliferative stage, at 9 DAS. At this time point, the mesh was transferred to plates with one-half-strength MS medium containing 25 mM D-mannitol (plant culture tested; Sigma), 5  $\mu\text{M}$  DEX (Sigma), or a combination of both. For expression analysis and growth experiments, all seedlings were

transferred to DEX, including the GFP:IOE control lines, to account for the possible effects of DEX on growth or gene expression.

### Growth Analysis

Growth analysis was performed on the third true leaf harvested at different time points after transfer to DEX. After clearing with 70% ethanol, leaves were mounted in lactic acid on microscope slides. For each experiment, about 15 to 20 leaves were photographed with a binocular microscope, and abaxial epidermal cells (100–200) were drawn for three representative leaves with a DMLB microscope (Leica) fitted with a drawing tubus and a differential interference contrast objective. Photographs of leaves and drawings were used to measure leaf area and cell size, respectively, using ImageJ version 1.370 (<http://rsb.info.nih.gov/ij/>), and average cell numbers were calculated by dividing leaf area by cell area.

### Sampling RNA for Expression Analysis

Samples were obtained from three independent experiments and from multiple plates within the experiment. Whole seedlings were harvested rapidly in an excess of RNAlater solution (Ambion) and, after overnight storage at 4°C, dissected using a binocular microscope on a cooling plate 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. RNA was extracted with Trizol (Invitrogen) and further purified with the RNeasy Mini Kit (Qiagen). DNA digestion was done on columns with RNase-free DNase I (Roche).

### Genome-Wide Expression Changes

For the identification of genome-wide expression changes, samples of the strong *ERF6*-overexpressing line (*ERF6*<sup>IOE-S</sup>) and the control line (GFP:IOE) were harvested 4 h after transfer to DEX. Two micrograms of pure RNA samples was hybridized to AGRONOMIC51 Arabidopsis Tiling Arrays (Rehauer et al., 2010) at the VIB Microarray Facility. Obtained expression data were processed with Robust Multichip Average (background correction, normalization, and summarization) as implemented in BioConductor (Irizarry et al., 2003a, 2003b; Gentleman et al., 2004). The Brainarray “*agronomics1air9genecdf*” Chip Definition File was used to assign probes to genes (Brainarray). The BioConductor package Limma as well as the Rank Products method were used to identify differentially expressed genes (Breitling et al., 2004; Smyth, 2004). To compare gene expression with and without *ERF6* induction (*ERF6*<sup>IOE-S</sup> × GFP:IOE), moderated Student's *t* test statistics were calculated using the eBayes function, and *P* values were corrected for multiple testing using topTable (Hochberg and Benjamini, 1990). False discovery rate-corrected *P* < 0.05 was used as a cutoff.

### Flow Cytometry

For flow cytometry analysis, 16 leaves were chopped with a razor blade in CyStain UV Precise P buffers (Partec) according to the manufacturer's instructions. The nuclei were analyzed with a CyFlow flow cytometer with FloMax Software (Partec).

### Quantitative Reverse Transcription-PCR

For complementary DNA synthesis, the iScript cDNA Synthesis Kit (Bio-Rad) was used according to the manufacturer's instructions using 1  $\mu\text{g}$  of RNA. Quantitative reverse transcription-PCR was done on a LightCycler 480 (Roche Diagnostics) on 384-well plates with LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's instructions. Melting curves were analyzed to check primer specificity. Normalization was done against the average of housekeeping genes AT1G13320, AT2G32170, and AT2G28390:  $\Delta\text{Ct} = \text{Ct}(\text{gene}) - \text{Ct}(\text{mean}[\text{housekeeping genes}])$  and  $\Delta\Delta\text{Ct} = \Delta\text{Ct}(\text{control line}) - \Delta\text{Ct}(\text{line of interest})$ . Ct refers to the number of cycles at which SYBR Green fluorescence reaches an arbitrary value during the exponential phase of amplification. Primers were designed with the QuantPrime Web site (Arvidsson et al., 2008; Skirycz et al., 2010). Primers used in this study are as follows: *ERF5*, 5'-AAATTCGCGCGGAGATTCGTG-3' and 5'-TCAAACGTCCCAAGCCAAACGC-3'; *ERF6*, 5'-TCGAATCCTCTCGCGTTACTG-3' and 5'-TTCGGTGGTGGCATCTTCAACG-3'; *GA2-OX6*, 5'-TGGATCCCA-

ATCCCATCTGACC-3' and 5'-TCTCCCATTCGTCGAATGCCTGAAG-3'; *MYB51*, 5'-GCCCTTCACGGCAACAAATG-3' and 5'-GGTTATGCCCTTGT-GTGTAACTGG-3'; *STZ*, 5'-TCACAAGGCAAGCCACCCTAAG-3' and 5'-TTGTCGCCGACGAGGTTGAATG-3'; *WRKY33*, 5'-CTTCCACTTGTTCAGTCCCTCTC-3' and 5'-CTGTGGTTGGAGAAGCTAGAACG-3'.

## RGA:GFP Quantification

Amounts of RGA:GFP protein in either DEX-treated or nontreated ERF6<sup>1OE-S</sup> plants were quantified by western blotting. Complete seedlings were harvested in liquid nitrogen 48 h after transfer to DEX or control medium and ground with a Retsch machine. Protein extraction was done by adding extraction buffer (Van Leene et al., 2007) to ground samples, followed by two freeze-thaw steps and two centrifugation steps (20,817g, 10 min, 4°C), whereby the supernatant was collected each time. Western-blot analysis was performed with primary rabbit anti-GFP antibodies (Santa Cruz; diluted 1:200) and secondary horseradish peroxidase-conjugated donkey anti-rabbit antibodies (GE Healthcare; diluted 1:10,000). A chemiluminescence procedure (NEN Life Science Products) was used for detection.

## Long-Term Stress Exposure with IGIS

For long-term exposure to abiotic stress in combination with automated phenotypic analysis, plants were grown on the IGIS platform in the same conditions as described in "In Vitro Plant Growth Conditions." The one-half-strength MS medium contained 9 g L<sup>-1</sup>, and stresses were applied by adding 25 mM mannitol (osmotic stress), 50 mM NaCl (salt stress), or 1.5 mM hydrogen peroxide (oxidative stress). The platform allows for a detailed rosette growth analysis of in vitro-grown Arabidopsis plants and can hold up to 10 petri dishes. On each plate, the *erf5erf6* mutant was grown next to the appropriate control (azygous for both transfer DNA constructs). Images were captured on an hourly basis, using near-infrared technology to visualize plants in the dark. Individual rosettes were extracted automatically by image analysis processing. A data analysis pipeline compiles the measurements and constructs rosette growth curves. Details about the IGIS platform will be published later.

## Protoplast Activation Assay

The protoplast activation assay was performed as described previously (De Sutter et al., 2005; Pauwels et al., 2010). All transformation constructs were obtained using the Gateway cloning system, and all liquid handlings were done on the Tecan Genesis automated platform (De Sutter et al., 2005). The protoplast activation assay was performed in a 3-d-old tobacco BY-2 cell culture, subcultured from a 6- to 10-d-old culture. BY-2 cells were protoplasted using a 1% cellulase (Kyowa Chemical Products) and 0.1% pectolyase (Kyowa Chemical Products) enzyme solution in a 0.4% mannitol (Sigma) buffer. Protoplasts were then washed, counted, and diluted to 500,000 mL<sup>-1</sup>. For every transcription factor-promoter combination, 100 μL (50,000 protoplasts) was used. To confirm direct binding of ERF5/ERF6 on the promoters of *STZ*, *MYB51*, and *WRKY33*, protoplasts were cotransfected with 35S-*ERF5* or 35S-*ERF6* (in p2GW7) and p*STZ*-*FLUC*, p*MYB51*-*FLUC*, or p*WRKY33*-*FLUC* (in pM42GW7). Promoters were defined as the 2,000 bp upstream of the start codon. *FLUC* encodes the firefly luciferase enzyme. Every protoplast sample was transfected with 2 μg per construct as well as with 2 μg of normalization construct expressing the *Renilla* luciferase (rLUC) enzyme. Transformed protoplasts were further grown by gentle shaking overnight in BY-2 medium to allow expression of the constructs. The next day, the BY-2 medium was removed and protoplasts were lysed in Cell Culture Lysis Reagent (Promega). Protoplast content was transferred to Nunc plates (Thermo Scientific), and *FLUC* and rLUC activities were measured using the Dual Luciferase Assay (Promega) and the LumiStar Galaxy (De Sutter et al., 2005). Measured *FLUC* activities were then normalized to rLUC activities.

Microarray data from this article were deposited in the Gene Expression Omnibus database (GSE45830).

## Supplemental Data

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

**Supplemental Figure S1.** *ERF5* and *ERF6* are induced by abiotic stresses.

**Supplemental Figure S2.** *ERF5* and *ERF6* are homologous genes and share highly conserved functional domains.

**Supplemental Figure S3.** Gene structure and expression analysis of *erf5* and *erf6* mutants.

**Supplemental Figure S4.** Gene expression of *ERF5*, *ERF6*, and their targets under osmotic, salt, and oxidative stress.

**Supplemental Figure S5.** Cellular measurements of ERF6<sup>1OE-S</sup> and ERF6<sup>1OE-W</sup>.

**Supplemental Figure S6.** Endoreduplication index in third leaves of plants overexpressing ERF6.

**Supplemental Figure S7.** BiNGO analysis of the putative ERF6 target genes.

**Supplemental Figure S8.** Expression pattern of the multiple *GA2-OX* genes upon ERF6 overexpression and under osmotic stress.

**Supplemental Figure S9.** Induction of the ERF6 targets upon *ERF6* overexpression in ERF6<sup>1OE-S</sup> and ERF6<sup>1OE-W</sup>.

**Supplemental Figure S10.** Induction of *GA2-OX6* in the *erf5erf6* double mutant following osmotic stress exposure.

**Supplemental Figure S11.** *ERF6* is transcriptionally induced by ERF6 itself and not by EIN3 and EIL1.

**Supplemental Table S1.** Measurements of *erf5erf6* rosettes under different long-term abiotic stress conditions.

**Supplemental Table S2.** Measurements of *erf5erf6* third leaves under control and osmotic stress conditions.

**Supplemental Table S3.** List of putative ERF6 target genes.

**Supplemental Table S4.** Genes induced within 3 h of osmotic stress exposure.

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