AtERF#111/ABR1 is a transcriptional activator involved in the wounding response

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SUMMARY
AtERF#111/ABR1 belongs to the group X of the ERF/AP2 transcription factor family (GXERFs) and is shoot specifically induced under submergence and hypoxia. It was described to be an ABA-response repressor, but our data reveal a completely different function. Surprisingly, AtERF#111 expression is strongly responsive to wounding stress. Expression profiling of ERF#111-overexpressing (OE) plants, which show morphological phenotypes like increased root hair length and number, strengthens the hypothesis of AtERF#111 being involved in the wounding response, thereby acting as a transcriptional activator of gene expression. Consistent with a potential function outside of oxygen signalling, we could not assign AtERF#111 as a target of the PRT6 N-degron pathway, even though it starts with a highly conserved N-terminal Met–Cys (MC) motif. However, the protein is unstable as it is degraded in an ubiquitin-dependent manner. Finally, direct target genes of AtERF#111 were identified by microarray analyses and subsequently confirmed by protoplast transactivation assays. The special roles of diverse members of the plant-specific GXERFs in coordinating stress signalling and wound repair mechanisms have been recently hypothesized, and our data suggest that AtERF#111 is indeed involved in these processes.

Keywords: ERF/AP2 transcription factors, PRT6 N-degron pathway, hypoxia, submergence, wounding, abscisic acid, Arabidopsis thaliana.

INTRODUCTION
During their life cycle, plants are continuously subjected to an immense number of abiotic (e.g. too much or too little water, salt, cold, heat) and biotic (e.g. pathogenic bacteria, insects, fungal) stress factors that impair growth, development and reproduction. Due to a changing climate, the number of heavy rainfalls and floods has markedly increased in recent decades, impacting dramatically plant performance (Bailey-Serres et al., 2012). Model-based large-scale projections predict a four-fold increase in flood hazards in most areas of the world by the end of this century (Hirabayashi et al., 2008, 2013; Alifanti et al., 2017). Submergence of plants caused by flooding events leads to restricted gas diffusion between the plant and its environment. Consequently, the gaseous plant hormone ethylene accumulates, whereas a shortage in O2 and CO2 limits aerobic respiration as well as photosynthesis, resulting in a severe energy crisis and carbohydrate deficit (Bailey-Serres and Voesse, 2008; Sasidharan et al., 2018).

As sessile organisms, plants rely on faithful perception of the low-oxygen stress (hypoxia) and have to timely translate it into adaptive responses by reprogramming gene expression and transcriptional regulation. In Arabidopsis thaliana, oxygen sensing is achieved by the homeostatic regulation of the stability of the Ethylene Response Factor family (ERF), subgroup VII (GVIIERFs) transcription factors via the Cys branch of the PRT6 N-degron pathway of ubiquitin-mediated proteolysis (Gibbs et al., 2011, 2014; Licausi et al., 2011; Dissmeyer, 2019). The five GVIIERFs – RELATED TO APETALA2.2 (RAP2.2), RAP2.3, RAP2.12, HYPOXIA-RESPONSIVE ERF1 (HRE1) and
HRE2 – are characterized by a highly conserved N-terminal MCGGAII/L motif (amino acid single letter code), whose second amino acid cysteine (Cys2) determines their stability dependent on the availability of molecular oxygen and nitric oxide (NO). In brief, METHIONINE AMINOPEPTIDASES 1 and 2 (MAP1/2) constitutively expose an N-terminal Cys2 by removal of the initiator methionine (Met1). An oxidation of Cys2 by plant cysteine oxidases (PCOs) (Weits et al., 2014; White et al., 2017, 2018) makes the protein accessible for further modifications catalyzed by ARGINYL-tRNA PROTEINTRANSFERASES (ATEs) (White et al., 2017) and the downstream acting E3 Ub ligase PROTEOLYSIS6 (PRT6), which is suggested to poly-ubiquitinate the protein and mark it for degradation by the 26S proteasome (Gibbs et al., 2011; Licausi et al., 2011; summarized in Dissmeyer, 2019). In contrast, hypoxic conditions or an inhibition of NO accumulation are sufficient for the stabilization of the GVIIFErfs, which control the transcription of hypoxia-responsive genes (HRGs) by binding to the 12 base pairs (bp) long hypoxia-responsive promoter element (HRPE) (Bui et al., 2015; Gasch et al., 2016).

In Arabidopsis, 122 ERF genes, divided into 12 subgroups I–X, VI-L and Xb-L, were identified (Nakano et al., 2006). These plant-specific transcription factors share an APETALA2 (AP2) DNA binding domain and have various functions during developmental and physiological processes in plants. Aside from the GVIIFErfs, more than 200 proteins in the Arabidopsis genome initiate with Met/Cys (MC), making them potential PRT6 N-degron pathway substrates. Among these, the transcription factor (TF) AtERF#111, ABSCISIC ACID REPRESSOR 1 (ABR1), gained our interest. AtERF#111 is one of eight members of the subgroup X of the ERF/AP2 family (Nakano et al., 2006). Recently, two other proteins with an N-terminal MC motif, VERNALISATION2 (VRN2) (Gibbs et al., 2018) and LITTLE ZIPPER 2 (ZP2R) (Weits et al., 2019) were demonstrated to be oxygen-sensitive targets of the Cys branch of the PRT6 N-degron pathway, thereby linking oxygen availability to the epigenetic control of plant development and shoot meristem activity, respectively.

PRT6 N-degron pathway mutants have been described to show altered ABA sensitivity (Holman et al., 2009; Gibbs et al., 2014). Among these, ged1, a mutant defective in PRT6, as well as prt8-1 and the double mutant ate1 ate2, exhibited enhanced sensitivity to ABA during germination (Holman et al., 2009). Also, microarray analysis of ged1 showed downregulation of ABA-responsive genes already upon control conditions (Choy et al., 2008; Riber et al., 2015). Interestingly, AtERF#111 was suggested to be induced upon drought stress and involved in ABA signalling (Pandey et al., 2005; Ha et al., 2014). In addition, the expression of AtERF#111 was induced upon hypoxia and submergence (Tsai et al., 2014; van Veen et al., 2016; Yeung et al., 2018). However, knowledge of AtERF#111 expression under different stress conditions was very limited so far due to the lack of this sequence on the widely used Affymetrix ATH1 microarray chip.

Here we show that AtERF#111 is shoot specifically induced upon submergence and hypoxia. Even though AtERF#111 starts with an N-terminal MC motif, we could not confirm its degradation by the PRT6 N-degron pathway. Nevertheless, protein stability experiments showed ubiquitin-dependent degradation. AtERF#111 was described to be an ABA-response repressor (Pandey et al., 2005), but our data suggest a completely different function. We could not confirm an involvement of AtERF#111 in ABA signalling or the drought response. However, we revealed a strong induction of AtERF#111 upon wounding. A microarray analysis of AtERF#111-overexpression (OE) plants showed a pronounced overlap between genes induced by AtERF#111 and by wounding. Interestingly, AtERF#111-OE led to a clear phenotype in root hair length and number which correlates with the AtERF#111 transcript level. We were able to identify direct target genes of AtERF#111 using a glucocorticoid-inducible prooplast assay, which also showed a link to wounding stress.

RESULTS

The expression of AtERF#111 is induced upon hypoxia and submergence

Earlier expression analyses on plants under hypoxia or submergence did not contain information on AtERF#111 (AT5G64750) expression. However, new technologies such as RNA-seq as well as the use of the Agilent Arabidopsis 4×44k chip have revealed interesting expression patterns of this gene. The expression of AtERF#111 was shown to be induced during submergence in datasets of RNA-seq as well as ribosome sequencing (van Veen et al., 2016; Yeung et al., 2018). In detail, the RNA-seq data analysis was performed after 4 h of submergence in darkness and indicated a shoot-specific upregulation of AtERF#111 in all eight tested Arabidopsis accessions (Figure S1). Therefore, we analyzed the AtERF#111 transcript level by RT-qPCR after 24 h of submergence of 3-week-old plants and could confirm an induction of AtERF#111 already upon dark treatment (AD) in comparison with illuminated control conditions (AL), as well as an increased induction by the compound stress of darkness and submergence (SD) (Figure 1).

In addition to that, we analyzed the AtERF#111 transcript level in hypoxia-stressed seedlings within a time course experiment. The expression of AtERF#111 was significantly induced after 8 h of hypoxia treatment and remained upregulated after 1 h of re-aeration (both in light) (Figure 1b). When separating roots and shoots of hypoxia-treated seedlings, we could confirm a shoot-specific upregulation of AtERF#111 (Figure S2). Interestingly, there
were no differences in AtERF#111 transcript level when comparing wildtype (WT) Col-0 and the PRT6 N-degron pathway mutant prt6-1, indicating that the AtERF#111 gene is not a target of the GVIIERFs. According to that, we could not identify any HRPE in the region comprising 3 kb upstream of the transcription start site by using the RSA tool matrix-scan.

To test whether an altered expression of the low-oxygen responsive AtERF#111 had an impact on post-submergence survival, two T-DNA insertion lines were isolated, SALK_094151C (erf#111-1) and SALK_012151C (erf#111-2). As annotated (http://www.arabidopsis.org), we could confirm the T-DNA insertion of erf#111-2 in the intron and of erf#111-2 in the second exon by sequencing. Only for erf#111-2, no transcript was detected after hypoxia treatment, which therefore displays a true null allele, whereas the intronic T-DNA insertion of erf#111-1 is most likely removed by splicing processes (Figure S3). Three-week-old plants of the erf#111-2 mutant did not show an altered survival after short-term submergence under dark conditions (4–7 days), followed by 2 weeks recovery relative to Col-0 (Figures 1c and S4). Furthermore, we generated stable AtERF#111-OE Arabidopsis plants in the WT background, having an N-terminal His6-FLAG epitope to mask AtERF#111 from potential degradation by the PRT6 N-degron pathway (see below, OEI and OEII). OEII only displayed a slightly decreased survival capacity after submergence in comparison with Col-0 and erf#111-2, but this could also be due to phenotypic differences of the OE lines already under normoxic conditions (see below).

AtERF#111 is not a target of the PRT6 N-degron pathway, but is degraded in an ubiquitin-dependent manner

As the N-terminus of AtERF#111 initiates with the amino acids MC, it represents a possible PRT6 N-degron pathway substrate. Interestingly, the N-terminal region (including the first eight amino acids) of AtERF#111 seems to be highly conserved in homologous proteins of different Brassicaceae species, for example Arabidopsis lyrata, Arabidopsis halleri or Capsella rubella (Figure S5). The GVIIERFs, however, show a motif at their N-terminus (consensus MCGGAI/L) which is different from the N-terminus of AtERF#111 and homologs. It was shown that a substitution of Cys with Ala is sufficient to inhibit protein degradation by the PRT6 N-degron pathway, leading to a stabilization of the GVIIERFs under normoxic conditions (Gibbs et al., 2011, 2014). Therefore, constructs containing AtERF#111 with the natural (MC) and the mutated (MA) N-terminal residues were generated as fusion constructs with C-terminal epitope tags. In accordance with the function as
a TF, a localization of both AtERF#111 constructs fused with a C-terminal green fluorescent protein (GFP)-tag could be detected in the nucleus of transiently transformed tobacco leaves (Figure 2). As there was no clear difference in fluorescence intensity and therefore in protein stability of the two AtERF#111 versions, we performed a cycloheximide (CHX)-chase experiment in Arabidopsis WT protoplasts to evaluate protein stability in vivo. The CHX-chase assay showed that both (MC)AtERF#111 and (MA) AtERF#111 (fused with C-terminal haemagglutinin (HAI), disappeared independently of their N-termini within 3 h in the presence of the translational inhibitor CHX (Figure 2b). Therefore, under the tested conditions, AtERF#111 could not be assigned as a target of the PRT6 N-degron pathway. However, co-incubation with the 26S proteasome inhibitor MG132 clearly resulted in a stabilization of the AtERF#111 protein, and protein steady-state levels were markedly increased (Figure 2c). Hence, these results demonstrated that AtERF#111 – despite its N-terminal MC motif – does not seem to be a target of the PRT6 N-degron pathway, but is still a target of the 26S proteasome.

AtERF#111 does not repress the ABA response

Pandey et al. (2005) suggested for AtERF#111 a role as a repressor of ABA signalling, and therefore named it ABSCI- SIC ACID REPRESSOR 1 (ABR1). This hypothesis was based on experiments with two erf#111 T-DNA insertion lines in the Col-0 background (SAIL140_G06 and SALK_012151C). In addition to an induction of AtERF#111 expression upon cold, high salt and drought stress, an increase in transcript level upon ABA treatment as well as a higher transcript accumulation of selected ABA-marker genes in the mutant lines compared with the WT were observed. Furthermore, erf#111 mutant lines showed a hypersensitive ABA-mediated response in comparison with the WT regarding seed germination at 0.7 μM ABA and root growth at 10 μM ABA.

We aimed at confirming these findings and to further evaluate the function of the TF. To that end, we used erf#111-2 (SALK_012151C), one of the two T-DNA insertion lines analyzed by Pandey et al. (2005). Additionally, we made use of two His6-FLAG-AtERF#111-OE lines (OEI and OEII) originating from independent T-DNA insertion events in the Col-0 background (see below). However, in our experiments we could not detect any differences between WT and erf#111-2, and the His6-FLAG-OE lines showed contradicting results to AtERF#111 being an ABA repressor (Figure 3). In detail, we performed the germination assay with varying ABA concentrations (0–0.7 μM ABA) and only used seeds of the same age. The latter is very important, as the sensitivity towards ABA can alter with increasing seed age (Holman et al., 2009). The ability to germinate decreased with increasing ABA concentrations, to a comparable extend for WT and erf#111-2 (Figure 3a). The OE lines showed partial yellowing of the cotyledons as well as uneven root lengths already under control conditions.

Figure 2. AtERF#111 is not a target of the PRT6 N-degron pathway, but is a target of the 26S-proteasome. (a) Confocal laser scanning microscopy visualization of tobacco plants transiently expressing p35S:(MC)AtERF#111-GFP and p35S:(MA)AtERF#111-GFP. Bar: 15 μm. White arrow indicates the nucleolus. Chlorophyll and GFP fluorescence and merged images of infiltrated tobacco leaves are shown. (b) Stability of AtERF#111 constructs with a modified N-terminus in Arabidopsis WT protoplasts. Protoplasts were either transfected with p35S:(MC)AtERF#111-HA or p35S:(MA)AtERF#111-HA and incubated with 100 μM cycloheximide (CHX) for the indicated time periods. Coomassie brilliant blue (CBB) staining was used as the loading control. The molecular weight of AtERF#111 3HA is 49 kDa. (c) CHX chase of p35S:(MC)AtERF#111-HA and p35S:(MA)AtERF#111-HA with or without the proteasome inhibitor MG132 (50 μM).
They did not display higher ABA insensitivity in comparison with Col-0, as one would expect if AtERF#111 was an ABA-response repressor. Similar results were obtained for the root growth assay: The root lengths decreased with increasing ABA concentrations, showing again no differences between erf#111-2 and Col-0 (Figure 3b). At concentrations of 50 and 100 μM ABA, the OE lines displayed shorter roots than the WT.

To verify the effectiveness of our ABA treatment, we repeated these two assays including the published ABA hypersensitive mutant pyt6-1 (Holman et al., 2009) as well as the ABA insensitive quadruple mutant of the ABA receptors pyrabactin resistance1/PYR1-like (PYR1/PYL) pyt1 pyt1 pyt2 pyt4 (Park et al., 2009) (Figures S6 and S7). To compare these mutants to Col-0 and erf#111-2, germination was assessed in more detail and scored into different categories with the criterion of no visible radicle protrusion (=dead), visible radicle protrusion (>1 mm length) as well as full seedling establishment (including the formation of green cotyledons) (Figure S6). Indeed, pyt6-1 displayed hypersensitive inhibition of germination in comparison with Col-0 and was not able to establish green cotyledons at any ABA concentration tested, whereas pyt1 pyt1 pyt2 pyt4 was ABA insensitive and showed full seedling establishment even at 0.7 μM ABA. In contrast with these lines, erf#111-2 behaved similar to Col-0 and showed an intermediate phenotype with 6–12 % of dead seeds and about 80–90% visible radicle protrusions at 0.7 μM ABA.

Regarding the root growth assay, the quadruple mutant pyt1 pyt1 pyt2 pyt4 displayed a higher relative root length as well as a significantly increased relative seedling weight than Col-0, whereas there were no detectable differences between Col-0, erf#111-2 and pyt6-1 (Figure S7). The latter was expected, as Holman et al. (2009) reported that pyt6 alleles show an ABA hypersensitivity of germination, but not a hypersensitivity regarding ABA inhibition of root elongation. Therefore, these data confirmed again our findings that erf#111-2 shows no modified ABA sensitivity in comparison with Col-0.

Furthermore, the ABA-responsive genes ARABIDOPSIS THALIANA DROUGHT-INDUCED 8 (RESPONSIVE TO ABA 18 (ATD18/RAB18; AT5G66400) and RESPONSIVE TO DESICCATION 22 (RD22; AT5G25610) did not show an altered expression between the erf#111-2 mutant, WT and the OE lines after ABA treatment. Even the AtERF#111 expression itself did not increase in response to ABA treatment in our hands (Figure 3c), as was reported by Pandey et al. (2005). These results are confirmed by transcriptome data from a time series RNA-seq experiment (Song et al., 2016) as well as by microarray analysis (Liu et al., 2013), which also displayed no differential expression of AtERF#111 in response to ABA treatment. Consequently, under the conditions used here, our experiments show that AtERF#111 is not involved in ABA signalling and therefore is no ABA repressor.

**AtERF#111 is not induced by drought, but strongly induced by wounding stress**

Microarray data by Ha et al. (2014) implied an induction of AtERF#111 expression in response to drought stress (Gene Expression Omnibus (GEO) accession no.: GSE48949). In that experiment, the aerial parts of 24-day-old plants were detached and exposed to dehydration on paper for 0 (control), 2 and 4 h. However, microarray data by Nishiyama et al. (2013) did not show an effect of drought stress on the expression of AtERF#111 (GEO accession: GSE42290). In the corresponding experiment, 3-week-old plants were grown in pots for 10 days without watering or grown under well watered conditions (control). These contradictory findings led us to repeat the different drought treatments. Firstly, when we subjected plants (8 leaf stage) to drought stress by letting them grow in pots for 9 days without watering, we observed a >250-fold induction of the drought-induced marker gene AtRAB18 in comparison with control plants by RT-qPCR (Figure 4; Figure S8). However, AtERF#111 showed no changes in transcript level in accordance with the data from Nishiyama et al. (2013). Secondly, when we exposed whole plants (8 leaf stage) to dehydration on papers for 3 h, AtRAB18 expression increased 30-fold in comparison with controls in soil. This time, also AtERF#111 showed a 75-fold induction of expression. Of note, while Ha et al. (2014) only detached the aerial parts, we chose to place the whole plants including roots onto paper. Importantly, this experimental setup did not interfere with the induction of AtERF#111 expression, as similar induction was observed in the shoots of plants when exposed to dehydration on paper with or without the roots (Figure S9). Consequently, we could also confirm the data from Ha et al. (2014).

However, given the artefact-prone stress treatment applied by Ha et al. (2014), we decided to introduce another control treatment, in which we covered the roots of the exposed plants with wet paper to avoid dehydration. Surprisingly, AtERF#111 expression increased under these conditions to the same amount as without moistening, whereas AtRAB18 expression did not alter between controls in pots and controls on papers (Figure 4a). Hence, we hypothesized that just removing the plant from the soil is sufficient to induce AtERF#111 expression, likely to be caused by wounding stress. To verify this assumption, we performed an independent time-resolved wounding experiment by slightly injuring the leaves with a needle and revealed that AtERF#111 expression is strongly induced by this treatment (Figure 4b). In detail, its expression reached a maximum (>300-fold increase) 1 h after wounding stress and decreased to basal levels after 6 h.
In addition, we generated stable Arabidopsis transgenics expressing firefly luciferase under control of the promoter of AtERF#111 (prAtERF#111:fLUC in pBGWL7). At 90 min after wounding, bioluminescence could only be observed in leaves of prAtERF#111:fLUC lines, and importantly, the signal was restricted to the wounded sites (Figure 5). In contrast, no signal was observed in leaves of Col-0, confirming a wounding-dependent response of prAtERF#111 in leaves (Figure 5).
Analysis of AtERF#111 expression upon other stress treatments

As we could observe an induction of AtERF#111 upon hypoxia, submergence and upon wounding, we wanted to evaluate other related stress conditions. When plants are flooded, they rapidly accumulate high levels of ethylene – a volatile plant hormone that triggers further signalling cascades (Sasidharan et al., 2018). However, we could not detect a change in AtERF#111 transcript levels after spraying 7-day-old seedlings with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Figure 6). Methyl jasmonate (MeJA) induces wound-responsive gene expression in plants and also H$_2$O$_2$ is systemically...
generated in leaves upon wounding stress (León et al., 2001). We could observe a 2.1-fold induction of \textit{AtERF#111} expression by \textit{H}_2\textit{O}_2 treatment and a 3.6-fold induction by \textit{MeJA} treatment. These findings endorse an involvement of \textit{AtERF#111} in the wounding response (Figure 6). Once again, we could not detect an \textit{AtERF#111} induction upon \textit{ABA} treatment in the context of this experiment (Figure 6, see also Figure 3c).

**ERF#111-overexpression lines show differences in root and shoot development**

We generated stable \textit{ERF#111-OE} plants with an N-terminal His\textsubscript{6}-FLAG epitope in the WT background to further investigate the function of the TF. Interestingly, \textit{ERF#111-OE} lines displayed noticeable phenotypes. Here, 5-week-old plants (8 h light regime) possessed smaller leaves and petioles than the WT and often produced only a small amount of seeds (Figure 7). At the seedlings stage, His\textsubscript{6}-FLAG-\textit{ERF#111} overexpression significantly increased elongation and production of root hairs in comparison with Col-0 (Figure 7b,c). Using standard RT-PCR we analyzed, whether there was a correlation between root hair formation and \textit{AtERF#111} transcript level of different \textit{ERF#111-OE} lines. Therefore, we used nine OE lines derived from independent transformation events, and grouped them according to their number and length of root hairs. Strikingly, we could identify a clear positive correlation between root hair formation and transcript level: OE lines that formed only M ACC, or 100 \textmu M \textit{MeJA}, which normally does not produce root hairs (Figure 7e). As expected, WT seedlings only formed root hairs in the hair positions.

**Gene expression profiling of \textit{ERF#111}-overexpressing plants**

We investigated the effect of \textit{ERF#111-OE} on global gene expression by microarray profiling using the Arabidopsis 4x44k array (Agilent Technologies, Waldbronn, Germany). We harvested roots and shoots of 7-day-old His\textsubscript{6}-FLAG-\textit{ERF#111-OEI} and -OEII seedlings separately and compared gene expression data to roots and shoots of WT seedlings. We identified 807 differentially expressed genes (DEGs), whose transcript expression significantly varied more than two-fold in comparison with WT samples (\textit{Signal-Log2-Ratio (SLR)} > 1, \textit{P} \textless 0.01, Figure 8, Data S1). Here, 450 of the 807 DEGs were significantly upregulated by \textit{ERF#111-OE}. Of these, 277 genes were only upregulated in shoots, 116 genes only in roots, and 57 genes in both shoots and roots. Furthermore, 357 of the 807 DEGs were significantly downregulated, 128 genes only in shoots, 222 in roots and seven in both shoots and roots.

When we compared all upregulated genes to the set of 49 core HRGs (Mustroph et al., 2009) in order to test for a possible link to the anaerobic response, we could identify only one core gene, \textit{RHODANASE} (\textit{AT2G17850}) that was upregulated by \textit{ERF#111-OE} in roots and shoots, and four more hypoxia core genes that were only upregulated in the shoots: two wound-responsive family proteins (\textit{AT4G33560}, \textit{AT4G10270}), \textit{PYRUVATE DECARBOXYLASE 1} (\textit{PDC1}, \textit{At4G33070}) and \textit{ETHYLENE RESPONSE2} (\textit{ETR2}, \textit{AT3G23150}). When comparing all DEGs with a SLR \textgreater 1 to microarray data from Arabidopsis seedlings that were submerged for 6 h in the dark (Hsu et al., 2013), or subjected to 4 h anoxia (Tsai et al., 2014), we could identify especially shoot-specific overlaps between the data sets (Figures 8b and S10). The overlaps between all \textit{ERF#111-OE} shoot-induced genes and the submergence and anoxia treatment were calculated as statistically significant and therefore greater than expected by chance (\textit{P} \textless 0.001, Fishers exact test), indicating a possible link to submergence and hypoxia. The hypoxia core gene \textit{RHODANASE} was upregulated in all data sets.
The genes most strongly induced by \textit{ERF#111}-OE in the shoots were the class I PLANT DEFENSINS PDF1.2c (AT5G44430; SLR 6.9), PDF1.3 (AT2G26010; SLR 6.5), PDF1.2b (AT2G26020; SLR 6.1), and PDF1.2a (AT5G44420; SLR 4.5). Those transcripts showed no change in expression in response to submergence or anoxia (references from Figure 8). As \textit{AtERF#111} expression is also highly induced upon wounding treatment (Figure 4b), we hypothesized that \textit{AtERF#111} might have a function in the defence/wounding response. Therefore, we compared our data with an already published microarray experiment employing the 4x44k array in which the expression was measured 3 h after wounding of Arabidopsis leaves (Wang et al., 2015) (Figure 8b). Consistent with our data, the microarray data by Wang et al. (2015) also included an induction of \textit{AtERF#111} expression upon wounding. Again, the overlap between the DEGs by \textit{ERF#111}-OE and the wounding arrays were calculated as being statistically significant ($P < 0.001$, Fishers exact test). Especially the genes most highly induced by \textit{ERF#111}-OE were also differentially expressed upon wounding, for example members of the plant defensins, but also Thioredoxin H-type 8 (TH8; AT1G69880) or Strictosidine synthase 3 (SS3; AT1G74000). Additionally, we used Gene Ontology (GO) analysis to find enriched GO categories (see Data S2). Most enriched GO terms were found in the shoot-specific DEGs in comparison with root-specific DEGs (Figure 8c), including the molecular functions peroxidase activity, oxidoreductase activity and strictosidine synthase activity as well as biological processes connected to external stimuli, for example response to chemical, stress, hormone, defence or response to other organism, supporting the idea of \textit{AtERF#111} being involved in the wounding response.
Identification of direct AtERF#111 target genes

As a next step, we aimed at identifying target genes that are likely to be regulated directly by the TF AtERF#111. We decided to compare gene expression after wounding between Col-0 and the erf#111-2 mutant line by performing a microarray experiment, in which we wounded 3-week-old Arabidopsis leaves and harvested plant material 3 h after the treatment, similar to the experiment performed previously (Wang et al., 2015). When comparing...
Overexpression of AtERF#111 identified for the shoot-specific genes induced by stable to other organism (Figure 9d), similar to the GO terms defence response, response to wounding and response to stress or in expression was also modified in response to wounding. We identified 15 direct target genes of AtERF#111, whose expression was also modified in response to wounding stress or in ERF#111-2 mutant plants directly. This could either be due to the possibility that we had chosen an inadequate time point to identify differences between erf#111-2 and WT, or and this seems more likely, that AtERF#111 is not the only regulator of putative targets in response to wounding and that the effect might be covered by redundantly acting TFs.

To solve this problem, we chose another approach to identify direct AtERF#111 target genes by using a glucocorticoid-inducible protoplast assay. Technically, we expressed a translational fusion of AtERF#111 to a glucocorticoid receptor (ERF#111-HBD) in Arabidopsis protoplasts of the erf#111-2 genotype. In this system, cytoplasmic-to-nuclear translocation of ERF#111-HBD is initiated by addition of the synthetic glucocorticoid dexamethasone (DEX) to the protoplast suspension. The effect of DEX treatment on target gene induction was measured in the presence or absence of the translation inhibitor cycloheximide (CHX), allowing for the distinction of direct and indirect target genes of AtERF#111. After 4 h of DEX treatment, protoplasts were harvested and RNA was isolated for subsequent microarray analysis. We identified 309 genes that are the sum of direct and indirect target genes (DEX treatment only) and 109 genes that were presumptive direct target genes (CHX + DEX treatment) (Figure 9 and Data S1). Among the direct target genes, all were significantly upregulated and none was significantly downregulated, supporting the conclusion that AtERF#111 is an activator of gene expression, and not a repressor as suggested by Pandey et al. (2005). By comparing the different microarray data, we could identify 15 direct target genes of AtERF#111, whose expression was also modified in response to wounding stress or in ERF#111-2 OE transgenic lines (Figures 8 and 9), again including the genes SS3 and TH8, but also for example the CYTOCHROME P450, CYP71B72 (AT3G26200). GO analysis of all identified direct AtERF#111 target genes revealed an enrichment of the biological processes response to external stimulus, defence response, response to wounding and response to other organism (Figure 9d), similar to the GO terms identified for the shoot-specific genes induced by stable overexpression of AtERF#111.

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AtERF#111 involved in wounding response

We used a protoplast transactivation system to study the transactivation potential of AtERF#111 on selected target promoters. To this end, an N-terminally HA-tagged AtERF#111 fusion (35S:HA-ERF#111) was cotransfected into Arabidopsis mesophyll protoplasts with the promoter of interest (a maximum of 2000 bp upstream of the start codon was used) fused to the firefly Luciferase gene (fLUC). Renilla Luciferase (rLUC) was used as an internal standard, and promoter activity was quantified by calculating fLUC activity relative to cotransfected rLUC activity (fLUC/rLUC). We chose the above-mentioned wounding and defence responsive genes PDF1.2a, TH8, SS3 and CYP71B22 as target promoter candidates (Figure 9e). Additionally, we also selected the hypoxia core gene RHODANASE, as well as the gene EXPANSIN1 (EXPA1), which is thought to be involved in cell wall loosening and could explain the root hair phenotype of the ERF#111-2 OE lines. Except for SS3, we were able to detect significant transactivation of the selected target promoters of PDF1.2a, TH8, CYP71B22, EXPA1 and RHODANASE by AtERF#111, demonstrating again that AtERF#111 positively regulates the transcriptional activity of these genes (Figure 10).

To further confirm the putative target genes of AtERF#111, we analyzed the expression of the selected genes in Col-0 and the erf#111-2 loss-of-function line under control as well as under stress conditions (Figure 11). RHODANASE and SS3 were induced upon wounding, but only a slightly lower expression was observed in erf#111-2 compared to the WT (Figure 11c,d). CYP71B22 showed no significant transcript changes 3 h after wounding stress in all genotypes (Figure 11e). To test the expression of the member of the plant defensins, we generated oligonucleotides amplifying PDF1.1 to PDF1.3 simultaneously, as their sequence is very similar. Interestingly, PDF1.1-1.3 expression was significantly lower in the erf#111-2 mutant in the control treatment compared with the WT, but the gene family was not induced by wounding under these experimental conditions (Figure 11a). The same could be observed for EXPA1, which also showed a lower expression in erf#111-2 than in WT plants under control conditions, suggesting that AtERF#111 controls the expression of these two genes already under normal conditions.

One gene that was significantly less induced in response to wounding stress in erf#111-2 than in the WT was TH8 (Figure 11b). This trend was also observed in our microarray data: TH8 was significantly induced in response to wounding in the WT, but not in erf#111-2. Nevertheless, the expression of TH8 was not reduced to basal levels in the RT-qPCR experiment, again suggesting that AtERF#111 is not the only regulator of this gene.

As the expression of RHODANASE is strongly responsive to hypoxia, we also analyzed the transcript level after 4

AtERF#111 transactivates selected target gene promoters
and 8 h of hypoxia as well as 8 h hypoxia and 1 h re-aeration. Again, we could not detect any significant differences in expression between WT and \textit{erf#111-2}, but we confirmed induction of this gene by hypoxia. These data suggest that AtERF#111 is not the only regulator of the wounding response. Indeed, several members of GXERFs in Arabidopsis are also strongly induced by wounding, among them \textit{ERF#108/RAP2.6}, \textit{ERF#109/RRTF1}, \textit{ERF#112}, \textit{ERF#113/RAP2.6L}, \textit{ERF#114}, and \textit{ERF#115} (Figure S11, Ikeuchi \textit{et al.}, 2017). Those TFs together with AtERF#111 might contribute to the transcriptional regulation of the wounding response.

**Figure 9.** Identification of direct AtERF#111 target genes. (a) Number of DEGs (SLR > 1, \textit{P} < 0.01) in the wounding microarray from Wang \textit{et al.} (2015) (wound I) and our own wounding microarray (wound II). Plant material was in both cases collected 3 h after wounding of WT leaves. (b) Number of direct and direct + indirect AtERF#111 target genes identified by using the glucocorticoid-inducible protoplast assay. (c) Venn diagram showing overlapping DEGs between wound I, direct AtERF#111 target genes and genes induced by \textit{ERF#111-OE} in the shoot. (d) Selected Gene Ontology (GO) categories for molecular function (MF) and biological process (BP), significantly overrepresented (\textit{P}-values calculated by GOHyperGAll) in all direct target genes of AtERF#111. (e) Heatmap of selected genes comparing direct targets, direct + indirect targets, \textit{AtERF#111-OE} in shoot and root as well as wound I and wound II (of WT and \textit{erf#111-2} mutant plants). Signal-log2-ratios are indicated by the intensity of the colour scale from −3 (blue) to 3 (yellow).
DISCUSSION

AtERF#111 is a target of the ubiquitin/proteasome system

As mutants of the PRT6 N-degron pathway display a range of pleiotropic defects (Yoshida et al., 2002; Graciet et al., 2009; Holman et al., 2009; Riber et al., 2015; Gibbs et al., 2016; Vicente et al., 2017, 2018) and >200 proteins of the Arabidopsis genome start with an N-terminal Met–Cys, it is anticipated that there might be other MC-initiated targets of the PRT6 N-degron pathway, aside from the GVIIERFs. Among these, we investigated the function of the transcription factor AtERF#111, whose N-terminal region is highly conserved in protein homologues of other Brassicaceae species and initiates with MC (Figure S5). However, the analysis of the protein stability of (MC)/(MA)-ERF#111 constructs in vivo showed that both AtERF#111 versions were unstable and were degraded independently from their N-termini within 3 h (Figure 2b). Consequently, AtERF#111 does not represent a major target of the PRT6 N-degron pathway.

Not all proteins initiated with MC are true PRT6 N-degron pathway substrates, as N-degrons have to have several features. Aside from a primary destabilizing residue and an optimally positioned downstream lysine, the N-terminal region has to be unstructured to be accessible (Gibbs et al., 2016; Dissmeyer et al., 2018; Dissmeyer, 2019). One prominent example for a protein that evades the PRT6 N-degron pathway, despite containing the N-terminal motif, is SUB1-A1, which is a major determinant of submergence tolerance in rice (Fukao et al., 2011; Gibbs et al., 2011). For this protein, it was recently demonstrated that the C-terminus protects it from degradation (Lin et al., 2019). The half-life of the AtERF#111 protein might therefore be affected by other post-translational mechanisms, for example SUMOylation or ubiquitination on different target sites. Interestingly, AtERF#115, another member of the GXERFs, was tested to be a proteasome target (Heyman et al., 2013), and an ubiquitination site was mapped to a lysine (K9) near the N-terminus (Walton et al., 2016). An alignment of the GXERFs 8-15 revealed a conservation of this site in AtERF#111, AtERF#112, AtERF#114 and AtERF#115, suggesting that AtERF#111 might also be ubiquitinated at this position (Figure S6d). Indeed, we were able to show that the degradation of AtERF#111 is likely to be dependent on the ubiquitin/proteasome system, as an inhibition of...
the proteasome by MG132 resulted in a stabilization of the AtERF#111 protein (Figure 2c).

AtERF#111 is an activator of gene expression that is not related to ABA signalling or drought stress

In the context of this work, we examined the role of AtERF#111 in relation to ABA signalling and drought stress. AtERF#111, previously named ABA REPRESSOR 1 (ABR1) was described to be strongly induced upon exogenous ABA treatment, acting as an inhibitor of the ABA response (Pandey et al., 2005). However, we were not able to confirm an involvement of AtERF#111 in ABA signalling. Essentially, we could not detect an induction of AtERF#111 expression after treating Arabidopsis seedlings with

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100 μM ABA (Figures 3c and 6). Song et al. (2016) monitored responses to ABA in the context of an RNA-seq time series experiment, including time points from 1 to 60 h after treating WT seedlings with 10 μM ABA in comparison with mock treatments. AtERF#111 expression was not significantly modified at any tested time point. Furthermore, also microarray analysis of seedlings treated for 6 h with 10 μM ABA showed no AtERF#111 induction (Liu et al., 2013).

In addition to an increase in AtERF#111 transcript level upon ABA treatment, Pandey et al. (2005) observed a higher expression of selected ABA-marker genes in erf#111 mutant lines in comparison with Col-0. In our experiment, the ABA-responsive genes RAB18 and RD22 did not show an altered expression between the erf#111-2 mutant and the WT after ABA treatment, and also the OE lines displayed no downregulation of RAB18 and RD22 expression, as one would expect if AtERF#111 was an ABA repressor (Figure 3c). GO analysis of DEGs in the ERF#111-OE lines or of direct ERF#111 target genes did not include enriched GO categories related to ABA or drought (see Data S2). Additionally, no ABA- or drought-responsive genes, for example RD29A, RD29B, RD22, RAB18, COR47, or DREB2A were modified in expression in our microarray experiment of the ERF#111-OE lines (Data S1).

Furthermore, we also could not detect any differences between WT and erf#111-2 in the germination assay on ABA-containing medium as well as in the root growth assay in the presence of ABA. In addition to Pandey et al. (2005), we included ERF#111-OE lines in our analysis, which showed no ABA insensitivity (Figure 3a,b, S6 and S7).

The synthesis of the phytohormone ABA is promoted by abiotic stresses that lead to a water deficit and osmotic stress, for example salt and temperature, but mainly drought stress. Consequently, many genes induced by exogenous ABA treatment are also drought-induced (Finkelstein et al., 2002; Seki et al., 2002; Cutler et al., 2010; Sah et al., 2016). In addition to an induction of AtERF#111 expression upon ABA treatment, Pandey et al. (2005) observed an increase in AtERF#111 transcript level also upon drought stress. However, previously published data revealed contradicting results: a microarray experiment by Nishiyama et al. (2013) did not show an effect of drought stress on AtERF#111 expression, whereas data by Ha et al. (2014) implied an induction of AtERF#111 expression in response to drought. Drought stress treatments varied methodically in the corresponding experiments: Nishiyama et al. (2013) let plants progressively dry in pots by withholding water, whereas Ha et al. (2014) detached the aerial parts of the plants and exposed them to dehydration on paper. When we repeated the different drought treatments, we observed an induction of the drought-induced marker gene RAB18 in both experiments, indicating that the plants are suffering from drought stress (Figure 4a). However, by letting plants dry in pots, we could not detect any changes in AtERF#111 expression, confirming the findings of Nishiyama et al. (2013). We also let plants dry on paper similar to Ha et al. (2014), but instead of comparing changes in gene expression to intact plants in soil, we induced another control treatment, in which we covered the roots of the exposed plants with wet paper to avoid dehydration (Figure 6a). Thereby, we revealed that AtERF#111 is strongly induced by mechanical stress, which occurs when the plant is removed from the soil and put on paper (Figure 4a). These findings highlight the importance of proper control treatments, which should be as similar to the actual stress treatment as possible. Therefore, the study from Ha et al. (2014) not only identified genes induced by drought treatment, but also those induced by mechanical stress, making a differentiation in this context impossible.

Apart from that, our experiments indicated that AtERF#111 is not a repressor, but an activator of gene expression, as all direct target genes of AtERF#111 identified by DEX-dependent nuclear localization of ERF#111 in the context of inhibited protein biosynthesis (Figure 9) were significantly upregulated and none was significantly downregulated in our microarray analysis. Furthermore, AtERF#111 was able to activate the promoters of the selected target genes (Figure 10). Taken together, our findings demonstrate that AtERF#111 is a transcriptional activator that seems to be neither involved in ABA signalling nor in the drought response.

AtERF#111 is involved in the wounding response

We were able to demonstrate that AtERF#111 expression is strongly responsive to mechanical stress: its transcript level increased more than 300-fold within 1 h after wounding and decreased to basal levels after 6 h (Figure 4b). Furthermore, luminescence at wounded rosette leaves of stably transformed fLUC reporter lines expressing fLUC under the control of the promoter of AtERF#111 was evident (Figure 5). In line with this, we detected an induction of AtERF#111 expression by H₂O₂ or MeJA, which are related to wounding stress (Figure 6). Additionally, the microarray analysis of ERF#111-OE plants showed a significant overlap of genes induced by ERF#111-OE in the shoot and by wounding (Figure 8b).

Also GO analysis of DEGs in ERF#111-OE transgenic plants highlighted responses to external stimuli, defence response or response to other organism (Figure 8c), supporting the hypothesis that AtERF#111 is involved in the wounding and defence response. We were able to identify a set of 109 genes that are directly regulated by AtERF#111 (Figure 9b). GO analysis of direct target genes included the GO-term ‘response to wounding’ (Figure 9d). By comparing the different microarray data, we could identify 15
direct target genes of AtERF#111, whose expression was also modified in response to wounding stress and by ERF#111-OE. In a protoplast transactivation assay, we showed that AtERF#111 activated the promoters of the selected target genes PDF1.2a, TH8, RHODANASE, CYP71B22 and EXPA1 (Figure 10).

When we compared gene expression after wounding between Col-0 and the erf#111-2 mutant line by microarray analysis, we could not identify genes that were significantly lower expressed in erf#111-2 in comparison with Col-0. Therefore, we hypothesized that AtERF#111 might not be the only regulator of putative targets in response to wounding. Additional RT-qPCR analysis of the selected target genes in Col-0 and erf#111-2 under control conditions and after wounding treatment showed that the only gene, which was significantly less induced in response to wounding stress in erf#111-2 was TH8 (Figure 11b), which is a h-type thioredoxin (TRX). In general, TRXs are small proteins that act as protein disulfide oxidoreductases and are involved in the regulation of the redox environment of the cell (Gelhaye et al., 2005). Arabidopsis TRXs are organized in at least five different families (f, m, x, o and h), whereas group h contains eight genes that are thought to encode for cystolic proteins in Arabidopsis (Meyer et al., 2002; Reichheld et al., 2002). For one member of this group, AtTRXh5, an upregulation during wounding, abscission and senescence as well as during contact with the bacterial pathogen Pseudomonas syringae was shown (Laloi et al., 2004). Additionally, AtTRXh5 is required for the response to victorin, a phytotoxin which induces programmed cell death in sensitive plants (Sweat and Wolpert, 2007; Lorang et al., 2012). Only very little information is available on TH8, but we observed a clear induction upon wounding and showed that it is a direct target gene of AtERF#111 (Figures 9e, 10 and 11). The expression of TH8 was not reduced to basal levels after wounding in the erf#111-2 mutant in comparison with the WT, suggesting again that the loss of AtERF#111 is covered by redundantly acting TFs.

Indeed, several of the eight members of GXERFs in Arabidopsis are also strongly induced by wounding, among these ERF#108/RAP2.6, ERF#109/RRTF1, ERF#112, ERF#113/RAP2.6L, ERF#114 and ERF#115 (Figure S11, Ikeuchi et al., 2017). Just recently, the hypothesis was published that members of the GXERF TFs coordinate stress signalling with the activation of wound repair mechanisms (Heyman et al., 2018). With the exception of ERF#112, they share a subfamily-specific conserved motif near the N-terminus (Figure S5d). This was shown, at least for ERF#114 and #115, to be important for the heterodimerization with TFs of the GRAS domain type – an interaction that turns these GXERFs into highly potent cell division activators (Heyman et al., 2016, 2018).

Interestingly, the expression of another member of the GXERFs, AtERF#108 – named REDOX RESPONSIVE TRANSCRIPTION FACTOR 1 (RRTF1) – is mediated by the WRKY TFs 18, 40, and 60, and is aside from wounding highly responsive to JA and reactive oxygen species (ROS), whereas the gene product itself enhances ROS production (Wang et al., 2008; Pandey et al., 2010; Matsuo et al., 2015). In the context of a genome-wide binding study, Birkenbihl et al. (2017) showed that AtERF#111 is also a target of the WRKY TFs 18, 33 and 40, which modulate pathogen-triggered immune responses in plants. This dataset included the information that ERF#111, #112, and #115 are targets of WRKY18, 33 and 40, and confirmed ERF#108 being a target of WRKY18 and 40. It was hypothesized that ERF#109 is important for controlling the balance of ROS within the cell (Matsuo et al., 2015). ERF#109-OE plants displayed enhanced susceptibility to the plant pathogen Alternaria brassicaceae, which could be weakened by applying antioxidants or free radical scavengers (Matsuo et al., 2015). In addition, rrf1 mutants did not show an obvious phenotype, whereas OE of ERF#109 led to the production of more and longer root hairs (Cai et al., 2014). Correspondingly, we observed that overexpression of AtERF#111 also significantly increased elongation and production of root hairs in comparison with Col-0 (Figure 7). Microtome sections of ERF#111-OE lines showed root hairs that were not only produced in root hair cells, but also in the non-hair cells that normally lack root hairs (Figure 7e).

The phenomenon of the ectopic development of root hairs in the non-hair positions has been shown to be caused by abiotic stresses, such as phosphorus or iron starvation (Müller and Schmidt, 2004). One candidate gene that might be responsible for the observed root hair phenotype of AtERF#111-OE plants is EXPA1, as we noticed no further root hair- or root epidermis-specific genes modified in expression (Data S1), and EXPA1 is also a direct target gene of ERF#111 (Figure 9e). Expansins are proteins without hydrolytic activity that participate in cell wall loosening (Cosgrove, 2000; Choi et al., 2006). AtEXPA7, another member of α expansins in Arabidopsis, was shown to influence root hair initiation and root growth (Cho and Cosgrove, 2002). EXPA1 was reported to be induced by cytokinin in the root, which is involved in controlling cell differentiation initiation (Bhar-gava et al., 2013; Pacifici et al., 2015). Interestingly, the development of root hairs was delayed in the expa1 mutant, indicating a setback in cell differentiation (Pacifici et al., 2018). These data support the hypothesis that EXPA1 could be connected to the root hair phenotype of AtERF#111-OE plants. Notably, the fact that the expression of other genes related to root cell differentiation was unchanged in the AtERF#111-OE genetic background hints towards a function of AtERF#111 in stress responsive
modulation of root morphology, rather than developmental hair cell specification.

Is the induction of AtERF#111 related to mechanical stress during submergence?

We showed that the expression of AtERF#111 is induced upon hypoxia and submergence (Figure 1a,b). Datasets of RNA- as well as ribosome sequencing confirmed its induction upon submergence, which is shoot-specific (van Veen et al., 2016; Yeung et al., 2018). AtERF#111 seems to be not a target of the GVIERFs, as its promoter sequence does not contain any HRPE and we could not detect differences in AtERF#111 expression in Col-0 and the PRT6 N-degron pathway mutant pr6-1 (Figure S2). In line with the assumption that various members of the GXRFS might act redundantly, we could not observe any variation in submergence survival of Col-0 and erf#111-2 (Figure 1c). Indeed, also other GXRFS show enhanced expression under submergence, for example ERF#108, ERF#112, ERF#113 and ERF#114 (Lee et al., 2011; Hsu et al., 2013; Yeung et al., 2018) and/or re-eration after hypoxic treatment, for example ERF#108, ERF#109, ERF#113 and ERF#114 (Branco-Price et al., 2008; Tsai et al., 2014).

Additionally, AtERF#111 seems to be no major regulator of the anaerobic response. Only one of the 49 core HRGs (Mustomph et al., 2009), RHODANESE, was upregulated by ERF#111-OE in roots and shoots and was identified to be directly regulated by AtERF#111 (Figure S10a, 9e and 11e). Expression analysis of RHODANESE revealed no differences in response to hypoxia between Col-0 and erf#111-2 (Figure 11c).

When comparing all DEGs caused by ERF#111-OE to submergence microarray data (Hsu et al., 2013), we found a significant overlap between the data sets (Figures 8b and S10b). Submergence is a compound stress, including not only low-oxygen availability, but also low light, nutrient deficiency, high risk of infection or mechanical stress, and therefore many genes are modified in expression. Interestingly, innate immunity marker genes as well as members of the WRKY TF family are strongly induced during submergence (Hsu et al., 2013). Among these, WRKY22 was shown to activate the immune response, thereby increasing the resistance towards the pathogen Pseudomonas syringae (Hsu et al., 2013). This is a good example how submergence can stimulate the immune response of the plant, as the risk of wounding or pathogen infection increases after flooding.

Aside from WRKY22, also WRKY18, WRKY33, and WRKY40 are significantly induced upon submergence (Hsu et al., 2013), and all three are also upregulated by anoxia (Tsai et al., 2014) and wounding stress (Wang et al., 2015). As AtERF#111 was shown to be regulated by WRKY18, 33 and 40 as mentioned above (Birkenbihl et al., 2017), we speculated that the regulation of AtERF#111 expression might be related to mechanical stress during submergence. As AtERF#111 is not only induced by submergence, but also by hypoxia, one could also imagine that submerged plants might expect to be mechanically stressed or wounded when the flood recedes, as the hypoxia treatment simulates the low-oxygen availability during submergence.

CONCLUSION

In the present study, we identified AtERF#111 as a wound- responsive TF, whose expression is also induced upon hypoxia and submergence. We could neither confirm AtERF#111 acting as a repressor of ABA signalling, nor an involvement in the drought response. Despite its N-terminal MC motif, this potential substrate could not be shown to be a target of the PRT6 N-degron pathway. By replacing the conserved Cys2 residue with Ala and comparing protein abundance, both (MC) and (MA)AtERF#111 demonstrated instability, whose degradation is yet dependent on the ubiquitin/proteasome system. By microarray analyses, we could define a set of genes that show a link to wounding stress and are directly regulated by AtERF#111, thereby acting as a transcriptional activator of gene expression. However, resolving the function of AtERF#111 in combining the responses to submergence and wounding remains a future challenge. The likely redundancy of AtERF#111 and other GXRFS in coordinating stress singaling makes it necessary to generate higher order mutants to further investigate their function.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis (Arabidopsis thaliana) ecotype Columbia (Col-0) was used as the WT. Seeds of the T-DNA insertion lines SALK_094151C (erf#111-1) and SALK_012151C (erf#111-2) were ordered from the Nottingham Arabidopsis Stock Centre, pr6-1 (SAIL_1278_H11) was obtained from Julia Bailey-Serres. Seeds of the quadruple mutant pyr1 pyl1 pyl2 pyl4 were obtained from Sean Cutler (Park et al., 2009). Seeds were surface-sterilized and sown on Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands, including 1% (w/v) sucrose, 1% (w/v) agar), stratified (3 days darkness, 4°C) and grown for the indicated time periods in phycobacteria under long-day (LD) conditions (23°C, 16 h/8 h light/dark cycle; 100 μmol of photons m⁻² sec⁻¹). For experiments with adult plants, 7-day-old seedlings were planted into soil (soil:vermiculite, 2:1; for submergence experiments one-part sand was added to two-parts of the soil mixture) and grown for 2-3 weeks under short-day (SD) conditions (23°C, 8 h/16 h light/dark cycle; 100 μmol of photons m⁻² sec⁻¹). For protoplast experiments, seeds were directly sown on soil and plants were grown for 4 weeks under SD conditions.

Hypoxia treatments and submergence experiments

For hypoxia treatments, 7-day-old seedlings grown on MS medium were used. 2 h after the onset of the photoperiod, open Petri dishes were placed into a desiccator for the indicated time periods.
and constantly flushed with 100% nitrogen under LD conditions in the light. For re-aeration treatments, the Petri dishes were removed from the desicator and placed under LD conditions in air. Controls were also kept under ambient LD conditions in air for the same time periods.

For submergence experiments followed by RT-qPCR analysis, Arabidopsis plants were grown until the 10-leaf stage under SD conditions. Two hours after the beginning of the photoperiod, plants were either kept under control conditions air + light (AL) or were transferred to air + darkness (AD) or to submergence + darkness (SD). After 24 h, leaf material (except cotyledons) was harvested (two plants were pooled per treatment). For submergence survival experiments, plants were submersed in plastic tubs with temperature adjusted water in darkness for 4, 5, 6 and 7 days, whereas control plants were kept in dark and air for the same time (10 plants per treatment). After 2 weeks of recovery under SD conditions, pictures were taken and the survival rate of the plants was scored, which was determined as the ability to form new leaves. After all treatments, plant material was immediately frozen in liquid nitrogen and stored at –80°C until further processing.

**ABA experiments**

For the germination assay in the presence of ABA, seeds of the same age of WT, erf#111-2, ERF#111-OEI and OEII, ptf6-1 and ptyr1 ppyl1 ppyl2 ppyl4 were placed on MS agar plates (for all ABA experiments described here, MS medium was used without sucrose) with 0, 0.3, 0.5 and 0.7 μM ABA (Duchefa, A0941.0100). The ability to establish germination was documented after 10 days (16 h photoperiod). For the expression analysis of ABA-responsive genes, 7-day-old seedlings were sprayed with 100 μM ABA (solvent ethanol) for 4 h and control plants were equally treated with a mock solution. For the root growth assay, seedlings were grown for 3 days on MS agar plates and then transferred on MS agar plates supplied with 0, 5, 10, 30, 50 or 100 μM ABA. The root lengths were measured after an additional 14 days (n > 10 per replicate and treatment).

**Wounding and drought treatments**

For the wounding experiments, all rosette leaves of 3-week-old plants grown on soil (eight leaf stage, 8 h photoperiod) were gently wounded with a needle, whereas non-wounded control plants were kept in parallel. Leaf material (except cotyledons; two plants were pooled per treatment) was harvested after the indicated time points. For the progressive drought treatments in pots, 3-week-old plants grown on soil were exposed to drought stress (D) by letting them grow for 9 days without watering, whereas controls (C) were well watered (C/D pot). For drought treatment on paper, the whole plants were removed from the soil and exposed to dehydration on papers for 3 h, whereas the roots of the exposed control plants were covered with wet paper (C/D paper).

**In vivo bioluminescence imaging**

For the imaging of fLuc activity, the leaves of intact plants of Col-0 and plants expressing prAtERF#111:fLuc (T1 generation) were cut with scissors. After an incubation time of 90 min, the rosettes of the plants were evenly sprayed with 2 mM α-luciferin (PKJ, Kleinblittersdorf, Germany) + 0.1% (v/v) Triton X-100. Pictures of bioluminescence were taken in a low-light imaging system (Intas) with a camera shutter time of 20 min.

**H2O2, MeJA and ACC treatment**

Seven-day-old WT seedlings grown on MS agar plates were sprayed with 10 mM H2O2, 50 μM MeJA (Sigma-Aldrich, Taufkirchen, Germany, 392707) or 500 μM ACC (Sigma-Aldrich, Taufkirchen, Germany, A3903). Control plates were equally treated with the according solvents (e.g., ethanol or water). For each treatment 0.01% Tween-20 was added and plant material was harvested after 1 h.

**Microtome sections and analysis of the root hair phenotype**

For microtome sections, 7-day-old roots of Col-0 and erf#111-OEI and OEII were used. For chemical fixation, roots were vacuum infiltrated for 30 min with fixation solution (2% (w/v) paraformaldehyde, 1% (v/v) glacial acetic acid, 1% (w/v) caffeine, 0.01% Triton X-100 in 0.1 mM phosphate buffer (pH 7.0)) and incubated overnight at 4°C. For subsequent mechanical fixation, the roots were washed two times with 0.1 mM phosphate buffer (pH 7.4) and dehydrated in baths of 50, 70, 90, 95 and 100% ethanol, 1-butanol/ethanol 1:1 (v/v), and 100% 1-butanol, for 30 min each. For embedding, the Technovit 7100 plastic embedding system (Kulzer Technique, Wehrheim, Germany) was used according to manufacturer’s instructions. Root sections (15 μm) were made using the 2050 SuperCut Microtome (Leica Microsystems, Wetzlar, Germany) and viewed under a DM1000 microscope (Leica Microsystems).

To calculate the length (n > 130) and number (n > 14) of root hairs at the root tip as well as at the root base, photographs of 7-day-old seedlings were taken with a RS Photometrics CoolSnap camera coupled to a M3B stereomicroscope (Wild Heerbrugg, Heerbrugg, Switzerland). Root lengths were measured using the ImageJ software (version 1.44p; https://imagej.nih.gov/ij/).

**RNA isolation and PCR analysis**

RNA isolation, cDNA synthesis, reverse transcription standard and quantitative PCR (RT-qPCR) were performed as described previously (Klecker et al., 2014). RT-qPCR was performed using the iQ SYBR Green Super mix and the CFX Connect Real-Time PCR Detection System (Bio-Rad, Feldkirchen, Germany). All primers are listed in Data S3. Three biological replicates, each with three technical repetitions, were measured. Relative expression values were determined by the 2^–ΔΔCT method and normalized to ELONGATION FACTOR 1A (EF1a).

**Plant transformation and confocal imaging**

To generate stable Arabidopsis lines as well as transiently transformed tobacco, binary expression vectors were transformed into Agrobacterium tumefaciens, strain GV3101 (Koncz et al., 1984). Tobacco leaves were transiently transformed with the Agrobacterium solution as described by Bendahmane et al. (2000) and after 3 days, leaf discs were collected for confocal imaging. For generation of stable overexpression lines, Arabidopsis plants were transformed by floral dip as described previously (Clough and Bent, 1998). After 4 weeks, the seeds were harvested and positive transformants were identified by antibiotic resistance (Kanamycin). Homozygous transgenic plants were obtained in the T3 generation.

For subcellular localization and imaging of transiently transformed tobacco, GFP fluorescence was analyzed by confocal laser scanning microscopy using LEICA TCS SP2 (Leica Microsystems, at λex 488 nm for GFP and chlorophyll excitation, λem 530-555 nm for GFP and 650-720 nm for chlorophyll emission).

**Vector construction and plasmid purification**

The N-terminal HA-tagged effector construct used for protoplast transfection p35S:HA-GFP has been described before (Klecker et al., 1998).
et al., 2014) and p35S:HA-AtERF#111 was constructed by recombin- ing the coding sequence of AtERF#111 into the Gateway vector p3SS:HA-GW (Ehlert et al., 2006). For the firefly Luciferase reporter constructs prPDF1.2a:lUC, prTH8:fLUC, prRHO3ANASE:lUC, prSS3:fLUC, prCYP71B22:fLUC, and prEXPA1:fLUC, the 5’ upstream sequences (a maximum of 2000 bp) from the start codon were amplified from Col-0 genomic DNA with specific primer pairs (Data S3). PCR products as well as the vector pBT10GA-L4UAS (Wehner et al., 2011) were digested with NcoI and BamHI before ligation, removing the GAL4UAS sequence. For the normal- ization of gene expression, the p35S promoter and the coding sequence of renilla Luciferase was isolated from p705RlUC (Stahl et al., 2004) and integrated into pBT10GAL4UAS, thereby removing the GAL4UAS sequence and the firefly coding sequence and generating the new construct pBT10-lUC.

For the construction of (MA)AtERF#111 and (MC)AtERF#111, the AtERF#111 coding sequence was amplified with a forward primer that introduced a mutation in the second codon, thereby replacing the N-terminal Cys2 with Ala to inhibit a potential degradation by the Cys branch of the PRT6 N-degron pathway. For C-terminal translational fusions, the reverse primer was designed without the stop codon. Products were recombined into the Gateway entry vector pDONR221 using BP clonase (Invitrogen, Karlsruhe, Germany). Entry clones were then recombined into different Gateway destination vectors using LR clonase (Invitrogen). For Agrobacterium-mediated plant transformation, the destination vectors pMDCS2-GFP (Curts and Grossniklaus, 2003) and p3SS:HF-GATA (Mustroph et al., 2010) were used.

To generate lUC reporters for stable plant genome integration, the promoter region of AtERF#111 (1302 bp) was amplified from Col-0 gDNA with specific Gateway recombination-compatible primers (Data S3). Products were first recombined into the Gateway entry vector pDONR201 and then recombined into the Gateway destination vector pBGWL7 (Karimi et al., 2005).

For the glucocorticoid-inducible protoplast assay, entry clones (GFP was used as a control) were recombined into p3SS:rfA-HBD (a kind gift from Monika Tomar). For protein stability analysis in protoplasts, (MC)AtERF#111 and (MA)AtERF#111 were recombined in the destination vector p3SS:GW-HA to gain a C-terminal HA-tag. Plasmids were purified using the NucleoBond PC 500 Midi Kit (Macherey-Nagel, Düren, Germany) and stored at −20°C until use.

Protoplast isolation, transient transformation and treatment

Protoplast isolation and transient transformation followed by luci- ferase activity measurements were performed as described previ- ously (Klecker et al., 2014). For each transformation, a concentration of approximately 3.5 × 10^5 Arabidopsis mesophyll protoplasts per ml was used. For promoter transactivation assays, 200 μl of protoplast solution was transformed with 4 μg of the reporter plasmid; 2 μg of the effector plasmid and 0.5 μg of the normalization vector pBT10-lUC. For measurements, 20 micro- liters of protoplast solution was mixed with 50 μl of the respective substrate solution. Light emission was measured with the GloMax 96 Microplate Luminometer (Promega, Mannheim, Germany) with an integration time of 3 sec. For the glucocorticoid-inducible protoplast assay followed by microarray analysis, 400 μl of protoplast solution was transformed with 20 μg of 3SS:AtERF#111-HBD or 3SS:GFP-HBD, incubated overnight for 18 h under LD conditions and then mixed with 50 μM CHX (Sigma-Aldrich, C7989), or the same amount of the solvent dimethyl sulfoxide (DMSO). After 30 min incubation with CHX, 10 μM DEX (Sigma-Aldrich, D4802) was added to the suspension and incubated for additional 4 h under LD conditions. Afterwards, cells were frozen in liquid nitrogen and RNA was isolated.

For protein stability assays by western blot analysis, protoplasts were transformed according to Wu et al. (2009). Here, 30 μg of plasmid DNA (effector plasmids encoding 35S:(MC)AtERF#111-HA or 35S:(MA)AtERF#111-HA, see above) were transformed in 600 μl reaction volumes containing each ~16.5×10^6 cells. After overnight expression, protoplast suspensions were split into 180 μl of samples and supplemented with 50 μM MG132 (UBP Bio, Aurora, CO, USA, F1101) or a DMSO mock control. For the 3 h chases, 100 μM CHX (Santa Cruz Biotechnology, Heidelberg, Germany, sc-3508) were added immediately after addition of proteasome inhibitor. For 1 h chases, samples were treated 2 h later and all samples were harvested after 1 h additionally by centrifugation (200 g, 1 min). Pellets were frozen in liquid nitrogen. For SDS-PAGE analysis, pellets were resuspended in 116 μl of extraction buffer (50 mM Tris–HCl, pH 7.6; 150 mM NaCl; 20 mM NaF; 1% (v/v) Nonidet P-40; 0.5% (w/v) deoxycholate; 10 mM NaPO4; 1 mM EDTA; 0.5 mM EGTA; 1 mM DTT; 1x Complete EDTA-free Protease Inhibitor Cocktail (Roche, Mannheim, Germany)), and incubated for 15 min at 68°C with 3x SDS sample buffer. Protein was detected using anti-HA antibody (Covance HA.11, MMS-101R; 1:1000 dilution) or anti-CHX antibody (Santa Cruz Biotechnology, Heidelberg, Germany, sc-8072). Probed blots were incubated overnight with secondary antibodies (anti-mouse IgG-HRP coupled anti-mouse secondary antibody (Pierce, 31437; 1/5000 dilution in the same blocking buffer as above).

Microarray analysis

For microarray experiments, total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For analysis of the effect of ERF#111 overexpression, we extracted RNA from 7- day-old seedlings from two independent OE lines, ERF#111-OE and ERF#111-OEI, which were separated in roots and shoots, and compared the expression with that of roots and shoots of Col-0. For the wounding microarray, RNA was extracted from whole rosettes (except cotyledons) of 3-week-old Col-0 and erf#111-2 mutant plants 3 h after wounding. For the glucocorticoid-inducible protoplast assay, RNA was extracted from p3SS:AtERF#111-HBD and 3SS:GFP-HBD transformed protoplasts isolated from erf#111-2 mutant plants.

The RNAs were processed by the Genomics and Bioinformatics core facility (University of Bayreuth). For hybridization of the probes, the Arabidopsis 4×44k array from Agilent Technologies was used (design ID G21169). Here, 150 ng of total RNA of each sample were labelled using the Low Input Quick Amp Labelling Kit as recommended by the manufacturer (manual G4140-90050, Agilent Technologies). Dye-swap experiments were included in the microarray design. The hybridization experiments were performed as recommended in the Two-Colour Microarray-Based Gene Expression Analysis protocol (manual G4140-90050, Agilent Technologies). Processed microarrays were scanned using a high- resolution microarray scanner (Agilent Technologies) and spot intensities were quantified using Agilent’s feature extraction soft- ware.

Data were analyzed with the limma package using the program R. Every array was background corrected and normalized with the Loess-algorithm, before the arrays the Quantile method was used for normalization. Significantly modified genes with a differ- ence in expression greater than 2 (Signal/Log2-Ratio >1) and a P- value < 0.01 were chosen for further analysis. For comparison of our microarray data to already published microarray experiments,
the data were downloaded from the GEO database and reanalyzed.

Gene ontology analysis

The lists of DEGs identified by microarray analysis were evaluated for an enrichment of the GO categories specific biological process, molecular function or cellular compartment by using the GOHyper-GAll function in the program R (Horan et al., 2008). GO categories with an adjusted P < 0.05 were classified as significantly enriched.

ACCESSION NUMBERS

Raw data have been deposited at the GEO database under accession number GSE121687. AGI codes for Arabidopsis genes studied are available in Data S3.

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AUTHOR CONTRIBUTIONS

JB, ND, MK, ARW, and AM planned and designed the experiments. JB, WR, MK, LM, and ARW performed experiments. JB and AM analyzed the data and wrote the manuscript. All authors read and commented on the manuscript.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Response to flooding stress in different Arabidopsis accessions.

Figure S2. Expression analysis of AtERF#111 upon hypoxia.

Figure S3. Analysis of AtERF#111 T-DNA insertion alleles.

Figure S4. Survival after submergence of erf#111-2 and erf#111-OEII in comparison with Col-0.

Figure S5. Protein multiple sequence alignments of AtERF#111.

Figure S6. Germination assay in the presence of ABA.

Figure S7. Evaluation of root growth and seedling weight in the presence of ABA.

Figure S8. Representative pictures of the drought/wounding treatments.

Figure S9. The expression of AtERF#111 is induced by mechanical stress.

Figure S10. Venn diagrams showing overlapping DEGs.

Figure S11. Expression of other GXERFs upon wounding.

Data S1. Microarray data presented in this publication.

Data S2. GO analysis of differentially expressed genes.

Data S3. Material used in this publication.

Data S4. References.

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