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RESEARCH PAPER

ERF-VII transcription factors induce ethanol fermentation in response to amino acid biosynthesis-inhibiting herbicides

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Abstract

Herbicides inhibiting either aromatic or branched-chain amino acid biosynthesis trigger similar physiological responses in plants, despite their different mechanism of action. Both types of herbicides are known to activate ethanol fermentation by inducing the expression of fermentative genes; however, the mechanism of such transcriptional regulation has not been investigated so far. In plants exposed to low-oxygen conditions, ethanol fermentation is transcriptionally controlled by the ethylene response factors-VII (ERF-VIIs), whose stability is controlled in an oxygen-dependent manner by the Cys-Arg branch of the N-degron pathway. In this study, we investigated the role of ERF-VIIs in the regulation of the ethanol fermentation pathway in herbicide-treated Arabidopsis plants grown under aerobic conditions. Our results demonstrate that these transcriptional regulators are stabilized in response to herbicide treatment and are required for ethanol fermentation in these conditions. We also observed that mutants with reduced fermentative potential exhibit higher sensitivity to herbicide treatments, thus revealing the existence of a mechanism that mimics oxygen deprivation to activate metabolic pathways that enhance herbicide tolerance. We speculate that this signaling pathway may represent a potential target in agriculture to affect tolerance to herbicides that inhibit amino acid biosynthesis.

Keywords: Amino acid biosynthesis-inhibiting herbicides, ERF-VII transcription factors, ethanol fermentation, glyphosate, imazamox, N-degron pathway.

Introduction

The use of herbicides that inhibit the biosynthesis of amino acids in plants is widespread due to their efficacy in removing weeds (Duke and Powles, 2008; Powles and Yu, 2010). Chemical engineering has led to the formulation of several types of herbicides whose primary sites of action are associated with the specific inhibition of the activity of enzymes involved in the amino acid biosynthetic pathways. Herbicides such as imazamox inhibit acetohydroxyacid synthase (AHAS, EC 2.2.1.6) in the branched-chain amino acid biosynthesis pathway (Ray, 1984; Shaner *et al.*, 1984), while glyphosate

Abbreviations:ADH, alcohol dehydrogenase; AHAS, acetohydroxyacid synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; ERF-VII, group VII ethylene response factor; Luc, luciferase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase.

inders the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) in the aromatic amino acid biosynthesis pathway (Steinrücken and Amrhein, 1980). Although the specific biochemical targets of the herbicides inhibiting amino acid biosynthesis are well known, the sequence of events triggered from herbicide application to plant death is still unclear. Even though the herbicides mentioned above target different pathways, they produce several common physiological effects in treated plants, which suggests that their toxicity shares certain characteristics (Orcaray *et al.*, 2010; Gil-Monreal *et al.*, 2017).

Both AHAS and EPSPS inhibitors have been shown to impair carbon metabolism (Orcaray *et al.*, 2012), and induce the alternative respiration pathway (Gaston *et al.*, 2003; Armendáriz *et al.*, 2016) and the pyruvate dehydrogenase (PDH) bypass (Gil-Monreal *et al.*, 2017). Moreover, an accumulation of free amino acids has been described in plants treated with these types of herbicides (Shaner and Reider, 1986; Wang, 2001; Zulet *et al.*, 2013), accompanied by a decrease in the soluble protein content (Gaston *et al.*, 2002; Zulet *et al.*, 2013; Maroli *et al.*, 2016) associated with increased proteolytic activity (Zulet *et al.*, 2013). These biochemical alterations cause growth arrest followed by slow plant death (Gruys and Sikorski, 1999; Wittenbach and Abell, 1999). The shared responses stimulated by AHAS and EPSPS inhibitors could be part of a general plant stress response, as proposed recently by a model for sublethal herbicide-induced stress and plant responses (Dyer, 2018). According to this model, sublethal herbicide exposure activates a common signal transduction cascade, which leads to translation or modification of stress-related proteins that play a role in defense, reactive oxygen species management and xenobiotic inactivation (Dyer, 2018).

Induction of ethanol fermentation is another common physiological consequence of the application of amino acid biosynthesis-inhibiting herbicides. An increase in the activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) and their protein contents has been widely described in herbicide-treated pea (Gaston *et al.*, 2002; Zabalza *et al.*, 2005; Orcaray *et al.*, 2012) and Arabidopsis plants (Zulet *et al.*, 2015). This induction is also known to be regulated at the transcriptional level in both species (Zulet *et al.*, 2015; Gil-Monreal *et al.*, 2018). However, which transcription factors are involved in the activation of this response has not been investigated. The induction of ethanol fermentation due to an accumulation of pyruvate as a consequence of AHAS inhibition can be hypothesized since this metabolite is a common substrate of PDC and AHAS. However, induction of fermentation in plants after treatment with glyphosate and other amino acid biosynthesis inhibitors cannot be directly related to an increase in the pyruvate availability because neither of these compounds inhibits a pyruvate-consuming enzyme (Zabalza and Royuela, 2014). Thus, induction of the ethanol fermentation pathway can also be regarded as a general physiological response after a stress situation, as has been reported after cold, osmotic stress, and drought (Dolferus *et al.*, 1994; Minhas and Grover, 1999; Kato-Noguchi, 2000; Kürsteiner *et al.*, 2003; Peters and Frenkel, 2004). Therefore, these two explanations for the induction of ethanol fermentation pathway as a consequence of exposure to herbicides inhibiting amino acid biosynthesis are not mutually exclusive and they may even act in coordination.

Transcriptional regulation of ethanol fermentation genes has been mainly studied in response to hypoxia, as a part of a conserved metabolic strategy to sustain glycolysis in the absence of oxygen as the terminal electron acceptor in the mitochondrial electron transport chain (Bui *et al.*, 2019). Indeed, *PDC* and *ADH* are among the core hypoxia-responsive genes described by Mustroph and colleagues (2009) and shown to be controlled by members of the group VII ethylene response factor (ERF-VII) family, whose activity is directly linked to oxygen availability. In brief, in the presence of oxygen, ERF-VII transcription factors are degraded by the 26S proteasome following the N-degron pathway, while in low-oxygen conditions they are stabilized and can activate the hypoxic genes (Gibbs *et al.*, 2011; Licausi *et al.*, 2011). The N-degron pathway consists of a series of post-translational modifications that relate the metabolic stability of a protein to the nature of its N-terminal amino acid residue (Bachmair *et al.*, 1986; Varshavsky, 2019). This pathway is present across prokaryotes and eukaryotes, and is part of the ubiquitin–proteasome system (Ingvardsen and Veierskov, 2001). ERF-VII transcription factors contain a highly conserved N-terminal MCGGAI motif (Nakano *et al.*, 2006) that is recognized by specific methionine amino peptidases that cleave off the initial methionine and leave the cysteine exposed as the N-terminal residue (Bradshaw *et al.*, 1998). The N-terminal cysteine can be oxidized by plant cysteine oxidases using oxygen as the substrate (Weits *et al.*, 2014) or by NO in a spontaneous reaction (Gibbs *et al.*, 2011), which allows its recognition by arginine transferase. Arginine transferase adds an arginine to the N-terminus of the ERF-VIIs generating a primary destabilizing residue (Graciet and Wellmer, 2010). Finally, the exposed N-terminal arginine is recognized by the E3 ligase PRT6 that polyubiquitinates the target protein and provokes its degradation by the 26S proteasome (Garzón *et al.*, 2007). By contrast, if the oxygen availability decreases, the N-terminal cysteine cannot be oxidized, and thus, the ERF-VIIs accumulate in the nucleus and can bind to the Hypoxia Responsive Promoter Element domain present in the promoter of the hypoxia marker genes and activate the anaerobic response (Gasch *et al.*, 2016).

Five members belong to the ERF-VII group in Arabidopsis: RAP2.2, RAP2.3, RAP2.12, HRE1, and HRE2. Upon hypoxia, the transcription factors RAP2.2 and RAP2.12 trigger the initial anaerobic response (Giuntoli *et al.*, 2017), while HRE1 and HRE2 have been proposed to maintain the anaerobic response (Licausi *et al.*, 2010) and the role of RAP2.3 in low-oxygen stress has not been deeply investigated.

Although the transcriptional regulation of ethanol fermentation by ERF-VIIs has been deeply studied in plants exposed to low-oxygen conditions, the role of these transcription factors in the induction of fermentation upon herbicide treatment has not been investigated in depth yet. The expression of ERF-VII transcription factors has been found to be affected by the application of amino acid biosynthesis-inhibiting herbicides (Manabe *et al.*, 2007; Das *et al.*, 2010) and the 26S proteasome has been reported to be involved in the physiological response of the plants to amino acid biosynthesis-inhibiting

herbicide application (Kurepa *et al.*, 2010; Zulet *et al.*, 2013). Altogether, these results prompted us to evaluate the role of ERF-VIIs in the transcriptional regulation of aerobic ethanol fermentation in plants upon herbicide treatment. To this end, the response to the herbicides of Arabidopsis wild-type and mutant seedlings lacking the five ERF-VIIs was monitored. Specifically, the activation of ethanol fermentation and the stabilization of ERF-VIIs was analysed in both genotypes. The role of fermentation in plants exposed to herbicide treatment was also evaluated.

Materials and methods

Plant material

The Arabidopsis Columbia-0 ecotype (Col-0) was used as wild-type background. The following mutant lines were also used. First, to evaluate the implication of ERF-VIIs in the induction of ethanol fermentation by herbicides in treated plants, a quintuple mutant *erfVII* (described in Abbas *et al.* (2015) and kindly provided to us by Michael Holdsworth (University of Nottingham)), and a chimeric *RAP2.12*$_{1\text{-}28}$*-Luc* reporter line (Weits *et al.*, 2014) were used. Second, to evaluate the physiological role of fermentation in the response of the plants to herbicide application, mutant lines with impaired ethanol fermentation or with expected increased fermentative activity were used. On the one hand, a line with a double T-DNA insertion for the *PDC1* and *PDC2* genes (*pdc1pdc2*, previously described in Gil-Monreal *et al.*, 2017) and a T-DNA insertion mutant for the *ADH1* gene (*adh1*) (Banti *et al.*, 2008; Zulet *et al.*, 2015) were used. On the other hand, two mutant lines with increased transcript levels of the fermentative genes, including *PDC1* and *ADH1*, were used: a double mutant line defective for *ATE1* and *ATE2* (*ate1ate2*, previously described in Graciet *et al.*, 2009), and a mutant line with insertion in the *PRT6* gene (*prt6*, line N684039 obtained from the NASC collection, previously described in Riber *et al.*, 2015).

Herbicide treatments and tolerance assays in six-well plates

Seeds of Arabidopsis wild-type, *erfVII*, and *RAP2.12*$_{1\text{-}28}$*-Luc* lines were surface sterilized with 70% ethanol for 30 s and rinsed six times with sterile deionized water. Seedlings were grown in sterile six-well plates in liquid medium under continuous shaking. The liquid medium was half-strength Murashige and Skoog medium (pH 5.7) (Sigma-Aldrich Co., St Louis, MO, USA) enriched with 1% (w/v) sucrose. In each well about 10 sterile seeds were placed and incubated for 3 d at 4 °C in darkness. Later, plates were moved to a growth chamber and seedlings were grown under 80 μmol m^{-2} s^{-1} photosynthetically active radiation, at 23 °C/18 °C day/night temperature and at a 12/12 h day/night photoperiod. When plants were 6 d old, the old growth medium was replaced with fresh sucrose-free half-strength Murashige and Skoog medium (pH 5.7), and the desired herbicide dose where needed. Commercial formulations of imazamox and glyphosate were used at a final concentration in the growth medium of 1.5 mg active ingredient l^{-1} (4.9 μM) of Pulsar®40 (BASF Española SA, Barcelona, Spain), in the case of imazamox, or 50 mg active ingredient l^{-1} (219.12 μM) of Fortin Green® (Industrial Química Key, SA, Tàrrega, Lleida, Spain), in the case of glyphosate (Gil-Monreal *et al.*, 2017).

The duration of the experiments aimed at measuring gene expression and fermentation pathway activity was chosen on the basis of the trend of ADH activity induction, preliminarily assessed over 5 d of herbicide treatment, setting the end point in correspondence with the maximum induction, observed at day 5 (data not shown). Accordingly, 5 d after treatment application, whole seedlings from one individual well were harvested as a biological sample and different wells were collected as replicates. Samples were collected 4 h after the beginning of the light period. The seedlings were immediately frozen, and frozen samples were ground under liquid nitrogen using a Retsch mixer mill (MM200, Retsch®, Haan, Germany) and stored at −80 °C for further analyses (chlorophyll extraction and quantitation, lipid peroxidation assay, PDC and ADH activities and protein contents, gene expression analysis, and luciferase activity).

To evaluate the lethality of the herbicides, a set of plants of the wild-type and *erfVII* mutants was maintained under herbicide treatment until death was observed. Untreated plants were used as controls. In addition, recovery of wild-type and *erfVII* mutants was evaluated by transferring the 5-d treated seedlings to herbicide-free half-strength Murashige and Skoog medium (pH 5.7) enriched with 1% (w/v) sucrose. The nutrient solution, with or without herbicides, was renewed every 4 d. In both lethality and recovery experiments, visual inspection was used as a marker of lethality and plates were scanned using a GS-800 densitometer (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Chlorophyll extraction and quantification

Total chlorophyll was extracted from homogenized seedlings using 80% (v/v) acetone buffered with 2.5 mM Na-phosphate at pH 7.8. Samples were incubated for 30 min in the dark at 4 °C with shaking. The debris was pelleted by centrifugation at 5000 *g* for 10 min. The chlorophyll content was spectrophotometrically measured in the supernatant according to Porra *et al.* (1989). Four biological replicates were used for each experimental condition.

Lipid peroxidation assay

The extent of lipid peroxidation was estimated spectrophotometrically by the amount of malondialdehyde in seedlings as described by Hodges *et al.* (1999). This method takes into account the possible interference generated by non-specific turbidity, thiobarbituric acid–sugar complexes and other non-thiobarbituric acid reactive substances absorbing at 532 nm. Four biological replicates were analysed for each experimental condition.

In vitro activities of PDC and ADH

The *in vitro* activities of PDC and ADH were assayed in ground seedlings as described in Gaston *et al.* (2002). Briefly, PDC and ADH activities were measured in a spectrophotometer monitoring NADH consumption or formation at 340 nm, respectively. Four biological replicates were analysed for each experimental condition.

PDC and ADH immunoblotting

PDC and ADH protein immunoblot assay was performed according to standard techniques (as in Zulet *et al.*, 2015). PDC and ADH antibodies from Agrisera (Vännäs, Sweden) were used at dilutions of 1:2500 and 1:500, respectively. Goat anti-rabbit IgG alkaline phosphatase (Sigma-Aldrich) was used as the secondary antibody at a dilution of 1:20 000, and bands were visualized using the Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot® Assay Kit (Bio-Rad Laboratories). Immunoblots were scanned using a GS-800 densitometer (Bio-Rad Laboratories), and protein bands were quantified using Quantity One software (Bio-Rad Laboratories). Four biological replicates were analysed for each experimental condition.

Total RNA extraction, cDNA synthesis and gene expression analysis

Total RNA was isolated from previously ground frozen seedlings using the Macherey-Nagel NucleoSpin® RNA Plant kit following the manufacturer's instructions. Extracted RNA concentration was measured with Gen 5.1.11 (Biotek Instruments, Inc., USA) and RNA quality was assessed by 1% agarose gel electrophoresis. The gels were visualized using a Gel Doc 2000 system (Bio-Rad Laboratories). Five hundred nanograms of RNA was reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories) following the manufacturer's instructions.

Quantitative real-time PCR amplification was performed using an ABI Prism 7300 sequence detection system (Applied Biosystems/

Life Technologies, Darmstadt, Germany) with iQ™ SYBR® Green Supermix (Bio-Rad Laboratories). Each reaction was performed using 15 ng cDNA template. The following thermal profile was used for all qRT-PCRs: 10 s 50 °C, 3 min 95 °C, followed by 40 cycles of amplification (15 s 95 °C, 30 s 60 °C) and a dissociation curve (15 s 95 °C, 30 s 60 °C and 15 s 95 °C).

Three reference genes (*ACTIN2*, At3g18780; *B-TUBULINE8*, At5g23860; and *PROTEIN PHOSPHATASE 2A SUBUNIT A3*, At1g13320) were assessed for stable expression using geNorm software (Vandesompele *et al.*, 2002) and BestKeeper (Pfaffl *et al.*, 2004). All candidate reference genes showed *M* values lower than the geNorm threshold of 1.5 and SD of C_t values lower than the BestKeeper threshold of 1, revealing stability (see Supplementary Table S1 at *JXB* online). The geometric mean of the expression ratios of the two most stable reference genes (*ACTIN2* and *B-TUBULINE8*) was used as the normalization factor in all samples. Primer 3 software and the QuantPrime Tool (http://quantprime.mpimp-golm.mpg.de/; Arvidsson *et al.*, 2008) were used to design the primers. The specific primer pairs used in the qRT-PCRs are presented in Supplementary Table S2. The relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All gene expression analyses were performed with four independent biological replicates.

Measurement of relative luciferase activity

Firefly luciferase (Luc) activity in *RAP2.12*$_{1-28}$*-Luc* seedlings was evaluated with the aid of the Dual-Luciferase® Reporter (DLR™) Assay System (Promega). Approximately 100 mg fresh tissue was extracted with 400 µl Passive Lysis Buffer and spun down at 5000 *g* for 5 s. Enzyme activity in the supernatant was quantified according to the manufacturer's recommendation, using a Lumat LB 9507 Tube Luminometer (Berthold Technologies). The luminescence signal (expressed in relative luminescence units, RLU) was normalized to the total protein amount of the sample, measured according to the Bradford protein assay (Bradford, 1976) with the related reagent (Bio-Rad Laboratories). Six biological replicates were used for each experimental condition.

Determination of root growth on agar plates

Seeds of Arabidopsis wild-type and *pdc1pdc2*, *adh1*, *ate1ate2*, and *prt6* mutants were used. Plants were grown in agar containing plates as described in Gil-Monreal *et al.* (2017). Briefly, seeds were surface sterilized and were then transferred to Petri dishes containing half-strength Murashige and Skoog medium (pH 5.7) enriched with 1% (w/v) sucrose and 0.7% (w/v) plant agar. Plants were incubated for 3 d at 4 °C in darkness before they were transferred to the growth chamber. Plants were grown under 150 µmol m^{-2} s^{-1} photosynthetically active radiation, 65% relative humidity at 23 °C/18 °C day/night temperature and 12/12 h day/night cycle. After 4 d in the growth chamber, when the root length was about 1 cm, seedlings were transferred to 12×12 cm plates containing half strength MS medium (pH 5.7) and 0.9% (w/v) plant agar (eight seeds per plate) and, where corresponding, the selected herbicide dose (Gil-Monreal *et al.*, 2017). Imazamox and glyphosate were applied as commercial formulations at a final concentration of 0.005 mg active ingredient l^{-1} (0.016 µM) for imazamox or 0.25 mg active ingredient l^{-1} (1.1 µM) for glyphosate. Both herbicides were sterilized using 0.20 µm filters and added to the medium before it solidified. Untreated plants were used as controls. Plates were scanned using a GS-800 densitometer (Bio-Rad Laboratories) and root elongation (n=24) was measured 15 d after the transfer to 12×12 cm plates. The experiment was done in triplicate.

Statistical analysis

Data (mean ±SE) were subjected to one-way or two-way analysis of variance (ANOVA; P<0.05). Before ANOVA, data were checked for normality and the homogeneity of variances, and log-transformed to correct deviations from these assumptions when needed. *Post hoc* comparisons were tested using the Bonferroni *post hoc* test at a significance level of P<0.05. Statistical analyses were conducted using the IBM SPSS Statistics (v.22) software package (IBM Corp., Armonk, NY, USA). Values that significantly differ from each other are indicated by different letters in figures. See Supplementary data Table S3 for details about the statistical analysis.

Results

Physiological characterization of Arabidopsis wild-type and erfVII *mutant under herbicide treatment*

The main objective of the present study was to ascertain whether, similar to low-oxygen stress, ERF-VII transcription factors might regulate the transcriptional induction of ethanol fermentation in plants upon herbicide treatment. We first analysed the expression level of *ERF-VII* (*RAP2.2*, *RAP2.12*, *RAP2.12*, *HRE1*, and *HRE2*) genes exploiting the transcript data available at the Genevestigator (Hruz *et al.*, 2008) database. Changes in the expression of the five *ERF-VIIs* were found in Arabidopsis after AHAS or EPSPS inactivation by herbicides (Fig. 1). While the expression of *RAP2.2*, *RAP2.12*, and *HRE2* increased as a consequence of AHAS or EPSPS inhibition, *RAP2.3* and *HRE1* were downregulated (Fig. 1). These results suggested the potential role of ERF-VIIs in the induction of the fermentative response after herbicide application.

To evaluate the actual implication for ERF-VIIs, we compared the expression of ethanol fermentation genes in wild-type plants and a quintuple *erfVII* mutant (Abbas *et al.*, 2015) upon treatment with two herbicides that inhibit amino acid biosynthesis. Effectiveness of both treatments was confirmed by repression of growth and extended chlorosis after 5 d of herbicide application (Fig. 2), as such symptoms have been reported after inhibition of amino acid biosynthesis (Cobb and Reade, 2010). Both herbicide treatments were lethal for the two genotypes (see Supplementary Fig. S1) and visual inspection of the seedlings showed that none of the studied genotypes recovered when they were transferred to herbicide-free medium (Supplementary Fig. S2). Interestingly, the *erfVII* mutant was more susceptible to either herbicide application and died within 15 d after treatment, while wild-type seedlings died within 19 d after herbicide application (Supplementary Fig. S1).

The drop in total chlorophyll concentration (Fig. 3A) and the extent of lipid peroxidation (Fig. 3B) were measured as general stress markers. In both genotypes, the chlorophyll concentration decreased in response to herbicide application, although the effect was not statistically significant in all cases (Fig. 3A). In addition, both herbicides provoked an increase in the malondialdehyde concentration, indicating the occurrence of lipid peroxidation in the treated seedlings (Fig. 3B). Both AHAS and EPSPS inhibitors affected the two parameters equally in both genotypes (Fig. 3).

Ethanol fermentation in Arabidopsis wild-type and erfVII *mutant*

Induction of the enzymatic activity of PDC and ADH, an increase of their protein content, and an increase in transcript levels of the genes coding for these enzymes have been reported in pea and Arabidopsis after the inhibition of EPSPS or AHAS activity by herbicides (Gaston *et al.*, 2002; Zabalza

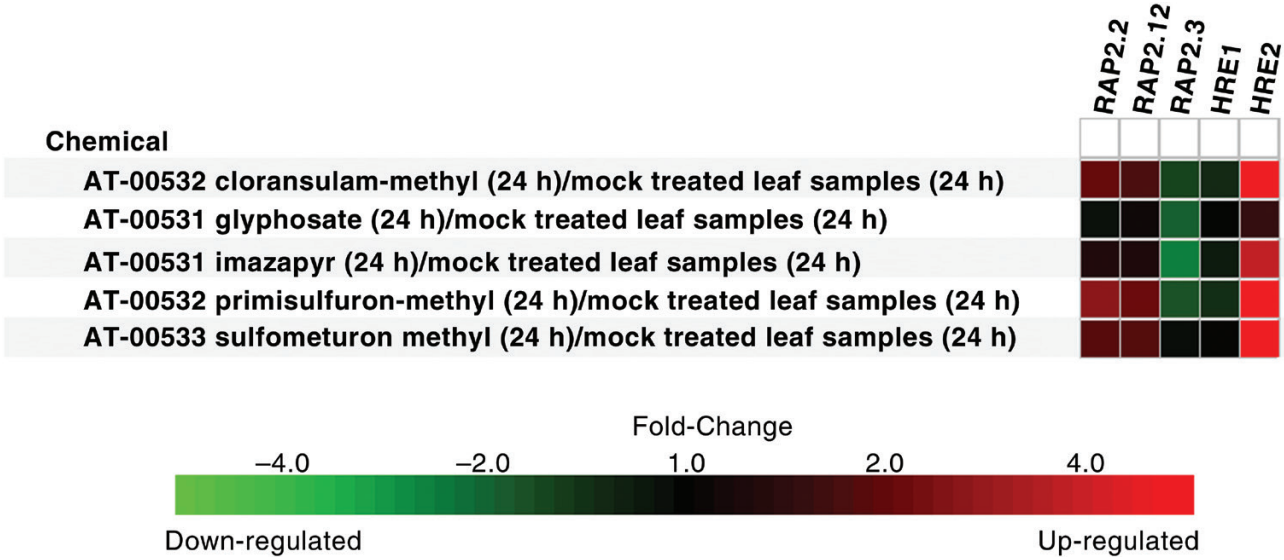

Fig. 1. Effect of herbicides on the expression of ERF-VIIs. The effect of different branched-chain or aromatic amino acid biosynthesis-inhibiting herbicides on the relative transcript levels of the five ERF-VII transcription factors in Arabidopsis. Data extracted from the experiment of Das *et al.*, 2010 from Genevestigator (Hruz *et al.*, 2008).

Fig. 2. Seedlings growing in six-well plates. Arabidopsis wild-type and *erfVII* mutant seedlings were untreated or treated with imazamox (1.5 mg l^{-1}) or glyphosate (50 mg l^{-1}) for 5 d.

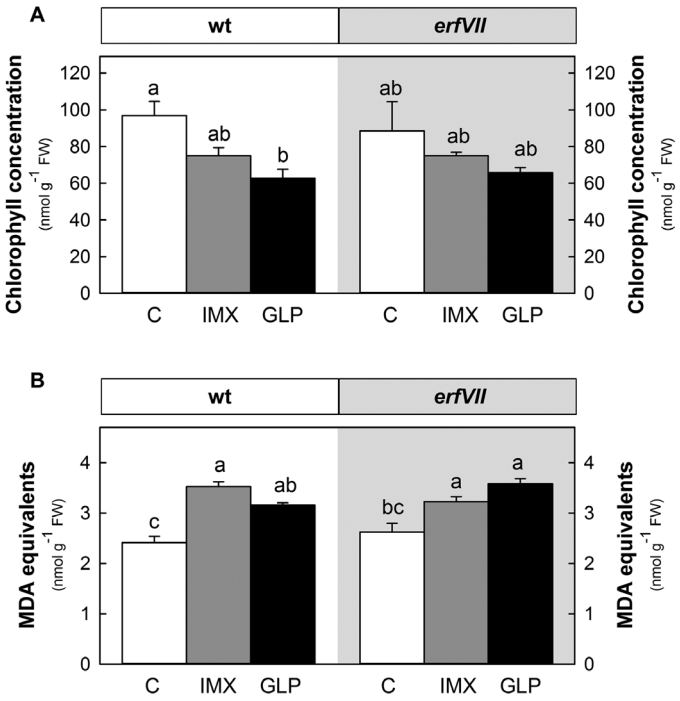

Fig. 3. Chlorophyll concentration and lipid peroxidation as general stress markers. Total chlorophyll concentration (A) and MDA concentration (B) were measured in Arabidopsis wild-type and *erfVII* seedlings untreated (control) or treated with imazamox (1.5 mg l^{-1}) or glyphosate (50 mg l^{-1}) for 5 d. Data are presented as mean±SE (n=4). Different letters refer to statistically significant differences between treatments (two-way ANOVA followed by the Bonferroni *post hoc* test (P<0.05). C, control; GLP, glyphosate; IMX, imazamox; MDA, malondialdehyde.

et al., 2005; Zulet *et al.*, 2015; Gil-Monreal *et al.*, 2017). These previous studies have been performed in the roots of adult plants grown in hydroponic systems. To evaluate whether this response also occurs in seedlings, the *in vitro* activities of PDC and ADH (Fig. 4), their protein amount (Fig. 5), and their transcript levels (Fig. 6) were measured in Arabidopsis seedlings non-treated or treated with imazamox or glyphosate for 5 d. In addition, the effects of the two classes of herbicides on the ethanol fermentation pathway were compared between the wild-type and the *erfVII* mutants, in order to evaluate whether the lack of ERF-VIIs modifies the effects of AHAS or EPSPS inhibitors on this pathway.

Both herbicides activated the ethanol fermentation pathway in wild-type plants to a similar extent. PDC and ADH activity increased as a consequence of imazamox or glyphosate application (Fig. 4), but protein levels were less affected (Fig. 5). These results confirmed that, as occurs in adult plants, ethanol fermentation is also activated in herbicide-treated Arabidopsis seedlings. The effect of herbicides on the ethanol fermentation pathway was milder on the *erfVII* mutant compared with the effect observed in wild-type seedlings. Levels of PDC and ADH protein in the *erfVII* mutant were not significantly affected by herbicide treatment (Fig. 5), but their activities were stimulated by 2- to 5-fold (Fig. 4).

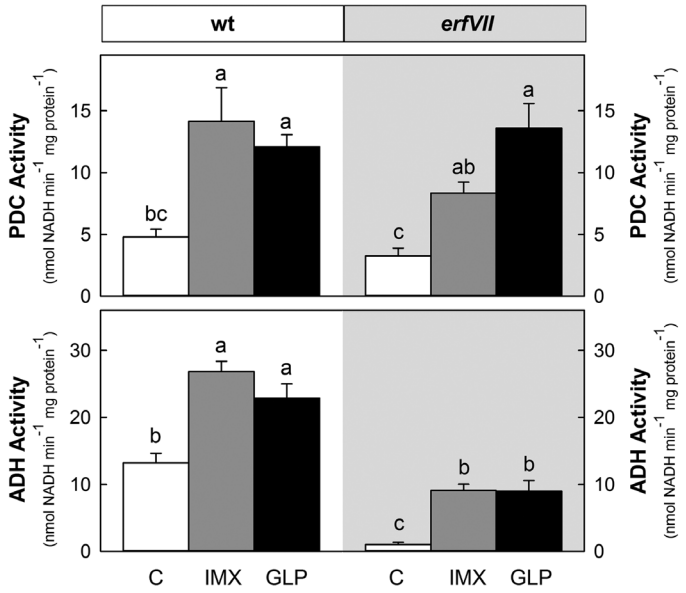

Fig. 4. Effect of herbicides on the activity of the enzymes involved in ethanol fermentation. The enzymatic activities of PDC and ADH were measured in Arabidopsis wild-type and *erfVII* seedlings untreated (control) or treated with imazamox (1.5 mg l^{-1}) or glyphosate (50 mg l^{-1}) for 5 d. Data are presented as mean ±SE (n=4). Different letters refer to statistically significant differences between treatments (two-way ANOVA followed by the Bonferroni *post hoc* test (P<0.05). ADH, alcohol dehydrogenase; C, control; GLP, glyphosate; IMX, imazamox; PDC, pyruvate decarboxylase.

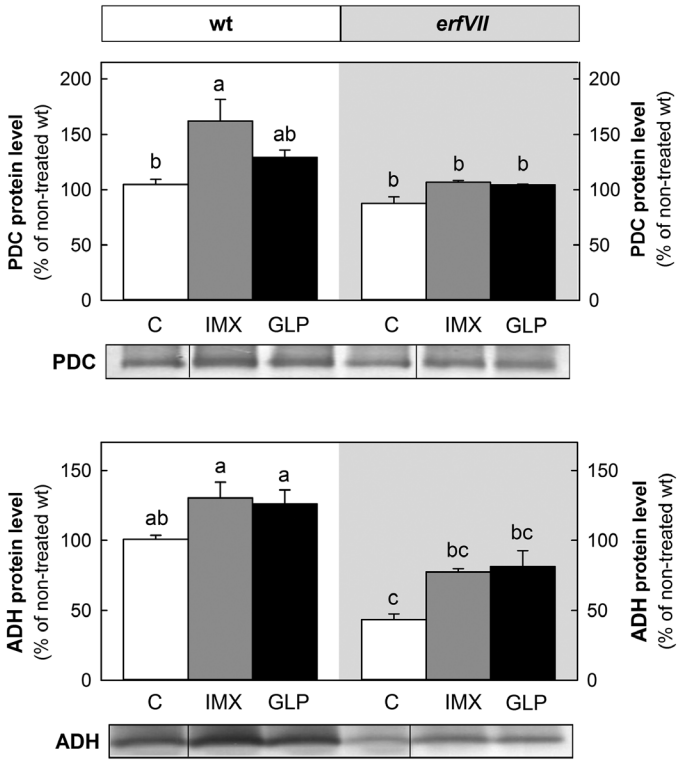

Fig. 5. Effect of herbicides on the protein content of the enzymes involved in ethanol fermentation. Immunoblot detection of PDC and ADH in Arabidopsis wild-type or *erfVII* mutant seedlings untreated (control) or treated with imazamox (1.5 mg l^{-1}) or glyphosate (50 mg l^{-1}) for 5 d. For each protein one representative blot is shown (n=4) and each lane contains 30 µg of protein. In blots, each vertical dividing line indicates one complete lane removed from the original blot to avoid treatment repetition. Analyses of band intensity on blots are presented in graphs as the relative ratio of the wild-type control (mean ±SE). Control is arbitrarily presented as 100%. Different letters refer to statistically significant differences between treatments (two-way ANOVA followed by the Bonferroni *post hoc* test (P<0.05)). ADH, alcohol dehydrogenase; C, control; GLP, glyphosate; IMX, imazamox; PDC, pyruvate decarboxylase.

The increase in the activity of PDC and ADH and their protein content by the two classes of herbicides could be attributed to an upregulation of the genes coding for these enzymes. Thus we measured the relative transcript levels of three genes belonging to the PDC gene family in Arabidopsis (Kürsteiner *et al.*, 2003). Statistically significant accumulation of *PDC1*, *PDC2*, and *PDC4* mRNAs was detected in herbicide-treated wild-type plants, with the exception of *PDC4* in the glyphosate-treated seedlings (Fig. 6). Also the only Arabidopsis ADH-encoding gene, *ADH1* (Chang and Meyerowitz, 1986; Bui *et al.* 2019), was induced when plants were treated with herbicides (Fig. 6). The effect of imazamox on the upregulation of *PDC1* and *PDC4* was more severe than that provoked by glyphosate. Moreover, milder induction of the ethanol fermentation pathway in the *erfVII* mutant in response to the two herbicides was measured (Fig. 6). Lower accumulation of *PDC1* was observed as a consequence of imazamox or glyphosate application in *erfVII* mutant as compared with wild-type seedlings, and no accumulation of *PDC4* was found after imazamox application to *erfVII* mutants. These results suggest that ERF-VII transcription factors are implicated in the regulation of ethanol fermentation in plants treated with herbicides.

Expression of other genes regulated or non-regulated by ERF-VIIs in Arabidopsis wild-type and erfVII *mutant*

Apart from the ethanol fermentation genes, ERF-VII transcription factors regulate the expression of other core hypoxia-responsive genes, whose induction is restrained in the *erfVII* mutant during hypoxia (Giuntoli *et al.*, 2017). To further check the implication of ERF-VII transcription factors in the activation of anaerobiosis-like response upon herbicide treatment, we measured the expression of other representative ERF-VII target genes in our conditions. We took into consideration lactate dehydrogenase (*LDH*), non-symbiotic hemoglobin (*HB1*), and acyl-CoA desaturase (*SAD6*) (Branco-Price *et al.*, 2008; Mustroph *et al.*, 2009, 2010). Both herbicides activated the expression of the three hypoxia markers in wild-type seedlings (Fig. 7A). Interestingly, this effect was attenuated in the mutant defective for the ERF-VII transcription factors (Fig. 7A). The effect of both AHAS and EPSPS inhibitors on the expression of *SAD6* was attenuated in the *erfVII* mutant; a reduction of the effect of imazamox on *HB1* and *LDH* expression was also observed, although it was non-significant (Fig. 7A). These results correlate with the ones observed for the ethanol fermentation genes, reinforcing the hypothesis that the herbicides under evaluation can elicit transcriptional responses that overlap to some extent with those triggered by oxygen deficiency. These results also demonstrate that ERF-VIIs still regulate their hypoxic targets following herbicide treatment. To evaluate whether ERF-VIIs can also mediate herbicide-specific responses, which

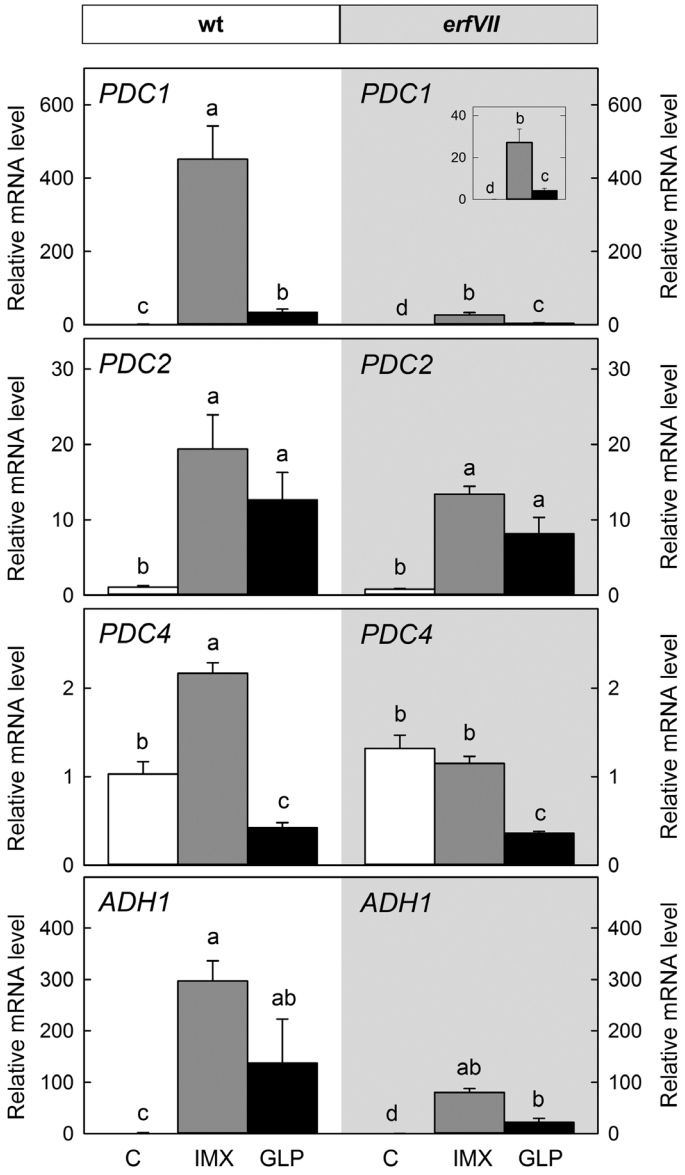

Fig. 6. Effect of herbicides on the expression of the genes encoding ethanol fermentative enzymes. Relative transcript levels of the genes *PDC1*, *PDC2*, *PDC4*, and *ADH1* in Arabidopsis wild-type or *erfVII* mutant seedlings untreated (control) or treated with imazamox (1.5 mg l^{-1}) or glyphosate (50 mg l^{-1}) for 5 d. Data are presented as mean +SE (n=4). Different letters refer to statistically significant differences between treatments (two-way ANOVA followed by the Bonferroni *post hoc* test (P<0.05)). ADH, alcohol dehydrogenase; C, control; GLP, glyphosate; IMX, imazamox; PDC, pyruvate decarboxylase.

are unrelated to low oxygen-induced transcriptional response, we monitored the expression of other genes not participating in core hypoxia responses. Specifically, we measured the expression of the genes encoding group S1 basic Leucine Zipper transcription factors 1 and 53 (*bZIP1* and *bZIP53*) and the gene coding for the protein kinase General Control Non-repressible 2 (*GCN2*). bZIPs regulate branched-chain amino acid, proline, and asparagine metabolism (Dietrich *et al.*, 2011), while GCN2 can promote the action of glyphosate, by an unknown mechanism (Faus *et al.*, 2015). Both herbicides induced the expression of *bZIP53* in the wild-type line and this effect was maintained in the *erfVII* mutant (Fig. 7B). In contrast, while AHAS or EPSPS inhibitors did not affect the expression of *bZIP1* or *GCN2* in the wild-type seedlings, the two classes of herbicides upregulated the expression of both genes in the *erfVII* mutant (Fig. 7B). These results show that the induction of *bZIP53* observed in wild-type seedlings does not rely on ERF-VIIs.

Stabilization of ERF-VII transcription factors in the presence of herbicides

Although transcriptionally induced by herbicides (Fig. 1), under fully aerated conditions ERF-VII proteins would be expected to undergo continuous degradation through the 26S proteasome. However, the pieces of evidence presented in the previous paragraphs hinted at ERF-VII accumulation in air, when imazamox or glyphosate were supplied. To obtain insights into the actual ERF-VII protein levels after application of the herbicides, we took advantage of Arabidopsis seedlings expressing a chimeric reporter, consisting of 28 N-terminal amino acids of RAP2.12, which contain its oxygen destabilization domain, coupled to firefly luciferase (Weits *et al.*, 2014). Given that this translational reporter is expressed under control of the constitutive 35S CaMV promoter, its output can be used as a proxy for the stability of the endogenous RAP2.12 protein. Luciferase activity increased as a consequence of herbicide application, suggesting that also RAP2.12 degradation was impaired (Fig. 8). This result indicates that ERF-VII transcription factors are likely to be stabilized upon herbicide treatment, and thus are amenable to activate the core set of hypoxia response genes, including the ethanol fermentation pathway.

Physiological significance of ethanol fermentation in the response to amino acid biosynthesis-inhibiting herbicides

The possible physiological implications of the induction of ethanol fermentation in the toxicity of amino acid biosynthesis-inhibiting herbicides was evaluated, to understand whether this response could constitute a stress-induced mechanism that protects the viability of the plant, or rather it contributes to herbicidal toxicity. To this end, wild-type Arabidopsis seedlings, a *pdc1pdc2* double mutant, and the *adh1* mutant were grown on vertical agar plates and root growth was measured to assess sensitivity to herbicides in the different genotypes.

The three genotypes were severely affected by imazamox and even more by glyphosate application (Fig. 9A) and root growth was hindered in all cases (Fig. 9B). Plants impaired in the ethanol fermentation pathway exhibited a more severe arrest of root elongation provoked by the herbicides (Fig. 9C).

The role of fermentation in the tolerance to herbicide application was furthermore explored in a less direct way, by use of mutants in which the ethanol fermentation pathway is expected to be stimulated. Specifically, we tested two mutants impaired in the N-degron pathway: a line defective for the ATE1 and ATE2 enzymes, responsible for the arginylation of N-degrons exposing acidic residues, and another line with abolished expression of the N-recognin PRT6, which labels the ERF-VIIs for degradation via polyubiquitination (see Supplementary Fig. S3).

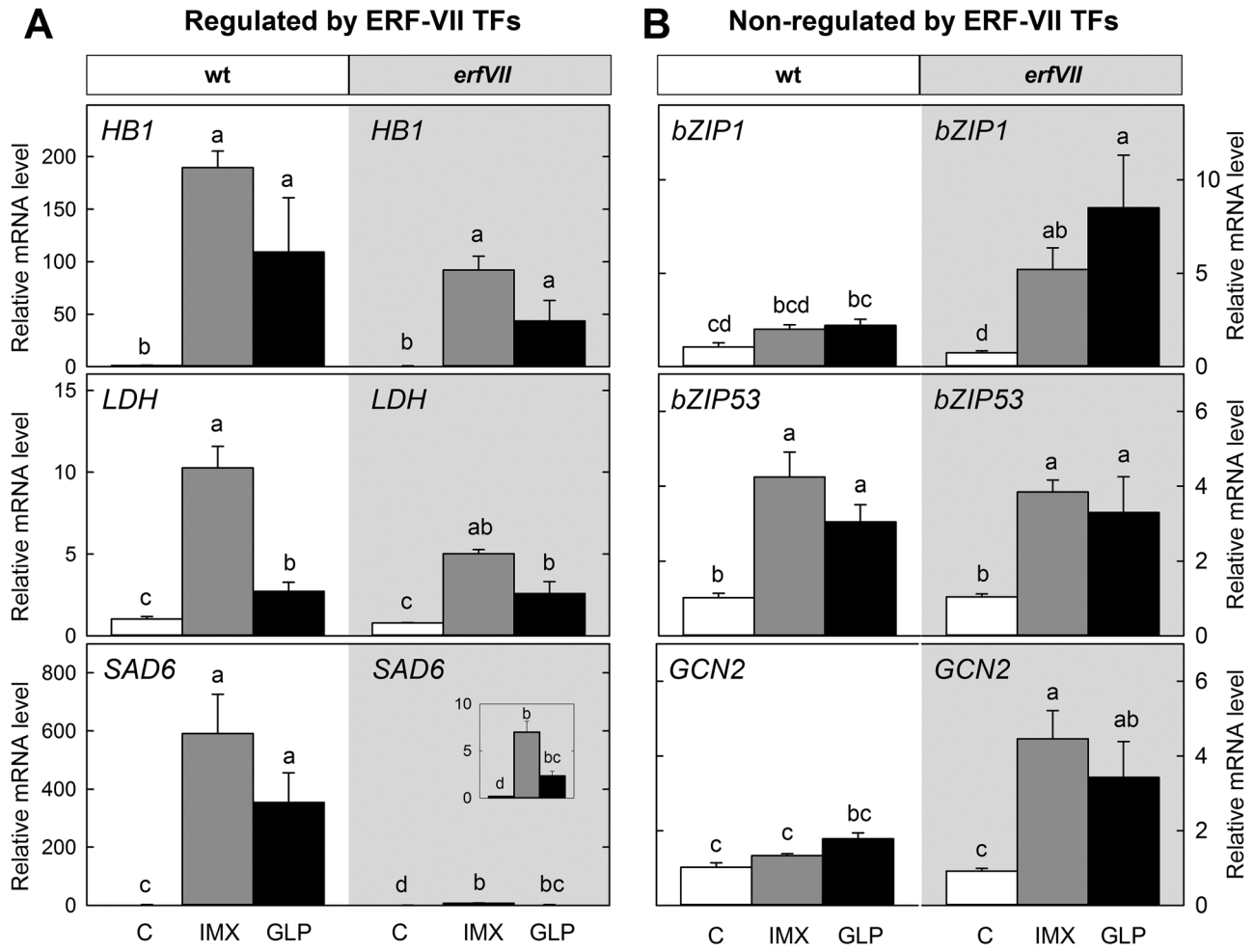

Fig. 7. Effect of herbicides on the expression of genes regulated and non-regulated by ERF-VII transcription factors. Relative transcript levels of genes regulated by ERF-VII transcription factors (A) and non-regulated by ERF-VII transcription factors (B) in Arabidopsis wild-type or *erfVII* mutant seedlings untreated (control) or treated with imazamox (1.5 mg l^{-1}) or glyphosate (50 mg l^{-1}) for 5 d. Data are presented as mean +SE (n=4). Different letters refer to statistically significant differences between treatments (two-way ANOVA followed by the Bonferroni *post hoc* test (P<0.05)). C, control; GLP, glyphosate; IMX, imazamox. TFs, transcription factors.

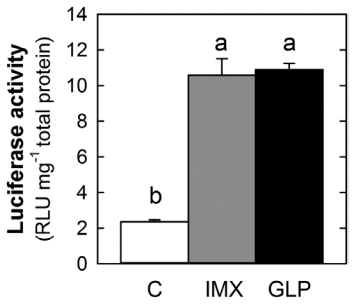

Fig. 8. Stabilization of ERF-VII transcription factors by herbicides. Firefly Luc activity in Arabidopsis *RAP2.12*$_{1\text{-}28}$*-Luc* chimeric line, untreated (control) or treated with imazamox (1.5 mg l^{-1}) or glyphosate (50 mg l^{-1}) for 5 d. The luminescent signal is expressed in RLU normalized to the total protein amount of the sample. Six biological replicates were used for each experimental condition (mean +SE (n=6)). Different letters refer to statistically significant differences between treatments (one-way ANOVA followed by the Bonferroni *post hoc* test (P<0.05)). C, control; GLP, glyphosate; IMX, imazamox; Luc, luciferase; RLU, relative luminiscence units.

These two mutants show ERF-VII stabilization in aerobic conditions and present increased transcript levels of the fermentative genes, including *PDC1* and *ADH1* (Gibbs *et al.*, 2011, Licausi *et al.*, 2011). However, no differences were found regarding the effects of the two herbicides in the root length of *ate1ate2* or *prt6* mutants, compared with the effects observed in the wild-type seedlings (Supplementary Fig. S3), suggesting the existence of additional compensatory effects, taking place downstream of the Arg/N-degron pathway and acting in parallel to ERF-VII target processes.

Discussion

Transcriptional regulation of ethanol fermentation in plants exposed to low oxygen stress has been studied in detail (Licausi *et al.* 2010; Bui *et al.* 2015, 2019). However, the regulation of this pathway in aerobic stress conditions has not been as extensively investigated. Here, we examined whether ERF-VII transcription factors are involved in the transcriptional regulation of ethanol fermentation in plants after herbicide treatment. We selected imazamox and glyphosate, two distinct herbicides that inhibit branched-chain and aromatic amino acid biosynthesis, respectively. At the concentration employed in this study, both herbicides caused lethality (see Supplementary Fig. S1), after

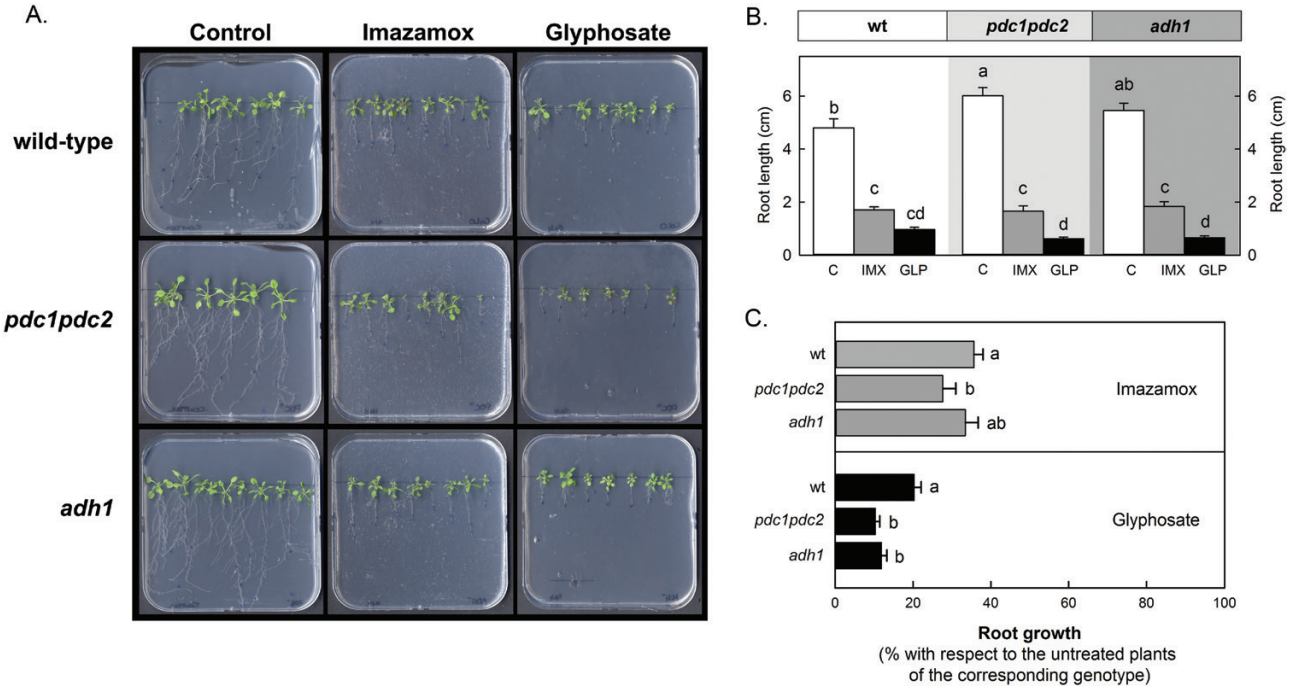

Fig. 9. Attenuation of herbicide toxicity by ethanol fermentation. As a marker of sensitivity to the herbicide, root length of seedlings was monitored in wild-type Arabidopsis Col-0 and in *pdc1pdc2* and *adh1* mutants, untreated (control) or treated with imazamox (0.005mg l^{-1}) or glyphosate (0.25 mg l^{-1}) (A) Representative example photographs of the treatments. (B) Root length measured in seedlings of wild-type (wt) Arabidopsis Col-0, *pdc1pdc2* and *adh1* mutant plants, untreated (C) or treated with IMX or GLP. (C) Comparison of the inhibitory effect of each herbicide with respect to the untreated seedlings of each genotype. Values represent the mean ±SE (n=24, biological replicates). Different letters refer to statistically significant differences between treatments (two-way ANOVA (B) or one-way ANOVA (C) followed by the Bonferroni *post hoc* test (P<0.05)). C, control; GLP, glyphosate; IMX, imazamox.

triggering previously known symptoms including growth arrest (Fig. 2), decrease in chlorophyll content (Fig. 3A) and increased malondialdehyde content (Fig. 3B; Cobb and Reade, 2010; Gil-Monreal *et al.*, 2017).

We first confirmed that imazamox or glyphosate could stimulate the ethanol fermentation pathway in Arabidopsis seedlings, acting at the transcriptional level (Fig. 6), with a consequent increment in protein (Fig. 5) and activity levels (Fig. 4) for both PDC and ADH enzymes. These results also indicated that, opposite to low-oxygen responses, where only *PDC1* and *PDC2* genes are involved (Mithran *et al.*, 2014), the *PDC4* isoform is also implicated in the response to imazamox application.

Overexpression of ERF-VIIs can enhance stress tolerance to low oxygen conditions in Arabidopsis and rice (Xu *et al.*, 2006; Hinz *et al.*, 2010; Licausi *et al.*, 2011). Besides, ERF-VIIs are also implicated in the tolerance to a range of other stress responses, including osmotic and oxidative stresses (Fukao *et al.*, 2011; Papdi *et al.*, 2015), salt stress tolerance (Jung *et al.*, 2007; Park *et al.*, 2011), and tolerance to different pathogenic fungi (Jung *et al.*, 2007; Zhao *et al.*, 2012). Our results also show a role of ERF-VIIs in the response of the plants to herbicides. Indeed, mutant seedlings lacking the ERF-VII transcription factors showed increased sensitivity to both AHAS- and EPSPS-inhibiting herbicides as compared with wild-type seedlings (see Supplementary Fig. S1). Herbicide-treated *erfVII* mutants presented lower induction of fermentation at transcriptional (Fig. 6), protein content (Fig. 5) and enzymatic activity level (Fig. 4). The fact that the effect of herbicides on the ethanol fermentation pathway was not completely abolished in plants with reduced ERF-VII activity correlates with the results obtained in previous studies working with the same *erfVII* mutant. Indeed, Giuntoli *et al.* (2017) showed that the induction of fermentative genes in plants exposed to low-oxygen stress was not completely prevented, since in this mutant a residual amount of *RAP2.2* transcript is produced. Alternatively, in the case of herbicides, it can be hypothesized that other factors than ERF-VIIs are involved in the mild induction of fermentation observed in treated *erfVII*. For instance, pyruvate has been described to activate the enzymatic activities of PDC and ADH and increase their protein levels (Zabalza *et al.*, 2011; Gil-Monreal *et al.*, 2018).

In addition, our results show that, apart from regulating the ethanol fermentation pathway in herbicide-treated seedlings, ERF-VIIs are also involved in the herbicide-induced up-regulation of other hypoxia-target genes, including *LDH*, *HB1*, and *SAD6* (Fig. 7A). Moreover, lack of differential regulation of specific herbicide-responsive genes in the *erfVII* mutant (Fig 7B) supports the conclusion that ERF-VII transcription factors can trigger specific hypoxia-like responses to herbicide treatments.

Herbicidal control of ERF-VII activity might occur at the transcription or post-transcriptional level. Induction of *RAP2.2*, *RAP2.12*, and *HRE2* by herbicides was indeed reported (Fig. 1). Here, we also observed the stabilization of ERF-VIIs upon herbicide treatment by measuring the luciferase activity of a RAP2.12-luciferase reporter line (Fig. 8). A role for ERF-VIIs in various abiotic and biotic stresses has been proposed in plants, including dehydration, salt stress, exposure to heavy metals, heat, and several pathogens (for reviews, see

Gibbs *et al.*, 2015; Giuntoli and Perata, 2018). The fact that ERF-VIIs play a role in the response to stresses that are in principle not related to oxygen limitation might call for oxygen-independent ERF-VII-stabilizing mechanisms. On one hand, decreased oxygen levels in pea roots treated with AHAS inhibitors (Zabalza *et al.* 2011) has been reported and ascribed to lower oxygen diffusion to the inside of the tissues due to root thickening (Zabalza *et al.*, 2011). Internal hypoxia could therefore explain ERF-VII stabilization in herbicide-treated plants (Fig. 8). The *RAP2.12*$_{1\text{-}28}$*-Luc* reporter revealed that also in this case the post-transcriptional ERF-VII regulation involves the same N-terminal domain that responds to oxygen. However, taking into account the structure of the roots of the Arabidopsis seedlings used in the present study, limitations to oxygen diffusion to the roots are not expected. Evidence for the existence of other mechanisms that target ERF-VIIs for proteasome-dependent proteolysis has been reported. An N-terminal modified RAP2.12 version that cannot be recognized by the N-degron pathway is still degraded through the 26S proteasome (Papdi *et al.*, 2015). That study has also revealed that RAP2.12 amount is affected by the putative E3 ubiquitin ligases SINAT1 and 2, suggesting that they might target RAP2.12 for proteasomal degradation independently of the N-degron pathway (Papdi *et al.*, 2015). In addition, SINAT2 has been observed to be involved in the regulation of different stress responses (Kim *et al.*, 2006; Ning *et al.*, 2011; Bao *et al.*, 2014). On the other hand, a reduction in nitrate reductase activity and subsequent decrease in NO levels have been proposed to stabilize ERF-VII proteins during salinity (Vicente *et al.*, 2017). Further investigations evaluating how ERF-VIIs are stabilized upon herbicide treatment and whether SINAT proteins and/or nitrate reductase activity are involved in this stabilization are needed.

Ethanol fermentation is induced in response to a number of stressful aerobic conditions. The activity of ADH increased in maize seedlings exposed to osmotic stress (Kato-Noguchi, 2000), and the activity and transcript levels of this enzyme also increased in maize and rice seedlings exposed to low temperature conditions (Christie *et al.*, 1991). Additionally, the expression of *ADH1* increased in Arabidopsis plants and rice seedlings exposed to several abiotic stresses (Dolferus *et al.*, 1994; Minhas and Grover, 1999; Kürsteiner *et al.*, 2003) and it has been suggested that fermentation improves cold stress tolerance (Peters and Frenkel, 2004) and that this pathway might act as an overflow regulating carbohydrate metabolism (Tadege *et al.*, 1999). Remarkably, the existence of additional ethanol biosynthetic pathways has been proposed in light of studies conducted on Arabidopsis *adh1* null mutants and the absence of ADH-like sequences in primordial plant species (Bui *et al.* 2019).

In this study, we examined the significance of the induction of fermentation in response to the herbicides to evaluate if it is part of the plant defense against herbicides or by contrast it contributes to their chemical toxicity. Aromatic and branched-chain amino acid biosynthesis is impaired as a consequence of AHAS or EPSPS inhibition by herbicides. Under this condition, *de novo* synthesis of proteins is abolished (Zabalza *et al.*, 2006), while protein synthesis is not interrupted as proteolysis of existing proteins is activated and the released free amino acids are used for the synthesis of new proteins after AHAS or EPSPS inhibition (Zabalza *et al.*, 2006; Zulet *et al.*, 2013). Higher ADH and PDC protein contents are detected after herbicide treatment (Fig. 5), which addresses the importance of ethanol fermentation in the response of the plants to herbicides, as more scavenged amino acids from pre-existing proteins are being re-used for the preferential synthesis of fermentative enzymes.

To ascertain the role of fermentation upon herbicide treatment, root elongation was used as an overall marker to compare sensitivity to the herbicides among wild-type, *pdc1pdc2* and *adh1* seedlings. Root elongation has been frequently used as an overall marker of the sensitivities to different abiotic stresses such as osmotic stress (Fujii *et al.*, 2011), exposure to heavy metals (Freeman and Salt, 2007; Song *et al.*, 2010*a*,*b*; Tang *et al.*, 2014), or herbicide application (Teixeira *et al.*, 2007; Gil-Monreal *et al.*, 2017). Both genotypes impaired in the ethanol fermentation pathway were more severely affected by the herbicides than the wild-type plants (Fig. 9), indicating that ethanol fermentation contributes to the herbicide stress tolerance. These results correlate with the results observed in the evaluation of the lethality of the herbicides, where it was found that *erfVII* mutants were more susceptible to application of both herbicides and died earlier than the wild-type seedlings (see Supplementary Fig. S1). On the other hand, the effect of the herbicides was not modified in genotypes with increased fermentation activity (Supplementary Fig. S3). Alleviation of the effects provoked by amino acid biosynthesis-inhibiting herbicides on different parameters (carbohydrate and amino acid accumulation) was previously observed in the *adh1* mutant (Zulet *et al.*, 2015), but this alleviation could not be related to a decreased sensitivity to the herbicides of *adh1* mutants because lethality of the herbicides was not monitored in that study. The details of the contribution of ethanol fermentation to the alleviation of the effects provoked by the herbicides remain to be elucidated. It has been suggested that ethanol fermentation could serve to metabolize the accumulated pyruvate after the inhibition of AHAS or EPSPS (Zabalza *et al.*, 2011; Gil-Monreal *et al.*, 2017). Indeed, other pyruvate consuming enzymes, including lactate dehydrogenase and alanine aminotransferase, are known to be activated after the inhibition of AHAS by herbicides (Gaston *et al.*, 2002; Zulet *et al.*, 2015). Both imazamox and glyphosate trigger pyruvate accumulation in the tissues (Armendáriz *et al.*, 2016) and it has been demonstrated that pyruvate participates in the regulation of ethanol fermentation in herbicide-treated plants (Gil-Monreal *et al.*, 2018). Other pathways could metabolize the acetaldehyde produced by PDC during ethanol fermentation in the *adh1* mutant. For instance, the induction of the PDH bypass has been described in plants upon herbicide treatment (Gil-Monreal *et al.*, 2017). This pathway, which produces acetyl-CoA from pyruvate in an alternative pathway to the PDH complex, begins with pyruvate decarboxylation to acetaldehyde through the action of PDC. This alternative pathway would help in the detoxification of the products of ethanol fermentation and/or may serve to metabolize the accumulated pyruvate after the inhibition of AHAS or EPSPS. Interestingly, plants overexpressing the PDH bypass were less sensitive to

these herbicides (Gil-Monreal *et al.*, 2017) supporting the hypothesis that induction of PDC contributes to herbicide stress tolerance.

In conclusion, the present study reveals that ethanol fermentation is induced by amino acid biosynthesis-inhibiting herbicides in Arabidopsis seedlings at the transcript level. Mutants with reduced fermentative potential exhibited higher sensitivity to herbicide treatments. ERF-VII transcription factors appeared to be key regulators in this process, in turn being stabilized by herbicide treatment. Altogether, our results confirm that ERF-VIIs participate in the transcriptional regulation of ethanol fermentation in plants after herbicide treatment, as is the case for low oxygen stress. How these transcription factors are stabilized remains unclear, since no oxygen limitation is expected in treated plants.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Survival of Arabidopsis seedlings to herbicide application.

Figure S2. Recovery of Arabidopsis seedlings from herbicide application.

Figure S3. Herbicide toxicity in seedlings with enhanced ethanol fermentation pathway.

Table S1. Expression stability of the reference genes calculated by geNorm and BestKeeper software.

Table S2. Gene specific primers used in the qRT-PCRs.

Table S3. Results of the two-way analysis of variance.

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