

HRE1 and HRE2, two hypoxia-inducible ethylene response factors, affect anaerobic responses in *Arabidopsis thaliana*

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SUMMARY

Plants often experience challenging hypoxic conditions imposed by soil waterlogging or complete flooding. In rice, *Sub1A*, a flooding-induced ethylene responsive factor (ERF) plays a crucial role in submergence tolerance. In this study, we examined two *Arabidopsis* Hypoxia Responsive ERF genes (*HRE1* and *HRE2*), belonging to the same ERF group as *Sub1A*. Transgenic *Arabidopsis* plants, which over-expressed *HRE1*, showed an improved tolerance of anoxia, whereas a double-knockout mutant *hre1hre2* was more susceptible than the wild type. *HRE1* over-expressing plants showed an increased activity in the fermentative enzymes pyruvate decarboxylase and alcohol dehydrogenase together with increased ethanol production under hypoxia, but not in normoxia. Whole-genome microarray analyses suggested that an over-expression of *HRE1*, but not *HRE2*, increased the induction of most anaerobic genes under hypoxia. Real-time quantitative (q)PCR analyses confirmed a positive effect of *HRE1* over-expression on several anaerobic genes, whereas the double-knockout mutant *hre1hre2* showed a decreased expression in the same genes after 4 h of hypoxia. Single-knockout mutants did not show significant differences from the wild type. We found that the regulation of *HRE1* and *HRE2* by low oxygen relies on different mechanisms, since *HRE1* requires protein synthesis to be induced while *HRE2* does not. *HRE2* is likely to be regulated post-transcriptionally by mRNA stabilization. We propose that *HRE1* and *HRE2* play a partially redundant role in low oxygen signalling in *Arabidopsis thaliana*, thus improving the tolerance of the plant to the stress by enhancing anaerobic gene expression and ethanolic fermentation.

Keywords: hypoxia, anoxia, ERF, ADH, transcription factor, *Arabidopsis*.

INTRODUCTION

Plants often have to cope with oxygen shortages during their lifetime. In fact, even with normal oxygen concentrations in the atmosphere, plant tissues can experience hypoxia because of impediments to intracellular oxygen transport (Geigenberger, 2003; van Dongen *et al.*, 2004). Moreover, hypoxia can occur when external oxygen availability decreases if the whole plant or non-photosynthetic organs become flooded, since gas diffusion is four-orders of magnitude slower in water than in a gaseous atmosphere (Armstrong, 1979).

Lack of oxygen causes a reduction in respiratory efficiency and, as a consequence, in energy production (Gupta *et al.*, 2009). Therefore, under anoxia ATP synthesis is mostly provided by glycolysis coupled with NAD regenerative

pathways, including ethanolic fermentation and alanine production (Ismond *et al.*, 2003; Ricoult *et al.*, 2006). Additionally, in rice (*Oryza sativa*), nitrite can substitute for O₂ as the final electron acceptor in the mitochondrial electron transport chain (Stoimenova *et al.*, 2007).

Both the transcriptional and translational regulations of specific genes play a crucial role in the adaptation of plants to oxygen limitation (Licausi and Perata, 2009). However, despite several previous studies that have carried out detailed overviews of the global transcriptome and proteome, only a few transcription factors regulating these events have been described so far.

Analyses of the promoter sequences of hypoxia-induced genes, such as alcohol dehydrogenase (*ADH*), pyruvate

decarboxylase (*PDC*) and sucrose synthase (*SUSY*) in several plant species have contributed to the identification of several DNA elements putatively responsible for hypoxia inducibility (Dolferus *et al.*, 1994; Mohanty *et al.*, 2005). Transcription factors belonging to the MYC, MYB, AP2/ERF and NAC families have been reported to be able to up-regulate *ADH* expression in *Arabidopsis* (Abe *et al.*, 2003; Papdi *et al.*, 2008; Christianson *et al.*, 2009). However, only MYB2 has been directly related to hypoxic stress (Hoeren *et al.*, 1998) since it binds the GT motif, a DNA element that is necessary for induction of *ADH* under hypoxic conditions. More recently, *Sub1A*, a transcription factor belonging to the VII group of the ethylene responsive factor (ERF) family (Nakano *et al.*, 2006), has been demonstrated to play a major role in the submergence tolerance of lowland rice varieties (Fukao *et al.*, 2006; Xu *et al.*, 2006). According to the model proposed, the induction of *Sub1A* reduces gibberellin (GA) responsiveness, thus leading to shoot elongation, by means of a process involving ethylene (which induces *Sub1A*) and an increase in the level of GA signalling repressors such as SLR1 and SLRL1 (Fukao and Bailey-Serres, 2008). In this way, *Sub1A* promotes a 'quiescent strategy' in order to avoid any unnecessary energy consumption caused by hormone-mediated elongation in the submerged tissues (Perata and Voeselek, 2007). In the opposite fashion, the ERF transcription factors *SNORKEL1* and *SNORKEL2* promote GA-mediated internode elongation in deep-water rice varieties (Hattori *et al.*, 2009), promoting an 'escape strategy' to survive flooding (Voeselek and Bailey-Serres, 2009).

Apart from *Sub1A*, which is not conserved in all rice varieties, other members of the ERF group VII have been reported to be up-regulated under low-oxygen conditions in *Arabidopsis*, rice and poplar (*Populus trichocarpa*) (Loreti *et al.*, 2005; Lasanthi-Kudahettige *et al.*, 2007; Kreuzwieser *et al.*, 2009). These data suggest that this family of transcription factors is a strongly conserved element in the regulation of hypoxic responses in higher plants.

The ERF transcription factors are so called because the first members of this family to be studied showed ethylene responsiveness (Ohme-Takagi and Shinshi, 1995). However, further characterization of the whole family clearly demonstrated that each ERF is regulated in a specific manner and, therefore, this acronym is nowadays associated with a conserved domain of 60–70 amino acids responsible for DNA binding (Sakuma *et al.*, 2002; Nakano *et al.*, 2006).

Transcriptional regulators belonging to the AP2/ERF family play a role in many processes, encompassing development (Elliott *et al.*, 1996; Boutillier *et al.*, 2002) and responses to both biotic (Yamamoto *et al.*, 1999) and abiotic stresses (Stockinger *et al.*, 1997; Liu *et al.*, 1998; Xu *et al.*, 2006). Several members of the ERF family from different plant species have been demonstrated to regulate genes involved

in scavenging reactive oxygen species (ROS) (Ogawa *et al.*, 2005; Wu *et al.*, 2008). When over-expressed, many of these members are also able to increase tolerance to different stress conditions (Weiste *et al.*, 2007; Wu *et al.*, 2008; Youm *et al.*, 2008).

A DNA element named the GCC box (AGCCGCC) has been shown to be recognized by most members of the AP2/ERF family (Ohme-Takagi and Shinshi, 1995), even though a number of other motifs have been recently demonstrated to be specifically bound by different ERF transcription factors, despite the high conservation of the amino acid sequence of the DNA-binding domain (Sasaki *et al.*, 2007; Welsch *et al.*, 2007; Maeo *et al.*, 2009).

This conservation among species and the extent of the hypoxic induction of some group VII ERF genes point to their involvement in low-oxygen signalling in plants. In this paper, we describe two group VII ERFs and highlight their role in hypoxic responses in *Arabidopsis thaliana*.

RESULTS

HRE1 and *HRE2* encode two nuclear-localized ERFs specifically up-regulated under low oxygen in *Arabidopsis*

There are five group VII ERF genes in the *Arabidopsis* genome, namely *At1g53910* (*RAP2.12*), *At1g72360*, *At2g47520*, *At3g14230* (*RAP2.2*) and *At3g16770* (*RAP2.3* also known as *AtEREBP*). These ERFs are characterized by a conserved DNA-binding domain, by the extremely conserved N-terminal motif MCGGAV/IISD and by the leucine/isoleucine and tryptophan residues at the C-terminus (Figure 1a). In order to estimate the evolutionary relatedness among the members of this group, we used a neighbour joining method to build a phylogenetic tree of the ERF VII proteins from *Arabidopsis*, rice, maize (*Zea mays*), grapevine (*Vitis vinifera*) and poplar (Figure 1b). A separation between dicot and monocot genomes appeared evident. *At1g72360*, *At3g13230* and *At1g53910* cluster together with at least another group VII ERF belonging to the dicot genomes considered. *At2g47520* and *At3g16770* also cluster together with sequences from grapevine and poplar, although in different groups. The rice *Sub1* and *Snorkel* proteins, which regulate the submergence response, cluster together in a rice-specific group.

We carried out a detailed analysis of the expression of the five *Arabidopsis* members of ERF group VII under hypoxia (Figure 2a). The expression of *RAP2.3* was unaffected by hypoxia, while the mRNA level of *RAP2.2* and *RAP2.12* declined following the low-oxygen treatment (Figure 2a). *At1g72360* (*AtERF#73*, according to Nakano *et al.*, 2006) and *At2g47520* (*AtERF#71*) were induced by low oxygen and were therefore named *HRE1* and *HRE2* (Hypoxia Responsive ERFs; Figure 2a). The *HRE1* expression pattern under hypoxia highlighted a peak of expression 2 h after the onset of hypoxia (Figure 2a). *HRE2* mRNA was barely detectable in

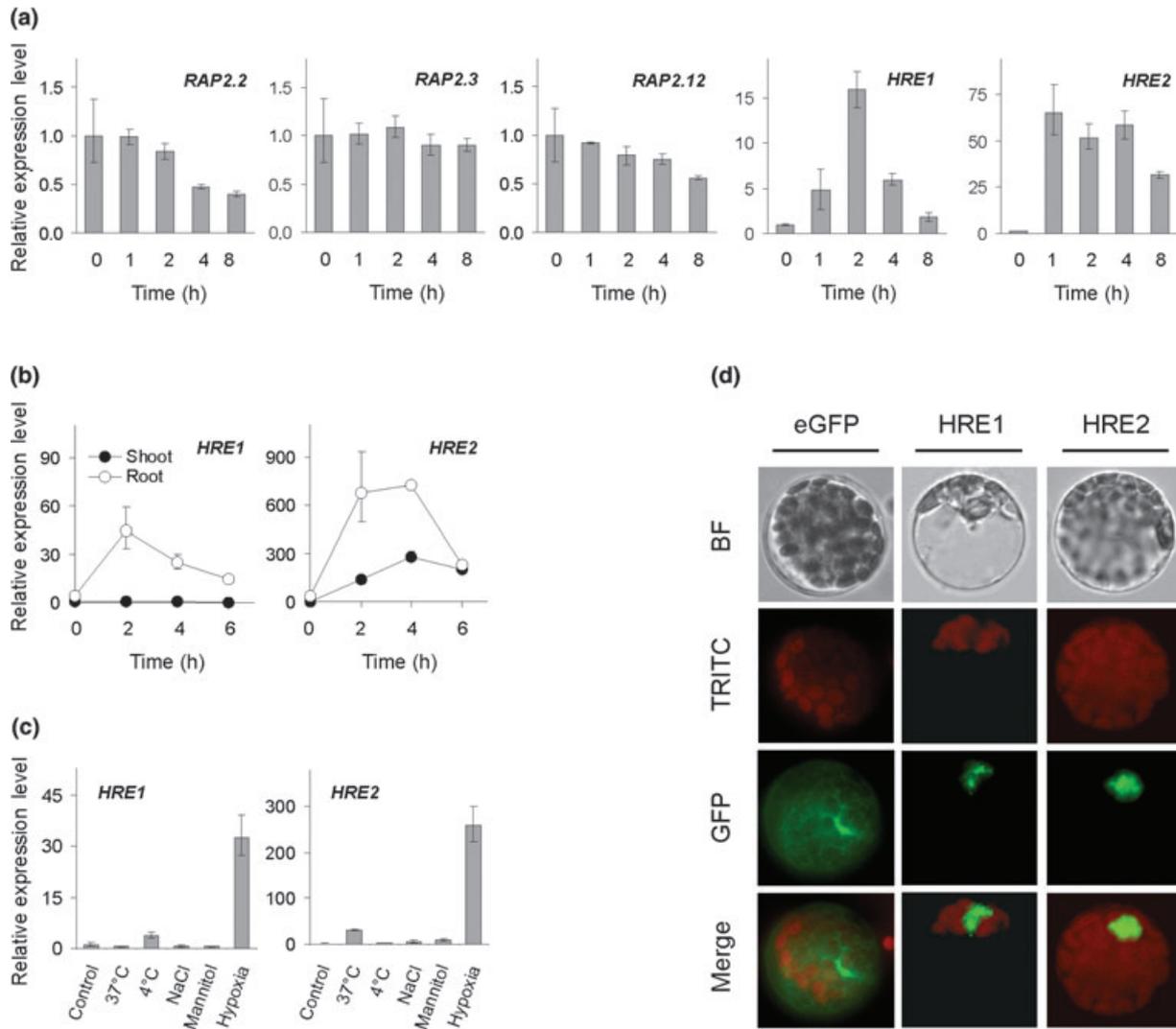


Figure 2. Expression profile of Arabidopsis group VII ethylene responsive factor (ERF) genes.

(a) Effects of hypoxia on the expression of group VII ERFs. Seven-day-old *Arabidopsis* seedlings were treated under hypoxia (1% O₂). Relative expression levels of *RAP2.2* (*At3g14230*), *RAP2.3* (*At3g16770*), *RAP2.12* (*At1g53910*), *HRE1* (*At1g72360*) and *HRE2* (*At2g47520*) were measured. Relative expression levels are shown as fold change values (1 = time 0). Data are mean \pm SD ($n = 3$).

(b) Pattern of expression of *HRE1* and *HRE2* under hypoxia. Fifteen-day-old seedlings were treated under hypoxia (1% O₂). The mRNA level in shoots (filled circles) and roots (empty circles) was measured by real-time quantitative (q)PCR. Relative expression levels are shown as fold change values (1 = shoot, time 0). Data are mean \pm SD ($n = 3$).

(c) Relative expression levels of *HRE1* and *HRE2* in response to different abiotic stresses. Fifteen-day-old seedlings were treated under various abiotic stress conditions for 3 h. The mRNA level in whole seedlings was measured by real-time qPCR. Relative expression levels are shown as fold change values (1 = control). Data are mean \pm SD ($n = 3$).

(d) Subcellular localization of *HRE1* and *HRE2* proteins in transiently transformed *Arabidopsis* mesophyll protoplasts. Pictures were taken using bright-field (BF), chlorophyll (TRITC) and GFP (GFP) filters.

normal air (data not shown) but its mRNA abundance increased rapidly under hypoxia (Figure 2a). Previous microarray analyses reported high *HRE2* expression under prolonged (48 h) hypoxia (1 and 4% oxygen) but its expression declined when the oxygen availability was higher than 4% (van Dongen *et al.*, 2009).

In general, most of the typical anaerobic genes are either predominantly or exclusively expressed in the roots, where hypoxia is experienced more frequently. Indeed, both *HRE1*

and *HRE2* were mostly expressed in the hypoxic root (Figure 2b). *HRE1* is not expressed in shoots, even under hypoxia, while *HRE2* is up-regulated ubiquitously, even though the expression level in the root is higher than in the shoot (Figure 2b).

The most obvious effect of an oxygen shortage is an energy deficit. However, energy deprivation is a common consequence of almost all abiotic stresses that negatively affect photosynthesis and/or respiration (Baena-González

and Sheen, 2008). To verify the hypoxia specificity of the induction of both *HRE1* and *HRE2*, we analysed the effects of a set of abiotic stresses on Arabidopsis seedlings by means of qPCR. The results showed that hypoxia was the strongest trigger for the expression of both *HRE1* and *HRE2* (Figure 2c). In addition to hypoxia, cold and heat also had a modest effect on the expression of *HRE1* and *HRE2*, respectively (Figure 2c).

Some group VII members of the ERF family have been reported to function as positive regulators of transcription, and RAP2.3 has been shown to be localized in the nucleus (Pan *et al.*, 2001). Transient transformation of mesophyll protoplasts with a construct bearing a GFP-tagged version of *HRE1* and *HRE2* showed that both gene products are indeed localized in the nucleus (Figure 2d).

HRE1 and HRE2 contribute to tolerance of anoxia in Arabidopsis seedlings

In rice, *Sub1A*, a group VII ERF, plays a crucial role in submergence tolerance (Xu *et al.*, 2006). Both *HRE1* and *HRE2* may play a similar role in Arabidopsis.

We decided to investigate the low-oxygen tolerance of Arabidopsis lines with altered expression of *HRE1* and *HRE2*. We therefore produced Arabidopsis plants expressing *HRE1* and *HRE2* under control of the constitutive 35S CaMV promoter. We also obtained homozygous T-DNA insertion Salk lines from the European Arabidopsis Stock Center (NASC). A mutant bearing an insertion in the 5' untranslated region (UTR) of *HRE1* was selected (Salk_023445) while for *HRE2* a mutant characterized by T-DNA insertion in the middle of the second exon was available (Salk_052858). Real-time qPCR analysis of *HRE* expression confirmed that production of the mature mRNA coding for the full-length protein was abolished in all the Salk lines investigated (Figure S1a in Supporting Information). *hre1* and *hre2* lines were crossed to generate a double homozygous knockout mutant (*hre1hre2*). Neither the over-expressers nor the mutant lines obtained showed an evident phenotype at any developmental stage examined (see Figure S1b for the rosette stage).

Tolerance tests using hypoxia showed that Arabidopsis can easily survive several days in a 1% O₂ atmosphere in the dark (not shown). Moreover, the phenotypes observed after this period resembled those of prolonged darkness and carbon starvation stress, which are distinct from oxygen deficiency stress. We therefore decided to expose Arabidopsis HRE over-expressers and mutants to a few hours' anoxia to compare their survival. Wild-type plants (Columbia-0, Col-0) showed a 70% survival after 8 h under anoxia, but their tolerance dropped to about 10% after 10 h (Figure 3a,b). The tolerance of the individual mutants (*hre1* and *hre2*) to anoxia was comparable to the wild type, while the *hre1hre2* mutant showed a significantly lower tolerance after 8 h of anoxia. Conversely, the *35S::HRE1*, but not the

35S::HRE2, plants showed enhanced anoxia tolerance, with 92% surviving after 8 h of anoxia and 67% after 10 h (Figure 3a,b).

A detailed anoxic time-course confirmed the lower tolerance displayed by the *hre1hre2* genotype (Figure 3c,d). Enhancement of tolerance to anaerobiosis in plants often relies, at least partly, on the ability to regenerate NAD⁺ through ethanol fermentation (Bailey-Serres and Voesenek, 2008). The increased tolerance in *35S::HRE1* plants could be due to an enhancement of the alcoholic fermentative pathway. Indeed, the *35S::HRE1* roots showed ADH and PDC activities that were significantly higher than the wild type (Col-0) (Figure 4a). Interestingly, activities of both ADH and PDC in *35S::HRE1* plants were already higher than in the wild type in aerobic conditions. To verify whether the increased PDC and ADH activities correlated with enhanced fermentation *in vivo*, we measured ethanol production. *35S::HRE1* plants showed an increased hypoxic ethanol production, whereas the double mutant displayed a lower ethanol accumulation (Figure 4a). Under aerobic conditions, no significant difference was found among the genotypes (Figure 4a). Additionally, we analysed the time-course of ethanol production in the wild type, double mutant and *35S::HRE1* seedlings (Figure 4b). The results indicated that the *hre1hre2* mutant produced less ethanol than the wild type at all time points, while *35S::HRE1* seedlings showed increased ethanol production after 12 and 24 h under hypoxia (Figure 4b).

HRE1 and HRE2 affect the expression of anaerobic genes under low oxygen

The phenotypic and biochemical changes that we observed in *35S::HRE1* plants suggested that *HRE1* is involved in the transcriptional regulation of anaerobic genes, thus playing a role in mechanisms of adaptation to low oxygen. A microarray analysis was carried out on the *35S::HRE1* and *35S::HRE2* lines compared with the wild type (Col-0) under aerobic and hypoxic conditions (4 h at 1% O₂). In air, *HRE1* over-expression significantly influenced (adjusted *P*-value ≤ 0.05) five genes in addition to *HRE1* itself (Table S1). Interestingly, *ADH* was among the up-regulated genes (Table S1). Under hypoxia, 22 genes were significantly up-regulated (twofold or more) and 15 were down-regulated (twofold or less) in *35S::HRE1* seedlings compared with the wild type (Table S2). Among the up-regulated genes, *At1g55810* (putative uracil phosphoribosyltransferase), *At4g30380* (expansin-like B2 precursor), *At5g54740* (lipid transfer family protein), *At4g32840* (putative phosphofructokinase), *At1g77200* (AP2 transcription factor TINY) and *At2g22880* (VQ domain-containing protein) have also been previously reported to be induced by low oxygen in other microarray analyses (Branco-Price *et al.*, 2005, 2008; Loreti *et al.*, 2005). Among the 15 genes significantly down-regulated, *At5g24210*, encoding for a lipase class 3 protein,

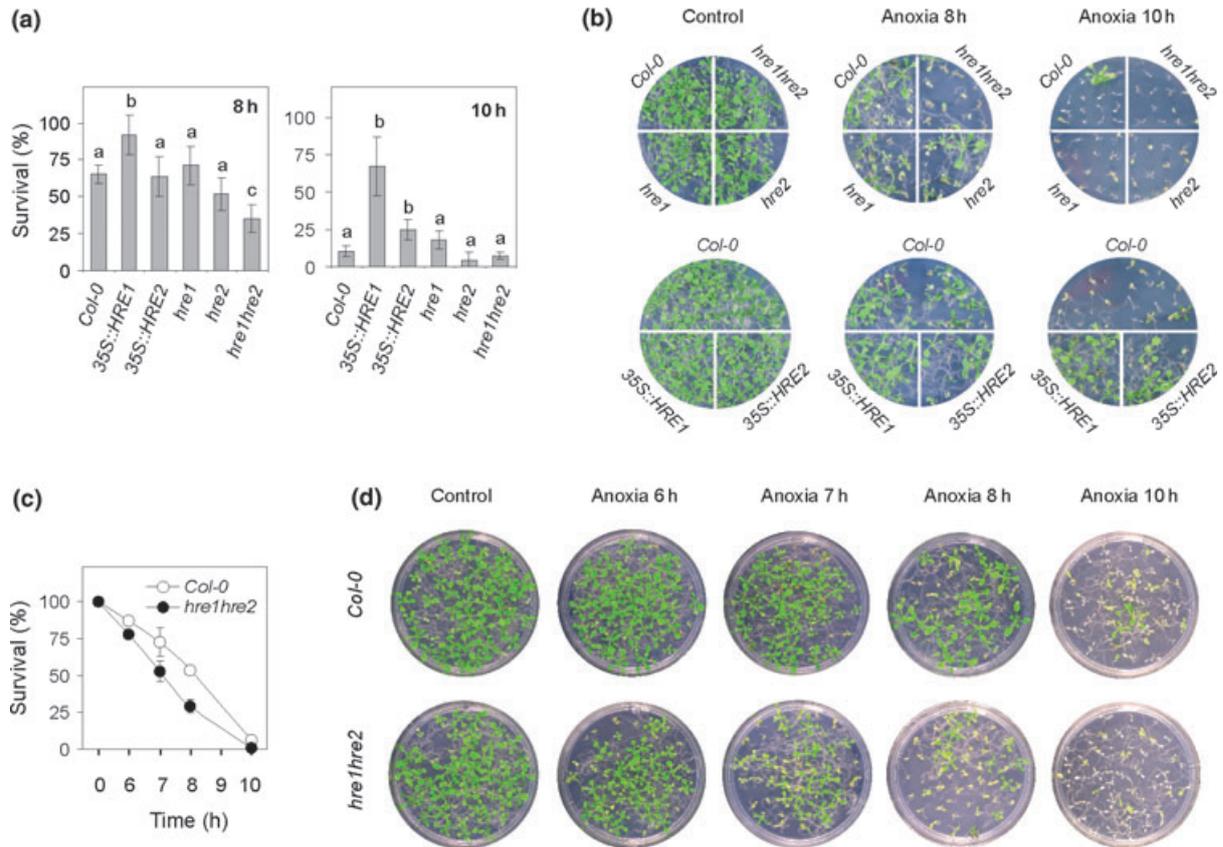


Figure 3. Effects of *HRE1* and *HRE2* on tolerance of anoxia.

(a) Survival rates of 7-day-old *Arabidopsis* plants treated under anoxia for 8 and 10 h. Fifteen seeds were sown in each plate sector. After the anoxic treatment, plants were transferred to air and surviving plants were counted after a 10-day recovery period. Survival rates were calculated as a percentage. Data are mean survival rates \pm SD ($n = 5$ plates containing at least 15 seedlings each).

(b) Phenotypes of the wild-type, mutant and over-expressor plants after a 14-day recovery after the anoxic treatments.

(c) Survival rates of 7-day-old *Arabidopsis* plants treated under anoxia for 6, 7, 8 and 10 h. Fifty seeds were sown in each plate ($n = 4$ for each time point). After the anoxic treatment, plants were transferred to air and surviving plants were counted after a 10-day recovery period. Survival rates were calculated as a percentage. Data are mean survival rates \pm SD ($n = 4$ plates containing at least 50 seedlings each).

(d) Phenotypes of the wild type (Col-0) and the double mutant *hre1hre2* after a 14-day recovery after the anoxic treatments.

At3g04290, called *ATLTL1*, a lipase involved in cadmium tolerance, *At2g47000*, a P-glycoprotein (PGP4), *At5g45950*, a GDSL-lipase, and *At2g04040* (*ATDTX1*) are involved in lipid metabolism and membrane remodelling related to stress tolerance. In addition *ELIP2*, *PLA2A* and *CSD2*, encoding enzymes involved in scavenging and protection against ROS production, are less expressed in the 35S:*HRE1* seedlings.

In aerobic conditions, the over-expression of *HRE2* significantly affects the expression of 31 genes, among which 26 are down-regulated (Table S3). Surprisingly, included in this list are *ADH*, non-symbiotic haemoglobin (*HB1*), *PDC*, *At5g66985*, *At4g33560* and *At3g48100* that have been shown to be up-regulated by low oxygen in several experiments (Branco-Price *et al.*, 2005; Loreti *et al.*, 2005; van Dongen *et al.*, 2009). However, the hypothesis that *HRE2* acts as a negative regulator of anaerobic gene expression cannot be accepted. In fact, under hypoxic conditions, *HRE2* over-expression does not affect the expression of typical anaerobic

genes (Table S4). To gain a further overview of anaerobic genes, we selected those genes whose expression was increased by hypoxia more than eightfold in the wild type. This group was then compared with previous microarray analyses on the anaerobic transcriptome in similar conditions (Branco-Price *et al.*, 2005, 2008; Loreti *et al.*, 2005; van Dongen *et al.*, 2009). All 48 genes were induced by low-oxygen treatments and were therefore designated as an anaerobic cluster (Table S5). A two-way ANOVA analysis of the anaerobic cluster showed a significant increase in gene expression in 35S:*HRE1* compared with the wild-type under hypoxia, but not in 35S:*HRE2* (Figure 5). We investigated the possible effects of *HRE1* and *HRE2* on the expression of anaerobic genes under hypoxia by analysing a detailed time-course using a more sensitive real-time qPCR procedure. We selected *ADH*, *HB1*, *SUCROSE SYNTHASE1* (*SUS1*) and *SUCROSE SYNTHASE4* (*SUS4*) as good markers of the anaerobic response and tested their expression at different

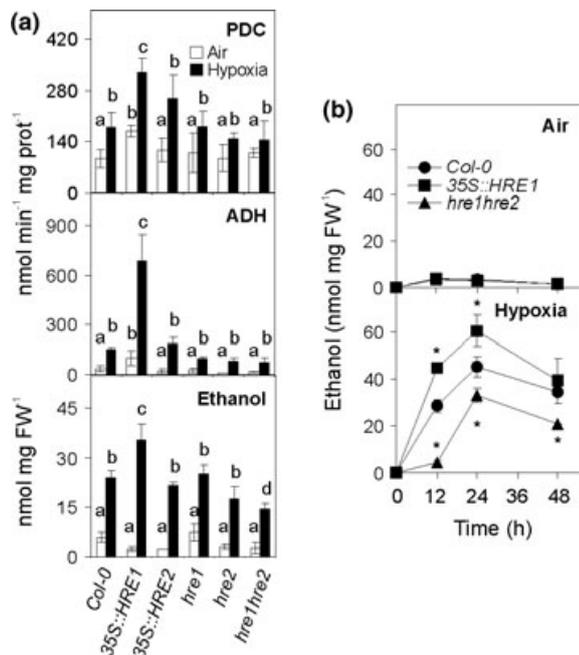


Figure 4. Effects of *HRE1* and *HRE2* on ethanolic fermentation.

(a) Fermentative enzyme activities [pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH)] and ethanol accumulation in roots from 15-day-old plants and in 7-day-old seedlings, respectively, treated for 12 h with air (empty bars) and 1% O₂ (filled bars) in the dark. The results shown are mean \pm SD ($n = 3$). A two-way ANOVA (P -value ≤ 0.01) pairwise comparison was used to assess the significance of the differences found between the '*HRE*' genotypes and the wild type (Col-0) under aerobic and hypoxic conditions. (b) Ethanol production over 48 h in 7-day-old seedlings treated with air and 1% O₂ in the dark. The results shown are mean \pm SD ($n = 3$). A two-way ANOVA (P -value ≤ 0.01) pairwise comparison was used to assess the significance of the differences found between the '*HRE*' genotypes and the wild type (Col-0) under hypoxic conditions at the time points considered. The asterisk (*) indicates an ethanol production significantly from the wild type at the time point examined.

time points during the course of 8 h of hypoxia (Figure 6). Real-time qPCR analysis showed that all four genes were significantly up-regulated in the *35S::HRE1* plants compared with the wild type. Interestingly, *HRE1* over-expression also positively affects the expression of *HRE2* under hypoxia. Apparently, over-expression of *HRE1* only enhanced the effect of the hypoxic treatment, since it did not significantly alter the expression of *SUS1*, *SUS4* and *HB1* under aerobic conditions. Only *ADH* expression was higher in normoxic *35S::HRE1* plants compared with the wild type (Col-0), although to only one-tenth of the levels induced by low oxygen. No significant differences were observed in the *35S::HRE2* plants. Null mutants of *HRE1* and *HRE2* (*hre1* and *hre2*) were not impaired in the expression of the four markers, but the double mutant *hre1hre2* showed a drastic decrease in the expression of anaerobic genes 4 h after the onset of hypoxia (Figure 6).

To identify DNA sequences that act as candidate *cis*-elements bound by HRE1, we focused on the 22 genes that showed a significantly higher hypoxic increase in *35S::HRE1*

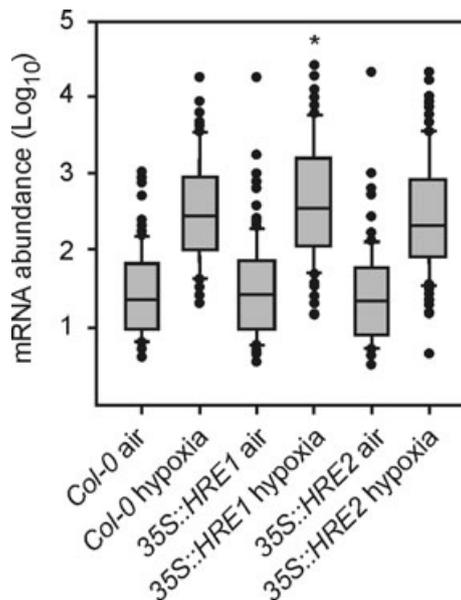


Figure 5. Effects of *HRE1* and *HRE2* over-expression on anaerobic genes. The box plot shows the overall effects of hypoxia on the expression of the anaerobic cluster (Table S5) in the different genotypes. P -value ≤ 0.01 .

compared with the wild type (Col-0) (Table S2). An analysis of the region upstream of the start of transcription (up to 1000 bp), using Toucan software (Stein *et al.*, 2003, 2005), revealed no significant ($P \leq 0.01$) over-representation of any known *cis*-element bound by transcription factors, including the GCC box which represents the putative ERF-binding site (Wang *et al.*, 2009). On the other hand, a search for new motifs led to the identification of A- (or T-) rich regions that might represent a structural requirement for the binding of the HREs. Interestingly, A- and T-rich motifs have already been reported to be present in the promoters of low-oxygen responsive genes in several species (Mohanty *et al.*, 2005).

***HRE1* and *HRE2* are regulated at transcriptional and post-transcriptional levels, respectively**

The very first events in the molecular responses to environmental stimuli are likely to be independent of gene transcription or protein synthesis since the regulatory proteins involved should be constitutively present in the cell in an inactive form and quickly activated by post-translational modifications to allow a fast response. Since *HRE1* and *HRE2* are already induced after 30 min of imposition of hypoxia (van Dongen *et al.*, 2009), we tested whether they require newly synthesized proteins to be up-regulated. A previous microarray analysis of transcriptional changes in response to cycloheximide (CHX), an inhibitor of the ribosome release from mRNA, showed that CHX treatments increase mRNA levels for *HRE2* (NASCArrays-189). We used CHX and emetine (EMT), two different inhibitors of protein synthesis, to test whether these two compounds affect induction of *HRE1*

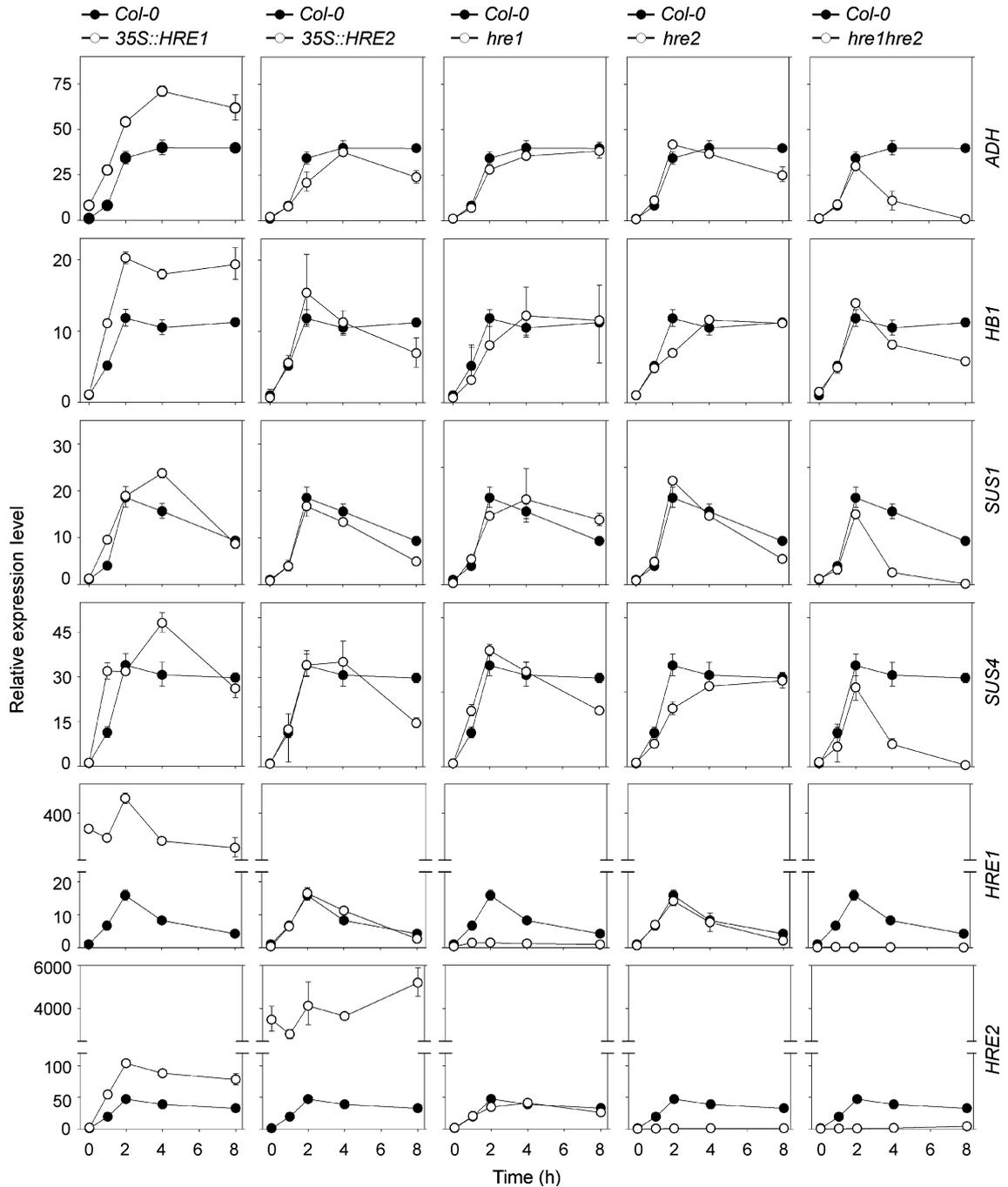


Figure 6. Effects of *HRE1* and *HRE2* on the expression of anaerobic genes.

The expression of four anaerobic genes (*ADH*, *HB1*, *SUS1* and *SUS4*) and of *HRE1* and *HRE2* was analysed under hypoxia (1% O_2) in 7-day-old *Arabidopsis* seedlings. Five genotypes were used: Col-0 (wild type), *35S::HRE1*, *35S::HRE2*, *hre1*, *hre2* and *hre1hre2*. The mRNA level in whole seedlings was measured by real-time quantitative PCR. Relative expression levels are shown as fold change values (1 = Col-0, time 0). Data are mean \pm SD ($n = 3$). In each individual graph, empty circles represent the transgenic or mutant genotypes (as indicated in the legend) and the filled circles identify the wild-type (Col-0) plants.

and *HRE2* under hypoxia. Cycloheximide and EMT prevented the induction of both *ADH* (used as a control) and *HRE1* under low oxygen in *Arabidopsis* seedlings, but not *HRE2*, whose expression was increased to a higher extent in aerobic conditions (Figure 7a). This suggests that the

induction of *HRE1* and *ADH* requires the translation of one or more transcription factors, which should take place during the first 30 min after stress imposition. Dolferus *et al.* (1994) identified *MYB2* (*At2g47190*) as a transcription factor that plays a major role in the induction of *ADH* and other genes

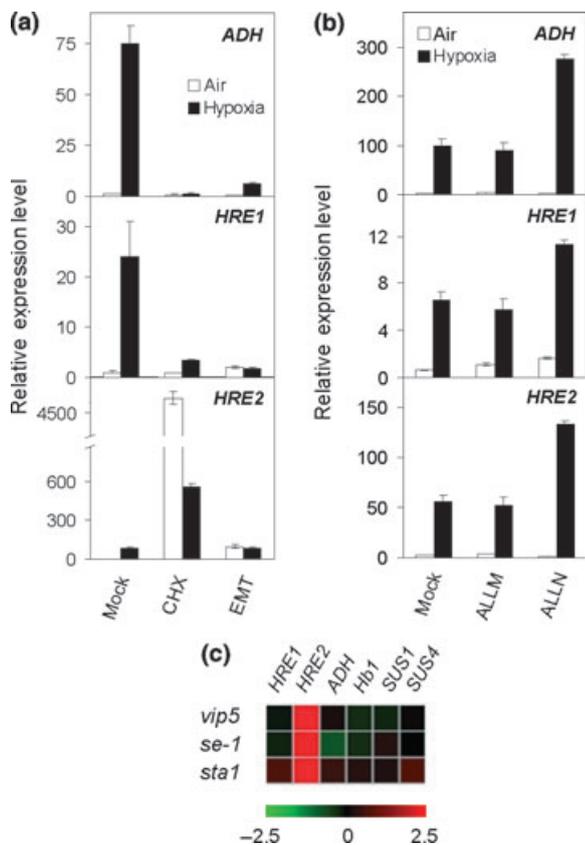


Figure 7. Regulation of *HRE1* and *HRE2* expression.

(a) Effects of protein synthesis inhibitors. Expression of *HRE1*, *HRE2* and *ADH* genes in the absence and presence of 10 μM emetine (EMT) or 50 μM cycloheximide (CHX), under aerobic and hypoxic conditions. The EMT and CHX were added 1 h before the beginning of the hypoxic treatment (1% O_2 , 4 h). The mRNA level in whole seedlings was measured by real-time quantitative PCR. Relative expression levels are shown as fold change values (1 = mock air). Data are mean \pm SD ($n = 3$).

(b) Effects of proteasome inhibitors. Expression of *HRE1*, *HRE2* and *ADH* genes in the absence (mock) and presence of 100 μM *N*-acetyl-Leu-Leu-methioninal (ALLM) or 100 μM *N*-acetyl-Leu-Leu-norleucinal (ALLN) under aerobic (empty bars) and hypoxic (1% O_2 , 2 h) conditions (filled bars). Ethanol 10 mM was used as a mock control. The mRNA level in whole seedlings was measured by real-time quantitative PCR. Relative expression levels are shown as fold change values (1 = mock, time 0). Data are mean \pm SD ($n = 3$).

(c) Effects of mRNA-processing and micro (mi)RNA–small interfering (si)RNA production mutants on the expression of *HRE2*. The Geneinvestigator heat map (Hruz *et al.*, 2008) shows the expression values of *HRE1*, *HRE2* and four anaerobic genes (*ADH*, *Hb1*, *SUS1* and *SUS4*) in Arabidopsis mutants involved in mRNA processing and miRNA–siRNA production under aerobic conditions: *vip5* (*vernalization independent 5*), *se-1* (*serrate-1*), *sta1-1* (*stabilized1-1*). Microarray data have been retrieved from the ArrayExpress (*vip5*, E-MEXP-838) and GEO (*se-1*, GDS1047, *sta1-1*, GDS2102) databases.

upon anaerobiosis. MYB2 binds the GC box present in the promoter region of *ADH* (Hoeren *et al.*, 1998). A reduction in *MYB2* expression in the *myb2* mutant, however, did not affect the expression of either *HRE1* or *HRE2* (Figure S2).

The observed induction of *HRE2* by CHX and EMT (Figure 7a) led us to speculate about the existence of a negative regulatory mechanism acting in the air which is suppressed by low oxygen. Treatment with 10 μM MG132,

an inhibitor of the 26S proteasome, was shown to increase the mRNA levels of *HRE2* (NASCArrays-190). Two different proteasome inhibitors [*N*-acetyl-Leu-Leu-norleucinal (ALLN) and *N*-acetyl-Leu-Leu-methioninal (ALLM)] did not affect or even increase *HRE2* expression under anaerobic conditions (Figure 7b). We therefore ruled out the involvement of the proteasome in the hypoxic regulation of *HRE2*. Three mutants for the genes involved in mRNA processing, *vip5*, *sta1-1* and *se1*, showed higher expression of *HRE2* in air (Figure 7c), suggesting that *HRE2* is regulated post-transcriptionally at the level of mRNA stability. According to this model, *HRE2* is constitutively transcribed at a high level but its mRNA is highly unstable under normoxia, while under anaerobic conditions it suddenly becomes stable.

DISCUSSION

The ERF family encodes for proteins that belong to the AP2 superfamily, characterized by a single ERF DNA-binding domain (Nakano *et al.*, 2006). Previous characterization of orthologs in several species, including hot pepper (*Capsicum annuum*), tobacco (*Nicotiana tabacum*), pepper (*Piper nigrum*) and wheat (*Triticum aestivum*), described these proteins as nuclear-localized which were able to positively regulate gene transcription because of a single or multiple activation domains (Lee *et al.*, 2004, 2005; Xu *et al.*, 2007). Both *HRE1* and *HRE2* are indeed localized in the nucleus (Figure 2d). There are five members of ERF group VII in the Arabidopsis genome: *At1g53910* (*RAP2.12*), *At1g72360* (*HRE1*), *At2g47520* (*HRE2*), *At3g14230* (*RAP2.2*) and *At3g16770* (*RAP2.3*, also known as *AtEREBP*). In dicots, such as poplar (Zhuang *et al.*, 2008) and grape (FL and PP, unpublished), only a few members of this ERF group were identified, while in grasses the number of ERF group VII members is at least twice (rice) that in dicots or even more (maize) (Figure 1b). Rice *Sub1A*, an ethylene and flooding inducible group VII ERF, strongly affects submergence tolerance in rice (Xu *et al.*, 2006). Among the effects of *Sub1A*, it is worth highlighting its role as an enhancer of fermentative metabolism, mostly through the induction of *ADH* (Fukao *et al.*, 2006). *Sub1A*, however, also acts as a repressor of the genes involved in carbohydrate mobilization through sucrose synthase (Fukao *et al.*, 2006) and in gibberellin-mediated adaptation, such as cell expansion (Fukao and Bailey-Serres, 2008).

We identified two hypoxia-inducible group VII ERFs in Arabidopsis (named *HRE1* and *HRE2*) whose regulation and effects during anaerobiosis partly resemble those of *Sub1A*. Unlike *Sub1A* and *Sub1C*, *HRE1* and *HRE2* are not induced by ethylene (NASCArrays-32) but respond rapidly to oxygen deprivation (van Dongen *et al.*, 2009). Induction of *HRE1* and *HRE2* genes under hypoxia is very likely to result in an increase of the respective protein levels, since their mRNAs have been shown to be associated with polyribosomes under conditions of oxygen deprivation

(Branco-Price *et al.*, 2005, 2008). Both genes appear to be required for a prolonged anaerobic response, thus maintaining the expression of some anaerobic genes under low oxygen. Indeed, the double mutant *hre1hre2* displayed a normal induction of anaerobic genes but failed to maintain the high expression 4 h after the onset of hypoxia (Figure 6). When over-expressed, only *HRE1* was able to increase the hypoxic induction of the anaerobic genes, whereas *35S::HRE2* does not show any difference compared with the wild type. Moreover, expression of the *ADH* gene is higher under aerobic conditions in *35S::HRE1* plants, compared with the wild type. *RAP2.12*, another member of ERF group VII, has been reported to induce *ADH* through the interaction with its promoter (Papdi *et al.*, 2008). However, it is unclear whether the induction triggered by *RAP2.12* over-expression is comparable with that caused by hypoxia. Remarkably, over-expression of the most similar paralogue of *RAP2.12*, *RAP2.2*, did not affect the expression of *ADH* or any other anaerobic gene (Welsch *et al.*, 2007) in air. *RAP2.2* and *RAP2.12* were not up-regulated in hypoxic *Arabidopsis* seedlings (Figure 2a). In addition to *ADH*, *SUS1* and *SUS4* were also up-regulated in *35S::HRE1* (Figure 6), suggesting that the analogies with *Sub1A*, a repressor of sucrose synthase (Fukao *et al.*, 2006), are limited.

It is thus unlikely that *HREs* play a role in carbohydrate conservation, suggesting different survival strategies in rice and *Arabidopsis*. Indeed, tolerance to anoxia is enhanced in *35S::HRE1* plants and reduced in *hre1hre2* plants. The lower expression of the genes involved in lipid modifications associated with oxidative stress tolerance in *35S::HRE1* plants (Table S2) suggests that in these plants the consequences of hypoxia are attenuated, thus preserving membrane integrity and resulting in a reduced need for ROS scavenging and protective enzymes. Lipid peroxidation and membrane damage are associated with flooding and anoxic stress, although it is not clear whether this is mainly a consequence or a cause of cell death under oxygen deprivation (Licausi and Perata, 2009).

HRE1 appears to act as an enhancer rather than a trigger of the anaerobic response(s), since the induction of *ADH* and other anaerobic genes is preserved in the *hre1* mutant. Although *HRE2* did not appear to trigger the same effects as *HRE1*, only the double mutant *hre1hre2* displayed a reduced (but not abolished) anaerobic response (Figure 6). This suggests that these two ERF proteins have a partially redundant molecular function, although *HRE2* alone was not able to up-regulate the anaerobic genes (Figure 6). The hypoxic induction patterns of *HRE1* and *HRE2* were different (Figure 2), suggesting that their hypoxic regulation relies on different mechanisms. *HRE1* was only transiently induced with a peak 2–4 h after the onset of hypoxia (Figure 2a), while *HRE2* was rapidly induced and its mRNA steady state was high throughout the (8 h) duration of the stress (Figure 2a). Furthermore, while *HRE2* did not require

de novo protein synthesis to be up-regulated, *HRE1* did. In fact, the translation inhibitors EMT and CHX prevented the expression of many anaerobic genes such as *ADH* and *HRE1* but not *HRE2*, which is instead induced by the addition of both inhibitors, even in aerobic conditions (Figure 7a).

It is tempting to speculate that *HRE2* is repressed under aerobic conditions by a labile negative regulator which, under hypoxic conditions, is not synthesized. This would fit nicely with the observation of a general repression of translation under low-oxygen conditions aimed at saving energy (Branco-Price *et al.*, 2008). However, proteasome inhibitors could not prevent induction of *HRE2* under low oxygen (Figure 7b). It therefore seems more likely that regulation of this gene occurs at the mRNA level. Indeed mutants impaired in mRNA maturation and micro (mi)RNA production, such as *vip5*, *se* and *sta1-1*, accumulated *HRE2* in air but not other typical hypoxic genes. This thus indicates that secondary effects on plant morphology caused by the mutation do not affect the availability of oxygen for the cell (Figure 7c). *VIP5* (*Vernalization Independent 5*) encodes for the orthologue of yeast RTF1, a protein that plays a role in post-transcriptional events acting on mRNA stability (Mueller *et al.*, 2004; Oh *et al.*, 2004). *STA1* (*Stabilized1*) is part of a nuclear surveillance system that selectively degrades specific, dangerous, mRNAs before they exit the nucleus (Lee *et al.*, 2006). *Serrate1* (*SE-1*) encodes for a zinc-finger protein involved in pre-mRNA splicing and pre-miRNA processing (Yang *et al.*, 2006). All this evidence suggests that *HRE2* is constitutively transcribed but its mRNA is degraded in air. No other mutant listed in Genevestigator (Hruz *et al.*, 2008) and involved in the biogenesis or action of miRNAs or *trans*-acting small interfering (si)RNAs exhibited a significant difference in *HRE2* mRNA (data not shown). The positive effect of CHX and EMT on *HRE2* could then be explained by the stabilizing effect of these substances on mRNA, since protein synthesis inhibitors interfere with specific mRNA decay mechanisms (Lopez *et al.*, 1998). Assuming that *HRE2* mRNA is unstable in air, this should reside in its non-translated regions. This is because *HRE2* is accumulated at high levels in transgenic plants that over-express its coding sequence devoid of untranslated regions. *HRE1* might be involved in the up-regulation of *HRE2*, since the hypoxic induction of *HRE2* is higher in *35S::HRE1* plants.

A possible explanation for the different effects of *HRE1* and *HRE2* lies in an analysis of their protein structure (Figure 1a). The other group VII ERFs *RAP2.2*, *RAP2.3* and *RAP2.12* act as positive regulators of gene transcription (Buettner and Singh, 1997; Welsch *et al.*, 2007; Papdi *et al.*, 2008) and *RAP2.2* possesses a DNA-binding domain followed by an activation domain (Welsch *et al.*, 2007). *HRE2* is shorter than the other members [171 amino acids (aa) compared with an average length of the group of 284 aa] since it lacks a large part of the region located downstream

of the DNA-binding domain (Figure 1a). It is tempting to speculate that HRE2 lost the activating domain and requires an interaction with a partner with a *trans*-activating activity. Putative interactors could be selected from the large number of proteins up-regulated by low oxygen and whose function has not yet been described or to which gene transcription regulation is putatively attributed. Work is in progress to identify the possible partners of HRE2.

Under aerobic conditions, the effect of *HRE1* on the expression of anaerobic genes is negligible, apart from a higher *ADH* mRNA level in the aerobic *35S::HRE1* plants. It is thus likely that HRE1 also requires interaction with one or more proteins, either to form a complex or to be activated. Since *35S::HRE1* displays an enhanced expression of several anaerobic genes under hypoxia (Figure 6), its putative partner could also be more expressed under oxygen deprivation, thus enhancing the response as a positive loop. It is unlikely that HRE1 and HRE2 might interact since they share a conserved DNA-binding domain and have been reported to bind to DNA as monomers. On the other hand, interactions with different classes of transcription factors are highly probable. In rice, OsEBP89 interacts with OsBP5, a MYC transcription factor, to regulate the *WXY* gene (*Wx*) (Zhu *et al.*, 2003) and RAP2.3 has been reported to interact with OBF4, a protein belonging to a specific class of basic-region leucine zipper (bZIP) transcription factors (Buettner and Singh, 1997). Interestingly OsEBP89 alone binds weakly to the *Wx* promoter and requires OsBP5 to strongly activate gene transcription (Zhu *et al.*, 2003).

In this study, we have shown that HRE1 and HRE2, two ERF transcription factors which are part of the conserved response to low oxygen in plants, are indeed involved in the regulation of the molecular response to oxygen starvation. Both HRE1 and HRE2 are required to ameliorate plant survival under anoxia. The increased tolerance in plants that over-express *HRE1* is possibly due to the enhanced expression of the genes involved in the fermentative pathway. Increased ethanol production correlates positively with low-oxygen tolerance in plants over-expressing *PDC* (Ismond *et al.*, 2003). *HRE1* over-expression not only positively affects both *PDC* and *ADH* but also up-regulates ethanolic fermentation. It is tempting to propose that the enhanced tolerance of anoxia in *35S::HRE1* plants arises from a more efficient fermentative metabolism. Furthermore, the observed tolerance in *35S::HRE1* plants might be also due to the enhanced expression of a large set of anaerobic genes, including those involved in the fermentative pathway (Figure 5). Additional experiments are now required to identify the direct targets of HRE1 and HRE2. Production of plant lines expressing epitope-tagged versions of HRE1 and HRE2 coupled to chromatin immunoprecipitation (ChIP) would provide a valuable tool for the identification of target promoters and DNA motifs required for the molecular action of the two HREs. Moreover,

immunopurification could also be used to identify post-translational modifications and partners of HRE1 and HRE2 proteins. Future analyses on the post-transcriptional regulation of their molecular function, as well as the identification of putative partners, will lead to the identification of other components of the anaerobic signalosome.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana, Col-0 ecotype, was used in all the experiments. Seeds were sterilized with 70% ethanol (1 min), 0.85% sodium hypochlorite (10 min) and rinsed with sterile water (six times). For experiments with 4-day-old seedlings, seeds were sown in liquid Murashige–Skooog half-strength medium supplemented with 1% sucrose. Seeds were stratified for 72 h in the dark at 4°C and then transferred at 23°C with a 12-h light (100 $\mu\text{E m}^{-2} \text{sec}^{-2}$ intensity) photoperiod with shaking. To obtain 7- and 15-day-old plants, seeds were germinated on agar (0.9%) medium supplemented with 1% sucrose using the same conditions reported above. The T-DNA insertion mutants *hre1* (Salk_023445) and *hre2* (Salk_052858) were obtained from the European Arabidopsis Stock Centre (NASC). Homozygous lines were identified via PCR screening of genomic DNA using gene-specific primers together with T-DNA-specific primers (Table S6). Double homozygous lines were obtained by crossing the two single mutants and then screening the F_2 generation as described above. Oxygen treatments were applied as described by van Dongen *et al.* (2009) and treating plants in the dark with normal air containing 21% oxygen (normoxia) or 1% oxygen (hypoxia) and 0.035% CO_2 for the time indicated in the figure legends. All treatments were started at the beginning of the light phase.

Constructs and transgenic plant preparation

Whole coding sequences of *At1g72360* (*HRE1*) and *At2g47520* (*HRE2*) were obtained by PCR from hypoxic cDNA using a Pfu polymerase kit (Fermentas, <http://www.fermentas.com/>) and cloned into pENTR-D-TOPO (Invitrogen, <http://www.invitrogen.com/>) to generate the entry vectors pENTR-*HRE1* and pENTR-*HRE2*. These two entry vectors were recombined into the destination vector pK7WG2 (Karimi *et al.*, 2002) using the LR reaction mix II (Invitrogen) to obtain the expression vectors *35S::HRE1* and *35S::HRE2*.

Transgenic plants were obtained using the floral dip method (Zhang *et al.*, 2006). T_0 seeds were screened for kanamycin resistance and single-insertion lines were identified using real-time qPCR on genomic DNA with primer pairs annealing to the CAMV 35S promoter, the *NPTII* gene and *At3g10040* and *At5g10040* as wild-type, single-copy genes. Homozygous T_3 or subsequent generations were used in the following experiments.

To obtain GFP tagged expression of two ERF proteins, pENTR-*HRE1* and pENTR-*HRE2*, were recombined into p2FGW7 (Karimi *et al.*, 2002). *Arabidopsis* mesophyll protoplasts were prepared and transformed with 10 μg plasmid DNA according to Yoo *et al.* (2007). Fluorescence was observed with a Nikon ViCo videoconfocal microscope (<http://www.nikon.com/>) using a GFP filter.

Real-time qRT-PCR analyses

RNA was extracted from the seedlings, which were grown as indicated in the figure legends. Total RNA, extracted using the RNeasy kit (Qiagen, <http://www.qiagen.com/>) according to the manufacturer's instructions, was subjected to DNase treatment using the TURBO DNA-free kit (Ambion, <http://www.ambion.com/>). Five micrograms of RNA was reverse transcribed into cDNA using the

Superscript III reverse transcriptase kit (Invitrogen). Real-time PCR amplification was carried out with the ABI Prism 7900 sequence detection system (Applied Biosystems, <http://www.appliedbiosystems.com/>), using a power sybr-green master mix (Applied Biosystems) and the primers described in Table S6. *Ubiquitin10* (*At4G05320*), *PPR* (*AT1G62930*), *SAND* family protein (*At2g28390*) and *PDF2* (*At1g12320*), selected from the list reported in Czechowski *et al.* (2005), were used as housekeeping genes. Relative quantification of the expression of each individual gene was performed using the comparative threshold cycle method, as described in the ABI PRISM 7900 sequence detection system user bulletin no. 2 (Applied Biosystems).

Anoxia tolerance assay

Anoxic treatments were carried out in the dark. An enclosed anaerobic workstation (Anaerobic System model 1025; Forma Scientific, <http://www.thermo.com>) was used to provide an oxygen-free environment for seedling incubation. This chamber uses palladium catalyst wafers and desiccant wafers to maintain strict anaerobiosis to less than $10 \mu\text{g ml}^{-1} \text{O}_2$ (according to the manufacturer's specifications). High-purity N_2 was used to initially purge the chamber, and the working anaerobic gas mixture was $\text{N}_2:\text{H}_2$ with a ratio of 90:10. Agar plates were used to evaluate anoxia tolerance. Treatments were performed by transferring the plates containing the 7-day-old *Arabidopsis* seedlings to anoxia. The plates were then transferred to the light for the post-anoxic recovery (12-h/12-h photoperiod at $100 \mu\text{E m}^{-2} \text{sec}^{-2}$). Surviving and dead plants were counted 10 days after the treatments.

Metabolite and biochemistry assays

Fourteen-day-old plants grown in vertical plates were treated in air or 1% O_2 (hypoxia) for 12 h. Proteins were extracted from frozen root material and PDC (EC 4.1.1.1) and ADH (EC 1.1.1.1) activities were assayed as described by Ismond *et al.* (2003). Ethanol production by *Arabidopsis* seedlings was analysed enzymatically as described by Banti *et al.* (2008) using 7-day-old plants transferred into liquid medium and then treated in air or hypoxia (1% O_2) with constant shaking.

Microarray analysis

Total RNA was extracted from whole 7-day-old plants using the RNeasy kit (Qiagen). The quality of the RNA was assessed by agarose gel electrophoresis and spectrophotometry. RNA was processed for use on Affymetrix *Arabidopsis* Genome GeneChip arrays, as previously described (Loreti *et al.*, 2005). Hybridization, washing, staining and scanning procedures were performed by Genopolis (University of Milano-Bicocca, <http://www.genopolis.it/>), as described in the Affymetrix technical manual. Microarray analysis and data quality control were performed using Robin (developed at MPI-MP Golm, <http://bioinformatics.mpimp-golm.mpg.de/projects/own/robin/>). Normalization of the raw data and an estimation of signal intensities were carried out using the Genechip Robust Multichip Average (GC-RMA) methodology (Wu *et al.*, 2004). Average expression values and their adjusted *P*-values were calculated using the Benjamini-Hochberg adjustment method (Reiner *et al.*, 2003). Microarray datasets were deposited in a public repository with open access (accession no. GSE17099; <http://www.ncbi.nlm.nih.gov/geo/>).

Chemical treatments

Four-day-old seedlings were treated with 50 μM CHX, 10 μM EMT, 100 μM ALLM and 100 μM ALLN for 1 h before applying the low-oxygen treatments. Distilled water was used as a mock control for

the CHX and EMT treatments while 10 mM ethanol was used as a mock control for the ALLM and ALLN treatments. Plant material was sampled at the time points indicated in the figure legends.

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SUPPORTING INFORMATION

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

Figure S1. Molecular and morphological phenotypes of *hre1*, *hre2*, *hre1hre2*, *35S::HRE1* and *35S::HRE2* genotypes.

Figure S2. Expression of *HRE1* and *HRE2* in the *myb2* mutant.

Table S1. Genes significantly (adjusted *P*-value ≤ 0.05) up- or down-regulated in *35S::HRE1* plants compared with wild-type (Col-0) plants under aerobic conditions.

Table S2. Genes significantly (adjusted *P*-value ≤ 0.05) up- or down-regulated in *35S::HRE1* plants compared with wild-type (Col-0) plants under hypoxic (1% O_2 , 4 h) conditions.

Table S3. Genes significantly (adjusted *P*-value ≤ 0.05) up- or down-regulated in *35S::HRE2* plants compared with wild-type (Col-0) plants under aerobic conditions.

Table S4. Genes significantly (adjusted *P*-value ≤ 0.05) up- or down-regulated in *35S::HRE2* plants compared with wild-type (Col-0) plants under hypoxic (1% O_2 , 4 h) conditions.

Table S5. Cluster of anaerobic genes identified in the study as up-regulated at least eightfold by hypoxia (1% O_2 , 4 h) in wild-type (Col-0) plants.

Table S6. List of primers used in the study.

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