

# The AP2/ERF Transcription Factor AtERF73/HRE1 Modulates Ethylene Responses during Hypoxia in Arabidopsis<sup>1</sup>[W][OA]

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A number of *APETALA2* (*AP2*)/*ETHYLENE RESPONSE FACTOR* (*ERF*) genes have been shown to function in abiotic and biotic stress responses, and these genes are often induced by multiple stresses. We report here the characterization of an *AP2/ERF* gene in *Arabidopsis* (*Arabidopsis thaliana*) that is specifically induced during hypoxia. We show that under normoxic conditions, the expression of *AtERF73/HRE1* can be induced by exogenous addition of 1-aminocyclopropane-1-carboxylic acid and that a combination of hypoxia and 1-aminocyclopropane-1-carboxylic acid results in hyperinduction of *AtERF73/HRE1* expression. In addition, hypoxic induction of *AtERF73/HRE1* is reduced but not completely abolished in ethylene-insensitive mutants and in the presence of inhibitors of ethylene biosynthesis and responses. These results suggest that, in addition to ethylene, an ethylene-independent signal is also required to mediate hypoxic induction of *AtERF73/HRE1*. To assess the role of *AtERF73/HRE1*, we generated three independent RNA interference (RNAi) knockdown lines of *AtERF73/HRE1*. Under normoxic conditions, the *AtERF73/HRE1-RNAi* seedlings displayed increased ethylene sensitivity and exaggerated triple responses, indicating that *AtERF73/HRE1* might play a negative regulatory role in modulating ethylene responses. Gas chromatography analyses showed that the production of ethylene was similar between wild-type and RNAi lines under hypoxia. Quantitative reverse transcription-polymerase chain reaction analyses showed that hypoxia-inducible genes could be affected by *AtERF73/HRE1-RNAi* lines in two different ways: hypoxic induction of glycolytic and fermentative genes was reduced, whereas induction of a number of peroxidase and cytochrome P450 genes was increased. Taken together, our results show that *AtERF73/HRE1* is involved in modulating ethylene responses under both normoxia and hypoxia.

Oxygen deficiency (hypoxia) is an abiotic stress encountered by plants during flooding in soil. The consequences of hypoxia, such as a decrease in cellular energy charge, drop in cytoplasmic pH, and accumulation of toxic end products from anaerobic respiration and of reactive oxygen species during recovery, are responsible for the slowed growth and reduced yield of many agriculturally important crops in the event of flooding (Subbaiah and Sachs, 2003). Plants have developed adaptive mechanisms to sense oxygen deficiency in their environments and make coordinated physiological and structural adjustments to enhance their hypoxic tolerance (Liu et al., 2005; Huang et al., 2008).

Several microarray studies showed that genes coding for enzymes of sugar metabolism, glycolysis, and fermentation are up-regulated in *Arabidopsis* (*Arabidopsis thaliana*) under low oxygen concentrations (Klok et al., 2002; Liu et al., 2005; Loreti et al., 2005; van Dongen et al., 2009). Hypoxia-induced genes can be controlled at transcriptional, posttranscriptional, and translational levels (Manjunath et al., 1998; Conley et al., 1999; Peng et al., 2005). In particular, the aerobic-anaerobic switch results in the induction of two genes: *ALCOHOL DEHYDROGENASE* (*ADH*) and *PYRUVATE DECARBOXYLASE* (*PDC*). Studies of *ADH* genes in *Arabidopsis* and maize (*Zea mays*) have identified a cis-acting element, designated anaerobic-responsive element, that is essential for hypoxia induction (Dolferus et al., 1994; Kyojuka et al., 1994; Hoeren et al., 1998). It was shown that ethylene is necessary but not sufficient for hypoxic induction of *ADH* in *Arabidopsis* (Peng et al., 2001, 2005). It was also reported that ethylene regulates aerenchyma formation in root tips of maize plants exposed to hypoxic conditions (He et al., 1996). These observations suggested that ethylene plays an essential role in hypoxia signaling pathways.

The *APETALA2* (*AP2*)/*ETHYLENE RESPONSE FACTOR* (*ERF*) gene family encodes plant-specific transcription factors, which are often involved in

<sup>1</sup> This work was supported by the National Science Council, Taiwan, by Academia Sinica, and by the National Natural Science Foundation of China (grant no. NSFC 90717105 to N.-N.W.).

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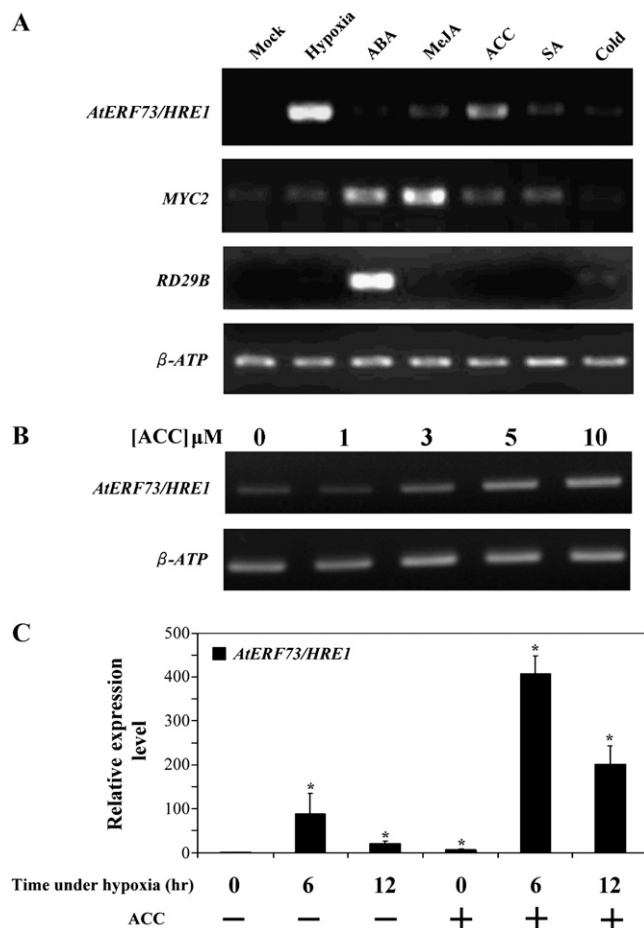
regulating plant responses to biotic and abiotic stresses (Dubouzet et al., 2003; Gutterson and Reuber, 2004; Nakano et al., 2006). Expression of many AP2/ERF genes has been shown to be regulated by a variety of external stimuli, such as wounding, jasmonic acid (JA), salicylic acid (SA), ethylene, and infection by pathogens (McGrath et al., 2005; Pré et al., 2008). ERF proteins that bind to the GCC box, an ethylene-responsive element, have been identified from several plant species (Gu et al., 2000; Ohta et al., 2000; Zhang et al., 2004). Constitutive overexpression of Arabidopsis ERF1 (At3g23240) activates the expression of *PLANT DEFENSIN1.2* (*PDF1.2*) and *BASIC CHITINASE* (*ChiB*) genes (Lorenzo et al., 2003). *ORA59* (At1g06160), another member of the Arabidopsis AP2/ERF family, could also activate *PDF1.2* gene expression and was shown to be involved in the cross talk between the JA and ethylene signal transduction pathways (Pré et al., 2008). In addition to positive regulatory roles, some AP2/ERF factors have negative regulatory functions. For example, ERF4 (At3g15210) down-regulates the expression of *PDF1.2* (McGrath et al., 2005).

AP2/ERF genes have been reported to be involved in signaling pathways associated with abiotic stresses such as cold and drought; however, studies relating to their roles in hypoxia are very limited. In rice (*Oryza sativa*), the *Sub1* locus contains two or three ERF-like genes whose transcripts are regulated by submergence and ethylene (Xu et al., 2006; Perata and Voesenek, 2007). The cultivars with *Sub1A-1* are tolerant of submergence. In deepwater rice, a pair of ERF factors, *SNORKEL1* (*SK1*) and *SK2*, also play a key role in allowing rice to adapt to flooding terrain (Hattori et al., 2009). In this study, we have investigated the functional roles of ethylene in hypoxia response in Arabidopsis. To mimic natural flooding conditions, we have adopted an "open system" treatment (Drew, 1997), in which only roots are subjected to hypoxia treatment. Using microarray analysis, we identified a number of AP2/ERF genes in Arabidopsis that are induced at different stages of hypoxia treatment. One of these genes, *AtERF73/HRE1* (At1g72360), which is induced strongly by hypoxia but not by cold or dehydration, was chosen for further studies. Very recently, a study of the same gene was reported (Licausi et al., 2010). The authors showed that overexpression of this gene resulted in increases of *PDC* and *ADH* expression during hypoxia but not under normoxia, suggesting a positive regulatory role of *AtERF73/HRE1* during hypoxia. In addition, it was shown that another member in the same subfamily, *RAP2.2*, might also be important for survival of Arabidopsis under hypoxia and that its expression is mainly affected by darkness and ethylene (Hinze et al., 2010). However, we show here that *AtERF73/HRE1* was involved in modulating ethylene responses under both normoxia and hypoxia. In addition, our results also indicate that two pathways, one ethylene dependent and the other ethylene independent, are involved in hypoxia induction of *AtERF73/HRE1*.

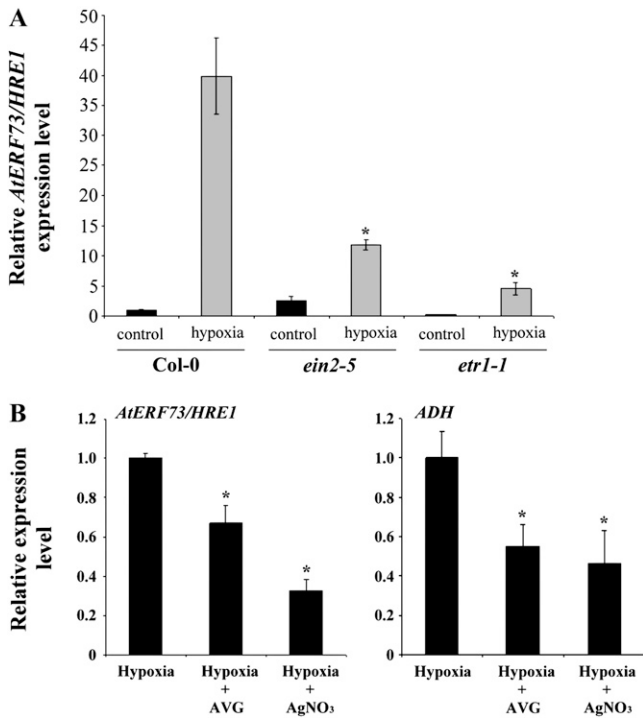
## RESULTS

### *AtERF73/HRE1* mRNA Accumulation Is Controlled by Hypoxia and Ethylene Signal Transduction Pathways

By comparing our microarray data with published microarray data, we found that *AtERF73/HRE1*, corresponding to At1g72360, could only be induced during hypoxia but not by other abiotic stresses, including cold, drought, and dehydration. Recently, it was shown that among group VII ERF genes, *AtERF73/HRE1* and



**Figure 1.** Effects of abiotic stresses and hormones on *AtERF73/HRE1* expression. A, RT-PCR analysis of *AtERF73/HRE1* expression. The mRNA levels of *AtERF73/HRE1*, *MYC2*, and *RD29B* in roots of 14-d-old seedlings were determined after mock treatment (lane 1) and after treatment with hypoxia (lane 2), 5  $\mu$ M ABA (lane 3), 50  $\mu$ M MeJA (lane 4), 5  $\mu$ M ACC (lane 5), 50  $\mu$ M SA (lane 6), or cold (4°C; lane 7) for 6 h.  $\beta$ -ATP was used as a control. Experiments were carried out in triplicate with similar results. B, RT-PCR analysis of the effects of ACC. mRNA levels of *AtERF73/HRE1* in 14-d-old seedlings were determined after treatment with 0, 1, 3, 5, or 10  $\mu$ M ACC for 6 h. C, Quantitative RT-PCR analysis of the effects of combined ACC and hypoxic treatment. Levels of *AtERF73/HRE1* mRNA in 14-d-old seedlings were determined after treatment with hypoxia for 6 or 12 h in the presence (+) or absence (-) of 5  $\mu$ M ACC, respectively. The relative amounts of transcripts were calculated and normalized with  $\beta$ -ATP mRNA. Values represent means  $\pm$  SD from three biologically independent experiments. \*  $P < 0.05$  versus the wild type (one-way ANOVA with Dunnett's  $t$  test).



**Figure 2.** Effects of ethylene-responsive mutations and inhibitors of ethylene biosynthesis or response on the hypoxic induction of *AtERF73/HRE1*. A, Quantitative RT-PCR analysis of *AtERF73/HRE1* expression in *ein2-5* and *etr1-1* mutants under hypoxic conditions. Total RNAs were isolated from roots of Col-0, *ein2-5*, and *etr1-1* after hypoxia treatment for 6 h. Levels of *AtERF73/HRE1* gene expression were determined. B, Expression levels of *AtERF73/HRE1* and *ADH* in Arabidopsis seedlings after 6 h of hypoxia in the presence of 10  $\mu$ M AVG and 10  $\mu$ M AgNO<sub>3</sub> were determined by quantitative RT-PCR, and the relative amounts of transcripts were calculated and normalized with  $\beta$ -ATP mRNA. Values represent means  $\pm$  SD from three biologically independent experiments. \*  $P < 0.05$  versus the wild type (one-way ANOVA with Dunnett's  $t$  test).

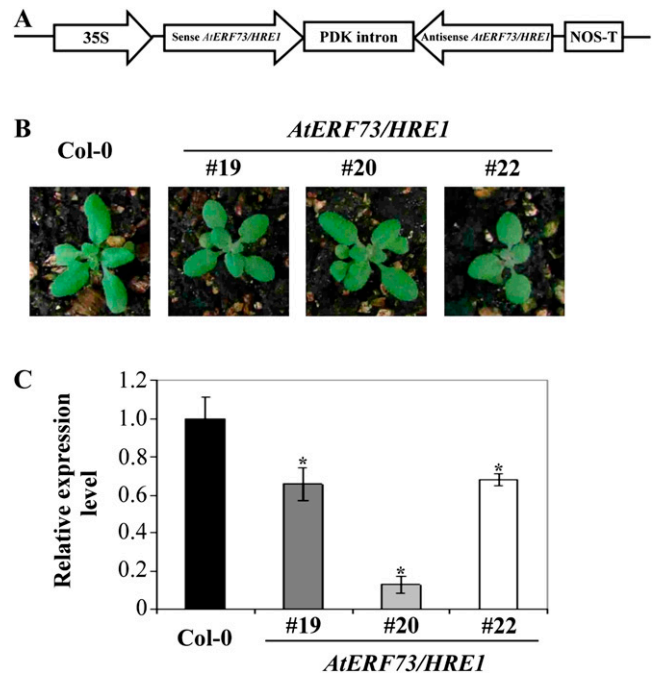
*AtERF71/HRE2* could be induced by hypoxia treatment, in which the entire seedlings were subjected to low-oxygen conditions (Licausi et al., 2010). Similarly, under our hypoxia treatment conditions, *AtERF73/HRE1* and *AtERF71*, but not *RAP2.2*, *RAP2.12*, and *RAP2.3*, could be induced in roots (Supplemental Fig. S1). However, no significant change of *AtERF73/HRE1* transcripts was observed in the shoots (Supplemental Fig. S2).

To investigate the effects of various signaling molecules, we used reverse transcription (RT)-PCR to compare the transcript levels of *AtERF73/HRE1* from roots of Arabidopsis plants under hypoxia, abscisic acid (ABA), methyl jasmonate (MeJA), 1-aminocyclopropane-1-carboxylic acid (ACC; a precursor of ethylene), SA, or cold treatment. The data showed that *AtERF73/HRE1* was highly induced during hypoxia, moderately induced by ACC treatment, and weakly induced upon MeJA or SA treatment (Fig. 1A, top panel). By contrast, *RD29B* was highly induced by ABA, while *MYC2* could be induced by ABA and MeJA (Fig. 1A, middle panels). The expression patterns for *RD29B* and *MYC2*

are consistent with published results (Nakashima et al., 2006; Dombrecht et al., 2007). Next, we examined the dosage effect of ACC on *AtERF73/HRE1* expression. The results showed that the mRNA accumulation of *AtERF73/HRE1* reached a maximal level at ACC concentrations between 5 and 10  $\mu$ M (Fig. 1B). To investigate whether there is a synergistic effect of hypoxia and ethylene, we determined by quantitative RT-PCR the level of *AtERF73/HRE1* transcript after different combinations of hypoxia and ACC treatment. The *AtERF73/HRE1* mRNA level was induced after 6 h of hypoxia treatment and decreased after treatment for 12 h. Plants subjected to both hypoxia and ACC treatments displayed a significant increase in the level of *AtERF73/HRE1* transcripts as compared with those treated with hypoxia alone (Fig. 1C). These results demonstrate that hypoxia and ethylene have additive effects on *AtERF73/HRE1* mRNA accumulation.

### Hypoxic Induction of *AtERF73/HRE1* mRNA Accumulation Is Affected in *ein2-5* and *etr1-1*

We have previously shown that ethylene is essential for hypoxic induction of *ADH* in Arabidopsis (Peng



**Figure 3.** *AtERF73/HRE1* gene expression levels in transgenic *AtERF73/HRE1*-RNAi lines. A, Structure of the *AtERF73/HRE1*-RNAi construct. B, Phenotypes of 2-week-old seedlings of wild-type Col-0 and transgenic *AtERF73/HRE1*-RNAi lines. C, Quantitative RT-PCR analysis. The mRNA levels of *AtERF73/HRE1* in roots of 2-week-old seedlings of Col-0 and *AtERF73/HRE1* lines RNAi-19, RNAi-20, and RNAi-22 were determined after hypoxia treatment for 6 h by quantitative RT-PCR, and the relative amounts of transcripts were calculated and normalized with  $\beta$ -ATP mRNA. Values represent means  $\pm$  SD from three biologically independent experiments. \*  $P < 0.05$  versus the wild type (one-way ANOVA with Dunnett's  $t$  test).

et al., 2001). To further investigate the role of ethylene in hypoxic induction of *AtERF73/HRE1*, we examined the expression pattern of *AtERF73/HRE1* during hypoxia in two ethylene signaling mutants, *ethylene insensitive2-5* (*ein2-5*) and *ethylene resistant1-1* (*etr1-1*). EIN2 was shown to be a bifunctional transducer and may mediate the cross talk between ethylene and stress responses (Alonso et al., 1999). The *ETR1* gene encodes an ethylene receptor protein with homology to two-component regulators (Chang et al., 1993). As shown in Figure 2A, the level of *AtERF73/HRE1* transcript in the wild type increased 40-fold after 6 h of hypoxia treatment. By contrast, the levels of *AtERF73/HRE1* in *ein2-5* and *etr1-1* increased only 3-fold and 8-fold, respectively, as compared with the levels under control conditions. We also examined the effects of aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, and silver nitrate ( $\text{AgNO}_3$ ), an inhibitor of ethylene responses, on the expression of *AtERF73/HRE1*. The levels of *AtERF73/HRE1* and *ADH* mRNA accumulation were reduced under hypoxia in the presence of either AVG or  $\text{AgNO}_3$  (Fig. 2B). These results indicate that ethylene has a positive role in regulating hypoxic induction of *AtERF73/HRE1* mRNA accumulation. However, the fact that hypoxic induction of *AtERF73/HRE1* in both ethylene-insensitive mutants and in the presence ethylene inhibitors is only reduced, but not completely abolished, suggests that an additional ethylene-independent signaling pathway is also involved in the induction of *AtERF73/HRE1* during hypoxia.

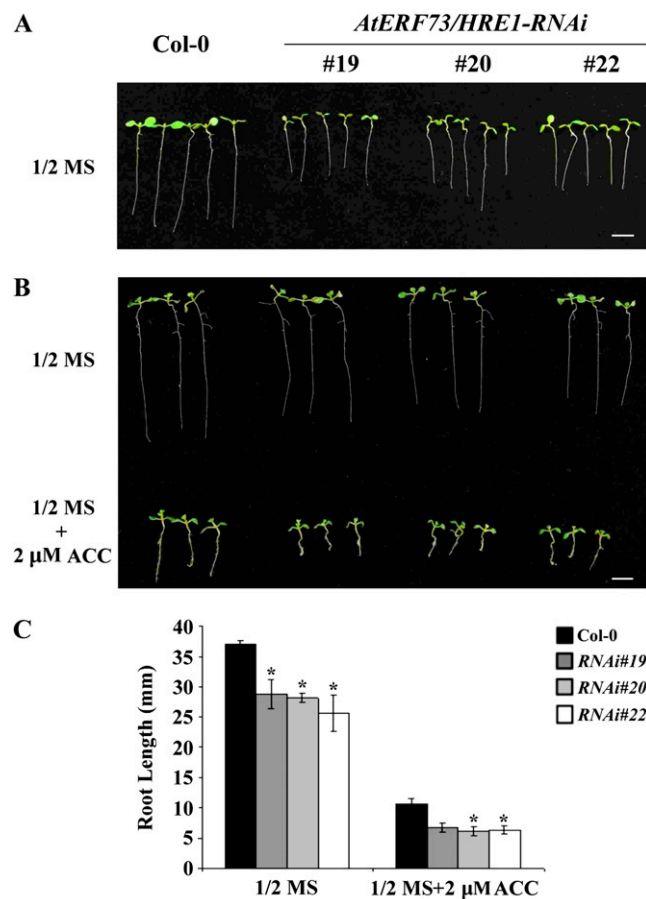
#### Generation of *AtERF73/HRE1* RNA Interference Transgenic Plants

To assess the role of *AtERF73/HRE1*, we decided to use RNA interference (RNAi) to generate transgenic plants with reduced *AtERF73/HRE1* mRNA accumulation. We generated an RNAi construct that contains the cauliflower mosaic virus 35S promoter fused to an inverted repeat of the *AtERF73/HRE1* open reading frame (Fig. 3A). T1 and T2 kanamycin-resistant lines harboring this construct were recovered. We selected three T3 homozygous *AtERF73/HRE1*-RNAi lines, designated *RNAi-19*, *RNAi-20*, and *RNAi-22*, for further analysis. Under normal growth conditions, these *AtERF73/HRE1*-RNAi lines show slightly smaller rosette leaves compared with 14-d-old *Arabidopsis* wild-type (ecotype Columbia [Col-0]) plants (Fig. 3B). Quantitative RT-PCR showed that the *AtERF73/HRE1* mRNA levels in roots of hypoxia-treated plants were reduced by approximately 2-fold in *RNAi-19* and *RNAi-22* lines and by approximately 10-fold in the *RNAi-20* line in comparison with the wild type (Fig. 3C). *AtERF73/HRE1* belongs to the group VII ERF subfamily, which has five members in the *Arabidopsis* genome (Licausi et al., 2010). We used quantitative RT-PCR to determine whether our RNAi construct also resulted in the reduction in expression of other members in the VII ERF subfamily in transgenic lines. The

results show that the expression of four other genes in the VII subfamily is not affected in the three RNAi lines (Supplemental Fig. S3).

#### *AtERF73/HRE1*-RNAi Transgenic Lines Display Increased Ethylene Sensitivity

To determine whether *AtERF73/HRE1* functions as a regulator of ethylene signaling pathways, seeds from the wild type and the three *AtERF73/HRE1*-RNAi lines were germinated in the presence or absence of ACC. We found that the root lengths in 7-d-old seedlings, grown on one-half-strength Murashige and Skoog (MS) medium in the absence of ACC, were significantly shorter in the *AtERF73/HRE1*-RNAi lines in comparison with the wild type (Fig. 4A). When seedlings were grown for 10 d, transgenic plant lines de-



**Figure 4.** Comparison of root growth in Col-0 and *AtERF73/HRE1*-RNAi lines with or without ACC treatment. A, The phenotypes of seedlings grown on one-half-strength MS medium for 7 d. Bar = 5 mm. B, The phenotypes of seedlings grown for 10 d on one-half-strength MS medium in the absence or presence of ACC (2  $\mu\text{M}$ ). Bar = 5 mm. C, Average root length for seedlings grown on one-half-strength MS or medium containing ACC (2  $\mu\text{M}$ ). The data represent average values  $\pm$  SD from 50 seedlings of each genotype obtained from three biologically independent experiments. \*  $P < 0.05$  (Student's *t* test).

veloped roots with average lengths of 28.7, 28.2, and 26.0 mm, respectively, while the average root length of wild-type plants was 37.0 mm (Fig. 4C). Addition of ACC resulted in inhibition of root elongation in both wild-type and transgenic lines; however, the root lengths in *AtERF73/HRE1-RNAi* lines were shorter than those in the wild type (Fig. 4, B and C).

To further assess the ethylene sensitivity of *AtERF73/HRE1-RNAi* lines, we measured the degrees of triple response to ethylene in etiolated seedlings. No difference in the phenotype between 4-d-old etiolated seedlings of the wild type and three *AtERF73/HRE1-RNAi* lines was observed in the absence of ACC (Fig. 5A, top panel). However, in the presence of 2  $\mu\text{M}$  ACC, etiolated seedlings of *AtERF73/HRE1-RNAi* lines showed exaggerated curvature of the apical hook compared with wild-type seedlings (Fig. 5A, middle and bottom panels). The level of apical curvature was analyzed for more than 100 seedlings each and classified according to an index scale ranging from 1 to 4, where index 1 and 2 represent curvatures of 90° and 180°, respectively, and index 3 and 4 represent the beginning of hook formation and the presence of a full hook, respectively (Leclercq et al., 2002). As shown in Figure 5B, about 95% of the seedlings of *AtERF73/HRE1-RNAi* lines displayed a phenotype with hook formation and full hook (index 3 and 4), whereas a significant fraction of wild-type seedlings (27%) lacked hook formation but displayed 180° apical curvature (index 2). The root and hypocotyl elongation in the wild type and *AtERF73/HRE1-RNAi* lines did not show any apparent difference (Supplemental Fig. S4). Taken together, these results suggest that *AtERF73/HRE1* negatively regulates a

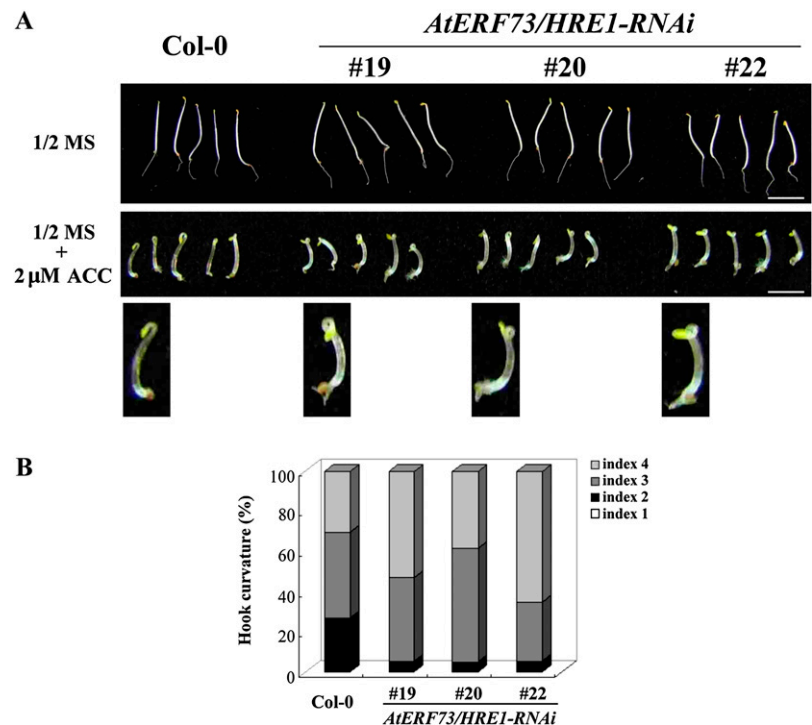
subset of ethylene responses in Arabidopsis seedlings under normoxic conditions.

### Effects of *AtERF73/HRE1* Knockdown Lines on Hypoxia-Inducible Genes

To determine how a decrease in *AtERF73/HRE1* expression affects hypoxia responses, we examined the expression of glycolytic and fermentative genes, including *ADH*, *PDC1*, *PDC2*, *SUS1*, *SUS4*, and *LDH*, in *AtERF73/HRE1-RNAi* lines. Quantitative RT-PCR analyses showed that, under hypoxic conditions, the mRNA levels for all of these genes, with the exception of *LDH*, were reduced significantly in *AtERF73/HRE1-RNAi* lines (Fig. 6). The levels of reduction in three *AtERF73/HRE1-RNAi* lines roughly correlated with the degrees of *AtERF73/HRE1* knockdown, with *RNAi-20* having the most pronounced effect on hypoxic induction of these genes. The data suggest that *AtERF73/HRE1* positively regulates a subset of glycolytic and fermentative genes during hypoxia.

In order to identify additional genes whose expression is regulated by *AtERF73/HRE1*, we performed microarray analysis to compare gene expression profiles of the wild type and *AtERF73/HRE1-RNAi20*, in which *AtERF73/HRE1* expression was severely knocked down. Arabidopsis Affymetrix GeneChip arrays were probed with RNAs isolated from roots of untreated plants (controls) and plants treated with hypoxia for 6 h. Up-regulated genes in wild-type plants were identified from four independent experiments at a 5-fold change cutoff and a *P* value cutoff of  $P \leq 0.05$ . The results showed that there were 285

**Figure 5.** Effects of ethylene on the apical hook curvature in *AtERF73/HRE1-RNAi* lines. A, The phenotypes of seedlings grown on one-half-strength MS medium with or without ACC (2  $\mu\text{M}$ ) for 4 d in the dark. Bars = 5 mm. B, The level of apical curvature was estimated visually for at least 100 seedlings using a scale ranging from 1 to 4 (1, 90° curvature; 2, 180° curvature; 3, beginning of hook formation; 4, full hook).



genes up-regulated in the wild type upon hypoxic treatment (Supplemental Table S1). Among these hypoxia-inducible genes, 13.3% had higher induction levels in the wild type, 7.4% had higher induction levels in the *AtERF73/HRE1-RNAi20* line, and 79.3% had about the same induction level (Supplemental Table S1). A list of 24 genes, which exhibited a change in raw intensity above 1.5-fold and displayed different levels of hypoxic induction between the wild type and the *AtERF73/HRE1-RNAi20* line, was generated (Table I). Among these 24 genes, most genes had higher levels of hypoxic induction in the *AtERF73/HRE1-RNAi20* line than in the wild type. We noticed that, among the genes that had higher induction levels in the *AtERF73/HRE1-RNAi20* line, peroxidase and cytochrome P450 genes were overrepresented. These genes, including *At3g03670* (putative peroxidase), *At1g14540* (putative anionic peroxidase), *At1g14550* (putative anionic peroxidase), *At5g39580* (putative peroxidase), *At4g31970* (CYP82C2), *At5g57220* (CYP81F2), and *At2g30750* (CYP71A12), were selected to confirm their expression patterns by quantitative RT-PCR analysis (Fig. 7). Consistent with the microarray data, five of the genes were induced at significantly higher levels in the *AtERF73/HRE1-RNAi20* line, whereas the induction level of *At1g14550* did not differ significantly between the wild type and *AtERF73/HRE1-RNAi20*. These results show that AtERF73/HRE1 could nega-

tively modulate hypoxic induction of these peroxidase and cytochrome P450 genes in Arabidopsis.

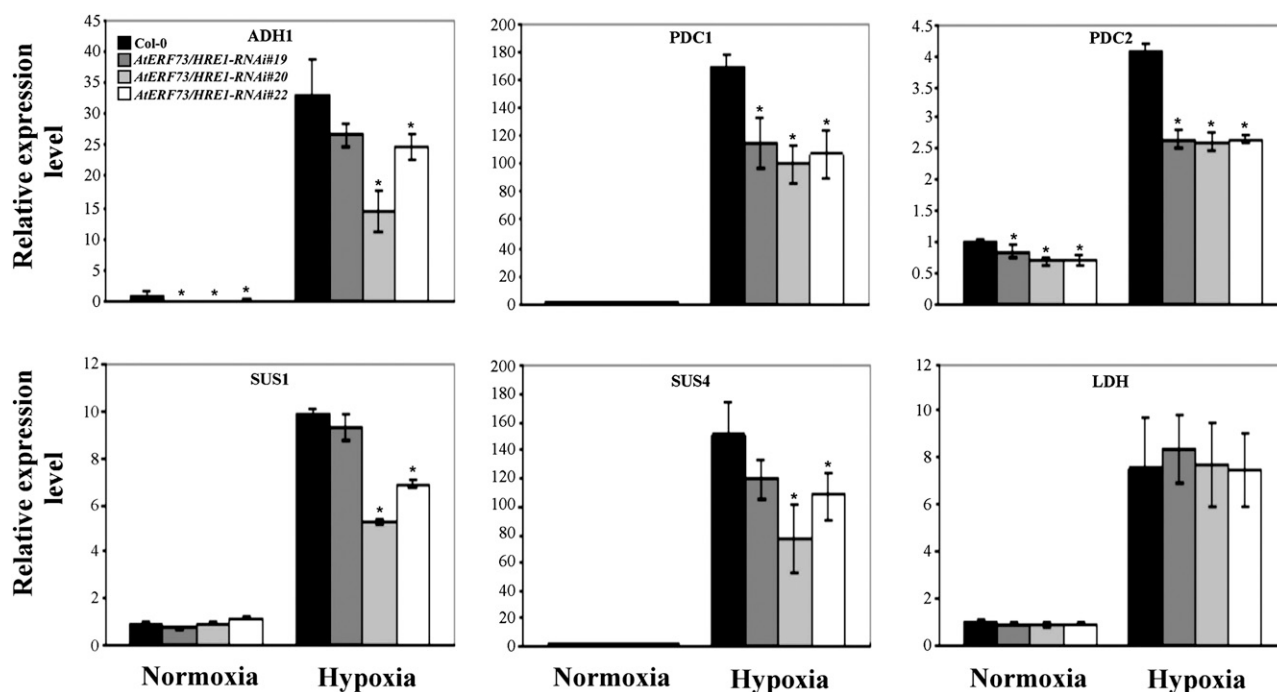
#### Effects of *AtERF73/HRE1* Knockdown Lines on Ethylene Production

Since we have shown previously that ethylene production is increased during hypoxia in Arabidopsis (Peng et al., 2001), the negative effect of AtERF73/HRE1 on ethylene responses could be due to its inhibition of ethylene synthesis or its effects on ethylene signaling pathways. To distinguish between these two possibilities, we measured the level of ethylene production during hypoxia by gas chromatography. The results show that the wild type and *AtERF73/HRE1-RNAi* lines exhibited similar levels of ethylene production under normoxic and hypoxic conditions (Fig. 8).

## DISCUSSION

#### Effects of Hypoxia and Ethylene on *AtERF73/HRE1* mRNA Accumulation

Microarray studies have shown that a number of Arabidopsis *AP2/ERF* genes are induced during hypoxia (Branco-Price et al., 2005; Gonzali et al., 2005; Liu et al., 2005). However, the regulatory functions of



**Figure 6.** Effects of *AtERF73/HRE1* lines on fermentative and related carbohydrate metabolic genes under normoxic or hypoxic conditions. Total RNAs were extracted from roots of 14-d-old seedlings exposed to normoxic or hypoxic conditions for 6 h. The mRNA levels of *ADH*, *PDC1*, *PDC2*, *SUS1*, *SUS2*, and *LDH* genes were determined by quantitative RT-PCR, and the relative amounts of transcripts were calculated and normalized with  $\beta$ -*ATP* mRNA. Data represent means  $\pm$  SD from three biologically independent experiments. \*  $P < 0.05$  versus the wild type (one-way ANOVA with Dunnett's *t* test).

these transcription factors remained largely unknown. *AtERF73/HRE1* transcription factor belongs to the AP2/ERF subfamily group VII of plant transcription factors. Members within this subfamily displayed differential expression patterns, with *AtERF73/HRE1* and *HRE2 (AtERF71)* being induced at different stages of hypoxia (Licausi et al., 2010; Supplemental Fig. S1). It was reported that overexpression of *HRE1* improved the tolerance of Arabidopsis plants to anoxia (Licausi et al., 2010). However, whether ethylene is involved in regulating *AtERF73/HRE1* during hypoxia was not analyzed.

This report focused on the interplay between *AtERF73/HRE1* and ethylene during hypoxia. When we tested the effects of other abiotic stresses and hormones, such as ABA, MeJA, ACC, SA, or cold treatment, our data showed that *AtERF73/HRE1* expression could be induced not only by hypoxia but also by ACC treatment (Fig. 1, A and B). We further demonstrated that hypoxia and ACC treatments had an additive effect on the mRNA accumulation of *AtERF73/HRE1* (Fig. 1C). However, we have shown previously that production of ethylene is increased in hypoxia-treated Arabidopsis seedlings (Peng et al., 2001). This raises the question of why exogenous addition of ethylene could further enhance the induction of *AtERF73/HRE1* during hypoxia. Two possible

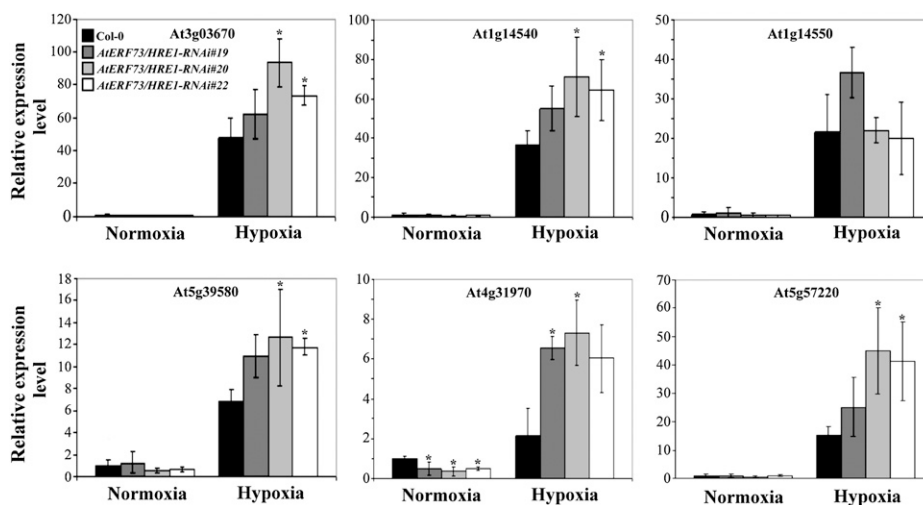
explanations can be offered: the level of ethylene production has not reached maximum under our hypoxia treatments, or there is a feedback control to prevent the effects of ethylene reaching a maximal level. Results from our studies are consistent with the latter explanation, since *AtERF73/HRE1-RNAi* lines resulted in exaggerated ethylene responses under both normoxic and hypoxic conditions. Additionally, the wild type and *AtERF73/HRE1-RNAi* lines had the same ethylene production under normoxic and hypoxic conditions. This result established the *AtERF73/HRE1* gene was involved in ethylene signaling but not in the biosynthesis pathway.

Interestingly, another member of the ERF subfamily VII, *RAP2.2*, is constitutively expressed at a high level in roots and at a lower level in shoots, but it is induced by dark and ethylene treatments (Hinz et al., 2010). Although *RAP2.2* was not induced during hypoxia, its overexpression resulted in the improvement of plant survival under hypoxic stress (Hinz et al., 2010). In addition, transgenic lines containing T-DNA knockout mutations of *RAP2.2* also have lower survival rates than the wild type. These results suggest that although *RAP2.2* might not play a direct role in regulating hypoxic signaling pathways, it controls the expression of a set of genes whose products are involved in the survival of Arabidopsis under hypoxic stress.

**Table 1.** Microarray-derived expression of selected genes with differential expression between the wild type and *AtERF73/HRE1-RNAi* upon hypoxia

Accession No.	Affymetrix No.	Gene Description	Wild-Type Fold Change <sup>a</sup>	<i>p</i> <sup>b</sup>	<i>AtERF73/HRE1-RNAi</i> Fold Change <sup>c</sup>	<i>p</i> <sup>b</sup>
AT4G30380	253616_at	Encodes a plant natriuretic peptide (PNP)	180.8	2.48E	28.4	1.43E
AT4G37710	253060_at	VQ motif-containing protein	101.8	2.59E	68.3	1.65E
AT3G03670	259197_at	Peroxidase	51.7	1.19E	334.6	4.49E
AT1G14540	261474_at	Peroxidase	48.5	1.03E	239.6	2.82E
AT4G31970	253505_at	Cytochrome P450 (CYP82C)	36.5	2.03E	414.4	3.24E
AT5G57220	247949_at	Cytochrome P450 (CYP81F2)	30.5	2.71E	201.0	3.50E
AT1G26410	261006_at	FAD-binding domain-containing protein	26.3	1.21E	66.5	1.93E
AT5G13320	250286_at	Encodes PBS3 (avrPphB susceptible)	21.1	3.81E	65.2	8.77E
AT2G23270	245082_at	Unknown protein	19.6	2.23E	74.5	2.16E
AT1G57630	246405_at	Disease resistance protein (TIR class), putative	19.2	2.51E	23.6	1.16E
AT4G19520	254587_at	Disease resistance protein (TIR-NBS-LRR class)	16.4	5.86E	18.1	2.36E
AT5G26920	246821_at	Calmodulin-binding protein CBP60g	15.9	2.16E	28.1	1.31E
AT2G26560	245038_at	Lipid acyl hydrolase	15.8	2.78E	42.1	4.11E
AT5G25250	246927_s_at	Membrane-associated protein	15.4	4.36E	66.4	2.18E
AT5G57510	247913_at	Unknown protein	15.2	1.25E	9.4	4.81E
AT2G26150	266841_at	Heat shock transcription factor A2 (ATHSFA2)	14.3	2.13E	3.14	5.74E
AT1G14550	261475_at	Peroxidase	14.1	4.38E	38.3	9.65E
AT1G19250	256012_at	Flavin-dependent monooxygenase 1 (FMO1)	13.8	9.24E	7.9	1.23E
AT2G44800	266875_at	Oxidoreductase, 2OG-Fe(II) oxygenase family	13.2	2.24E	1.62	8.11E
AT1G26380	261021_at	FAD-binding domain-containing protein	12.8	8.30E	130.0	7.63E
AT3G55790	251755_at	Unknown protein	12.5	5.31E	64.4	2.34E
AT1G05880	261249_at	ARIADNE12 (ARI12)	12.1	5.82E	34.8	1.48E
AT5G64905	247215_at	Elicitor peptide 3 precursor (PROPEP3)	11.4	1.39E	28.2	3.85E
AT1G53620	260971_at	Unknown protein	11.1	2.15E	21.7	6.64E

<sup>a</sup>Relative expression of wild-type fold change upon normoxia conditions compared with hypoxia conditions. <sup>b</sup>The *P* value was determined by *t* test with fold change of the wild type and *AtERF73/HRE1-RNAi* upon hypoxia conditions. <sup>c</sup>Relative expression of *AtERF73/HRE1-RNAi* line fold change upon normoxia conditions compared with hypoxia conditions.



**Figure 7.** Effects of *AtERF73/HRE1* lines on hypoxia-inducible peroxidase and cytochrome P450 genes under normoxic or hypoxic conditions. Total RNAs were extracted from roots of 14-d-old seedlings exposed to normoxic or hypoxic conditions for 6 h. The mRNA levels of peroxidase (*At3g03670*, *At1g14540*, *At1g14550*, and *At5g39580*) and cytochrome P450 (*At4g31970* and *At5g57220*) genes were determined by quantitative RT-PCR, and the relative amounts of transcripts were calculated and normalized with  $\beta$ -*ATP* mRNA. Data are means  $\pm$  SD from three biologically independent experiments. \*  $P < 0.05$  versus the wild type (one-way ANOVA with Dunnett's *t* test).

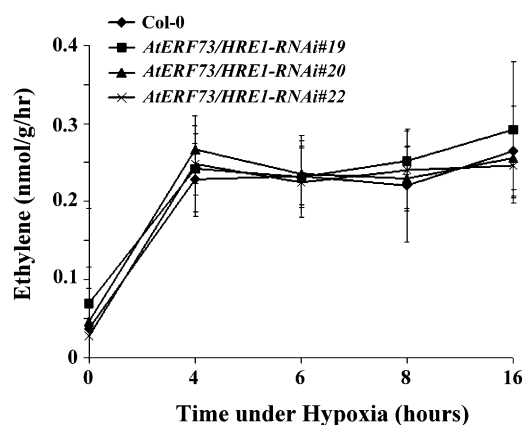
#### Hypoxia-Responsive *AtERF73/HRE1* Expression Is Mediated by Ethylene-Dependent and Ethylene-Independent Pathways

We have previously reported that the production of ethylene is increased during hypoxia and that ethylene is necessary but not sufficient in the hypoxic induction of *ADH* in *Arabidopsis* (Peng et al., 2001, 2005). We showed here that hypoxic treatment for 6 h could induce 12- and 5-fold increases in the expression of *AtERF73/HRE1* in *ein2-5* and *etr1-1* mutants. However, the levels of induction in these mutants were much lower than that determined in the wild type (Fig. 4). These results indicate that ethylene is required for the mRNA accumulation of *AtERF73/HRE1* during hypoxia. On the other hand, our results also show that hypoxic induction of *AtERF73/HRE1* is not completely abolished in both *ein2-5* and *etr1-1*. Indeed, the obtained patterns are similar to those observed for the hypoxic induction of *ADH* in *ein* mutants (Peng et al., 2005). Similarly, addition of inhibitors of ethylene (AVG and AgNO<sub>3</sub>) resulted in only partial reduction in hypoxic induction of *AtERF73/HRE1*, suggesting that ethylene is necessary but not sufficient for the hypoxic induction of *AtERF73/HRE1*.

#### *AtERF73/HRE1* Functions as a Positive and Negative Regulator

Ethylene has been shown to regulate a number of cellular responses, including inhibition of root elongation and elicitation of triple responses in dark-grown seedlings (Huang et al., 2003; Swarup et al., 2007). The triple-response phenotype, which consists of shorten-

ing of the hypocotyl and root, radial swelling of the hypocotyl, and exaggerated curvature of the apical hook, is a well-known effect of ethylene on etiolated dicotyledonous seedlings (Guo and Ecker, 2004). In our studies, seedlings of *AtERF73/HRE1-RNAi* lines displayed an ethylene-oversensitive phenotype under normoxic conditions (Figs. 4 and 5), suggesting that *AtERF73/HRE1* might negatively regulate ethylene responses.



**Figure 8.** Production of ethylene in Col-0 and *AtERF73/HRE1-RNAi* lines during hypoxia. The Col-0 (diamonds) and *AtERF73/HRE1-RNAi* lines 19 (squares), 20 (triangles), and 22 (crosses) subjected to hypoxic treatment for different time periods were harvested and assayed for ethylene production as described in "Materials and Methods." The means of six independent hypoxic treatments are plotted. Bars represent SD.



Our analyses showed that *AtERF73/HRE1-RNAi* lines could affect hypoxia-regulated gene expression in two ways. For one group of genes, including *ADH* and a subset of glycolytic and fermentative genes, levels of hypoxic induction were significantly reduced in three *AtERF73/HRE1-RNAi* lines (Fig. 7), indicating that *AtERF73/HRE1* positively regulates the induction of these genes during hypoxia. However, it has to be pointed out that in the report by Licausi et al. (2010), hypoxic induction of *ADH* was affected by the double mutation of *AtERF73/HRE1* and *AtERF71/HRE2* but not by the single mutation of *AtERF73/HRE1* in the single knockout line. In contrast, the expression of *ADH* was increased in the *AtERF73/HRE1* overexpression line. The difference in the effects of *AtERF73/HRE1-RNAi* lines might reflect differences in hypoxia treatment conditions, in which an open system was used in our experiments. Our analyses also showed that hypoxic induction of several peroxidase and cytochrome P450 genes was enhanced in the *AtERF73/HRE1-RNAi* lines, suggesting that *AtERF73/HRE1* could inhibit the hypoxic induction of these genes. The Arabidopsis genome contains as many as 286 different cytochrome P450 genes, some of which play crucial roles in the biosynthesis of a variety of endogenous lipophilic compounds and cross talk in the responses to abiotic and biotic stresses (Paquette et al., 2000; Noordermeer et al., 2001; Narusaka et al., 2004). Peroxidases are known to be involved in oxidative cross-linking of cell wall (e.g. lignification) and in hydrogen peroxide ( $H_2O_2$ ) detoxification. Numerous studies have shown that hypoxia stress triggers the formation of reactive oxygen species and induces oxidative stress in plants (Geigenberger, 2003; Garnczarska and Bednarski, 2004). It was reported that the level of  $H_2O_2$  increased in response to oxygen deprivation (Baxter-Burrell et al., 2002) and that there is a mechanism to maintain the homeostasis of  $H_2O_2$ . Therefore, it might be possible that *AtERF73/HRE1* plays a role in maintaining the homeostasis of  $H_2O_2$  by regulating the expression of genes involved in antioxidation. Consistent with this hypothesis, we found that expression of *AtERF73/HRE1* could also be induced by  $H_2O_2$  (data not shown).

#### Potential Roles of *AtERF73/HRE1* and Ethylene in Hypoxia Signal Pathways

The results we have reported here indicate that *AtERF73/HRE1* plays an essential role in hypoxia signaling pathways. We showed that hypoxia triggers ethylene-dependent and ethylene-independent pathways, both of which are required for the accumulation of *AtERF73/HRE1* transcripts. The induced *AtERF73/HRE1* in turn participates in the activation of *ADH* and other genes related to fermentation and carbohydrate metabolism. In addition, we showed that *AtERF73/HRE1* could modulate ethylene responses under both normoxic and hypoxic conditions. We also showed that *AtERF73/HRE1* negatively regulated a group of peroxidase and cytochrome P450 genes through its

inhibitory effects on ethylene signaling responses. The functions of *AtERF73/HRE1* are very similar to those of the rice *Sub1A* and *SK* genes, which play a central role in submergence tolerance of lowland and deepwater rice, respectively (Xu et al., 2006; Hinz et al., 2010). In rice, the *Sub1* locus encodes three submergence-inducible ERF genes, *Sub1A*, *Sub1B*, and *Sub1C* (Fukao et al., 2006; Xu et al., 2006; Voesenek and Bailey-Serres, 2009). *Sub1B* and *Sub1C* exist in all rice cultivars that have been examined. The *Sub1A* has two alleles among *indica* cultivars. The submergence-tolerant cultivars, such as FR13A, carry the *Sub1A-1* allele, whereas the submergence-sensitive cultivars carry the *Sub1A-2* allele. In FR13A, induction of *Sub1A-1* resulted in the inhibition of *Sub1C* expression as well as the retardation of root and shoot elongation during the submergence of rice plants (Fukao et al., 2006; Xu et al., 2006). It has been demonstrated that ethylene is involved in the induction of *Sub1A-1* during submergence and that *Sub1A* in turn suppresses ethylene production and GA responses, which allow rice plants maintain a low metabolic state to survive long periods of submergence (Fukao et al., 2006; Perata and Voesenek, 2007). In deepwater rice, which does not carry *Sub1A*, a pair of ERF factors, *SK1* and *SK2*, are induced under submergence conditions through the action of ethylene signaling pathways (Hattori et al., 2009). In contrast to the lowland rice, the activation of *SK1* and *SK2* resulted in enhanced GA responses to stimulate shoot elongation, which allows the deepwater rice to adapt to flooding conditions (Hattori et al., 2009). Despite the similarity of the proposed functions of *AtERF73/HRE1*, *Sub1A*, and *SK*, induction of *Sub1A* and *SK* genes results in submergence tolerance in rice, whereas induction of *AtERF73/HRE1* probably results in only temporary survival of Arabidopsis. We are currently determining the downstream targets of both *AtERF73/HRE1* and *Sub1A* to identify transcriptional regulatory pathways mediated by these two factors to determine the molecular basis of the difference in hypoxia sensitivity between Arabidopsis and rice.

## MATERIALS AND METHODS

### Plant Materials, Growth Conditions, and Stress Treatment

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 was used in this study. Two ethylene-insensitive mutants, *ein2-5* and *etr1-1*, were obtained from Dr. L.-C. Wang, and their phenotypes were confirmed before use. All seeds were sterilized and kept for 3 d at 4°C in the dark to break dormancy. Seeds were germinated on 0.8% (w/v) agar plates containing one-half-strength MS medium supplemented with 0.5% (w/v) Suc at pH 5.7. The plates were incubated at 22°C under a 16-h-light (81  $\mu\text{mol s}^{-1} \text{m}^{-2}$ )/8-h-dark cycle in a growth chamber for 7 to 10 d. For morphological observations, seeds were sown on a 2:1:6 mixture of vermiculite, perlite, and peat moss and irrigated with water for 2 weeks.

For stress treatments, 14-d-old seedlings were treated with 5  $\mu\text{M}$  ABA, 50  $\mu\text{M}$  MeJA, 5  $\mu\text{M}$  ACC, or 50  $\mu\text{M}$  SA at 4°C for 6 h in the dark. For hypoxia treatment, 14-d-old Arabidopsis seedlings were placed on filter paper presoaked with one-half-strength MS solution supplemented with 0.5% (w/v) Suc at pH 5.7 for 1 h in the dark and then transferred onto a floating

platform with roots dipped into one-half-strength MS solution containing 0.5% (w/v) Suc at pH 5.7, which was constantly bubbled with 3% (v/v) oxygen and 97% (v/v) nitrogen, in the dark for the times indicated. For hypoxia treatment with the presence of AVG and AgNO<sub>3</sub>, Arabidopsis seedlings were treated in the same way except that 10 μM AVG or 10 μM AgNO<sub>3</sub> was added to the MS solution. To measure root lengths, Arabidopsis seeds were germinated on 0.8% (w/v) agar plates containing one-half-strength MS and 0.5% (w/v) Suc with or without 2 μM ACC at pH 5.7. The plates were kept at 4°C in the dark for 3 d and then transferred to 22°C under a 16-h-light (81 μmol s<sup>-1</sup> m<sup>-2</sup>)/8-h-dark cycle in a growth chamber for 7 to 10 d. The root lengths were measured for 50 seedlings of each genotype. The measurements were performed three times with independently germinated seedlings. Photographs were taken at 7 or 10 d of treatment. For apical hook observations, seeds were grown on 0.8% (w/v) agar plates containing one-half-strength MS and 0.5% (w/v) Suc supplemented with or without 2 μM ACC at pH 5.7. The plates were kept at 4°C for 3 d and then transferred to 22°C for 4 d in the dark. The level of apical curvature was estimated visually for at least 100 seedlings. Photographs were taken at the end of the treatment.

### Constructs and Plant Transformation

For the *AtERF73/HRE1-RNAi* lines, the *AtERF73/HRE1* (*At1g72360*) open reading frame was amplified by PCR from the Col-0 ecotype using the following primer pair: 5'-GTAGAGGAGAATCATGAGGCTGATC-3' (forward) and 5'-CTCGAGGGTACCCAAAGAGACTACTCAGAGACGTTGA-3' (reverse). The reverse primer contains the recognition sites for *KpnI* and *XhoI*. The amplified product was cloned as an inverted repeat with a pyruvate-orthophosphate dikinase artificial intron into the binary vector pBI121. The recombinant plasmid was then transformed into *Agrobacterium tumefaciens* strain GV3101. The resulting *Agrobacterium* was used to generate transgenic Arabidopsis by vacuum infiltration. The seeds were planted on one-half-strength MS agar plates containing kanamycin (50 μg mL<sup>-1</sup>) to identify T1 transgenic plants. T1 seeds were generated on the kanamycin plates to select homozygous T2 lines.

### RT-PCR and Quantitative RT-PCR Analyses

RNA samples were isolated from frozen tissues of Arabidopsis using the TRIzol reagent (Invitrogen). For RT-PCR analysis of Arabidopsis RNAs, total RNA samples from roots (2 μg per reaction) were first treated with DNase I and then reverse transcribed into cDNAs by Moloney murine leukemia virus reverse transcriptase. Primers used for RT-PCR are listed in Supplemental Table S2. The RT-PCR products were separated on a 1.0% agarose gel by electrophoresis.

Quantitative RT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems) with the Power SYBR Green PCR Master Mix according to the manufacturer's recommendations. Amplification of *β-ATP* cDNA under identical conditions was used as an internal control to normalize the level of cDNA. The data obtained were analyzed with the 7500 System SDS software. Amplification conditions were as follow: 95°C for 10 min, and then 40 cycles at 95°C for 15 s and 60°C for 1 min. The specificity of primer pairs (Supplemental Table S2) was based on an analysis of dissociation curves (75°C–99°C). Quantitative RT-PCR experiments were repeated three times independently, and the data were averaged.

### Ethylene Measurements

The 14-d-old seedlings of the wild type and *AtERF73/HRE1-RNAi* were treated with hypoxia as described above. Ten plants were transferred to a 12- × 75-mm test tube and capped for 1 h at room temperature in the dark. Ethylene was measured using a gas chromatograph (6890N Agilent GC system). The experiments were repeated six times independently, each time in triplicate.

### Microarray Analysis

All microarray experiments were carried out using the ATH1 Arabidopsis GeneChip microarray (Affymetrix) containing a set of 22,810 probes. Four independent biological replicates were performed using cDNA obtained from control and hypoxia-treated (6 h) samples from 14-d-old seedlings. Fluorescence labeling of cDNA probes starting from mRNA was performed by in

vitro transcription followed by fragmentation following the manufacturer's suggestion (GeneChip Expression Analysis Technical Manual rev5; Affymetrix). Microarray experiments were performed at the DNA microarray core facility of the Institute of Plant and Microbial Biology at Academia Sinica in Taiwan. The raw microarray data from all hybridization assays were analyzed and normalized by the GeneSpring GX 7.3 software. Normalization was performed, as values below 0.01 were set to 0.01, per chip was divided by the 50th percentile, and per gene was normalized by the median of corresponding gene measurements in normoxic samples. GeneSpring was used with a 5-fold change and  $P \leq 0.05$  cutoffs to identify up- and down-regulated genes for further analysis.

The microarray Gene Expression Omnibus accession number is GSE27475.

### Supplemental Data

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

**Supplemental Figure S1.** The expression levels of group VII *ERF* genes after hypoxic treatment.

**Supplemental Figure S2.** The shoot mRNA levels of *AtERF73/HRE1* after hypoxic treatment.

**Supplemental Figure S3.** The expression levels of group VII *ERF* genes in *AtERF73/HRE1-RNAi* lines after hypoxia treatment.

**Supplemental Figure S4.** The hypocotyl and root lengths on one-half-strength MS medium with ACC for 4 d in the dark.

**Supplemental Table S1.** List of up-regulated genes in the wild type upon hypoxic treatment.

**Supplemental Table S2.** Primers for PCR and quantitative RT-PCR.

### ACKNOWLEDGMENTS

We thank Dr. L.-C. Wang (Institute of Plant and Microbial Biology, Academia Sinica) for providing seeds of *ein2-5* and *etr1-1* mutants. Affymetrix GeneChip assays were performed by the Affymetrix Gene Expression Service Laboratory (<http://ipmb.sinica.edu.tw/affy/>), supported by Academia Sinica.

Received January 11, 2011; accepted March 6, 2011; published March 10, 2011.

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